

Running title:

Mannitol in olive adaptation to salinity and drought

Corresponding author:

Hernâni Gerós, Departamento de Biologia

Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Phone: + 351 253 604048

Fax: + 351 253678980

e-mail: geros@bio.uminho.pt

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Mannitol transport and mannitol dehydrogenase activities are coordinated in *Olea europaea* under salt and osmotic stresses

Artur Conde^{1,2}, Paulo Silva^{1,2}, Alice Agasse², Carlos Conde³ and Hernâni Gerós^{1,2}

¹Centro de Investigação e de Tecnologias Agro-Ambientais e Biológicas (CITAB),
Portugal

²Departamento de Biologia, Universidade do Minho, Campus de Gualtar, 4710-057
Braga, Portugal

³IBMC, Instituto de Biologia Molecular e Celular, Universidade do Porto; 4150-180,
Porto, Portugal

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Abstract

The intracellular accumulation of organic compatible solutes functioning as osmoprotectants, such as polyols, is an important response mechanism of several plants to drought and salinity. In *Olea europaea* a mannitol transport system (OeMaT1) was previously characterised as a key player in plant response to salinity. In the present study, heterotrophic sink models, such as olive cell suspensions and fruit tissues, and source leaves were used for analytical, biochemical and molecular studies. The kinetic parameters of mannitol dehydrogenase (MTD) determined in mannitol-growing cells, at 25 °C and pH 9.0, were as follows: K_m , 54.5 mM mannitol and V_{max} , 0.47 $\mu\text{mol h}^{-1} \text{mg}^{-1}$ protein. The corresponding cDNA was cloned and named *OeMTD1*. *OeMTD1* expression was correlated with MTD activity, *OeMaT1* expression and carrier-mediated mannitol transport, in mannitol- and sucrose-growing cells. Furthermore, sucrose-growing cells displayed only residual OeMTD activity, even though high levels of OeMTD1 transcription were observed. There is evidence OeMTD is regulated at both transcriptional and post-transcriptional levels. MTD activity and *OeMTD1* expression were repressed after Na^+ , K^+ and PEG treatments, both in mannitol- and sucrose-growing cells. In contrast, salt and drought significantly increased mannitol transport activity and *OeMaT1* expression. Altogether, these studies support that olive tree copes with salinity and drought by coordinating mannitol transport with intracellular metabolism.

Keywords:

Environmental stress, mannitol, mannitol dehydrogenase, membrane transport, osmoprotectant

Introduction

High salinity and drought reduce plant growth and consequently agricultural productivity more than any other environmental stress (reviewed by Hussain et al. 2008). The negative effects of soil salinity are a consequence of ion cytotoxicity (mostly due to Na^+ , Cl^- and SO_4^-), hyperosmotic stress (reviewed by Zhu, 2002), nutritional imbalance (Grattan and Grieve, 1999) and oxidative stress (Hernández et al. 2001). Plants living in habitats where soil salinity and drought are significant have developed various morphological, physiological and molecular adaptations to cope with these stresses (Winicov, 1998; Munns and Tester, 2008). The intracellular accumulation of organic compatible solutes functioning as osmoprotectants, such as polyols, proline and ammonium quaternary compounds represents an important metabolic adjustment of several stress-tolerant plants (Rathinasabapathi, 2000).

Polyols are the reduced form of aldoses and ketoses and can be found in all living forms (Noiraud et al. 2001a). In some plants these sugar alcohols are, together with sucrose (as in celery), or raffinose saccharides (as in olive), direct products of photosynthesis and serve similar functions as translocation of carbon skeletons and energy between source and sink organs. Mannitol is the most common polyol in nature and has been observed in over 100 vascular plant species of several families including the Apiaceae (celery, parsley, carrot), Rubiaceae (coffee) and Oleaceae (olive, privet) (reviewed by Conde et al. 2008). Mannitol synthesis takes place in mature leaves from mannose-6-phosphate by the joint action of a NADPH-dependent mannose-6-phosphate reductase (M6PR) and a mannose-6-phosphate phosphatase. It is then translocated through the phloem to heterotrophic sink tissues where it can be either stored or oxidised to mannose through a NAD^+ -dependent mannitol dehydrogenase (MTD) and

used as carbon and energy source (reviewed by Stoop et al. 1996; Noiraud et al. 2001b; Conde et al. 2008).

Olive is an evergreen tree traditionally cultivated in the Mediterranean basin (Loumou and Giourga, 2003) where summer months are characterised by high temperatures, high light levels, high vapour pressure deficit (VPD) and lack of precipitation. Under these climate conditions, often combined with an increasing salinisation of the soils, olives have to cope with limited water availability (Angelopoulos et al. 1996). Mannitol is one of the primary photosynthetic products in *O. europaea* (Drossopoulos and Niavis 1988; Cataldi et al. 2000) and its content in olive pulp increases during maturation reaching values of 8 mg g⁻¹ DW in the full ripe fruit (Marsilio et al. 2001). The elucidation of the role of mannitol as carbon and energy source for plant growth, as well as a protecting solute against biotic and abiotic stresses assumes critical importance towards the enhancement of yield potential of *O. europaea* and other plants. Indeed, significant knowledge has been achieved in understanding the protective role of mannitol in several plants, including *O. europaea* (Yancey et al. 1982; Tarczynsky et al. 1993; Williamson et al. 1995; Shen et al. 1997b; Jennings et al. 1998, Patonnier et al. 1999; Hu et al. 2005; Voegelé et al. 2005; Conde et al. 2007a; Seckin et al. 2009).

During leaf maturation in celery, the proportion of mannitol increases dramatically, and mannitol exported to the phloem and sink tissues is directly correlated to the increased mannitol biosynthesis whereas sucrose remains fairly constant (Davis et al. 1988). Due to the similarities between celery and olive regarding the use of mannitol, this is expected to be also the case in *O. europaea*. In celery, there is a close relationship between the development of photosynthetic capacity, mannitol synthesis, and M6PR activity (Everard et al. 1993; 1997). Root-zone salinity increased the activity of M6PR

up to 6-fold in celery leaves and promoted changes in photosynthetic carbon partitioning from sucrose to mannitol facilitating its accumulation in leaf tissues, providing improved stress tolerance (Everard et al. 1994). Similarly, in peach (*Prunus persica*), the *in vitro* activity of aldose-6-phosphate reductase, the key enzyme in sorbitol synthesis, increased linearly in response to drought stress, as did the partitioning of newly-fixed carbon into sorbitol and its extrusion and concentration in the phloem sap (Escobar-Gutiérrez et al. 1998). A significant shift in photosynthetic carbon partitioning to mannitol under salt or drought stress has also been clearly demonstrated in leaves of *O. europaea* confirming the role of mannitol as a potential osmoregulator in leaf mesophyll (Tattini et al. 1996; Gucci et al. 1996; 1997; 1998; Dichio et al. 2009; Melgar et al. 2009; Remorini et al. 2009; Cimato et al. 2010).

Studies conducted in our laboratory have yielded some information concerning the biochemical and molecular mechanisms involved in sugar and polyol uptake and metabolism in olive (Oliveira et al. 2002; Conde et al. 2007a, Conde et al. 2007b; Conde et al. 2007c). In particular, the relevance of mannitol transport regulation for osmotic adjustments of *O. europaea* cells in response to NaCl stress was addressed (Conde et al. 2007a). It was shown that the addition of 500 mM NaCl promoted the increase of *OeMaT1* (*Olea europaea* Mannitol Transporter 1) transcription and mannitol transport activity over time when compared with control cells. The parallel between V_{\max} of mannitol transport and *OeMaT1* message levels supported that the expression of *OeMaT1* is responsible for the increase of mannitol transport capacity under salt stress conditions.

In the present study, we performed a detailed biochemical characterisation of Olive NAD⁺-dependent mannitol dehydrogenase, identified its respective potential encoding cDNA (*OeMTD1*), and provided a solid body of evidence that indicates that

its activity is tightly coordinated with mannitol transport capacity in order to regulate the cellular mannitol pool, which proved to be critical for olive cells to cope with salinity and drought conditions.

Results

1- Mannitol dehydrogenase activity, OeMTD1 cloning and expression and coordination with mannitol transport activity and OeMaT1 expression

The activity of mannitol dehydrogenase (MTD) measured in homogenates from 2%-mannitol-growing cells of *O. europaea* is in line with a previous kinetic characterisation (Conde et al. 2007a). In the present study, MTD activity was determined in mannitol-growing cells at different temperatures, ranging from 20°C to 50°C, and pH values, ranging from 7.5 to 10.5, and subsequently compared with the activity measured at 25°C and pH 9 in homogenates from 2%-sucrose-growing cells (Figure 1), allowing for the subsequent correlation with gene expression in both growth conditions. The kinetic parameters, at 25 °C and pH 9.0, were as follows: K_m , 54.5 mM mannitol and V_{max} , 0.47 $\mu\text{mol h}^{-1} \text{mg}^{-1}$ protein. Temperature and pH dramatically affect enzyme activity, the V_{max} being highest at pH 10 and 45°C. An activation energy value of 68.9 kJ mol^{-1} was estimated (data not shown).

The cloning of an 816 bp cDNA named *OeMTD1* (accession nr. ABR31791.1) allowed us to confirm by means of CLUSTAL W alignment its high similarity with other plant mannitol dehydrogenases (Figure 2). Although we cannot exclude that other mannitol dehydrogenase isoforms may operate in *Olea europaea* that could, at least theoretically, cross-hybridise with the probe in the gene expression studies, the signals observed in northern experiments were associated with *OeMTD1* expression. However,

it is well demonstrated that only one isoform of MTD exists in celery, mainly located in the cytoplasm and to a lesser extent in the nucleus, or secreted as in other plants (Williamson et al. 1995; Stoop et al. 1995; Stoop et al. 1996; Yamamoto et al. 1997; Zamski et al. 2001; Blackburn et al. 2010).

Figure 3 depicts the correlation between *OeMTD1* and *OeMaT1* transcript levels and mannitol transport activity throughout cell growth with 0.5% (w/v) mannitol (initial concentration) as carbon and energy source. In terms of mannitol availability in the extracellular medium, these conditions are equivalent to those from the mid-late exponential growth phase to the stationary phase in cultures, with an initial mannitol concentration of 2%. Results indicate that cells keep *OeMaT1* transcription at basal levels when mannitol is present in measurable amounts, in line with previous work by Conde et al. (2007a). At the fourth day, when the external concentration of mannitol fell below approx. 0.1% (w/v), the activity of the polyol transport system increased and its maximal activity was reached at the day 6, when mannitol was completely exhausted from the culture medium. Although high mannitol levels seem to repress carrier-mediated mannitol transport, a different correlation is observed for *OeMTD1* transcription because *OeMTD1* expression decreases together with the substrate levels in the culture, from day 1 to day 5.

2- Mannitol transport and metabolism under salt and drought stresses in mannitol- and sucrose-growing cells

Mannitol transport activity and MTD activity were correlated with *OeMaT1* and *OeMTD1* expression under salt (NaCl or KCL) and drought (PEG) stress both in 2%-mannitol-growing cells and 2%-sucrose-growing cells, which were collected at the mid-late exponential growth phase. Results showed that non-treated 2%-mannitol-growing

cells (control *mannitol*-cells) display basal mannitol transport activity and *OeMaT1* transcript levels, together with a high MTD activity and high level of *OeMTD1* transcription (Figure 4), in accordance with the data from figure 3 (day 1-4 after sub-culture). Conversely, in non-treated sucrose-growing cells (control *sucrose*-cells), both the mannitol transporter and MTD activities are kept at basal levels, but *OeMTD* expression is high (Figure 4).

The addition of 250 mM NaCl to mannitol-growing cells caused a severe decrease in MTD activity associated with a repression of *OeMTD1* transcription. A similar repression of *OeMTD1* transcription was observed after KCl addition, but the enzyme activity was not completely repressed ($V_{\max} = 1.3 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ D.W.}$) (Figure 4). In contrast, mannitol transport activity paralleled the high increase of *OeMaT1* transcript levels in both situations. To mimic drought conditions without the ionic cytotoxic component, 250 mM PEG was added to the culture. PEG caused mannitol-growing cells to repress *OeMTD1* transcription, and consequently MTD activity was also reduced to non-detectable levels. However the up-regulation of *OeMaT1* expression and activity was less significant than under salt-stress conditions. In agreement with these results, fluorescence microscopy analysis revealed that both NaCl and KCl induced loss of cell viability within 24 h, as assessed by fluorescein diacetate (FDA) and propidium iodide (PI). Sucrose-growing cells, however, were shown to be much more sensitive than mannitol-growing cells to the deleterious effect of both salts (not shown).

When the effects of NaCl, KCl and PEG were evaluated in sucrose-growing cells there was also a substantial increase of mannitol transport activity and of *OeMaT1* transcript levels, together with a decrease of MTD activity from basal levels to total repression. *OeMTD1* transcription was strongly repressed in all experimental stress conditions. Moreover, mannitol addition substantially protected sucrose-growing cells

from the deleterious effect of NaCl: the reduction of cell viability in cells incubated in the presence of 500 mM NaCl during 24 h (> 90%) diminished in cells incubated with 500 mM NaCl + 50 mM mannitol (~50%), as assessed after cell incubation with the fluorescent dyes FDA and PI (Figure 5).

Discussion

This study provides a description of how the coordination between the mannitol transport and oxidation steps operating in olive suspension-cultured cells is crucial for salt and drought stress tolerance in *O. europaea*. A wealth of information is already available on the involvement of mannitol or other solutes in abiotic stress plant tolerance (see Introduction section), whereas much less is known about the regulation of transport activity and metabolism in relation to gene expression and its contribute in the tolerance process. The drawback is that plant organs are often not accessible to such studies. The use of suspension-cultured cells to study mannitol transport and oxidation and its regulatory mechanisms offers a number of distinct advantages over the intact plant where bulk diffusion, tissue penetration barriers and cell heterogeneity impair kinetic studies. Moreover, in suspension-cultured cells, the plasma membrane is readily amenable for coping with exogenous substrates such as mannitol or sucrose, which are abundant photoassimilates in *O. europaea* leaves and fruits. Despite the necessary cautions needed to extrapolate the results to a tissue or whole-plant level, cell suspensions provide a useful and convenient experimental system that has already yielded a lot of information that matched observations outside the laboratory scale in actual field conditions. Extremely useful information on sugar transport or metabolic mechanisms and its regulation (Roitsch and Tanner, 1994; Ehness and Roitsch, 1997; Oliveira et al. 2002; Cakir et al. 2003; Conde et al. 2006; Conde et al. 2007a), as well as

in other key physiological processes, such as hormonal signaling (Cakir et al. 2003), cell cycle (Riou-Khamlichi et al. 1999) and regulation of gene expression (Graham et al. 1994; Cheng et al. 1999) has been obtained using this model, thus reinforcing that our observations in olive cell suspensions are indeed physiologically relevant. In line with this assumption, both *OeMaT* and *OeMTD* expression was observed in olive fruits (not shown) during ripening where they should play an important role in mannitol transport and metabolism.

In heterotrophic suspension-cultured cells of *O. europaea*, carrier-mediated mannitol uptake occurs through a 1:1 polyol:H⁺ symport system (K_m of 1.3 mM mannitol). *OeMaT1* is down-regulated by high levels of mannitol and up-regulated by high salinity (Conde et al. 2007a). Following transmembrane transport, oxidation to mannose catalysed by an NAD⁺-dependent mannitol dehydrogenase (MTD) is the first step of the mannitol metabolism in heterotrophic tissues (Stoop et al. 1996). MTD activity measured in homogenates from cultured cells grown with mannitol was substantially higher (up to 10-fold) than in homogenates from sucrose-growing cells, but the transcription of *OeMTD1* was high in both conditions (Figure 1 and figure 5). Moreover, in mannitol-growing cells the expression of *OeMTD1* was completely repressed when mannitol in the culture medium declined to undetectable values, at the end of the exponential growth phase (Figure 3). These data strongly suggest that mannitol dehydrogenase activity is up-regulated by its own substrate, at a transcriptional or post-transcriptional level. The significantly higher MTD activity in mannitol-growing cells when compared to sucrose-growing cells is consistent with the observations of Stoop and Pharr (1993), although in celery this increment was only up to 4-fold. The same group observed that the repression of MTD activity by sucrose was linked to a down-regulation of MTD transcription (Pharr et al. 1995). The high amount

of *OeMTD1* transcripts found in sucrose-growing cells suggests that *OeMTD1* activity may also be negatively regulated by sucrose at the protein level.

The K_m of *OeMTD1* is physiologically similar to the one of the MTD of celery roots (72 mM mannitol). The specific activity of *OeMTD1* is very close to the $0.62 \mu\text{mol h}^{-1} \text{mg}^{-1}$ protein determined for the celery MTD in crude extracts of suspension-cultured cells and substantially different than the $0.8 \mu\text{mol h}^{-1} \text{mg}^{-1}$ protein displayed in celery roots (Stoop and Pharr, 1992; Stoop et al. 1995). Moreover, the V_{max} observed for *OeMTD1* is very similar to the one of the *Vicia faba* enzyme in normal conditions (Voegelé et al. 2005). The negative correlation observed in Figure 3 between *OeMaT1* and *OeMTD1* transcription suggested that the availability of the enzyme is higher at lower amounts of intracellular mannitol. However, it must be stressed that in a mannitol-rich medium, while *OeMaT1* expression and carrier-mediated mannitol transport activity are maintained at basal levels, energy-independent diffusional uptake is the preferred mode of mannitol absorption, as shown before (Conde et al. 2007a). The nature of this non-saturable mechanism widely reported in the literature is still poorly understood, but the involvement of channel-like proteins has been recently proposed (Conde et al. 2007b; Conde et al. 2010). This low affinity and high capacity mode of mannitol transport ensures high mannitol concentrations that sustain an elevated MTD activity (resulting from high *OeMTD1* transcription), which converts mannitol to mannose in order to feed the glycolytic pathway of exponentially growing cells. As external mannitol decreases to residual levels, the linear transport component no longer sustains mannitol uptake at a rate sufficient to allow cell growth/maintenance, and the involvement of a concentrative, energy-dependent transport system becomes critical. In these conditions *OeMaT* transcript levels and polyol:H⁺ symport activity increase, but the net amounts of intracellular mannitol are probably lower, which may explain the

decrease observed in *OeMTD1* expression. However, we may not exclude that at this stage substantial amounts of intracellular mannitol are still present as well as a sustained biochemical activity of OeMTD1, depending on the turnover rate of the protein.

The analysis of Figure 4 regarding the activity of OeMTD and *OeMTD* expression in control cells cultivated with mannitol and sucrose as sole carbon and energy sources suggests that OeMTD is regulated at both transcriptional and post-transcriptional level. Remarkably, gene transcription was completely abolished, and consequently a strong reduction of mannitol oxidation activity was observed after salt, K^+ and PEG treatments, both in mannitol- and sucrose-growing cells, suggesting that these stress factors inhibit MTD at the transcriptional level.

Carrier-mediated mannitol transport was up-regulated at a transcriptional level by NaCl, KCl, and PEG in both mannitol-growing cells and sucrose-growing cells. In all the experimental conditions there was an increase of the transcript levels of *OeMat1* and an increase of the V_{max} of carrier-mediated mannitol transport over the control that paralleled the repression of OeMTD. Taken together, these observations suggest that olive cells display an integrated response that leads to a nearly universal reaction to salt and osmotic stresses: intracellular osmolyte accumulation. This is thought to allow osmotic adjustment in order to compensate for the decrease of external water potential and allow for oxidative detoxification. As this highly integrated response occurred even in the absence of extracellular mannitol, the stress signalling pathways involved are epistatic over sugar-mediated regulation of gene expression. Thus, mannitol addition to sucrose-growing cells substantially alleviated the damage caused by salt, as shown by fluorescence microscopy with vital fluorescent stains.

An important question that needs further investigation regards the protective role of mannitol relatively to its intracellular compartmentation. That is, how could mannitol

protect the cells if a significant part of it is compartmented in the vacuole in normal conditions? In common ivy (*Hedera helix*), the branched-chain sugar alcohol hamamelitol is predominantly present in the vacuole of mesophyll cells and in lower levels in the cytosol, but it undergoes a significant redistribution from the vacuole to the cytosol under drought stress (Moore et al. 1997). Moreover, in other plants such as celery, peach, and *Plantago major* (common plantain) mannitol and other sugar alcohols like sorbitol are present in significant quantities in the cytosol, vacuole and even stroma of mesophyll cells (Keller and Matile, 1989; Nadwodnik and Lohaus 2008). Despite the shared cytosolic and vacuolar presence of mannitol in a mannitol-synthesizing plant like celery, which may also be the case in *O. europaea*, immunolocalization of mannitol dehydrogenase (MTD) in both celery suspension-cultured cells and plants showed that MTD is primarily a cytoplasmic enzyme, with a less significant presence observed in the nucleus (Zamski et al. 1996; Yamamoto et al. 1997). This fact may suggest and reinforce that mannitol is clearly present in high levels in the cytosol of cells from mannitol-producing plant species, so that it can be readily oxidized in its metabolic pathway for energy gain. This seems also to be inferred from several other studies on celery (Williamson et al. 1995; Stoop et al. 1995; Stoop et al. 1996; Yamamoto et al. 1997; Zamski et al. 2001; Cheng et al. 2009). Nonetheless, if a vacuolar localisation of mannitol significantly occurs in olive cells under normal conditions, there is no doubt that rapid and efficient mechanisms providing its translocation to the cytosol are present at the tonoplast, so that mannitol can be here accumulated to act as osmoregulator and osmoprotectant under abiotic stress. In future experiments, it would be undoubtedly interesting to study in more focus the compartmentalisation of mannitol in olive cells, and, in particular, an eventual redistribution in its subcellular localisation in response to osmotic stress.

In conclusion, in the present study conducted with heterotrophic cells as sink models, we have studied how cells coordinate the activity of mannitol/H⁺ symporter with the activity of NAD⁺-dependent mannitol dehydrogenase at gene expression and protein activity level under salinity and drought stress. By adjusting mannitol transport and intracellular metabolism, the olive tree should be able to cope with increased salinity and drought stress, typical of the Mediterranean basin (Table 1). In agreement, the large increase in mannitol concentration in salt-treated leaves of *O. europaea* indicates that it may function primarily as a compatible solute, like in salinity adaptation of *P. latifolia* (Tattini et al. 2002). However, in *O. europaea* mannitol may also function as an antioxidant osmoprotectant against oxidative stress resulting from salt/drought stress and even solar irradiance (Melgar et al. 2009; Remorini et al. 2009, Cimato et al. 2010). Mannitol accumulation has been recently suggested to protect salt-treated leaves in full sunshine from heat stress-induced oxidative damage to a greater extent than leaves growing under partial shading (Cimato et al. 2010). Concordantly, mannitol has been described as a potent scavenger of hydroxyl radicals that may be resultant from salt or drought stress (Shen et al. 1997a; 1997b). In plants, it is suggested that the antioxidant function of mannitol may be to shield susceptible thiol-regulated enzymes like phosphoribulokinase (PRK), thioredoxin, ferredoxin, and glutathione from inactivation by hydroxyl radicals in plants (Shen et al. 1997b). Thus, accumulation of mannitol may have dual functions: providing osmotic adjustment and supporting redox control (Shen et al. 1999). In the case of *O. europaea*, and under the lights of our results, the coordination of mannitol transport via OeMaT1 with the significantly lowered mannitol oxidation via OeMTD1 suggests that the mannitol function in salt or drought stress tolerance (whether resulting from excessive concentration of Na⁺ or K⁺ ions, or from actual low water availability) is mainly as osmoregulator, but a function as

osmoprotectant against oxidative damage is also perfectly plausible. However, it is not yet completely clear if the abiotic stress-induced accumulation of such osmoprotectant compounds may by itself allow an increased tolerance directly, or if it is just a participant in a much more complex and intricate mechanism of stress-tolerance by acting synergistically with other key intervenients. Given the hugely importance of mannitol accumulation in abiotic stress tolerance, a coordination of mannitol transport and oxidation allowing that osmoregulator/antioxidant pool is extremely logical in sink cells and its tight regulation is critical, as demonstrated in the present work. The continued study of the signalling pathways responsible for responses to salt, drought and other abiotic stresses may allow for the treatment of plants with exogenous compounds, such as mannitol and other osmoprotectants and antioxidants, without recurring to genetic manipulation, thus avoiding the introduction of genetically manipulated plants in nature. The protective role of mannitol when added to salt stressed sucrose-growing cells of *O. europaea* (present study) or its enhancing effect on several antioxidant enzymes of wheat, which does not produce mannitol, under high salinity conditions (Seckin et al. 2009) confirms that this research topic requires continuous investment from the scientific community.

Materials and methods

Cell suspensions and growth conditions

Cell suspensions of *O. europaea* L. var. Galega Vulgar were grown in 250 ml flasks on a 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 0.5% or 2% (w/v) mannitol or 2% (w/v) sucrose. Cells were sub-cultured weekly by transferring 10 ml

aliquots to 70 ml of fresh medium. Cell growth was assayed as previously described (Conde et al. 2006). Salt (and consequently osmotic) stress was induced by addition of NaCl or KCl at final concentrations of 250 mM. To mimic drought conditions without the ionic cytotoxic component polyethylene glycol (PEG) was added to provide the osmotic potential in MPa reached with 250 mM NaCl. The stress treatments were carried out for a 24h period at the mid-exponential growth phase of the cells.

Determination of the Mannitol Dehydrogenase (OeMTD1) activity

Protein extraction and OeMTD1 activity determination was assessed as described by Stoop and Pharr (1993). *O. europaea* suspension-cultured cells were harvested as described above and ground with a chilled mortar and pestle in an approximately 1:1 (v/v) powder:buffer ratio. The protein extraction buffer contained 50 mM MOPS (pH 7.5), 5 mM MgCl₂, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT) and 1% (v/v) Triton X-100. The homogenates were then centrifuged at 20000x g for 20 min and the supernatants were maintained on ice and used in the enzymatic assays. MTD activity assays, except for the temperature effect evaluation, were performed at room temperature (25°C), in a total volume of 1 ml. The reaction mixture contained enzyme extract, 100 mM Bis-Tris propane (pH 9.0, or the desired one in the case of the pH effect evaluation), 2 mM NAD⁺, and D-mannitol at the desired final concentration. The reduction of NAD⁺ was evaluated spectrophotometrically at 340 nm. All reactions were initiated by the addition of mannitol. For the assessment of the effects of temperature and pH on OeMTD1 activity, 200 mM of D-mannitol were used to ensure the V_{\max} of the enzyme. Total protein concentrations of the extracts were determined by the method of Bradford (1976) using BSA as a standard.

Cloning of the *O. europaea* mannitol dehydrogenase gene *OeMTD1*

In order to identify and clone putative cDNA sequences encoding mannitol dehydrogenases in *O. europaea*, conserved regions of several plant mannitol dehydrogenases were identified and subsequently used to design degenerated primers. RT-PCR was performed on mRNA extracted from suspension-cultured cells grown with 2% (w/v) mannitol as carbon and energy source. An 816 bp cDNA sequence was amplified and cloned into pGEM T-Easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions and subsequently sequenced and submitted to GenBank (accession nr. ABR31791.1) and named *OeMTD1*.

Transport studies with radiolabelled mannitol

Harvested cells were centrifuged, washed twice with ice-cold culture medium without sugar at pH 4.5, and re-suspended in the same medium at a final concentration of 5 mg DW ml⁻¹. To estimate the initial uptake rates of D-[¹⁴C]mannitol, 1 ml of cell suspension was added to 10 ml flasks, with shaking at 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 radiolabelled mannitol 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). Washing, radioactivity measurements and calculations were performed as described by Conde et al. (2006).

RNA gel blot analysis

Total RNAs from olive suspension-cultured cells were obtained by phenol extraction combined with a 2 M LiCl precipitation step (adapted from Howell and Hull 1978). RNA blot analysis was performed for both *OeMaT1* and *OeMTD1* as described by Conde et al. (2006), using partial [³²P]*OeMaT1* and [³²P]*OeMTD1* probes, respectively.

Mannitol quantification by High Performance Liquid Chromatography (HPLC) analysis

The quantification of mannitol in the culture medium was performed in a HPLC system from Gibson (132 RI Detector) using a Hyperrez H⁺ column (Hypersil), at a flow rate of 0.5 ml min⁻¹, with 2.5 mM H₂SO₄ as the mobile phase. Before each experiment, the column was balanced for 30 min at 30 °C. The standard solution contained glucose, sucrose, fructose, raffinose and acetate (internal standard), all at 0.5% (w/v). The samples were diluted 1:1 in an acetate solution of 1% (w/v), and 25 µL of sample and standard solutions were injected in the column.

Determination of cell viability

FDA and PI double staining was used to evaluate cell viability, as described in Jones and Senft (1985). Stock solutions of FDA (500 mg ml⁻¹, Sigma St Louis, MO, USA) and PI (500 mg ml⁻¹, Sigma) were prepared in dimethylsulfoxide and water, respectively. 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 for 10 min at room temperature in the dark. Stained cells were observed under a Leitz Laborlux S epifluorescence microscope with a 50W 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 management and archiving (AxioVision Version 3.0, Carl Zeiss Vision, GmbH).

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Table 1: *O. europaea* copes with salt and osmotic stresses by adjusting mannitol transport and metabolism as pinpointed in the present study and several previous publications (see the references in the text).

	no salt/drought	salt/drought
Source tissues	important photoassimilate	increased mannitol synthesis
Sink tissues	stored sugar; carbon and energy source	Osmolyte/antioxidant (increased transport; decreased oxidation)

Figures

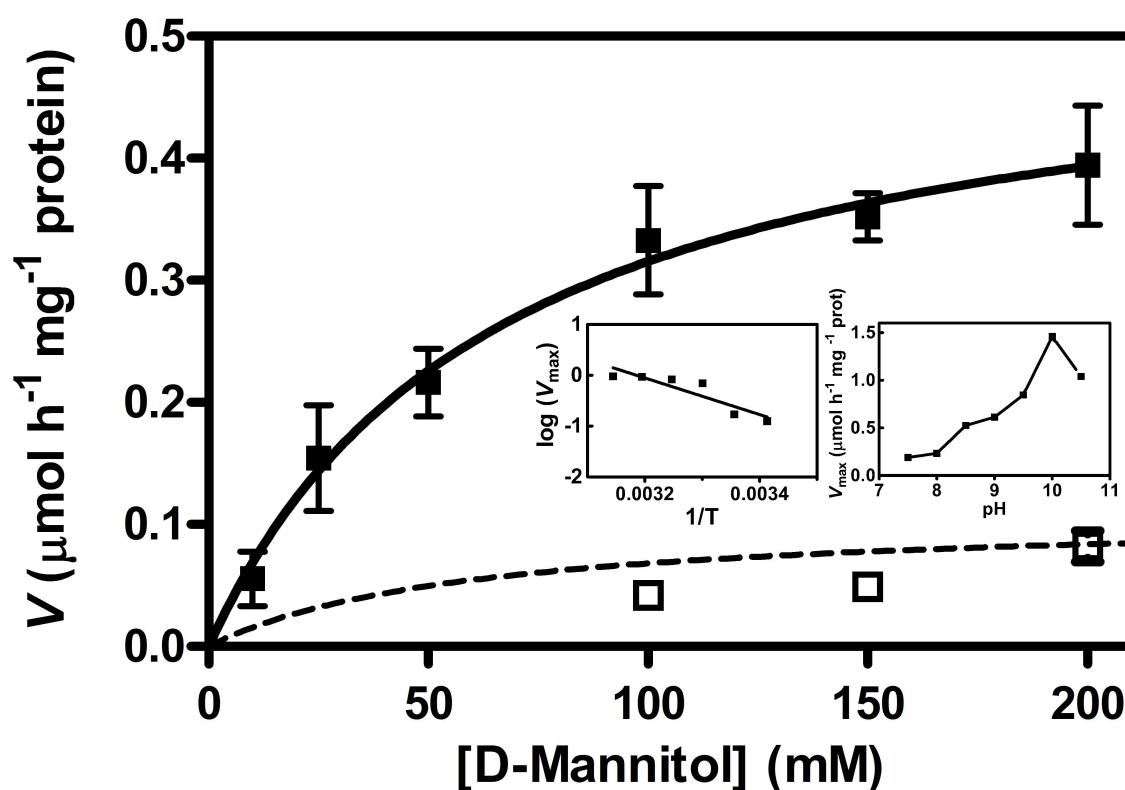


Figure 1. Mannitol dehydrogenase (MTD) activity, measured at 25 °C and pH 9.0, in extracts of *O. europaea* suspension-cultured cells cultivated up to the mid-exponential growth phase with 2% (w/v) mannitol (closed squares) and 2% (w/v) sucrose (open squares). Insets: dependence of V_{\max} of mannitol oxidation in extracts of mannitol-growing cells on the temperature of the assay medium, at pH 9.0 (left graph) and dependence of V_{\max} on pH, at 25 °C (right graph). Expression of *OeMTD1* (*Olea europaea* mannitol dehydrogenase 1) in both experimental conditions is depicted in figure 4. Error bars denote SD from the mean, n=3.

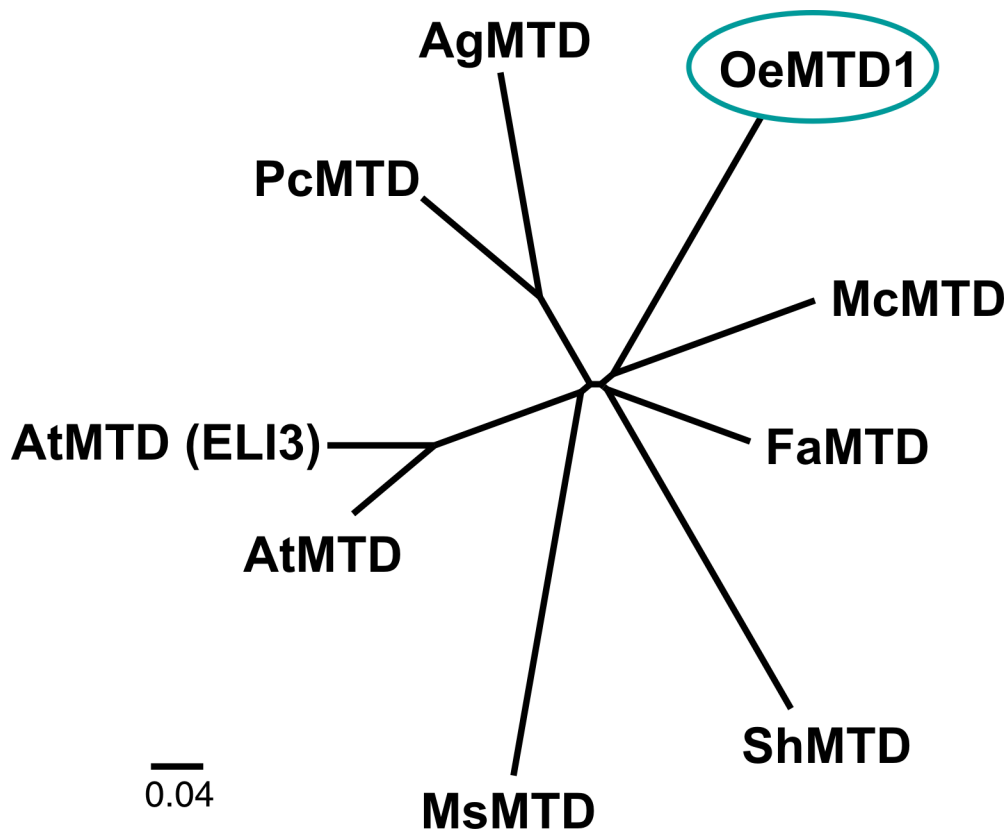


Figure 2. Phylogenetic tree showing the relation between *OeMTD1* and other mannitol dehydrogenase genes from other plants. Sequence analysis, multiple sequence alignments (using the ClustalW algorithm) and the unrooted phylogenetic tree were performed using the Phylip 3.68 software package, and the final tree was built using the FigTree 1.1.2. software package. GenBank accession numbers are as follows: *Olea europaea* mannitol dehydrogenase (*OeMTD1*), ABR31791.1; *Apium graveolens* mannitol dehydrogenase (*AgMTD*), 2117420A; *Petroselinum crispum* mannitol dehydrogenase (*PcMTD*), P42754.1; *Fragaria ananassa* mannitol dehydrogenase (*FaMTD*), Q9ZRF1; *Mesembryanthemum crystallinum* (*McMTD*), P93257; *Stylosanthes humilis* mannitol dehydrogenase (*ShMTD*), Q43137.1; *Medicago sativa* mannitol dehydrogenase (*MsMTD*), O82515.1; *Arabidopsis thaliana* putative mannitol dehydrogenase (*AtMTD*), also known as cinnamyl-alcohol dehydrogenase 8 (*AtCAD8*), Q02972; and *Arabidopsis thaliana* putative mannitol dehydrogenase (*AtMTD*) elicitor-activated 3 (*ELI3*), NP_001031805.1.

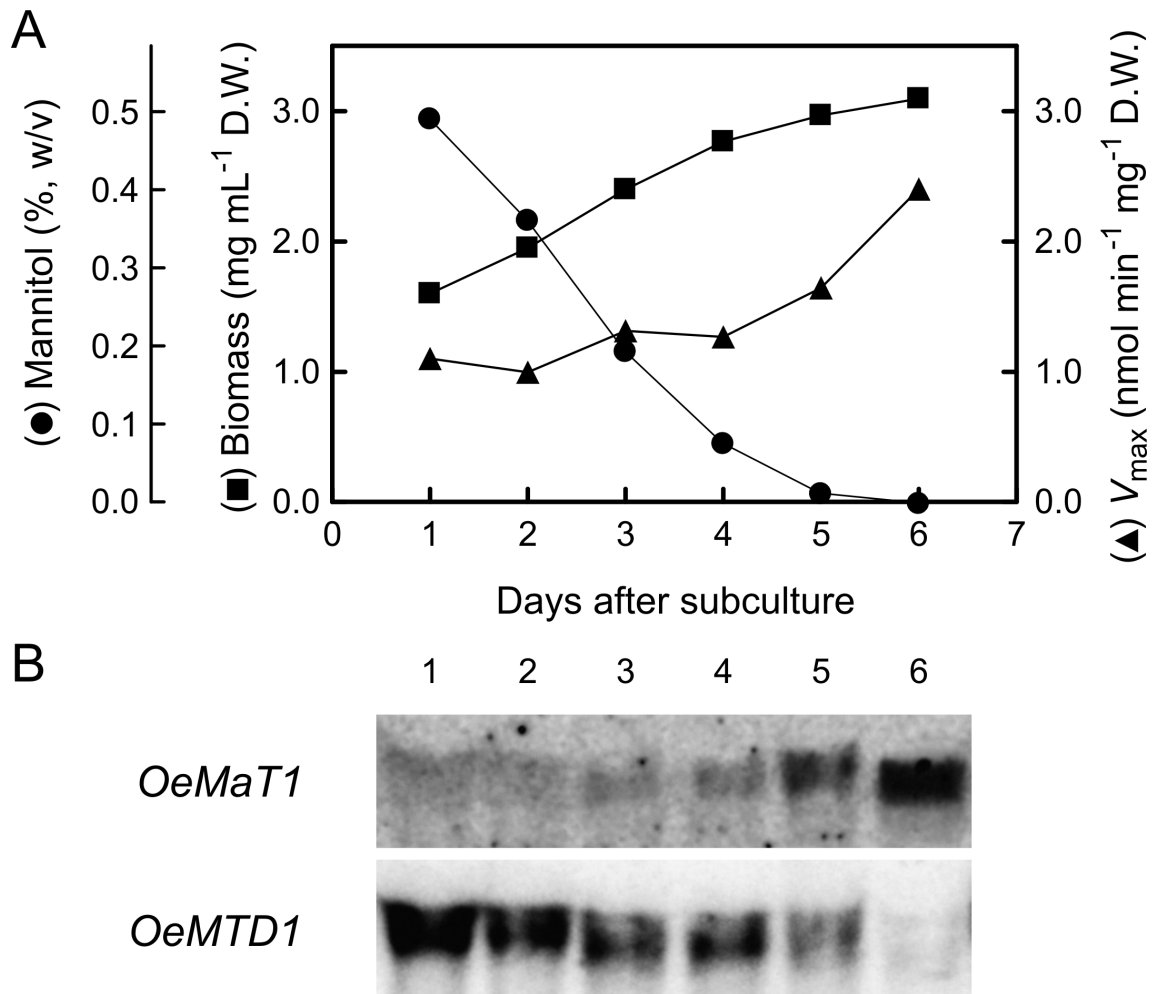


Figure 3. Growth, mannitol/H⁺ symport activity and *OeMaT1* and *OeMTD1* expression in *O. europaea* cell suspensions. (A) Cells were cultivated with 0.5% (w/v) mannitol (initial concentration), and D-[¹⁴C]mannitol uptake (closed triangles) was measured in cell aliquots harvested from the culture at the times indicated. Values are represented by the mean of two independent experiments. (B) Northern blot analysis of *OeMaT* and *OeMTD1* expression. In each condition 50 µg of RNA were used as in Conde et al. (2007a).

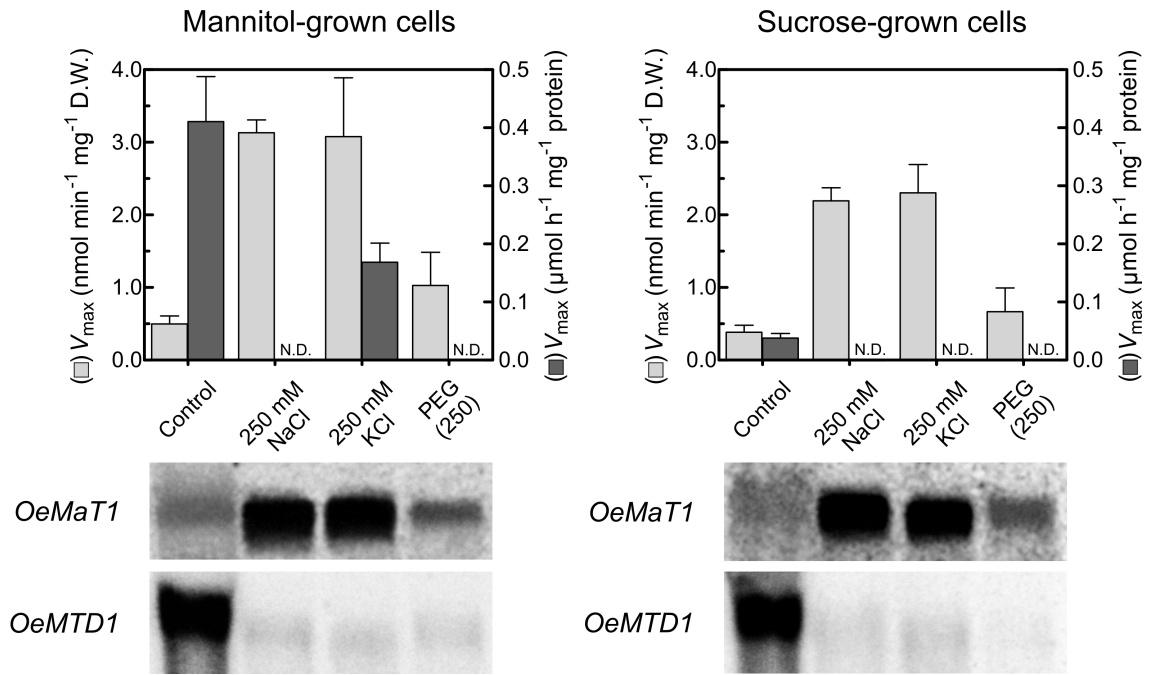


Figure 4. Effect of NaCl, KCl, PEG on the V_{max} of mannitol transport (■) and the V_{max} of mannitol oxidation (■) in mannitol grown cells and sucrose-growing cells of *O. europaea*, and expression of *OeMaT1* and *OeMTD1*. Cells were collected at mid-exponential growth phase and subjected to salt and drought treatment during 24 h. Error bars denote SD from the mean, n=3. In each lane 50 μ g of RNA were used.

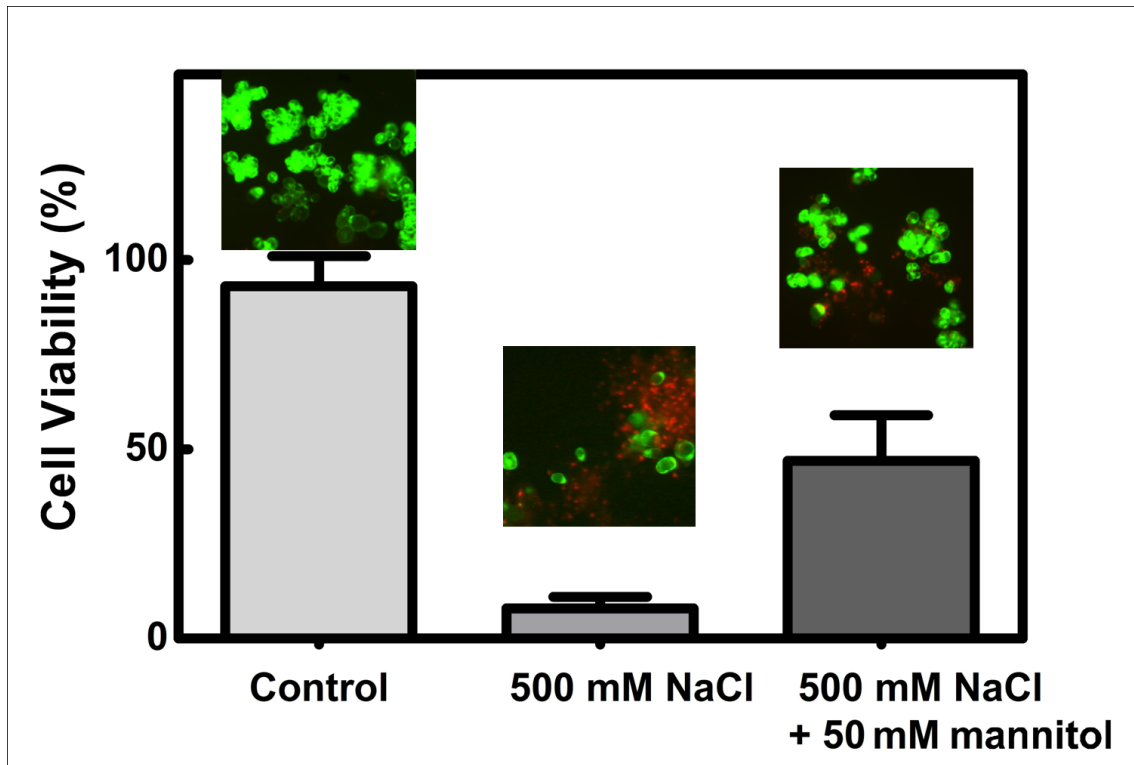


Figure 5. Protective role of mannitol from the deleterious effect of NaCl in *O. europaea*. Cell viability assays in suspension-cultured cells cultivated with sucrose in the absence of salt (control), 24 h after the addition of 500 mM NaCl, and 24 h after the addition of 500 mM NaCl + 50 mM mannitol. Fluorescence was measured after incubation with fluorescein diacetate (FDA, green fluorescence) and propidium iodide (PI, red fluorescence).