Biochemical Systematics and Ecology, Vol. 18, No. 2/3, pp. 107–110, 1990.
Printed in Great Britain.

0305-1978/90 $3.00+0.00

Comparison of Free Sugars in Growing and Desiccated Plants of *Selaginella lepidophylla**

ROBERT P. ADAMS, E. KENDALL† and K. K. KARTHA†
CSFFA, BU Box 7372, Baylor University, Waco, TX 76798, U.S.A.;
†Plant Biotechnology Institute, National Research Council, Saskatoon, Canada S7N 0W9

Key Word Index—*Selaginella lepidophylla*; Selaginellaceae; trehalose; sucrose; sugars; extraction; desiccation.

Abstract—The free sugars are growing and desiccated *Selaginella lepidophylla* plants were examined. Growing and desiccated plants differed significantly in their percentage of glucose (3.1, 0.2%), sucrose (6.9, 23.1%) and trehalose (89.8, 75.6%). The apparent stoichiometry between the decrease in sucrose and increase in trehalose concentration after rehydration suggests that sucrose serves as the carbon source for trehalose synthesis. A comparison of extraction methods revealed that using "Boileezers" in Soxhlets resulted in sugar degradation ranging from 25 to 75%. Extraction by shaking in 80% ethanol appeared to be the best extraction method.

Introduction

Selaginella lepidophylla (Hook. and Grev.) Spring (Selaginellaceae) is a xerophytic species which is known for its ability to desiccate, curl up and survive dry periods. It is native to the Chihuahuan desert of Texas and neighbouring Mexico. The taxon has been the subject of several studies on desiccation tolerance [1], the effects of rehydration on enzyme dynamics [2], protein synthesis [3] and solute leakage during desiccation [4]. Bergtrom *et al.* [5] suggest that the basis of recovery from drought stress is due, at least in part, to conserved cellular (membrane) organization and enzymatic integrity during desiccation. One possible factor for the ability of *S. lepidophylla* to withstand desiccation may be the accumulation of the α-α-disaccharide trehalose as a storage sugar. Examination of extracts from xerophytic *S. repanda* and mesophytic *S. rajasthanensis* showed the xerophyte to be higher in soluble sugars, soluble proteins and phenols [6]. In anhydrobiotic organisms, trehalose stabilizes dry membranes [7]. Trehalose has been found to be one of the most effective sugars for preserving structural and functional integrity of membranes and proteins at low water concentrations [8–10]. Trehalose has also been used to protect against freeze induced dehydration during cryopreservation of carrot and tobacco cells [11]. In yeast, where trehalose occurs naturally, Coutinho *et al.* [12] found that mutant yeast strains with higher trehalose synthase activities had an enhanced capacity to survive drying and freezing. In fact, we were able to cryopreserve entire desiccated *S. lepidophylla* plants in liquid nitrogen and then resurrect the plants by merely thawing at room temperature and rehydrating.

Trehalose was first isolated in vascular plants from *S. lepidophylla* in 1913 [13], although it was discovered in a water extract of ergot of rye in 1832 [14]. In spite of the long period since the isolation of trehalose from *S. lepidophylla,* there appears to be no information on the composition and yields of the free sugars in growing and desiccated plants. Here we report on the changes in soluble sugars in such plants.

During the course of our analyses, we experienced degradation of internal standards and this prompted the examination of extraction methods. Free sugars have been extracted by several methods, including a Soxhlet with 80% ethanol [15], refluxing with 85% ethanol [16], steeping in 80% ethanol on a shaker [16, 17], and extraction with hot water [18]. In addition, a number of modifications to the above procedures incorporate additional extractions with water or the use of additives such as KN_3 [16]. In this paper we report on the effects of the use of

*NRCC Publication No. 30915.

(*Received* 17 *October* 1989)

white boiling stones vs glass beads in Soxhlet extractions and compare steeping by shaking vs using a water bath.

Results and Discussion

Percentage dry weight of the growing leaves and the desiccated leaves were 39.15 and 93.10% DM, respectively. These factors were used to obtain g DM for yield determinations.

The major effects of the different extraction methods are given in Table 1. Total sugars (mg g DM^{-1}) were significantly lower using the white boiling stones ("Boileezers"), as were the yields of sucrose and trehalose. No significant differences were found between using the Soxhlet with glass beads, water bath and the shaker for either total sugars or trehalose. The yield of sucrose is significantly lower for the bath vs Soxhlet with glass beads (Table 1).

Reduced yields obtained using the Soxhlet with "Boileezers" were due to the degradation of both di- and monosaccharides with the degradation products appearing to be 2- and 3-carbon compounds. Similar degradation, ranging from 25 to 75%, was detected when the sugars arabinose, cellobiose, fructose and gentiobiose were individually refluxed using the "Boileezers" in the boiling flask. The "Boileezers" varied in colour from stone to stone and successive lots yielded varying amounts of degradation of sugar standards. These boiling stones should not be used in the Soxhlet extractions. Interestingly, drying the extracts or sugar standards in an 80% ethanol solution at 100°C did not lead to degradation of the sugars even though the chlorophyll in the leaf extracts was degraded.

Extractions using the Soxhlet ("Boileezers" and glass beads), steeping in a water bath and shaking resulted in only a few differences in the compositional data (Table 1). There were no significant differences for the percentages of α-glucose, β-glucose or sucrose. The proportion of mannitol was significantly larger using the "Boileezers" and that of trehalose was significantly smaller with the "Boileezers" but using the glass beads also lessened the trehalose yield (Table 1). An unidentified trisaccharide present in all the extracts was highest using the "Boileezers" and lowest in the shaker extraction.

A comparison was made between growing plants and desiccated plants. Although the total sugars did not differ significantly (Table 2), the change in concentration of sucrose (mg g DM^{-1}) was highly significant. Trehalose yields tended to be greater in growing materials, but the differences were not quite significant at the 5% level. The trehalose yields in the growing leaves had a narrow range, from 121 to 127 mg g DM^{-1}, in contrast to the yields from desiccated leaves

TABLE 1. COMPARISONS OF FOUR EXTRACTION PROCEDURES ON THE YIELDS AND COMPOSITIONS OF FREE SUGARS FROM DESICCATED LEAVES OF *S. LEPIDOPHYLLA*

	Extraction method			
	Soxhlet		Ground in liquid nitrogen	
	1. White stones	2. Glass beads	3. Bath 60°C	4. Shaker 22°C
Total sugars (mg g^{-1})	51.4	118.6[a]	119.5[a]	130.8[a]
Sucrose (mg g^{-1})	15.6	32.8[a]	27.6[b]	29.5[a,b]
Trehalose (mg g^{-1})	33.7	83.8[a]	88.9[a]	99.3[a]
% Fructose	0.05 (NT)	0.00 (NT)	0.03 (NT)	0.00 (NT)
% α-Glucose	0.14[a]	0.10[a]	0.07[a]	0.10[a]
% Mannitol	0.35	0.08[a]	0.14[a]	0.10[a]
% β-Glucose	0.29[a]	0.14[a]	0.12[a]	0.10[a]
% Inositol	0.09 (NT)	0.10 (NT)	0.03 (NT)	0.03 (NT)
% Sucrose	30.03[a]	27.64[a]	23.59[a]	23.10[a]
% Trehalose	66.72[a]	70.68[a,b]	74.22[b]	75.62[b]
% Trisaccharide	0.46[a]	0.26[a,b]	0.39[a,b]	0.18[b]

Any means in a row that do not share the same superscript are significantly different ($P=0.05$) by the SNK (Student–Newman–Keuls) multiple range test. Two trace components (fructose and inositol) were not tested (NT) for significant differences because they were present only at the level of detection in many cases so quantitative data were not obtained.

TABLE 2. COMPARISON OF YIELDS AND COMPOSITION OF SUGARS FROM GROWING AND DESICCATED *S. LEPIDOPHYLLA*

	S. lepidophylla		
Component	Growing	Desiccated	Significance
Total sugars (mg g^{-1})	139.0	130.8	0.55 n.s.
Sucrose (mg g^{-1})	9.7	29.5	0.0004**
Trehalose (mg g^{-1})	124.8	99.3	0.09 n.s.
% Fructose	0.1 (NT)	0.0 (NT)	
% α-Glucose	1.2	0.1	0.0003**
% Mannitol	0.0 (NT)	0.1 (NT)	
% β-Glucose	1.9	0.1	0.0005**
% Inositol	0.1 (NT)	0.03 (NT)	
% Sucrose	6.9	23.1	0.005**
% Trehalose	89.8	75.6	0.003**
% Trisaccharide	0.1	0.2	0.37 n.s.

Significance: **$P=0.01$. The percentages of fructose, mannitol and inositol were too small for meaningful statistical testing and were not tested (NT).

which varied from 71 to 106 mg g DM^{-1}. Because *S. lepidophylla* curls up into a dry brownish, pale green ball when desiccated, it is difficult to separate the dead outer branches from the interior ones that will revive upon rehydration. This may be the source of the large variability seen in the desiccated materials.

The shift in the free sugar pool is clearly seen in the compositional data. In particular, a relatively large increase in glucose was observed (Table 2). The percentage of trehalose increased upon rehydration and growth while a concomitant decrease in sucrose level was observed. This appears to confirm the work of White and Towers [19] who fed $^{14}CO_2$ to actively growing *Selaginella wallacei, S. kraussiana* and *S. densa.* After 2 h, between 81 and 98% of the labelled $^{14}CO_2$ was found in trehalose, except for one sample of *S. kraussiana* which had 42% in trehalose, 1% in sucrose and 56% in an unidentified sugar [19]. The stoichiometric decrease in sucrose concentration following rehydration and growth (Table 1) suggests that sucrose may provide the carbon source for trehalose synthesis during the early stages of rehydration and growth. Experiments are underway in our laboratory to determine if *Selaginella* does indeed accumulate trehalose immediately after photosynthesis. This would explain our findings. Thus, these preliminary results suggest that while a demonstrated path for trehalose degradation [19] exists in *Selaginella,* sucrose rather than trehalose is used as a carbon/energy source prior to full photosynthetic competency.

Several studies have suggested that during periods of water and cold stress, plants accumulate more sugars [15, 18, 20, 21]. One might expect that *S. lepidophylla* would accumulate more free sugars as it becomes desiccated. On face value, our data do not support that idea. However, since *S. lepidophylla* has evolved in a desert environment where rain is unpredictable, both in frequency and duration, the plant must be constantly prepared for drought and desiccation. Therefore, the plants may maintain a large pool of trehalose, even during times of growth. A second possibility is that some trehalose is phosphorylated during desiccation, and thus our estimates of trehalose in desiccated leaves would be too low. Additional research should clarify this point.

Experimental

Plants of *S. lepidophylla* were obtained from Carolina Biological Supply in the desiccated state. Six of the plants were rehydrated (24 h) and planted in potting mix in the greenhouse. Six plants were kept as received for use as "desiccated" plants. Percentage dry matter was determined by weighing growing and desiccated material before and after drying at 100°C for 48 and 120 h. Growing material was obtained by pooling a branch from each of the six greenhouse plants, one week after planting (three replicates). The desiccated leaves were sampled (three replicates) by peeling off the exterior (completely brown) branches and removing four to six pale green branches from the interior of the ball.

Four extraction methods were investigated, each using 80% ethanol (v/v) as the extraction solvent, and approximately 0.4 g of desiccated leaves. (1) The plant material was ground to a fine powder in liquid nitrogen with a pestle and mortar, then transferred to a 10×50 mm Whatman thimble-A, and extracted (4 h) by Soxhlet with a 30 ml flask, using "Boileezers" (Fisher, white boiling stones), 10 mg arabinose as an internal standard (5 ml of 2 mg ml^{-1} 80% ethanol solution) was added to the extraction thimble on top of the plant material and 5 ml of 80% ethanol added to the boiling flask. (2) Soxhlet [as method (1)] but using glass beads (3 mm) in the boiling flask. (3) Leaves were ground in liquid nitrogen with pestle and mortar, with 5 ml of the arabinose standard solution and 5 ml of 80% ethanol added to the mortar during grinding. The slurry was then transferred to a centrifuge tube and incubated (60°C, 60 min) in a water bath. The slurry was then centrifuged through a Whatman extraction thimble. (4) Same as method (3), except the slurry was transferred to a centrifuge tube and placed on a rotary shaker (300 rpm, 22°C, 60 min) then centrifuge-filtered through a Whatman extraction thimble. Method (4) (shaking) was used to extract the growing and desiccated materials for use in the analysis of differencs between growing and desiccated *Selaginella.* The plant material was extracted by first grinding the leaves to a fine powder in liquid nitrogen, after which 10 ml of 80% ethanol was added and the slurry shaken for 60 min.

The extracts were concentrated to dryness by rotary evaporation at 24°C under reduced pressure. The material was redissolved in dimethylformamide (Piece 20672). A 100 μl aliquot was removed and silylated with 400 μl Tri-Sil "Z" (Sigma Chem. Co. 49231).

The silylated extracts were analysed by GLC on a J & W DB1701 (7% cyanopropylphenyl silicone), 0.15 μm coating, 15 m, 0.25 mm i.d. capillary column on a Hewlett-Packard HP 5710A gas chromatograph operated as follows: helium carrier gas 30 cm s^{-1}; injector 250°C, FID 250°C; temperature program 150°C for 2 min, then 8°C min^{-1} to 270°C and held for 8 min at 270°C. The extract (2–4 μl) was injected and split 1:20. The injector liner was packed with silylated glass wool to absorb the non-volatile materials (chlorophyll, etc.). No differences were found in detector response to internal standards analysed in solvent vs those added to plant extracts containing chlorophyll, etc. Thus, there do not appear to be interactions between the degraded products and the sugars in the injector port. Direct analysis of the crude extract saves considerable sample manipulations that have been used in other studies to "clean-up" the extract before chromatography. However, it is necessary to clean the injector liner about every 100 to 200 runs and a short portion of the column

may need to be removed occasionally if peak tailing becomes a problem. Quantitation was by use of an electronic digital integrator (Spectra Physics 4270). Identifications were based on comparisons of retention times with standards and GC–MS analysis. Data were analysed by analysis of variance and the Student–Newman–Keuls (SNK) multiple range tests [22].

Acknowledgements—The authors wish to thank Doug Olson for mass spectral analyses.

References

1. Eickmeier, W. G. (1986) *Can. J. Bot.* **64**, 611.
2. Harten, J. B. and Eichmeier, W. G. (1986) *Pl. Physiol.* **82**, 61.
3. Eickmeier, W. G. (1988) *Can. J. Bot.* **66**, 2574.
4. Leopold, A. C. (1981) *Pl. Physiol.* **68**, 1222.
5. Bergstrom, G., Schaller, M. and Eickmeier, W. G. (1982) *J. Ultrastruct. Res.* **78**, 269.
6. Sharma, S. N., Varma, S. and Bhardwaja, T. N. (1987) *Bionature* **7**, 1.
7. Crowe, J. H., Crowe, L. M. and Chapman, D. (1984) *Science* **223**, 701.
8. Crowe, L. M., Mouradian, R., Crowe, J. H., Jackson, S. A. and Womersley, C. (1984) *Biochim. Biophys. Acta* **769**, 141.
9. Crowe, L. M. and Crowe, J. H. (1988) *Biochim. Biophys. Acta* **946**, 193.
10. Crowe, J. H., Crowe, L. M., Carpenter, J. F. and Wistrom, C. A. (1987) *Biochem. J.* **242**, 1.
11. Bhandal, I. S., Hauptmann, R. M. and Widholm, J. M. (1985) *Pl. Physiol.* **78**, 430.
12. Coutinho, C., Bernardes, E., Felix, D. and Panek, A. D. (1988) *J. Biotechnol.* **7**, 23.
13. Kandler, O. and Hopf, H. (1980) *The Biochemistry of Plants* (J. Preiss, ed.) Vol. 3, p. 221. Academic Press, New York.
14. Harding, T. S. (1923) *Sugar* **25**, 476.
15. Green, D. G. (1983) *Can. J. Pl. Sci.* **63**, 415.
16. Kindel, P. K., Liao, S.-Y., Liske, M. R. and Olien, C. R. (1989) *Carbohydrate Res.* **187**, 173.
17. Olien, C. R. and Lester, G. E. (1985) *Crop Sci.* **25**, 288.
18. Tschaplinski, T. J. and Blake, T. J. (1989) *Can. J. Bot.* **67**, 1681.
19. White, E. and Towers, G. H. N. (1967) *Phytochemistry* **6**, 663.
20. Alberdi, M., Meza-Basso, L., Fernandez, J., Rios, D. and Romero, M. (1989) *Phytochemistry* **28**, 759.
21. Green, D. G. and Ratzlaff, C. D. (1975) *Can. J. Bot.* **53**, 2198.
22. Steele, R. G. D. and Torrie, J. H. (1980) *Principles and Procedures of Statistics.* McGraw–Hill, New York.