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Evaluation of freezing tolerance in olive cultivars by stomatal density and freezing stress

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Abstract
Selection of frost tolerant cultivars and understanding the mechanisms of frost hardiness could help to improve freezing resistance in olive plants. Olive cultivars may differ in frost hardiness due to differential survival of specific organs. The aim of this study was to screen different olive cultivars based on their stomatal density and metabolic modifications under cold conditions. The 'Zard' cultivar had the lowest while 'Derak' had the highest stomatal density, respectively. In another experiment, where entire potted olive plants were subjected to freezing stress (0, -6, -12 and -18 ˚C), 'Zard' and 'Dehghan' were found to be the most tolerant cultivars. They showed the lowest starch content, ionic leakage and wood injury. They also had the highest reducing sugar, phenolic and proline contents among studied cultivars. We concluded that 'Zard' and 'Dehghan' are the most tolerant cultivars and 'Derak', 'Dakal' and 'Shiraz' are the most sensitive cultivars to freezing injury.

Keywords: hardiness, ion leakage, proline, wood injury.

Introduction

Olive (*Olea europaea* L.) is one of the most important evergreen trees in the Mediterranean area. The olive tree grows mostly between latitudes of 30 and 45° in both hemispheres (Candev *et al*. 2009). However, in recent years increasing demand for better quantity and quality of olive oil has resulted in the cultivation of olive trees. This resulted in expanding olive cultivation areas into higher latitudes rather than its origin cultivation area in the Mediterranean basin (Mancuso, 2000)

A major limiting factor for growing olives at higher latitudes is exposure to minimum temperature especially in winter and early spring. When winter temperature drops below -7 °C, the aerial parts of olive tree can be damaged. This leads to yield reduction and in extreme situation threatens the life of the plant (Palliotti and Bongi, 1996). At temperatures below -12 °C, olive trees would face to severe damages which decrease its survival (Gomez del Campo and Barranco, 2005). Selection and use of tolerant cultivars are the most effective way for avoiding frost damage in olive trees. Plant physiological features such as stomatal density (Roselli *et al*., 1989), phenolic compound concentration (Roselli *et al*., 1992), ionic leakage (Barranco *et al*., 2005), and carbohydrate and starch concentrations (Lavee, 1986) have been used for selection of frost tolerant genotypes in olive.

In this study, changes in ionic leakage, reducing sugars, starch, proline and phenolic concentrations as biochemical features together with stomatal density as a

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morphological feature, were assessed in eight olive cultivars with contrasting levels of cold hardiness. Changes associated with freezing stress were analyzed to identify differences among cultivars in order to find suitable criteria for selection of tolerant cultivars in cold-winter regions.

Materials and Methods

Plant material and application of freezing stress

Eight olive cultivars ('Amygdalolelia', 'Conservallia', 'Dakal', 'Shiraz', 'Dehghan', 'Zard','Dezful' and 'Tokhme-Kabki') were obtained from the Experimental Agricultural Station and Natural Resources of Kazeroon, Iran, in 2012. Two-year-old plants (three plants for each cultivar) were propagated by semi-hardwood cuttings and were grown in 2 l plastic bags under greenhouse condition before exposure to freezing temperature. The plants were exposed to low temperatures (4 °C, 0°C, -6 °C, -12 °C, and -18 °C) for one hour. The temperature was gradually decreased to -6 °C by approximately 1.5 °C h^{-1} and by 5 °C h^{-1} thereafter. To thaw slowly, plants were removed from each low temperature treatment and were exposed to 4 °C overnight. To estimate the extent of freezing injury to the leaves, electrolyte leakage and biochemical changes were determined.

Electrolye leakage

Ionic leakage was measured as described by Bartolozzi and Fontanszza (1999). Five discs with 10 mm diameter were collected from leaves of three plants that had been subjected to the different freezing treatments and placed in a test tube containing 25 ml manitol (0.2 M) and incubated in a shaker for 4 h. Electrolytic conductivity (EC1) was measured using digital conductivity meter (Model HI8633, USA). Solutions and samples were then autoclaved to kill the cells. Once the solution was cooled, conductance was again measured (EC2). Ionic leakage (EC%) was calculated based on the Equation (1).

$$EC\% = EC1/EC2 \times 100 \quad (1)$$

Proline concentration

Twenty four hour after removing from freezer, young fully-expanded leaves (0.2 g) were used for determination of proline. Samples were homogenized in 3% sulfosalicylic acid. After addition of acid-ninhydrin, proline content was determined according to the method described by Bates *et al*. (1973). The absorbance of the fraction with toluene was determined at 520 nm, using a spectrophotometer (Model UV-120-20, Japan).

Total Phenolic Concentration

Total phenolic concentration (TPC) was measured using Folin-Ciaceleture reagent. Five mg of dry leaf powder was mixed with distilled water: methanol (50:50) and extracted for 15 min. The extract was then centrifuged at 13400 rpm for 15 min and the remaining filtrate was re-extracted twice using 5% aqueous methanol (Gutfinger, 1981). One ml of extract was mixed with one ml of 2% sodium carbonate (w/v) and was kept at room temperature for 30 min under dark conditions. The absorbance of the mixture was measured by spectrophometer at 750 nm. TPC was calculated as mass of gallic acid equivalents (GAE) per fresh weight (FW) mass of sample (g kg^{-1}).

Soluble carbohydrate concentration

To determine soluble carbohydrate concentration, 0.1 g of leaf powder was twice extracted with ethanol (80%). The samples were then centrifuged at 5000 rpm for 10 min and supernatant was adjusted to 25 ml. Soluble carbohydrate concentration was measured according to Dubois *et al*. (1956). One ml of 18% phenol and five ml of sulfuric acid were added to one ml supernatant in a test-tube. The mixture was immediately shacked and its absorption was recorded at 490 nm using a

spectrophotometer (Model UV-120-20, Japan).

Starch concentration

Starch concentration in the leaf samples was measured using anthrone reagent (Mc Cready *et al*., 1950). To do this, five ml cold water and 6.5 ml perchloric acid (52%) were added to the pallet material collected from the sample which was used for sugar analysis and mixed for 15 min. Approximately, 20 ml water was added to the mixture and the samples was centrifuged at 5000 rpm for 10 min. The supernatant was separated and the same procedure was repeated. The supernatants were combined and left for 30 min at 0 °C. After filtration, the volumes of supernatants were adjusted to 100 ml. About 2.5 ml of cold anthrone solution (2%) was added and the sample was heated at 100 °C for 7.5 min, then transferred immediately to an ice bath and cooled down to the room temperature. Absorption at 630 nm was recorded using a spectrophotometer (Model UV-120-20, Japan).

Stomatal density

Stomatal density was measured according to Roselli *et al*. (1989). Twenty leaves from midpoint of one-year-old wood were taken from each of the 10 cultivars (previous cultivars + Derak and Roughani cultivars). The stellate hairs were removed from the lower surface of each leaf using adhesive tape. A thin film of Actrifix was then painted on the clean surface, allowed to dry at room temperature and then peeled from the leaves. The films from each leaf were mounted on a glass microscope slide. The stomatal density was recorded with a light microscope using a 10X acular, 40X objective in a field area of 0.49 mm^2. Three stomatal counts were made from each leaf position (apex, center and base) for nine observations per leaf, and in total 180 observations were recorded per each cultivar.

Determination of wood injury

To evaluate cold hardiness of olive cultivars, plants were held at 22±1 °C for 24 h after removing them from freezer (Whirl pool, IRAN). For this evaluation 10 percent of stem per each cultivar was randomly harvested at each temperature. Individual pieces of stem were sectioned with rozar blade with 10 mm diameter and examined under a binocular microscope. The samples were checked for necrosis of the wood and samples with dull, straw or black/brown appearances were considered as dead samples. This experiment was repeated three times for each cultivar (Rekika *et al*., 2004).

Statistical analysis of freezing treatments was conducted as a factorial experiment using a completely randomized design with three replications (plants). Measurements of stomatal density were conducted in a completely randomized design. Twenty leaves per cultivar and nine observations per leaf were analyzed using SAS software and means with significant differences ware compared using DMRT (P= 0.05)

Results

Electrolyte leakage

At -18 °C, the highest leakage rates were observed in ‘Dakal’ and ‘Shiraz’ while the lowest electrolyte leakage was observed in ‘Dehghan’ cultivar. At -12 °C, ‘Dehghan’ and ‘Zard’ showed the lowest electrolytic leakage (Table 1). Based on the obtained results for electrolytic leakage, olive cultivars can be ranked as the following:

‘Shiraz’ ≥ ‘Dakal’ ≥ ‘Dezful’ ≥ ‘Amygdalolelia’≥ ‘Tokhme-Kabki’≥ ‘Conservallia’ ≥ ‘Zard’ ≥ ‘Dehghan’.

Starch concentration

The analysis of results indicated that starch concentration in olive leaf tissue was 74.7 mg g^{-1} DW following freezing stress at 0 °C, while it was 44.6 mg g^{-1} DW following stress at -18 °C. Leaf tissue of ‘Shiraz’ cultivar had the highest starch concentration, whereas, ‘Dehghan’, ‘Tokhme-Kabki’, and ‘Zard’ had the lowest starch concentrations following freezing stress (Table 2). Based on the

obtained results for starch concentration, olive cultivars can be ranked as the following:

'Shiraz'> 'Dakal'> 'Amygdalolelia'≥ 'Dezful'> 'Consercvallia'> 'Zard' ≥'Tokhme-Kabki' > 'Dehghan'.

Reducing sugar concentration

Following freezing stress conditions, 'Dehghan' had the highest and 'Shiraz' had the lowest reducing sugar concentrations (Table 3). Reducing sugar concentrations were significantly higher (60.9 mg g^{-1} DW) following stress at 0 °C, while the amount of reducing sugar was 90.2 mg g^{-1} DW following freezing stress at -18 °C. Among the studied cultivars, 'Shiraz' had the lowest and 'Dehghan' had the highest reducing sugar concentrations (Table 3).

Based on the obtained results for concentration of reducing sugar, olive cultivars can be ranked as the following:

'Dehghan' > 'Zard' ≥ 'Tokhme-Kabki'≥ 'Conservallia' > 'Amygdalolelia' > 'Dezful' ≥ 'Dakal'> 'Shiraz'.

Proline concentration

Leaf proline concentrations following freezing stress were significantly increased as freezing temperature increased (Table 4). Proline concentration was 23.6 (µmol g^{-1} FW) following stress at 0 °C and 79.9 µg mg^{-1} FW at -18 °C. The highest proline concentration was recorded in 'Dehghan' (80.3 µg mg^{-1} FW) while the lowest concentrations were observed in 'Shiraz' and 'Dakal' (27.7 and 34.5 µg mg^{-1} FW, respectively) (Table 4).

Based on the obtained results for concentration of reducing sugar, olive cultivars can be ranked as the following:

'Dehghan' ≥ 'Zard' ≥ 'Tokhme-Kabki' ≥ 'Conservallia' ≥ 'Dezful' ≥ 'Amigdalolelia' ≥ 'Dakal' ≥ 'Shiraz'.

Table 1. Ionic leakage (%) in the leaves of olive cultivars following exposure to different freezing temperatures

Cultivar	Temperature (°C)				Mean
	0	-6	-12	-18	
Amygdalolelia	39.0 Ab	61.7Bba	72.1ABd	81.7 Ab	63.6 B
Conservallia	32.3 bc	37.1Cd	58.0 Bef	74.3 Abc	50.4 C
Dakal	44.3 Da	63.3 Cab	92.7Bb	109.3 Aa	77.4 A
Dehghan	22.7Dd	29.0 Cd	50.3 Bf	61.3 Ac	40.8 D
Dezful	28.9Cd	52.3 Bbe	83.4 Ac	96.3Aa	65.2 B
Shiraz	45.3 Ca	72.0 Ba	102.7Aa	103.3 Aa	80.8 A
Tokhme-Kabki	27.5 Dd	42.8 Ccd	61.0 Bc	83.4 Ab	53.7C
Zard	21.6Cd	30.5 Cd	50.9 Bf	72.5 Abc	43.7D
Mean	32.7 D	48.6C	71.3B	85.1 A	

Means with same capital letters in columns and small letters in rows are not significantly different at 5% DMRT.

Table 2. Starch concentrations (mg g^{-1} DW) in the leaves of olive cultivars following exposure to different freezing temperatures

Cultivar	Temperature (°C)				Mean
	0	-6	-12	-18	
Amygdalolelia	67.7Acd	65.0 Abc	63.7 Aab	59.5Aab	64.0 Bc
Conservallia	65.0 Bcd	64.0 Abcd	63.4 Aab	51.3 Bbc	60.9 C
Dakal	86.4Aab	76.3 ABb	63.3 Bab	49.7Cbc	68.9 B
Dehghan	60.8Ad	41.0 Be	30.4 Ce	26.7Ce	39.8E
Dezful	82.8 Aab	68.0 Bb	57.7 Cbc	44.5Ccd	63.3BC
Shiraz	95.0 Aa	89.9ABa	78.2Aba	62.7 Ba	81.4 A
Tokhme-Kabki	74.0 Abc	52.9 Bcde	39.67 Cde	27.5Ce	48.5 D
Zard	67.7 Acd	50.3 Bde	47.3 Bcd	34.7Cde	49.5 D
Mean	74.7 A	63.4 B	55.5C	44.6 D	

Means with same capital letters in columns and small letters in rows are not significantly different at 5% DMRT.

Table 3. Soluble sugar concentrations (mg g^{-1} DW)in the leaves of olive cultivars following exposure to different freezing temperatures

Cultivar	Temperature (°C)				Mean
	0	-6	-12	-18	
Amygdalolelia	58.1 Ccd	63.3 BCcd	74.1 ABb	86.0Ac	70.4 C
Conservallia	65.3 Cbc	84.2 Bb	95.3 ABba	102.3 Ab	86.8B
Dakal	55.0 Acd	60.8 Acd	61.7Ab	66.3 Ad	60.92 D
Dehghan	86.7Aa	101.3 Aa	110.7Aa	114.3 Aad	103.3A
Dezful	59.3 Acd	60.0 Acd	62.3 Ab	70.0 Ad	62.9 CD
Shiraz	43.0 Be	49.0 ABd	56.3 Ab	58.0 Ad	51.6E
Tokhme-Kabki	48.7Cde	75.9 Bbc	108.1Aa	119.8 Aa	88.1 B
Zard	70.6 Cb	81.7BCd	95.7ABa	104.0 Aab	88.2 B
Mean	60.9D	72.0 C	83.0 B	90.2 A	

Means with same capital letters in columns and small letters in rows are not significantly different at 5% DMRT.

Table 4. Total proline concentration (µmol g^{-1} FW) in the leaves of olive cultivars following exposure to different freezing temperatures

Cultivar	Temperature (°C)				Mean
	0	-6	-12	-18	
Amygdalolelia	26.7 dab	33.4 cd	39.0 bd	53.0 acd	38.1 D
Conservallia	22.5 cbc	45.0 bb	63.0 bb	107.7ab	59.6B
Dakal	15.0 bd	39.6 ab	41.7ad	41.5 ade	34.5D
Dehghan	31.8 ca	84.1 ba	92.7ba	112.5aab	80.3A
Dezful	27.0 cab	39.8 bb	52.2ac	57.4ac	44.1 C
Shiraz	18.3 bcd	20.8 bc	34.9 ad	36.7ac	27.7E
Tokhme-Kabki	19.2 dcd	75.2ca	88.7ba	125.0 aa	77.0 A
Zard	28.4 cab	85.8ba	94.3 ba	106.2ab	78.7A
Mean	23.6 D	53.0 C	63.3 B	80.0 A	

Means with same capital letters in columns and small letters in rows are not significantly different at 5% DMRT.

Phenolic concentration

Following application of freezing stress, concentrations of total phenolic compounds in 'Dehghan' and 'Zard' cultivars were higher than their concentrations in other cultivars (Table 5). When freezing temperature increased from 0°C to -18°C, the subsequent phenolic concentrations in the leaves were increased from 12.1 to 18.6 mg g^{-1} DW. Highest and lowest total phenolic concentrations were measured in 'Dehghan' and 'Shiraz', respectively (Table 5).

Based on the obtained results for concentration of phenolic compounds, olive cultivars can be ranked as the following:

'Dehghan' > 'Zard' > 'Tokhme-Kabki' > 'Conservallia'> 'Amygdalolelia' > 'Dezful' > 'Dakal'> 'Shiraz'.

Stomatal density

Stomatal density was significantly different among the ten cultivars. The highest stomatal densities were found on the leaves of 'Derak' and 'Amygdalolelia' and the lowest Stomatal density was observed in 'Zard' cultivar (Table 6).

Based on the obtained results for stomatal density, olive cultivars can be ranked as the following:

'Derak' ≥ 'Amygdalolelia'≥ 'Shiraz' > 'Roughani'> 'Dezful'> 'Dehghan' ≥ 'Dakal' > 'Conservallia' > 'Tokhme-Kabki' > 'Zard'.

Determination of wood injury

Following freezing stress at -18 °C, highest percentage of dead wood was observed in 'Shiraz' and 'Dakal' cultivars. Following freezing stress at -6 °C, only small injuries were observed on 'Shiraz', 'Dakal' and 'Amygdalolelia' cultivars, while no necrosis or other wood injuries were observed in other cultivars. Exposure to -

12 °C and -18 °C caused damage to the wood of all cultivars and the highest symptoms of injury was observed in 'Shiraz' and 'Dakal' cultivars. Except 'Dehghan', other cultivars showed 50% wood injury at -12 °C (Table 7).

The order of wood hardiness among studied cultivars was as the following:

'Dehghan'> 'Tokhme-Kabki'≥ Zard' ≥ 'Dezful'> 'Conservallia' ≥ 'Amygdalolelia' >'Dakal' ≥ 'Shiraz'.

Table 5. Total phenolic concentration (mg g^{-1} Dw) in the leaves of olive cultivars following exposure to different freezing temperatures

Cultivar	Temperature (°C)				Mean
	0	-6	-12	-18	
Amygdalolelia	9.8 ad	10.8 abc	15.0 abc	16.9acd	13.1 CDE
Conservallia	13.1abc	13.4 ab	14.3abc	17.3acd	14.5 CD
Dakal	11.2 abcd	11.5 abc	11.7ac	12.6 ad	11.7 EF
Dehghan	20.5ba	25.6 aBa	27.0 aba	29.2aa	25.6A
Dezful	11.1abcd	11.3 abc	13.2ac	12.6 ad	12.1EDF
Shiraz	8.4 ad	10.3 abc	11.2ac	11.7 ad	10.4 F
Tokhme-Kabki	8.4bd	9.4bc	20.8aab	22.0 abc	15.1 C
Zard	14.6bb	23.0 aa	25.7aa	26.3 aab	22.4 B
Mean	12.1 C	14.4 B	17.4A	18.6A	

Means with same capital letters in columns and small letters in rows are not significantly different at 5% DMRT.

Table 6. Stomatal density of olive cultivars

Cultivar	Stomata density (number per 0.49 mm^2 field area)
Amygdalolelia	45.5a
Conservallia	38.6 e
Dakal	40.4 d
Dehghan	41.6cd
Drak	46.7a
Dezful	42.4bc
Roughani	43.0 bc
Shiraz	43.6 b
Tokhme-Kabki	33.4 f
Zard	31.0 g

Means in column with same letters are not significantly different at 5% DMRT.

Table 7. Wood injury (%) in potted plant of olive cultivars following exposure to different freezing temperatures

Cultivar	Temperature (°C)				Mean
	0	-6	-12	-18	
Amygdalolelia	0 Ca	6.67Cb	66.67Bb	80 Ab	38.33 B
Conservallia	0 Ca	0Cb	63.33Bb	83.33 Ab	36.67 B
Dakal	0 Da	13.33 Ca	83.33 Ba	100 Aa	49.17 A
Dehghan	0 Ca	0 Cb	40 Bc	60 Ad	25 E
Dezful	0 Ca	0 Cb	63.33Bb	76.67Abc	35 BC
Shiraz	0 Ca	16.67 Ba	90 Aa	100 Aa	51.67 A
Tokhme-Kabki	0 Ba	0 Bb	56.67 Ab	63.33 Acd	30 D
Zard	0 Ca	0 Bb	53.33Bb	70 Abcd	30.83 CD
Mean	0 D	4.58 C	64.58B	79.17 A	

Means with same capital letters in columns and small letters in rows are not significantly different at 5% DMRT.

Discussion

In the current study, freezing temperature resulted in a significant increase in ion leakage (Table 1). This indicates that electrolytic leakage can be a useful tool for distinguishing frost resistance among olive cultivars. Cultivars such as 'Zard' and 'Dehghan' with the lowest electrolytic leakages can be likely grown in cold areas with less visual symptoms of damage under freezing temperatures. These results are in agreement with Bartolozzi and Fontanazza (1999) who reported that resistant cultivars due to low ionic leakage from leaves show slight negative responses to freezing temperatures.

When freezing temperature increased from 0 to -18 °C, concentration of reducing sugars in the olive leaves was increased from 60.85 mg g^{-1} DW to 90.21 mg g^{-1} DW (Table 3) while, the concentration of starch in the leaves was significantly decreased (Table 2). In our study, we found that soluble sugar concentrations in leaves of olive were increased to a maximum level during fall and winter, while opposite trend was observed for starch concentration (unpublished data).

Starch, is a type of polysaccharides that converts to simple sugars through activity of amylase and maltase enzymes. It has been shown that activity of these enzymes can be induced by cold temperatures (Hallwell 1980). In our study, the highest concentration of reducing sugars was concurrent withmaximum freezing resistance. Highest reducing sugar concentration was observed in resistant cultivars (e.g. 'Dehghan'), while sensitive cultivars had the lowest concentration of reducing sugars (Table 3). Reducing sugars play an important role in osmotic adjustment of cells, which helps to prevent intracellular freezing. The results of this study are in agreement with the other studies on walnut (*Juglans regia* L.) (Ameglio *et al*., 2004) and pomegranate (*Punica granatum* L.) (Ghasemi *et al*., 2012).

There was a strong relationship between cold hardiness and proline concentration in the leaves of olive. Exposure to freezing stresses led to activation of an osmotic adjustment mechanism through the accumulation of proline. In the present study, when the temperature was lowered from 0 °C to -18 °C, proline concentration increased from 23.64 µM g^{-1} FW to 79.99 µM g^{-1} FW, respectively. When exposed to different freezing temperatures, tolerant cultivars, such as 'Dehghan', 'Tokhme-Kabki' and 'Zard', had higher proline concentrations than sensitive cultivars such as 'Dakal' and 'Shiraz' (Table 4). Herber *et al*. (1973) reported that proline is capable of preventing freezing-induced membrane damages. Proline also contributes to solute accumulation, presumably by protecting proteins and membrane structures. Proline may act as a scavenger of reactive oxygen species (ROS) under stress conditions (Verslues *et al*., 2006).

Total phenolic concentrations in tolerant cultivars, such as 'Dehghan' and 'Zard', were higher than their concentrations in sensitive olive cultivars, especially at -18 °C. This is in agreement with Ortega-Garcia and Peragon (2009) who reported that exposure to strong stress conditions led to accumulation of phenolic content in leaf samples. Accumulation of phenolic compounds under low temperature stresses may be related to their antioxidant activities and therefore phenolic compounds may offer protection against oxidative damage induced by freezing stresses (Ortega-Garcia and Peragon, 2009).

The critical temperature that caused discrimination among studied olive cultivars was -12 °C. At this temperature there was a considerable difference in wood survival among olive cultivars (Table 7). At -12 °C, there were high rates of wood survival for 'Dehghan' and 'Zard' cultivars and low rates of wood survival for 'Dakal' and 'Shiraz' cultivars. At -18 °C, 100% wood injury was observed in 'Dakal' and 'Shiraz' cultivars and the lowest wood injury was observed in 'Dehghan' and

'Zard' cultivars. At this temperature, resistant cultivars such as 'Dehghan' and 'Zard' showed lower ionic leakage than other cultivars (Tables 1 and 7). In general, our results are in agreement with other reports on cold hardiness (Candev *et al*., 2009).

The results of our study showed that the more resistant cultivars, the lower stomatal density on their leaves. 'Derak' and 'Amygdalolelia' cultivars had the highest and 'Zard' cultivar had the lowest stomatal density. The number of stomata within a cultivar is usually affected by environment; therefore, evaluation of cultivars should only be made within a specific environment (Roselli *et al*., 1989).

In conclusion, we found that cultivars with lower stomatal densities ('Zard' and 'Dehghan'), higher concentrations of proline, phenolic compounds, reducing sugars, and lower level of starch and ionic leakage were more tolerant to freezing stress than cultivars with higher stomatal densities. These cultivars can be cultivated in colder areas to improve quality and quantity of their oil. Finally a link between stomatal density and biochemical measurements was found that can be used for screening of cold resistance among olive cultivars.

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