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SUGAR BIOTRANSFORMATIONS BY FUNGI ON LEAVES OF THE RESURRECTION PLANT *SPOROBOLUS STAPFIANUS**†

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Key Word Index—*Sporobolus stapfianus*; Gramineae; resurrection plant; water-stress; fungi; sugar biotransformations; trehalose; mannitol.

Abstract—Fresh, dried and rehydrated leaf samples of the resurrection plant, *Sporobolus stapfianus*, were analysed for their contents of low-M_r substances. Glucose, fructose and sucrose were the prevailing sugars in aqueous extracts from fresh leaves, whereas sucrose accumulated in dried leaves. After 72 hr rehydration, an unusual content of mannitol and trehalose was observed. The involvement of fungal species, able to perform sugar biotransformations in rehydrated leaves, is reported. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

In a previous paper on *Sporobolus stapfianus*, Gandoger resurrection plant which survives almost total dehydration, we reported the content of low M_r substances in epicuticular waxes, organic and aqueous extracts of fresh, 120 hr dried and 24 hr rehydrated leaves [1]. We were mainly interested in the variation of the sugars in aqueous extracts, as one of the factors related to drought-tolerance. In fresh leaves, galactose, glucose, fructose and sucrose were the prevailing sugars. Trehalose, several alditols (ribitol, xylitol and mannitol) and 2-*O*-β-D-glucopyranosylglycerol (lilioside B) were also present, although in lower amounts. Sucrose accumulated mainly in leaves during desiccation, whereas glucose was the dominant sugar after rehydration. The sugar changes observed in *S. stapfianus* leaves before and after water-stress were qualitatively similar to those reported earlier for other resurrection plants, *Boea hygroscopica* [2] and *Craterostigma plantagineum* [3], where the common trend was the transformation of monosaccharides, present in fresh leaves, into sucrose in dried leaves and vice versa in restored leaves.

The RWC (relative water content) of *S. stapfianus* leaves, used in our previous experiments [1], decreased rapidly during dehydration, especially within the initial 7 hr. We had no information about these first hours, having analysed only the 120 hr dried leaf sample. In order to correlate in a more accurate way the carbohydrate change with the RWC variation of the leaves, we accomplished a closer timing of sampling with new leaves of *Sporobolus*. In the present paper, we report on the trend of fructose, glucose and sucrose occurring in aqueous extracts of leaves during water-stress and on the anomalous presence of mannitol and trehalose in rehydrated leaves of some *S. stapfianus* samples.

RESULTS AND DISCUSSION

Fresh leaves of *S. stapfianus* were excised from well-watered plants, grown in a glass-house at Capannori. Some leaves were dehydrated for 3, 7, 24 and 120 hr, and some of the 120 hr dried leaves were rehydrated for 24 and 72 hr. Chloroform treatment and methanol–H_2O extraction of all leaf samples, following the described procedure [1], gave epicuticular waxes and aqueous and organic extracts as separated fractions. Table 1 shows the content of the sugars occurring in aqueous extracts, analysed by GC as trimethylsilyl derivatives. With respect to the previous results [1], qualitative and quantitative differences can be observed.

As expected, fructose and glucose were present in reasonable amount in fresh leaves, along with sucrose. After 7 hr desiccation, the monosaccharides decreased, while sucrose started increasing, and its increase continued until the end of the stress (120 hr). Quite surprisingly, an unusual sugar composition was observed in the leaves after treatment with water. In 24 and 72 hr rehydrated samples, mannitol and trehalose

*This paper is dedicated to Prof. Paolo Grünanger on the occasion of his 70th birthday.

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Table 1. Composition (mg g^{-1} of aqueous extracts)* of water-soluble substances in fresh, dried and rehydrated leaves of *Sporobolus stapfianus* (Capannori samples)

Components	Fresh	Dried				Rehydrated	
		3 hr	7 hr	24 hr	120 hr	24 hr	72 hr
Glycerol	1.07	1.12	tr	1.21	tr	6.44	3.22
Fructose	15.04	15.80	10.74	8.51	5.64	5.37	1.07
Glucose	29.01	32.74	12.84	15.79	14.58	42.53	4.06
Mannitol	tr†	1.12	3.22	tr	tr	29.16	69.85
Lilioside B	3.22	2.25	1.07	1.21	1.12	1.07	–
Sucrose	51.56	55.32	69.82	73.04	79.48	3.22	1.12
Trehalose	tr	tr	tr	tr	tr	10.88	21.45
Raffinose	3.22	tr	3.22	4.86	3.64	tr	tr
Unidentified	4.29	4.51	6.44	7.29	5.64	18.26	7.90

*Calculated by GC analyses (external standard).
†Trace amounts.

appeared in high amounts, whereas the other sugars strongly decreased. The presence of mannitol and trehalose was clearly evidenced through GC, GC-mass spectrometry and NMR comparison with authentic samples.

These data were not consistent with the reported trend of sugars in the leaves of the resurrection plants [1–4]. To the best of our knowledge, the prevalence of mannitol and trehalose in desiccation-tolerant angiosperms is unprecedented.

To get more information, we decided to analyse a new set of *S. stapfianus* leaf samples. The fresh leaves were excised from plants grown in a glass-house in Florence, where environmental conditions were different (see Experimental) from the ones occurring in the glass-house of Capannori. As in the first experiments [1], samples of fresh, 120 hr dried and 24 hr rehydrated leaves were analysed. The content of sugars present in aqueous extracts is reported in Table 2.

Also in these samples, fructose, glucose and sucrose were the dominant sugars in fresh leaves. Water-stress enhanced sucrose, whereas after 24 hr rehydration, glucose and fructose became the predominant ones. No trehalose was determined in any of the analysed samples and only traces of mannitol were detected in fresh and rehydrated leaves.

Table 2. Composition (mg g^{-1} of aqueous extracts)* of water-soluble substances in fresh, dried and rehydrated leaves of *Sporobolus stapfianus* (Florence samples)

Components	Fresh	Dried (120 hr)	Rehydrated (24 hr)
Glycerol	1.65	tr†	4.95
Fructose	12.42	5.64	16.93
Glucose	25.78	11.71	50.38
Mannitol	1.52	tr	3.04
Inositol	1.08	1.03	—
Lilioside B	1.92	tr	1
Sucrose	61.98	91.07	8.85
Raffinose	2.98	4.47	1.49
Unidentified	9.01	7.2	30.07

*Calculated by GC analyses (external standard).
†Trace amounts.

The disaccharide trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) has been found in a wide variety of lower organisms [5] but in vascular plants it is a rare sugar [6]. When trehalose is present in plants, it often exceeds sucrose in concentrations [7] and sometimes appears to replace sucrose as translocated disaccharide [6]. In the past, trehalose was not reproducibly identified in angiosperms [8, 9], with a few reported exceptions [6], in *S. stapfianus* [1] and in *Myrothamnus flabellifolia* [10, 11], where this disaccharide was present in significant amounts, along with sucrose, in hydrated and dried leaves.

The detection of both mannitol and trehalose in plants has been related to the presence of fungi [6, 12–13]. The production of the reduced sugar, mannitol, by different microorganisms through conversion of glucose and other sugars is well known [14–16]. Trehalose is a very common sugar in plant symbioses and mycorrhizal symbioses are the most frequent [6]. Excised Ascomycete ectomycorrhizas have been shown to convert glucose and fructose into the 'fungus-specific' metabolites, trehalose and mannitol [6].

To explain the presence of mannitol and trehalose in the rehydrated leaves of the resurrection plant *S. stapfianus*, we also assumed an involvement of fungal species, naturally present on the leaf surface, able to perform biotransformations.

A similar doubt about the presence of trehalose in leaves of *M. flabellifolia* was previously reported [11], but anatomical and ultrastructural research did not show the presence of endophytes on the plant, thus excluding the possibility of a fungal origin for the trehalose detected. Moreover, the reported [11] significant increase of trehalose and sucrose in dried leaves, with respect to the fresh ones, was in reasonable agreement with the sugar trend observed in the resurrection plants [2–4].

In spite of the chance that the extraction procedure used had partially killed the fungi on the leaves, to confirm our hypothesis we incubated on PDA (potato dextrose agar) the residues of aqueous methanolic extracts of fresh, 24 and 120 hr dried and 24 and 72 hr rehydrated leaves (Capannori samples). For compari-

son, residues of extracts of Florence leaf samples were incubated under identical conditions. Significant growth of fungi and bacteria was observed on agar plates where all residues from Capannori samples were incubated for seven days, whereas only bacteria grew from the residues of Florence samples. *Cladosporium cladosporioides*, *Alternaria alternata*, *Fusarium* sp., *Penicillium* sp., *Rhizopus stolonifer* and *Trichoderma* sp. were identified and isolated. To verify whether these fungi may convert sugars, they were inoculated separately into aqueous solutions, each one containing excess of glucose, fructose or sucrose.

After seven days incubation, fungal growth was evident. GC analyses of the sugars (as trimethylsilyl derivatives) in filtrates showed that different starting sugars were differently converted by fungi, mannitol and trehalose being among the products of biotransformation.

Growth conditions of fungi on the leaf surface are different from those artificially produced in the laboratory, either as far as sugar concentrations are concerned or because of the presence of other components naturally occurring in vegetable materials. In an attempt to reproduce at least the sugar concentration conditions occurring in the leaves where a fungi spontaneously could have converted the sugars, we inoculated the fungal pool into a medium having a sugar content similar to that of 120 hr dried leaves. The results of the transformations performed by the fungal pool are given in Fig. 1.

GC determinations of starting sugars in the pool showed a 3:6:41 ratio between fructose, glucose and sucrose, respectively. After one day of treatment, no significant variations were observed in sugar composition (8:11:81). A three-day incubation caused an increase of fructose, near disappearance of glucose and a decrease in sucrose (37:2:41). Little mannitol (3) was detected, along with a reasonable amount of inositols (17). These cyclitols, whose structure of chiro-, muco-, epi-inositol was suggested by GC-mass spectral data, could be the cyclic precursors of the sugar alcohol, mannitol. After seven days, sucrose was completely converted into its hydrolysis products, fructose and glucose, which increased parallel to each other, fructose being the prevailing sugar compared to glucose (1:60:21). Significant amounts of mannitol (10) and trehalose (8) were also detected. Also, bacterial strains isolated from extracts of both Capannori and Florence samples were incubated in aqueous solutions of glucose, fructose and sucrose, separately. After seven days only unchanged starting sugars were present.

The isolation of fungi on some of the *S. stapfianus* leaf samples, confirming our hypothesis about the presence of mannitol and trehalose in aqueous extracts of rehydrated leaves, prompted us to consider the reason why only the plants grown in Capannori were contaminated, a problem which does not occur when the resurrection plants (*S. stapfianus*, as well as *B. hygroscopica*) were grown in the Florence glasshouse. In the heated glass-house at Capannori, plants experienced very high relative humidities and an everyday water spray (see Experimental). These growth conditions could have favoured a high occurrence to fungi on the fresh leaves of *Sporobolus*. In Capannori samples, the differences between sugar composition of fresh or dried leaves and the rehydrated ones (Table 1) could be due to the different capability of the leaves to resist fungal attack. Although each plant species is affected by *ca* 100 different kinds of fungi and bacteria, normally the plants defend themselves against pathogens either by means of physical barriers or biochemical reactions [17]. Fresh leaves on the plant might have been able to resist fungal penetration, and the excised dried leaves, because of water scarcity, are not an ideal habitat for fungal growth. Therefore, the presence of fungi on fresh and dried leaves does not affect the sugar content of these leaves. The slow decrease of fructose and glucose and the gradual increase of sucrose in the 3, 7, 24 and 120 hr dried leaves are well related to RWC variation (Fig. 2) and agree with the trend reported for resurrection plants [1–4].

When dried leaves were rehydrated by floating on water, fungal growth could easily take place. During rehydration, leaves absorbed water, but the physiological parameters were not restored, because detached leaves do not become desiccation-tolerant as attached ones do [18], then they could be considered decaying leaves. The continued degradation of structural lipids

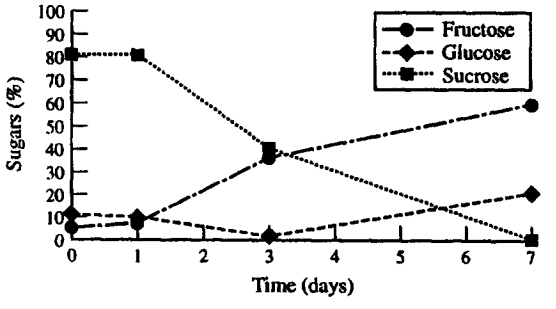

Fig. 1. Biotransformations of fructose, glucose and sucrose by fungal pool of *Sporobolus stapfianus* leaves.

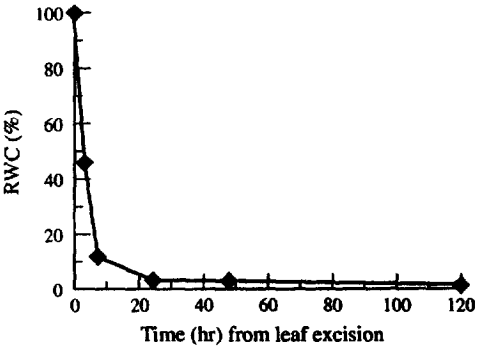

Fig. 2. Relative water content (RWC) of *Sporobolus stapfianus* leaves (Capannori samples) determined 3, 7, 24, 48 and 120 hr after excision.

during rehydration, along with accumulation of neutral lipids recently observed [19], could be a clear indication that *S. stapfianus* does not revive when both dehydration and rehydration occur on detached leaves. Thus, in decaying leaves, mannitol and trehalose could really be the product of a fungal biotransformation.

EXPERIMENTAL

Material and methods. All plants of *S. stapfianus* Gandoger were obtained from seeds sown in January 1992. Plants were grown inside heated glass-houses in Capannori (Lucca) or in Florence at the Department of Agronomy and Crop Production of Florence University.

Growth conditions for plants grown in Capannori (experiments of October 1993) were as follows: temp. inside glass-house was 18–20° (min. occasionally occurring, 16°), plants were set out on benches heated to 20–22°; in summer the glass-house temp. could increase to 35°. Relative humidity (RH) was *ca* 90–95% in autumn and winter and 35–40% in spring and summer. Sunlight was reduced because of shadowing of the glass ceiling and walls. Plants were grown in 12 cm diam. pots on peat and expanded clay (1:3) (pH 5.5–6) and were watered every day by spraying; every 15 days they were fertilized with a N–P–K (18:17:18) fertilizer. Growth conditions for plants grown in Florence (experiments of November 1994) were as follows: temp. inside glass-house was 18–23° (min. occasionally occurring, 16°), RH was not controlled. In summer, plants were grown outside, in full sun conditions; plants were grown in rectangular trays (40 cm width, 60 cm long, 25 cm height) on leaf mould and sand (1:3) and, when necessary, were irrigated with tap water; they were not fertilized.

Dehydration and rehydration experiments were carried out on excised leaves in a controlled environment: irradiance 21.2 W m^{-2}; light/dark temp. 27°; photoperiod 16 hr. Excised leaves were dehydrated in 1 l sealed jars over activated silica gel (*ca* 0% RH) for up to 120 hr. Leaves dehydrated for 120 hr were rehydrated by floating on dist. H_2O. Freshly excised leaves were controls. Leaf samples for each treatment were freeze-dried and stored at −20° until the time of use.

Extraction and solvent partitioning. These were accomplished on all samples as previously reported [1], giving epicuticular waxes and aq. and organic extracts as separate frs; wts are reported in Table 3.

Aqueous extracts. For GC analyses, sugars were converted into TMSi derivatives with pyridine–hexamethyldisilazane–trimethylchlorosilane (2:1:1). Derivatized sugars were kept in iso-octane for GC and GC-MS analyses. The ^{1}H NMR spectrum of the acetylated (Ac_2O–Pyridine) aq. extract from the Capannori 72 hr rehydrated leaves was recorded at 300 MHz in $CDCl_3$ (TMS at int. standard); chemical shifts are expressed in δ, and coupling constants (J) in Hz. The spectrum was almost superimposable on those of commercial trehalose and mannitol after acetylation (Ac_2O–Pyridine), with signals at δ 5.52 (*dd*, $J = 9.5$, 10 Hz, H-3 trehalose), partially overlapped by signal of equivalent H-3 and H-4 mannitol, *m* at 5.45; 5.3 (*d*, $J = 4$ Hz, H-1 trehalose); 5.08 (*m*, H-2 and H-5 mannitol); 5.03 (*dd*, $J = 4$, 10 Hz, H-2 trehalose), overlapped to H-4 trehalose (*t*, $J = 9$ Hz); 4.24, 4.05 (both *dd*, $J = 3$, 13 Hz; $J = 5$, 13 Hz, CH_2-1 and CH_2-6 mannitol), partially overlapped to H-5 and methylenic protons of trehalose; 2–2.1 (acetyl singlets). GC *and* GC-MS analysis conditions were as previously reported [1]. Characteristic ions for the TMSi derivatives of mannitol, trehalose and identified cyclitols were as follows: *Mannitol. m/z* (rel. int.) 614 $[M]^+$ (1), 421 (2), 345 (5), 332 (3), 319 (100), 305 (20), 217 (30), 205 (40), 147 (30), 103 (10), 73 (60). *Trehalose. m/z* (rel. int.) 533 (1), 477 (1), 451 (1), 361 (100), 319 (6), 281 (11), 271 (10), 217 (17), 204 (12), 191 (23), 169 (10), 147 (13), 103 (7), 73 (40). *Chiro-inositol. m/z* (rel. int.) 612 $[M]^+$ (1), 432 (2), 393 (2), 367 (4), 318 (30), 305 (25), 265 (15), 217 (35), 204 (15), 191 (20), 147 (50), 103 (5), 73 (100). *Muco-inositol. m/z* (rel. int.) 612 $[M]^+$ (1), 432 (4), 393 (6), 367 (1), 318 (30), 305 (30), 265 (3), 217 (40), 204 (20), 191 (25), 147 (50),

Table 3. Weights (% in parentheses) of starting samples, surface waxes, total MeOH–H_2O extract and organic and aqueous extracts of fresh, dried and rehydrated levels of *Sporobolus stapfianus*

Compound	Lyophylized sample	Waxes	MeOH–H_2O extract	Organic extract	Aqueous extract
Capannori samples					
Fresh	1000	7 (0.7)	238 (23.8)	18 (7.5)	188 (79)
3 hr dried	994	6 (0.6)	210 (21)	19 (9)	172 (82)
7 hr dried	1128	11 (0.97)	280 (25)	18 (6.4)	138 (49)
24 hr dried	988	8 (0.8)	171 (17.3)	16 (9.35)	122 (71)
120 hr dried	915	7 (0.76)	165 (18)	10 (6)	134 (81)
24 hr rehydr.	844	6 (0.7)	80 (9.5)	16 (20)	62 (77.5)
72 hr rehydr.	1228	11 (0.9)	80 (6.5)	8 (10)	67 (84)
Florence samples					
Fresh	554	7 (1.3)	60 (11)	8 (13)	50 (83)
120 hr dried	386	7 (1.8)	55 (14)	8 (14.5)	44 (80)
24 hr rehydr.	333	5 (1.5)	26 (7.8)	3 (11.5)	21 (80)

103 (8), 73 (100). *Epi-inositol. m/z* (rel. int.) 612 $[M]^+$ (1), 432 (2), 393 (5), 367 (8), 318 (15), 305 (40), 265 (15), 217 (50), 204 (15), 191 (35), 147 (50), 103 (5), 73 (100).

Fungal and bacterial growth. Fungal and bacterial cultures were isolated from residues of *S. stapfianus* leaf samples from Capannori and Florence after aq. MeOH extraction. From each sample, 100 mg was collected and distributed on PDA medium poured in to Petri dishes and incubated at 25° for 7 days. Isolated fungi, were maintained on PDA and used for testing their capacity to transform sugars.

Liquid growth media for evaluating sugar biotransformations. Fungal isolates were grown on liquid media containing different sugars as carbon source and peptone as nitrogen source; the compositions of the media were A: sucrose, 2 g; peptone, 1 g; dist. H_2O, 100 ml; B: glucose, 2 g; peptone, 1 g; dist. H_2O, 100 ml; C: fructose, 2 g; peptone, 1 g; dist. H_2O 100 ml; D = sucrose, 70 mg; glucose 10 mg; fructose, 5 mg; peptone, 0.8 g; dist. H_2O 100 ml. Each fungal isolate was grown on media A–C for evaluating their capability of biotransformation. Medium D was the most similar to the natural conditions of the 120 hr dried leaves as far as sugar composition was concerned; it was used for growing the pool of fungal isolates. Spores were diluted to 10^6 ml^{-1} with the above mentioned media; 1 ml was inoculated into 99 ml growth medium in a 250 ml flask and incubated at 25° for 1, 3 and 7 days.

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