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Desiccation increases sucrose levels in *Ramonda* and *Haberlea*, two genera of resurrection plants in the Gesneriaceae

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A few genera of angiosperms are known as 'resurrection plants' since their leaves withstand complete desiccation. In many organisms, including some resurrection plants, desiccation tolerance is associated with the accumulation of special carbohydrates. We examined whether this is also true for the two European angiosperm genera of resurrection plants, *Ramonda* and *Haberlea* in the Gesneriaceae. Using gas chromatography, non-structural carbohydrates were determined as a percentage of the dry weight in leaves of *Ramonda nathaliae* subjected to various desiccation regimes. Sucrose was the predominant soluble carbohydrate in all samples, and its level steadily increased from 2 to 10% during desiccation. Starch amounted to ca 2% in control leaves and disappeared completely within 8 days of desiccation. Considerable amounts (1–2.5%) of raffinose and smaller amounts of its precursor galactinol (1-*α*-galactosyl-*myo*-inositol) were present in control leaves; these carbohydrates showed only minor changes upon desiccation. Similar results were obtained when excised leaves of *Ramonda nathaliae*, *Ramonda myconi* and *Haberlea rhodopensis* were subjected to desiccation. These data indicate that sucrose accumulation is connected to desiccation tolerance in Gesneriaceae; the presence of raffinose may be a pre-adaptation since this sugar prevents crystallization of sucrose during drying.

Key words – Carbohydrate metabolism, compatible solutes, desiccation, drought stress, galactinol, raffinose, resurrection plants, starch, sucrose.

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Introduction

Many lower organisms are desiccation-tolerant, but there is only a small number of higher plants, the so-called resurrection plants, that withstand complete desiccation during vegetative growth (Bewley and Krochko 1982, Ingram and Bartels 1996). Is this resistance towards desiccation linked to a rapid accumulation of compatible solutes, especially carbohydrates? In the case of lower organisms, besides other compatible solutes, the disaccharide trehalose is thought to play a central role as a protectant against desiccation (Crowe et al. 1984, Wiemken 1990). Interestingly, while trehalose is generally absent from plants (Müller et al. 1995), three resurrection plants have been reported to accumulate trehalose upon desiccation, the pteridophyte *Selaginella lepidophylla* (Adams et al. 1990), the angiosperms *Myrothamnus flabellifolius* (Bianchi et al. 1993, Drennan et al. 1993), and *Sporobolus stapfianus* (Albini et al. 1994). However, other resurrection plants do not accumulate trehalose but show different responses with regard to carbohydrate metabolism. *Craterostigma plantagineum*, the best-studied example, contains the uncommon monosaccharide 2-octulose in its hydrated state which, upon drought, is rapidly degraded and transformed to sucrose, indicating that sucrose might also have a protective potential (Bianchi et al. 1991, 1992). Experimental evidence suggests that sucrose also can act as a protectant of membranes and proteins (Crowe et al. 1987), and high amounts of sucrose were indeed shown to be

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correlated with the survival of pollen (Hoekstra et al. 1989) and embryos (Black et al. 1996), two phases in the life cycle of higher plants where usually complete desiccation occurs. The protective effect of sucrose is promoted by the presence of raffinose which prevents sugar crystallization and facilitates vitrification (Koster 1991, Lin and Huang 1994, Black et al. 1996). Thus, it has been shown in model experiments that lipids dried in mixtures of sucrose and raffinose have lamellar structures similar to those associated with the lipids in water (Caffrey et al. 1988). Molar sucrose/raffinose-ratios of less than 20:1 have been found to be sufficient to prevent crystallization of sucrose during desiccation (Lin and Huang 1994).

There are only two European genera of angiosperms that are resurrection plants, namely *Ramonda* and *Haberlea* of the Gesneriaceae (Bewley and Krochko 1982, Stefanov et al. 1992, Markovska et al. 1994), both of which have not yet been examined with respect to the possible role of sugars in desiccation tolerance. Here, we show that leaves of these Gesneriaceae accumulate large quantities of sucrose during desiccation, which they metabolize rapidly upon re-watering, and that they contain considerable amounts of raffinose even in the unstressed state, a feature that might pre-adapt them to the protective effect of sucrose.

Materials and methods

Growth of plants

Plants of *Ramonda nathaliae* Panc. & Petrov., *Ramonda myconi* (L.) Reichenb., and *Haberlea rhodopensis* Friv. were grown in the Botanical Garden of the Univ. of Basel (Switzerland). For desiccation experiments with excised leaves, mature leaves were taken from plants and transferred to open petri dishes (9-cm diameter) with one layer of filter paper (Schleicher & Schuell, Basel, Switzerland) kept dry or wetted with 10 ml of water. After incubation for one day, the leaves were prepared for carbohydrate extraction as indicated below.

For drought stress experiments with intact plants, *R. nathaliae* plants were transferred to plastic pots (7-cm diameter) with a rapidly drying soil mixture consisting of clay, sand and peat in a 2:1:1 (v/v/v) ratio, grown in a greenhouse with natural lighting at about 22°C (photoperiod ca 14 h), and watered daily with tap water for one month. Then, some of the plants were kept without watering for up to 10 days, and others left unwatered for 5 days and then watered again, while controls continued to be watered daily. At intervals, leaf samples were cut from the plants, immediately chilled and lyophilized.

Identification and analysis of soluble carbohydrates

Carbohydrates were analyzed as described previously (Müller et al. 1994). Briefly, soluble carbohydrates were extracted by grinding the leaves (ca 10 mg dry weight) in 80% (v/v) methanol (50 ml g^{-1} dry weight) containing 1% (w/v) insoluble polyvinylpyrrolidone and mannoheptulose (50 µg per sample) as internal standard. The homogenized samples were incubated at 60°C for 10 min followed by centrifugation (13 000 *g*, 10 min). The extraction was repeated three times, and the supernatants were collected and vacuum-dried. After resuspending the pellets in 0.6 ml distilled water, charged compounds were removed using a mixed-bed ion exchanger (Serdolit micro blue and red 2:1 [v/v] mixture; Serva, Heidelberg, Germany) of which 50 µl were added to the samples. After vortexing and centrifugation of the samples,

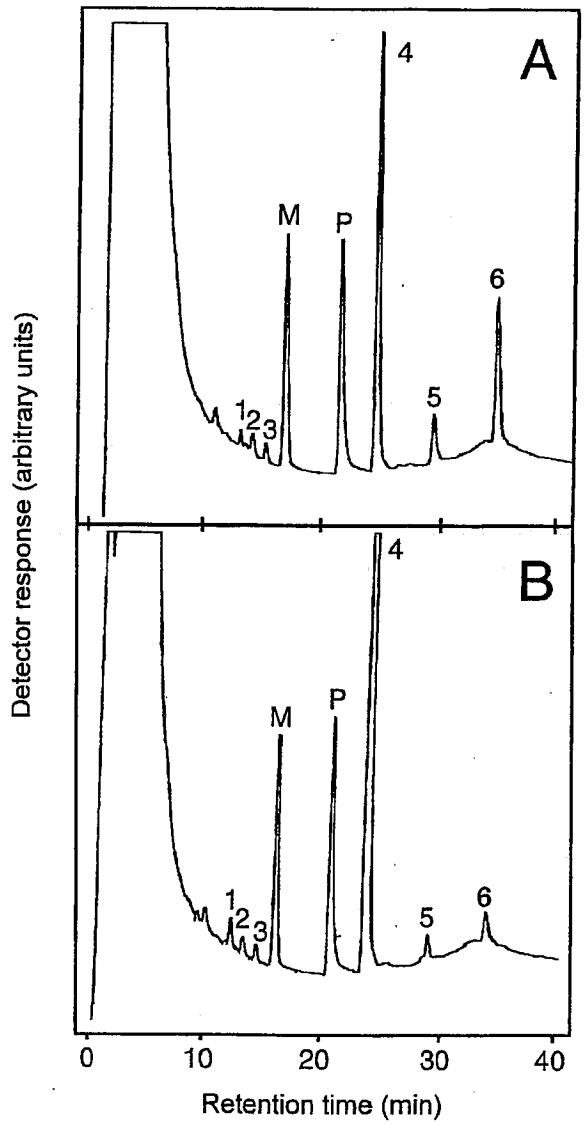

Fig. 1. Typical gas chromatographs of silylized, soluble carbohydrates extracted from control or dried leaves of *Ramonda nathaliae*. A, Sample from control leaf (5.3 mg dry weight); B, sample from leaf dried for 5 days (4.2 mg dry weight); M, mannoheptulose (internal standard); P, phenylglucopyranoside (derivatization standard); 1, fructose; 2, glucose; 3, *myo*-inositol; 4, sucrose; 5, galactinol; 6, raffinose.

the supernatants were lyophilized. After redissolving and transferring the pellets to gas chromatography vials, silylation and gas chromatography of the silylized carbohydrates was performed as described (Müller et al. 1994), using authentic standards for identification. The galactinol standard was a gift of Dr F. Keller, Univ. of Zurich, Switzerland.

Analysis of starch

After removing the soluble carbohydrates, starch in the lyophilized samples was solubilized by heating in 0.2 ml NaOH (0.5 *M*) at 60°C for 1 h. Then, 0.2 ml HCl (0.5 *M*) was added in order to neutralize the preparation, and starch was quantified by addition of 1 U glucoamylase (Boehringer, Mannheim, Germany) in 0.4 ml 0.2 *M* acetate (Na^+) buffer adjusted to pH 4.5 (Wagner and Wiemken 1989). After overnight incubation at 37°C, the glucose released was quantified by the glucose-peroxidase method using a test kit (Boehringer) according to the manufacturer's instructions.

Results

Carbohydrates in fully-hydrated and desiccated leaves of *Ramonda* and *Haberlea*

As shown in Fig. 1A, the main carbohydrate peak from well-watered leaves of *R. nathaliae*, aside from standards, corresponded to sucrose (ca 2% of the dry weight). Glucose, fructose, and inositol were present only in low quantities (ca 0.1% of dry weight), but raffinose was present at high levels (ca 1% of dry weight), as was its precursor galactinol (ca 0.3% of dry weight). Desiccation of the leaves lead to an increase in sucrose but to a decrease in raffinose and galactinol; no new peaks appeared (Fig. 1B).

Similar carbohydrate profiles were detected in leaf samples from *R. nathaliae*, *R. myconi* and *H. rhodopensis*. They all showed a marked increase in sucrose levels in desiccated leaves as compared to control leaves, reaching values of 5–7% of the dry weight corresponding to 85–91% of the total amount of soluble carbohydrates (Tab. 1). In the well-watered stage, all contained raffinose and galactinol at relatively high levels that corresponded to 0.3–2% of the dry weight each, and these compounds decreased during desiccation. The ratio of sucrose/raffinose was low in control plants (2:1 to 5:1 on a mass basis) and increased to about 10:1 upon desiccation (Tab. 1).

Tab. 2. Typical water contents and appearances of desiccating *Ramonda nathaliae* leaves. The water content of the leaves was determined by weighing before and after lyophilization and is expressed as percent of the total weight. Means ± SE for three independent plants.

Days after starting drought	Appearance of the leaves	Water content (%)
0	Normal	75.4 ± 0.5
1	Normal, a few leaves start rolling up	69.1 ± 0.7
2	Most leaves start rolling up	62.6 ± 2.9
3	Completely rolled up, but still green	17.3 ± 1.8
> 4	Brownish	10.3 ± 1.4

Dynamics of the carbohydrate pools of *R. nathaliae* during a drying-re-watering cycle

A more detailed study was undertaken with intact *R. nathaliae*, which grew best under greenhouse conditions, after replanting it into a quickly drying soil. Under these conditions, the sucrose pool in leaves of control plants was about 2% of the dry weight at the beginning and decreased to about 1.5% during the course of the experiment. When *R. nathaliae* plants were left without water, they showed the first symptoms of desiccation within two days, namely a characteristic rolling of the leaves. In this state, the water content of the leaves was 62% as compared to 75% in control leaves (Tab. 2). The sucrose pool increased strongly during this phase to about 6% of the dry weight (Fig. 2). When leaves were left without water for one week, their water content dropped to 10% of the total weight (Tab. 2), and their sucrose content reached more than 10% of the dry weight (Fig. 2). Five days after starting the experiment, some desiccated plants were re-watered. After one day, the leaves had already regained the color and shape of control leaves. Their sucrose pool dropped to values comparable to control plants within three days (Fig. 2).

The starch pool in leaves of control plants was initially 1.2% of the dry weight and increased to 2.2% in

Tab. 1. Soluble carbohydrates in excised leaves from various resurrection plants. The leaves were incubated for 24 h, either on wet filter paper (control) or on dry filter paper (dry). The carbohydrate contents were determined by gas chromatography. Values represent means from two independent samples; variation between duplicates was less than 10%.

Plant	Treatment	Soluble carbohydrates (mg g^{-1} dry weight)					
		Sucrose	Raffinose	Galactinol	Glucose	Fructose	Inositol
R. nathaliae	Control	22.1	10.6	3.1	1.8	2.3	0.5
	Dry	52.6	5.3	1.4	0.4	0.5	0.4
R. myconi	Control	33.5	19.9	13.6	0.1	1.5	0.9
	Dry	70.2	8.2	1.4	0.9	0.6	1.1
H. rhodopensis	Control	20.5	4.5	10.9	3.8	1.2	0.2
	Dry	56.9	3.3	0.6	0.4	0.4	0.8

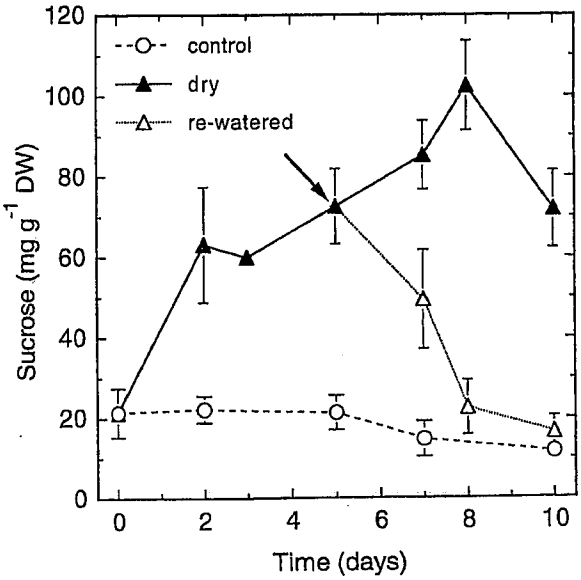

Fig. 2. Sucrose content of leaf disks of *Ramonda nathaliae*. Plants were either watered normally (control), not watered (dry) during the whole experiment or not watered during 5 days and then re-watered (re-watered.), The arrow indicates the point when re-watering started. Sucrose was measured by gas chromatography. Means ± SE for 5 independent plants.

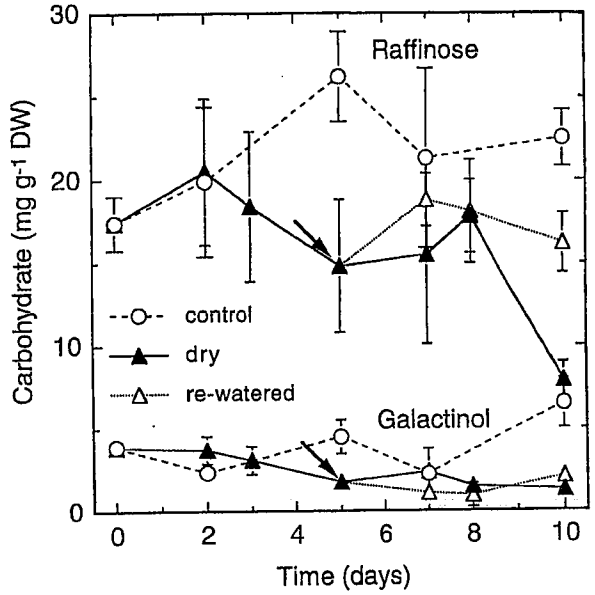

Fig. 4. Levels of raffinose and galactinol in leaf disks of *Ramonda nathaliae*. Plants were treated as described in the legend of Fig. 2. Means ± SE for 5 independent plants.

the course of the experiment. It started to decrease after three days without water, approaching the limits of the detection system used at the end of the desiccation experiment. Upon re-watering, starch quickly re-accumulated, reaching control values within 1 day, at the time when the leaf had regained normal appearance (Fig. 3).

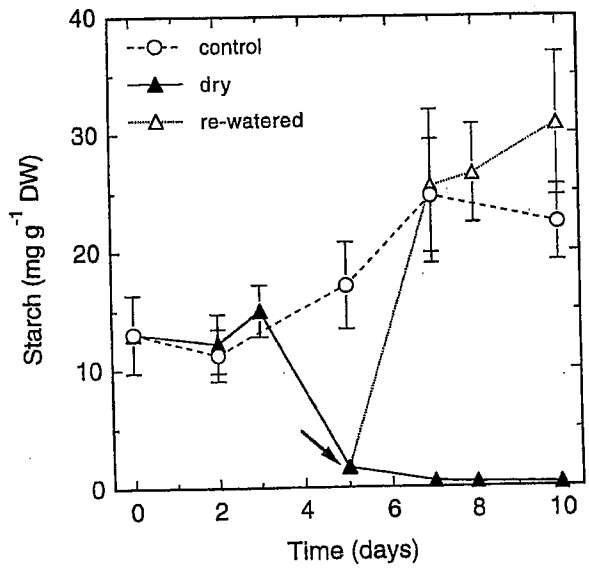

Fig. 3. Starch content of leaf disks of *Ramonda nathaliae*. Plants were treated as described in the legend of Fig. 2. Starch was determined enzymatically. Means ± SE for 5 independent plants.

The pool size of raffinose was 1.8–2.6% of dry weight in control plants. In contrast to sucrose, it did not increase upon desiccation, but decreased considerably to reach values of less than 1% of dry weight. Upon re-watering, this tendency was reversed (Fig. 4). The pool size of galactinol, the galactosyl donor for raffinose, amounted to 0.4–0.7% of dry weight; it decreased in parallel with raffinose during desiccation. The galactinol content of the leaves did not rise after re-watering, but remained as low as in dry leaves, at ca 0.2% of dry weight (Fig. 4).

In addition, *R. nathaliae* leaves contained small amounts (less than 0.1% of the dry weight) of glucose, fructose and *myo*-inositol. The pools of these three monosaccharides tended to increase in leaf samples of plants desiccated for more than 5 days and dropped back to control levels upon re-watering (Tab. 3).

Discussion

The resurrection plant, *R. nathaliae*, withstood desiccation for several weeks without apparent harm. After re-watering, the plant recovered quickly and re-greened within one day. More detailed physiological studies, performed with *Ramonda serbica*, have shown that the photosynthetic apparatus of desiccated plants was as active as in control plants already within one week after re-watering (Markovska et al. 1994). Besides the expression of desiccation-related polypeptides (Schneider et al. 1993), the accumulation of high amounts of compatible solutes, especially carbohydrates, could account for the desiccation resistance of resurrection plants (see Ingram

Tab. 3. Pool sizes of glucose, fructose and *myo*-inositol in leaf disks of *Ramonda nathaliae*. The plants were either normally watered (control), not watered (dry) during the whole experiment or not watered during 5 days and then re-watered (re-watered; days after re-watering in brackets). Means (mg g^{-1} fresh weight) ± SE are given for 5 independent plants.

Time (days)	Treatment	Glucose	Fructose	Inositol
0	Control	0.80 ± 0.12	0.52 ± 0.09	0.50 ± 0.08
2	Control	0.60 ± 0.10	0.34 ± 0.07	0.41 ± 0.03
	Dry	0.78 ± 0.20	0.48 ± 0.20	0.47 ± 0.05
5	Control	0.50 ± 0.07	0.32 ± 0.09	0.44 ± 0.04
	Dry	0.84 ± 0.20	0.40 ± 0.13	0.60 ± 0.04
7	Control	0.50 ± 0.06	0.24 ± 0.12	0.22 ± 0.06
	Dry	1.73 ± 0.74	1.10 ± 0.50	0.39 ± 0.10
	Re-watered (2)	0.58 ± 0.18	0.18 ± 0.12	0.20 ± 0.12
10	Control	1.02 ± 0.20	0.48 ± 0.10	0.43 ± 0.04
	Dry	1.80 ± 0.60	1.80 ± 0.80	0.67 ± 0.07
	Re-watered (5)	0.50 ± 0.16	0.30 ± 0.10	0.35 ± 0.08

and Bartels 1996). Indeed, in *R. nathaliae* leaves, the pool of sucrose increased quickly from levels of ca 2% to 10% of the dry weight upon drying. About one-fifth of this increase appeared to occur at the expense of starch which was completely depleted, and another fifth at the expense of raffinose. We could not detect any other carbohydrate whose degradation could account for the increase in sucrose, and we therefore postulate that a considerable part of the sucrose accumulated represents de novo carbohydrate synthesis. This is different from the situation in maturing, desiccating wheat embryos, where the increases in sucrose and raffinose are fully explained by the decrease of the starch pool (Black et al. 1996), and from the situation in *Craterostigma* where, upon drought stress, the synthesis of sucrose occurs mainly at the expense of the uncommon monosaccharide octulose (Bianchi et al. 1991).

It is well known that sucrose increases in many desiccation-sensitive plants upon water stress (Kameli and Lösel 1993, Bohnert et al. 1995). In the desiccation-resistant resurrection plants of the Gesneriaceae, two factors may contribute to a particularly efficient protective effect of sucrose: (1) the sucrose content increased quickly upon water loss reaching about 10% of the dry weight; high amounts of sucrose being necessary for a protective effect on membranes (Crowe et al. 1987); and (2) raffinose, a sugar that facilitates vitrification instead of crystallization of sucrose and thereby promotes the protective effect of sucrose (Koster 1991, Lin and Huang 1994, Black et al. 1996), was present in high amounts (0.5–2% of the dry weight) in non-stressed leaves, yielding a molar ratio of sucrose/raffinose of about two. Upon drought, the raffinose pool decreased; however, the molar ratio never exceeded 20, so that the protective effect of raffinose may still be guaranteed (Black et al. 1996). Raffinose commonly occurs in desiccation-tolerant resting tissues, e.g. in seeds (see Blackman et al. 1992), or seasonally during winter in woody stems and roots of trees (*Cornus sericea*, Ashworth et al. 1993; *Picea abies*, Wiemken and Ineichen 1993) but normally only in very small amounts in leaves. In addition to the high constitutive pools of raffinose, we found galactinol in *Ramonda* and *Haberlea* leaves. Concerning higher raffinosides, only traces of stachyose could be detected in leaf samples from *R. nathaliae* (F. Keller, personal communication). *R. nathaliae* did not contain sizable amounts of other rare or unusual soluble carbohydrates.

We conclude that the resurrection plants of the Gesneriaceae accumulate sucrose as a protectant against desiccation stress. The constitutive presence of raffinose might be considered a pre-adaptation, such that vitrification of the accumulated sucrose occurs under anhydrobiotic conditions.

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