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Uptake of mannitol from the media by *in vitro* grown plants

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Abstract

Higher plants grown *in vitro* are very seldom fully autotrophic. Therefore, such cultures are usually supplied with exogenous sugars. However, at higher sugar concentration a decrease in dry matter accumulation is observed which can be explained by a decrease in osmotic potential of the medium.

To test this explanation a series of experiments with mannitol, a sugar alcohol often used for simulation of osmotic stress, were performed with excised wheat embryos, rape seedlings and potato stem segments grown *in vitro*. As the presence of mannitol in the medium caused a significant decrease in dry matter accumulation, the content of mannitol in the shoot tissues was determined using HPLC analysis to estimate the uptake and transport of mannitol from roots to shoots. Mannitol contents up to 30% of dry weight in wheat and 20% in rape and potato shoots were found, indicating that mannitol is easily taken up by *in vitro* plants and transported to shoots. There were no large changes in the content of glucose, fructose and sucrose caused by the presence of mannitol in the tissues. These data show that mannitol can not be used as an inert osmoticum in *in vitro* studies.

Introduction

The growth of plants under *in vitro* conditions is normally supported by exogenous sugar supply. Increasing the concentration of sugars in the medium not only increases the availability of a carbon source, thus stimulating growth, but it also results in a more negative water potential of the medium, which might inhibit growth. To mimic osmotic stress, mannitol or another non-metabolized sugar alcohol is often added to the medium. In such experiments it is assumed that these osmotic compounds are not taken up by the plants (Oparka & Wright, 1988; Wright & Oparka, 1990; Do & Cormier, 1991). To check whether this assumption is valid, we performed a series of experiments using several plant species grown *in vitro* on media with different concentrations of sucrose or mannitol. The growth of plants, as judged from dry matter accumulation, and the content of mono- and disaccharides and mannitol in the shoots were determined.

The aim of the study was to characterize the rate of mannitol uptake from the medium in three different *in vitro*-grown cultures to estimate water stress of mannitol-exposed tissues.

Material and methods

Plant material

Single-node stem segments from *in vitro*-grown potato (*Solanum tuberosum* L., cv Bintje) plantlet were placed on membrane rafts (Sigma M4380, Sigma, St Louis, USA) floating on the surface of Murashige and Skoog's (1962) liquid medium modified to contain 1% sucrose. After 10 days of growth, the segments had formed roots and the rafts with segments were transferred to liquid media with different sucrose concentrations or with 1% of sucrose plus different concentrations of mannitol. Cultivation was continued for 6 days thereafter.

Rape (*Brassica napus* cv. Darmor) seeds were surface-sterilized by immersion in 3% commercial bleach for 15 min and then washed with sterile water and sown on Murashige and Skoog's medium solidified with 0.7% agar (w/v) and different sucrose concentrations or with 1% sucrose plus different mannitol concentrations. The cultures were grown for 21 days.

Wheat (*Triticum aestivum*, cv. Minaret) seeds were surface-sterilized by immersion in 2% commercial bleach for 30 min, washed three times with sterile water and allowed to imbibe overnight at 25°C in sterile Petri dishes in darkness. Embryos were excised and transferred to membrane rafts floating on the surface of Murashige and Skoog's liquid medium modified to contain 1% (w/v) sucrose. After 24 hours, the rafts with the embryos were transferred to liquid media with different concentrations of sucrose or with 1% sucrose plus different concentrations of mannitol. The plants were harvested after 7 days of growth.

Growth conditions for all plants were: 20°C, 16-h photoperiod, 21 μmol m^{-2} s^{-1}, SON-T 400W Agro lamp, HPI-T 400W Lamp (Philips)

Sample preparation

Harvested plants were washed thoroughly with running water for about 5 seconds, shoots and roots were separated and frozen in liquid nitrogen, freeze-dried and the dry weight was determined. Every sample contained roots or shoots from three plants. Average values of 10 sample determinations are given in figures showing growth curves. The samples were boiled with 80% methanol (0.5 ml) at 75°C for 10 min and then the methanol was evaporated. The residue was dissolved in water in an ultrasonic bath for 10 min. The samples prepared for sugar determination were stored at −80°C.

Sugar content determination

The samples were diluted 50–100 times with water and 20 μl was used for HPLC analysis (High pH anion exchange chromatography with pulsed amperometric detection, Dionex, Sunnyvale, USA; column: CarboPac PA1 (4×250 mm) with companion guard column (4×50 mm), flow rate 1 ml min, ambient temperature, eluent 100 mM NaOH). Three independent samples were measured for each treatment.

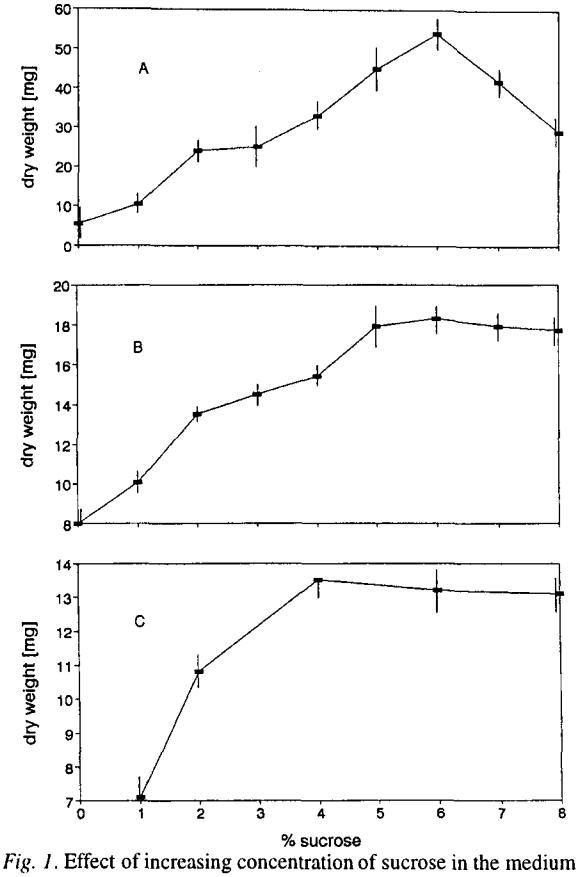

Fig. 1. Effect of increasing concentration of sucrose in the medium on dry matter accumulation (mg/plant) in *in vitro*-grown plants of (*A*) potato, (*B*) rape,(*C*) wheat. Bars indicate standard deviations.

Results and discussion

Growth of *in vitro*-cultivated plants is strongly influenced by exogenous sugars, normally added to the media. There is a positive correlation between sugar concentration in the medium and total dry matter accumulation (Hyndman *et al.*, 1982; Galzy & Compan, 1992). This relationship, however, is only valid for low sugar concentrations. At higher concentrations a decrease in dry matter accumulation is observed. In Fig. 1A such a situation is demonstrated with potato stem segments grown on media with different concentrations of sucrose. With the increasing concentration of sucrose the dry weight of the plantlets increased reaching a maximum at 6% sucrose. Similar patterns were found for cultures of rape seedlings and wheat embryos grown *in vitro* (Fig. 1B,C).

Sugars added to medium can be taken up and used as the source of carbon and energy but, at the same time, they change the water potential of the medium. The decrease in dry matter accumulation of cultures

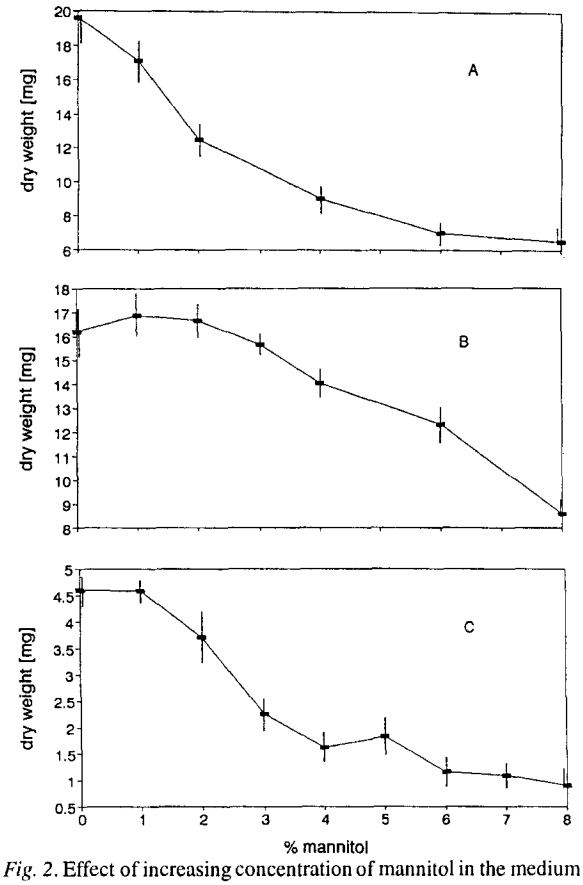

Fig. 2. Effect of increasing concentration of mannitol in the medium on dry matter accumulation (mg/plant) in *in vitro*-grown plants of (*A*) potato, (*B*) rape,(*C*) wheat. Bars indicate standard deviations

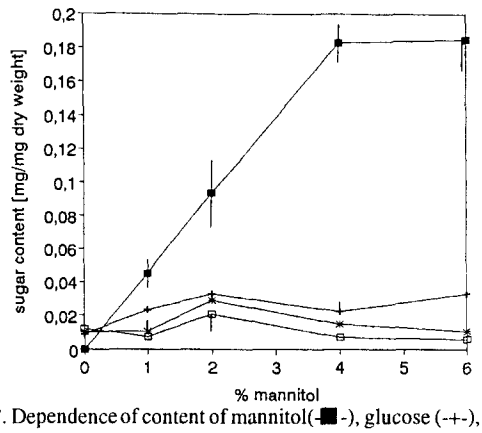

Fig. 3. Dependence of content of mannitol(-■-), glucose (-+-), fructose (-*-) and sucrose (-□-) in the shoots of potato on the concentration of mannitol in the medium. Bars indicate standard deviations. Only the highest standard deviation for each curve is given for glucose, fructose and sucrose.

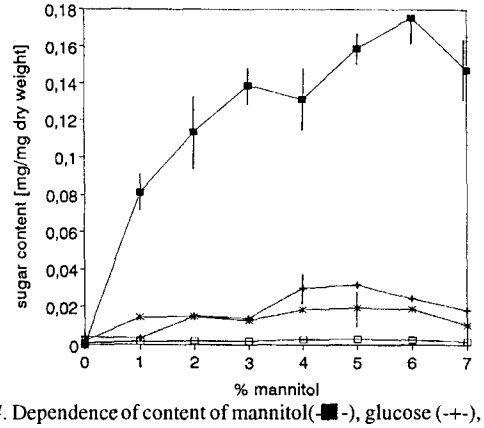

Fig. 4. Dependence of content of mannitol(-■-), glucose (-+-), fructose (-*-) and sucrose (-□-) in the shoots of rape on the concentration of mannitol in the medium. Bars indicate standard deviations. Only the highest standard deviation for each curve is given for glucose, fructose and sucrose.

grown under higher sugar supply might be due to this decrease of water potential of the medium. To test this explanation we determined dry matter accumulation in the presence of a constant low level of sucrose combined with different concentrations of mannitol, a sugar alcohol frequently used for adjustment of water potential of media mainly in experiments simulating drought stress (Do & Cormier, 1991; Pritchard *et al.*, 1991; Ribaut & Pilet, 1991; Williams *et al.*, 1991; Gulati & Jaiwal, 1993). Figure 2 shows the results for cultures of potato nodal segments, rape seedlings and wheat embryos. We found rather dramatic decreases in dry matter accumulation at higher concentrations of mannitol but also significant decreases at concentrations corresponding to the sugar concentrations frequently used as exogenous sugar supply of *in vitro*-grown plants (around 3%). The dry weight:fresh weight ratio changed in response to different mannitol contents in the medium. For wheat cultures, the values were 0.06, 0.07, 0.08 0.12 and 2.6 for 1, 3, 5, 7, 9%, respectively. The pattern of changes was similar for rape and potato cultures.

Mannitol is a sugar alcohol which is produced by some plants as primary photosynthetic product and some plants can metabolize it (Trip *et al.*, 1964; Tholakalabavi *et al.*, 1994). Numerous other plant species are considered not to be able to metabolize this sugar and cells of these plants are supposed to take up mannitol only very slowly (Diamond & Wright, 1969; Cram, 1984; Ribaut & Pilet, 1991).

To interpret our results we need to know whether mannitol was taken up by the plants used in our experiments and the rate of transport of mannitol from roots to leaves. The concentrations of mannitol in root and shoot tissue of the three species under investigation, grown on media with different mannitol concentra-

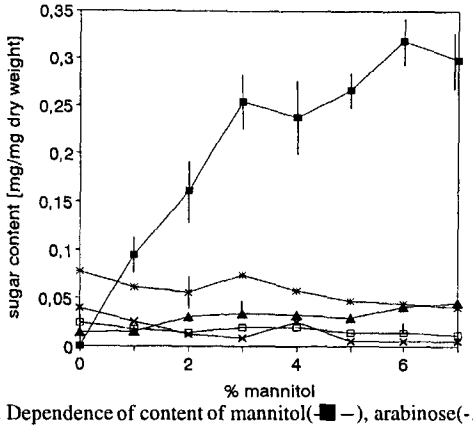

Fig. 5. Dependence of content of mannitol(-■-), arabinose(-▲-), glucose (-+-), fructose (-*-) and sucrose (-□-) in the shoots of wheat on the concentration of mannitol in the medium. Bars indicate standard deviations. Only the highest standard deviation for each curve is given for arabinose, glucose, fructose and sucrose.

tions were determined using HPLC analysis. The highest mannitol accumulation was found in experiments with wheat embryos (Fig. 5). The content of mannitol exceeded the concentration of glucose - the major sugar compound in wheat shoot tissue - even at 1% of mannitol in the medium and reached more than 30% of dry matter at a concentration of 6% of mannitol in the medium. The values determined in root tissue are even higher (40%). Do & Cormier (1991) did not find any changes in mannitol concentration in the medium during 12 days of cultivation of grape suspension culture and so no mannitol accumulation in the cells can be expected. Suspension cultures of poplar (Tholakalabavi *et al.*, 1994) were able to absorb and metabolize some of mannitol from the medium. However, the presence of most of the mannitol(82–92% of the initial amount) in the medium on the day 12 indicated that mannitol was not taken up by these cells in substantial amount. As there is no mannitol-supported growth which can be estimated from the comparison of changes in dry matter accumulation in cultures grown on sucrose or mannitol (see Fig. 1 and Fig. 2), we cannot suppose a high rate assimilation of mannitol by the cultures under study. We find it difficult to compare the results obtained with cell suspension cultures and cultured tissues. When tissues are cultured, mannitol does not necessarily enter the living cells and its accumulation can take place mainly in the apoplast.

Soluble sugars, mainly glucose, fructose and sucrose are referred to many times as the substances responsible for osmotic adjustment in tissues under osmotic stress (Premachandra *et al.*, 1992; Irigoyen *et al.*, 1992; Virgona & Barlow, 1991). Therefore, it was

Table 1. Uptake of mannitol into shoots of *in vitro*-grown wheat plants

% mannitol in the medium	mannitol concentration (mg/mg dry weight)	
	day 1	day 2
1	0.02	0.05
3	0.06	0.14
6	0.15	0.20

expected that the levels of these sugars would increase with the increasing level of mannitol in the medium. Surprisingly no dramatic changes in concentrations of glucose, arabinose, fructose and sucrose were observed in wheat leaf tissue.

Similar results, accumulation of mannitol, without drastic changes in the levels of mono- and disaccharides, were obtained with quite different cultures of potato stem segments (Fig. 3) and rape seedlings (Fig. 4). The highest endogenous concentrations of mannitol were slightly lower in these cases, reaching about 20% of dry weight.

The rate of mannitol uptake from the medium was studied using wheat plants derived from excised embryos. Table 1 shows that even at a low concentration of mannitol in the medium, *viz* 1% , the endogenous mannitol content equalled the level of glucose after two days of cultivation. At higher concentrations of mannitol, the endogenous levels exceeded the levels of glucose after one day of cultivation.

Conclusions

The results clearly show that mannitol is taken up by the plants under investigation and that it is transported to shoots rather quickly. As there are probably great differences in the ability of various plants to accumulate mannitol and use it as the source of carbon and energy it is necessary to carefully check the response of a particular plant to mannitol when this compound is used for water potential adjustment or simulation of drought stress. Although it is highly probable that the growth of plants *in vitro*, supplied with exogenous sugars, is stimulated by the influx of sugars and at the same time is inhibited by low water potential of the medium, it is not possible to demonstrate this idea unequivocally by experiments comparing the effects of sucrose and mannitol, since mannitol can be taken up by *in vitro*-grown plants.

Acknowledgement

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