



Research paper

The cost of avoiding freezing in stems: trade-off between xylem resistance to cavitation and supercooling capacity in woody plants

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Received February 4, 2017; accepted May 24, 2017; handling Editor Maurizio Mencuccini

Stems and leaves of *Olea europaea* L. (olive) avoid freezing damage by substantial supercooling during the winter season. Physiological changes during acclimation to low temperatures were studied in five olive cultivars. Water relations and hydraulic traits, ice nucleation temperature (INT) and temperatures resulting in 50% damage (LT_{50}) were determined. All cultivars showed a gradual decrease in INT and LT_{50} from the dry and warm summer to the wet and cold winter in Patagonia, Argentina. During acclimation to low temperatures there was an increase in leaf cell wall rigidity and stomatal conductance (g_s), as well as a decrease in leaf apoplastic water content, leaf water potential (Ψ), sap flow and stem hydraulic conductivity (k_s). More negative Ψ as a consequence of high g_s and detrimental effects of low temperatures on root activity resulted in a substantial loss of k_s due to embolism formation. Seasonal stem INT decrease from summer to winter was directly related to the xylem resistance to cavitation, determined by the loss of k_s across cultivars. Thus the loss of freezable water in xylem vessels by embolisms increased stem supercooling capacity and delayed ice propagation from stems to the leaves. For the first time, a trade-off between xylem resistance to cavitation and stem and leaf supercooling capacity was observed in plants that avoid extracellular freezing by permanent supercooling. The substantial loss of hydraulic function in olive cultivar stems by embolism formation with their high repair costs are compensated by avoiding plant damage at very low subzero temperatures.

Keywords: cold acclimation, hydraulic conductivity, ice nucleation temperature, *Olea europaea*, sap flow, stomatal conductance.

Introduction

Low temperatures are, together with drought, the two most important environmental constraints affecting the distribution of plants and their productivity worldwide (e.g., Levitt 1980, Boyer 1982). Plants in general can avoid or tolerate extracellular freezing according to the intensity and duration of the freezing events as well as their inherent physiological characteristics (Levitt 1980, Gusta and Wisniewski 2013). Freezing avoidance, which prevents ice formation through freezing point depression or by supercooling, has been found in plant species previously acclimated to mild subzero temperatures (Goldstein et al. 1985, Pearce 2001, Reyes-Diaz

et al. 2006). Exposure of plants to gradually low, but not freezing, temperatures initiates physiological and biochemical adjustments that protect them from damage when subzero temperatures occur in late autumn and winter. Thus, this increase in the ability to resist freezing is the consequence of acclimation of plants to low temperatures (Levitt 1980, Larcher 2003). Cold acclimation is usually assumed to be a process leading to the development of freezing tolerance in plants, i.e., ice seeding in extracellular spaces without intracellular freezing. It is a complex process that requires a programmed and integrated genetic capacity to activate the appropriate mechanisms needed to withstand harsh

winter conditions (Welling et al. 2002). However, plants that avoid freezing damage by supercooling may also require physiological changes during the transition period from summer to winter to be able to avoid harmful subzero temperatures.

Key traits for surviving to subzero temperatures by tolerance and or avoidance include: (i) an increase in cell wall rigidity (Mancuso 1998, Dichio et al. 2003, Solecka et al. 2008, Arias et al. 2015, Le Gall et al. 2015, Zhang et al. 2016); (ii) an increase in the amount of cryoprotectant substances combined with low amount of intra- and/or extracellular water; and (iii) a decrease in osmotic potentials (Arora and Wisniewski 1996, Arora et al. 2004, Charrier et al. 2013, Pramsöhler and Neuner 2013). In general, leaf water potentials of woody plants are lower during the dry season than during the wet season, which is a typical plant response to drought. Nevertheless, some species exhibit a pronounced drop of water potential during the transition from the dry to the wet and cold season (Pavel and Fereres 1998, Mayr and Charra-Vaskou 2007, Iniesta et al. 2009, Barkatky et al. 2013, Arias et al. 2015, Lopez-Bernal et al. 2015). These low water potentials are likely caused by an imbalance between water absorption by roots and water loss by transpiration (Lopez Bernal et al. 2015, Garcia-Tejera et al. 2016) as well as by an increase in water viscosity at lower temperatures (Bloom et al. 2004, Melkonian et al. 2004). This may result in low stem water transport efficiency due to cavitation and embolism formation.

Olea europaea L. (olive) is an evergreen woody plant species that is highly drought resistant and moderately resistant to low temperatures (Arias et al. 2015). The traditional latitudinal range for olive cultivation is from 30° and 45° in the Northern and Southern hemisphere (Bongi and Palliotti 1994); however, new orchards are being established more recently in habitats with an appreciable risk for experiencing subzero temperatures. Olive tissues do not tolerate ice formation, but permanently supercool to avoid ice formation at low temperatures (Bongi and Palliotti 1994, Arias et al. 2015). The ability to maintain water in a liquid phase at subzero temperatures is acquired during acclimation between summer and winter (Arias 2015).

Cold acclimation-induced changes that enhance tolerance to subzero temperatures have been investigated in many studies (Sakai and Larcher 1987, Guy 1990, Thomashow 1999, 2010, Ball et al. 2004, Roden et al. 2009, Ruelland et al. 2009, Charrier and Ameglio 2011); however, few studies have focused on cold acclimation-induced changes associated with the acquisition of increased supercooling capacity (Wisniewski and Ashworth 1986, Arora et al. 1992, Fujikawa and Kuroda 2000). In addition, few studies were done with other tissues, besides leaves, such as stems. In this context, the aim of this study was to evaluate the changes in plant water relations during cold acclimation and their relationship with supercooling capacity in stems and leaves of five olive cultivars. Acclimation should result in coordinated changes in physiological and anatomical traits related to freezing avoidance. We ask if the strong decrease in leaf water potentials observed

during winter in olive trees (Arias 2015) represents an adaptive advantage for these plants to avoid damage by subzero temperatures, and if there is a trade-off between xylem resistance to hydraulic failure and supercooling capacity. We hypothesized that a decrease in soil temperatures during winter affects leaf water potentials, which in turn impair the hydraulic function of the vascular tissue at the same time as enhancing the supercooling capacity of stems and leaves. We also ask if there are coordinated changes in stem and leaf responses to low temperatures during the process of acclimation.

Materials and methods

Site and experiment design

The research was carried out near Comodoro Rivadavia city, Patagonia, Argentina (45°47'53" S; 67°24'59" W). Rain falls mostly in autumn and winter (April to September) and the mean annual value is 300 mm. The mean annual air temperature is 9.0 °C and daily mean temperature varies from 14 °C during summer (December to February) to 3 °C during winter (June to August). Five *O. europaea* cultivars (Arbequina, Changlot Real, Frantoio, Hojiblanca and Manzanilla) were selected for this study. Forty individuals of 2-year-old plants per cultivar were grown outdoors in a common garden during 1 year in 20-dm³ pots (one plant per pot) and irrigated bi-weekly. This study was done during 2011–12.

Environmental variables

Air relative humidity and temperature were measured continuously with a HOBO datalogger (Pro series, Onset Computer Corporation, Pocasset, MA, USA). Pot soil temperature at 7 cm depth was determined with thermocouples (Type T) connected to a CR10X datalogger (Campbell Scientific, Logan, USA). Pot volumetric water content was determined at 5 cm depth using ECH₂O probes (10 HS, Decagon devices, Inc., Pullman, USA).

Leaf water relations

Leaf water potentials (Ψ) at predawn and midday from four trees per cultivar ($n = 10$ leaves) were determined with a pressure chamber (PMS System, Corvallis, OR, USA) during summer, autumn and winter. Pressure–volume analysis (Tyree and Hammel 1972) was used to determine seasonal pressure–volume relationships from all olive cultivars plants. Five exposed and mature leaves from different 2-year-old plants per cultivar were sampled at predawn and immediately recut under water and covered with plastic bags. The petiole cuts were kept submerged for 2 h. The leaves were then weighed to obtain full turgor mass and immediately the initial leaf water potential was determined. Measurements were repeated many times while the leaves were allowed to dehydrate slowly on a laboratory bench. Finally, the leaves were dried at 60 °C for 72 h to obtain their dry mass. Osmotic potential at full turgor (π^{100}), osmotic potential at turgor loss point (π^0), apoplastic water fraction (AWF) and

volumetric bulk modulus of elasticity (ϵ) were determined from pressure–volume relationships, which were obtained by plotting $1/\Psi$ vs relative water deficit ($1 - \text{relative water content, RWC}$).

Volumetric bulk modulus of elasticity (ϵ) was determined over the full range of positive turgor (Evans et al. 1990):

$$\epsilon = (\Delta\Psi/\Delta\text{RWC}) \times \text{FS}$$

where $\Delta\Psi$ is the change in turgor pressure, ΔRWC is the change in relative water content and (FS) is the symplastic water fraction.

Thermal analysis and tissue damage

Thermal analyses were done on sun-exposed mature leaves and stems ($n = 3-4$) of 2-year-old plants. Leaves and stem segments 2 cm long were collected in the field and immediately were placed in a freezer. Copper-constantan thermocouples placed in close contact with samples were used to determine tissue temperature. Samples with thermocouples attached were positioned in test tubes within the freezer. Temperature was lowered at a rate of $5\text{ }^{\circ}\text{C h}^{-1}$ from ambient to $-20\text{ }^{\circ}\text{C}$. This rate is similar to maximum rate of temperature drop in the field at night in the study site, and similar to those used in other studies (e.g., Arias et al. 2015, Zhang et al. 2016). Temperatures were recorded at 4-s intervals with a data logger (CR10X, Campbell Scientific). The ice nucleation temperatures (INT) were detected from the tissue temperature kinetics. A sudden increase in temperature (exotherm) indicates heat release from water during extracellular ice nucleation.

During thermal analysis we did not inoculate the samples to promote freezing close to the equilibrium freezing temperatures, because *O. europaea* does not tolerate ice formation. The ice inoculation will underestimate the freezing avoidance capacity because this species exhibits freezing resistance by permanent supercooling (Arias et al. 2015),

The electrolyte leakage method was used to determine tissue damage (Wilner 1960). Leaf, stem and root samples from five olive cultivars were collected at predawn, kept in plastic bags to prevent water loss, and then transported to the laboratory for analysis. Five to seven leaf discs, and 2–4 cm stem or root samples were placed into sealed tubes and incubated in the freezer. The freezer was cooled at the same rates used to determine INT to reach different target temperatures (5, 0, -2 , -4 , -6 , -8 , -10 , -12 , -14 , -16 , -18 and $-20\text{ }^{\circ}\text{C}$). After maintaining the samples at a particular target temperature for 15 min (see Lipp et al. 1994), three tubes per cultivar containing leaf, stem or root samples were removed from the freezer and thawed at $4\text{ }^{\circ}\text{C}$. Ten milliliters of deionized water was added to each tube, which was held in a shaker for 24 h at ambient temperature. Electric conductivity (EC) of the solution was then measured with an electrical conductance/resistance meter (Hanna HI 98,311, Hanna instruments, Woonsocket, USA). The tubes were enclosed in an autoclave to achieve a complete electrolyte leakage of each sample. Electric conductivity of the solution was

measured after removing the samples from autoclave. The relative EC (an indicator of membrane damage or ion leakage) was calculated as a percentage:

$$\text{Relative EC} = \left(\frac{\text{EC after the temperature treatment}}{\text{EC autoclave}} \right) \times 100$$

The % EC was plotted as a function of the incubation temperature. The temperature at the 50% relative EC was defined as the tissue damage temperature (LT_{50}).

Stomatal conductance

Stomatal conductance to water vapor (g_s) was measured during summer and winter using a steady-state porometer (LI-1600; LICOR Inc., Lincoln, NE, USA). Measurements were done on three to five fully expanded and sun-exposed leaves from each cultivar (from three to five plants per cultivar) in the morning between 10:00 and 11:00 h and in the early afternoon between 13:00 and 14:00 h during three typical days of each season.

Sap flow

A heat pulse system based on the work developed by Burgess et al. (1998) and modified by Scholz et al. (2002) was used to measure sap flow in the trunk of three potted trees per cultivar during summer and winter. The temperature sensors were connected to a data logger (CR10X, Campbell Scientific) sealed inside an insulated box to minimize temperature differences between the temperature sensors inputs. The heat pulse velocity (V_h , cm h^{-1}) was calculated according to Marshall (1958) as

$$V_h = (Dt/x)\ln(v_1/v_2)$$

where Dt is thermal diffusivity of wet wood, x is distance between the heat source (line heater) and the temperature sensors, and v_1 and v_2 are the increase in temperature after the heat pulse, at equidistant points downstream and upstream, respectively, from the heater. Heat pulses were released every 30 min and calculations were based on measurements obtained between 60 and 100 s after the release of the heat pulse, when the ratio of v_1 and v_2 was most stable (Burgess et al. 2001).

Stem hydraulic conductivity

Five branches per cultivar and season were collected at predawn by cutting large branches in air, and then re-cutting under water in order to relax xylem tensions and to avoid the formation of non-native embolism (Wheeler et al. 2013). Branches with the cut ends under water were covered with black plastic bags and transported to the laboratory. Then, stem segments were recut under distilled water to a length of 20 cm and attached to the hydraulic conductivity apparatus (Tyree and Sperry 1989). This segment length was selected in order to minimize the number of xylem vessels cut open at both ends of stem segments because the maximum vessel length is about 18 cm (Trifilò et al. 2014a, 2014b).

A constant hydraulic head of 60 cm was maintained during the measurements and distilled water was used as perfusion liquid. Hydraulic conductivity ($\text{kg m s}^{-1} \text{MPa}^{-1}$) was calculated as $k_h = J_v / (\Delta P / \Delta X)$, where J_v is the flow rate through the stem segment (kg s^{-1}) and $\Delta P / \Delta X$ is the pressure gradient across the segment (MPa m^{-1}). Then, specific hydraulic conductivity (k_s , $\text{kg m}^{-1} \text{s}^{-1} \text{MPa}^{-1}$) was calculated as the ratio of k_h and the cross sectional area of the active xylem. The active xylem area (A_s) was obtained by introducing indigo carmine dye to stem segments from one cutting end (Bucci et al. 2003).

Stem saturated water content

Saturated water content of lateral branches was measured on five stems per cultivar. Stem fresh mass (FM) was determined and then samples were allowed to equilibrate overnight in deionized water. The samples were weighed to determine saturated mass (SM) and then oven-dried to a constant mass to obtain dry mass (DM). Stem saturated water content was calculated as:

$$\text{SWC} = \frac{\text{SM} - \text{DM}}{\text{DM}}$$

Statistical analysis

All physiological trait data were normally distributed (Kolmogorov–Smirnov test). Differences between cultivars in ϵ , INT, LT_{50} , π^0 , AWF, Ψ_{\min} , Ψ_{\max} and k_s were examined using an ANOVA test. Seasonal differences within a cultivar were evaluated using Student test. The SPSS 11.5 statistical package (SPSS Inc., Chicago, IL, USA) was used. Linear regressions were fitted to INT against AWF, ϵ and π^0 during winter, autumn and summer and to stem INT against leaf INT during winter. We used the Sigma Plot software (Systat Software Inc., San Jose, CA, USA) to fit the functions shown in Figures 4, 5, 7 and 8.

Results

Environmental conditions

Air temperature and air saturation deficit (D) varied seasonally in the area close to the common garden used to grow the plants (data not shown). In the study period, mean monthly temperature ranged

from 22 °C in January to 3.5 °C in July. Absolute minimum air temperature was –6 °C in July. The air saturation deficit reached maximum values of 5 kPa in summer, while in winter it decreased to 1 kPa. The soil temperature at 7 cm depth inside the pot was always higher than the air temperature, and in the coldest day of the study period the lowest soil temperature observed was –1.8 °C.

Seasonal changes in leaf ice nucleation temperature and cell membrane damage

Leaf ice nucleation (leaf INT) and leaf membrane damage (leaf LT_{50}) decreased significantly from summer to winter within each cultivar. Leaf INT in Arbequina decreased from –6.5 to –11.9 °C and leaf LT_{50} decreased from –3.5 to –13.1 °C (Table 1). During summer when plants were not acclimated to low temperatures, leaf LT_{50} occurred before leaf INT, except in Hojiblanca. For example, in Manzanilla leaf damage in the summer occurred at 5 °C higher than INT. However, LT_{50} and INT nicely converged in autumn and winter as a result of plant acclimation to lower temperatures. Thus, the differences between leaf INT and leaf LT_{50} observed during autumn and especially during winter were very small in all cultivars (Table 1).

Ice nucleation temperature and damage of stems and roots across cultivars during winter

The stem ice nucleation temperature (stem INT) varied, depending on the cultivar, between –9.3 and –10.8 °C (Table 2). Stem tissue damage (stem LT_{50}) occurred at about 1 °C below stem INT across all cultivars (from –10.4 to –11.9 °C), but no significant differences in stem INT and LT_{50} were found between cultivars during winter. At the minimum soil temperature experienced during the study period (–2 °C) all cultivars exhibited root tissue damage less than 50% of the maximum damage (Table 2). The lowest percentage of root damage was observed in Changlot Real (22%). The other study cultivars did not differ substantially in root damage at –2 °C, varying between 34% and 44%. The root damage was substantially lower at temperatures higher than –2 °C (results not shown). The stem saturated water content (SSWC) ranged from 86% to 98% and was linearly correlated to the stem INT (stem INT = –20 + 0.10 × SSWC; $R^2 = 0.78$, $P \leq 0.05$). Cultivars with higher SSWC exhibited higher stem INT (Table 2).

Table 1. Summer, autumn and winter leaf ice nucleation temperature (INT, °C), and leaf temperature at which 50% of membrane leakage occurred (LT_{50} , °C). Values are means ± SE of three trees per cultivar. Different capital letters indicate significant differences within each cultivar across seasons and different small letters indicate significant differences among cultivars.

| Cultivar | Summer | | Autumn | | Winter | |
|---------------|----------------|----------------------------|----------------|----------------------------|-----------------|----------------------------|
| | Leaf INT (°C) | Leaf LT_{50} (°C) | Leaf INT (°C) | Leaf LT_{50} (°C) | Leaf INT (°C) | Leaf LT_{50} (°C) |
| Arbequina | –6.5 ± 0.35aA | –3.8 ± 0.3bA | –6.00 ± 0.2aA | –5.90 ± 0.24aB | –11.86 ± 0.65aB | –13.08 ± 0.65aC |
| Changlot Real | –6.43 ± 0.46aA | –3.9 ± 0.25bA | –8.00 ± 0.5aB | –8.80 ± 0.05aB | –11.39 ± 0.75aC | –12.68 ± 0.03aC |
| Frantoio | –6.36 ± 0.43aA | –3.6 ± 0.35bA | –7.02 ± 0.35aA | –8 ± 0.44aB | –10.74 ± 0.92aB | –12.03 ± 0.61aC |
| Hojiblanca | –5.83 ± 0.68aA | –5.8 ± 0.07aA | –10 ± 0.25aB | –10.12 ± 0.3aB | –11.60 ± 1.38aC | –12.57 ± 0.7aC |
| Manzanilla | –7.19 ± 0.45aA | –2 ± 0.45bA | –7.2 ± 0.4aA | –8 ± 0.44aB | –10.13 ± 0.41aB | –11.25 ± 0.07aC |

Table 2. Stem ice nucleation temperature (INT), stem temperature at which 50% of membrane leakage occurred (LT_{50} , °C), root damage at -2 °C (minimum soil temperature) and stem saturated water content (SWC) during winter in five olive cultivars. Values are means ($n = 3$ or 4 , \pm SE). Different letters indicate significant differences among cultivars.

| Cultivars | Stem INT (°C) | Stem LT_{50} (°C) | Root damage at -2 °C (%) | Stem SWC (%) |
|---------------|--------------------|---------------------|----------------------------|-------------------|
| Arbequina | $-10.81 \pm 0.02a$ | $-11.90 \pm 0.65a$ | $35.87 \pm 0.98a$ | $86.3 \pm 7.33a$ |
| Changlot Real | $-10.4 \pm 0.48a$ | $-11.50 \pm 0.03a$ | $22.14 \pm 0.90b$ | $89.2 \pm 8.89a$ |
| Frantoio | $-9.83 \pm 0.64a$ | $-10.94 \pm 0.61a$ | $37.29 \pm 1.68a$ | $97.68 \pm 7.51a$ |
| Hojiblanca | $-10.58 \pm 0.03a$ | $-11.67 \pm 0.7a$ | $34.24 \pm 1.02a$ | $87.13 \pm 9.44a$ |
| Manzanilla | $-9.29 \pm 0.51a$ | $-10.41 \pm 0.07a$ | $44.07 \pm 1.81a$ | $95.54 \pm 9.14a$ |

Seasonal changes in leaf water potentials, sap flow and leaf gas exchange

Predawn (Ψ_{max}) and midday (Ψ_{min}) leaf water potentials exhibited significant seasonal differences (Figure 1). The seasonal differences between summer and winter Ψ_{min} varied between 0.8 MPa and 1.7 MPa depending on cultivar (Figure 1B). Predawn Ψ_{max} was lower during winter than summer in three of the five study cultivars while Ψ_{min} were significantly lower in winter than summer in all cultivars ($P < 0.05$) (Figure 1). There were no differences in Ψ_{min} across cultivars (winter $F = 0.428$; $P = 0.93$; summer $F = 0.345$; $P = 0.93$) (Figure 1).

Stomatal conductance was higher in winter than in summer across all cultivars ($P < 0.01$) (Figure 2). Whereas in summer maximum g_s ranged between 90 and 100 $\text{mmol m}^{-2} \text{s}^{-1}$ across cultivars, in winter g_s ranged between 120 and 180 $\text{mmol m}^{-2} \text{s}^{-1}$. A consistent stomatal decrease from morning to early afternoon was only observed during summer across cultivars (Figure 2A). Manzanilla was the cultivar with the largest g_s reduction after midday (33%). However, during the winter some cultivars tended to increase g_s at midday, but the differences were not significant (Figure 2B).

Representative daily courses of sap flow velocity for each cultivar during summer and winter are shown in Figure 3. Significant differences in sap flow velocity were found between seasons in all study cultivars ($P < 0.001$). During summer, sap flow increased rapidly during the morning, briefly attained a clearly defined maximum value and then tended to decline (Figure 3). During winter this pattern was not well defined. Very small daily fluctuations were observed in winter, and in several days the sap flow was negligible (Figure 3).

Midday water potentials were correlated to leaf and stem INT across cultivars and seasons (Figure 4). There was a linear positive relationship between Ψ_{min} and stem and leaf INT across cultivars and seasons (Figure 4). The water potentials and INT were significantly more negative in winter compared with summer ($P < 0.05$). During autumn both variables were substantially higher than winter values, but they were relatively close to summer values. Leaf and stem INT observed during winter were highly correlated; however, stem ice nucleation occurred about 1 °C before leaf ice nucleation (Figure 5).

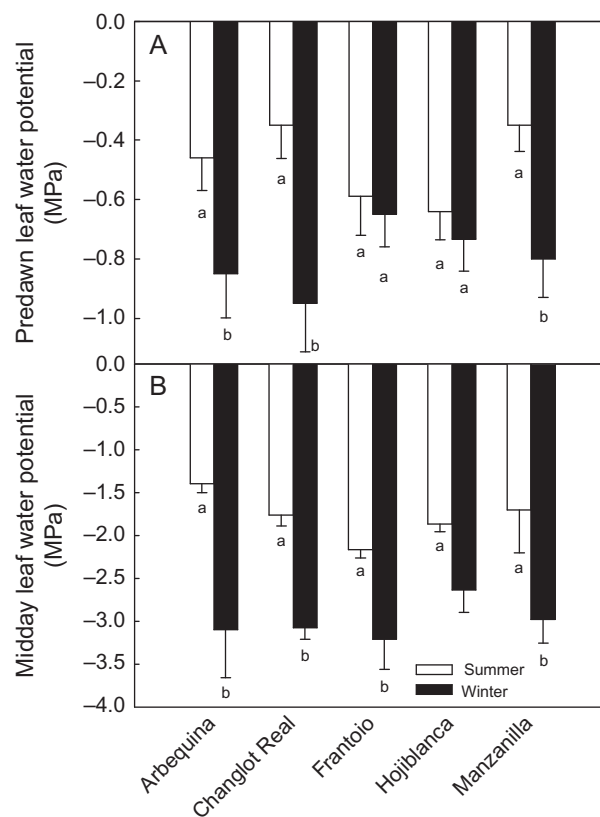


Figure 1. Predawn (A) and midday (B) leaf water potential of five olive cultivars during summer (white columns) and winter (black columns). Each bar represents the mean value ± 1 SE of four trees per cultivar and season. Different letters indicate significant differences between seasons within cultivar.

Stem-specific hydraulic conductivity (k_s) was significantly higher in summer than in winter in all cultivars (Figure 6). The percentage loss in k_s from summer to winter (a measure of xylem resistance to cavitation) varied between 90% in Manzanilla and 98% in Hojiblanca. Native k_s measured in summer (Figure 6) and winter stem INT (Table 2) were linearly correlated (stem INT = $-0.54k_s - 9.15$; $R^2 = 0.52$, $P < 0.05$). The winter k_s was correlated to the percentage of root damage at the minimum soil temperature recorded during winter across cultivars (winter $k_s = 0.004 \times \% \text{ root damage} + 0.197$; $R^2 = 0.90$; $P < 0.05$). Cultivars with lower winter k_s exhibited less root damage at low

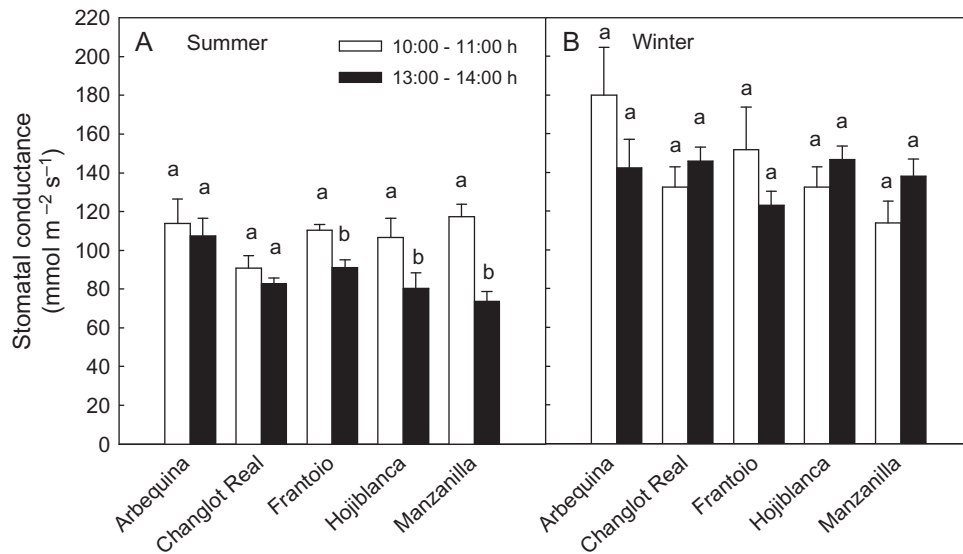


Figure 2. Stomatal conductance (g_s) measured in the morning (10:00–11:00 h) and in the afternoon (13:00–14:00 h) during summer (A) and winter (B) for the five olive cultivars. Bar are means \pm SE of three to five trees per cultivar. Different letters indicate significant differences between seasons within cultivar.

temperatures. The decrease in stem and leaf INT from summer to winter was functionally related to the decrease in k_s from summer to winter across cultivars in an asymptotic fashion (Figure 7). Hojiblanca exhibited the largest decrease in stem INT and the largest loss of k_s , and thus it was the cultivar with the lowest resistance to cavitation and the highest supercooling capacity.

Leaf RWC was maintained between 77% and 90% depending on the cultivar during both seasons (results not shown). Apoplastic water fraction was lower and bulk elastic modulus (ϵ) was substantially higher (i.e., more rigid cell wall) during winter compared with summer and autumn, with the exception of Hojiblanca and Changlot Real cultivars, which exhibited intermediate values between both seasons (Figure 8A and B). Apoplastic water content was positively correlated to leaf INT (Figure 8A), while ϵ and π^0 were negative correlated to leaf INT in all cultivars across seasons (Figure 8B). Osmotic potential at the turgor loss point (π^0) was significantly higher (less negative) during winter than autumn and summer in all cultivars ($P < 0.05$; Figure 8C). The π^0 in summer ranged between -4.1 and -4.7 MPa, while in winter the range observed was -1.2 to -1.7 MPa. The same pattern was observed in the osmotic potential at saturation (π^{100}) between summer and winter in all cultivars ($P < 0.01$; data not shown).

Discussion

Seasonal changes in water relations traits enhance freezing resistance

Cold acclimation in *O. europaea* cultivars resulted in variations in water relations traits, similar to the responses of some woody plants to water deficits (e.g., Scholz et al. 2012, Bucci et al. 2013). Leaf water potential, cell wall elasticity, stem hydraulic conductivity and sap flow decreased during the gradual drop in

air temperature from summer to winter. Nevertheless, there were some striking responses that differed from those observed during drought. Stomatal conductance at the end of the cold acclimation period to low temperatures was higher than during summer and osmotic adjustment did not occur. All these changes during acclimation to low temperatures were of adaptive value for enhancing freezing avoidance by supercooling in *O. europaea*.

More negative leaf water potentials are commonly observed in woody plants when soil and atmospheric water availability are low (Clifford et al. 1998, Bucci et al. 2008, Sofo et al. 2008, Rousseaux et al. 2009, Scholz et al. 2012, Torres Ruiz et al. 2015). However, in this study substantially low water potentials occurred when soil water availability was high but ambient temperature was low. Osmotic potentials increased (less negative) from summer to winter in all olive cultivars. These seasonal changes in osmotic potential indicate that osmotic adjustment (i.e., a solute buildup either by active or passive increase in osmotically active solutes) was not a response to a decrease in leaf water potentials during winter. In an earlier study with olives trees, Arias et al. (2015) observed a rapid decrease in osmotic potential during days with lower temperatures (-5 °C) than average air temperature conditions, probably related to short-term carbohydrate responses.

Decrease in leaf water content during cold acclimation (Li et al. 2005, Gusta and Wisniewski 2013) is beneficial in plants exposed to freezing temperatures, as tissues containing a large amount of free water are highly susceptible to ice formation (Guy 2003). Although, leaf water content remained without significant changes during cold acclimation in all *O. europaea* cultivars, there was a substantial water migration from apoplastic to symplastic compartments. Apoplastic water fraction decreased

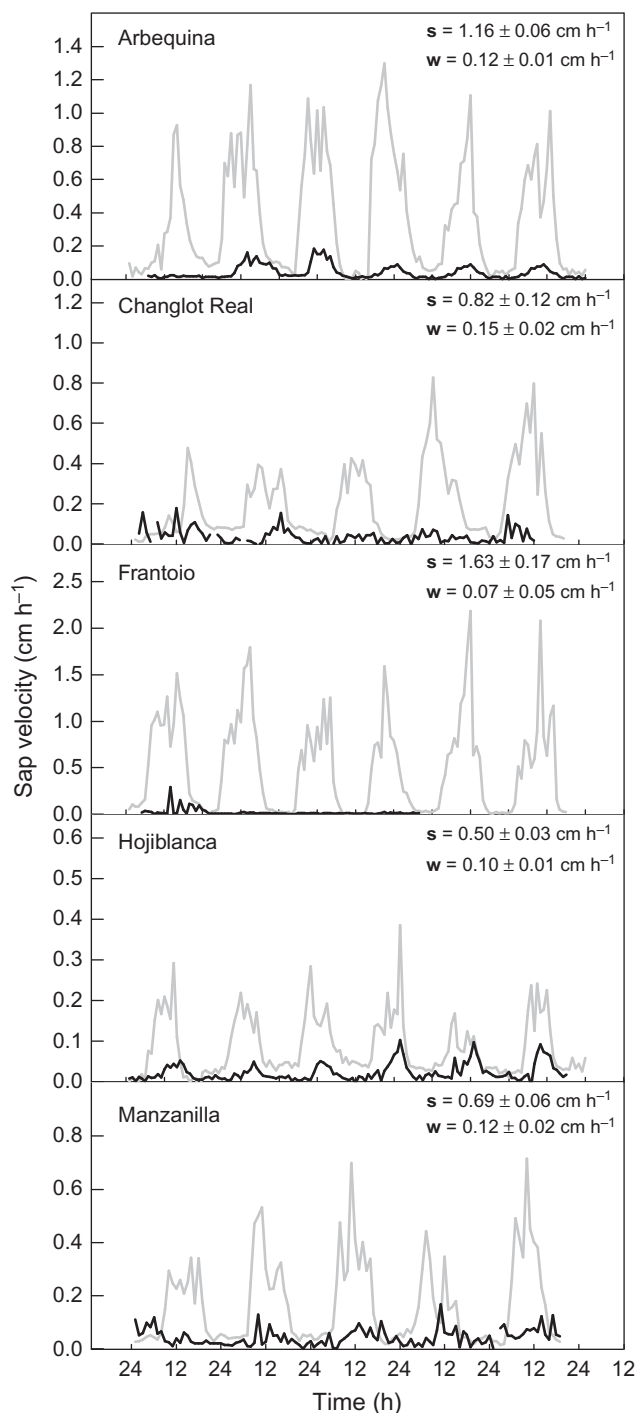


Figure 3. Diurnal courses of sap flow velocity (cm h^{-1}) during summer (solid grey line) and winter (solid black line) for about four to six consecutive representative days in five olive cultivars. Inside each panel the mean maximum sap velocity \pm SE per cultivar is shown for summer (S) and winter (W).

about 40% in some cultivars from summer to winter. Because ice seeding usually starts in extracellular spaces, supercooling capacity is enhanced by low apoplastic water content (Goldstein et al. 1985, Cordell et al. 1998, Arias et al. 2015). The water movement to the intracellular compartments during acclimation

also may contribute to the osmotic water potential increase observed during winter despite the very low water potential.

Cold acclimation of plants also involves biochemical changes in cell walls including lipids and pectin deposition (Baldwin et al. 2014) and phenolic cross-linking between cell wall polymers (Fry 1986), which may result in more rigid cell walls. *Olea europaea* exhibited an increase in the wall rigidity as denoted by a higher bulk elastic modulus after exposure to low, but non-freezing-temperatures. One of the functional advantages of rigid walls in freezing-tolerant plants is their higher mechanical resistance to physical pressure exerted by extracellular ice growth. In freezing-avoidance species, such as olives, small pore size, low water content and uniform cell surface of rigid cell walls can result in a lower probability for ice nucleation (Arias et al. 2015).

Stomata, in general, tend to respond to changes in soil water potential, air saturation deficit and/or hydraulic conductance (Bucci et al. 2005, Buckley 2005, Domec et al. 2006). Stomatal closure following temperature decreases has been observed in several species (Ameglio et al. 1990, Wilkinson et al. 2001), as a consequence of a decrease in hydraulic conductance. In our study, there was no stomatal closure in response to almost total loss of the stem water transport capacity in winter in all *O. europaea* cultivars. High stomatal conductance during winter was also observed in other studies with olives in the Mediterranean region (Pavel and Fereres 1998, Perez Lopez et al. 2010, López Bernal et al. 2015), and in Northern Argentine (Rousseaux et al. 2008). The effects of soil water status on stomatal conductance via hydraulic or chemical signals, as suggested in other studies with this species (e.g., Jones 1992, Giorio et al. 1999, Moriana et al. 2002), is not considered a plausible explanation in our case because the pots where the olive plants grew were well irrigated during the study period. Consequently, we assume that *O. europaea* stomata respond mainly to changes in air saturation deficit, which is lower in winter compared with summer, thus explaining the observed high winter stomatal conductance. We are intrigued by the high stomatal conductance during winter when leaf water potentials were very low and cell turgor was lost. However, lack of stomatal closure even in leaf tissues without turgor is common in other species (Kolb and Sperry 1999, Scholz et al. 2012), and suggests that guard and epidermal turgor pressure can be decoupled (Turner and Jones 1980, Buckley 2005). In evergreen plants, this behavior may be advantageous for continuous carbon assimilation during the cold season (Arias 2015). *Olea europaea* can maintain high stomatal conductance and photosynthesis even at leaf water potentials below -5 MPa (Gucci et al. 2002, Perez-Martin et al. 2009, Arias 2015).

Frost-drought-induced embolisms and supercooling capacity: a new trade-off between xylem resistance to cavitation and freezing avoidance

Low temperatures could affect hydraulic functions at a whole-plant level by its effects on cell metabolism, membrane properties and

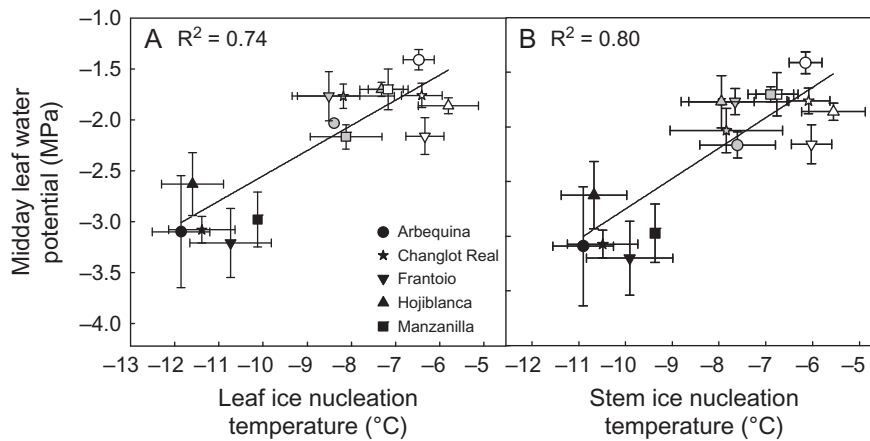


Figure 4. Relationships between midday leaf water potential (MPa) and (A) leaf ice nucleation temperature (°C) and (B) stem ice nucleation, during summer (open symbols), autumn (grey symbols) and winter (black symbols) in five olive cultivars. Each symbol represents the mean value ± 1 SE of four trees per cultivar. The solid lines indicate the linear regressions fitted to the data: (A) $y = -0.13x + 0.24$; $P < 0.001$; (B) $y = -0.28x + 0.07$; $P < 0.001$.

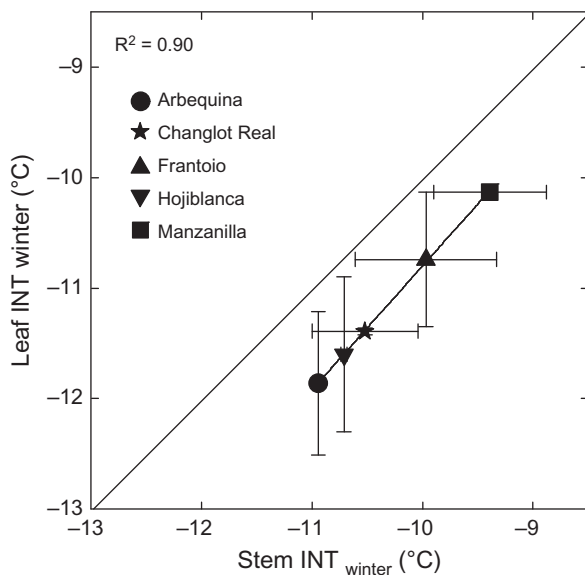


Figure 5. Stem ice nucleation temperature (stem INT) in relation to leaf ice nucleation temperature (leaf INT) across five olive cultivars during winter. Each symbol represents the mean value ± 1 SE of four trees per cultivar. The line is the linear regression fitted to the data ($R^2 = 0.90$; $y = -20.14 + 0.11x$; $P < 0.05$). The solid line is the 1 to 1 relationship between both variables.

water viscosity (Torrez Ruiz et al. 2015). Although increase in water viscosity could contribute to the low water transport efficiency in winter, viscosity alone cannot explain the substantial drop of stem water flow observed in all *O. europaea* cultivars, similar to the findings in other studies (Ameglio et al. 1990, Murai-Hatano et al. 2008, Lopez-Bernal et al. 2015). Then, the decrease in stem hydraulic capacity observed in our study was probably the result of an increase in radial root hydraulic resistance due to cell membrane damage by low temperatures. The relationship observed between winter k_s and root damage

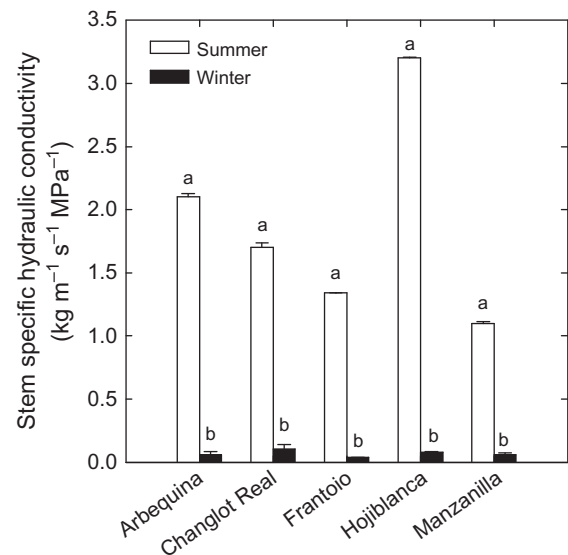


Figure 6. Stem hydraulic conductivity (k_s) during summer (open bars) and winter (filled bars) in five olive cultivars. Each bar represents the mean value ± 1 SE of four trees per cultivar and season. Different letters indicate significant differences between seasons within cultivar.

provides strong evidence for this pattern of behavior, which was reflected in the very low sap flow during winter in all olive cultivars. These results are consistent with findings in other studies (Pavel and Fereres 1998, Rousseaux et al. 2009, López Bernal et al. 2015). The decrease in root hydraulic efficiency resulted in an imbalance between water uptake from roots and water loss from leaves, affecting water transport along the soil-to-leaf continuum. High stomatal conductance during winter and high resistance to water flow in the soil-root pathway led to a substantial drop in leaf water potentials. More negative leaf water potentials during winter in olives had been found in other studies (Pavel and Fereres 1998, Rousseaux et al. 2008, Perez Lopez et al. 2010,

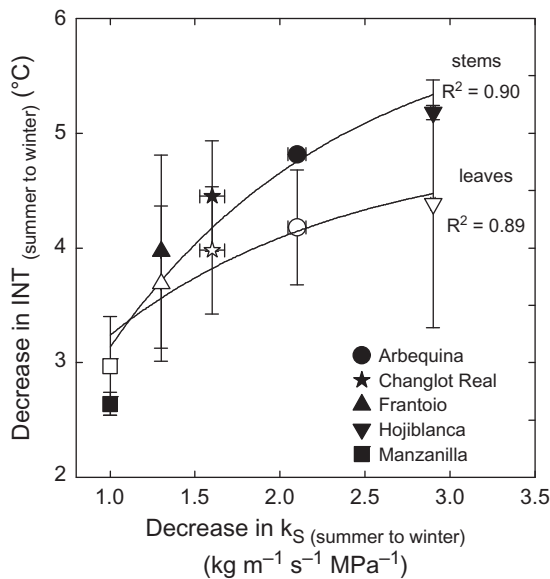


Figure 7. Decrease in ice nucleation temperature (INT) from summer to winter as a function of the decrease in stem hydraulic conductivity (k_s) from summer to winter in stem (filled symbols) and leaves (open symbols). Exponential rise to maximum functions were fitted to the data. For stems: $y = 6.07 (1 - \exp(-0.72x))$, $P < 0.05$; and for leaves $y = 6.78 (1 - \exp(-0.71x))$, $P < 0.05$.

Pierantozzi et al. 2013, Lopez Bernal et al. 2015), nevertheless the rationality for this behavior was not fully explained.

The freeze-induced embolism can be ruled out as the cause of a marked loss of stem water transport capacity because *O. europaea* does not tolerate extracellular ice formation (Arias et al. 2015). We are assuming in this study that the large loss of stem hydraulic conductivity, in winter, was strongly related to embolism formation, and it was the consequence of high xylem tension despite relative high soil water content and low air saturation deficits during the cold season. Leaf water potentials observed in our study are low enough to trigger embolisms according to olive branches vulnerability curves (Hacke et al. 2015; but see Cochard et al. 2015). Several species, including *O. europaea*, which experience embolism formation, are able to repair daily or seasonally their xylem vessels (see Broderson and McEnrone 2013, Trifilò; et al. 2014a, 2014b). Some mechanisms of vessel refilling imply an energy cost for plants (Bucci et al. 2003, Salleo et al. 2006, Secchi et al. 2011), requiring the use of osmotically active solutes obtained from starch depolymerization in xylem parenchyma cells. This carbon cost could be paid back because olive trees maintain photosynthetic activity during winter ($6\text{--}12 \mu\text{mol m}^2 \text{s}^{-1}$ depending on cultivar; Arias 2015). Despite the high metabolic costs involved in the hydraulic function recovery, having low cavitation resistance is helpful for enhancing stem and leaf supercooling capacity. Higher losses of k_s were associated to lower stem and leaf ice nucleation temperatures across cultivars and thus improved stem supercooling capacity. This represents a trade-off between

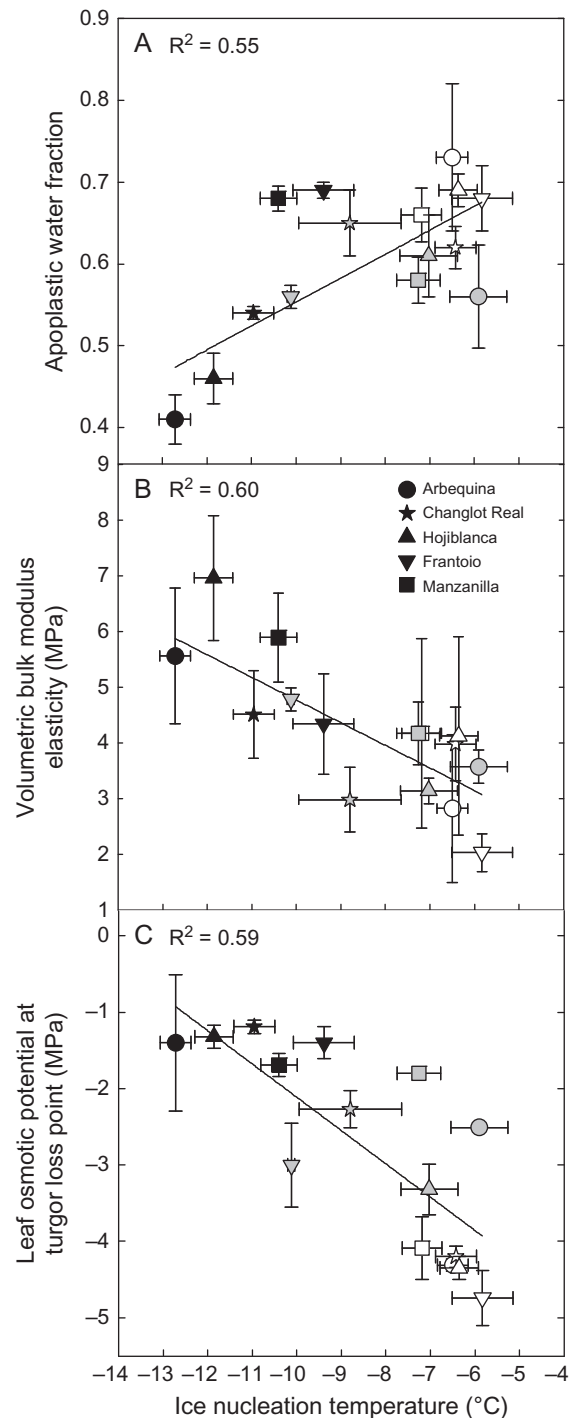


Figure 8. (A) Apoplastic water fraction, (B) bulk elastic modulus and (C) leaf osmotic potential at turgor loss point and in relation to leaf ice nucleation temperature ($^{\circ}\text{C}$) of five olive cultivars during summer (open symbols), autumn (grey symbols) and winter (black symbols). Each symbol represents the mean value ± 1 SE of three to four trees per cultivar. Solid lines in A, B and C indicate the linear regressions fitted to the data ($y = 0.845 + 0.029x$; $P < 0.01$; $y = 0.698 - 0.407x$; $P < 0.001$ and $y = -6.475 - 0.437x$; $P < 0.001$, respectively).

resistance to cavitation and freezing avoidance. In freezing-tolerant species, Lintunen et al. (2013) observed a positive correlation between ice nucleation temperatures in the xylem and

the long distance water transport efficiency across species. This relationship was explained by the xylem vessel size because the temperature at which the xylem freezes depends on conduits diameter. Wider vessels contain larger amount of water and thus it have higher chance to freeze (Zhang et al. 2016). However, we cannot consider changes in conduit size as determinants of the trade-off between xylem resistance to cavitation and supercooling in olive trees because changes in pit pore size from summer to winter within each plant did not occur (Arias 2015).

Some studies have shown similar relationships between supercooling capacity and leaf water potential to the ones observed in this study (Rada et al. 1987, Goldstein et al. 1994, Rada 2016); however, the nature of the positive relationship between these two variables is not known. Although pressure decreases the ice nucleation temperature, when water is in a double metastable state (under tension and under subzero temperatures) it has a higher chance for the formation of a new phase (ice or bubbles) (Pallares et al. 2016). Consequently, according to theory, there is not a causal relationship between higher tension and lower ice nucleation temperatures. We think that the loss of water transport efficiency induced by very low water potentials contributed to a decrease in stem tissue water content. Thus, the loss of freezable water from xylem vessels by massive embolism formation increased the stem supercooling capacity of olive cultivars and delayed ice propagation to the leaves. Leaf ice nucleation started approximately at a temperature of 0.9 °C lower than in the stems. Vessels without liquid water may act as a barrier preventing ice from spreading to leaves (Pramsohler and Neuner 2013). A similar pattern between leaf water potential and ice nucleation temperature was observed in these olive cultivars when they were subjected experimentally to water deficits (Arias 2015). Cold and dehydrated leaves exhibited enhanced supercooling capacity compared with warm and well hydrated leaves (Arias 2015). Therefore, our findings highlight the key role of water deficit in providing resistance to freezing temperatures.

Conclusions

We observed substantial freezing avoidance by supercooling in stems and leaves of olive cultivars. Changes in cell wall elasticity and water movement between tissues compartments (symplastic vs apoplastic) as a consequence of acclimation to low temperatures contributed to decrease in leaf INT and thus, increased supercooling. There was a coordinated response to low temperatures during acclimation in stems and leaves. More negative stem INT after acclimation was the result of strong loss of hydraulic conductivity due to an imbalance between root water uptake and leaf water loss during the cold season. This imbalance resulted in massive embolization of stem xylem, and consequently, substantial loss of k_s and the lowering of ice nucleation temperatures (higher supercooling capacity). For the first time, a

trade-off between xylem resistance to cavitation and supercooling capacity was observed in stems of freezing-intolerant woody plants. Our results suggest that the substantial loss of xylem k_s during winter desiccation, and the significant repair costs this likely represents, can be compensated for via the avoidance of ice formation in sap and cytoplasm.

Acknowledgments

We are grateful to Carlos Pasquet from CIASA nursery garden from San Juan, Argentina for donating the olive plants used in this study. We thank Sean M. Gleason and anonymous referees for helpful comments on the manuscript.

Conflict of interest

We have no conflict of interests.

Funding

This study was partially supported by Secretaria de Ciencia, Tecnologia e Innovacion Productiva Chubut, Argentina, Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET) (PIP grant) and Agencia Nacional de Promocion Cientifica y Tecnologica, Fondo para la Investigacion Cientifica y Tecnologica (ANPCYT-FONCYT) (PICT grants). This work complies with Argentinean Law.

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