Freezing avoidance by supercooling in Olea europaea cultivars: the role of apoplastic water, solute content and cell wall rigidity

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Abstract. Plants can avoid freezing damage by preventing extracellular ice formation below the equilibrium freezing temperature (supercooling). We used Olea europaea cultivars to assess which traits contribute to avoid ice nucleation at subzero temperatures. Seasonal leaf water relations, non-structural carbohydrates, nitrogen and tissue damage and ice nucleation temperatures in different plant parts were determined in five cultivars growing in the patagonian cold desert. Ice seeding in roots occurred at higher temperatures than in stems and leaves. Leaves of cold acclimated cultivars supercooled down to -13 ºC, substantially lower than the minimum air temperatures observed in the study site. During winter, leaf ice nucleation and leaf freezing damage (LT_{50}) occurred at similar temperatures, typical of plant tissues that supercool. Higher leaf density and cell wall rigidity were observed during winter, consistent with a substantial acclimation to sub-zero temperatures. Larger supercooling capacity and lower LT_{50} were observed in cold-acclimated cultivars with higher osmotically active solute content, higher tissue elastic adjustments and lower apoplastic water. Irreversible leaf damage was only observed in laboratory experiments at very low temperatures, but not in the field. A comparative analysis of closely related plants avoids phylogenetic independence bias in a comparative study of adaptations to survive low temperatures.

Key-words: freezing resistance, ice nucleation, LT_{50}, non-structural carbohydrate**,** olive.

Introduction

Cold and drought are two of the most important environmental stresses that affect growth, productivity and distribution of plants worldwide (Levitt 1980; Boyer 1982), particularly in cold deserts such as the patagonian steppe. Plants can survive freezing temperatures by either, avoiding extracellular ice formation or tolerating extracellular ice formation. Plants that tolerate extracellular ice formation are able to cope with cellular dehydration for long periods of time without apparent cell injury (Lipp et al. 1994; Scholz et al. 2012). Plants can avoid extracellular ice formation by thermal insulation of freezing- sensitive organs (Rada et al. 1985; Neuner et al. 2011) or by supercooling, preventing ice formation below the tissue specific equilibrium freezing temperature (Goldstein et al. 1985; Larcher 1982; Pearce 2001; Reyes et al. 2006).

Plants increase their freezing resistance (avoidance or tolerance) upon exposure to low nonfreezing temperatures, a phenomenon known as cold acclimation. Cold acclimation is the result of highly complex biochemical processes including the induction of genes encoding stress proteins (e.g. dehydrins), increases in sugar concentration, enhancements of antioxidative mechanisms and changes in lipid and protein composition (Ball et al. 2004; Gusta & Wisniewski 2013; Quellet & Charron 2013).

Plant parts that exhibit permanent supercooling (*sensu* Larcher 1982) avoid the potentially damaging effects of cell dehydration during freezing, but exhibit tissue damage when freezing is induced. All physical and biological systems supercool to some degree but there are few animal or plant species whose ice nucleation temperatures are very low which allow those organisms to survive cold environmental conditions. In this study we will use the term supercooling hereafter to refer to plants that avoid freezing by permanent supercooling. These plant parts are generally tissues with reduced intercellular spaces, low water content in the apoplastic spaces and without nucleators, which are active close to the equilibrium

freezing temperature (Goldstein et al. 1984; Sakai & Larcher 1987; Goldstein & Nobel 1991; Melcher et al. 2000). Active decrease in osmotic potential through the production of low-molecular-weight solutes can increase the degree of supercooling (Sakai & Larcher 1987; Kasuga et al. 2007). The substantial accumulation of osmotically active solutes helps to prevent intracellular freezing-induced dehydration as well as to provide non-colligative protection of cell membranes in freezing tolerant plant species (Steponkus et al. 1977; Levitt 1980; Thomas & James 1993; Wanner & Junttila 1999; Xin & Browse 2000; Kosová et al. 2007). This behavior has been observed in plants of alpine ecosystems (Rada et al. 1986; Kasuga et al. 2007), whereas tissue elastic adjustments (more rigid cell wall) was one of the responses found in shrubs from cold deserts to cope with sub-zero temperatures during winter (Scholz et al. 2012). Deposition of lipids and others structural changes in the cell wall during cold acclimation may increase cell wall rigidity preventing cell contraction and collapse when ice nucleation occurs in intercellular spaces, and thus enhancing tolerance to extracellular ice formation..

Supercooling has been observed in plant tissues such as xylem ray parenchyma, leaf buds and flower buds (George et al. 1974; Sakai 1979; Hong & Sucoff 1980; Neuner 2014), and in leaves of tropical alpine plants (e.g. Goldstein et al. 1985). It is also the main avoidance mechanism observed in most tissues of Olea europaea (olive) cultivars (Fiorino et al. 2000; Pearce et al. 2000). This makes olive trees an interesting model system to study biophysical and biochemical processes involved in frost avoidance. The objective of this study was to assess the ice nucleation temperature of roots, stems, and leaves and the role of apoplastic water fraction, leaf cell wall rigidity and chemical compounds in the freezing avoidance of five cultivars of O. europaea growing in the patagonian steppe of southern Argentina. We also compared seasonal patterns of those traits in acclimated and non-acclimated leaves and estimated ice seeding in roots, stems and leaves. Our hypothesis was that cultivars with lower

apoplastic water content due to the increase in intracellular solutes content and higher cell rigidity exhibit higher supercooling capacity during the winter than cultivars with higher apoplastic water fraction and more elastic cell walls.

Materials and methods

Site and experiment design

The research was carried out near the coast of Chubut Province, Argentina, close to Comodoro Rivadavia city. Mean annual rainfall is 300 mm falling mostly in autumn and winter (April to September) and the mean annual air temperature is 9.0 ^{0}C. Average summer (December to February) and winter (June to August) temperatures are 14°C and 3°C, respectively. One-year old plants of five Olea europaea cultivars (Arbequina, Changlot Real, Frantoio, Hojiblanca and Manzanilla) were grown outdoors for one year in 20-dm^3 pots (one plant per pot) filled with a mixture of clay and sand soil and irrigated bi-weekly. Forty plants per cultivar were used in this study. Cultivars were selected according to its frost hardiness and resistance to low soil water availability (Fiorino et al. 2000; Bartolozzi et al. 2009). Changlot, Arbequina and Hojiblanca are considered frost and drought resistance and Manzanilla a frost sensitive cultivars. They are some of the most important olive cultivars in Argentina and can growth under harsh conditions in the patagonian desert; nevertheless there is no information neither on its resistance to low temperature or on its acclimation capacity.

Environmental variables

Relative humidity and air temperature were monitored continuously with HOBOs Pro series (Onset Computer Corporation, Pocasset MA, USA). Soil temperature was measured with thermocouples (Type T) installed at 7 cm depth in the 20dm^3 pots and connected to a CR10X

datalogger (Campbell Sci.). Soil volumetric water content was determined using ECH_2O probes (10 HS, Decagon devices, Inc.) installed at 5 cm soil depth.

Leaf pressure-volume curves

Pressure–volume (P–V) relationships were developed using the dehydration technique (Koide et al. 1989) to estimate bulk leaf water characteristics on a seasonal basis for all five cultivars. Measurements were performed on exposed and mature leaves of two year old plants. Five leaves per cultivar (five trees per cultivar) were sampled at predawn when leaf water potential was close to 0 MPa and transferred immediately to the laboratory in sealed plastic bags. The leaves were first weighted to obtain full turgor mass and immediately placed in a pressure chamber (PMS system, Corvallis) to obtain the initial leaf water potential. The procedure was repeated many times while the leaves were allowed to dehydrate slowly in the laboratory (20–25 $^{\circ}$C). Finally, leaves were dried in an oven at 60 $^{\circ}$C for 72 h and their dry mass were recorded. The tissue water relations parameters calculated from the curves were osmotic potential at full turgor, apoplastic water fraction (AWF), volumetric bulk modulus of elasticity (ε) and solute content (N_s) per dry mass. To obtain solute content per dry mass (mosmol g^{-1}), osmotic potential at full turgor was converted to osmolality by multiplying by 410 milliosmol MPa^{-1}. Osmolality was then multiplied by the symplastic water volume and divided by the dry mass of the sample (Tyree et al. 1978). Volumetric bulk modulus of elasticity (ε) was calculated over the full range of positive turgor as described by Evans et al. (1990):

$$\varepsilon = (\Delta\Psi P/\Delta RWC)^* FS$$

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

Leaf osmotic potential

Leaf osmotic potentials were determined on ten leaves per tree (n=4) and cultivar during summer (day with mean temperature of 25°C), winter (days with mean temperature of 8°C and -5°C) and spring (days with 18°C). Cell sap was extracted from samples collected every two hours during one day in each season and immediately submerged in liquid nitrogen and stored for analysis of osmotic potential. To obtain sap for osmotic potential determinations, leaf samples were thawed for one hour before pressing the tissue between plastic tubes using a vise. Then the sap sample was placed on a filter paper and introduced in the chamber of a vapor-pressure osmometer (Wescor 5600) to determine sap osmolatility. Sap osmolality was converted to osmotic potential using the Van't Hoff equation (Nobel 1991).

Leaf tissue density and leaf relative water content

The leaf tissue density was measured in 10 leaves per plant per cultivar. Leaf tissue density (δ) was determined by dividing the leaf dry mass by the leaf fresh volume of the sample as described in Scholz et al. (2007). Leaf relative water content was determined in 10 leaves per plant per cultivar at pre-dawn. The leaves were first weight to obtain the fresh mass (FM) and then immersed in distilled water for 24 h and weight to obtain the saturated mass (SM). Finally, leaves were dried in an oven at 60 $^{\circ}$C for 48 h and their dry mass were recorded. Leaf relative content was calculated as: (FM - DM)/(SM −DM) *100

Thermal analysis

Thermal analyses were conducted with different plant organs (roots, stems and leaves) in one year plants and in leaves of two years old plants to determine ice nucleation temperature (INT). One year old plants (n= 3 per cultivar) and sun-exposed mature leaf samples of two years old plants (n= 3 to 4) were collected in the early morning. Both whole plant and leaf samples were placed in a freezer and temperature were lowered at a rate of 5 °C h^{-1} from

ambient to -25°C. Tissue temperature (roots, stems and leaves) were monitored with copper-constantan thermocouples placed in close contact with the tissue by using small pieces of surgical tape. The thermocouples were connected to a datalogger (CR10X, Campbell Scientific, USA), and temperatures were recorded at 4-s intervals. The tissue ice nucleation temperatures (INT) were detected from the tissue temperature kinetics. A rapid increase in temperature indicated heat release from water during extracellular ice nucleation.

In the present study we decided not to inoculate the samples with ice to prevent supercooling in the leaves (that is to freeze the samples as close to the equilibrium freezing temperatures as possible), because this species do not tolerate ice formation. Ice inoculation would underestimate the freezing avoidance capacity as these plants resist freezing injury mainly through substantial supercooling, but not through tolerance to extracellular freezing. When we experimentally nucleated leaves with ice at 0°C we observed differences of about 2°C between INT of dry and inoculated leaves (higher INT in inoculated leaves). On the other hand, during days with air temperatures below -6 °C (data not shown) we did not observe neither wet leaves nor ice formation on the leaf surface, consequently experimentally ice seeding the plant material would not represent freezing under natural conditions. In addition the wetness sensor installed at the field (Dielectric Leaf Surface Wetness Sensor Decagon Devices) indicated lack of dew formation when air temperatures were below 0 °C.

Leaf tissue damage

The electrolyte leakage method was used to assess the influence of low temperatures on leaf tissue damage (Wilner 1960). Mature leaves from two years old plants and from the five studied cultivars were collected from the field in the early morning, kept in plastic bags with moist paper towels to prevent water loss (moist paper towels were not in contact with the samples, and then transported to the laboratory. Leaf samples were placed into sealed tubes (5

to 7 leaf discs from different plants per tube) and incubated in a freezer. The freezer was then cooled down at a rate of 5 ^{o}C h^{-1} to reach different target temperatures (5, 0, -2, -4,-6, -8,-10, -12, -14, -16,-18 and -20 ^{o}C). After maintaining the samples at a particular target temperature for 15 min (see Lipp et al. 1994), three tubes with leaf samples per cultivar were taken out from the freezer and thawed at 4 ^{o}C for 2 h and then 10 ml of deionized water was added to each tube. The solution with leaf samples were held at 4^{o}C for 24 h in a shaker. Electric conductivity (EC) of the solution was then measured with an electrical conductance/ resistance meter (Hanna HI 98311, Hanna instruments). After EC measurements, the tubes with the samples were moved into an autoclave to obtain the maximum ion leakage. Electric conductivity of the solution with leaf samples was measured again after 24 h of mixing and shaking. The relative EC, as an indicator of relative ion leakage, was calculated for each sample as:

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

The temperature at the 50% relative EC was defined as the lethal temperature of acclimated and non acclimated tissues (LT_{50}). Relative leakage is not the same as relative injury. Nevertheless, relative leakage can be used as measure of tissue injury. The difference between INT obtained by thermal analysis and LT_{50} was used to determine the freezing resistance mechanism of the study olive cultivars (tolerance or avoidance).

Leaf soluble carbohydrates and nitrogen content

Non-structural carbohydrate content per mass dry was determined by the anthrone method (Yemm & Willis 1954) and the absorbance was measured at 600nm in a spectrophotometer (Spectrum SP 1105).To determine nitrogen leaf content per dry mass, the Kjeldahl method was used (Miller & Miller 1948). For these measurements were used three acclimated and three non-acclimated leaves per tree (n=4) and cultivar

Data analysis

All physiological and morphological trait data were normally distributed (Kolmogorov–Smirnov test). Differences between cultivars in leaf density, ε, INT, LT_{50}, solute content, apoplastic water fraction and carbohydrate content were examined using one-way ANOVA and Tukey post-hoc test. Seasonal differences in the study variables within a cultivar were evaluated using Student test. The SPSS 11.5 statistical package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Linear regressions were fitted to LT_{50} against solute content, relative water content and apoplastic water fraction during winter and sigmoid curves were fitted to the relationship between relative EC and treatment temperature for all cultivars with Sigma Plot software (Systat Software Inc.).

Results

Environmental conditions

Air temperature varied seasonally in the study site. Mean monthly temperature ranged from 22 ^{o}C in January to 3.5°C in July. Absolute air minimum temperature was -6 ^{o}C in July and the absolute maximum temperature was 37 ^{o}C in January. During the experimental period, soil volumetric water content was maintained around 20 to 35 %, corresponding to a soil water potential around 0 MPa. The soil temperature at 7 cm depth was always above the air temperature and in the coldest day of the study there was a difference of about 4°C with air temperature; whereas lowest air temperature was -6 °C, the lowest soil temperature was -1.8°C. This soil temperature was the lowest recorded during the study period.

Ice nucleation across plant organs and plant age

Root, stem and leaf ice nucleation temperatures during winter, in one year old plants, varied across cultivars, and ice seeding (INT) occurred at temperatures substantially higher in the roots than in the stems and leaves (Table 1). Although there were no significant differences (except in Frantoio cultivar), INTs of stems were higher than those in leaves. Two year old plants of all cultivars had lower (more negative) leaf ice nucleation temperature (INT) than those in one year old plants ($p<0.05$), except in Frantoio cultivar (Table 1 and 2). For example, Arbequina cultivar had an INT difference of about 5 °C between one and two year old plants.

Seasonal and cultivar variations in INT and LT_{50}

Seasonal variations in water relations traits and leaf density were observed in all olive cultivars (Fig.1). Bulk elastic modulus (ε) tended to increase in winter (cell wall become more rigid) in all cultivars, but only Hojiblanca cultivar exhibited significant differences between seasons, with an increase of 5 MPa from summer to winter (Fig. 1 A). Leaf density increased significantly during the winter in all cultivars (Fig. 1 B). The apoplastic water fraction did not show seasonal differences in Manzanilla and Frantoio cultivars, while in the other three cultivars it was significantly lower in winter compared to summer (Fig.1 C).

Non-structural soluble carbohydrate content per dry mass in leaves was significantly higher in summer than in winter across all cultivars (Fig 2A). Arbequina was the cultivar with highest seasonal changes in non-structural carbohydrates (about 10 mg g^{-1} DLM). Leaf nitrogen content per dry mass on the other hand significantly increased from summer to winter in Arbequina, Changlot and Hojiblanca, and marginally increased in Manzanilla (Fig. 2B)

Leaf osmotic potentials varied significantly within each cultivar between summer and winter (Fig. 3), with higher values during typical winter conditions (8°C) and with very lower

values during a cold day in winter (-5°C) .While during typical summer conditions, osmotic potentials were lower than -2.5 MPa, in winter they were close to -1 MPa in all cultivars. Nevertheless when air temperature was -6 ºC throughout the day, leaf osmotic potentials exhibited an abrupt decrease, reaching values within the -4 to -5 MPa range in all cultivars.

Differences in leaf osmotically active solute content were observed across cultivars ($F=4,092$; $p<0.05$) with Hojiblanca and Arbequina cultivars showing the highest values (Fig. 4). There was a positive linear relationship between winter solute content per dry mass of leaf tissue and the magnitude of cultivar-specific seasonal changes in ε from winter to summer (Fig. 4A). Cultivars with higher solute content per dry mass exhibited higher seasonal changes in tissue elasticity. Apoplastic water fraction, which differed significantly among cold acclimated cultivars ($F=3.05$, $p<0.05$), was also correlated to solute content across cultivars with cultivars having lower apoplastic water fraction at higher solute content (Fig. 4B).

Thermal analysis of leaf tissues in all cultivars showed that there was only one event of freezing indicated by one exotherm due to the release of latent heat down to -20°C (data not shown). Seasonal changes in leaf INT were observed in two year old cultivars (Table 2). Ice nucleation temperatures from non-acclimated leaves (during summer) were about 6°C higher than INTs in winter acclimated leaves. The relationship between electrical conductivity (EC), as a measure of membrane damage, and the tissue temperature was well described by a sigmoid function for all cultivars, with low EC values at temperatures around 0 ^{o}C and with a rapid EC increase at a certain subzero threshold temperatures raising to a plateau at even lower temperatures, particularly for summer acclimated leaves (Fig. 5). Leaf temperatures at which 50% of membrane damage occurred (LT_{50}), varied significantly between seasons for all cultivars ($p<0.05$) and ranged from -2 to -5.8 ^{o}C during summer (non-acclimated leaves) and from -10.13 to -13.3 °C during winter (low temperature acclimated leaves) (Table 2; Fig. 5).

The LT_{50} differed significantly among cultivars during winter ($F=4.01$, $p<0.05$) and had a maximum difference of 2 ^{o}C, with Arbequina exhibiting the lowest LT_{50} and Manzanilla the highest LT_{50} (Table 2). Summer leaf INTs were negatively correlated with summer leaf LT_{50} values across cultivars ($R^2 = 0.879$, $y= - 2.68x – 20.97$, $p <0.05$), while in cold acclimated leaves, INT and LT_{50} were positively correlated (Table 2, Fig. 6). During summer, leaf LT_{50} values were higher than leaf INTs, however during winter ice nucleation and tissue damage temperature were similar across all cultivars. Ice nucleation temperatures for all cultivars started before than LT_{50}, but the differences in temperature were very small.

Solutes and water content in relation to leaf LT_{50} and INT across cultivars in winter

Osmotically active solute content was linear and negatively correlated to leaf LT_{50} and leaf INT across cold acclimated cultivars (Fig 7A and B). An inverse relationship was observed between apoplastic water fraction and leaf LT_{50} and between apoplastic water fraction and leaf INT (Fig 7C and D). Cold acclimated cultivars that exhibited higher solute content and lower water fraction in the apoplastic compartment experienced ice nucleation and leaf damage at lower temperatures than leaves of cultivars with lower solute content and higher apoplastic water fraction. Leaf relative water content was linear and positively correlated to INT and LT_{50} ($p<0.05$, data not showed).

Discussion

Variation in ice nucleation temperature between roots, stems and leaves

Thermal analysis on one year old plants indicated that ice nucleation temperature was 1 to 4 ºC higher in roots than in stems and leaves in all the study cultivars. This relatively high root INT may not be a limitation for pant functioning in the cold desert of Patagonia as soil temperatures in winter rarely drops below 0^{o}C despite low subzero air temperatures.

In this study INTs observed in different parts of one year old plants suggest that the order of cold resistance was leaves > shoots > roots. Although the INTs in stems were higher than in leaves, the differences were very small, except in Frantoio. Ice seeding started first in stems, probably in largest vessels, and then ice was immediately propagated to the leaves.

Freezing cell injury and supercooling in acclimated and non-acclimated plants and across cultivars

During winter leaves of all cultivars exhibited lower LT_{50} and ice nucleation temperatures compared to summer. The regression line between INT and LT_{50} in winter was, close to a one to one relationship between both variables suggests that cell membrane damage occurred shortly after ice nucleation. However, during summer the slope of this relationship was different from 1 and leaf damage occurred before INT as result of the lack of acclimation to low temperatures during the warm season. Our results indicate that cold acclimated cultivars developed substantial supercooling in leaf tissues.

During winter, cultivars with higher supercooling capacity (i.e. lower INT) had less apoplastic water fraction in their leaves. It is likely that acclimation to low temperature before winter decreases the amount of apoplastic water content in the leaves and thus enhances its supercooling capacity. High elevation giant rosette plants of the genus Espeletia have low apoplastic water content in the leaves and very low INT (about -15 °C) (Goldstein et al. 1985; Rada et al. 1985) while lower elevation members of the same genus exhibit substantial higher apoplastic water content in the leaves and higher INT, suggesting that a low amount of water in the intercellular spaces may delay ice seeding in the leaves enhancing supercooling capacity. Gusta et al. (2004) also showed that non-acclimated leaves of Brassica napus with higher water content did not supercool as much as acclimated leaves. In our study, Manzanilla and Frantoio cultivars had highest apoplastic water fractions among the study

cultivars and did not exhibit significant seasonal changes in this variable, and thus were the cultivars that less improved its supercooling capacity after acclimation and those with the highest INTs. Across cold acclimated olive cultivars, supercooling capacity increased with the decrease of apoplastic water fraction in leaf tissue compartments. The lower water content in winter compared to summer in three of the study cultivars was also reflected in the higher tissue density, which could contribute to higher tissue rigidity observed in cold acclimated cultivars. Higher cell wall rigidity (higher ε) allows the decrease in cell water potential with a small change in the cell water volume, and may help reduce the water content in their walls (Scarth & Levitt 1937). In addition, in tissues where cells are tightly packed and the walls are rigid and probably with low porosity, the intercellular spaces are small and retain low amount of water, which could alter the freezing processes, resulting in enhanced supercooling (Ashworth & Abeles 1984).

Scholz et al. (2012) found anegative correlation between the observed percentage of leaf damage at -20^{o}C and ε in shrub species of cold deserts that are tolerant to extracellular freezing, indicating that the elastic adjustment from summer to winter plays a key role in the ability to resist low temperatures. Olive cultivars do not tolerant extracellular freezing, as Patagonian shrubs do, but instead avoid ice formation by supercooling. Nevertheless elastic adjustment from summer to winter was also observed, in particular in Arbequina, Frantoio and Hojiblanca cultivars (ε increased 1.5 to 3.5 folds). We are assuming that changes in the modulus of elasticity have different roles in freezing tolerance and in freezing avoidance. While higher wall rigidity could confer greater mechanical resistance to physical pressure exerted by extracellular ice growth in tolerant species, higher cell wall rigidity could be associated to smaller micro-pores and more uniform cell surface resulting in a lower chance of ice nucleation. Enhanced pectin synthesis has been observed in the cell walls of other species during cold acclimation (Kubacka-Zebalska & Kacperska 1999; Baldwin et al. 2014)

358 which contributes to lower porosity of the cell wall, and possibly to increase ε. Also, changes
359 in phospholipids composition in plasmatic membrane contribute to tissue rigidity during cold
360 acclimatation (Larcher et al. 1982; Griffith & Brown, 1982; Gulen et al. 2009).
361 Olea europaea is an evergreen species that produces a large diversity of endogenous
362 carbohydrates such as sucrose, glucose, mannitol and rafinose (Reskjoba et al. 2005). It is
363 known that osmotically active solute content, such as sucrose and other simple molecules may
364 provide non-colligative protection to cell membranes (Strauss & Hauser 1986; Anchordoguy
365 et al. 1987, Lipp et al. 1994). On the other hand, higher solute content within the cell may
366 modify the pattern of distribution of water between apoplastic and symplastic compartments
367 enhancing influx of water into the symplasm. Consistent with this, we observed that olive
368 cultivars with higher solute content, NSC, and lower apoplastic water content (such as
369 Arbequina, Changlot Real and Hojiblanca) during winter had high supercooling capacity and
370 low LT_{50}. Nevertheless, leaf osmotic potentials and NSC observed during typical days of the
371 winter season indicated lower concentration of solutes respect to summer, probably due to
372 relatively low metabolic activity and CO_2 assimilation during winter. Despite this, large starch
373 reserves could be hydrolyzed to soluble sugars when temperatures drop substantially below
374 0°C (Rada et al. 1985). Consistent with findings of Rada et al (1985), leaf osmotic potentials
375 during winter days with temperatures close to -5°C were as low as -4 MPa, in all olive
376 cultivars, while during days with mean winter air temperature of 8°C the osmotic potentials
377 were substantially higher (around -1.5MPa), suggesting fast hydrolysis of starch at low
378 temperatures in Olive cultivars. This short-term carbohydrate dynamic (rapid conversion of
379 starch to osmotically active solutes) could lower the ice nucleation temperature by about 3°C
380 or 4°C (Rada et al. 1985). In line with these findings, Bartolozzi et al. (1999) also observed an
381 abrupt increase in sucrose in olive leaves after a short period with subzero temperatures.

In acclimated plants, in addition to osmotically active solutes, other cryoprotectant molecules can be synthesized, such as low-molecular weight nitrogenous compounds (e.g., proline, glycine betaine) and dehydrin proteins. Four of the olive cultivars studied exhibited an increase in nitrogen concentration during winter, which may be associated with amino acids and proteins that can help to stabilize both membrane phospholipids and proteins as well as cytoplasmic proteins (D'Angeli & Altamura, 2007; Eris et al. 2007; Janska et al. 2009, Fernandez-Escobar et al. 2011,Wisniewski et al. 2001).

In conclusion, acclimation to low temperatures modified leaf temperature at which freezing damage occurs in olive cultivars. During winter, supercooling was the mechanism exhibited in all study cultivars to avoid extracellular ice formation. In the Patagonian steppe, leaf temperatures may not reach the dew point and thus leaves can supercool below their equilibrium freezing temperatures. In this study, enhanced permanent supercooling was partially explained by chemical and structural leaf traits. An important finding of this study was that differences in supercooling capacity among cultivars were associated with differences in solute content (e.g. soluble carbohydrates and nitrogen), apoplastic water content and cell wall elastic adjustments. A novel role for cell wall rigidity is suggested for plants that avoid freezing damage by supercooling. A potential role for osmotic active solutes on the redistribution of apoplastic and simplastic water fractions in leaves was also hypothesized. Overall, the results suggest that all the olive cultivars studied are adapted to withstand the low subzero temperatures, typical of the largest cold desert in South America, by supercooling as irreversible damage in leaves was not observed under field conditions.

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565 **Table 1** Ice nucleation temperature (INT) of lateral roots, stems and leaves, and the difference
566 between leaf INT and stem INT in one year old plants. Values are means ± SE of three plants
567 per cultivar. Different letters within a cultivar indicate significant differences in INT between
568 plant parts.

Cultivar	Lateral Roots INT (°C)	Stem INT (°C)	Leaf INT (°C)	Stem INT – Leaf INT (°C)
Arbequina	-2.097±0.15**a**	- 6.08±0.02**b**	- 6.77±0.65**b**	0.69
Changlot Real	-3.31±0.25 **a**	- 6.78±0.48 **b**	- 7.06±0.03 **b**	0.28
Frantoio	-6.84±0.3 **a**	- 10.84±0.84 **b**	- 11.89±0.61 **c**	1.05
Hojiblanca	-3.23±0.08 **a**	- 8.1±0.03 **b**	- 8.4 ±0.7 **b**	0.3
Manzanilla	-4.8±0.45 **a**	- 5.77±0.51 **a**	- 6.07±0.07 **a**	0.3

Table 2. Summer and winter leaf ice nucleation temperature (INT; °C), leaf lethal temperature at which 50% of membrane leakage occurred ($LT_{50;}$ °C) and the difference between leaf INT and leaf LT_{50}. Values are means ± SE of three trees per cultivar. Different lower case letters indicate significant differences between leaf INT and leaf LT_{50} values within a cultivar and upper case letters indicate significant differences in INTs or LT_{5O} values for a cultivar between seasons.

Cultivar	Leaf INT (°C)	Leaf LT_{50} (°C)	Leaf INT – Leaf LT_{50} (°C)
Summer			
Arbequina	-6.5±0.35 a A	-3.8±0.3 b A	-2.7
Changlot Real	-6.43±0.46 a A	-3.9±0.25 b A	-2.53
Frantoio	-6.36±0.43 a A	-3.6±0.35 b A	-2.73
Hojiblanca	-5.83±0.68 a A	-5.8±0.07 a A	-0.03
Manzanilla	-7.19±0.45 a A	-2±0.45 b A	-5.19
Winter			
Arbequina	-11.86±0.65 a B	-13.08±0.65 a B	1.22
Changlot Real	-11.39±0.75 a B	-12.68±0.03 a B	1.29
Frantoio	-10.74±0.92 a B	-12.03±0.61 a B	1.29
Hojiblanca	-11.60±1.38 a B	-12.57±0.7 a B	0.97
Manzanilla	-10.13±0.41 a B	-11.25±0.07 a B	1.12

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570 **Legends of figures**

571

572 Figure 1. (A) Leaf tissue bulk elasticity modulus, (B) leaf density and (C) apoplastic water
573 fraction during summer (white bar) and winter (black bar) of five cultivars of Olea europaea.
574 Each bar represents the mean value + 1SE of four trees per cultivar and season. Significant
575 differences between seasons within each cultivar are indicated as: *<0.05, ** <0.01,
576 ***<0.001.

Figure 2. (A) Total non-structural carbohydrate content and (B) leaf nitrogen content of five olive (Olea europaea) cultivars during summer and winter. Each bar corresponds to the mean value + 1SE of three trees per cultivar and season. Significant differences between seasons within each cultivar are indicated as: *<0.05, ** <0.01, ***<0.001.

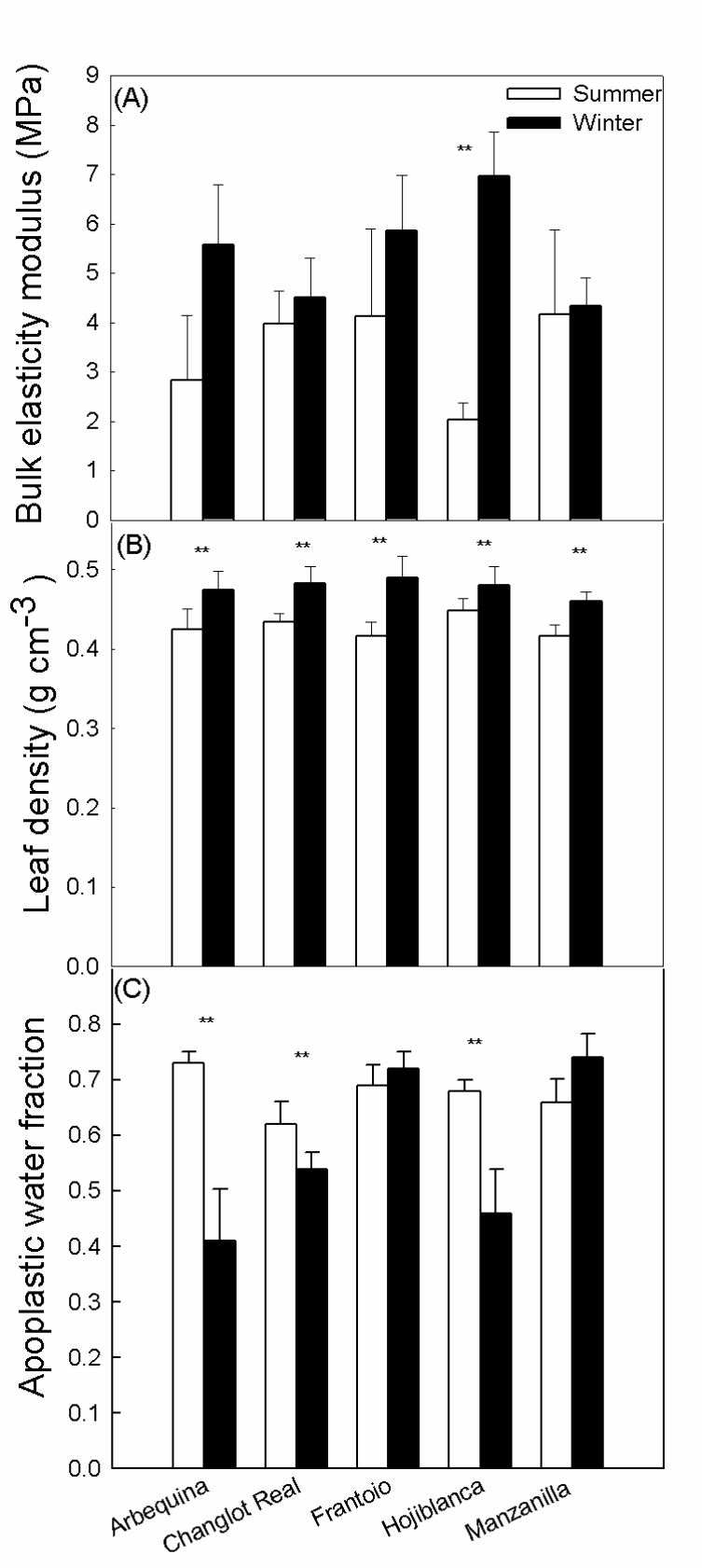
Figure 3. Leaf osmotic potential in five olive cultivars measured on days with mean temperatures of summer, winter and spring and during one winter day with an air temperature of -5 ºC. Bars are mean values -SE of 10 leaves per tree (n=4) and per cultivar. Different letters indicate significant differences between seasons within cultivar.

Figure 4. (A) Increase in leaf bulk elasticity modulus from summer to winter (e winter –e summer) and (B) winter apoplastic water fraction in relation to leaf osmotically active solute content in winter in five olive cultivars. Each symbol represents the mean value ± 1SE of four trees per cultivar. The solid lines indicate the linear regressions fitted to the data: (A) $y = 0.2021 – 0.044 x$; $p<0.05$, (B) $y = 0.95 – 1.35x$; $p<0.05$.

Figure 5. Relative electrical conductivity (%) (which indicates the relative percent of cell membrane leakage) for acclimated and non-acclimated olive leaves as a function of tissue temperature. Sigmoid functions were fitted to the data ($p < 0.001$) for all the regressions.

Figure 6. Leaf temperature at which 50% of membrane leakage occurred (LT_{50}) in relation to leaf ice nucleation temperature across five olive cultivars during winter. Each symbol represents the mean value ± 1SE of four trees per cultivar. The line is the linear regression fitted to the data ($R^2 = 0.963$, $y= 0.988x -1.311$, $p < 0.05$). The dash line is the one to one relationship between both variables.

Figure 7. Leaf osmotically active solute content and apoplastic water fraction in relation to (A, C) leaf temperature at which 50% of membrane leakage occurred (LT_{50}) and in relation to (B, D) leaf ice nucleation temperature, across five cold acclimated olive cultivars. Each symbol represents the mean value ± 1SE of three to four plants per cultivar. Solid lines indicate the linear regressions fitted to the data: (A) $y = -0.622+ - 0.08 x$; $p<0.05$; (B) $y = -1.21 + -0.13 x$; $p<0.01$; (C) $y = 0.622 + 0.08 x$; $p<0.05$, (D) $y = 2.81 +0.20 x$; $p<0.01$.



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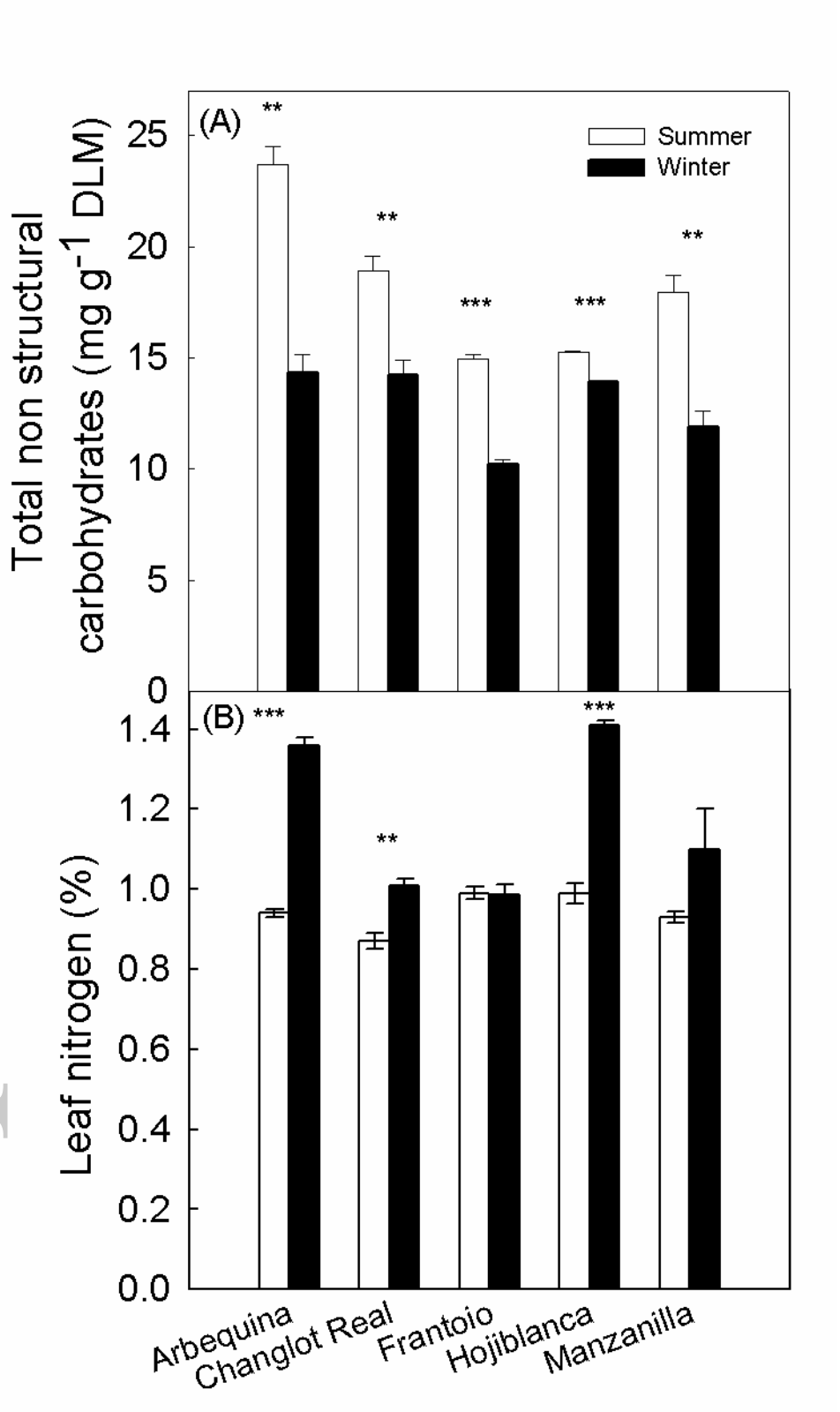

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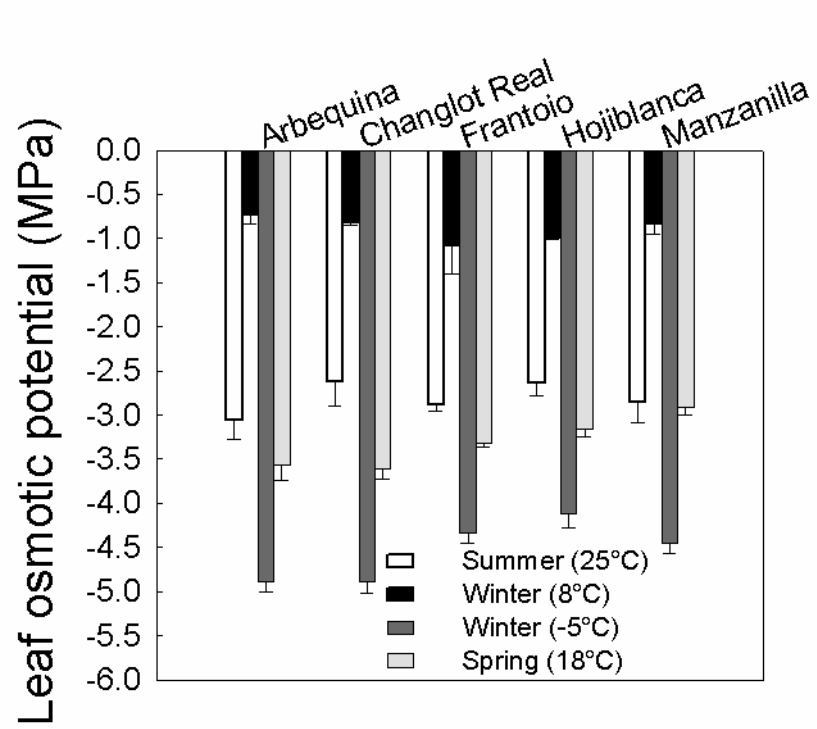

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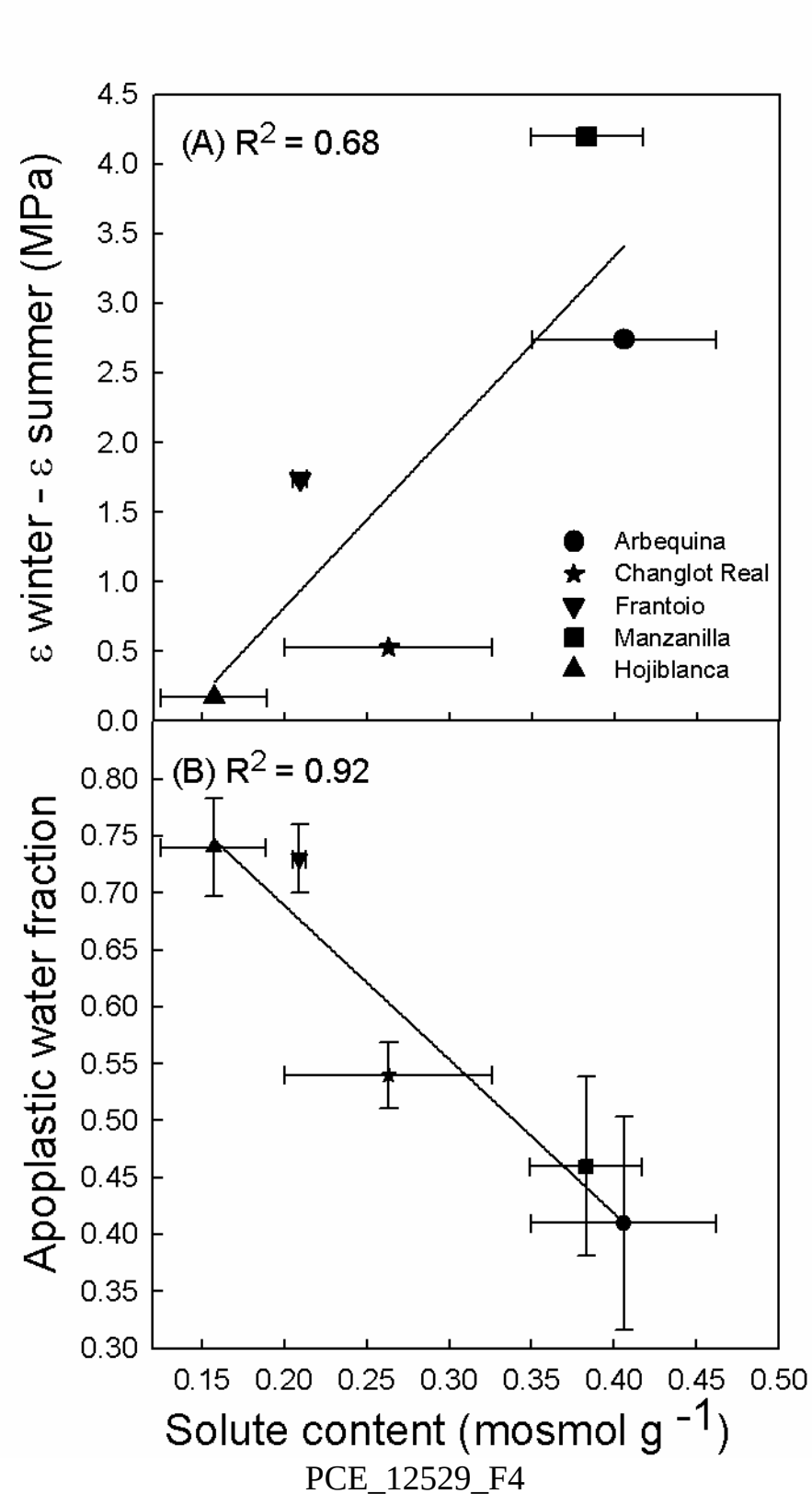

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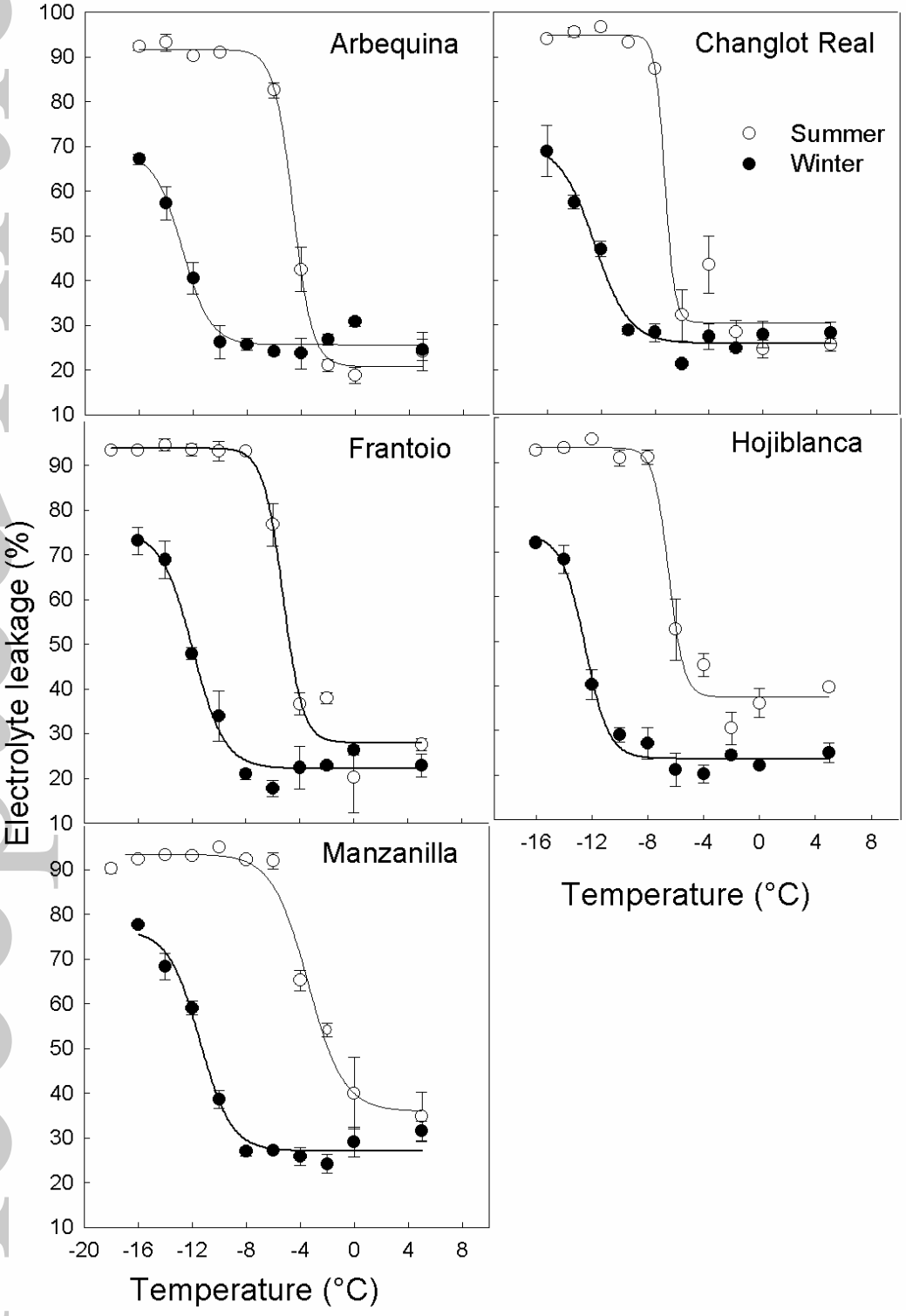

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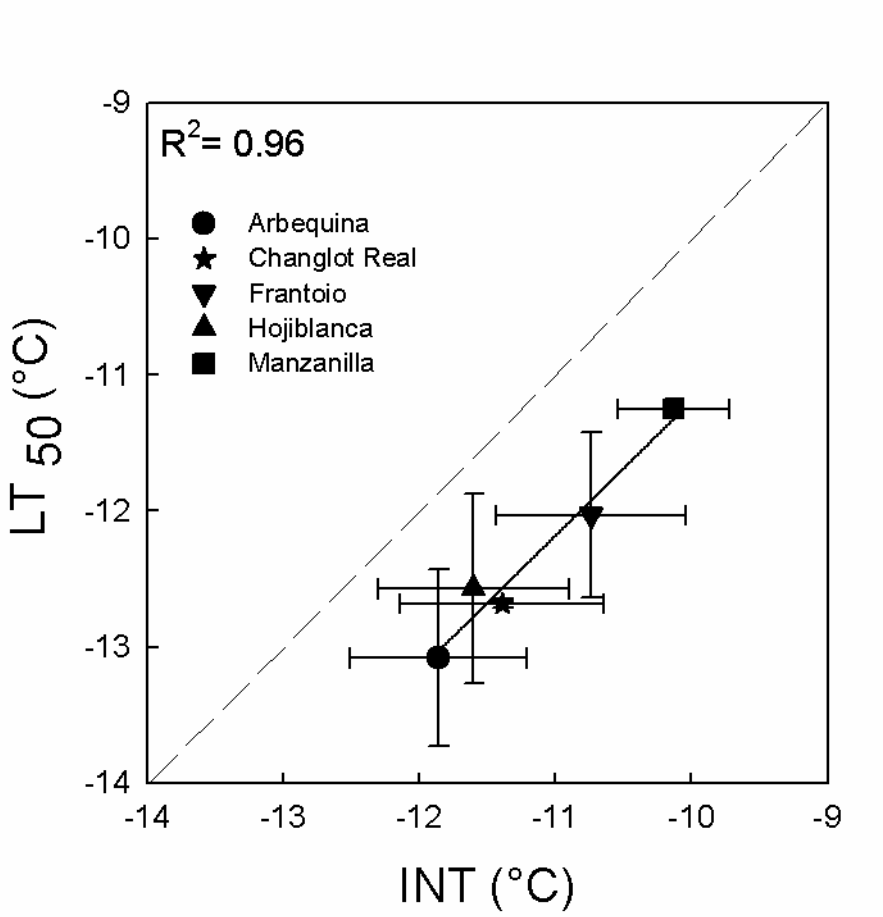

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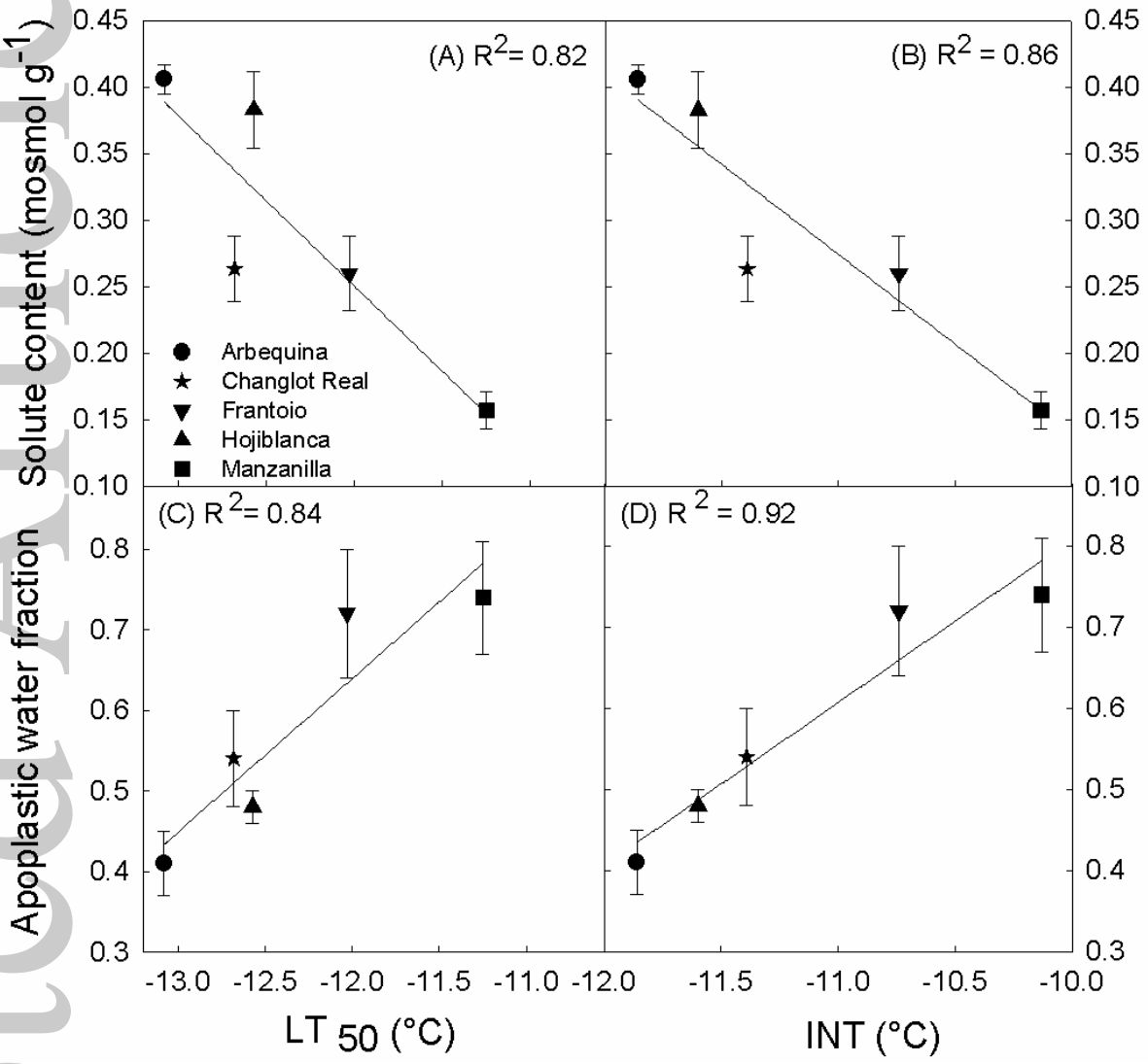

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