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Utilization of exogenous saccharides by protocorms of two terrestrial orchids

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ABSTRACT

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Orchid protocorms are completely mycoheterotrophic structures. Although saccharides are proposed as the main energy and carbon (C) sources provided by fungi, there is only limited knowledge on their effects. For the first time, utilization of a wide range of saccharides by *in vitro* axenic protocorms of two terrestrial orchids from two subfamilies, *Ophrys iricolor* subsp. *lojaconoi* and *Oeceoclades*, was tested. Protocorm size and, in the first of these also rhizoid length and soluble saccharide contents, were analysed. The endogenous saccharide spectra reflected the supplied saccharides and their metabolism. In both species, sucrose supported protocorm growth best. Surprisingly, fructose inhibited *O. iricolor* subsp. *lojaconoi* protocorm growth while *O. decaryana* ones grew well on it. Interestingly, mannitol abundant in mycorrhizal fungi was not utilized while sorbitol not found in fungi was usable. Galactose was toxic at pre-germination stage. Protocorm rhizoid length correlated with protocorm size but revealed several signalling effects of some saccharides. In conclusion, the orchid's ability to utilize various saccharides reflects more likely species life strategy rather than phylogenetic relations or saccharide abundance in mycorrhizal fungi.

Keywords: Orchidaceae; sugar alcohols; cultivation medium; maltose; raffinose

Orchidaceae, the largest plant family, comprises nearly one tenth of higher plant species. Despite enormous diversification, this family comprises many endangered species. To preserve this diversity, efficient propagation techniques are needed. Moreover, some species are of great horticultural, pharmaceutical or food interest (Gutiérrez 2010). Extremely small orchid seeds contain little reserves (Arditti and Ghani 2000) and germinate into specific structures, protocorms, living mycoheterotrophically below ground. For research or propagation, *in vitro* axenic cultures on saccharide-rich media are employed. Importantly, studies have rarely compared different exogenous saccharides, with only sucrose and a few other saccharides tested. More complex studies are scarce and only few species were studied: *Phalaenopsis*, *Dendrobium* and *Galeola* (Ernst 1967, Nakamura 1982), all tropical representatives of two orchid subfamilies: Epidendroideae and Vanilloideae.

Because of the strict mycoheterotrophy in early stages, the knowledge of orchid ability to utilize diverse saccharides is of special ecophysiological interest. The nature of compounds transferred in mycorrhiza is largely unknown. Amino acids (Cameron et al. 2008) and saccharides (Smith 1967) are proposed to participate in carbon (C) transfer from fungi to orchids, however, the evidence is only indirect. Nevertheless, saccharides are regarded as better candidates. In all associations between fungi and plants investigated to date, saccharides

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transferred majority of energy; mutualistic associations as ectomycorrhiza (Nehls 2008) and arbuscular mycorrhiza (Doidy et al. 2012) or interaction with pathogenic fungi (Talbot 2010) were studied. Importantly, the determination of suitable saccharide type for support of particular orchid species is an essential precondition for further studies on energy transfer mechanisms between symbionts as well as for successful axenic orchid cultivation.

In this study, the effects were characterized of selected saccharides on protocorm growth of two distantly related orchids not yet tested. *Ophrys iricolor* subsp. *lojaconoi* was selected as a representative of subfam. Orchidoideae. Smaller saccharide subset was tested also in *Oeceoclades decaryana* of Epidendroideae subfam. Both species share terrestrial life style. This is the first detailed study on both, saccharide utilization by subfam. Orchidoideae representative and strictly terrestrial orchid species.

MATERIAL AND METHODS

Plant material. Two orchid species, *Ophrys iricolor* subsp. *lojaconoi* (P. Delforge) Kreutz and *Oeceoclades decaryana* (H. Perrier) Garay & P. Taylor; designated as *Ophrys* and *Oeceoclades* hereafter. Seeds for experiments were collected from glasshouse-cultivated plants after manual self-pollination (Prague Botanical Garden, Prague, Czech Republic) and stored with silica gel at +25°C. *Ophrys* plants originated from seeds collected at southern Italy (Puglia, Mattinata). *Oeceoclades* plants were purchased from Palkowitschia company (Prague, Czech Republic).

***In vitro* cultivation**. It was performed according to Ponert et al. (2011). Briefly, the seeds were treated with 70% ethanol (5 min), washed with distilled water, exposed to 20% w/v chlorinated lime with Tween 20 (5 min), and then washed three times with sterile distilled water. The seed suspension was poured on solid medium in Petri dishes (diameter 9 cm); the dishes were sealed with Parafilm M foil and kept in the dark at 23°C. Cultivation medium SMS (Ponert et al. 2013) consisting exclusively of defined substances was used. Soluble D-saccharides were added prior to autoclaving (100 mmol monosaccharides, 50 mmol disaccharides, 33 mmol raffinose). pH was adjusted with 1 mol/L NaOH to 5.8 prior to autoclaving.

Protocorm size analysis. After 3 months culture, the protocorms (Figure 1) were photographed with camera Canon EOS 60D with lens Canon Macro EF 100 mm 1:2,8 L IS USM (Canon, Tokio, Japan). Protocorm size was expressed as the maximum protocorm diameter (Ponert et al. 2013). The length of one randomly selected rhizoid on each protocorm was measured using the ImageJ software (Schneider et al. 2012).

Saccharide content analysis. The freeze-dried samples were incubated with 80% methanol, then vacuum dried (Eppendorf Concentrator Plus, Eppendorf, Hamburg, Germany). Soluble saccharides were dissolved in Milli-Q ultrapure water (Millipore, Bedford, USA) in ultrasonic bath, then purified following the protocol of Kubeš et al. (2014). The samples were kept at –20°C until analyses using an HPLC system with refractometric detection following the protocol of Vojtíšková et al. (2006).

Statistical evaluation. Protocorm size and rhizoid length were analysed with the Kruskall-Wallis test and pair-wise comparisons using the Wilcoxon rank sum test with the Bonferroni correction. Saccharide contents were analysed with ANOVA and the Tukey-Kramer test.

R 3.1.2. statistical software package (R Core Team 2014), α = 5% probability level.

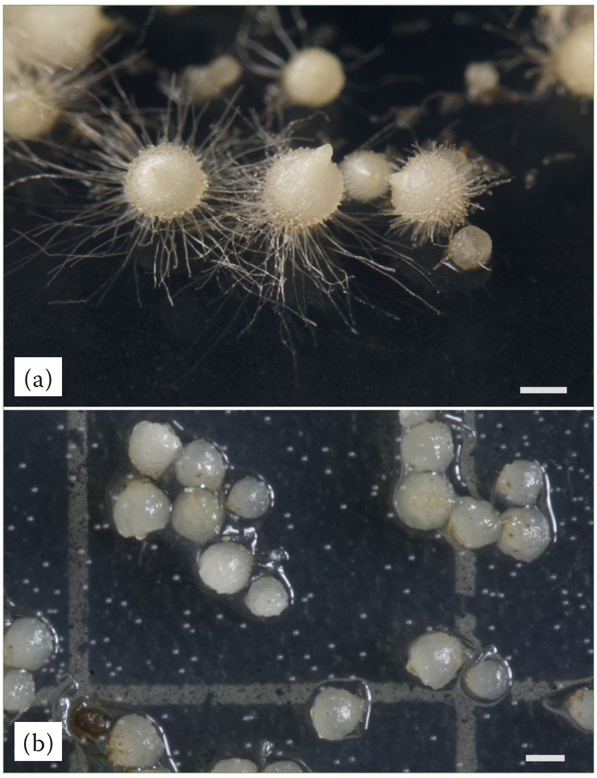

Figure 1. Protocorms after 3 months of cultivation on medium with sucrose. (a) *Ophrys iricolor* subsp. *lojaconoi*; (b) *Oeceoclades decaryana*. Scale bars 1 mm

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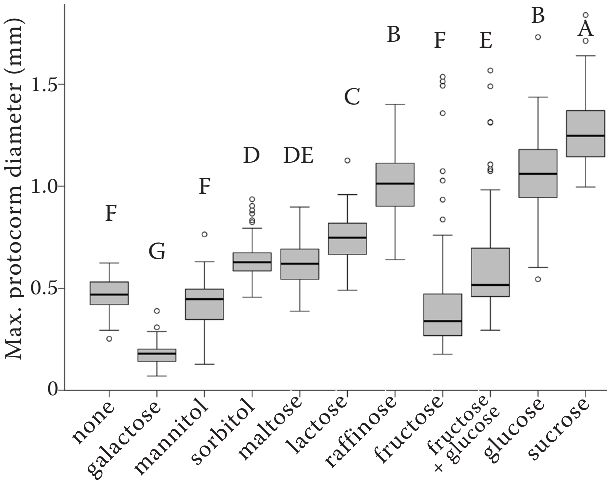

Figure 2. Effect of saccharide type in the medium on *Ophrys iricolor* subsp. *lojaconoi* protocorm size after 3 months of cultivation; 100 mmol monosaccharides, 50 mmol disaccharides, 33.3 mmol raffinose. Median values are given (n = 103–123). Different letters indicate significantly different groups of data according to pair-wise comparisons using the Wilcoxon rank sum test with the Bonferroni correction (α = 0.05)

RESULTS

Effect of exogenous saccharides on *Ophrys iricolor* subsp. *lojaconoi* protocorms. Protocorm size was significantly affected by exogenous saccharide supply (Kruskal-Wallis: $\chi^2_{(10)}$ = 996.9, $P < 2.2 \times 10^{-16}$). On the saccharide free medium, the seeds germinated, embryos ruptured testae, protocorms developed rhizoids and stopped growth at very small size. No soluble saccharides were detectable in protocorm tissues (Figures 1–4). Saccharides tested can be divided according to their ability to support protocorm development and utilization rates in *O. iricolor* subsp. *lojaconoi* protocorms (designated as *Ophrys* hereafter) (Figures 2–3): (i) Galactose – toxic, killing embryos in seeds before germination, dark brown necrotic imbibed embryos with galactose as the sole endogenous saccharide. (ii) Mannitol – not lethal, but no protocorm growth stimulation. The protocorms accumulated mannitol and interestingly also contained low levels of sucrose and other saccharides indicating partial mannitol utilization. (iii) Maltose and sorbitol – partly utilized by protocorms, supporting protocorm growth only slightly, accumulating in tissues. Interestingly, a range of endogenous saccharides developed in sorbitol-supported protocorms with a significant level of sucrose, while in the maltose-supported ones only glucose and an unidentified saccharide were detectable. (iv) Lactose – moderately utilized, supporting protocorm growth better than maltose or sorbitol, still at low degree, accumulating in tissues with small amounts of sucrose and glucose. (v) Raffinose – utilized by protocorms, supporting growth better than lactose, however, still less than sucrose. Protocorms contained significant levels of sucrose. (vi) Sucrose – readily utilized by protocorms, supporting the best protocorm growth. Protocorms contained the highest levels of endogenous saccharides with spectrum formed mainly

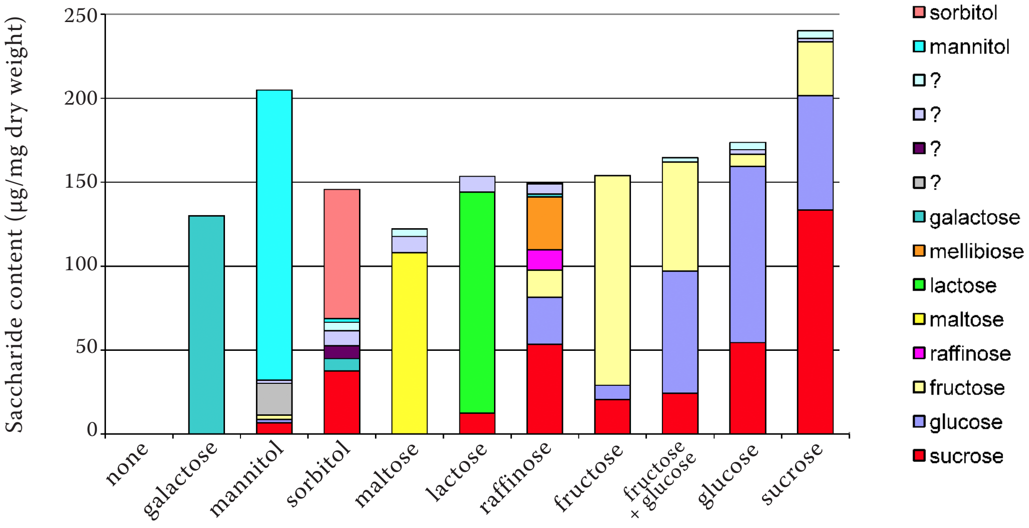

Figure 3. Effect of saccharide type in the medium on endogenous saccharide contents in *Ophrys iricolor* subsp. *lojaconoi* protocorms after 3 months of cultivation; 100 mmol monosaccharides, 50 mmol disaccharides, 33.3 mmol raffinose. Mean values of 2 to 3 replicates are given

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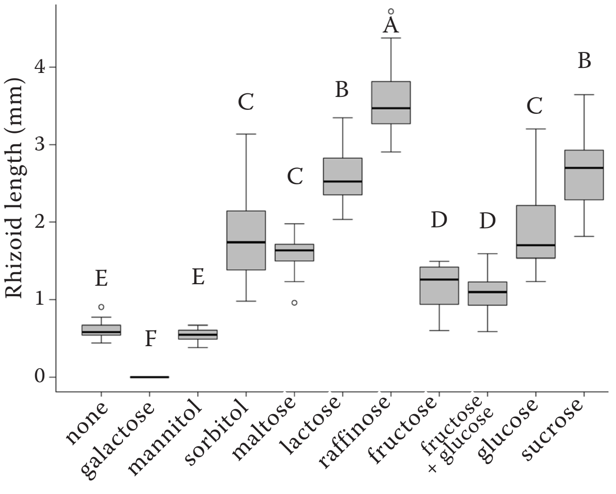

Figure 4. Effect of saccharide type in the medium on rhizoid length of *Ophrys iricolor* subsp. *lojaconoi* protocorms after 3 months of cultivation; 100 mmol monosaccharides, 50 mmol disaccharides, 33.3 mmol raffinose. Median values are given ($n = 23$). Different letters indicate significantly different groups of data according to pair-wise comparisons using the Wilcoxon rank sum test with the Bonferroni correction ($\alpha = 0.05$)

from sucrose, glucose and fructose. (vii) Hexoses – slightly ambiguous effects. Protocorms contained relatively high levels of endogenous saccharides with prevailing sucrose, glucose and fructose. However, the protocorm size varied significantly between treatments with the smallest protocorms on fructose and the largest ones on glucose. Surprisingly, some fructose-supported protocorms reached similar size as those on glucose or sucrose. Combination of equimolar glucose and fructose resulted in protocorm size markedly lower than hypothetical middle between glucose- or fructose-supported variants. However, the proportion of endogenous glucose to fructose was in-between glucose- and fructose-supported variants.

The length of rhizoids (Figures 1 and 4) was also significantly affected by exogenous saccharide supply (Kruskal-Wallis: $\chi^2_{(10)} = 229.8$, $P < 2.2 \times 10^{-16}$) and strongly positively correlated with protocorm size (Figure 5). Some treatments, however, significantly contributed to residual variation. The galactose-supported seeds did not germinate producing no rhizoids. The protocorms cultivated on glucose, mannitol and saccharide-free medium produced markedly shorter rhizoids while those cultivated on raffinose, lactose and fructose much longer ones than would correspond to their size. Interestingly, a comparison of variants grown on fructose and its mixture with glucose revealed similar rhizoid length, while much longer rhizoids were found in variant with sole glucose (Figure 4).

Effect of exogenous saccharides on *Oeceoclades decaryana* protocorms. Protocorm size of *O. decaryana* (designated as *Oeceoclades* hereafter) was significantly affected by exogenous saccharide supply (Kruskal-Wallis: $\chi^2_{(7)} = 639.2$, $P < 2.2 \times 10^{-16}$), no rhizoids were produced in any variant. On saccharide-free medium, seeds germinated, embryos swelled up, ruptured the testae and stopped growth at very small size (Figure 6). The saccharides tested were divided according to their ability to support the *Oeceoclades* protocorms growth (Figures 1 and 6): (i) Lactose – not supporting protocorm growth and not metabolizable, embryos similar as those without saccharide supply. (ii) Maltose and raffinose – supporting protocorm growth only slightly, probably metabolized at very low rate. (iii) Fructose – supporting protocorm growth well, probably easily utilized, though the protocorms were significantly smaller compared to the sucrose- or glucose-supported ones. (iv) Glucose and sucrose – supporting the best protocorm growth, readily utilized by protocorms (Figure 6). The equimolar mixture of glucose and fructose supported protocorms growth similarly as the sole fructose (Figure 6).

DISCUSSION

Saccharides play multiple roles in higher plants; though they primarily serve as C and energy sources

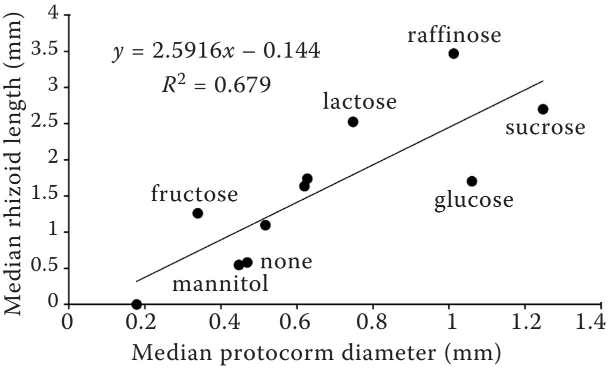

Figure 5. Correlation between protocorm size and rhizoid length of *Ophrys iricolor* subsp. *lojaconoi* under various exogenous carbohydrate supplies after 3 months of cultivation. For treatments significantly contributing to residual variability the saccharide type is indicated

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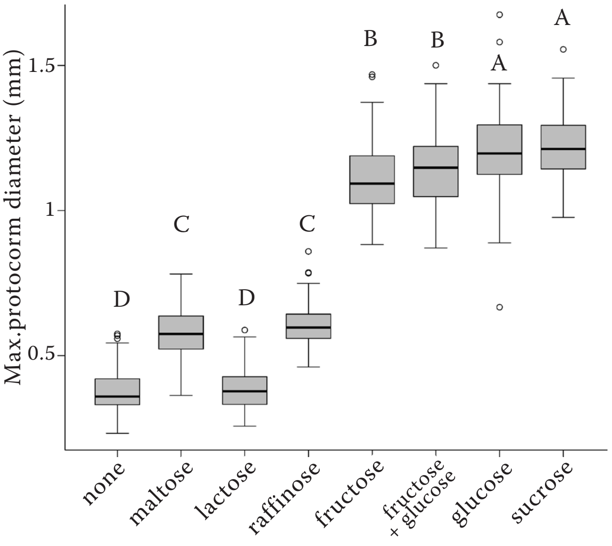

Figure 6. Effect of saccharide type in the medium on *Oeceoclades decaryana* protocorm size after 3 months of cultivation; 100 mmol monosaccharides, 50 mmol disaccharides, 33.3 mmol raffinose. Median values are given. Different letters indicate significantly different groups of data according to pair-wise comparisons using the Wilcoxon rank sum test with the Bonferroni correction (α = 0.05). *n* = 103–111, except for saccharide-free (*n* = 80) and lactose (*n* = 58) variants

to support growth. In recent decades, the important role of signalling molecules has also been proven in plants (Smeekens and Hellmann 2014). In both orchids studied, large differences in saccharide nutritive efficiencies were found. Galactose was toxic for *Ophrys* embryos, which corresponds well with effects in other orchids (e.g. Quednow 1930, Ernst et al. 1971, Nakamura 1982). Generally, galactose is known to be toxic to plant tissues likely through inhibition of β-galactosidase action in cell wall loosening during organ elongation (e.g. Cheung and Cleland 1991). Interestingly, galactose killed *Ophrys* embryos already at pre-germination stage.

Mannitol was negligibly utilized by *Ophrys* and protocorms did not grow on it. Similar results were achieved with several other orchids (e.g. Ernst 1967, Johnson and Kane 2013) except for *Galeola* (Nakamura 1982) and *Phalaenopsis* (Ernst 1967) which grew well on it. Inability of some orchids to grow on mannitol is surprising as it is a common fungal endogenous saccharide (e.g. Patel and Williamson 2016) also in orchid mycorrhizal fungi (e.g. Hadley 1984). Thus, mannitol represents a part of fungal C and energy pool not universally beneficial to orchids.

In contrast, sorbitol was utilized, though at low rate, by *Ophrys* protocorms as indicated by a complex endogenous saccharide spectrum with high sucrose proportion. However, the protocorms grew slowly and sorbitol accumulated in tissues. Sorbitol utilization differs among orchid species, ranging from a good growth support in *Phalaenopsis*, *Doritaenopsis* and *Galeola* (Ernst et al. 1971, Nakamura 1982, Rahman et al. 2005) to no growth in *Bletia* (Johnson and Kane 2013). As *Bletia* is a terrestrial orchid of subfam. Epidendroideae, sorbitol utilization is probably not related to phylogenetic position. Sorbitol was used as an osmoticum in *Phalaenopsis* protoplast regeneration (e.g. Shrestha et al. 2007), which is rather questionable as, being metabolizable, it is not an inert osmoticum. Our results indicate that the problem is valid for other orchids as well.

Maltose-supported protocorms of both *Ophrys* and *Oeceoclades* grew only slightly. *Ophrys* protocorms grew similarly as on sorbitol; accumulation of maltose indicated low utilization rate. Contrastingly, other orchids readily grow on maltose, sometimes even better than on sucrose (e.g. Rahman et al. 2005, Sopalun et al. 2010). All these species belong to the subfam. Epidendroideae and thus, low *Ophrys* (Orchidoideae) utilization might reflect its distant phylogenetic position. However, very slow growth was observed also in *Oeceoclades* belonging to the subfam. Epidendroideae. Both species are obligately terrestrial taxa while maltose-utilizing ones are epiphytes and thus it might be speculated that epiphytism is connected with orchid ability to utilize maltose.

Lactose did not support *Oeceoclades* protocorm growth. In *Ophrys*, however, it was superior to maltose or sorbitol, which is surprising, as inability to grow on lactose was reported in *Phalaenopsis* and only very slow growth in *Dendrobium* (Ernst 1967), both Epidendroideae.

Knudson (1924) regarded lactose unusable for any orchid species, though he tested only a few Epidendroideae taxa. In contrast, *Vanilla* (Vanilloideae) was able to readily grow on lactose (Bouriquet 1947). All orchid species tested to date and failing to grow on lactose belong to the subfam. Epidendroideae, which could indicate phylogenetically related lactose utilization capabilities.

Raffinose partly supported growth of both species. While *Oeceoclades* protocorms grew only slowly, being much smaller than on sucrose or glucose, *Ophrys*

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protocorms grew relatively well, comparably with the glucose supported ones and significantly better than on maltose or lactose. Similarly to *Oeceoclades*, two other orchid taxa, *Dendrobium* and *Phalaenopsis*, utilized raffinose only slightly (Ernst 1967). These taxa are much closely related to *Oeceoclades*, while *Ophrys* belongs to different orchid subfam. Thus, the difference between *Ophrys* and other species tested might be attributed to its distant phylogenetic position.

Remaining three saccharides tested, i.e. fructose, glucose and sucrose, are commonly classified as readily utilizable and suitable for orchid protocorm growth (e.g. Burgeff 1936, Arditti 1967). In our experiments, sucrose proved to be the best from all saccharides tested in both species. *Oeceoclades* protocorms grew readily on all three mentioned saccharides. However, in the presence of fructose, they were slightly smaller. Surprisingly, *Ophrys* protocorms, thriving very well on sucrose, were strongly inhibited by fructose and slightly also by glucose. On the medium with fructose + glucose mixture, the protocorms were much closer to fructose-supported variant than to hypothetical average between the glucose- and fructose-supported ones. This might be a result of fructose signalling inhibition even in the presence of utilizable saccharide. Moreover, the rhizoids of fructose-supported protocorms resembled those grown on fructose + glucose mixture. Another evidence for fructose inhibitory signalling could be an unusual protocorm size distribution in fructose variants, not observed elsewhere: a small proportion of protocorms grew well, similarly as on sucrose, probably overcoming a regulatory checkpoint controlled by fructose. Inhibitory effect of fructose has not been observed in orchids previously. Fructose can support orchid growth, sometimes even better than glucose (Arditti 1967, Ernst 1967). All orchid species that belong to a range of tropical epiphytical species from the subfam. Epidendroideae grow readily on fructose, thus the *Ophrys* response could result from its distant phylogenetic position. However, for *Habenaria* protocorms (Orchidoideae), fructose was a good carbon source comparable with sucrose and glucose, exhibiting only weak inhibitory effect (Stewart and Kane 2010). Clearly, other experiments are needed to clarify fructose inhibitory effect nature. Furthermore, though a strong overall correlation between rhizoid length and protocorm size was observed, some saccharides induced saccharide-specific variability in rhizoid length, which indicates certain additional effects, connected probably with carbohydrate signalling. Unfortunately, nothing is known about protocorm rhizoid length regulation and further studies will be necessary to shed some light on this issue.

Based on our results and comparison with available literature data it can be proposed that: (i) There is no close correlation between abundance of particular saccharide in orchid mycorrhizal fungi and its usability by symbiotic orchid (e.g. mannitol frequently present in mycorrhizal fungi not utilized by several orchids; sorbitol not found in fungi but utilized by some orchid taxa). (ii) Further, orchid ability to utilize various saccharides differs substantially between species often without close correlation with phylogenetic relationship. (iii) There might be a correlation between particular saccharide utility and species life strategy, assumed e.g. for maltose and epiphytic life form. Generally, the utilization of different saccharides in heterotrophic organs/phases of higher plants commonly varies not only between families but also between genera. It can be quite nicely demonstrated on the example of sugar alcohols that represent prevailing type of saccharide to support growth of sinks in one species and is not utilizable by closely related one (e.g. Bieleski 1982, Loescher et al. 1992). When regarding slow degradation of e.g. mannitol by plants, the ability of moderate degradation can be found even in species that did not produce it. In this case, the degradation activity is linked to stress response or pathogen defense strategies of plant not to basic metabolic arrangement (Sengupta et al. 2015, Patel and Williamson 2016). These characteristics probably reflect more the life strategy than the phylogenetic relationship. However, more species have to be tested before final conclusions on the life strategy responsibility for different saccharide utilization are made.

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