Utilization and transport of mannitol in *Olea europaea* and their implications on salt stress tolerance

C. Conde[1], P. Silva[1], A. Agasse[2], R. Lemoine[2], S. Delrot[2], R. M. Tavares[1] and H. Gerós[1]

[1]Centro de Biologia | Departamento de Biologia | Universidade do Minho | Campus de Gualtar | Braga | Portugal
[2]Laboratoire de Physiologie et Biochimie Végétales | Bâtiment Botanique | Université de Poitiers | Poitiers | France

INTRODUCTION

Polyols (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, polyols are direct products of photosynthesis in mature leaves in parallel with sucrose (Noiraud *et al.*, 2001). They serve similar functions such as translocation of carbon skeletons and energy between autotrophic source tissues and heterotrophic sink organs. Mannitol, the most widely distributed alditol, is present in over 100 higher plant species.

Olea europaea L. represents an important and widespread crop in the Mediterranean basin. Mannitol is one of the main soluble components in olive tissues and plays important roles, acting as an osmoprotectant in salt and water stress tolerance and providing a carbon and energy source for sink tissues (Marsilio *et al.*, 2001). In this way, membrane transport of mannitol is a major determinant of olive tree growth and productivity. The aim of this work was to contribute for the elucidation of the biochemical mechanisms associated with the transport and utilization of mannitol in *O. europaea* using heterotrophic cell suspensions as biological model.

RESULTS

Growth in batch cultures with mannitol and glucose

Figure 1 depicts the growth of *O. europaea* cell suspensions with 1% mannitol (Fig.1A) and 0.5% mannitol plus 0.5% glucose (Fig.1B), and the consumption of the carbon and energy sources from the medium. Mannitol specific transfer rate (q), estimated as the ratio μ_{max}/Y from Figure 1A, is compared in Table 1 with data from mannitol uptake and intracellular mannitol oxidation. The utilization of mannitol by heterotrophic cell suspensions of *O. europaea* requires its transport across the plasma membrane, which, in turn, may constitute an important step for the control of cell growth (Oliveira *et al.*, 2002).
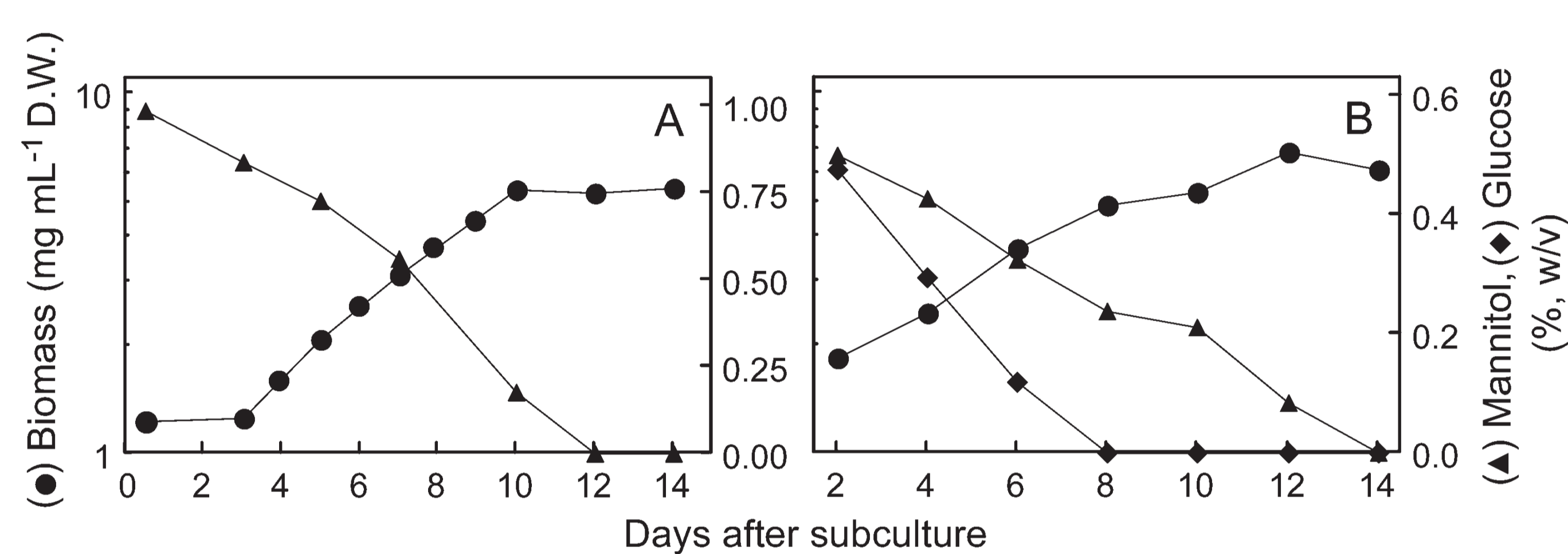

Figure 1. Dry weight and glucose and mannitol concentration in suspension cultured cells of *O. europaea* grown in MS medium with 1% (w/v) mannitol (A) and 0.5% glucose plus 0.5% mannitol (B).

Mannitol uptake experiments

In *O. europaea* cell suspensions harvested at the end of exponential growth phase, initial uptake rates of D-[14C]mannitol followed Michaelis-Menten kinetics (Figure 2A), suggesting carrier-mediated transport; $K_m = 1.3 \pm 0.15$ mM and $V_{max} = 1.29 \pm 0.04$ nmol min^{-1} mg^{-1} D.W.

Cells collected at midexponential growth phase also displayed capacity to transport mannitol; however, the Eadie-Hofstee plot of D-[14C]mannitol initial uptake rates was biphasic (Figure 2B), suggesting the presence of two distinct uptake mechanisms: carrier mediated and "diffusion-like" transport. The following values were estimated for the maximal capacity of the polyol carrier and for the first-order rate constant: $V_{max} = 0.25 \pm 0.05$ nmol mannitol min^{-1} mg^{-1} D.W.; $k_d = 0.06 \pm 0.01$ µL min^{-1} mg^{-1} D.W.

Table 1. Specific rate of mannitol transfer (q), D-[14C]mannitol uptake and MDH activity in cultured cells of *O. europaea* grown with 1% (w/v) mannitol. Units are in nmol mannitol min^{-1} mg^{-1} D.W.

	q	D-[14C]mannitol uptake	MDH activity[c]
Midexponential growth phase ([mannitol]$_{medium}$ 0.5%)	1.28	1.40 ± 0.07[a] (0.25 ± 0.05)[b]	0.22 ± 0.02
Late exponential growth phase ([mannitol]$_{medium}$ << 0.1%)		1.29 ± 0.04[b]	0.47 ± 0.11
Salt stressed[d]	-	4.00 ± 0.08[d]	0.09 ± 0.01

[a] extrapolated from Figure 2B
[b] V_{max} of mannitol/H$^+$ symporter
[c] V_{max} of MDH (Figure 7)
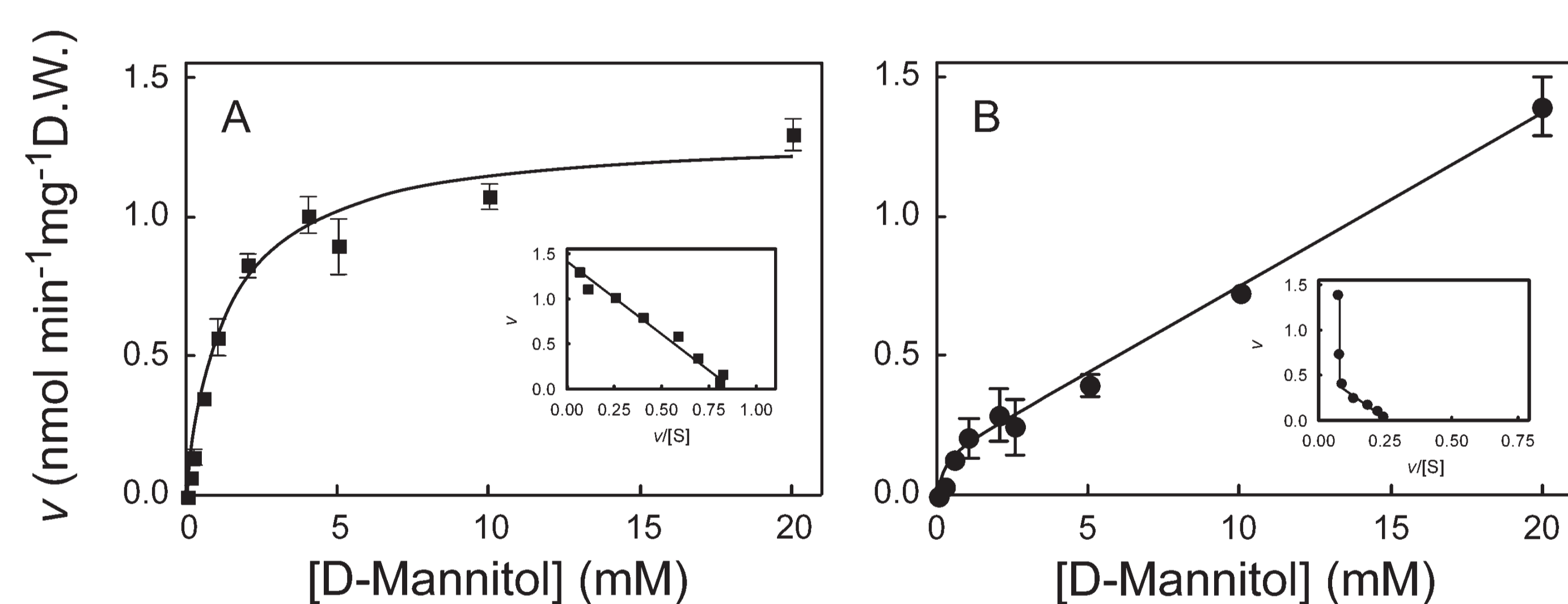[d] 19h after 500 mM NaCl addition, as indicated in Figure 6

Figure 2. Mannitol transport by suspension cultured cells of *O. europaea* cultivated with 1% mannitol as in Figure 1A. Initial uptake rates of D-[14C]mannitol, at pH 4.5, by cells collected at the end of exponential growth phase ([mannitol]$_{medium}$<<0.1%) (A) and at midexponential growth phase ([mannitol]$_{medium}$ 0.5%) (B). *Inserts:* Eadie-Hofstee plot of the initial D-[14C]mannitol uptake rates.

The carrier was specific for acyclic polyols, being able to transport, beyond mannitol, dulcitol, sorbitol and xylitol, but not *myo*-inositol, monosaccharides, and sucrose (Figure 3A).

O. europaea cell suspensions grown in similar conditions are able to transport glucose according to a carrier-mediated mechanism with $K_m = 67 \pm 30$ µM glucose and $V_{max} = 1.45 \pm 0.26$ nmol glucose min^{-1} mg^{-1} D.W. (Figure 3B). As shown, 20 mM mannitol did not inhibit 0.02 - 0.5 mM D-[14C]glucose uptake.
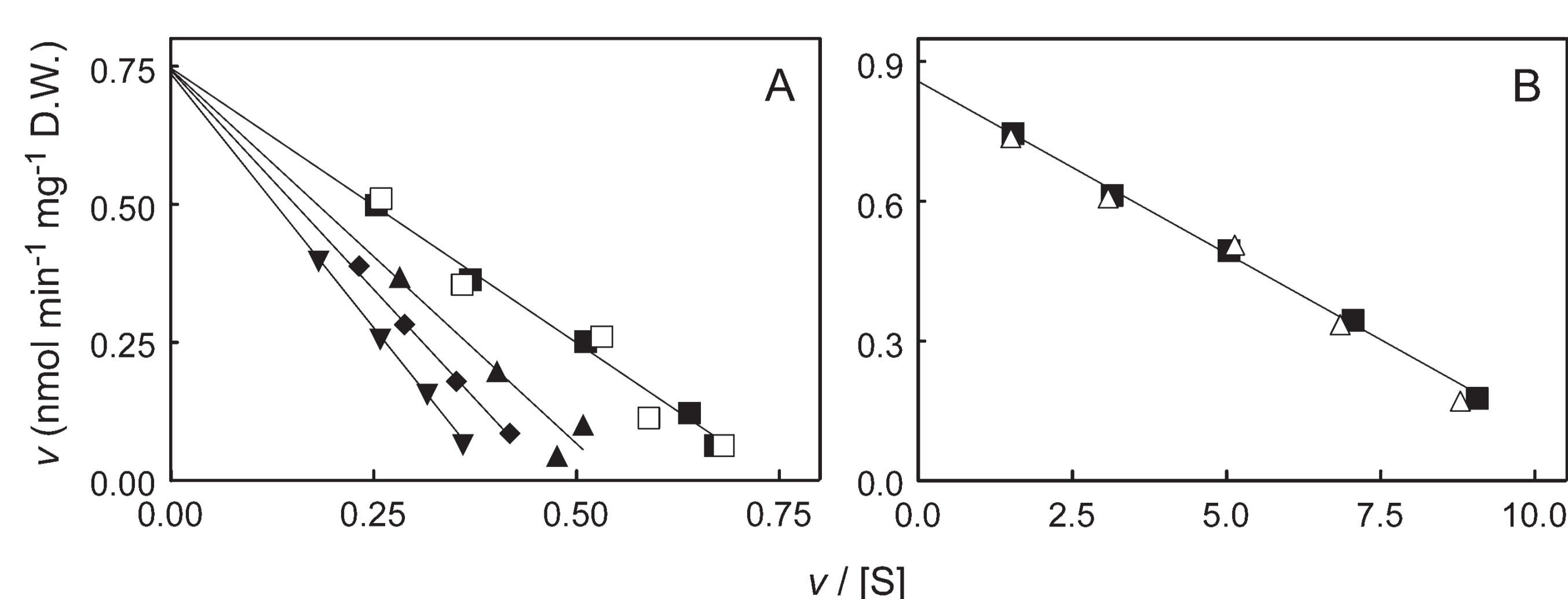

Figure 3. Eadie-Hofstee plots of the initial uptake rates, at pH 4.5, of D-[14C]mannitol (A) and D-[14C]glucose (B) by suspension cultured cells of *O. europaea*. Transport was measured in the absence of other sugars and polyols (■) and in the presence of unlabelled 5 mM sorbitol (▼), 5 mM dulcitol (▲), 5 mM xylitol (◆), 20 mM mannitol (△) and 20 mM glucose (□). Cells were cultivated with 1% mannitol as in Figure 1A and collected at the end of exponential growth phase as described in Figure 2A.

Taken together, the results suggest that in O. europaea *monosaccharides and polyols are transported via two distinct carriers with different K_m, which could account for the results of Figure 1B: glucose is the first substrate to be consumed when growth occurred in a medium with glucose and mannitol.*

Data from energetic studies on mannitol transport in *O. europaea* are presented in Figure 4. Results are consistent with the involvement of a mannitol/H$^+$ symporter with a stoichiometry of 1 mannitol:1 H$^+$.
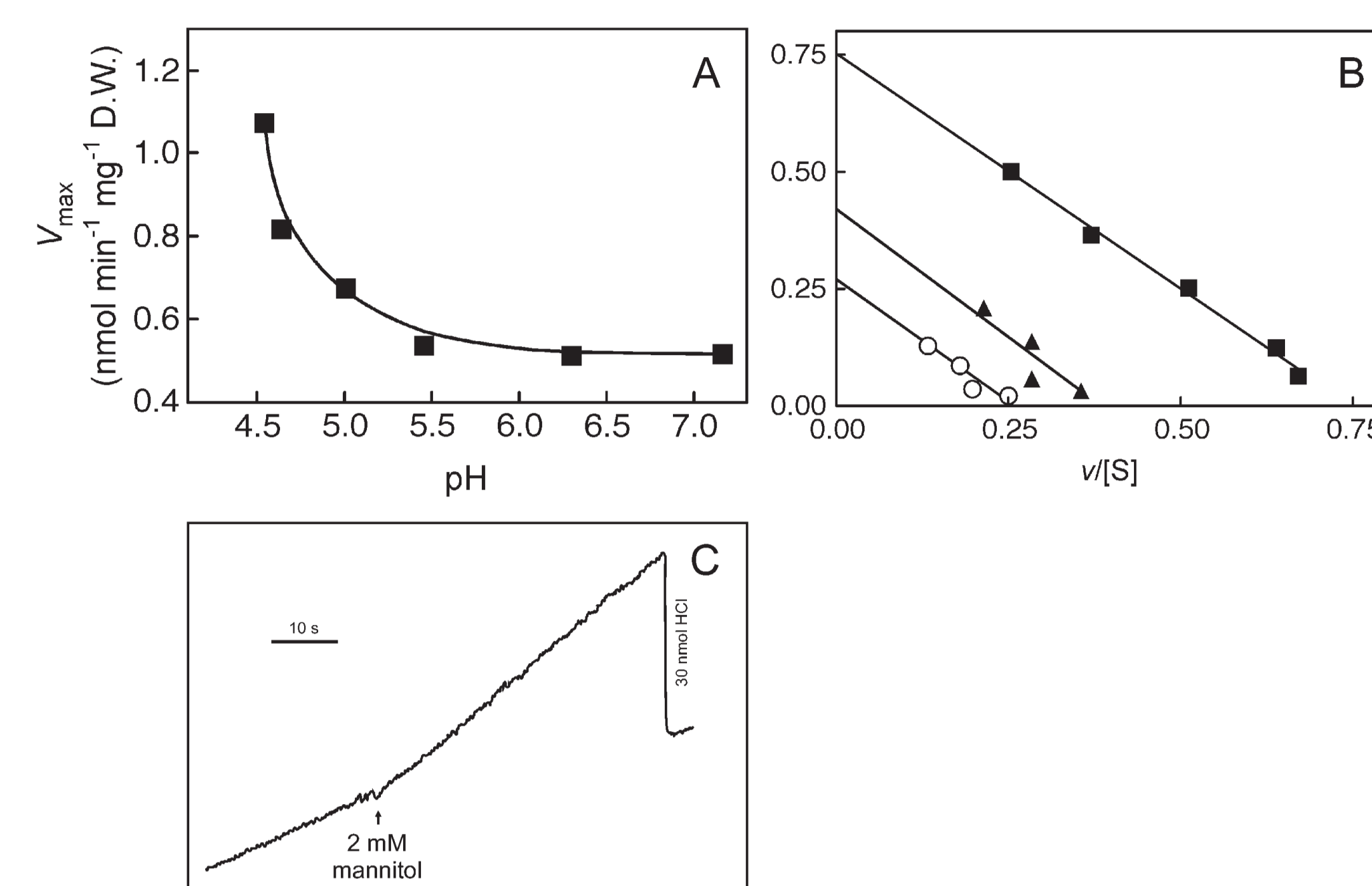

Figure 4. Energetics of *Olea europaea* polyol carrier. (A) pH dependence of mannitol transport; (B) Eadie-Hofstee plots of the initial uptake rates of D-[14C]mannitol in the absence (■) or in the presence of 50 µM CCCP (○) and 10 mM TPP$^+$ (▲); (C) Proton movements, at pH 4.5, associated with the addition of 2 mM mannitol to cell suspensions. Cells were cultivated with 1% mannitol as in Figure 1A and collected at the end of exponential growth phase as described in Figure 2A.

Effect of sugar levels on the activity of mannitol/H$^+$ symporter

Data from Figure 2 suggest that V_{max} of *O. europaea* mannitol/H$^+$ symporter increased along mannitol depletion from the medium. As shown in Figure 5A, when the external levels of mannitol falls below 0.05% (day 13), the activity of the polyol carrier increased abruptly. Cyclohexamide (0.05 mg mL^{-1}) maintained carrier capacity at basal levels.

RT-PCR performed on mRNA extracted from *O.europaea* suspension cultured cells exhibiting high mannitol transport activity resulted in the cloning of a 700 bp cDNA with extensive homology with the celery mannitol transporter AgMaT2. The full length cDNA sequence was obtained by RACE-PCR and denominated *OeMaT1*. Registration in Databank is under going. *OeMaT1* expression was evaluated by Northern-blot analysis (Figure 5B). For the first time, a tight regulation of a mannitol/H$^+$ symporter expression by external levels of its own substrate is reported.
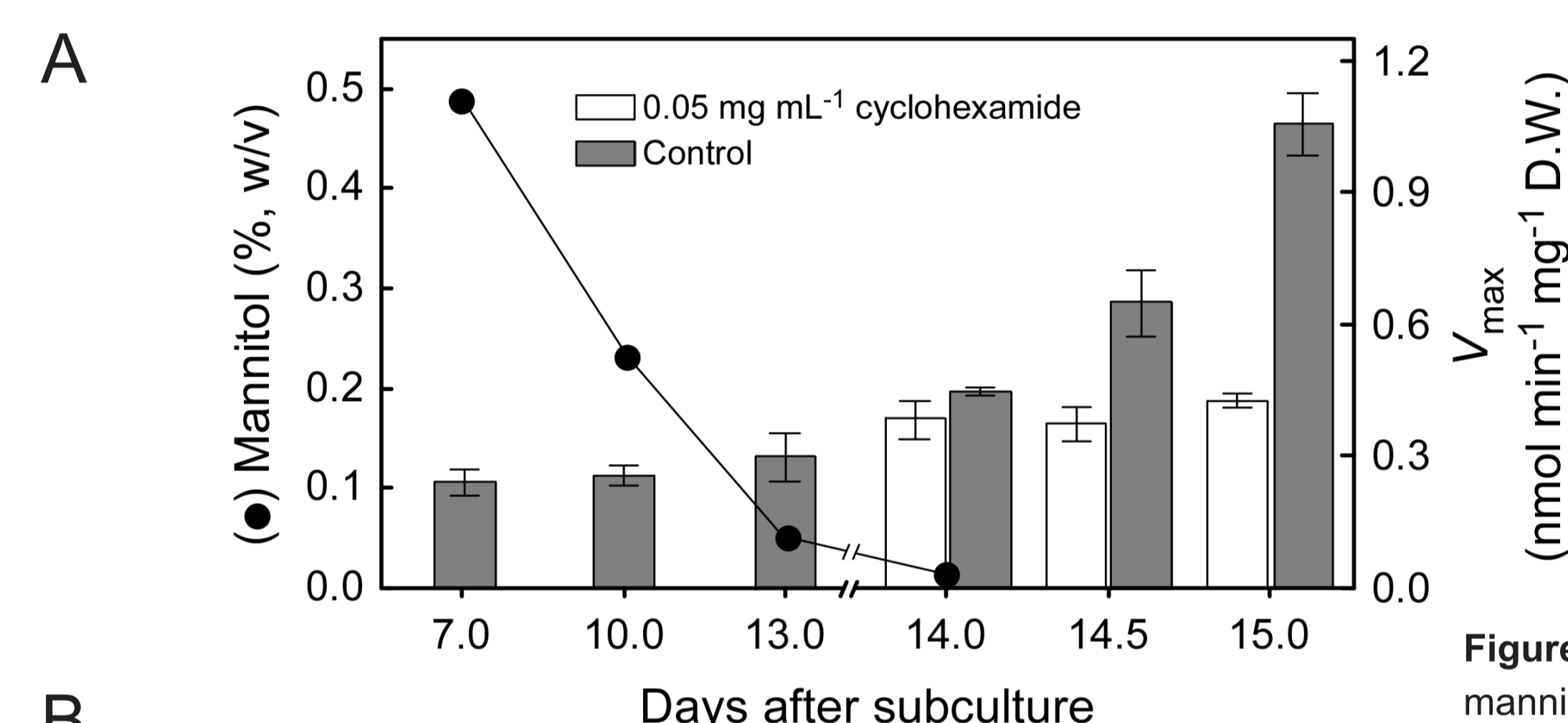

Figure 5. Regulation of *O. europaea* mannitol/H$^+$ symporter activity by mannitol levels in culture medium. Growth occured with 1% mannitol and D-[14C]mannitol uptake was measured in cell aliquots harvested from culture at the times indicated. Cyclohexamide (0.05 mg mL^{-1}) was added to a 25 mL aliquot of the culture at day 14 (A). Northern-blot analysis of *OeMaT1* expression (B).

Effect of salt stress on activity of mannitol/H$^+$ symporter

The transport capacity of the mannitol/H$^+$ symporter was enhanced in salt stressed cells: a 2-4 fold increase of the V_{max} was observed when D-[14C]mannitol uptake was measured in cell suspensions 19h after addition of 250-500 mM NaCl to the culture (Figure 6).
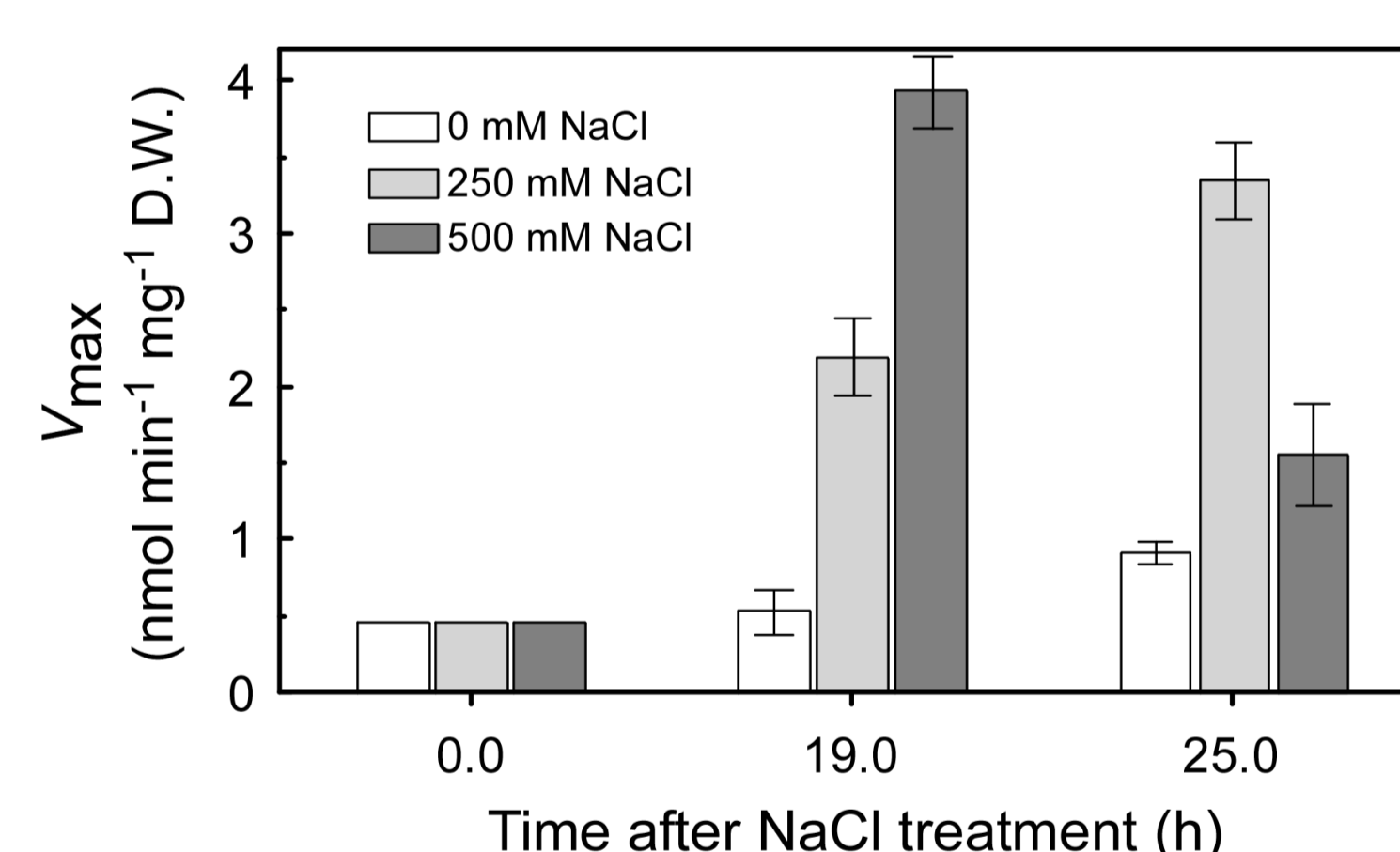

Figure 6. Effect of salt stress on *O. europaea* polyol/H$^+$ symporter activity. Cells were grown with 1% mannitol as in Figure 5 and 250 mM and 500 mM NaCl were added to 25 mL aliquots of the culture at day 14. D-[14C]mannitol uptake was measured in cell aliquots harvested from the cultures at times indicated.

Activation of mannitol catabolism by mannitol dehydrogenase (MDH)

MDH activity was enhanced along with mannitol depletion from culture medium (Figure 7), which correlates with the observed increase of mannitol carrier capacity. In mannitol-grown cells subjected to salt stress MDH activity was strongly repressed.

Figure 7. MDH activity of *O. europaea* suspension cultured cells. Cells were grown with 1% mannitol as in Figure 1 and collected at midexponential growth phase, as described in Figure 2B ([mannitol]$_{medium}$ 0.5%) (●) and at the end of exponential growth phase, as indicated in Figure 2A ([mannitol]$_{medium}$<<0.1%) (■). MDH activity of salt stressed cells measured 19 h after 500 mM NaCl addition, as described in Figure 6 (▲).
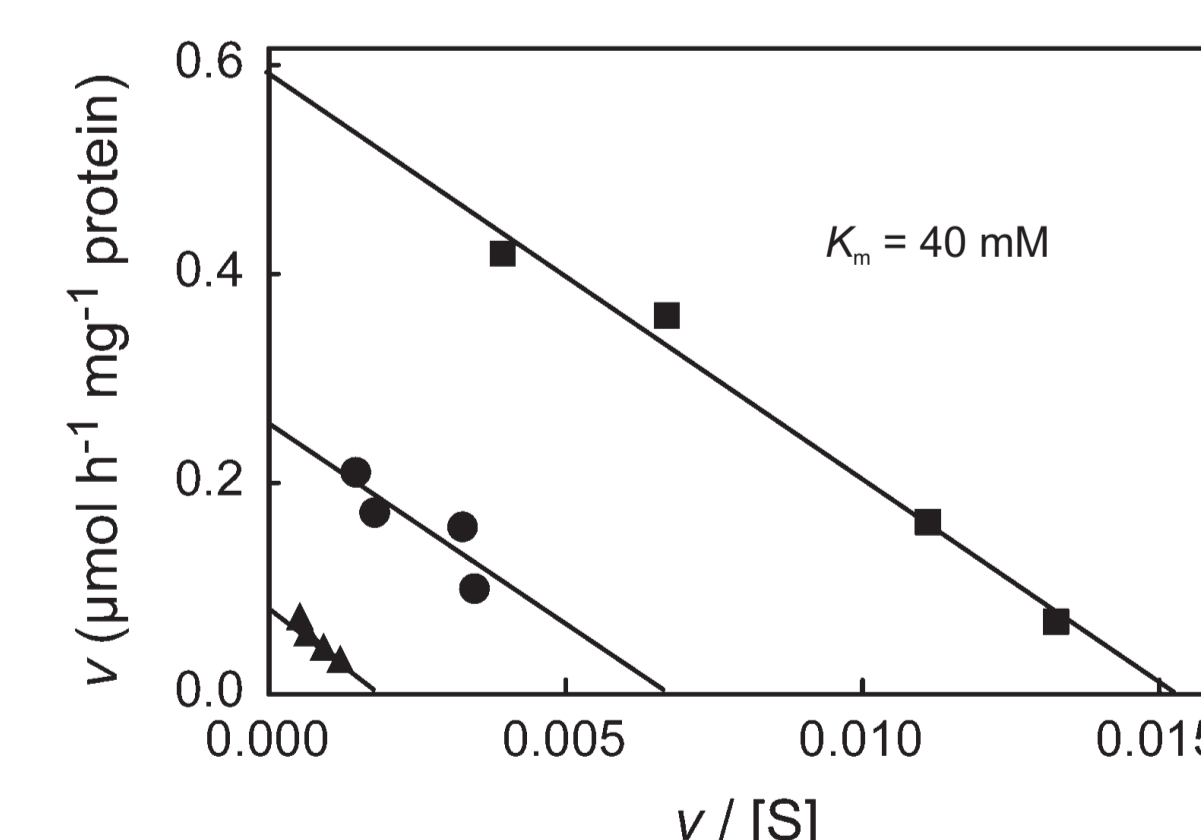
Concluding remarks

- *Olea europaea* cultured cells displayed activity for a mannitol/H$^+$ symporter with a stoichiometry of 1 mannitol:1 H$^+$ and $K_m = 1.3$ mM, able to accept, beyond mannitol, dulcitol, sorbitol and xylitol, but not glucose and sucrose.

- Mannitol seemed to regulate carrier activity because V_{max} increased along with polyol depletion from the medium; the parallel between *OeMaT1* transcripts and V_{max} suggests that carrier expression is mainly regulated at transcriptional level.

- In salt-stressed cells, down-regulation of MDH activity associated with enhanced mannitol uptake capacity, allows the intracellular accumulation of mannitol to compensate the decrease of external water activity, providing tolerance to salinity in *O. europaea*.

References
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