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Drought-induced metabolic adjustments in woodland strawberry leaves: the role of soluble carbohydrates and starch

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Abstract: Water deficit is one of the most important abiotic factors limiting crop yields. To better understand the link between carbohydrate balance and drought stress response in strawberry plants (*Fragaria vesca*), we monitored by liquid chromatography the carbohydrate status in leaves during exposure to drought of different duration and intensity as well as subsequent recovery. In two greenhouse experiments that differed in the rate of reaching the target water deficit, strawberry leaves showed osmotic adjustment, with gradual increases in glucose and fructose content, likely provided by observed starch degradation. At the point of the most severe stress, proline content increased, while stress markers, such as malondialdehyde content and chlorophyll fluorescence, showed no significant changes. It indicates the defence mechanisms' ability to protect cellular structures effectively. Strawberry, a member of the Rosaceae family, motivated us to investigate the role of sorbitol in the stress response. However, we found no sorbitol in any stress or control situations. Finally, testing sorbitol's ability to support strawberry plant or non-green callus growth *in vitro* did not indicate that sorbitol could be used as a carbon and energy source. In conclusion, strawberries exhibit marked changes in soluble carbohydrate and starch content as an efficient defence against drought, without apparent involvement of sorbitol.

Keywords: antioxidant; abiotic stress; malodialdehyde; prolin; saccharides; wild strawberry

Woodland strawberry (*Fragaria vesca* L.) is a perennial herb native to Eurasia and North America, prized for its fruit. This wild species is often used as a comparative model for the closely related octoploid strawberry (*Fragaria* × *ananassa* Duch.), one of the most important horticultural crops. As there is a high degree of collinearity between the *F. vesca* and *F.* × *ananassa* genomes (Rousseau-Gueutin et al. 2008), their metabolic regulations are likely to be very similar (Rohloff et al. 2012), which, together with the smaller genome size, makes the woodland strawberry a convenient model. Due to their shallow root systems and relatively large leaf areas, both species have high water demands, and water scarcity can significantly affect growth and productivity. Therefore, understanding the physiological responses of woodland strawberry leaves to water deficit would be very useful in developing effective strategies to mitigate these effects.

As water deficit is a common stress faced by plants in the natural environment, many of them have developed various mechanisms how to cope with it. One of the critical aspects of this stress response is the regulation of carbohydrate metabolism, which plays a key role in plant survival under water-limited conditions (Thomas and Beena 2021). Structural polysaccharides (e.g. cellulose) are the basic building blocks of the plant body, nonstructural ones serve as carbon and energy sources, but also as signalling molecules, that influence various physiological processes. In ad-

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dition, soluble carbohydrates can act as osmolytes/compatible solutes, providing osmotic adjustment and helping protect functional cellular structures from the adverse effects of water deficit (Valluru and Van Den Ende 2008, Koehler et al. 2015). Therefore, their accumulation is a common response to drought across various species (Amoah and Adu-Gyamfi 2024). In strawberry plants, osmotic adjustment was often observed during different stress treatments (Razavi et al. 2008, Ghaderi and Siosemardeh 2011, Rohloff et al. 2012, Davik et al. 2013, Sun et al. 2015), which raises the question of to what extent carbohydrates are involved in the response of strawberry leaves to adverse conditions. Under drought stress, the production of reactive oxygen species (ROS) is increased, leading to enhanced oxidative stress. Several sugars and sugar alcohols have been shown to play a critical role in mitigating ROS damage by acting as antioxidants and true ROS scavengers (Keunen et al. 2013, Peshev et al. 2013). The most reactive and dangerous ROS, hydroxyl radicals, cannot be detoxified enzymatically because of their extreme reactivity and short lifespans. Therefore, they can only be scavenged by low molecular compounds, like soluble carbohydrates, that are already present in sufficient quantities at a given location and quench the hydroxyl radical at the site of its origin (Peshev et al. 2013, Matros et al. 2015). It is important to note that individual soluble carbohydrates have different ROS-scavenging capacities (Peshev et al. 2013) and differ significantly in their ability to protect cellular membranes (Valluru and Van Den Ende 2008).

Plants often contain a broad spectrum of soluble carbohydrates, and strawberry is no exception. (Koehler et al. 2015). In the family Rosaceae, to which the genus *Fragaria* belongs, many plants produce high amounts of sugar alcohol sorbitol (Pleyerová et al. 2022). Moreover, sorbitol often accumulates in those species as an adaptive response to stress (Lo Bianco et al. 2000, Jiménez et al. 2013). While many economically important members of the Rosaceae family, such as apple, peach, and cherry, primarily utilise sorbitol as their main photosynthetic product and transport carbohydrate, strawberry stands out. It primarily translocates sucrose to its sinks, and only trace amounts of sorbitol are typically detected in some cultivar varieties in fruits (Ogiwara et al. 1998, Sutsawat et al. 2008) and leaves (Akšić et al. 2019), but significantly lower than in sorbitol-accumulating Rosaceae trees. Importantly, strawberries possess several genes for sorbitol metabolism (catabolism as well as anabolism), and some of the related enzymatic activities in fruits and leaves have been confirmed experimentally (Duangsrisai et al. 2007, Sutsawat et al. 2008). Therefore, studying sorbitol metabolism in strawberry, particularly under drought stress, is a highly desirable and logical area of research. The fact that strawberry possesses genes for sorbitol metabolism and that this pathway can be influenced opens significant avenues for genetic improvement. As expected, overexpression of a plum *PsS6PDH4* gene (a key sorbitol synthesis enzyme) in strawberry fruits successfully promoted the accumulation of sorbitol, while virus-induced gene silencing using a fragment of the same gene decreased sorbitol content (Du et al. 2024). This provides compelling evidence that the sorbitol pathway in strawberry is amenable to manipulation, even if its native activity is low. This metabolic divergence makes strawberry a unique model for studying the evolution and regulation of carbohydrate metabolism within a single plant family and raises questions about the functions of individual carbohydrates across different plants.

Reports monitoring stress-induced saccharide accumulation often use simple methods with low specificity and accuracy, and/or quantify only total sugars. It is clear that although total sugar levels are important for plant resistance to stress, shifts in the carbohydrate spectrum are also critical. Studies devoted in detail to the dynamics of individual components of the carbohydrate spectrum are relatively rare. In addition, many reports do not include the information on starch content and dynamics, making the predictive value of soluble carbohydrate-only analyses problematic. On top of that, saccharide levels are often monitored only once under stress, or at a limited number of time points, and the vital recovery phase is often omitted altogether.

The aim of this work is to expand the current knowledge on the physiological response of source leaves to water deficit in wild strawberry plants. The potted plants cultivated in a greenhouse were exposed to drought, achieved by two different rates of reaching the target soil water deficit. The effect of stress was evaluated by measuring proline, a stress-protective compound; malondialdehyde, an indicator of stress-induced membrane damage; and chlorophyll fluorescence. The analyses focused on carbohydrate metabolism and its role in drought stress, with particular attention paid to identifying signs of sorbitol involvement. The plant material's ability to integrate sorbitol into its metabolism was examined *in vitro*.

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MATERIAL AND METHODS

Plant material and growth conditions. The study was conducted with woodland strawberry (*Fragaria vesca* cv. Rujana) at Charles University, Prague, Czech Republic (50°4'18.214"N, 14°25'26.739"E, altitude 227 m a.s.l.). For greenhouse experiments, the seeds (Moravoseed CZ a.s., Mikulov, Czech Republic) were sown in pots with horticultural substrate and cultivated from spring to autumn. Then the ramets (plantlets from runners) were cut and planted individually to new 3.4 dm^3 pots with identical substrate. After overwintering in the greenhouse, the well-established plants were selected and divided into three groups: control (C); mild stress (MS), and severe stress (SS), and randomly assigned to a single block. The first experimental setup (fast desiccation, FD) took place from June to July, and the second (slow desiccation, SD) from August to October, both in a greenhouse under natural photoperiod.

Sampling time points were chosen as follows: first – start of the experiment (day 0); second – five days from the start (day 5); third – when the relative humidity of soil in SS variant dropped to 40% (day 11 and 32 in FD and SD respectively); fourth – when the relative humidity of soil in SS variant dropped to 20% (day 14 and 42 in FD and SD, respectively), and fifth – fourteen days after the fourth time point (day 28 and 58 in FD and SD respectively). Moisture in the SS variant was continuously monitored by PlantCare Mini-Logger (PlantCare Ltd., Russikon, Switzerland). In the MS variant, the moisture matched that of the SS variant until the third sampling point (after which the MS variant was irrigated – see below). Plant analyses were performed with 3–5 biological replications per treatment and sampling point. Only fully developed intact leaves were selected for analyses.

At the start of the experiment, all plants were fully irrigated (0.5 L water/plant, fully saturated substrate). Then, in the SS group, irrigation was withheld completely until the 4th sampling point. In the MS group, irrigation was withheld until the 3rd sampling point, and then irrigation was applied once with 0.1 L of water/plant. After the 4th sampling point, both stress groups were fully irrigated and kept that way during the whole recovery phase. The control group was irrigated as needed to keep the soil wet during the whole experiment.

Sorbitol utilisation experiments. For the *in vitro* experiments, the seeds were surface sterilised (96% ethanol for 1 min followed by 0.7% sodium hypochlorite for 15 min) and sown on sugar-free MS medium (8 g/L of agar; pH 5.7) (MS Basal Salt Mixture, M 5519 Sigma-Aldrich, Burlington, USA) supplemented with vitamins (100 mg/L of inositol, 0.5 mg/L of nicotinic acid, 0.5 mg/L of pyridoxine:HCl, 0.5 mg/L of thiamine:HCl and 2 mg/L glycine). All plants were cultivated under a 16 h photoperiod with approximately 50 µmol/m^2/s PAR (daylight fluorescent tubes, Osram AG, Premstaetten, Austria) and a temperature of 23 ± 1 °C. The cultivation vessels (250 mL Erlenmeyer flasks) were covered with transparent polypropylene foil bearing two semipermeable polypropylene filter discs (diameter 8 mm, porosity 0.04 µm, Sigma-Aldrich, Burlington, USA) to ensure gas exchange.

At the beginning of the experiment with fully *in vitro*-grown plants, seedlings at the five-leaf stage (8 plants per variant) were transferred to fresh MS medium supplemented with 3% sucrose, 1.5% sucrose + 1.5% sorbitol, 3% sorbitol, or no soluble carbohydrate, depending on the variant. The variants containing carbohydrate in medium were closed with aluminium caps instead of polypropylene foil. All plants were cultivated in the above-described conditions for 3 weeks.

The callus cultures were derived from *F. vesca* petiole cuttings, using MS medium with 3% sucrose, 4 mg/L 1-naphthaleneacetic acid (NAA) and 2 mg/L 6-benzylaminopurine (BAP), and were cultivated in 100 mL Erlenmeyer flasks sealed with aluminium caps, in darkness at 23 ± 1 °C. At the beginning of the experiment, the callus cultures (8 pieces per variant, approx. 0.5 cm^3 each) were transferred to fresh MS medium supplemented with 3% sucrose, 1.5% sucrose + 1.5% sorbitol or 3% sorbitol, according to the variant, and cultivated for 2 weeks under the same conditions.

Fast fluorescence kinetics measurement. Plants were dark-adapted (in a nontranslucent box) for 30 min to ensure full oxidation of photosystem reaction centres. The portable fluorimeter FluorPen 2 (Photon Systems Instruments, Drásov, Czech Republic) was used to measure the rapid onset of fluorescence induced by a saturating blue light pulse. The maximum quantum yield of photochemistry in Photosystem II was calculated as $(F_m - F_0)/F_m$, where F_m is the maximum value of chlorophyll fluorescence under saturating radiation pulse, and F_0 is the initial value of the fluorescence in the dark-adapted state.

Malondialdehyde content determination. Lipid peroxidation was estimated by measuring the malon-

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dialdehyde (MDA) level as described by Hodges et al. (1999) with modifications. Briefly, 80 mg of fresh leaf was homogenised in 2 mL of 80% ethanol (*v*/*v*) containing 1 mg/L of butylated hydroxytoluen and centrifuged at 9 000 × *g* for 20 min at 4 °C. Supernatant was divided into two microtubes (0.7 mL each). The first part was mixed with 0.7 mL of 20% trichloroacetic acid (*v*/*v*) containing 6.5 g/L thiobarbituric acid, and the second part was mixed with 20% trichloroacetic acid without thiobarbituric acid. Samples were heated in a dry block heater at 95 °C for 25 min, then cooled rapidly in an ice bath, and centrifuged again at 9 000 × *g* for 20 min at 4 °C. The absorbance of the mixture was measured at 450, 532, and 600 nm using a UV/Vis spectrophotometer (Evolution 201, Thermo Scientific, Waltham, USA). The amount of MDA was calculated using equations described by Kofroňová et al. (2020).

Proline content determination. The proline content was determined according to Bates et al. (1973). Fresh leaves (0.5 g) were homogenised in 2 mL of 3% sulfosalicylic acid (*v*/*v*) and centrifuged at 4 472 × *g* for 5 min at room temperature. The supernatant was mixed with an equivalent volume of ninhydrin and glacial acetic acid and incubated in boiling water for 1 h. Proline was extracted with 3 mL of toluene, and its concentration was quantified spectrophotometrically at 520 nm (Evolution 201, Thermo Scientific, Waltham, USA).

Soluble carbohydrate and starch content determination. Leaf samples (approx. 100 mg) were freeze-dried, and their dry weight was determined. The samples were incubated in a dry block at 75 °C for 15 min with 0.5 mL of 80% methanol. Methanol was vacuum-evaporated, and ultrapure water (Milli-Q, Millipore, Burlington, USA) was added to extract the soluble carbohydrates. The samples were placed in an ultrasonic bath for 15 min and then centrifuged for 10 min at 14 000 × *g*. The supernatant was filtered through membrane filters (pore size 0.22 µm). The content of soluble carbohydrates was determined by high-performance liquid chromatography (HPLC; mobile phase: ultrapure water) with refractive index (RI) detection (Shodex RI-71), column: SP0810 (Shodex, Tokyo, Japan). Standard dilutions with known concentrations of sucrose, glucose, fructose, sorbitol and inositol were used as references. The data were collected using Clarity 7.2 software (DataApex, Prague, Czech Republic). The remaining pellets from soluble carbohydrate extraction were used to determine the starch content. They were rinsed four times with ultrapure water, the starch was hydrolysed by α-amylase (Sigma-Aldrich, 30 U, Burlington, USA) and amyloglucosidase (Sigma-Aldrich, 60 U, Burlington, USA) for 1 h at 40 °C, and the released glucose was quantified by HPLC (see above). The starch content was expressed as the amount of glucose after enzymatic cleavage.

Data analysis. Data are presented as means of 3–5 independent biological replications. For treatment comparisons, one-way analysis of variance (One-way ANOVA) and Tukey-Kramer Test (for normally distributed data) or Kruskal-Wallis Multiple-Comparison Z-Value Test (for data not normally distributed) were applied using NCSS 9.0 software (NSCC LLC, Kaysville, USA). Differences were examined at $\alpha < 0.05$ (marked as asterisk *) and $\alpha < 0.1$ (marked as bold dot •).

RESULTS

Water content in soil and plants. The potted strawberry plants were subjected to water stress induced by irrigation withdrawal at two levels of severity: mild stress and severe stress, differing in the drought intensity achieved before rewatering (for details, see materials and methods). On top of that, the reduction in substrate moisture occurred in two setups: slow desiccation and fast desiccation, differing in the rate at which the target soil water deficit was achieved. The external conditions affecting plants are illustrated in Figure 1, which shows soil moisture decline in the severe stress variant in both experiments. There is a notable difference in the soil drying speed between the FD and SD experiments. This difference was likely caused by several factors (air temperature, photoperiod, and irradiance affecting evapotranspiration rates) that varied due to the semi-controlled design of the experiments. The shortage of water in the soil is reflected by the water status of strawberry plants, as shown in Figure 2. The greatest decrease (and the only statistically significant one compared to controls) in leaf water content was observed at the end of the stress period (day 14 and 42 in FD and SD, respectively) in the SS variant. The average leaf water contents in the controls at this point were 68.7% and 72.6%, while in SS variants, the values fell to 59.7% and 65.2% in FD and SD, respectively.

Carbohydrate content. Plant response to water shortage was primarily monitored at the level of endogenous carbohydrate content and spectra dy-

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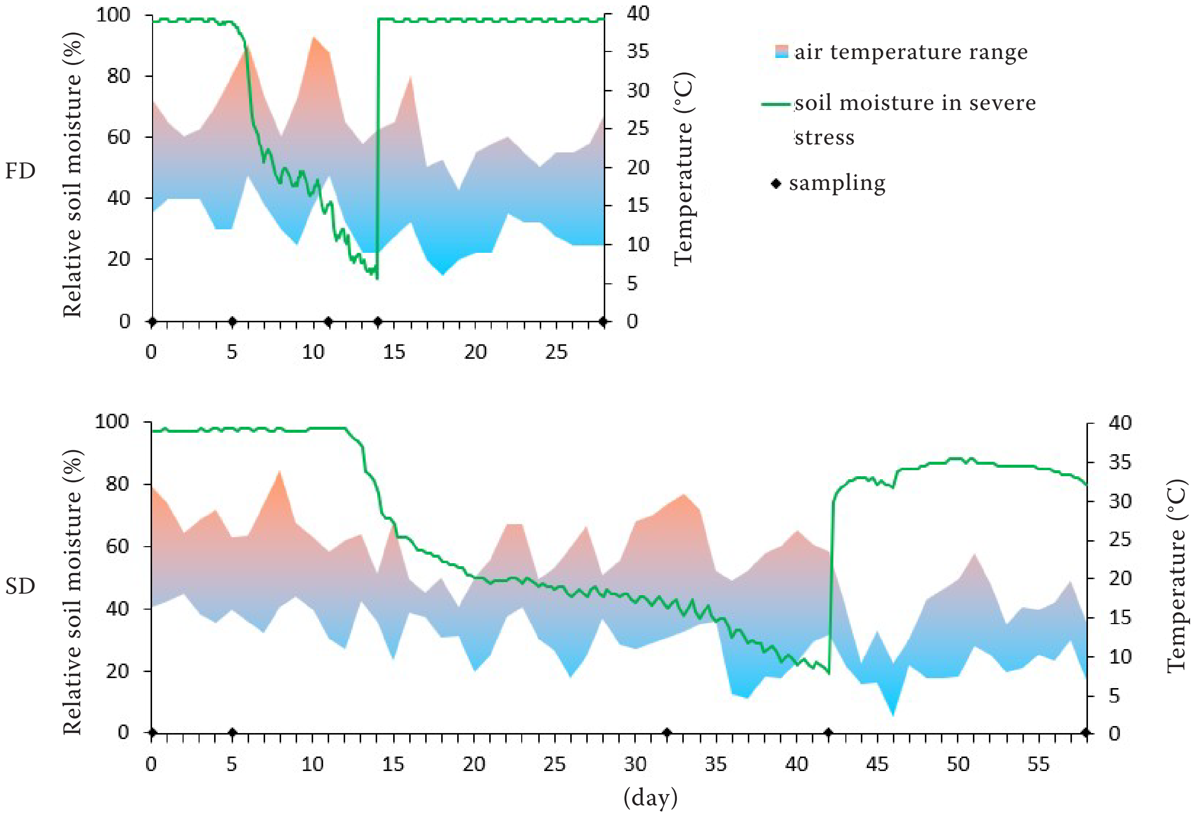

Figure 1. The course of relative soil moisture (primary *Y* axis) and the range of daily minimum and maximum air temperatures (secondary Y axis) in severe stress (SS) variant during both, fast desiccation (FD) and slow desiccation (SD) experiments. The black dots on *X* axes mark sampling points

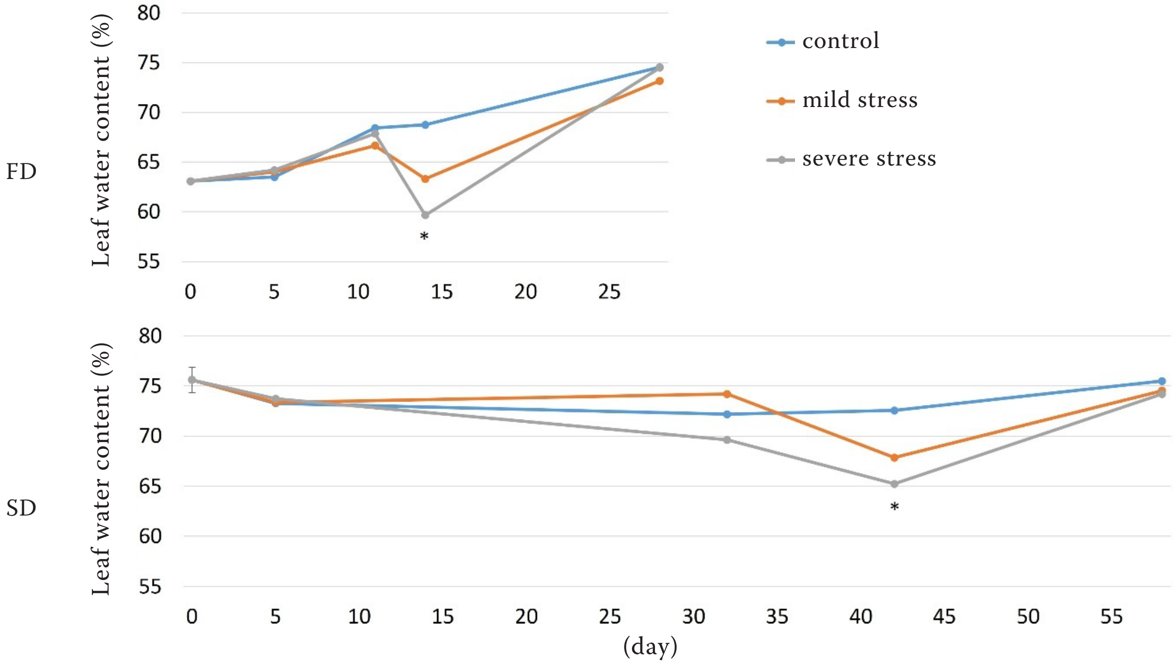

Figure 2. Leaf water content (as a proportion of fresh weight) under different drought set-up and intensity. The only statistically significant difference (α = 0.05, marked by asterisk) was observed in both experiments between control and severe stress (SS) variant at the end of stress period. Bars indicate standard deviations. FD – fast desiccation; SD – slow desiccation

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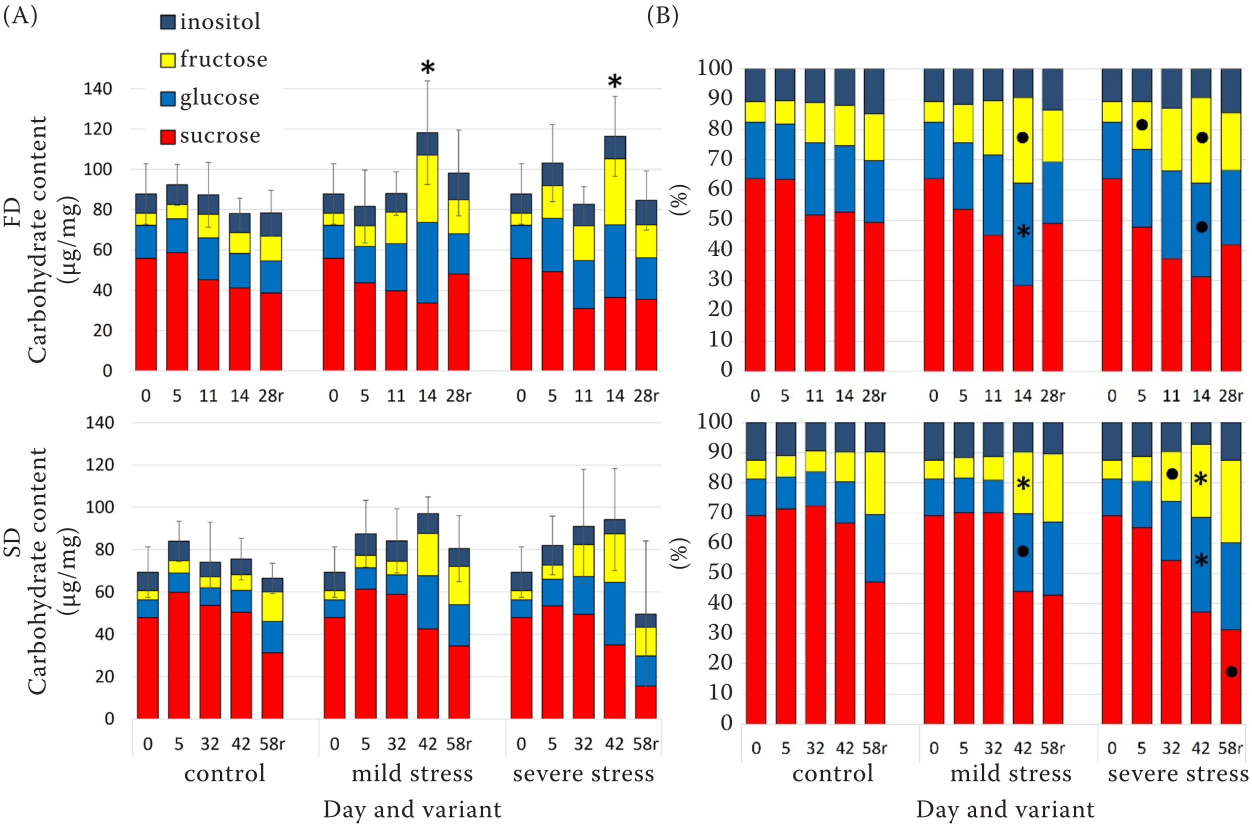

Figure 3. Carbohydrate contents and spectra in leaves under drought stress of varying duration and intensity. (A) Total content of soluble carbohydrates and (B) percentage representation of individual carbohydrates. Statistically significant differences from control are marked by asterisks (*α = 0.05) and dots (•α = 0.1). Bars indicate standard deviations. FD – fast desiccation; SD – slow desiccation

namics (Figure 3). The changes in leaf carbohydrate status of strawberry plants showed trends common to both the FD and SD set-ups. However, there were a few differences as well. Total soluble carbohydrate content was significantly increased at the end of the stress period (day 14) in MS and SS variants of the FD experiment (Figure 3A). In the corresponding variants and at the time point (day 42) of the SD experiment, a slight increase in total soluble carbohydrates was also observed, although not significant. As regards the carbohydrate spectrum, the above-mentioned increase was almost entirely due to glucose and fructose. At the same time, sucrose and inositol levels in stressed variants remained relatively stable compared to the control. The significance of the increase in glucose and fructose content varied across variants and experiments, but the trend is quite apparent (Figure 3B). The shift in the ratio of hexoses (glucose + fructose) to sucrose is demonstrated in Figure 4A. The trend toward increased values was present in both stressed variants of both experiments, even though, due to data variability (indicated by high standard deviations), it was statistically significant only in the SS variant of FD. There was also a difference in the recovery phase between FD and SD (days 28 and 58, respectively). In the first case (FD), the ratio in the MS and SS variants decreased after recovery. In contrast, in the second case (SD), the ratio in both stressed variants remained elevated, and the control also showed a slight increase.

Starch content. Starch content determination revealed further differences between the FD and SD experiment set-ups. However, in both setups, a significant decrease in starch level was observed during the stress period in the SS variant (Figure 5). In FD, a similar trend is visible even in the MS variant. After recovery, there is a notable increase in starch content across all FD variants, including the control. On the other hand, in SD, no such increase was present after recovery. The starch content in all variants and time points in the SD experiment was much higher than in the FD experiment. Figure 4B, depicting the ratio of total soluble carbohydrates to starch, further demonstrates changes in carbohydrate

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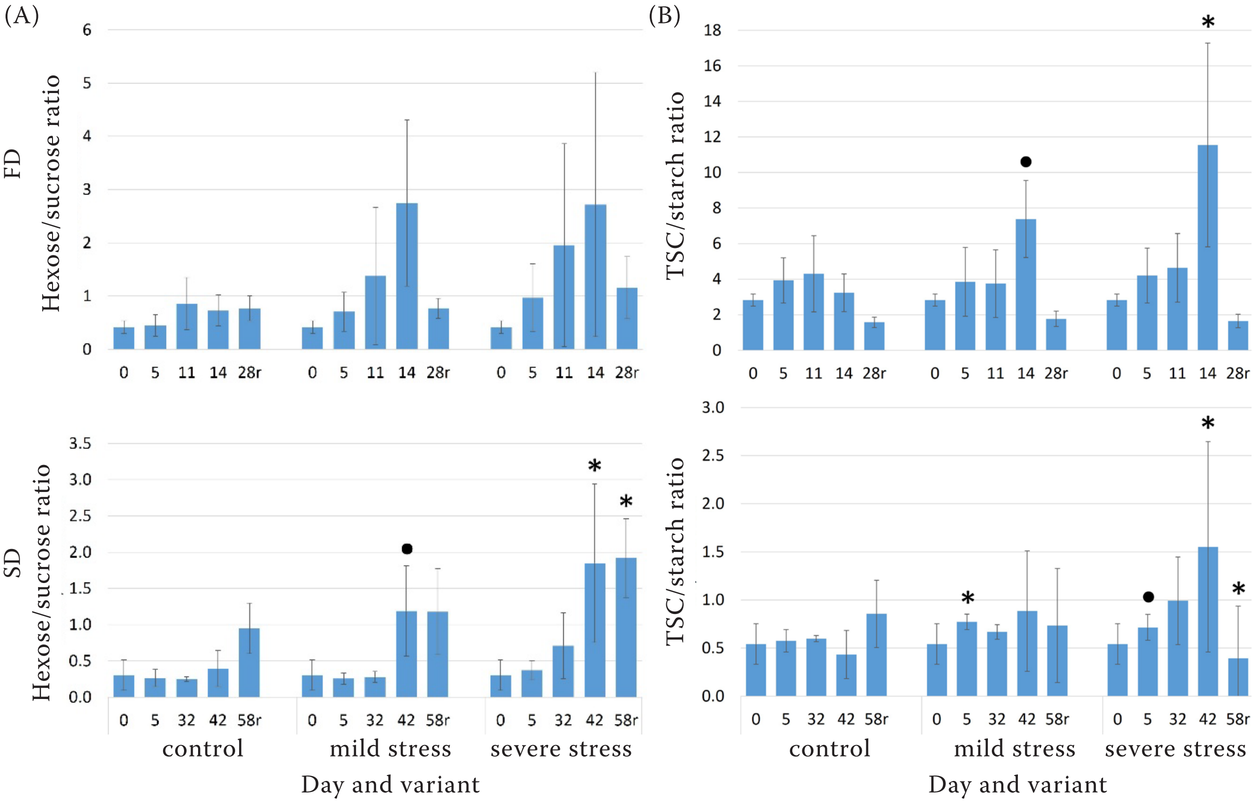

Figure 4. (A) The ratio of hexoses to sucrose contents and (B) the ratio of total soluble carbohydrates (TSC) to starch contents in leaves under drought stress of varying duration and intensity. Statistically significant differences from control are marked by asterisks (*α = 0.05) and dots (•α = 0.1). Bars indicate standard deviations. FD – fast desiccation; SD – slow desiccation

status under drought stress. A several-fold increase in this ratio at the end of the stress period was significant in the SS variant in both experiments. In contrast, in recovery, the ratio returned to the control level or even dropped further in the SD variant.

Proline content. Plants in control and MS variant exhibited very little changes in free proline content (Figure 6). The only considerable change was present in the SS variant at the end of the stress period (days 14 and 42 in FD and SD, respectively). In the first case (FD), the proline content was significantly increased by more than 16-fold. In the second case (SD), the increase was not statistically significant, although, compared to the control, the tendency to increase at day 42 was quite apparent.

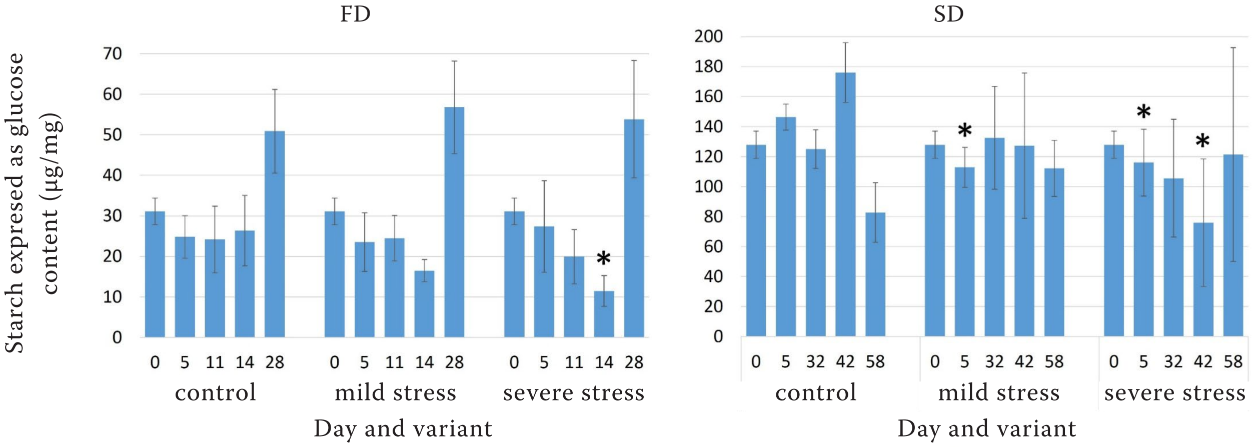

Figure 5. Starch content in leaves under drought stress of varying duration and intensity. Statistically significant differences from respective controls are marked by asterisks (α = 0.05). Bars indicate standard deviations. FD – fast desiccation; SD – slow desiccation

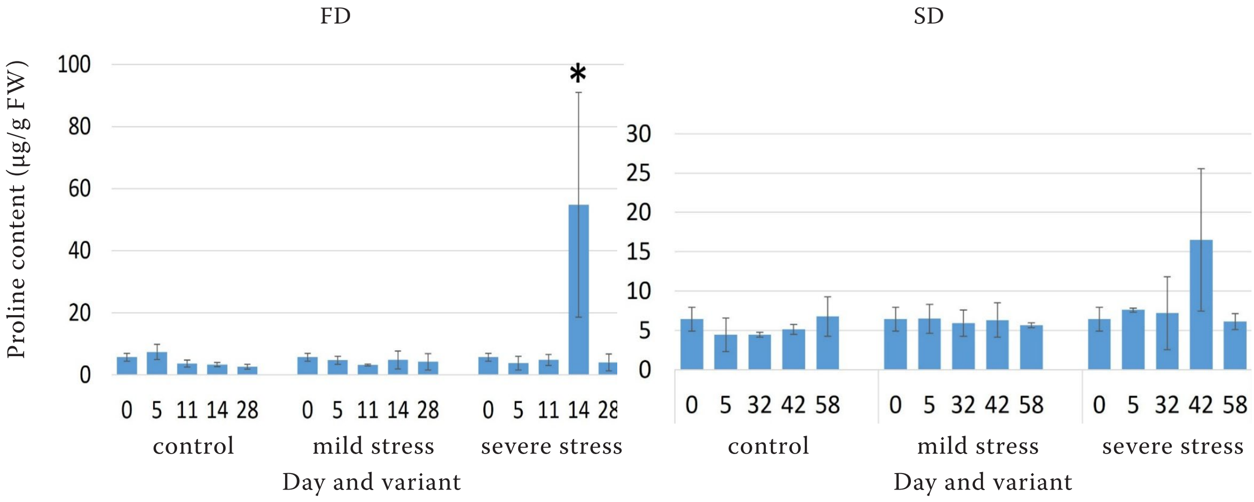

Figure 6. Proline content in leaves under drought stress of varying duration and intensity. Statistically significant differences from the control are marked by asterisks (α = 0.05). Bars indicate standard deviations. FD – fast desiccation; SD – slow desiccation; FW – fresh weight

Levels of stress markers – lipid peroxidation and photosystem fluorescence. The malondialdehyde content, an indicator of membrane damage, showed no treatment-dependent changes. In Table 1, we present MDA values at the beginning and end of the stress period for all variants in both experiments. Although there seemed to be an increase at days 14 and 42 in FD and SD, respectively, this increase was present to some extent even in the control variant, which, together with high variation among repetitions, caused none of the changes to be significant. As another stress marker, maximum quantum efficiency of photosystem II photochemistry (F_v/F_m) was measured (Table 2). The values ranged from 0.65 to 0.77. Control plants exhibited high temporal variability, probably due to semi-controlled experimental conditions. The effect of drought stress alone was assessed by comparing it with the respective controls at specific sampling points, treatments, and experimental types. However, no significant differences were observed, indicating that severe stress damage was not present.

Experiments *in vitro* – utilisation of carbohydrates. As in carbohydrate spectra obtained during previously presented experiments, no traces of sorbitol were detected, we decided to check the strawberry's ability to take up and utilise exogenously supplied sorbitol during *in vitro* cultivation. Plants at the five-leaf stage, cultivated photoautotrophically, were transferred to media with different carbohydrates and then cultivated mixotrophically (see materials and methods) for three weeks. Photoautotrophic cultivation on sugar-free medium was used as a control. Strawberry plants cultivated on the medium with sorbitol accumulated this sugar alcohol in the leaves (Figure 7), indicating that it penetrates the plants and is effectively transported from roots to leaves. However, sorbitol growth was very limited (Figure 8). The best growth was achieved with photoautotrophic cultivation, and those plants, despite having no sugar in the medium, had higher leaf sucrose levels than plants cultivated on 3% sucrose or 3% sorbitol.

The cultures used in previous *in vitro* experiments were grown under mixotrophic conditions, mak-

Table 1. Malondialdehyde (MDA) values at the beginning and at the end of drought period in both, fast desiccation (FD) and slow desiccation (SD) experiments

	MDA concentration (nmol/mL)						
	FD			SD			
	control	mild stress	severe stress		control	mild stress	severe stress
Day 0	0.12	0.12	0.12	day 0	0.25	0.25	0.25
Day 14	0.26	0.51	0.47	day 42	0.29	0.44	0.44

No statistically significant differences were found

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Table 2. The maximum quantum yield of photochemistry in photosystem II (F_v/F_m) in both, fast desiccation (FD) and slow desiccation (SD) experiments.

	F_v/F_m									
	FD					SD				
	day 0	day 5	day 11	day 14	day 28	day 0	day 5	day 32	day 42	day 58
Control	0.727 ± 0.015	0.743 ± 0.022	0.729 ± 0.032	0.674 ± 0.026	0.708 ± 0.025	0.747 ± 0.011	0.729 ± 0.006	0.748 ± 0.007	0.654 ± 0.042	0.758 ± 0.006
Mild stress	0.727 ± 0.015	0.728 ± 0.015	0.744 ± 0.019	0.703 ± 0.019	0.726 ± 0.024	0.747 ± 0.011	0.728 ± 0.017	0.752 ± 0.008	0.687 ± 0.037	0.769 ± 0.007
Severe stress	0.727 ± 0.015	0.717 ± 0.064	0.747 ± 0.021	0.735 ± 0.010	0.701 ± 0.082	0.747 ± 0.011	0.743 ± 0.008	0.737 ± 0.028	0.684 ± 0.022	0.75 ± 0.016

The ± values show standard deviations. No statistically significant differences were found

ing it difficult to draw a clear conclusion about the strawberry's ability to utilise sorbitol. Therefore, the following experiment used non-green callus cultures, which are fully heterotrophic. As a result, the photoautotrophic variant could not be used, and the 3% sucrose variant served as the control instead. The results (Figure 9) provided no support for the idea that callus can utilise sorbitol. The variant supplied with sorbitol as the sole carbon source exhibited only trace amounts of other carbohydrates. The increase in fresh weight in this variant was very small, and accumulated sorbitol itself apparently substantially contributed to the weight of the callus.

DISCUSSION

Causes of variability in stress response. Plant responses to abiotic stresses represent very complex processes. Under natural or field conditions, plants face multiple stresses that can act simultaneously or sequentially, with effects that can be both additive or cumulative and ameliorative. Their intensities often change over time, and these external stress burdens also interact with plant natural physiological changes and/or development. To survive and reproduce under given circumstances, plants must integrate all signals and information about external

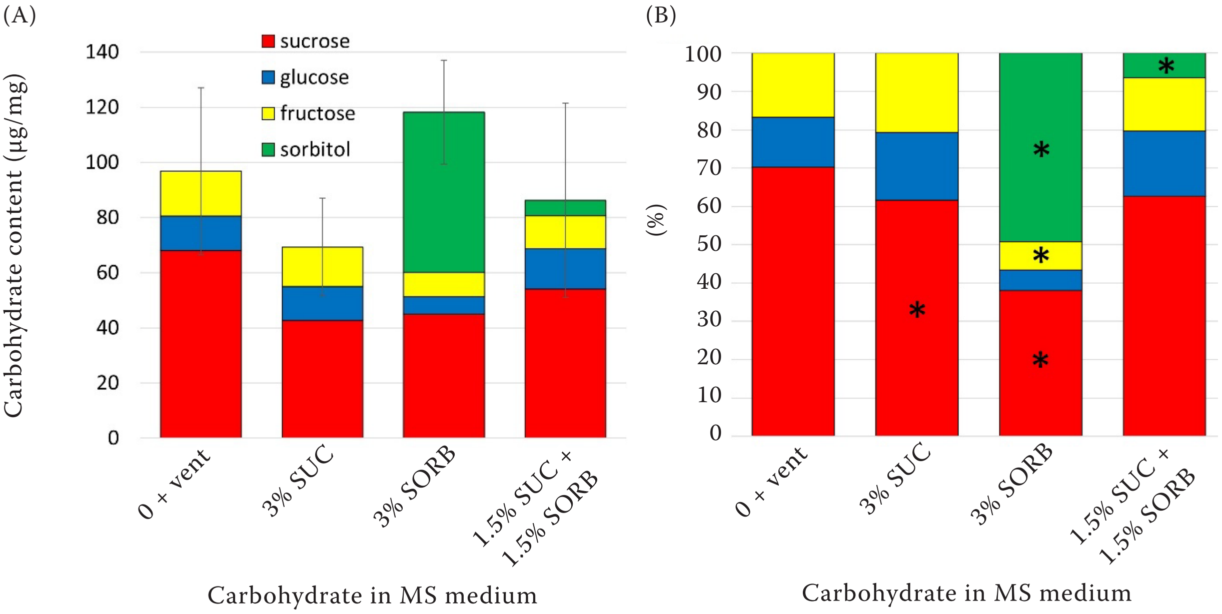

Figure 7. Effect of different carbohydrates supplied to the culture medium on carbohydrate content and composition of carbohydrate spectrum in leaves of *Fragaria vesca* grown *in vitro*. (A) Total content of soluble carbohydrates and (B) proportion of individual carbohydrates. Statistically significant differences from control are marked by asterisks (α = 0.05). Bars indicate standard deviations. SUC – sucrose; SORB – sorbitol

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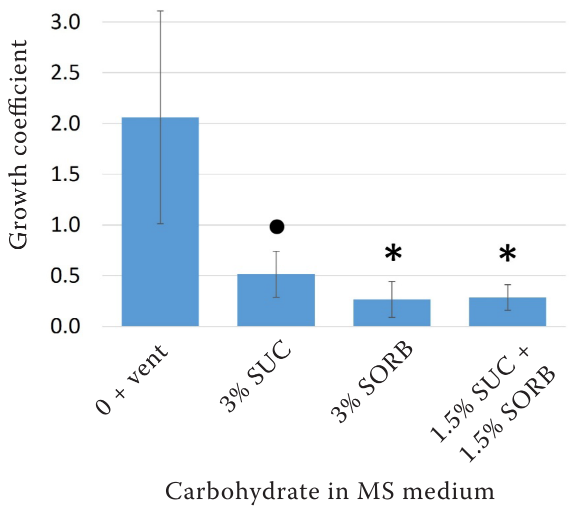

Figure 8. Effect of different carbohydrates supplied to the culture medium on growth of *Fragaria vesca* plants *in vitro*. Growth coefficient was calculated as increase in plant fresh weight at the end of the experiment divided by initial weight of plants. Statistically significant differences from control are marked by asterisks (*α = 0.05) and dots (•α = 0.1). Bars indicate standard deviations. SUC – sucrose; SORB – sorbitol

and internal conditions and find the best metabolic settings that will help them cope with the situation. Therefore, we focused on the identification of common components of draught stress responses induced by different conditions (Table 3).

To cover a wider range of circumstances that can occur during natural drought periods, the potted plants were cultivated in a greenhouse with drought induced by soil drying at two different rates. Those two experiments, conducted under semi-controlled conditions, were very similar in design but had a few additional distinctions. Most importantly, they were carried out at a slightly different time of year, with several consequences. On top of that, the semi-controlled experiments did not fully exclude the influence of ever-changing weather. Taken together, it is important to keep in mind that plants were affected by different photoperiods, irradiance, temperature, humidity, and so on. The time of the year also determines the developmental stage of strawberries, as they are adapted to life in temperate climates. In our results, we observed discrepancies between the control variants in both experiments, which we attribute to the above-mentioned factors. The water content of control plants increased during the fast desiccation experiment, whereas in the slow desiccation experiment, it remained quite stable at levels similar to those at the last sampling point in FD. This might be caused by the different ages of the leaves. In the FD experiment, they were younger and possibly

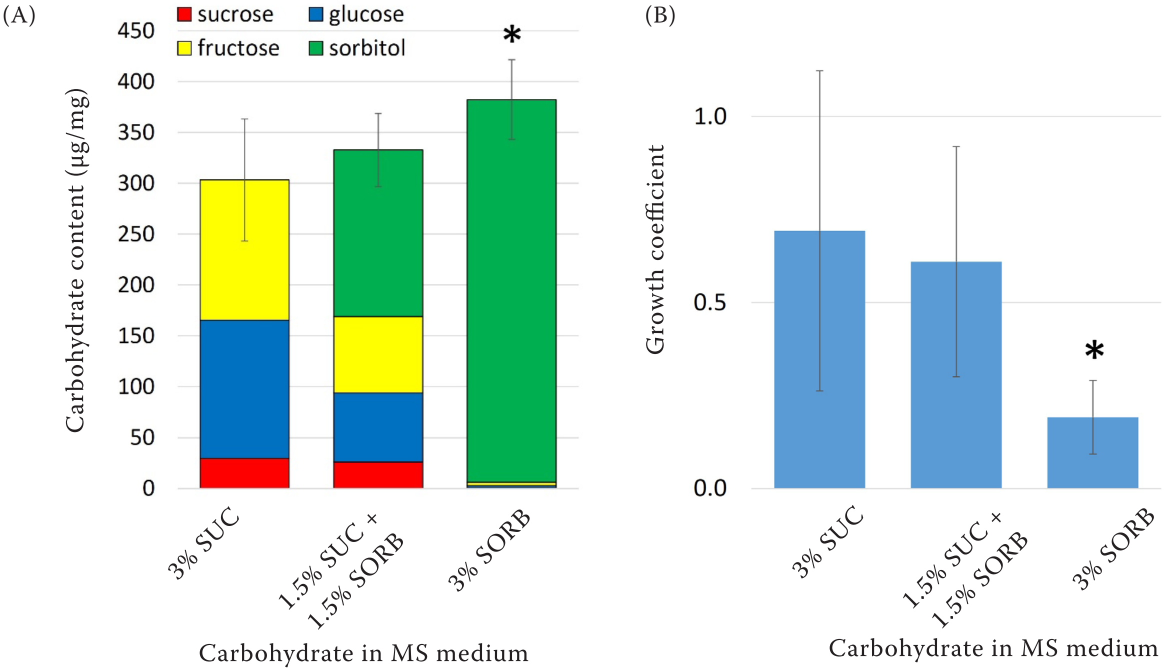

Figure 9. Effect of different carbohydrates supplied to the culture medium on (A) carbohydrate content (B) and growth of *Fragaria vesca* callus cultures. Growth coefficient was calculated as increase in callus fresh weight at the end of the experiment divided by initial weight. Statistically significant differences from 3% SUC variant are marked by asterisks (α = 0.05). Bars indicate standard deviations. SUC – sucrose; SORB – sorbitol

Table 3. Summary of results in both, fast desiccation (FD) and slow desiccation (SD) experiment

	FD					SD				
	0	5	11	14	28	0	5	32	42	58
	(day)									
Water content	–	–	–	–	–	–	–	–	–	–
	–	–	–	*	–	–	–	–	*	–
Total soluble carbohydrates (TSC)	–	–	–	*	–	–	–	–	–	–
	–	–	–	*	–	–	–	–	–	–
Sucrose	–	–	–	–	–	–	–	–	–	–
	–	–	–	–	–	–	–	–	–	•
Glucose	–	–	–	*	–	–	–	–	•	–
	–	–	–	•	–	–	–	–	*	–
Fructose	–	–	–	•	–	–	–	–	*	–
	–	•	–	•	–	–	–	•	*	–
Inositol	–	–	–	–	–	–	–	–	–	–
	–	–	–	–	–	–	–	–	–	–
Starch	–	–	–	–	–	–	*	–	–	–
	–	–	–	*	–	–	*	–	*	–
Hex/suc ratio	–	–	–	–	–	–	–	–	•	–
	–	–	–	–	–	–	–	–	*	*
TSC/starch ratio	–	–	–	•	–	–	*	–	–	–
	–	–	–	*	–	–	•	–	*	*
Proline	–	–	–	–	–	–	–	–	–	–
	–	–	–	*	–	–	–	–	–	–
Malondialdehyde	–	–	–	–	–	–	–	–	–	–
	–	–	–	–	–	–	–	–	–	–
Maximum quantum efficiency of photosystem II photochemistry (F_v/F_m)	–	–	–	–	–	–	–	–	–	–
	–	–	–	–	–	–	–	–	–	–

Light grey background marks mild stress (MS) and dark grey severe stress (SS) variants. Statistically significant differences from the control are marked by asterisks (*α = 0.05) and dots (•α = 0.1), dash symbol (–) means no significant difference was determined

not yet fully developed, although they had already reached their final size and looked mature. Younger leaves may vary in their physiological characteristics and also in their response to external conditions, as shown, for example, by Cechin et al. (2010). If this were true in our case, it would make sense: the water content of leaves in FD changed with their development, and at the end of the period, it reached a level similar to that in the SD experiment, where water content remained more or less constant in already fully developed leaves. Since differences between experiments affected the control variants, they likely affected the stressed variants in a similar manner. However, compared with the respective controls, the water contents in the stressed variants decreased as expected in both experiments. Despite reduced water content, levels of MDA, a widely used internal stress marker, showed only minor changes, none of which were significant. Another stress marker we followed, maximum quantum efficiency of photosystem II photochemistry (F_v/F_m), also showed no difference between stressed and control plants. Although the optimal F_v/F_m value for healthy plants is considered to be around 0.83 (Maxwell and Johnson 2000) and our values ranged from 0.65 to 0.77, this was probably not caused by drought, since values in the control group were within this range as well. Moreover, these values correspond well with those measured in *Fragaria* × *ananassa* by Zahedi et al. (2023), who also did not observe any treatment-dependent decrease. Taken together, both stress markers indicated that plants effectively coped with the level of drought stress they were exposed to and preserved the integrity of cellular structures and processes.

Shift in soluble carbohydrate contents across experiments. Changes in carbohydrate metabolism in response to drought stress have been reported several times in *Fragaria* × *ananassa*. In most cases, there was some increase in the content of total soluble carbohydrates or individual sugars. Sun et al. (2015) reported up to 2.5-fold higher sugar content in leaves of *F.* × *ananassa* exposed to drought stress compared to control, while Zahedi et al. (2023) reported 44% and Ghaderi and Siosemardeh (2011) only about 10% increase under drought. Razavi et al. (2008) observed approximately 1.5-fold higher levels of sucrose in drought-stressed strawberry plants, and Giné-Bordonaba and Terry (2016) found a slight increase in fructose but no significant change in sucrose or total sugar content. The differences among these results might be due to different experimental designs and, consequently, different levels and durations of stress. Another factor might be the use of various cultivars, which were confirmed to behave differently in adverse conditions (Ghaderi and Siosemardeh 2011, Giné-Bordonaba and Terry 2016). Despite the differences, common trends are observable across many studies, as confirmed by our work on *F. vesca*. The response of carbohydrate metabolism to drought appears gradual and proportional to the duration and intensity of the stress, but there is often an increase in total soluble carbohydrate content. In our case, this increase was relatively small and was almost entirely due to hexoses, i.e., glucose and fructose. This is in contradiction with the above-mentioned results of Razavi et al. (2008) but corresponds with those of Giné-Bordonaba and Terry (2016) and Zhang and Archbold (1993), who studied drought response in *F. chiloensis* and *F. virginiana*, the direct progenitors of *F.* × *ananassa*. In *F. chiloensis*, they found a 2-fold increase in both glucose and fructose content under water deficit, whereas in *F. virginiana*, there was only a slight increase. Since *F. chiloensis* is considered more drought-resistant than *F. virginiana*, the authors hypothesised that these soluble sugars play an important role in osmotic adjustment, leading to higher drought resistance. Our results confirm the role of hexoses in stress response. The increase, or at least the tendency to increase, in glucose and fructose content, associated with a shift in the hexose-to-sucrose ratio, was the main change in sugar metabolism we observed under water deficit, and it might be more important than the changes in total soluble carbohydrate content. As previously suggested by Giné-Bordonaba and Terry (2016), this shift in carbohydrate ratio may be beneficial since it contributes to osmotic adjustment. By accumulating hexoses instead of sucrose, the plant doubles the molar concentration of osmolyte without changing the amount of carbon needed. Simultaneously, this allows for an increase in the total amount of soluble sugar while maintaining a relatively stable sucrose level, which may help avoid metabolic disruptions. A very similar pattern of change in carbohydrate metabolism was already observed in *F. vesca* during cold acclimation by Rohloff et al. (2012), suggesting a certain degree of universality of this stress response. These authors also identified starch as a very important source of accumulated hexoses. This is in line with our results, where we found decreased starch content in stressed plants. It seems likely that, since photosynthesis is usually limited under stress, the plant increases its hexose pool by breaking down starch.

Role of carbohydrates in the mitigation of oxidative stress. The tendency to increase glucose and fructose content raises questions about their other roles in the stress response, beyond simple osmotic adjustment. One such role could be mitigating secondary oxidative stress. Nevertheless, Peshev et al. (2013) found that both these hexoses have approximately half lower •OH scavenging capacity compared to sucrose. So, the overall scavenging capacity of the soluble carbohydrate pool is not increased by splitting sucrose to glucose and fructose. For this purpose, strawberries would need to produce other, better scavenging carbohydrates, e.g., fructans. Since we have not found such carbohydrates in stressed plants, the protection against oxidative stress does not seem to be the purpose of the observed changes in carbohydrate metabolism, although the increase in total sugar content may play a minor role.

Sorbitol enigma. One of the objectives of our work was to verify whether strawberry makes use of sorbitol in any way. It was shown many times, that it can be beneficial under stress (Pleyerová et al. 2022) and it is abundant in many Rosaceae species, where its level often increases during drought (Lo Bianco et al. 2000, Jiménez et al. 2013, Yang et al. 2019). Keeping in mind that strawberry belongs to the Rosaceae family, and moreover, they possess the genes for sorbitol metabolism, we may ask what purpose they serve. However, our experiments showed no signs of sorbitol either in stress or control conditions. This means it was either absent in *F. vesca* plants or its content was below our detection limit.

In any case, it seems that sorbitol does not play an important role in the strawberry stress response. To further address the question, we performed *in vitro* experiments testing the ability of whole plants and heterotrophic callus cultures to utilise exogenously supplied sorbitol as a sole carbon source. Carbohydrate analysis showed that sorbitol is taken up and accumulated in large amounts by both whole plants and callus cultures. Moreover, the plants transported sorbitol from roots to leaves, although this transport was probably mediated by passive xylem flow and therefore not targeted. The utilisation of sorbitol for growth seemed to be very limited. In the case of whole plants, there was very little increase in biomass dry weight, and some of this increase can undoubtedly be attributed to photosynthesis, which can still take place even in closed vessels *in vitro*. Heterotrophic callus cultures, in which photosynthesis is impossible, showed even lower increases in biomass when cultivated on sorbitol. After subtracting the weight of the accumulated sorbitol, the increase in biomass was negative. Since we ruled out the direct role of sorbitol in drought stress response as well as in energy supply, the meaning of genes for its metabolism remains unclear. In strawberry, sorbitol dehydrogenase has a high affinity for sorbitol but quite low catalytic activity (Sutsawat et al. 2008). This might be why high levels of sorbitol cannot be metabolised. Expression of this enzyme was found to be high in mature fruit (Duangsrisai et al. 2007), and recently, sorbitol-6-phosphate dehydrogenase, the main synthetic enzyme, was highly expressed in young fruit (Yang et al. 2024). Together, these data suggest that the main role of sorbitol metabolism in strawberry is in fruit development, possibly primarily in its regulation. This is in line with numerous published reports on the roles of sucrose and hexoses in signalling pathways, as well as, so far, only indirect similar evidence for sorbitol.

Proline is the last response. Apart from carbohydrates, we investigated changes in levels of proline, which is also one of the stress-induced compounds used by strawberry plants to fight stress (Sun et al. 2015, Zahedi et al. 2023). In both our experiments, proline content increased at the highest level of stress (end of the stress period in the SS variant), though only in FD was the increase significant. Interestingly, in all other time points and variants, proline content was quite stable. This is a notable difference compared to the dynamic response of carbohydrate metabolism. While sugar and starch contents changed gradually with increasing stress levels, proline content seemed relatively stable until a threshold of stress intensity was reached, at which point it sharply increased. A greater increase in FD than in SD might be due to higher temperatures, which create an additional stress factor.

What happens during recovery. An important part of stress response evaluation is the analysis of the recovery phase following stress treatment. It shows whether the plants "forget" the stress and return to their initial state, or whether there is some kind of memory and the plants set their metabolism to the new "learned" state. The balance between resetting metabolism and maintaining a memory trace is crucial for the plant's future survival. Mechanisms of stress memory, however, range from epigenetic to hormonal and metabolic changes (Crisp et al. 2016, Staacke et al. 2025). We focused on the metabolic level, namely, proline content and carbohydrate balance after stress release. Regarding proline and total soluble carbohydrate content, we observed a return to the control levels in both experiments, which indicates a return to the previous setting. However, for the hexose-to-sucrose ratio, the picture was not so clear. It may be particularly important because glucose and sucrose have already been shown to be involved in stress memory signalling pathways that regulate transcription or chromatin modification (Sharma et al. 2019, Olas et al. 2021). There was a difference between the two experiments, while in FD the values returned to control levels, in SD they remained increased. The ratio in the severe stress variant of SD was significantly higher than in the control, even though the control itself showed a trend toward increasing compared to previous time points. We can speculate on a possible explanation: in the SD experiment, the duration differed significantly, and the experiments were conducted at different times during the vegetation season. Since it took longer to achieve the desired level of stress, the plants may have taken longer to "forget" it and reset their metabolism (as shown, for example, by Knüver et al. 2025). On top of that, responses to short- and prolonged-drought stress may differ significantly. Spieß et al. (2012) even found that carbohydrate accumulation varied with the duration and intensity of stress. Further long-term experiments will be needed to clarify these relationships. Another possible explanation we can propose for this difference between experiments is some kind of shift in carbohydrate metabolism, naturally occurring during later stages of the vegetative season, since the SD experiment was carried out at a later time of the year. This shift (serving, for example, as preparation for lower temperatures) could interact with changes caused by drought stress, which would act as a form of priming and lead to different behaviour in both stress-treated variants. Though this explanation might seem less likely, there are some clues pointing towards it. The first is the trend toward an increase in the hexose-to-sucrose ratio even in control plants during recovery, and the second is the dynamic of starch, which also differed between experiments; both might be associated with the phase of the vegetation cycle. In FD, starch content increased during the recovery phase in all variants, including the control. When we compared the amount of starch across different time points within each variant, the increase between the end of the stress period and the end of the recovery phase was significant in each. On the other hand, no such increase was observed in SD. However, when we compare the overall values from both experiments, we see that they are much higher in SD than in FD (up to several-fold). This could be caused by the natural development of plants during the growing season, where at the beginning most resources are used for growing and later, they are used for creating storage substances like starch. If this transition occurred in July and August, it would explain why starch content increased at the end of the FD experiment, even in the control, and why, in SD, starch levels were already higher from the start of the experiment and did not increase at the end.

In conclusion, a notable response to drought stress in wild strawberry was an increase in glucose and fructose content and a shift in the hexose/sucrose ratio. This was accompanied by a decrease in starch, which likely served as a source of hexoses. Changes in carbohydrate content started early, were gradual and could contribute to osmotic adjustment and protection of cellular structures. On the other hand, proline accumulation is a late response to water shortage in strawberry leaves under severe stress. Slight changes in physiological markers of damage indicated a high ability of the defence mechanisms to protect successfully cellular structures. We observed no signs of sorbitol involvement in the stress response, nor any evidence of strawberries' ability to utilise sorbitol as a carbon and energy source.

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