Trees (2009) 23:159–167
DOI 10.1007/s00468-008-0264-1

ORIGINAL PAPER

Comparing image (fractal analysis) and electrochemical (impedance spectroscopy and electrolyte leakage) techniques for the assessment of the freezing tolerance in olive

Elisa Azzarello · Sergio Mugnai · Camilla Pandolfi · Elisa Masi · Elettra Marone · Stefano Mancuso

Received: 7 January 2008 / Revised: 21 August 2008 / Accepted: 26 August 2008 / Published online: 12 September 2008

Abstract Olive growth and productivity are limited by low temperatures mainly during winter, but sometimes also in spring and fall. The most effective way to avoid these damages in areas subjected to these climatic conditions is to select least susceptible varieties, but the choice of the right method to determine cold hardiness is extremely difficult. The aims of the work were (1) to assess LT_{50} (lethal temperature at which 50% of damage in plants subjected to low temperatures occurs) of some olive varieties in two seasons (summer and winter) and (2) to assess the reliability of different methods to evaluate cold hardiness. LT_{50} was determined on 21 different olive (*Olea europaea* L.) Italian varieties by leaf and shoot electrolyte leakage, shoot impedance spectroscopy and leaf color determination of fractal spectrum. All the experiments were conducted on non-acclimated and cold-acclimated plants. Our results showed that all the three methods were able to detect damages on olive plants after exposure to low temperatures, with leaves appearing more sensitive to cold stress than shoots. Among these methods, fractal analysis could be very useful in assessing cold hardiness of plants on the basis of visible injury, without sophisticated or expensive instruments and in a reliable and cost-effective way, using only a scanning device, a personal computer and dedicated freeware software.

Keywords Cold hardiness · Fractal spectrum · Image analysis · *Olea europaea* L.

Introduction

Olive (*Olea europaea* L.) growth and productivity are limited by low temperatures mainly during winter, but sometimes also in spring and fall. These climatic conditions can frequently occur mainly in Italy and Spain, but also in other Mediterranean-climate zones of Europe, America and Australia (Denney et al. 1993). Although olive is moderately freezing tolerant, temperatures below a certain threshold (−7°C) can damage the plant (Palliotti and Bongi 1996), while at −12°C damage may be serious enough to threaten the life of the tree (Larcher 1970). The most effective way to avoid these damages in areas subjected to low temperatures is to select and use least susceptible varieties. A number of surveys and field trials provided tolerance data for several olive varieties (Fiorino and Mancuso 2000; Barranco et al. 2005), but most of these studies were based on isolated observations in areas with different cold intensity, which led to contradictory findings. Visual observation is often subjective and subordinate to other influential factor such as wind, air humidity, exposure, water status and health conditions of the plant, and they do not consider, for example, damages that can occur at the root level. For these reasons, the assessment of objective methods to evaluate cold hardiness of olive varieties becomes important to screen their possible cultivation in areas where low temperatures could negatively affect the production.

Communicated by J. Major.

E. Azzarello · S. Mugnai (✉) · C. Pandolfi · E. Masi · S. Mancuso
Department of Horticulture, University of Florence,
Viale delle Idee 30, 50019 Sesto Fiorentino (FI), Italy
e-mail: sergio.mugnai@unifi.it

E. Marone
Department of Food Science, University of Teramo,
P.zza Aldo Moro 45, 64100 Teramo, Italy

Unlike herbaceous plants, in which cold injury symptoms are visually discernible within hours or days, long-living evergreen woody plants like olive (Sutinen et al. 2001) exhibit a more complex symptomatology and may need weeks to express a visible injury. Several techniques [i.e. electrolyte leakage (EL) test, differential thermal analysis, chlorophyll fluorescence, measurement of the impedance] are currently used to assess damage caused by low temperatures. Unfortunately, the use of just one method is normally not sufficient (Palta et al. 1977; Dehayes and Williams 1989). Thus, the use of concurrent techniques in the assessment of cold hardiness can be justified despite the large labour investment it needs. Until now, lab methods developed to discriminate between freezing-tolerant and freezing-sensitive olive genotypes have been based on the measurement of electrical resistance by impedance spectroscopy (Mancuso 2000), by differential thermal analysis (Fiorino and Mancuso 2000), by vital stain (Fiorino and Mancuso 2000), by the release of phenolic compounds and leaf tissue browning (Roselli et al. 1989) and by EL (La Porta et al. 1994; Bartolozzi and Fontanazza 1999; Fiorino and Mancuso 2000). Such methods have not always been effective, and even if successful, they tend to be costly and/or laborious. Among the afore-cited methods, electrical impedance spectroscopy is considered a very fast and effective technique, since the samples can be measured immediately after a freeze-and-thaw cycle without a delay of days or weeks, but the need of expensive equipment and skilled staff makes the technique not as economic or reliable. Recently, fractal spectrum analysis technique has been developed by the authors to assess leaf cold hardiness in *Callistemon* and *Grevillea* (Mancuso et al. 2004), as fractal parameters decrease with low temperature damage (Mancuso et al. 2003). However, the use of the fractal analysis method and the comparison among this technique and others for the assessment of cold hardiness in olive has not been performed yet.

The main aim of this study was to screen some olive Italian varieties to evaluate the level of injury in cold-stressed plants by three different techniques: leaf EL and stem impedance spectroscopy, both based on the concept that injured cells are unable to maintain the chemical composition of their contents and release electrolytes through damaged membranes, and leaf fractal analysis, which quantify changes in the colour patterns on damaged leaf surfaces. On the basis of the results, a classification of the olive varieties was performed, grouping them into three clusters: *hardy*, *semi-hardy* and *non-hardy*. Finally, a brief comparison among the aforementioned methods was performed in terms of reliability and effectiveness.

Materials and methods

Plant material and treatments

The experimental site was located at Montepaldi Farm (inland Tuscany, Italy, 43°40′N, 11°09′E, 266 m a.s.l.), where twenty-one different olive (*Olea europaea* L.) varieties originated from different Italian regions were grown: ‘Ascolana Tenera’ and ‘Carbona’ from Marche (42°51′N, 13°34′E); ‘Bologna 2’ from Emilia Romagna (44°30′N, 11°21′E); ‘Campeglio’, ‘Diana’, ‘Frantoio’, ‘Leccino’, ‘Maurino’, ‘Moraiolo’, ‘Pendolino’, ‘Selezione clonale 06 (SC06)’, ‘Selezione clonale 07 (SC07)’, ‘Selezione clonale 08 (SC08)’ and ‘Urano’ from Tuscany (43°47′N, 11°15′E); ‘Coratina’ from Apulia (41°07′N, 16°52′E); ‘Parco Polcenigo 1 (PP1)’, ‘Parco Polcenigo 2 (PP2)’, ‘Rocca Bernarda’ and ‘Zamarian San Rocco (ZSR)’ from Friuli Venezia Giulia (46°04′N 13°14′E); ‘Vescovo’ from Garda Lake (45°28′N, 10°32′E). More, a cold-tolerant variety from Croatia (45°48′N, 15°58′E), ‘Simjaca’, was tested. All the experiments were conducted on leaves and shoots picked from 10-year-old plants during June on non-acclimated plants and during January on cold-acclimated plants. Both non-acclimated and acclimated samples were packed in polyethylene bags and subjected to a temperature-controlled cold treatment in an air-cooled chamber by a stepwise multiple-temperature regime (eleven different test temperatures 0, −2, −4, −6, −8, −10, −12, −15, −18, −20 and −24°C). In detail, temperature into the chamber started from 24°C and decreased by a rate of 2°C h^{-1} until reaching the predetermined test temperature, which was maintained for 4 h to establish a thermodynamic equilibrium. Recovery was performed by rising the temperature at the same rate (2°C h^{-1}) until reaching again the temperature of 24°C. After 4 h of recovery, samples were removed from the chamber and the physiological response was evaluated.

Electrochemical (electrolyte leakage and impedance spectroscopy) techniques

Five replicates per variety were randomly selected for their uniformity of appearance, growth habit and exposure. At each collecting season, samples from current-year shoots with similar length, diameter and internode length and from mature leaves were removed from the third node starting from the top of the plants, and brought into a laboratory 4 h before starting cold treatments. The EL was determined at each test temperature following the technique already described by Fiorino and Mancuso (2000) on olive leave (10–15 cm^2) and shoot (15 mm long and 2–4 mm in diameter) samples. The electrical conductivity of distilled water (20 ml) in which a leaf disc (10 mm of diameter) was incubated for 24 h at room temperature was measured using a digital conductivity meter (GLP 31, Crison, Spain). After that, the same disc was subjected to a freeze–thaw cycle, performed at −30°C for 4 h followed by a 4 h period at room temperature. EL was calculated as indicated by Eq. 1:

$$\mathrm{EL}\ (\%) = \frac{C_1}{C_2} \times 100 \qquad (1)$$

where C_1 is the electrical conductivity of the solution at room temperature and C_2 is the electrical conductivity of the solution after a freeze–thaw cycle.

The procedure for the shoot electrical resistance measurement by impedance spectroscopy was previously described in detail by Mancuso et al. (2004). Briefly, alternating current was applied to a portion of a shoot (10 mm long and 2–3 mm in diameter), and the impedance spectra were measured by an impedance meter (1920 Precision LCR Meter, Quadtech) connected to two Ag/AgCl electrodes placed in contact with the samples. The device was calibrated using an OPEN/SHORT circuit correction to eliminate the polarisation impedance of the electrode/paste interface. The absolute impedance value and the phase angle were then measured within a frequency range from 100 Hz to 1 MHz with an interval of 20 KHz at 14 frequency points. In this study, the impact of temperature injury was estimated as the change in impedance ratio (low/high frequency) before and after the treatment (Eq. 2):

$$\mathrm{DZ}_{\mathrm{ratio}} = \left(Z_{\mathrm{low}}/Z_{\mathrm{high}}\right)_{\mathrm{after}} - \left(Z_{\mathrm{low}}/Z_{\mathrm{high}}\right)_{\mathrm{before}} \qquad (2)$$

where $\mathrm{DZ}_{\mathrm{ratio}}$ is the change in the impedance ratio to cold temperatures, $(Z_{\mathrm{low}}/Z_{\mathrm{high}})_{\mathrm{after}}$ the impedance ratio at 1 and 20 kHz after cold treatments and $(Z_{\mathrm{low}}/Z_{\mathrm{high}})_{\mathrm{before}}$ the impedance ratio at 1 and 20 kHz before cold treatments.

Fractal analysis

From each variety, 20 fully expanded and healthy leaves were selected from both cold-acclimated and non-acclimated plants before exposure assessment and then subjected to cold treatments. Leaves were examined 2 days after the treatments to determine the effect of low temperatures on their fractal spectra. During this period, leaves were kept in dark. Digital images from collected leaves were obtained by an optical scanner (CanoScan D660U, 300 × 300 d.p.i., 16 million colours) and fractal parameters were determined by a fractal image analysis software (HarFA, Harmonic and Fractal Image Analyzer 4.9.1). In brief, each leaf image (16 million colours) was split in its three constituting colour channels (blue, green and red). Each channel was threshold for a colour intensity value between 0 and 255, and the fractal dimension (*D*) for each colour intensity value was then calculated. In fractal geometry, the fractal dimension is a statistical quantity that gives an indication of how completely a fractal appears to fill space, as one zooms down to finer and finer scales. In our case, *D* was assessed using the box-counting method (Mancuso et al. 2003; Pandolfi et al. 2006) and plotted against the colour intensity to obtain the fractal spectra of the three channels. A characteristic example of the spectra of the three colour channels (blue, green and red) obtained from each leaf analyzed is represented in Fig. 1. As blue channel seemed relatively unaffected to low temperatures, only green and red channel results were selected as 'informative' of the low temperatures colour modification of the leaves (Mancuso et al. 2004). Five fractal parameters (First *X*, *X* and *Y* coordinates of the peak, Last *X* and total peak area) univocally described each colour channel after drawing the baseline for the value of $D = 1$ that separates the fractal (>1) from the non-fractal (<1) zone of the spectrum (Fig. 2). The values of all the fractal parameters were sensitive to cold and were calculated for red and green colour channel at each tested temperature.

Cold hardiness assessment

Cold hardiness was expressed as LT_{50} (lethal temperature at which 50% of damage occurs) by fitting each response curve obtained by the three methods (EL, fractal parameters and electrical impedance) with the logistic sigmoid function (Eq. 3):

$$y = \frac{a}{1 + e^{b(x-c)}} + d \qquad (3)$$

where *x* is the treatment temperature; *b* is the slope at the inflection point; *c*, *a* and *d* determine the asymptotes of the

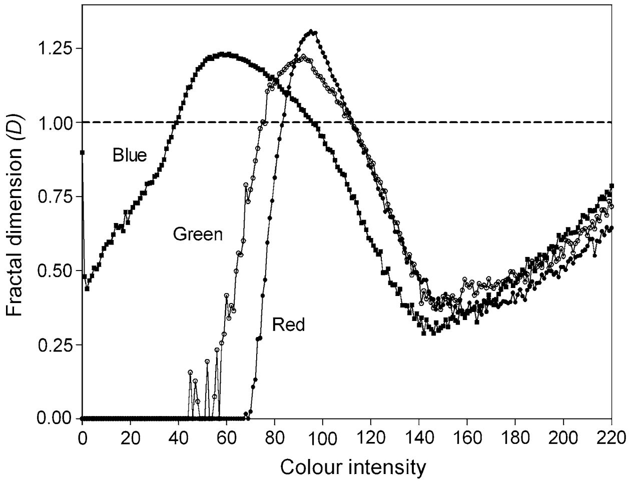

Fig. 1 Typical pattern of the fractal spectra for the *blue*, *green* and *red* colour channels determined on a single leaf. The *line* drawn at fractal dimension $D = 1$ separates the fractal (>1) from the non-fractal (<1) zone of the spectrum

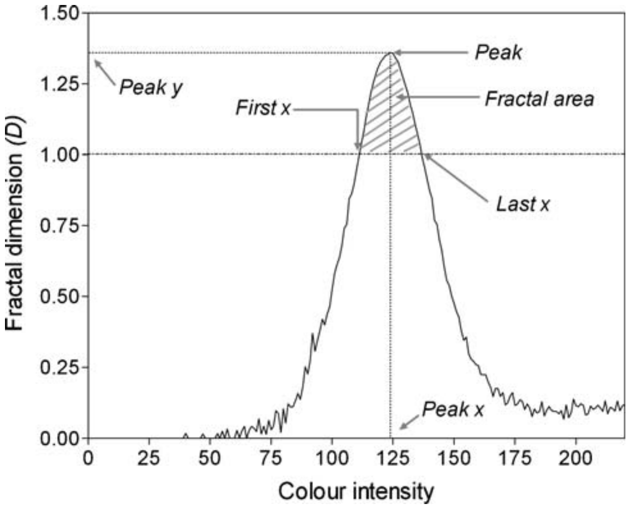

Fig. 2 Graphical representation of the five fractal parameters calculated from each colour channel: *First X*, *X* and *Y* coordinates of the peak, *Last X* and Total peak area (modified from Mancuso et al. 2003)

function. The best fit was determined by least squares (Ingram and Buchanan 1984). The temperature corresponding to the inflection point of the regression curve, which shows a 50% change compared with both the control and the totally damaged sample, was considered as the cold hardiness and represents the LT_{50} value.

Statistical analysis

LT_{50} data obtained by the three different methods were subjected to one-way ANOVA and means were separated by Duncan's multiple range test ($P < 0.05$, $n = 5$). The one-way ANOVA model used was (Eq. 4)

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij} \quad (4)$$

where μ is the general effect, α_i is the effect of the ith treatment and ε_{ij} are the errors. It is assumed that $\sum_{i=1}^{I} \alpha_i = 0$ and the ε_{ij} are normally and independently distributed $(0, \sigma^2)$. Statistical analysis was performed using GraphPad Prism ver. 5.0 (GraphPad software, San Diego, USA). For each organ (stem, impedance spectroscopy and EL; leaves, fractal analysis and EL), results were plotted and compared on a *XY*-graph, and a linear regression (95% confidence interval) was performed. To provide a conventional partitioning of the selected 21 accessions into three distinct and disjunct clusters, a non-hierarchical classification (the classical partitioning procedure of the *k*-means method, which minimises the sum of squares within classes) was used (Podani 2000), using SYN-TAX 2000 (Exeter software, Setauket, USA). The number of clusters (*k*) was chosen to comply with our main objective to divide the tested accessions into three groups of freezing tolerance: *hardy*, *semi-hardy* and *non-hardy*.

Results

Electrolyte leakage

Electrolyte leakage values and low temperatures were related by a sigmoid curve, which showed a clear increase in leave and shoot damage in relation to temperature decrease (Fig. 3). Cold-acclimated plants had greater freezing tolerance than non-acclimated plants (Table 1). As a general rule, shoots had lower LT_{50} temperatures when compared to leaves. Varieties with the lowest LT_{50}s were 'Rocca Bernarda' (−16.4°C in cold-acclimated shoots and −14.9°C in cold-acclimated leaves) and 'PP2' (−16.1 and −14.4°C, respectively), while 'Coratina' was the most sensitive with LT_{50} temperatures of −11.8°C in cold-acclimated shoots and −8.1°C in cold-acclimated leaves. As an example, the effect of low temperatures on EL in acclimated leaves and shoots collected from a tolerant ('PP2') and a sensitive variety ('Coratina') is reported in Fig. 3.

Impedance spectroscopy

Varietals LT_{50}s estimated by impedance spectroscopy showed a wide variation (Table 2). As was previously

Fig. 3 Effect of low temperatures on the electrolyte leakage (%) of leaves (*left*) and shoots (*right*) in acclimated plants of 'Coratina' and 'Parco Polcenigo 2 (PP2)'. Data are reported as means ± SD ($n = 5$)

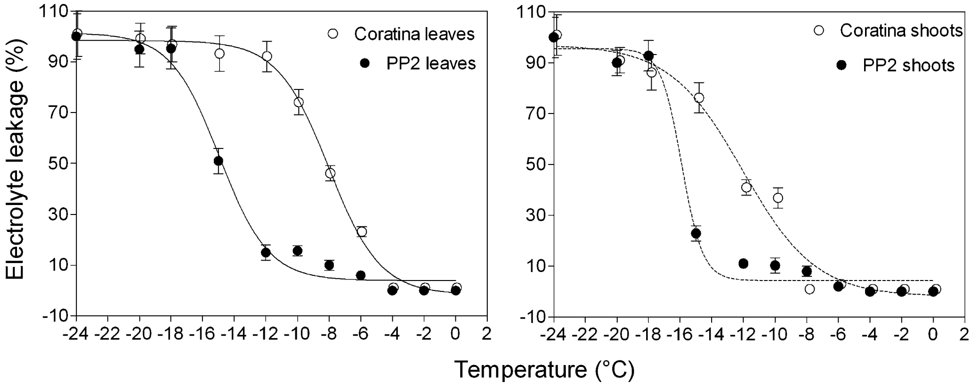

Table 1 LT_{50} (°C) estimated by electrolyte leakage in leaves and shoots on both non-acclimated and cold-acclimated plants

Varieties	Region of origin	Electrolyte leakage			
		Leaves		Shoots	
		LT_{50} non-acclimated	LT_{50} acclimated	LT_{50} non-acclimated	LT_{50} acclimated
Coratina	Apulia	−4.1 e	−8.1 e	−5.7 d	−11.8 c
Campeglio	Tuscany	−5.0 de	−10.1 d	−9.6 a	−12.1 c
Pendolino	Tuscany	−5.6 bcd	−9.9 de	−7.5 bc	−12.5 c
Vescovo	Garda lake	−5.3 cd	−11.5 bcd	−6.0 cd	−12.9 c
Zamarian San Rocco	Friuli Venezia Giulia	−5.6 bcd	−10.9 cd	−8.9 a	−12.9 c
Urano	Tuscany	−5.6 bcd	−11.8 bcd	−7.2 bc	−13.0 c
S.C. 07	Tuscany	−5.8 bcd	−11.3 bcd	−7.8 b	−13.8 bc
S.C. 08	Tuscany	−4.5 e	−11.0 cd	−7.0 c	−13.9 bc
S.C. 06	Tuscany	−5.7 bcd	−11.3 bcd	−6.8 c	−14.0 bc
Carbona	Marche	−5.6 bcd	−10.7 cd	−7.4 bc	−14.3 bc
Ascolana Tenera	Marche	–	−13.0 bc	–	−14.7 b
Maurino	Tuscany	−7.1 a	−12.9 abc	−8.4 b	−14.7 b
Moraiolo	Tuscany	−6.7 a	−13.1 ab	−8.4 b	−14.8 b
Frantoio	Tuscany	−5.4 cd	−13.5 b	−7.4 bc	−15.1 b
Diana	Tuscany	−6.0 bcd	−13.8 ab	−7.6 bc	−15.2 ab
Leccino	Tuscany	−7.3 a	−14.8 a	−7.7 bc	−15.5 ab
Simjaca	Croatia	−7.4 a	−15.1 a	−8.3 b	−15.9 a
Parco Polcenigo 2	Friuli Venezia Giulia	−6.7 a	−14.4 a	−9.4 a	−16.1 a
Bologna 2	Emilia Romagna	−6.3 bc	−12.8 bc	−8.0 b	−16.2 a
Parco Polcenigo 1	Friuli Venezia Giulia	−6.7 a	−14.8 a	−8.3 b	−16.2 a
Rocca Bernarda	Friuli Venezia Giulia	−6.7 ab	−14.9 a	−7.8 b	−16.4 a
Overall mean		−5.9	−12.4	−7.8	−14.4

Data are reported as means, and sorted from the least to the most tolerant variety following LT_{50} shoots values in acclimated plants. Different letters inside each column indicate a statistically significant difference when means were separated by Duncan's multiple range test ($P < 0.05$; $n = 5$)

shown using EL, 'Coratina' had the highest LT_{50} (−9.2°C) among the tested varieties, whereas 'PP1', 'PP2', 'Rocca Bernarda' and 'Bologna 2' had the greatest freezing tolerance (−13.9 to −13.7°C). The response of shoot impedance spectroscopy parameters to cold temperatures in the tolerant ('PP2') and sensitive ('Coratina') varieties was obtained by plotting DZ_{ratio} versus the test temperatures (Fig. 4).

Fractal spectrum

Low temperatures induced a marked decrease in fractal parameters values. As red and green channels showed similar trends, they have been averaged in a new spectrum, which was related to the test temperatures. As previously shown for EL and impedance spectroscopy, fractal parameters and low temperatures were related by a sigmoid curve (Fig. 5). Mean values of varietals LT_{50}s estimated by fractal analysis are reported in Tables 3 and 4. 'Coratina' confirmed to be the most sensitive species to cold temperatures, showing LT_{50} temperatures of −7.9°C (acclimated plants) and −5.82°C (non-acclimated plants). On the other hand, acclimated plants of 'Leccino' and 'Simjaca' showed the lowest LT_{50} values (−13.2 and −13.5°C, respectively).

Comparing methods

Varietals LT_{50} values calculated by EL method were compared to those obtained by the other two methods on the same organ (fractal analysis measured on leaves and impedance spectroscopy on shoots, Fig. 6), showing linear and highly significant relationships ($r^2 = 0.98$ and 0.95, respectively, for impedance spectroscopy and fractal analysis). Three clusters have been created based on the tolerance to cold temperatures of the selected varieties: *hardy*, *semi-hardy* and *non-hardy*. A high number of olive varieties were placed in the same cluster in spite of the different techniques used for the assessment of freezing tolerance, thanks to the good correspondence between EL

Table 2 LT_{50} (°C) estimated by impedance spectroscopy on shoots of non-acclimated and cold-acclimated plants

Varieties	Region of origin	Impedance spectroscopy	
		LT_{50} non-acclimated	LT_{50} acclimated
Coratina	Apulia	−8.0 bc	−9.2 e
Campeglio	Tuscany	−9.2 b	−9.5 e
Zamarian San Rocco	Friuli Venezia Giulia	−7.4 c	−9.7 e
Pendolino	Tuscany	−8.8 b	−10.0 de
Vescovo	Garda lake	−6.7 c	−10.1 de
Urano	Tuscany	−6.0 de	−10.2 d
S.C. 07	Tuscany	−8.4 bc	−10.5 cd
S.C. 08	Tuscany	−6.4 de	−11.0 cd
S.C. 06	Tuscany	−4.9 e	−11.1 c
Moraiolo	Tuscany	−9.4 ab	−11.3 c
Carbona	Marche	−5.8 de	−11.4 c
Diana	Tuscany	−5.3 d	−11.4 c
Ascolana Tenera	Marche	–	−11.7 bc
Maurino	Tuscany	−8.2 bc	−11.9 bc
Simjaca	Croatia	−7.0 cd	−12.5 bc
Frantoio	Tuscany	−8.1 bc	−12.9 b
Leccino	Tuscany	−6.5 d	−13.1 ab
Rocca Bernarda	Friuli Venezia Giulia	−6.0 de	−13.7 a
Parco Polcenigo 2	Friuli Venezia Giulia	−10.4 a	−13.7 a
Bologna 2	Emilia Romagna	−5.9 de	−13.9 a
Parco Polcenigo 1	Friuli Venezia Giulia	−8.3 bc	−13.9 a
Overall mean		−7.3	−11.6

Data are reported as means, and sorted from the least to the most tolerant variety following LT_{50} values in acclimated plants. Different letters inside each column indicate a statistically significant difference when means were separated by Duncan's multiple range test ($P < 0.05$; $n = 5$)

and the other two methods. All the three methods considered 'Coratina', 'Campeglio' and 'Pendolino' as the most sensitive varieties, whereas the *hardy* cluster comprised 'PP1', 'PP2' and 'Rocca Bernarda'. Moreover, 'Frantoio' could be considered as a borderline variety, as it seemed to be a tolerant variety when fractal analysis was performed, but is a medium tolerant variety when using shoot impedance spectroscopy method.

Discussion

Our results clearly indicated that (1) all the tested cultivars were more vulnerable to cold injury before acclimation to low temperatures and that (2) leaves were more sensitive to cold treatments than shoots, showing less negative LT_{50} values by all the tested varieties. The different physiological responses with or without acclimation can be basically attributed to the ability of a variety to modulate the physical state of its membranes to temperature changes (Orvar et al. 2000), which is indirectly appreciated by EL and impedance spectroscopy. Most of the LT_{50} values calculated on non-acclimated plants were about at least 5–6°C higher when compared to acclimated ones, but some cases of poor acclimation were reported ('Campeglio' and 'ZSR') with a difference in LT_{50} values of around 2–3°C. The difference between LT_{50} values in non-acclimated and acclimated plants can widely vary depending on the species. In olive, Bartolozzi et al. (2001) reported a difference around 3.5°C in LT_{50} values between non-acclimated and acclimated plants in a cold-sensitive variety. This is probably due to the fact that cold-sensitive olive cultivars, such as 'Campeglio' and 'ZSR', are unable to acquire a true acclimation because they are unable to block the increase in cytosolic [Ca^{2+}] (D'Angeli et al. 2003), which is considered an early biochemical marker for cold resistance in olive trees. Instead, the acclimation process is effective in other olive cultivars and correlated with a partial (*semi-hardy*) or total (*hardy*) block of cytosolic [Ca^{2+}] changes. In other woody species, such as *Betula pendula* L. (Li et al. 2002) and *Rubus idaeus* L. (Palonen and Junttila 1999), the difference among LT_{50} values in non-acclimated and cold-acclimated plants increased, ranging between 10 and 15°C depending on the cold acclimation treatments.

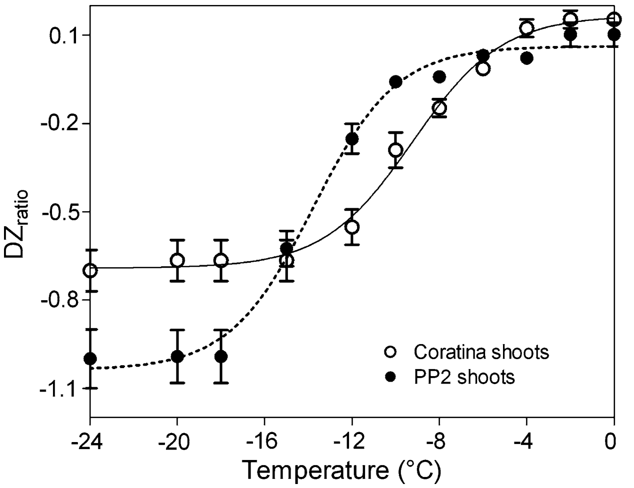

Fig. 4 Effect of low temperatures on DZ_{ratio} of shoots in acclimated plants of 'Coratina' and 'Parco Polcenigo 2 (PP2)'. Data are reported as means ± SD ($n = 5$)

Significant differences in freezing tolerance among olive varieties were observed by other authors (Bartolozzi et al. 2002; Barranco et al. 2005). Leaves are usually more sensitive to cold than shoots are, as observed by Denney

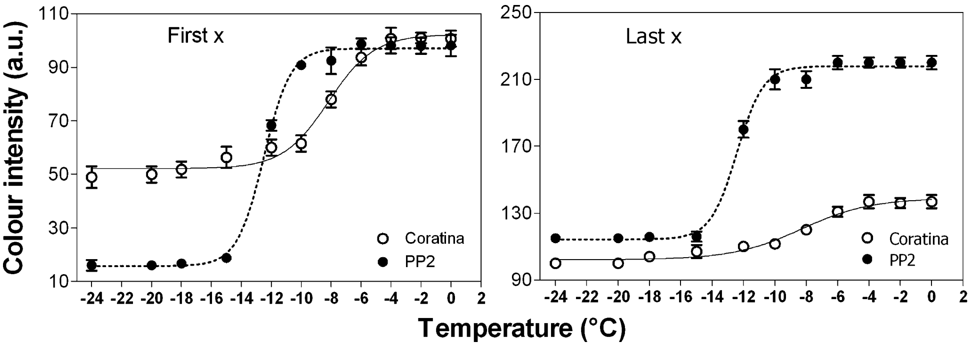

Fig. 5 Assessment of cold hardiness by two fractal parameters (*First X*, *left*; *Last X*, *right*) calculated from the fractal spectrum obtained by averaging the red and green channels in acclimated mature leaves of 'Coratina' and 'Parco Polcenigo 2 (PP2)'. Data are reported as means ± SD ($n = 5$)

Table 3 LT_{50} (°C) estimated by fractal analysis on leaves of non acclimated and cold acclimated plants

Varieties	Region of origin	Fractal analysis	
		LT_{50} non-acclimated	LT_{50} acclimated
Coratina	Apulia	−5.8 d	−7.9 d
Campeglio	Tuscany	−7.2 b	−8.5 d
Pendolino	Tuscany	−7.2 bc	−9.5 cd
Zamarian San Rocco	Friuli Venezia Giulia	−7.3 bc	−9.7 c
Carbona	Marche	−6.6 cd	−9.8 c
S.C. 07	Tuscany	−7.4 bc	−10.1 c
S.C. 08	Tuscany	−6.3 d	−10.3 bc
Urano	Tuscany	−6.2 d	−10.3 bc
Vescovo	Garda lake	−6.1 d	−10.3 bc
S.C. 06	Tuscany	−6.2 d	−10.4 bc
Moraiolo	Tuscany	−7.8 bc	−10.8 b
Bologna 2	Emilia Romagna	−8.0 ab	−11.1 b
Maurino	Tuscany	−8.7 a	−11.5 b
Ascolana Tenera	Marche	–	−11.7 b
Diana	Tuscany	−6.0 d	−12.0 b
Frantoio	Tuscany	−7.2 bc	−12.1 b
Parco Polcenigo 1	Friuli Venezia Giulia	−7.5 b	−12.5 ab
Parco Polcenigo 2	Friuli Venezia Giulia	−6.8 bcd	−12.5 ab
Rocca Bernarda	Friuli Venezia Giulia	−7.0 bcd	−12.9 ab
Leccino	Tuscany	−7.9 ab	−13.2 a
Simjaca	Croatia	−7.4 bc	−13.5 a
Overall mean		−7.0	−11.0

Results were obtained as an average of both green and red channels. Data are reported' as means, and sorted from the least to the most tolerant variety following LT_{50} values in acclimated plants. Different letters inside each column indicate a statistically significant difference when means were separated by Duncan's multiple range test ($P < 0.05$; $n = 5$)

et al. (1993), Fiorino and Mancuso (2000) and Mancuso (2000). Antognozzi et al. (1994) found that the LT_{50} of olive leaves collected from different varieties reached −12°C, while that of shoots reached −18°C. The higher sensitivity of leaves may be due to their greater exposure to cold resulting in a more rapid heat loss (Denney et al. 1993), even if other factors should be involved in olive-freezing tolerance. For example, Bongi and Long (1987) observed a light-dependent reduction in photosynthetic efficiency with increased chilling stress.

The classification of olive varieties as cold tolerant or cold sensitive is still under debate, and the investigation of olive-freezing tolerance should also consider the response of the same variety in different environments. As this kind of classification is widely based on the response of olive varieties to chilling temperatures (0–4°C, Fiorino and Mancuso 2000) instead of freezing temperatures (below 0°C), equivocals about genotypes' response can occur. For example, different results have been previously obtained in 'Frantoio', which showed a high cold tolerance (Pezzarossa 1985), a low cold tolerance (Gómez del Campo and Barranco 2005) or an intermediate cold tolerance (Bartolozzi and Fontanazza 1999). These differences are probably due to the fact that these experiments were carried out at different cold temperatures and/or with a different plant acclimation before the experiment. Another reason could be that 'Frantoio' is a generic term widely used to describe a large number of different olive genotypes (Barranco et al. 2005).

Our tested varieties were split into three clusters according to their freezing resistance in shoots and leaves, and grouped into '*hardy*', '*semi-hardy*' and '*non-hardy*' clusters. In spite of different LT_{50} values for the same variety obtained by the three different methods, each variety showed the same response to low temperatures compared to the others (Fig. 6). Results obtained by shoot analysis indicated that 'Bologna 2', 'Frantoio', 'Leccino', 'PP1' 'PP2', 'Rocca Bernarda' and 'Simjaca' could be classified as *hardy*; 'Ascolana', 'Carbona' together with 'Diana', 'Maurino', 'Moraiolo', 'SC06', 'SC07' and 'SC08 as *semi-hardy*. Finally, 'Campeglio', 'Coratina', 'Pendolino', 'Urano', 'Vescovo' and 'ZSR' were classified as *non-hardy*. It is interesting to note that 'Coratina' always showed the highest LT_{50} using all the three different

Table 4 ANOVA table for the methods of LT_{50} determination, showing the main effects of both plant organ and cold acclimation treatment

	Source of variation	MS	*df*	*P*
EL				
Leaves non-acclimated	Between groups	4.037	19	<0.001
	Within groups	0.2127	80	
	Total		99	
Leaves acclimated	Between groups	18.55	20	<0.01
	Within groups	0.2067	84	
	Total		104	
Shoots non-acclimated	Between groups	10.296	19	<0.01
	Within groups	0.122	80	
	Total		99	
Shoots acclimated	Between groups	4.828	20	<0.001
	Within groups	0.1275	84	
	Total		104	
IS				
Shoots non-acclimated	Between groups	11.39	19	<0.001
	Within groups	0.4339	80	
	Total		99	
Shoots acclimated	Between groups	11.67	20	<0.001
	Within groups	0.4468	84	
	Total		104	
FA				
Leaves non-acclimated	Between groups	4.394	19	<0.05
	Within groups	2.421	80	
	Total		99	
Leaves acclimated	Between groups	11.50	20	<0.001
	Within groups	0.3142	84	
	Total		104	

EL electrolyte leakage, *IS* impedance spectroscopy, *FA* fractal analysis

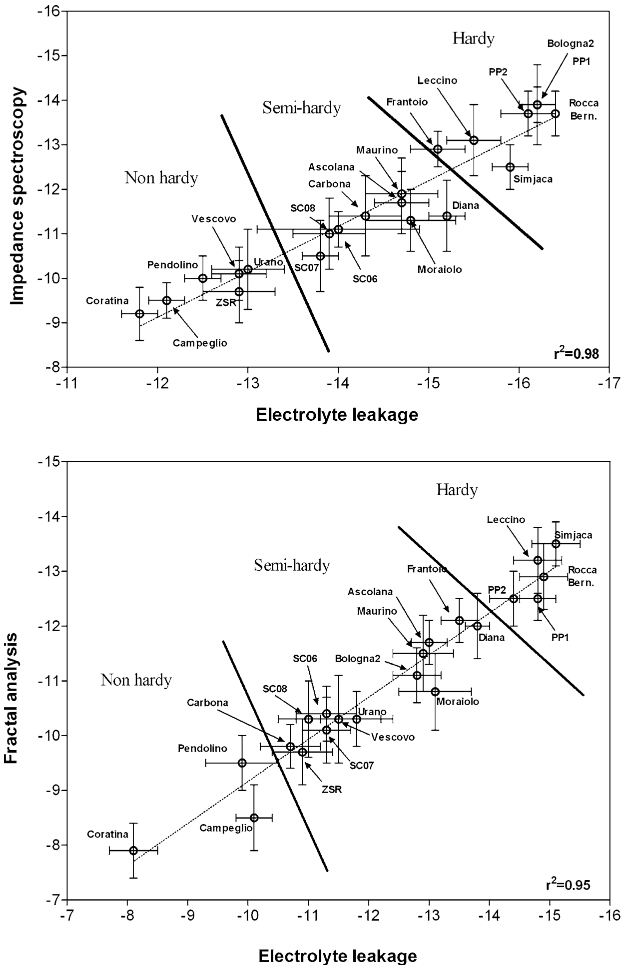

Fig. 6 Partitioning of the olive varieties into three clusters related to their freezing tolerance after comparison between electrolyte leakage and the other two methods in acclimated samples of shoots (impedance spectroscopy) and leaves (fractal analysis). Clusters refer to *hardy*, *semi-hardy* and *non-hardy* varieties. Data are reported as means ± SD ($n = 5$)

techniques. In fact, 'Coratina' is widely considered as a cold-sensitive variety (Mancuso 2000). This should be linked to its southern origin (Apulia, Italy) and to its cultivation in areas with higher average temperatures (Mancuso and Azzarello 2002). The assessment of freezing tolerance using olive leaves led to a very similar classification to that indicated by shoots, although some differences can be appreciated. For example, 'Bologna 2' could be considered as *semi-hardy* instead of *hardy* and 'Urano', 'Vescovo' and 'ZSR' as *semi-hardy* instead of *non-hardy*. This is probably due to the different responses and sensitivities of leaf and shoot tissues to low temperatures. In fact, according to Bartolozzi et al. (2002), the response of olive isolated tissues and cells to low temperatures may often differ from the whole plant system.

Our results confirmed that all the three methods were effective in detecting plant damage after exposure to cold stress, with a good match between the traditional method (EL) and the innovative ones (impedance spectroscopy and fractal analysis, Fig. 6). EL and impedance spectroscopy are two methods to measure the in vivo plant tissues conditions (Burr et al. 2000; Repo et al. 2000), and both are considered as fast tests and adequate in detecting freezing tolerance in tree species (Tsarouhas et al. 2000), but some considerations should be made regarding sample acclimation. Results obtained by using EL in acclimated olive plants indicated that LT_{50} values were significantly lower ($P < 0.05$) than those obtained by using both impedance spectroscopy and fractal analysis, contrary to other authors who detected injury at higher temperatures by EL than electrical impedance (Palta et al. 1977, on *Allium cepa*; Boorse et al. 1998, on *Rhus* spp. and *Ceanothus* spp.). Moreover, non-acclimated leaf samples showed higher LT_{50} values when determined by EL than those detected by fractal analysis. This should be related to a different sensitivity of EL applied on leaves compared to shoots, showing a wider range and higher values for the summer readings. In this case, the use of a more sensitive measure such as LT_{10} (Linden 2002) should be recommended.

In agreement with Mancuso et al. (2003), fractal analysis could be very useful in assessing cold hardiness of plants on the basis of visible injury, without sophisticated or expensive instruments and in a reliable and cost-effective way, using only a scanning device, a personal computer and dedicated freeware software. Although EL and impedance spectroscopy appeared to be effective, their use for the assessment of the cold hardiness is time consuming, also requiring expensive equipment and skilled staff. On the contrary, fractal analysis is a simple, objective and rapid method, also considering the cost of labour for collecting and scanning leaves plus processing the obtained information.

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