

# Differential thermal analysis, supercooling and cell viability in organs of *Olea europaea* at subzero temperatures

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*Key words:* chilling tolerance, electrolyte leakage, freezing temperature, *Olea europaea*, supercooling, visual score, vital stain.

**Abstract:** Cold hardiness in different organs and tissues of four different olive (*Olea europaea* L.) cultivars characterised in a previous work as different in frost tolerance, was assessed by four different methods (differential thermal analysis, visual score, vital stain and electrolyte leakage) to determine which method is more reliable for estimating freezing injury in olive. Results obtained with the different techniques consistently agreed. Among the four different procedures utilised, DTA was by far the fastest, whereas the visual score method the simplest, although not as quantitative as the other three methods. It would appear from the present study that the order of sensitivity in the different organs of olive is secondary roots > primary roots > apical leaves > basal leaves > shoots > vegetative buds. 'Ascolana' was the most chilling-tolerant variety, whereas 'Coratina' the most chilling-sensitive. The wide range of tolerance showed by olive (i.e. from -11.2 to -15.3°C for the leaves) together with the recurrent danger of frost in many areas of cultivation, made this species an ideal candidate to breeding for low-temperature-tolerant plants.

## 1. Introduction

Temperature extremes are one of the most important factors limiting plant distribution and productivity. For olive trees, low temperatures are more limiting than are high temperatures in both ecological and agronomic contexts. In particular, olive cultivars acclimated to high temperatures maintain 70-80% of their photosynthetic rate at 40°C (Bongi *et al.*, 1987) whereas the low temperature tolerance of most cultivars is not particularly noteworthy, as they generally succumb by -12°C (Mancuso, 2000).

Olive plants exposed to low temperatures can survive either by avoiding ice formation in their tissues (supercooling ability) or by developing a tolerance for it (frost hardening).

Frost hardening is a genetically-controlled trait which is driven by three key environmental factors: temperature, photoperiod and water stress (Tumanov and Krasavtsev, 1959; Christersson, 1978; Levitt 1980). According to the prevalent theory, certain specific genes are activated as a result of changes in these factors. These changes induce a metabolic hardening mechanism, which results in an increase in frost hardi-

ness in autumn and a decrease in spring (Weiser, 1970; Repo, 1992).

Experiments with different tree species and provenances have shown that the changes in frost hardiness at various stages during annual development are genetically determined, as well as the minimum hardiness level during the growing season (Sakai and Eiga, 1985; Sakai and Larcher, 1987; Toivonen *et al.*, 1991).

Many woody angiosperm species survive winter temperatures by deep supercooling tissue solution to temperatures as low as the homogeneous nucleation temperature of the aqueous solution (near -40°C for plant solution). The freezing of this fraction of water can be observed as an exotherm during cooling at a constant rate by differential thermal analysis (Ishikawa, 1984; Mancuso, 2000). Supercooling as a cold hardiness mechanism has been demonstrated in various temperate plant tissues such as xylem ray parenchyma, leaf buds and flower buds (George *et al.*, 1974; Sakai, 1979; Hong and Sucoff, 1980). In other words, supercooling is usually employed by limited tissues (xylem, buds, seeds). Compared with the majority of plants, olive seems rather particular as will be shown in the present paper that most of its organs, from leaf to roots, use supercooling as a mechanism of cold hardiness.

## 2. Materials and Methods

### *Plant material and growth conditions*

One chilling-tolerant ('Ascolana'), two chilling-sensitive ('Coratina' and 'Frantoio'), and one intermediate variety ('Leccino') of three-year-old olive trees, grown in 3-l pots containing a 50:50 (v/v) mixture of sandy gravel and peat, were used. Plants were grown in a field located in Pescia, Italy (43°54'N, 10°41'E, 30 m asl) and were brought into a laboratory 3 hr before the experiment. Samples of current year shoots of similar length, diameter and internode length, leaves removed from the third node from the top, vegetative buds, woody and non-woody roots, were used for the experiments. All the experiments were conducted in winter on cold acclimated plants.

### *Differential thermal analysis (DTA)*

Excised plant tissues were placed on one side of a thermopile plate (Peltier, Melcor CP 1.4-17-10L, USA) and a "dried sample of tissue" on the other used as a reference to detect differential temperature changes between the two samples during the freezing (Mancuso, 2000). A copper-constantan microthermocouple (0.2 mm diameter) was attached to the thermopile plate near the tissue fragment to measure ambient temperature and the whole plate was covered on both sides with a 2 mm thick layer of closed cell polystyrene tied to the plates with Parafilm to decrease loss of heat to the surroundings. The samples were then placed in a freezing cabinet and cooled at the rates of 5°C hr<sup>-1</sup>, usually down to -30°C. It had been ascertained in preliminary experiments that *Olea europaea* tissues did not produce exotherms between -28 and -50°C. Initially temperature was kept at 20°C, as in the laboratory. Three different plant samples were tested concurrently. The signals from the thermopiles were low-pass filtered, amplified and connected via a multichannel A-D convertor card (Lab-PC-1200 National Instrument, USA) to a P133 personal computer. Fresh tissues were used for each different rate of freezing.

### *Determination of the lethal freeze temperature*

Cold hardiness of leaves was estimated by measuring the conductivity of the leachate of leaf discs and by visual rating of injury after controlled freezing treatments carried out in an air cooled chamber. The initial and final temperature was 20°C, the rate of cooling and warming 7°C hr<sup>-1</sup> and the minimum temperature was maintained for 4 hr. About 50 leaf discs of 1 cm in diameter for each test temperature were set in a polyethylene bag. Thirty discs were incubated in 10 ml of distilled H<sub>2</sub>O at 26°C for 6 hr to measure the conductivity of the leachate. The remaining 20 discs were incubated for one week at room temperature for determination of injury performed by image analysis (Scion Image). Cold hardiness of shoots, leaf buds and roots were also measured by cooling samples at 5°C decre-

ments per 4 hr and storing them at the desired temperatures for one day. Injury was rated by image analysis after incubation at room temperature and by measuring the conductivity of the leachate as described above if necessary.

The relative electrolyte leakage (REL) was calculated as:

$$REL = [(L_1/L_2) \times 100]$$

where  $L_1$  is the first conductivity measurement, representing the ion leakage caused by freezing damage, also including background leakage, and  $L_2$  is the second conductivity measurement performed on heat-killed samples representing the total electrolyte content of the tissue.

Cold hardiness was expressed as LT<sub>50</sub> (lethal temperature at which 50% of the ion leakage occurs; or, in the case of visual score, the lethal temperature at which 50% of the tissues are damaged) by fitting the response curves with the following logistic sigmoid function:

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

where  $x$  = treatment temperature,  $b$  = slope at inflection point  $c$ ,  $a$  and  $d$  determine the asymptotes of the function.

### *Vital stain*

Five cross sections, two to three cells thick, were cut from near the middle of the leaf in deionised water using a new razor blade. The sections were taken at each temperature and placed for 20 min in 0.02% (by weight) neutral red [3-amino-7-dimethylamino-2-methylphenazine (HCl)]. Samples were stored at 5°C for 24 hr to maximise stain uptake (Didden-Zopf and Nobel, 1982). For each treatment temperature, about 100 cells were examined microscopically at a magnification of 200x in each of the five tissue samples, leading to a total of about 500 cells examined under each condition. Stain uptake occurs only for living cells and is detected by the intracellular localisation of the red dye.

## 3. Results and Discussion

### *Cold hardiness of Olea europaea*

Table 1 summarises cold hardiness (LT<sub>50</sub>) of various organs of *Olea europaea*, measured visually, by DTA or by electrolyte leakage test. The values are higher for the chilling-sensitive cultivars (Coratina and Frantoio) in comparison with chilling-tolerant 'Ascolana'. Moreover, such differences remain in all organs studied (with the exception of roots which, from this point of view, are the least meaningful) giving very similar LT<sub>50</sub> values among the different cultivars.

According to previous tests on the cold hardiness of

Table 1 - Frost hardness of olive trees estimated by relative electrolyte leakage (REL), by visual score (VS) and by differential thermal analysis (DTA), at the end of January in cold acclimated plants of four different cultivar

	'Ascolana'	'Leccino'	'Frantoio'	'Coratina'
<b>Leaves</b>				
REL	-15.3 ± 0.2 (n = 12) d	-13.4 ± 0.3 (n = 12) c	-12.6 ± 0.3 (n = 12) bc	-11.2 ± 0.4 (n = 12) a
VS	-14.2 ± 0.3 (n = 12) b	-12.0 ± 0.6 (n = 12) a	-11.5 ± 0.7 (n = 12) a	-11.2 ± 0.8 (n = 12) a
DTA	-14.5 ± 0.2 (n = 12) c	-12.9 ± 0.2 (n = 12) ab	-12.3 ± 0.2 (n = 12) ab	-11.8 ± 0.2 (n = 12) a
<b>Shoots</b>				
REL	-17.5 ± 0.3 (n = 10) d	-14.9 ± 0.4 (n = 11) c	-13.5 ± 0.4 (n = 12) bc	-11.9 ± 0.5 (n = 12) a
VS	--	--	--	--
DTA	-18.6 ± 0.6 (n = 10) c	-15.2 ± 0.5 (n = 11) b	-13.2 ± 0.5 (n = 12) a	-12.6 ± 0.4 (n = 12) a
<b>Buds</b>				
REL	-18.6 ± 0.4 (n = 9) d	-16.9 ± 0.3 (n = 10) bc	-17.8 ± 0.4 (n = 10) cd	-13.2 ± 0.4 (n = 10) a
VS	--	--	--	--
DTA	-19.3 ± 0.6 (n = 9) d	-16.2 ± 0.4 (n = 10) bc	-17.3 ± 0.5 (n = 10) c	-13.5 ± 0.4 (n = 10) a
<b>Roots</b>				
REL	-8.9 ± 0.4 (n = 12) a	-9.2 ± 0.3 (n = 12) a	-8.6 ± 0.4 (n = 12) a	-8.3 ± 0.4 (n = 12) a
VS	-10.6 ± 0.5 (n = 12) a	-10.3 ± 0.6 (n = 12) a	-10.5 ± 0.8 (n = 12) a	-10.1 ± 0.5 (n = 12) a
DTA	-9.1 ± 0.3 (n = 12) a	-8.8 ± 0.3 (n = 12) a	-9.0 ± 0.3 (n = 12) a	-8.6 ± 0.3 (n = 12) a

Rate of cooling is 5°C hr<sup>-1</sup>. The results are reported as means ± SEM.

olive made by electrical resistance measurements (Mancuso, 2000), we found that different organs in olive show different cold hardness. It would appear from the present study that the order of sensitivity is secondary roots > primary roots > apical leaves > basal leaves > shoots > vegetative buds.

Generally, electrolyte leakage test and image analysis determination are in good agreement for leaves and shoots (Fig. 1), whereas for roots electrolyte leakage test predicts a 1-2°C higher LT<sub>50</sub> for all the cultivars tested (Table 1).

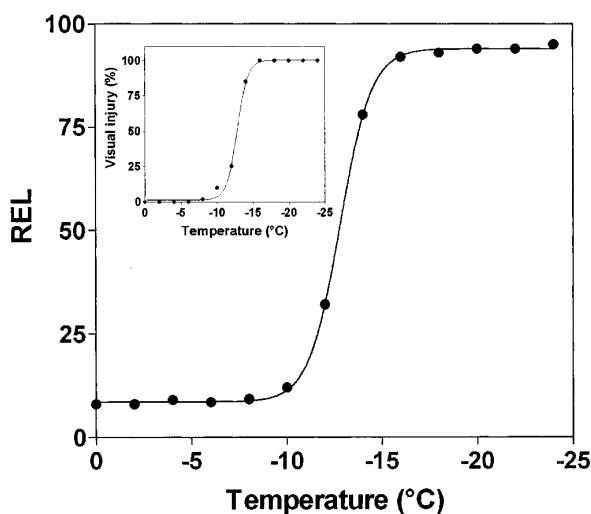


Fig. 1 - Assessment of cold hardness by electrolyte leakage method in *Olea europaea* leaf cooled at 5°C hr<sup>-1</sup>. The inset shows the visual rate of injury of *Olea europaea* leaf cooled at the same condition as above. The lines show symmetric non-linear regression curves for the shoots.

As the air temperature was lowered from 5°C at -20°C, the percentage of mesophyll cells of the leaves taking up the vital stain neutral red decreased, and again the pattern varied with the cultivar (Table 2). For instance, for a 2 hr treatment at -10°C, 86% of cells exhibited stain uptake for leaves from 'Ascolana' plants whereas in 'Frantoio' and 'Coratina' leaves the percentage of vital cells accounted for an average of 73% and 68%, respectively. Stain uptake following a 2

hr treatment at 0°C was indistinguishable from that of the control at 25°C, whereas no cells took up stain at -25°C in all the cultivars tested, indicating lack of survival at this low temperature.

Table 2 - Stain uptake at various subzero temperatures for mesophyll cells of four cultivars of *Olea europaea*

Cultivar	Stain uptake (% of control at 0°C)						
	0°C	-5°C	-10°C	-12°C	-14°C	-16°C	-20°C
'Ascolana'	100	100	86	82	79	18	0
'Leccino'	100	100	76	66	51	12	0
'Frantoio'	100	100	73	53	35	0	0
'Coratina'	100	100	68	51	36	0	0

Data are expressed as a percentage of the fraction of cells accumulating neutral red when treated at 25°C for 2 hr (control). Rate of cooling is 5 °C hr<sup>-1</sup>.

#### Differential thermal analysis

Typical DTA profiles of *Olea europaea* leaf are shown in figure 2a. When the leaf was moistened

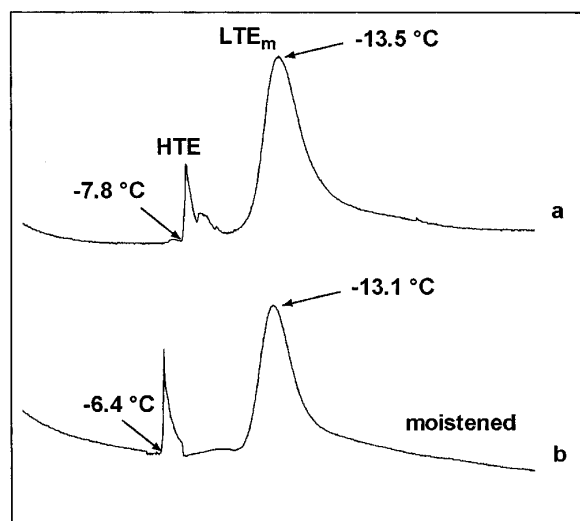


Fig. 2 - DTA profiles of *Olea europaea* leaf cooled at the rate of 5°C hr<sup>-1</sup> under different conditions. (a) Leaf without moistening (b) Leaf moistened with a little water on the surface prior to DTA. HTE is the High Temperature Exotherm, LTE<sub>m</sub> indicates the temperature at which 50% of the Low Temperature Exotherm (LTE) was produced.

about 2 hr before DTA, the HTE (High Temperature Exotherm) shifted to a slightly higher temperature and became larger (Fig. 2b). HTE was not related to injury of the leaf and might arise from the freezing of the water in the intercellular space. On the contrary, LTE (Low Temperature Exotherm) was not affected by moistening and seems to be closely related to injury in leaf.

All leaves showed sharp exotherms, however excised leaves of all four cultivars froze at exotherm temperatures significantly lower than leaves from intact plants (1.5-3°C lower). Ashworth and Davis (1984) and Andrew *et al.*, (1986) found in peach and cherry trees that there was a logarithmic increase in freezing temperature with an increase in specimen size. Ashworth (1990) suggests that this persistent pattern of increasing freezing temperature with increasing specimen size may result from a greater probability that large specimens have more ice nucleation sites. Nevertheless, we found this true only if portions of leaves were used, while no differences were found using intact leaves of different size (data not shown). Consequently, we directed our attention to the related possibility that the water in the petioles or in the xylem is more vulnerable to freezing than the water in the leaf tissue. To test the existence of differential ice formation in the different tissues, exotherm temperatures were recorded concurrently in leaf, petiole and shoot of the same branch cooled at rate of temperature changes of 5°C hr<sup>-1</sup>. We found that freezing was initiated in petioles 2-4 s earlier than leaves of the same branch (Fig. 3). This initial freezing of petiole water may then rapidly propagate to leaves which freeze at a higher temperature than when they were excised from the shoot. Our results are consistent with findings for a variety of fruit trees that plant organs (flowers, fruits), after detachment, freeze at a lower temperature than equivalent organs that remain a part of intact plants (Proebsting *et al.*, 1982; Andrew *et al.*, 1986).

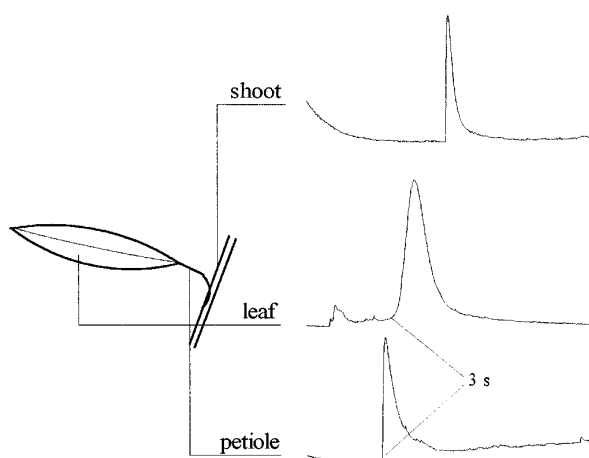


Fig. 3 - Representative result of a concurrent DTA in leaf, petiole and shoots of *Olea europaea* showing that freezing was initiated in petioles 3 s earlier than leaves of the same branch. This initial freezing of petiole water may then rapidly propagate to leaves.

HTE, which can indicate freezing of vascular and intercellular water, was not detected (Fig. 4) in shoots, roots and vegetative buds in agreement with previous findings (Mancuso, 2000). This was probably because free water accounted for a minimum of total water. The LTE<sub>m</sub> revealed by differential thermal analysis fit very well with the lethal temperatures (LT<sub>50</sub>) calculated from the electrolyte leakage measurements, thus confirming the presence of supercooling in leaves (Larcher, 1970) and other organs (Mancuso, 2000) of olive.

Woody plants frequently possess various freezing strategies ranging from extracellular freezing to extraorgans freezing and supercooling depending on tissues (Levitt, 1980). Thus, the occurrence in olive of a mechanism of “freezing avoiding” by supercooling generalised in most tissues is an exception worthy of study.

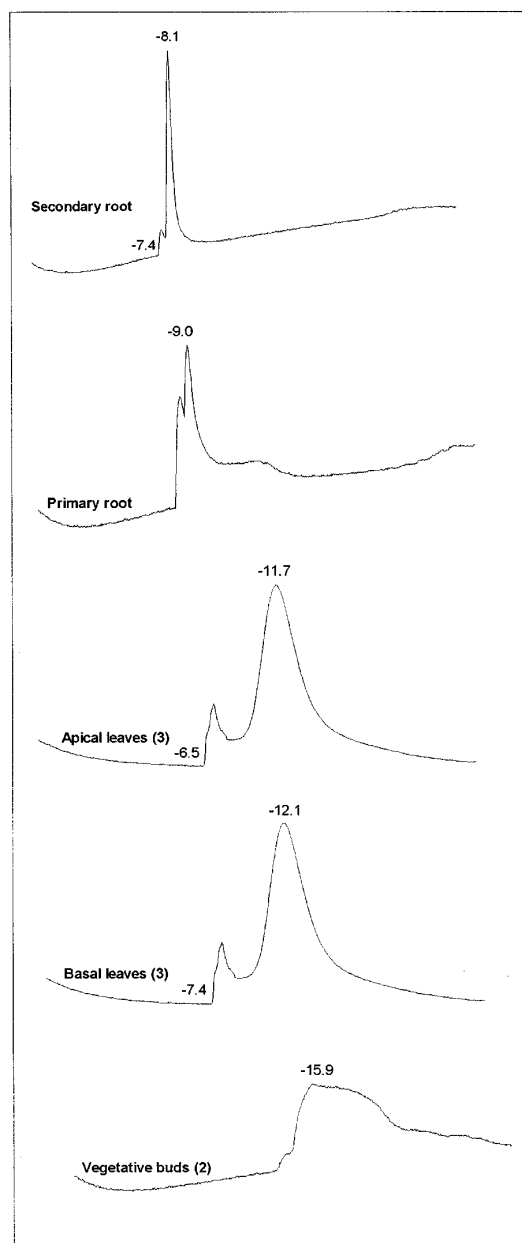


Fig. 4 - Differential thermal analysis in different organs of *Olea europaea* cooled at 5°C hr<sup>-1</sup>.

#### 4. Conclusion

In conclusion, the results reported in the present study, in addition to determining which methods are more reliable for estimating freezing injury in olive, show differential response to subzero temperatures among four cultivars characterised by different freezing tolerance.

Results obtained with differential thermal analysis, visual score and relative electrolyte leakage consistently agreed. Among the four different methods utilised, DTA was by far the fastest procedure, whereas the visual score method the simplest, although is not as quantitative as the other three methods.

Results were consistent with previous findings (Mancuso, 2000) and with anecdotal evidence that suggest that 'Ascolana' is one of the most tolerant olive cultivar, whereas 'Coratina' is one of the most sensitive. In contrast, to other woody species, results showed a generalised supercooling mechanism in olive which make olive interesting for the study of biophysical and biochemical aspects of frost tolerance.

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