Mannitol is one of the primary photosynthetic products and the major phloem-translocated carbohydrate in *Olea europaea* L., an important crop in the Mediterranean basin. Uptake of mannitol in heterotrophic cell suspensions of *O. europaea* was shown to be mediated by a 1:1 polyol: H⁺ symport system with a *Kₘ* of 1.3 mM mannitol and a *Vₘₐₓ* of 1.3 nmol min⁻¹ mg⁻¹ DW. Dulcitol, sorbitol, and xylitol competed for mannitol uptake, whereas glucose and sucrose did not. Reverse transcription–PCR (RT–PCR) performed on mRNA extracted from cultured cells exhibiting high mannitol transport activity allowed the cloning of a partial *O. europaea* mannitol carrier OeMaT1. The *Vₘₐₓ* of mannitol uptake and the amount of OeMaT1 transcripts increased along with polyol depletion from the medium, suggesting that the mannitol transport system may be regulated by its own substrate. Addition of 100–500 mM NaCl to cultured cells enhanced the capacity of the polyol: H⁺ symport system and the amount of OeMaT1 transcripts, whereas it strongly repressed mannitol dehydrogenase activity. Measurements of cell viability showed that mannitol-grown cells remained viable 24 h after a 250 and 500 mM NaCl pulse, whereas extensive loss of cell viability was observed in sucrose-grown cells. OeMaT1 transcripts increased throughout maturation of olive fruits, suggesting that an OeMaT is involved in the accumulation of mannitol during ripening of olive. Thus, mannitol transport and compartmentation by OeMaT are important to allocate this source of carbon and energy, as well as for salt tolerance and olive ripening.

**Keywords:** Mannitol — *Olea europaea* — Polyol transport — Salt stress.

**Abbreviations:** CCCP, carbonyl cyanide *m*-chlorophenyl-hydrazone; FDA, fluorescein diacetate; MTD, mannitol dehydrogenase; *mitD*, mannitol-1-phosphate dehydrogenase; PI, propidium iodide; TPP⁺, tetraphenylphosphonium; RT–PCR, reverse transcription–PCR.

**Introduction**

Polys (or sugar alcohols), the reduced form of aldoses and ketoses, can be either cyclic (cyclitols) or linear (alditols), and are present in all living forms. In some plant species, polys are direct products of photosynthesis in mature leaves, together with sucrose. Mannitol is the most widely distributed sugar alcohol in nature and has been reported in >100 species of vascular plants of several families, including the Rubiaceae (coffee), Oleaceae (olive, privet) and Apiaceae (celery, carrot, parsley). Mannitol is synthesized in mature leaves from mannose-6-phosphate, through the combined action of a NADPH-dependent mannose-6-phosphate reductase and a mannitol-6-phosphate phosphatase. It is transported to sink tissues where it can be either stored or oxidized to mannose by an NAD-dependent mannitol dehydrogenase (MTD) (reviewed by Stoop et al. 1996, Noiraud et al. 2001b).

In contrast to sucrose and monosaccharide transporters, little is known regarding the identity and regulation of polyol transporters either in sink or in source tissues in higher plants. The first cDNA encoding a mannitol transporter of a higher plant was identified and characterized in celery phloem (Noiraud et al. 2001a). This cDNA (*AgMaT1*, *Apium graveolens* mannitol transporter 1) was used to establish a heterologous expression system in yeast cells. In the past 5 years, polyol transporters from *Prunus cerasus* (Gao et al. 2003), *Plantago major* (Ramsperger-Gleixner et al. 2004), *Malus domestica* (Watari et al. 2004) and *Arabidopsis thaliana* (Klepek et al. 2005, Schneider et al. 2006) have been characterized.

Mannitol production may confer several potential advantages including more efficient carbon use (Stoop et al. 1996), resistance against oxidative stress (Smirnoff and Cumbes 1989, Williamson et al. 1995, Jennings et al. 1998) and salt tolerance. Thus, the mannitol concentration in celery grown in hydroponic nutrient solution progressively
increases as the total salinity of the growth solution increases (Stoop and Pharr 1994). Increased mannitol accumulation in leaves was also observed in plants irrigated with 0.3 M NaCl, as a consequence of a massive shift in partitioning of fixed carbon into mannitol instead of sucrose (Everard et al. 1994). The strong water stress tolerance of *Fraxinus excelsior* is in part related to an accumulation of malate and mannitol (Guicherd et al. 1997), and, in plants subjected to drought stress, the mannitol content of the leaf xylem sap increases (Patonnier et al. 1999). Additional evidence for a role for mannitol in salinity tolerance was obtained when *Nicotiana tabacum*, *Populus tomentosa* and other plants were genetically engineered to synthesize mannitol through introduction of an *Escherichia coli* mannitol-1-phosphate dehydrogenase (*mltD*), which catalyzes the biosynthesis of mannitol from fructose, resulting in more salt-tolerant plants (Tarczynsky et al. 1993, Hu et al. 2005). In Arabidopsis, *mltD* gene transfer and expression enhanced seed germination under salinity conditions (Thomas et al. 1995).

*Olea europaea* L. is an evergreen moderately salt-tolerant tree (Therios and Misopolinos 1988, Rugini and Fedeli 1990) traditionally cultivated in the Mediterranean basin. Olives and olive oil play an increasingly important nutritional role and are an essential part of what is now widely known as the ‘Mediterranean diet’. Sugars are the main soluble components in olive tissues and play important roles, providing energy and acting as precursors for olive oil biosynthesis. Glucose, fructose and galactose are the main sugars found in olive pulp, but appreciable quantities of mannitol are also present (Marsilio et al. 2001). Water deficit, freezing, salinity and air pollution are a few of the stress factors restricting plant growth, so that olive productivity at the end of the growing season expresses only a fraction of the plant’s genetic potential (Vitagliano and Sebastiani 2002). In this context, the elucidation of the role of mannitol as carbon and energy source for plant growth and as a protecting solute against salinity and drought is important for the improvement of yield potential of the plant. The present work characterizes a mannitol transporter expressed in cultured cells and intact fruits of *O. europaea*. For the first time in plants it is shown that mannitol transport is regulated by means of salt-mediated changes in the transcription of mannitol carrier(s). Altogether, the results showed that transmembrane transport of mannitol is a critical step in terms of osmotic adjustments and productivity in *O. europaea*.

**Results**

**Growth in batch cultures with mannitol**

Suspension cell cultures mimic heterotrophic plant tissues, where carbohydrates are imported from photosynthetically active source tissues, thus making them a suitable model system to study sugar transport into sink cells. The capacity of *O. europaea* cell suspensions to use sucrose, lactose, glucose, galactose, fructose, mannitol and glycerol as sole carbon and energy sources was studied previously (Oliveira et al. 2002). Among these substrates, only lactose and glycerol were unable to promote cell growth. Growth of *O. europaea* cell suspensions with 1\% (w/v) mannitol and its consumption are depicted in Fig. 1A. To ascertain whether mannitol transport constitutes the rate-limiting step of the growth of *O. europaea* cells on mannitol, the growth parameters were compared with the maximal capacity of mannitol uptake. The following values for the maximum specific growth rate ($\mu_{\text{max}}$) and yield coefficient ($Y$) were estimated: 0.2 d$^{-1}$ and 0.6 g biomass g$^{-1}$ mannitol, respectively. From the ratio $\mu_{\text{max}}/Y$, a value for the specific mannitol transfer rate ($q$) of 1.28 nmol min$^{-1}$ mg$^{-1}$ DW was estimated, similar to the corresponding $V_{\text{max}}$ of mannitol transport (Table 1), suggesting that mannitol uptake is an important metabolic step for the control of cell growth. Growth of *O. europaea* cells in a medium containing a mixture of 0.5\% (w/v) glucose and 0.5\% (w/v) mannitol is depicted in Fig. 1B. The rate of glucose

![Figure 1](https://academic.oup.com/pcp/article-abstract/48/1/42/2468937/fig1)

**Fig. 1** Representative growth curves of *O. europaea* cells in MS medium with 1\% (w/v) mannitol (A) and with 0.5\% (w/v) glucose and 0.5\% (w/v) mannitol (B).
depletion from the medium was higher than that of mannitol; after glucose exhaustion (day 8), mannitol sustained cell growth up to day 12.

Mannitol transport

Transport experiments were conducted in *O. europaea* cell suspensions harvested at the end of the exponential growth phase, after 10–15 d in culture, when the mannitol concentration had fallen to about 0.05% (w/v). Initial uptake rates of 0.2–20 mM D-[14C]mannitol followed Michaelis–Menten kinetics (Fig. 2A), suggesting carrier-mediated transport. By the application of a computer-assisted non-linear regression analysis (GraphPad Prism, version 4.0) to the data, the following kinetic parameters were obtained: $K_m = 1.3 \pm 0.15$ mM mannitol and $V_{max} = 1.29 \pm 0.04$ nmol mannitol min$^{-1}$ mg$^{-1}$ DW.

Cells of *O. europaea* cultivated with 1% mannitol, collected at the mid-exponential growth phase when the mannitol concentration in the medium is approximately 0.5% (w/v) (see Fig. 1A), also displayed the capacity to transport mannitol; however, the Eadie–Hofstee plot of D-[14C]mannitol initial uptake rates was biphasic (Fig. 2B). The computer-assisted non-linear regression analysis of the data agreed with the presence of two distinct transport modes: saturating transport associated with first order kinetics. The following values were estimated for the maximal capacity of saturating transport and for the diffusion-like component: $V_{max} = 0.25 \pm 0.05$ nmol mannitol min$^{-1}$ mg$^{-1}$ DW; $k_d = 0.06 \pm 0.01$ μl min$^{-1}$ mg$^{-1}$ DW.

To study the specificity of the transport system, the initial uptake rates of 0.1–2 mM D-[14C]mannitol were estimated in the presence of the following unlabeled putative competitors: dulcitol, sorbitol, xylitol, myo-inositol, glucose, fructose, mannose and sucrose. The acyclic polyols dulcitol, sorbitol and xylitol behaved as competitive inhibitors (Fig. 3A), suggesting that they share the same carrier; glucose and the remaining substrates (not shown) had no effect on the transport of mannitol, thus appearing not to be recognized by the permease. Although the identified transport system seems to be specific for polyols, *O. europaea* cell suspensions cultivated in similar conditions are able to transport glucose according
to a carrier-mediated mechanism with $K_m = 67 \pm 30 \mu M$ glucose and $V_{max} = 1.45 \pm 0.26 \text{nmol glucose min}^{-1}\text{mg}^{-1} \text{DW}$ (Oliveira et al. 2002). Here, inhibition experiments of d-[14C]glucose uptake by different polyols were performed to check that polyol uptake does not occur via the monosaccharide transport system. Mannitol at 20 mM did not inhibit 0.02–0.5 mMD-[14C]glucose uptake (Fig. 3B). Similar results were obtained with sorbitol and dulcitol (data not shown). Taken together, the results suggest that, in \textit{O. europaea}, monosaccharides and polyols are transported via two distinct transport systems with different $K_m$s. This could account for the results depicted in Fig. 1B, where glucose is the first substrate to be consumed when growth occurred in a medium with glucose and mannitol.

To study the energetics of mannitol transport in \textit{O. europaea} cultured cells, d-[14C]mannitol uptake was measured at different external pH values. $V_{max}$ decreased abruptly from pH 4.5 to 5.5, with little activity remaining above pH 5.5, consistent with the involvement of a proton-dependent transport system (Fig. 4A). Additionally, the uptake of 0.2–2.0 mM [14C]mannitol was strongly inhibited by 50 \mu M of the protonophore m-chlorophenylhydrazone (CCCP) (Fig. 4B). The occurrence of transient alkalinization of extracellular media upon addition of mannitol to cell suspensions (Fig. 4C) provided clear evidence for the involvement of a mannitol–proton symport system. The initial velocities of proton uptake were estimated from the slope of the initial part of the pH trace (the alkalinization curve) after 5 mM mannitol (saturating concentrations) had been added. A value of 1.1 nmol H$^+$ min$^{-1}$ mg$^{-1}$ DW can be obtained from the data of Fig. 4C, similar to the maximal capacity of the mannitol: H$^+$ symport system measured with d-[14C]mannitol, suggesting a 1 mannitol:1 proton stoichiometry. Since such a mechanism would be associated with a net influx of positive charges into the cells, the effect of the dissipation of transmembrane electric potential on mannitol transport was studied. Fig. 4B shows the effect of the lipophilic cation tetraphenylphosphonium (TPP$^+$) on the initial uptake rates of 0.2–2 mM d-[14C]mannitol. TPP$^+$ inhibited mannitol uptake, indicating that membrane potential makes a significant contribution to the driving force for substrate transport by the polyol carrier.

**Polyol: H$^+$ symport activity and OeMaT expression**

As the $V_{max}$ of the \textit{O. europaea} polyol: H$^+$ symport system increased along with mannitol depletion from the medium throughout the exponential growth (see Fig. 2), mannitol levels appear to have a regulatory effect. To study the induction of transport activity in response to mannitol concentration, the accurate dependence of permease activity on polyol levels in the medium was evaluated. Cells were grown in a medium with 1% (w/v) mannitol as in Fig. 1A and collected at the end of the exponential growth phase as described in Fig. 2A.

![Eadie–Hofstee plots of the initial uptake rates, at pH 4.5, of d-[14C]mannitol (A) and d-[14C]glucose (B) by suspension-cultured cells of \textit{O. europaea}. Transport was measured in the absence of other sugars or polyols (filled boxes) and in the presence of unlabeled 5 mM sorbitol (filled inverted triangles), 5 mM dulcitol (filled triangles), 5 mM xylitol (filled diamonds), 20 mM mannitol (open triangles) and 20 mM glucose (open squares). Cells were cultivated with 1% (w/v) mannitol as in Fig. 1A and collected at the end of the exponential growth phase as described in Fig. 2A.](https://academic.oup.com/pcp/article-abstract/48/1/42/2468937)
(RT–PCR) was performed on mRNA extracted from O. europaea suspension-cultured cells exhibiting high mannitol transport activity. This allowed the cloning of a 501 bp cDNA OeMaT1 (accession No. DQ059507) with extensive homology to the celery mannitol transporter AgMaT2. Low-stringency Southern blots of O. europaea genomic DNA digested with four different restriction enzymes and hybridized with OeMaT1 cDNA, identified one (Hind III and Xho I) and three (Xba I and Eco RI) bands (Fig. 5D). This suggests the presence of a multigene family of polyol transporters in olive tree, which is consistent with evidence available for other plant species such as P. cerasus (Gao et al. 2003), P. major (Ramsperger-Gleixner et al. 2004), M. domestica (Watari et al. 2004) and A. thaliana (Klepek et al. 2005). An OeMaT1 partial cDNA sequence was used as a probe for Northern analysis (Fig. 5B). Due to the impossibility of obtaining the full-length cDNA, we were not able to design a specific probe. We must therefore consider the possibility of a high level of sequence identity with the other potential mannitol transporters present in the O. europaea genome with the probable occurrence of cross-hybridization. For the rest of the work, we will refer to OeMaT to indicate that the signals observed in Northern experiments were either due to OeMaT1 or to other unidentified mannitol transporters present in O. europaea. The parallel between OeMaT transcripts and mannitol transport activity shown in Fig. 5B suggests that carrier expression is mainly controlled at the transcriptional level, although other forms of post-transcriptional regulation cannot be ruled out. These results show for the first time a tight regulation of a mannitol : H\(^{+}\) symport expression by external levels of its own substrate.

As referred to in the Introduction, substantial amounts of mannitol are present in the pulp of olive fruit, reaching maximum values in ripened olives of 8 mg g\(^{-1}\) DW (Marsilio et al. 2001). To study the involvement of the polyol : H\(^{+}\) symport system in mannitol unloading during olive fruit maturation, RNAs were isolated from olive fruits at the green, cherry and black stages, and the expression of OeMaT was studied. Although detectable throughout fruit development, OeMaT transcript levels strongly increased during the late stage of the ripening process (black stage) at the onset of mannitol accumulation (Fig. 5C).

**Effect of salt stress on polyol : H\(^{+}\) symport activity and OeMaT expression**

As referred to in the Introduction, mannitol can act as a compatible solute besides its role as a carbon and energy source. To study the influence of salt stress on mannitol...
transport capacity, NaCl was added to suspension-cultured cells at mid-exponential growth phase. Addition of 500 mM NaCl promoted the increase of OeMaT transcription and mannitol transport activity with time when compared with control cells, the maximal levels being achieved within 24 h (Fig. 6A). Additionally, the increase of salt concentration in the medium promoted a dose-dependent increase of OeMaT transcripts and polyol : H\(^+\) symport activity, measured after 24 h (Fig. 6B). The parallel between \(V_{\text{max}}\) of mannitol transport and OeMaT message levels suggests that the expression of OeMaT is responsible for the

![Graph showing the effect of NaCl on OeMaT expression and mannitol transport activity.](https://academic.oup.com/pcp/article-abstract/48/1/42/2468937)

![Image of Northern blots showing OeMaT expression in O. europaea.](https://academic.oup.com/pcp/article-abstract/48/1/42/2468937)
increase of mannitol transport capacity under salt stress conditions.

Regulation of mannitol catabolism by mannitol dehydrogenase

The activity of MTD has been fully characterized during the last decade by Pharr and co-workers in celery, where mannitol also represents an important carbon and energy source. Here, MTD activity was measured in crude extracts of *O. europaea* cells to correlate mannitol transport activity with the rate of intracellular mannitol conversion. Crude extracts were obtained from cells cultivated with 1% (w/v) mannitol up to mid- and late exponential growth phase as in D-[14C]mannitol transport experiments. The activity of MTD was also measured in homogenates from mannitol-grown cells subjected to salt stress. The Eadie–Hofstee plots of initial velocities of mannitol oxidation were linear over 5–150 mM mannitol, and a $K_m$ of 40 mM was obtained (Fig. 7). MTD activity was enhanced as mannitol was depleted from the culture medium and strongly repressed by salt. The decrease of MTD activity in salt-stressed cells associated with the increase of mannitol uptake capacity should allow the intracellular accumulation of mannitol to compensate for the decrease of external water activity.

Evaluation of the protective role of mannitol against salt stress

To assess the physiological role of mannitol in salt stress tolerance in *O. europaea*, we studied cell viability after salt addition to mannitol-grown cells and sucrose-grown cells. Cell aliquots were collected from each medium at mid-exponential growth phase, and 250 and 500 mM NaCl were added. Cell viability was assessed by fluorescein diacetate (FDA, green fluorescence) and propidium iodide (PI, red fluorescence). Fig. 8 shows that a large population of...
Mannitol-grown cells remained viable 24 h after a 500 mM NaCl pulse. Sucrose-grown cells were not able to display green fluorescence after the same treatment, and showed extensive loss of cell viability as evaluated by the PI red fluorescence. A similar result was obtained with glucose-grown cells (not shown). Taken together, these results suggest that mannitol plays essential roles in *O. europaea*, both providing a carbon and energy source for sink tissues and acting as an osmoprotectant in response to high salinity.

**Discussion**

Mannitol is the most abundant carbohydrate in olive tree leaves, accounting for 82–92% of the total soluble carbon (Drossopoulos and Niavis 1988), and is an important sugar in the olive fruit pulp (Marsilio et al. 2001). However, very little information is available regarding the utilization and transport of mannitol in this polyol-synthesizing plant. In the present work, mannitol transport mechanisms operating in *O. europaea* heterotrophic cells were investigated in detail. In addition, the regulation of the expression of the polyol transport system and its relevance in osmotic adjustments was also studied. The data obtained are particularly relevant because the olive tree is normally cultivated in areas in which water is the main limiting factor in agricultural production (Tattini et al. 1994). Although it is recognized that suspension-cultured cells may not be close to normal physiological conditions, they provide a convenient experimental system that has already yielded a lot of useful information on sugar transport mechanisms and regulation (Roitsch and Tanner 1994, Ehness et al. 1997, Oliveira et al. 2002, Cakir et al. 2003, Azevedo et al. 2006, Conde et al. 2006).

Mannitol-grown cells of *O. europaea* exhibit a specific growth rate ($\mu_{\text{max}}$, 0.2 d$^{-1}$) higher than cells cultivated with glucose ($\mu_{\text{max}}$, 0.07 and 0.11 d$^{-1}$ in media with 0.5 and 3% glucose, respectively; Oliveira et al. 2002) or 2% sucrose ($\mu_{\text{max}}$, 0.08 d$^{-1}$), possibly because mannitol catabolism produces a higher number of ATPs than the catabolism of an equal amount of glucose or sucrose. From the linear part of the growth curve, a value for the yield coefficient ($Y$) of 0.6 g biomass g$^{-1}$ mannitol was estimated, suggesting that most of the carbohydrate is respired and used as a carbon and energy source for exponential growth. Therefore, little or no sugar is channeled to internal stores, which is confirmed by the arrest of cell growth that was associated with the decline of sugar content. The fact that mannitol behaved as an efficient carbon and energy source in heterotrophic cells of *O. europaea* is consistent with the role of this compound as a major photoassimilate in this species.

The saturable transport observed in *O. europaea* cells involves a polyol:$H^+$ symport system with a stoichiometry of 1 mannitol:1 $H^+$ as indicated by the following observations: (i) mannitol addition to weakly buffered cell suspensions is associated with a transient alkalization of the extracellular medium; (ii) the $V_{\text{max}}$ of proton uptake is similar to the $V_{\text{max}}$ of carrier-mediated $\alpha$-mannitol uptake and depended on extracellular pH; (iii) dissipation of the proton-motive force by CCCP significantly inhibited the initial velocities of $\alpha$-mannitol uptake; and (iv) mannitol transport was sensitive to TPP$^+$, suggesting that the $\Delta \Psi$ is an important component of the proton-motive force involved in mannitol accumulation. Proton dependence and substrate affinity ($K_m$, 1.3 mM mannitol) are in good agreement with the data obtained for the celery mannitol transporter. In this polyol-producing plant, different membrane transport steps have been studied, from phloem loading to phloem unloading and storage in parenchyma cells. The cloned *AgMaT1* gave yeast cells the ability to grow on mannitol, and a $K_m$ value for mannitol uptake of 0.34 mM was obtained (Noiraud et al. 2001a), that correlates well with the value ($K_m$, 0.64 mM) determined in plasma membrane vesicles from phloem strands of celery (Salmon et al. 1995). The involvement of a co-transport with protons was proposed because the uptake of mannitol was almost abolished by CCCP and was maximal at acidic pH. Also, in storage parenchyma discs of celery leaves and in plasma membrane vesicles from parenchyma cells, $K_m$ values of 1 mM were obtained (Keller 1991, Salmon et al. 1995). In contrast, mannitol transport in vacuoles of celery parenchyma cells seems to be mediated by facilitated diffusion, because it was neither stimulated by energization with ATP and pyrophosphate, nor impaired by the dissipation of the proton-motive force (Greuet et al. 1998).

The selectivity of the *O. europaea* polyol:$H^+$ symport system is rather poor for mannitol because the transport system was able to accept, besides mannitol, dulcitol, sorbitol, and xylitol, as these acyclic polyols behaved as competitive inhibitors. In addition, monosaccharide uptake and polyol uptake are mediated by two distinct transport systems since glucose did not inhibit $\alpha$-$[^{14}C]$mannitol uptake. Likewise, mannitol did not affect $\alpha$-$[^{14}C]$glucose uptake mediated by a monosaccharide:$H^+$ symport system previously identified in mannitol-grown cells (Oliveira et al. 2002). Accordingly, glucose is not transported by *Saccharomyces cerevisiae* expressing the celery mannitol transporter *AgMaT1* (Noiraud et al. 2001a). This is in contrast to the polyol transporter from Arabidopsis AtPST5 which can also transport glucose (Klepek et al. 2005). Kinetic parameters may explain why $\alpha$-glucose is consumed before mannitol when the two substrates are present in the extracellular medium: the capacity of both...
transport systems is similar but the substrate affinity of the monosaccharide transport system is much higher than that of the polyol transport system. \( K_m \) 0.67 \( \mu \)M and 1.3 mM, respectively. However, one cannot exclude that the activity of MTD may be repressed by sugar as was reported in celery (Prata et al. 1997), impairing the intracellular conversion of mannitol before glucose depletion.

Since mannitol transport plays a key role in source-sink interaction in \( O. \) europaea, it is likely that the expression and activity of mannitol transporters are highly regulated by mannitol levels. Our data indeed show that in \( O. \) europaea suspension-cultured cells, alterations in mannitol levels have a pronounced effect on the expression and mannitol transport activity of \( OeMaT \). When high mannitol is present, energy-independent diffusional uptake is the preferred mode of mannitol absorption, and \( OeMaT \) expression and transport activity are maintained at basal levels. Whether non-saturable mechanisms involved in the diffusional uptake may play important roles in sink cells of olive in vivo needs further investigation, but it may be possible owing to the high sugar content in sink tissues (Patrick 1997). While non-saturable mechanisms of sugar and polyol transport were also reported in other plant cells and tissues (Delrot 1989, Keller 1991, Krook et al. 2000, Oliveira et al. 2002, Conde et al. 2006), its underlying mechanisms are still poorly understood. Several mechanisms or a combination of them could account for ‘diffusion-like’ kinetics: non-specific permeation of the sugar by free diffusion across the plasma membrane, involvement of carrier(s) or channels with very low affinity, or endocytic processes as reported by Etxeberria et al. (2005). When external mannitol decreases to residual levels, the linear transport component no longer sustains mannitol uptake at a rate sufficient to allow efficient activity of MTD, which exhibits a quite high \( K_m \) of 40 mM, and the involvement of a concentrative, energy-dependent transport system becomes critical. Following mannitol depletion, \( OeMaT \) transcript levels and polyol: \( H^+ \) symport activity increased, suggesting that mannitol regulates carrier expression at the transcriptional level, although other levels of regulation cannot be ruled out.

Salt stress can be regarded as a situation in which plants have to cope with both decreased water availability and ion toxicity (Lewitt 1980). Despite numerous studies indicating that, following polyol synthesis in mature leaves, there is an increase in polyol content in sink organs in response to drought or salt stress (see Introduction), and that salt alters enzyme activities related to polyol metabolism (Stoop and Pharr 1994, Williamson et al. 1995), little is known about the regulation of polyol transporters under such conditions. In \( Mesembryanthemum crystallinum \), where the genes \( MITR1 \) and \( MITR2 \) behave as \( myo \)-inositol: \( Na^+ \) symporters, \( myo \)-inositol could be a signal during the adaptation to salt stress (Nelson et al. 1999, Chauhan et al. 2000). In celery plants subjected to severe salt stress, the expression of \( AgMaT1 \) has not been studied yet, but it appears that the expression of the sucrose carrier \( AgSUT1 \) decreased in all organs (Noiraud et al. 2000), suggesting that transport of mannitol is favored. The present work provides clear evidence that \( O. \) europaea suspension-cultured cells exposed to high salinity display an increase of \( OeMaT \) expression and mannitol transport activity, together with a drastic decrease of mannitol oxidation by MTD. This should allow the intracellular accumulation of mannitol in order to compensate the decrease of external water activity, providing a tolerance mechanism to salinity in \( O. \) europaea (Fig. 9). Similarly, celery plants exposed to high salinity showed a specific down-regulation of MTD activity in sink tissues, resulting in decreased mannitol use and an accompanying increase in mannitol accumulation (Stoop and Pharr 1994, Pharr et al. 1995), and in cell suspensions, \( Mtd \) transcripts decreased in parallel with MTD activity upon addition of NaCl (Williamson et al. 1995). The present work demonstrates the importance of mannitol as a carbon and energy source in \( O. \) europaea, as well as its role in osmotic protection.

![Fig. 9](https://academic.oup.com/pcp/article-abstract/48/1/42/2468937) Regulation of mannitol transport and metabolism as a mechanism providing salt tolerance in \( O. \) europaea.
Indeed, after a salt pulse, a high percentage of mannitol-grown cells remained viable 24 h after addition of 250 and 500 mM NaCl, contrasting with the dramatic decrease of cell viability in sucrose-grown cells. Similarly, the growth rate of heterotrophic celery cell suspensions cultivated with sucrose was much more inhibited by NaCl than that of mannitol-grown cells, although it was demonstrated that both types of cells accumulated soluble sugars to the same osmotic potential (Pharr et al. 1995). Growing plants on soil with high salinity represents a challenge for the future, and it is therefore important to understand the strategies used by plants to cope with such stress. Given the apparent potential of mannitol for osmoprotection, the engineering of plants with mannitol metabolism is worth investigating.

Although it is clear that mannitol and sorbitol are translocated in phloem from their site of synthesis to their site of use, in a way very similar to sucrose in most plants, there is some controversy about the pathways involved in phloem loading and unloading. In the case of mannitol, unloading pathways have been poorly studied, although carrier-mediated mannitol uptake had been demonstrated in tissue discs and vacuoles of storage parenchyma of celery petioles (Keller and Matile, 1989, Keller 1991, Greutert et al. 1998). In the present work, we showed that OeMaT1 is expressed differentially during olive fruit maturation, suggesting apoplastic unloading. This is in contrast to the initial claim that polyol-transporting species such as the olive tree were symplastic loaders (Flora and Mandore 1993). However, the co-existence of symplastic connections between the sieve tubes and olive pulp cells cannot be ruled out. Thus, mannitol transport by OeMaT1 exerts an important role in the context of olive fruit maturation.

**Materials and Methods**

**Cell suspensions and growth conditions**

Cell suspensions of *O. europaea* L. var. Galega Vulgar were maintained in 250 ml flasks on a rotatory shaker at 100 r.p.m., in the dark, at 25 °C on modified Murashige and Skoog (MS) medium (Murashige and Skoog 1962), supplemented with 1% (w/v) mannitol, 0.5% (w/v) mannitol plus 0.5% (w/v) glucose, or 1% sucrose. Cells were subcultured weekly by transferring 10 ml aliquots into 70 ml of fresh medium. Growth was monitored as described previously (Conde et al. 2006).

**Transport studies of radiolabeled substrates and proton uptake**

Harvested cells were centrifuged, washed twice with ice-cold culture medium without sugar at pH 4.5, and resuspended in the same medium at a final concentration of 5 mg DW ml⁻¹. To estimate the initial uptake rates of D-[1-¹⁴C]mannitol, 1 ml of cell suspension was added to 10 ml flasks, with shaking (100 r.p.m.). After 2 min of incubation, at 25 °C, the reaction was started by the addition of 40 μl of an aqueous solution of radiolabeled sugar-alcohol at the desired specific activity and concentration. The specific activities were defined according to the final concentration of the polyol in the reaction mixture, as follows: 500 d.p.m. nmol⁻¹ (0.1–2 mM), 100 d.p.m. nmol⁻¹ (5–20 mM). Sampling times were 0, 60 and 180 s, time periods during which the uptake was linear. Washing, radioactivity measurements and calculations were performed as described by Conde et al. (2006).

**Competition between labeled substrates and other sugars and polyols was tested by running competitive uptake kinetics.** Inhibition of D-[1-¹⁴C]mannitol transport by non-labeled sugars and polyols was assayed by adding simultaneously the labeled and non-labeled substrate. The concentration range of labeled mannitol varied from 0.1 to 2 mM and the final concentration of the unlabeled substrate was at least 10-fold higher than the *Kₘ* value estimated for the transport system. Competitive inhibition of D-[1-¹⁴C]glucose transport (0.02–0.5 mM) was studied with 20 mM of either mannitol, sorbitol or dulcitol.

**Mannitol-induced proton uptake in the cells was measured as described earlier (Conde et al. 2006).**

**Cloning of a mannitol transporter gene**

To identify potential cDNA sequences encoding mannitol transporters in *O. europaea*, degenerated primers were designed based on conserved regions of plant polyol transporters. The primers were OeY5’ [forward, 5’-TTTATCTTTCAATGA(A/C)TTCC(A/C)-3’] and OeY3’ [reverse, 5’-CAAC(T/C)TCTTCTCA CACA(T/G)CC-3’]. RT–PCR was performed on RNA extracted from suspension-cultured cells exhibiting high mannitol transport activity. The amplified 501 bp cDNA was cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) according to the manufacturer’s instructions, sequenced and subsequently named OeMaT1.

**RNA gel blot analysis**

Total RNAs from olive fruits, harvested at green, cherry and black stages of the ripening process, and from olive cell suspension samples were isolated by phenol extraction and 2 M LiCl precipitation (adapted from Howell and Hull 1978). RNA blot analysis was conducted as described in Conde et al. (2006), using a partial [³²P]OeMaT1 probe.

**Southern blot analysis**

Genomic DNA of *O. europaea* was isolated from olive fruit according to Steenkamp et al. (1994). The DNA (10 μg) was digested with EcoRI, HindIII, XhoI and XhoI restriction enzymes. Digested genomic DNA was separated by electrophoresis in a 0.8% agarose gel, and blotted to a Hybond N membrane (Amersham, Little Chalfont, UK). The membrane was pre-hybridized for 3 h at 55 °C in 250 mM sodium phosphate buffer pH 7.2, 1% bovine serum albumin (BSA), 7% SDS, 1 mM EDTA. The membrane was hybridized overnight at 55 °C in the same buffer containing randomly primed [³²P]OeMaT1 probe (prime-a-gene, Promega). The membrane was washed twice in 2× SSC containing 0.1% SDS for 15 min and once for 5 min in 0.1× SSC and 0.1% SDS at 55 °C. The membrane was exposed to an autoradiographic film and imaged using a Bio-Imaging analyzer (Bio-Rad personal molecular imager FX).

**Determination of cell viability**

FDA and PI double staining was used to estimate cell viability, as described earlier (Jones and Senft 1985). A concentrated stock solution of FDA (500 μg μl⁻¹, Sigma, St Louis, MO, USA) was prepared in dimethylsulfoxide and of PI
(500 µg·µl⁻¹, Sigma) in water. For the double staining protocol, 1 ml of cell suspensions was incubated with 10 µl of FDA stock solution and 1 µl of PI stock solution in the dark for 10 min at room temperature. Cells were observed under a Leica L aborlux S epifluorescence microscope with a 50 W mercury lamp and appropriate filter settings. Images were acquired with a 3CCD color video camera (Sony, DXC-9100P), a frame grabber (IMAGRAPH, IMASCAN/Chroma P) and software for image managing and archiving (AxioVision Version 3.0, Carl Zeiss Vision, Gmbh).

Determination of mannitol dehydrogenase activity

Protein extraction and MTD activity assays were determined as described by Stoop and Pharr (1993). Olea europaea suspension-cultured cells were harvested as described above and ground in a chilled mortar using a 1:4 (v/v) powder:buffer ratio. The extraction buffer contained 50 mM MOPS (pH 7.5), 5 mM MgCl₂, 1 mM EDTA, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% (v/v) Triton X-100. Homogenates were centrifuged at 20,000 × g for 20 min and the supernatants were stored on ice. MTD activity was assayed by monitoring the reduction of NAD⁺ spectrophotometrically at 340 nm. Assays were conducted at room temperature (25°C) in a total volume of 1 ml. The reaction mixture contained 100 mM Bis-Tris propane (pH 9.0), 2 mM NAD⁺, enzyme extract, and D-mannitol at the desired final concentration. The reactions were initiated by the addition of mannitol. Protein concentrations were determined by the method of Bradford (1976) using BSA as a standard.

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