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J.P. Peltier · D. Marigo · G. Marigo

Involvement of malate and mannitol in the diurnal regulation of the water status in members of Oleaceae

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Abstract This study examines water status regulation in plants of the Oleaceae family and in some other co-occurring species that are exposed to high solar radiation, in the same habitat. *Fraxinus excelsior* L., one of the most studied Oleaceae in this field exhibited, during the growing season, a close relationship between diurnal variations in leaf water potential and changes in malate, mannitol and K^+ levels, depending on the weather conditions. On sunny days, similar variations can be observed in leaves of the other Oleaceae, with a concomitant decrease in the osmotic potential between predawn and solar noon. Malate, mannitol and the well-known osmoticum K^+, contribute greatly to the osmotic potential decrease. This mechanism, which can be related to the osmotic adjustment described for both drought and salt-affected plants, appears as a general response in plants of the Oleaceae family. Among the other co-occurring species investigated, only *Quercus robur* L. displayed a similar mechanism under the same environmental conditions, but two other organic compounds, quinic and shikimic acids, are presumably involved. *Alnus glutinosa* (L.) Gaertn. and *Robinia pseudacacia* L. responded to a vapor deficit by partial stomatal closure, as transpiration progressed through the morning.

Key words Osmotic potential · Oleaceae · Malate · Mannitol · K^+

Introduction

In response to adverse environmental conditions which modify their water status, such as salinity and edaphic or atmospheric drought, plants make use of a variety of strategies, including several biochemical and physiological mechanisms that are important to the maintenance of turgor (for review see Morgan 1984; Rhodes 1987; Ludlow 1989; Nobel 1991). A well-documented process involves the accumulation of solutes within the cells, which decreases the osmotic component of the water potential maintaining turgor and cell volume above a critical value (Munns 1988). This process, known as osmotic adjustment, generally involves soluble carbohydrates, amino acids or ions (Morgan 1984). It has been proposed as a possible explanation for plant tolerance to low tissue water content during drought conditions, and has been reported for many woody species (Osonubi and Davies 1978; Parker and Pallardy 1987; Abrams and Knapp 1986; Abrams et al. 1990), including, in particular, *Fraxinus excelsior* L. (Peltier et al. 1994). This ash species is of great interest in the study of plant water relations since two organic compounds, malate and mannitol, have been identified as the major constituents of a soluble carbon fraction, and the main active solutes that increase in the leaves after a summer drought in a mesoxerophilic mountain stand (Guicherd et al. 1997).

Malate is the commonest of the organic acids found in plant tissue (Lance and Rustin 1984; Martinoia and Reutsch 1994). It has several times been reported as being involved in osmotic adjustment in response to water deficit (Cutler and Rains 1978; Irigoyen et al. 1992; Tschaplinski and Tuskan 1994). Mannitol, on the other hand, is less widespread than malate in the plant kingdom, but is especially characteristic of some families such as Oleaceae (Trip et al. 1963, 1964; Lewis and Smith 1967; Loescher 1987; Drossopoulos and Niavis 1988). Recent experiments support a role for mannitol as osmoregulator and as a compatible solute which protects metabolic processes in the cytosol (Everard et al. 1992, 1993; Tarczynski et al. 1993). In *F. excelsior*, malate and mannitol are also present in great amounts in well-watered trees on flood plains and, for leaves exposed to high solar radiation, we have suggested that these two compounds are also involved in the diurnal regulation of the plant water status (Marigo and Peltier 1996). Unfortunately, the experiments performed to estimate the relative contribution of malate and mannitol to osmotic potential were unsuccessful presumably due to

J.P. Peltier · D. Marigo · G. Marigo (✉)
Centre de Biologie Alpine, University Joseph Fourier, BP 53,
F-38041 Grenoble cedex 9, France

experimental artefacts in the measurement of the osmotic potential by psychrometric technique, and the ability of Ca^{2+}, present at a high level in leaves, to form chelates with malate once the cells have been decompartmented by freezing and thawing (Marigo and Peltier 1996).

Since the presence of mannitol seems to be a common feature of the Oleaceae, we investigated in this work whether the osmotic involvement of these two compounds in the diurnal water regulation was a general response, for plants of this family, to both high temperature and evaporation demand under intense solar radiation. On summer days, the present study was also extended to some other woody species, co-occurring with ash trees in the same flood plain habitat. Finally, in the Oleaceae family, we investigated the contribution of malate and mannitol to osmotic potential and osmotic changes in the diurnal cycle.

Materials and methods

Study site and species

The study was conducted on leaves from different species growing on the Isere river plain (45° 20' N, 5° 30' E) at an elevation of 200m. The experimental site is located on the University Campus of Saint-Martin d'Hères, University Joseph Fourier, Grenoble. The pedology of the site has been described previously (Marigo and Peltier 1996). Six major species of Oleaceae were chosen for this study, *Fraxinus excelsior* L., *Fraxinus ornus* L., *Ligustrum vulgare* L., *Syringa vulgaris* L., *Jasminum fruticans* L, *Forsythia×intermedia* Zab cv *"Spring Glory* and also four co-existing trees, *Alnus glutinosa* (L.) Gaertn. *Acer pseudoplatanus* L., *Quercus robur* L., *Robinia pseudacacia* L., in order to determine the comparative responses with respect to the ecophysiological traits of water use. These experiments were also extended to another major species growing in this stand, *Reynoutria sachalinensis* (Schmidt Petrop.) Nakai, an invading plant.

Microclimatic factors measurements

Photosynthetic photon flux density (PPFD) was measured with a quantum sensor (Li-19OSB, Li-Cor, Lincoln, Neb.). The vapor pressure deficit (VPD) was calculated as:

$$VPD = (1 - RH/100) \times Vs$$

where RH is the relative humidity in % and Vs the saturation vapor pressure at air temperature.

Physiological measurements

Leaf water potential, stomatal conductance and transpiration were monitored periodically throughout the day, at different times, as indicated in the legends of the tables and figures. Stomatal conductance (Gs) and transpiration (E) were measured with a Li-Cor-1600 diffusive resistance porometer (Li-Cor, Lincoln, Neb.) and leaf water potential (ψw) by a Scholander pressure chamber (Scholander et al. 1965). Psychrometric methods were used to determine the osmotic potential ($\psi\pi$) according the dew-point method. Measurements were carried out with C30 chambers that were equipped with PST 55-15 thermocouple psychrometers and connected to a Wescor HR-33T microvoltmeter (Wescor, Logan, USA). Turgor pressure was estimated as the difference between the water potential and the osmotic potential.

Three south-facing leaves from the same position, which had been submitted to the same illumination level, were used, in the different species, for the determination of Gs, E and ψw. Simultaneously, similar leaves were collected and frozen in liquid nitrogen in order to measure $\psi\pi$ (five leaves) and to determine the solute content (four leaves).

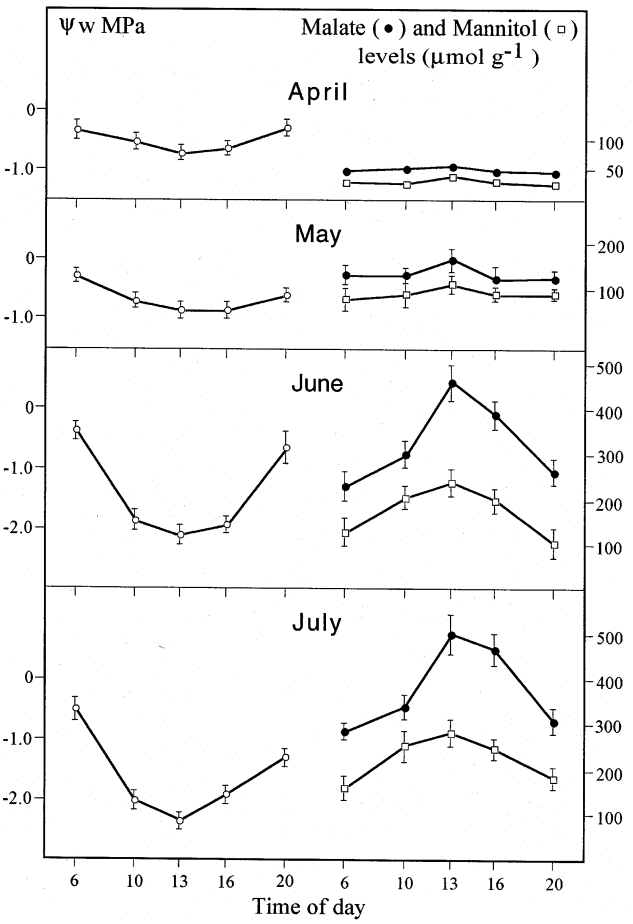

Fig. 1 Daily courses of tissue water potential (○), and malate (●) and mannitol (□) levels in leaves of *Fraxinus excelsior* during a growing season in 1995. Each point (± SD) represents the mean of four individual leaf measurements for malate and mannitol, and three individual leaf measurements for ψw

Other leaves were also sampled to determine the dry weight/fresh weight ratio and the water content (WC) of the leaves.

Dehydration changes in osmotic potential and solute potential calculation

The contribution of the measured solute changes was evaluated using the Boyle-Van't Hoff equation, $\psi s = RT \times ns/Vw$, where ψs is the solute potential, R is the gas constant, T the temperature in degrees K, ns the number of moles of solutes and Vw the total water volume of the cell.

Dehydration changes in osmotic potential, during the transpiration processes, were calculated using the Boyle-Van't Hoff relation from the decrease of the predawn osmotic potential due to the reduction of the tissue water content. For leaves showing an osmotic potential of $\psi\pi$ 6h at 0.6 h, a water loss of x% between predawn and solar noon would decrease the osmotic potential to $\psi\pi 13h = \psi\pi 6h / 1 - x\%$. The dehydration effect induced by the loss of water will be:

$$\Delta\psi\pi \text{ "dehydration"} = (\psi\pi 6h / 1 - x\%) - \psi\pi 6h$$

Analysis of solutes

Weighed samples of fresh leaves (1 g generally) were extracted in an Ultra turrax with a 20-fold amount of ice-cold water acidified to pH 3. The homogenate was centrifuged (20 min 5000g at 4°C) and the

Table 1 Changes in water potential (ψw), osmotic potential (ψπ), turgor pressure (ψp), water content (WC) and transpiration (E) in various species growing on the Isere river plain, from dawn (0600 hours) to solar noon (1300 hours). The experiments were conducted in July 1995. Values are the average of five measurements for ψp, three for ψw, WC and E (±SD; for ψw, SDs that are lower than 4% are not reported). The water content of the leaves is expressed in percentage of the tissue fresh weight. Different letters indicate significant differences ($P < 0.05$) according to the Student's *t*-test

	Oleaceae							Other species					
	Solar time TU	ψw	ψπ MPa	ψp	WC %FW	E mmol H_2O m^{-2} s^{-1}		Solar time TU	ψw	ψπ MPa	ψp	E mmol H_2O m^{-2} s^{-1}	
Fraxinus ornus	06	–0.3	–1.93 ± 0.07 a	1.63	55.8 ± 0.30	–	*Alnus glutinosa*	06	–0.22	–1.2 ± 0.08a	0.98	–	
	13	–2.15	–2.65 ± 0.05 b	0.50	50.9 ± 0.20	8.06 ± 0.75		13	–1.00	–1.35 ± 0.05a	0.35	1.46 ± 0.09	
Syringa vulgaris	06	–0.4	–2.00 ± 0.06 a	1.60	62.8 ± 0.40	–	*Robinia pseudacacia*	06	–0.14	–1.04 ± 0.05a	0.90	–	
	13	–2.1	–2.65 ± 0.06 b	0.55	58.7 ± 0.38	6.9 ± 0.70		13	–0.90	–1.21 ± 0.07a	0.31	1.23 ± 0.08	
Jasminum fruticans	06	–0.2	–1.60 ± 0.05 a	1.40	69.7 ± 0.30	–	*Quercus robur*	06	–0.15	–1.55 ± 0.05a	1.40	–	
	13	–1.85	–2.20 ± 0.08 b	0.35	66.0 ± 0.31	5.04 ± 0.62		13	–1.26	–2.15 ± 0.05b	0.89	4.6 ± 0.03	
Ligustrum vulgare	06	–0.6	–1.25 ± 0.05 a	0.65	74.8 ± 0.35	–	*Acer pseudo platanus*	06	–0.05	–1.26 ± 0.07a	1.11	–	
	13	–1.5	–1.80 ± 0.06 b	0.30	72.0 ± 0.38	9.35 ± 0.81		13	–0.25	–1.40 ± 0.07a	1.15	9.06 ± 0.8	
Forsythia x intermedia	06	–0.3	–1.5 ± 0.04 a	1.20	60.9 ± 0.21	–	*Reynoutria sachalinensis*	06	–0.07	–1.12 ± 0.06a	1.04	–	
	13	–1.5	–1.88 ± 0.06 b	0.35	57.2 ± 0.19	8.6 ± 0.63		13	–0.62	–1.18 ± 0.08a	0.56	9.31 ± 0.75	

supernatant used for the determination of malate, mannitol and the inorganic cations.

Malate and mannitol levels were determined enzymatically according to the method described by Hohorst (1970) and Lunn et al. (1989) using malate and mannitol dehydrogenase, respectively, following procedures reported previously (Marigo and Peltier 1996).

The concentrations of inorganic cations were measured with an atomic absorption spectrophotometer (model Varian AA 1275).

The student *t*-test ($P < 0.05$) was used to determine the significance of some data.

Results

Diurnal changes in the water potential and malate and mannitol levels in F. excelsior leaves at different periods of the growing season

Measurements of diurnal changes in the water potential and malate and mannitol levels in *F. excelsior* leaves were conducted in spring and summer, during the 1995 growing season, for expanded leaves in the field. April and May were characterised by considerable rainfall and moderate temperatures. Consequently, only variations of low amplitude were noticeable in the diurnal changes water potential, and no significant modification occurred in the malate and mannitol levels (Fig. 1). In contrast, in June and July, most days were completely sunny, with hot temperatures and no nightly rainfall. The water potential of the leaves which were exposed to high solar radiation decreased during the morning, reached a minimum between noon and 2 p.m., and then returned gradually to the initial value at the end of the afternoon. The lowest tissue water potential measured was in July, with a value as low as −2.15 MPa, when the transpiration rate was high (Besnard and Carlier 1990). Malate and mannitol levels increased with the age of the leaves, showing large diurnal variations in June and July, that were closely related to the changes in water potential.

Due to the presence of high levels of Ca^{2+} in *F. excelsior*, and its ability to form chelate with malate during the measurement of ψπ, it was impossible to determine the diurnal changes of ψπ in this species. These experiments were conducted in a subsequent way with other plants of the Oleaceae family containing Ca^{2+} in lower amounts.

Diurnal changes in tissue water potential components in plants of the Oleaceae family and in some other co-occurring species

As for *F. excelsior*, the water potential of most of the species studied decreased in July, during the morning, reaching a minimum at 1 p.m. (Table 1), and recovered in the afternoon (data not shown). In Oleaceae, the osmotic potential decreased to a greater or lesser extent, according to the species, but it never fell below the water potential, so that a positive turgor was maintened in all cases. For the other species studied (*Alnus glutinosa, Robinia pseudacacia, Quercus robur, Acer pseudoplatanus, Reynoutria sachalinensis*), turgor pressure also declined during the day, following the evolution of the water potential, but never approaching the point of turgor loss.

The extent of the water potential decrease was generally most pronounced in the case of the Oleaceae, which exhibited a high transpiration rate (Table 1), but a low

Table 2 Components of the osmotic potential in some members of the *Oleaceae* and their contribution to the osmotic ptoential changes from dawn (0600 hours) to solar noon (1300 hours). Dehydration changes ($\Delta\psi_\pi$ "dehydration") were determined using the Boyle-Van't Hoff equation, by considering the loss of water from leaves (in percentage of the initial water content). The solute potentials are deduced from the cumulative effects of malate and mannitol osmolalities, ψ_s (Mal+man); the former plus K^+, ψ_s (Mal+man+K^+); or only malate ψ_s (Mal) and malate plus K^+, ψ_s (Mal+K^+) for *Forsythia x intermedia*. The values in parentheses represent the contribution of ψ_s to ψ_π. The data used for the osmotic potentials and the solute concentrations are obtained from the results of Tables 1 and 3 respectively

Species	Water loss (%)	Total $\Delta\psi\pi$	$\Delta\psi_\pi$ "dehydration"	$\Delta\psi_s$ (Mal + man)	$\Delta\psi_s$ (Mal + man + K^+)
		MPa			
Fraxinus ornus	8.8	–0.72	–0.18 (25%)	–0.56 (80%)	–0.88 (122%)
Syringa vulgaris	6.5	–0.65	–0.14 (21%)	–0.41 (63%)	–0.58 (92%)
Jasminum fruticans	5.3	–0.60	–0.09 (15%)	–0.45 (75%)	–0.62 (92%)
Ligustrum vulgare	3.8	–0.55	–0.05 (9%)	–0.27 (50%)	–0.46 (76%)
				$\Delta\psi_s$ (Mal)	$\Delta\psi_s$ (Mal+K^+)
Forsythia x intermedia	6.0	–0.38	–0.09 (23%)	–0.11 (29%)	–0.34 (90%)

tissue water potential was also measured at 1 p.m. in *Q. robur.* In contrast, *R. sachalinensis* and *Acer pseudoplatanus* showed only slight variations in tissue water potential, despite a high transpiration rate. For *A. pseudoplatanus,* it was interesting to note the remarkable stability of the turgor pressure during the morning. The lowest osmotic potential measured was −2.65 MPa in *F. ornus* and *S. vulgaris.*

All members of the Oleaceae family show the same ability to decrease their osmotic potential between dawn and solar noon (Table 1), with different degree (Table 2). The one which showed the greatest efficiency in performing changes in osmotic potential is *F. ornus*, with an average of −0.72 MPa ($P < 0.05$); the minimum decrease ($\Delta\psi\pi = -0.38$ MPa, $P < 0.05$) was shown by *Forsythia* × *intermedia.* Among the other species studied, only oak trees demonstrated a similar capacity to decrease their osmotic potential ($\Delta\psi\pi = -0.6$ MPa, Table 1). At the opposite, there was little change in the osmotic potential between predawn and solar noon in leaves of *Alnus glutinosa* and *Robinia pseudacacia.* In fact, these two species showed, during sunny days, a decrease in their stomatal conductance after the first hours of the morning, as for *Q. robur* (from the time 10 h, Fig. 2). Stomatal conductance again reached a high level for *Q. robur* in the afternoon, when solar radiation decreased.

Analysis of tissue solutes and their diurnal variations between predawn and solar noon, in leaves of the Oleaceae

In agreement with data in the literature, mannitol was found in large amounts in leaves of the Oleaceae plant family, with the exception of *Forsythia* × *intermedia*, in which it could not be detected (Table 3). Malate was also present in all of the species considered, but its concentration was much lower than that found in leaves of *F. excelsior,* being about twice as much. K^+ was the most representative of the inorganic cations and was present in the greatest amounts. As for malate, Ca^{2+} was found in high concentrations in *F. excelsior*, but was poorly represented in the other species of the Oleaceae plant family in which its level did not change, moreover, between predawn and solar noon.

As in *F. excelsior*, the decline in leaf tissue water potential in the morning was accompanied, on a tissue dry-weight basis, by large changes in malate (46 µmol in *Forsythia* to 130 µmol in *Fraxinus ornus*), mannitol (107 µmol in *Jasminum fruticans* to 130 µmol in *F. ornus*) and K^+ contents (57 µmol in *F. ornus* to 137 µmol in *Ligustrum vulgare*), between predawn and solar noon, in the other Oleaceae (Table 3).

Contribution of malate, mannitol and K^+ to the osmotic potential decrease in members of the Oleaceae

The osmotic potential of leaves can be affected either by changing the molality of existing solutes in the cell, due to water loss, or by a net increase in the solute level in the tissue. Dehydration changes in osmotic potential induced by loss of water ($\Delta\psi\pi$ "dehydration") were estimated as described in Materials and methods, and the osmotic potential values considered were those reported in Table 1. The extent of the solute increase in changes of osmotic potential was determined by calculating the solute potential of the leaves, which was evaluated as indicated by the Boyle-Van't Hoff equation, and by comparing the changes in solute potential ($\Delta\psi s$) with the changes in osmotic potential measured by thermocouple psychrometry (Table 2). When compared with the total potential changes (total $\Delta\psi\pi$, Table 2), the changes due to dehydration ($\Delta\psi\pi$ "dehydration") were low, between 9 and 25%, depending on the species. Other factors must therefore be involved to explain the osmotic potential decrease, among which the increased concentrations of malate, mannitol and K^+, and their cumulative effects, seem to play a major role (Table 2). In fact, for each species considered, the cumulative effect of malate, mannitol and K^+ accounted for about 50% of the predawn osmotic potential (at 6 a.m. data not shown), but their contribution increases in controling the diurnal osmo-

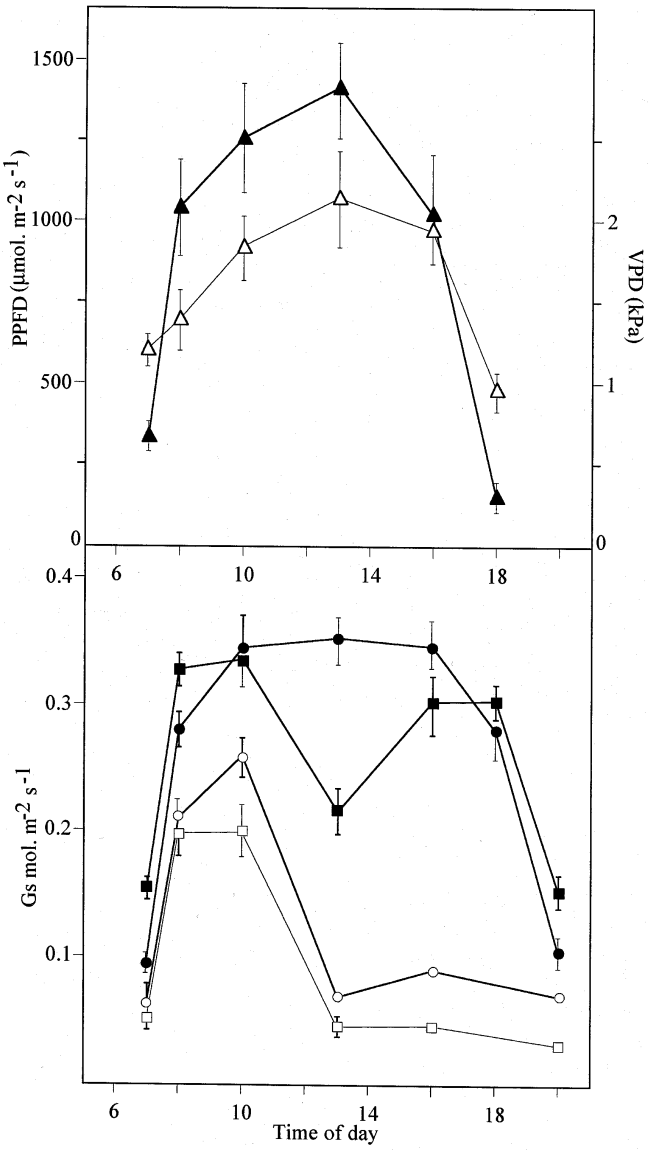

Fig. 2 Daily courses of stomatal conductance (*Gs*) for individual trees on the Isere river plain, during a sunny day in July 1995. Each data point is the mean of three measurements of canopy leaves from a single individual (± SD). For some points, the standard deviations are smaller than the symbols. *Alnus glutinosa* (○); *Quercus robur* (■); *Fraxinus ornus* (●); *Robinia pseudacacia* (□). Identical experiments, repeated on two differents occasions in July 1995, led to the same variations in Gs. Also shown are photosynthetic photon flux density (*PPFD*, ▲) and vapor pressure deficit (*VPD*, Δ) data. Each point is an average of the measurements carried out on leaves of the different species

tic potential changes, from about 76% (*Ligustrum vulgare*) to 92% (*Syringa vulgaris* and *Jasminum fruticans,*), depending on the species. The fact that the contribution of malate, mannitol, and K^+ was higher than 100%, in *F. ornus*, was due likely to some experimental errors in the solute determinations and the measurement of the water potential components. However, since Ca^{2+} at high concentrations can modify the osmotic potential of malate by chelation (Marigo and Peltier 1996), another possible explanation for this discrepancy could be a preferential localization of this cation in the vacuole as Ca-malate complexes.

Discussion

Osmotic adjustment is generally regarded as an important mechanism of adaptation to salinity or drought edaphic conditions. This term describes a change in the osmotic pressure of leaves or roots through an increase of soluble molecules per cell rather than from a lower cell volume (Munns 1988). In this way, for ash trees growing in a mesoxerophilic mountain stand, it has been shown, in previous studies, that two end-products of photosynthesis, malate and mannitol, play a large role in the osmotic adjustment that occurs in response to a summer drought (Guicherd et al. 1997). The involvement of a solute increase in the regulation of the water status of leaves, at a diurnal level, has received less attention (Acevedo et al. 1979; Davies and Lakso 1979). Recent experiments carried out in *F. excelsior* have indicated for species growing on flood plains, that changes in malate and mannitol could also be involved in the regulation of the water status, but here at a diurnal level, according to a mechanism similar to the osmotic adjustment described for drought and salt-affected plants (Marigo and Peltier 1996). In the present study, there is other evidence which supports this idea. In particular, in *F. excelsior,* it has been demonstrated that diurnal changes in malate and mannitol occur only in summer, during the growing season, when ash leaves are exposed to high solar radiation. Moreover, these variations in malate and mannitol levels are closely related to diurnal changes in leaf water potential. As for the osmotic adjustment described in droughted plants, these changes, which were evaluated on a tissue dry weight basis, were due to an increase of the number of molecules per cell. The same biochemical and physiological events have also been observed in other members of the Oleaceae, together with the characterization of an osmotic potential decrease between predawn and solar noon, thus confirming the hypothesis of the involvement of malate and mannitol in a diurnal osmotic potential control.

Since in droughted plants the osmotic adjustment does not occur without a decrease in growth rate, several authors (Munns and Weir 1981; Van Volkenburgh and Boyer 1985; Munns 1988) have proposed that solute build-up was related to a decrease in their utilization. The situation is quite different in the control of the osmotic potential which occurs in the daily cycle. First, the mechanism relies not only on an active accumulation of solutes in the morning to counteract the water evaporative demand, but also on a decrease in their content, in the afternoon, when the transpiration rate declined again (see Fig. 1 for *F. excelsior*). Secondly, malate and mannitol which accumulated in the morning were probably synthesized during the early stage of the light period and stored temporarily in the vacuolar space. Later, with the decrease of the transpiration, they were, in turn, remobilized from the vacuole and either

Table 3 Changes in the major solute concentrations in leaves of different members of the Oleaceae between dawn (0600 hours) and solar noon (1300 hours). This experiment was conducted in July 1995. Mean of four replicates (±SD). Values followed by different letters are significantly different ($P < 0.05$)

Species	Solar time (hours)	Malate	Mannitol µmol g^{-1} dry weight	K^+	Ca^{2+}
Fraxinus excelsior	0600	283 ± 15^a	150 ± 8^a	206 ± 9^a	385 ± 21^a
	1300	505 ± 26^b	280 ± 13^b	262 ± 7^b	465 ± 18^b
Fraxinus ornus	0600	180 ± 8^a	125 ± 9^a	191 ± 12^a	99 ± 8^a
	1300	310 ± 11^b	255 ± 12^b	248 ± 15^b	102 ± 11^a
Syringa vulgaris	0600	109 ± 6^a	122 ± 6^a	430 ± 22^a	213 ± 15^a
	1300	203 ± 9^b	250 ± 9^b	460 ± 25^a	233 ± 21^a
Jasminum fruticans	0600	92 ± 4^a	197 ± 12^a	533 ± 24^a	181 ± 13^a
	1300	199 ± 12^b	304 ± 18^b	624 ± 28^b	201 ± 18^a
Ligustrum vulgare	0600	170 ± 5^a	188 ± 6^a	440 ± 13^a	125 ± 12^a
	1300	244 ± 8^b	310 ± 14^b	577 ± 27^b	132 ± 14^a
Forsythia x intermedia	0600	88 ± 4^a	not detected	364 ± 21^a	204 ± 12^a
	1300	134 ± 5^b	not detected	437 ± 18^b	222 ± 14^a

translocated to other parts of the plant (for mannitol, see Lewis 1984; Keller 1989; Davis and Loescher 1990) or consumed (malate) in relation with the biosynthetic demand of the cells.

Diurnal control of the osmotic potential, as a mechanism preventing excessive loss of cell water during the transpiration processes, seems to be a general characteristic of the Oleaceae plant family. Indeed, for all of the Oleaceae species investigated, there was a correlation between the decrease in osmotic potential, and the minimum tissue water potential, suggesting that osmotic potential plays a major role in the maintenance of a critical turgor, in spite of the great transpiration rate. With the exception of *Forsythia* × *intermedia*, which did not contain mannitol, malate and mannitol were also the main solutes involved in this mechanism in all of the Oleaceae species with an inorganic cation, K^+.

By evaluating their contribution on a whole tissue basis, no distinction was made between the role of malate and mannitol in relation to their cellular distribution. In fact, malate accumulates preferentially in the vacuolar compartment (Martinoia and Rutsch 1994) whereas mannitol was found to be both vacuolar and cytosolic (Keller and Matile 1989). Due to its presence in the cytosolic compartment, some authors have postuled a role for mannitol in the osmotic potential regulation as compatible solutes which protect cell metabolic process in the cytosol (Everard et al. 1992, 1993; Tarczynski et al. 1993).

Determination of the leaf osmotic potential by the psychrometric method can lead to systematic errors due to wound reactions and failure to reach vapour equilibrium (Tyree and Richter 1981; Kikuta and Richter 1992) but its value may also be overestimated in plants containing high levels of Ca^{2+}, because of interactions between the positive charge of Ca^{2+} and the double charge of divalent anions, leading to the formation of chelate complexes above a critical Ca^{2+} concentration of 200 mM (Marigo and Peltier 1996). In leaves of *Fraxinus excelsior* which contain high levels of malate and Ca^{2+}, these chemical reactions induced experimental artefacts affecting the measurement of $\psi\pi$, preventing us from estimating osmotic potential changes (Marigo and Peltier 1996). Ca^{2+} is also one of the most important cations in leaves of other Oleaceae (Table 3), but, except in *F. excelsior*, its level does not exceed the critical value of 200 mM so that determination of $\psi\pi$ in the other Oleaceae species can be carried out without problem. A preferential Ca^{2+} vacuolar localization as Ca-malate complexes could explain however, in leaves of *F. ornus,* the unexpected contribution of solute potential (ψs) in the osmotic regulation (122% instead 100%, see Table 2).

The data on turgor pressure must be considered carefully since they may be subjected to potential errors, those described for the determination of the osmotic potential but also by the fact that they were determined by two different techniques, the pressure bomb and the psychrometer. These estimates were used, in this study, as a mean to determine the variations of turgor pressure and to indicate the point of turgor loss rather than to measure the absolute values of turgor.

A general procedure for evaluating the contribution of the major osmotically active compounds involves comparison of the osmotic potential measured by thermocouple psychrometry with the solute potential evaluated using the Boyle-Van't Hoff equation. With the exception of *Forsythia* × *intermedia* which does not contain mannitol, in the Oleaceae family, this procedure indicates that the organic compounds malate and mannitol were the main solutes involved in diurnal control of osmotic potential with an inorganic cation, K^+. Altogether, these three solutes, which accounted for about 50% of the osmotic potential for leaves sampled at predawn (data not shown), contributed greatly to the diurnal control of osmotic potential, between 75 and 100%, depending on the species. The water loss from the cell due to the high transpiration rate represented only a small part of the control process.

As seen previously, the osmotic involvement of $Ca2^+$, which can form chelates with the double charge of divalent anions, is not easy to understand. In the Oleaceae plant

family, since the diurnal Ca^{2+} level does not vary significantly during the daily cycle for the majority of the species, it may be assumed that Ca^{2+} is not involved in the diurnal control of the osmotic potential. In contrast, considerable changes in Ca^{2+} level occur, in *F. excelsior,* together with similar variations in malate levels. To explain the specific behaviour of Ca^{2+} and malate in this ash species, it may be suggested that, in parallel with its osmotic involvement, vacuolar malate accumulation in the leaves may serve to neutralize the excess of cellular Ca^{2+}, driven by the xylem sap, and to maintain cytosolic Ca^{2+} at a very low level (Bush 1995). A similar mechanism, involving the sequestration of calcium as oxalate crystals in cell vacuoles, has been postulated by Ruiz and Mansfield (1994) as an explanation for the regulation of Ca^{2+} in the vicinity of stomatal guard cells of *Commelina communis*. In addition, the free Ca^{2+} transported by the xylem sap may be also removed after its incorporation in the cell wall network.

Another common plant response to water deficit is stomatal closure, which reduces fluxes of both CO_2 and water vapor. In *Alnus glutinosa* and *Robinia pseudacacia* control of osmotic potential is inexistent or minimal during the daily cycle, but these species have very sensitive stomata which close under high solar radiation. This characteristic has already been reported for *A. glutinosa* trees growing on stony ground (P. Guicherd, unpublished data) and it enables them to avoid desiccation during the day, and to maintain leaf water potential at a relatively high level. In contrast, plants of the Oleaceae family respond to water vapor deficit by keeping their stomata open, and by maintaining turgor above a critical value according a control of their osmotic potential. In comparison to the mechanism described above, this latter process is much expensive in terms of energy consumption for malate and mannitol compartmentation, but it should result in a net carbon gain by photosynthesis in order to optimize leaf expansion during the day. *Q. robur* is an interesting species, since it brings into play a double strategy for the regulation of its water status, based on the previous processes. For *Quercus*, osmotic control cannot be attributed to malic acid which is present in small amounts (Boudet 1972) but two other organic acids, quinic and shikimic acids may be involved. In fact, in *Q. pedunculata* Boudet (1972) has observed that these two compounds, which together account for about 15% of the dry weight, were subject to large changes, on a tissue dry weight basis, during daylight hours in summer.

The final species investigated in this work, *Acer pseudoplatanus* and *Reynoutria sachalinensis* exhibited the same behaviour with respect to the regulation of their diurnal water status. Despite a high transpiration rate, their water potential decreased moderately during the morning, especially for the first species, but they did not show the ability to decrease their osmotic potential or to display stomatal regulation during the period of high evaporative demand. To explain these data, it may be hypothesized that these two species are characterized by a high hydraulic conductance, and that the xylem sap, driven from the soil to the aerial parts of the plant, is sufficient to compensate for the loss of water from the leaves.

In conclusion, this study shows that plants make use of multiple strategies to prevent excessive loss of cell water during the transpiration processes. One of those, which can be related to the osmotic adjustment described for droughted plants, seems to be a common feature of the Oleaceae*,* and in these species it involves malate, mannitol and K^+ as the major osmoticum.

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