

***IN VITRO* ANTARCTIC MOSSES:**

FRUCTAN SYNTHESIS UNDER ABIOTIC STRESS CONDITIONS

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Abstract

*Being widely used in medicine fructan polysaccharides are also considered the protective agents to various abiotic stresses. Antarctic mosses must have characteristics of extremal adaptation so studying their adaptation mechanisms including fructan synthesis appears promising. Moss different species tolerance to the effect of increased salinity (NaCl), increased nitrogen content (NH₄NO₃), low and high (+4 and +38°C) temperature abiotic stress factors was measured by the level of moss surviving, the coefficient of reproduction and the fructan content in plant extracts. *W.fontinaliopsis* plant samples appeared the most tolerant to modeled abiotic stress conditions, comparing to the other studied moss species. Salt and nitrogen stress effect led to fructan content increasing in *W.fontinaliopsis* plants comparing to control non-effected samples while high temperature stress effect caused nearly ten-time fructan content increasing. The neutral comet assay was performed in order to evaluate the moss extracts UV protective activity and showed the decreasing of the DNA damages level depending on the extract concentration.*

Key words: fructans, Antarctic mosses, abiotic stress tolerance, UV-protection.

1 INTRODUCTION

Fructans are carbohydrate fructose polymers known for their biological activity and usability in medicine. They are naturally found in cichory, artichokes, leeks, onion bulbs, garlic, watermelon and others (Pollock, CJ & Cairns, AJ 1991, Wei, JZ *et al.* 2002, Bancal, P, Carpita, NC & Gaudillère JP 1992, Bhatia, IS & Nandra, KS 1979, López, MG, Mancilla Margalli, NA & Mendoza Díaz, G 2003, Gupta, AK & Kaur, N 2000) though finding the new sources of fructans seems promising. The fructan polysaccharides are used as diet food additive in case of carbohydrate metabolism disorders, calcium and magnesium metabolism disorders (Abrams, SA *et al.* 2005.) dysbacteriosis, diabetes, blood-circulatory system diseases (Roberfroid, MB 2005). For example, inulin type fructan is considered a full glucose substitute with hypoglycemic effect while its usage causes the sugar level, cholesterol and triglyceride content decreasing significantly in blood of diabetic patients. (Kok, NN, Taper, HS & Delzenne, NM 1998, Delzenne, NM & Kok, N 2001, Delzenne, NM *et al.* 2002, Williams, CM & Jackson, KG 2002, Kaur, N & Gupta, AK 2002) As the long-chained fructans are not digested they are called dietary fibers, as sorbent they also cause toxic substances excretion from digestive system. (Kelly, G 2008) Moreover, the fructans are used as prebiotics as inulin stimulates the lacto- and bifidobacteria intestine microflora growth. (Özer, D, Akin, S & Özer, B 2005, Roberfroid, MB, Van Loo, J & Gibson, GR 1998) The short-chained fructans are used as additive medicine for curing some

forms of oncological diseases. (Pool-Zobel, BL 2005, Taper, HS, Lemort, C & Roberfroid, MB 1998, Taper, HS & Roberfroid, MB 2000)

Fructans are storage polysaccharides in plant and bacteria cells, they also act as osmoregulators and antifreezes. Fructans appeared the protective agents to various abiotic stresses. Fructan synthesis level was shown to increase greatly in case of low temperature (Portesa, MT et al. 2008), drought (Monti, A et al. 2005) and other abiotic stress effect due to fructan ability to change polymerization level. (Hinch, DK et al. 2002, Monti, A et al. 2005, Pilon-Smits, EAH et al. 1995)

Fructan synthesis is also carried out in case of plant *in vitro* cultivation. For chicory roots *in vitro* culture inulin concentration has been reported even higher comparing to the one of *in vivo* root culture. (Ranjitha Kumari, BD, Velayutham, P & Anitha, SA 2007) Besides the *in vitro* plant cultivation enables the conservation of rare or endangered plant species, quick plant biomass production and controlling the conditions of *in vitro* plant cultivation for regulation of valuable plant substance synthesis.

Antarctic mosses are considered the ones of great interest because of the extreme conditions of surviving in this region. Being the Antarctic plants, they, naturally, must have characteristics of extremal adaptation so that the studying of their adaptation mechanisms including fructan synthesis appears promising. Besides, Antarctic mosses are reported of various types of biological activity (Decker, EL & Reski, R 2007). They may become new source of biologically active substances, for example fructans.

Here we report the increasing of fructan level in Antarctic moss plants in response to the abiotic stress effect and discuss the UV- and DNA-protective activity of moss extracts.

2 MATERIALS AND METHODS

2.1 Plant material

We used the *in vitro* culture samples of *Polytrichum juniperinum* Hedw., *Warnstorfia fontinaliopsis* Müll.Hal., *Sanionia georgicouncinata* (Müll.Hal.) Ochyra and Hedenäs, *Bryum pseudotriquetrum* (Hedw.) P.Gaertn., *Bryum argenteum* Hedw., *Pohlia nutans* (Hedw.) Lindb., *Ceratodon purpureus* (Hedw.) Brid. Antarctic mosses. Antarctic mosses were obtained from several regions of Antarctica: Galindes Island, Dronning Maud Land, Enderby Land (Fig.1). We also used non-Antarctic *Physcomitrella patens* (Hedw.) Bruch & Schimp. moss samples from Ukraine (Lviv).

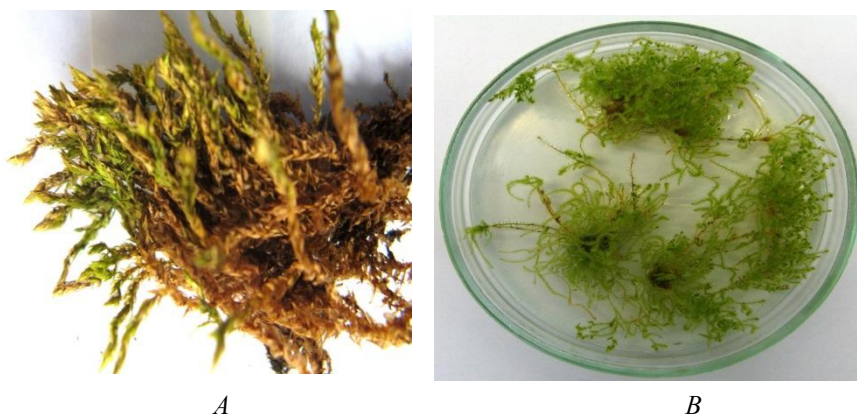


Figure 1 *Warnstorfia fontinaliopsis* (Müll.Hal.) Ochyra plants collected in Galindes Island (point 023, 65° 14' 56.73" S 64° 14' 42.53" W): a – native sample, b – *in vitro* cultivated samples

2.2 Stress conditions modeling and fructan content measurement

The plants were being cultivated *in vitro* on MS medium (Murashige, T & Skoog, F 1962) at 24°C under the conditions of 16-hour photoperiod and propagated by shooting induction. The effect of increased salinity (NaCl), increased nitrogen content (in NH_4NO_3 form), low and high (+4 and +38°C) temperature abiotic stress factors on fructan synthesis level was measured. For salt or nitrogen stress modeling the plants were being 30-day-cultivated on the media with 25, 50, 100 and 200 mM NaCl or 0,83, 1,65, 3,3, 6,6 and 13,2 g/l NH_4NO_3 respectively. For studying low and high temperature effect the plants were being cultivated at +4 °C for 2, 10 and 20 days or at +38 °C for 24 and 48 hours respectively, and then they were grown at +24 °C up to 30 days since the beginning of stress factor effecting. We used the method based of fructan ability to interact with rezorcin (in acidic environment) with the stained hydroxymethylfurfural formation for fructan content measurement on the 30th day.

2.3 Biological activity assay

The comet electrophoresis method was used for measurement of *W. fontinaliopsis* extract biological activity. This Antarctic moss extracts were obtained by incubation of weighed plants in phosphate buffer (pH 7,4) aliquots at +24°C for 60 minutes.

Human peripheral blood lymphocytes were used for comet assay. Cell suspension in RPMI 1640 medium was separated into four aliquots. The first one was exposed to UV-C light in dose of 40 J/m² to induce DNA damages (for example, thymine dimmers) and then cells were incubated at 37°C to allow excision repair proteins introduce single strand brakes at these sites (negative control). The second aliquot (suspension of interest), previously incubated at 37°C in RPMI 1640 medium with 100 µl and 200 µl of extract for one hour, was irradiated at 40 J/m² and then again cultivated for 15 min at the same condition. Cells from third sample were cultivated as the second one, but without UV-C exposure (control of extract action) and the fourth sample was exposed to any treatment (positive control).

Each aliquot of lymphocytes suspension (50 µl) was mixed with 100 µl 1% low-melting agarose and 25 µl of this mixture was used to prepare microscope slides covered with 1% high-melting point agarose. Slides were kept at 4°C until agarose polymerization and then placed into lysis solution (2,5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl (pH 8,0), 1% Triton X-100). Cells were exposed to the lysis solution for 2 hour at 4°C. After lysis treatment slides were washed with TBE buffer (0,089 M Tris, 0,089 M H_3BO_3 , 0,002 M EDTA, pH 7,5) and then neutral comet assay was performed for 20 min (4°C, 1 V/cm, 300 mA). After electrophoresis slides, without any fixation, were stained with 1 mg/mL 4',6-diamidino-2-phenylindole (Sigma) and immediately analyzed with fluorescence microscope (LOMO, Russia) connected with camera Canon A570. Totally 100–200 cells on each slide were analyzed with Comet Score software to determine the relative amount DNA in tail.

2.4 Statistical methods

The statistical design was carried on for the obtained results in way of fructan content measurement. The variance analytical models showing the median with the confidence ranges for the studied group rates with *p* value <0.05 were considered statistically significant and became the basis for comparisons. We also used Student criterion to evaluate the significance of comparisons of the obtained results.

3 RESULTS AND DISCUSSION

The fructan content was measured for the moss *P. juniperinum*, *W. fontinaliopsis*, *S. georgicouncinata*, *B. pseudotriquetrum*, *B. argenteum*, *P. nutans*, *C. purpureus* and *P. patens* *in vitro*

samples. The obtained data differed for the mosses of different species from $19,29 \pm 0,67$ mg/g FW for *P. juniperinum* to $34,12 \pm 3,81$ mg/g FW *B. pseudotriquetrum* cultivated at $+24^{\circ}\text{C}$ (fig. 2).

Moss different species tolerance to abiotic stress effect was measured by the level of moss surviving and the coefficient of moss reproduction under the stress conditions. *W. fontinaliopsis* plant samples appeared significantly the most tolerant to abiotic stress conditions, compared to the other studied moss species. For example the salt stress conditions showed even stimulating effect on the *W. fontinaliopsis* coefficient of plant reproduction while, for example, the *P. patens* coefficient of reproduction significantly decreased with NaCl concentration increasing (data unshown). This way the further studies were carried out mainly for *W. fontinaliopsis* plant samples as for the most promising ones. The *P. juniperinum* samples were taken for the control ones.

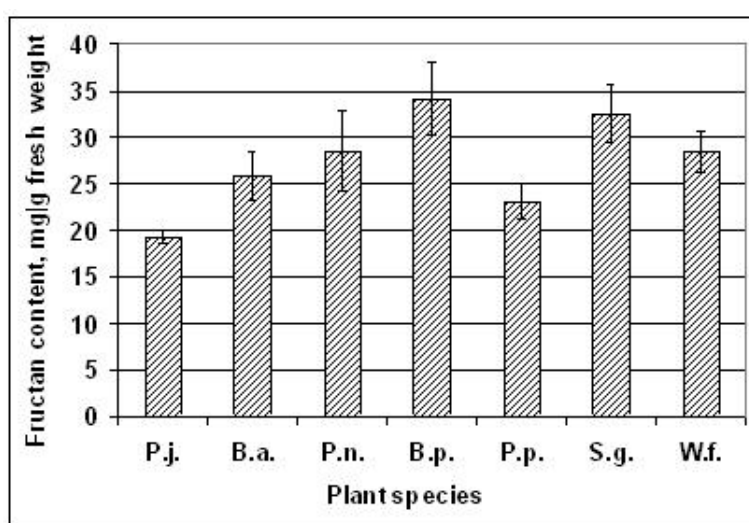


Figure 2 Fructan content in Antarctic *P. juniperinum*, *B. argenteum*, *P. nutans*, *B. pseudotriquetrum*, *S. georgicouncinata*, *W. fontinaliopsis* mosses and non-Antarctic moss *P. patens*

High temperature stress effect led to nearly ten-time fructan content increasing in *W. fontinaliopsis* moss extracts comparing to the control non-effected samples. The *P. juniperinum* sample high temperature treatment led to fructan content lowering under the same conditions (Fig 3).

The obtained results let us assuming the *W. fontinaliopsis* moss samples a promising source of fructan obtaining in case of modeling the certain conditions of moss cultivating.

Low temperature stress effect led to fructan content increasing in *W. fontinaliopsis* moss extracts comparing to the control non-effected samples while *P. juniperinum* samples low temperature treatment led to insignificant fructan content lowering under the same conditions (Fig 4).

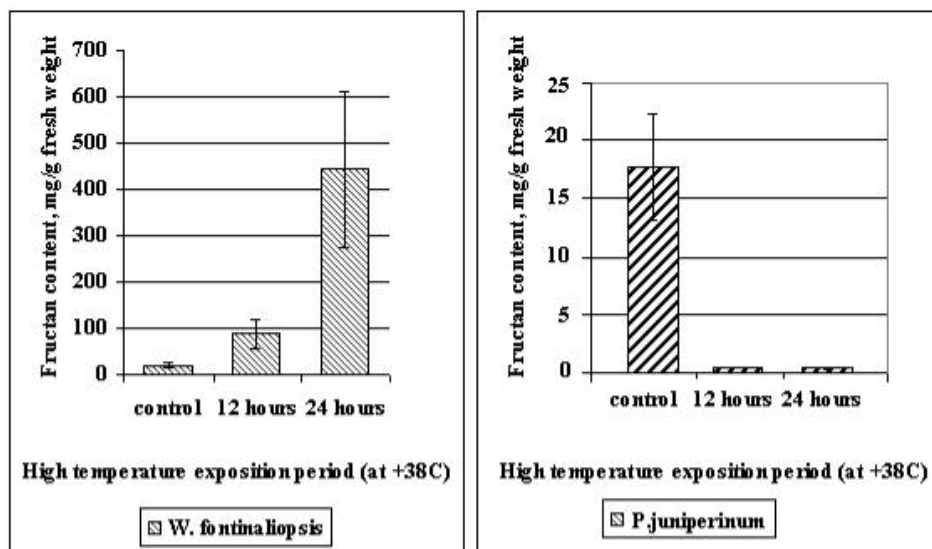


Figure 3 Fructan content in *P.juniperinum* or *W. fontinaliopsis* mosses under the high temperature stress conditions

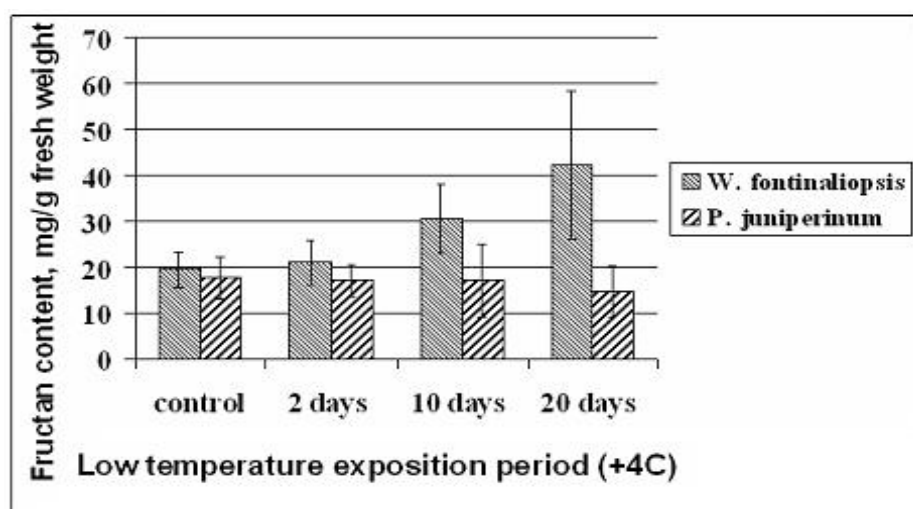


Figure 4 Fructan content in *P.juniperinum* or *W. fontinaliopsis* mosses under the low temperature stress conditions

Salt and nitrogen stress effect led to fructan content increasing in *W. fontinaliopsis* plants comparing to the control non-effected samples. So that the fructan content in case of 50 mM NaCl treatment increased in 1,64 times for *W. fontinaliopsis* mosses (fig. 5). The NH_4NO_3 concentration increasing from 0,82 to 3,3 g/l led to fructan content increasing in 2,14 times for these plants (fig.6). The fructan content of *P. juniperinum* samples was varying insignificantly under salt or nitrogen stress conditions.

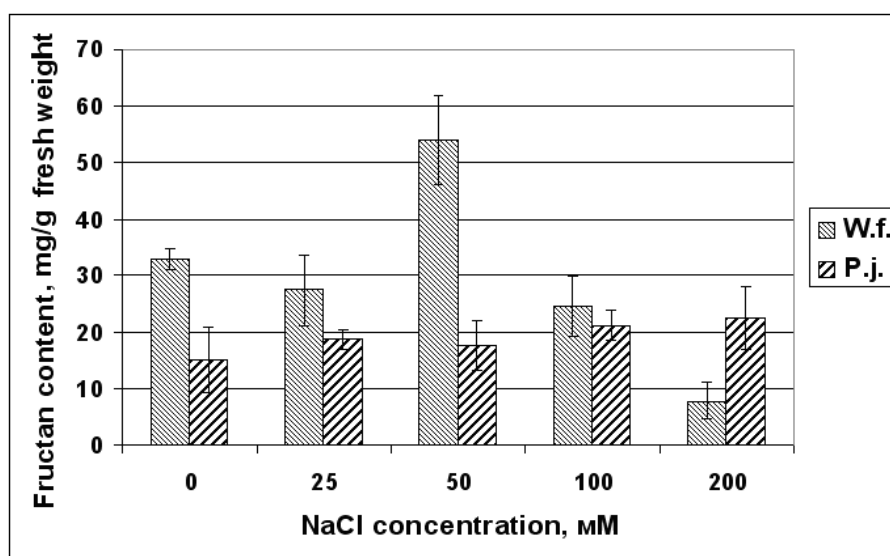


Figure 5 Fructan content in *W. fontinaliopsis* (W.f.) and *P. juniperinum* (P.j.) mosses under the NaCl stress conditions

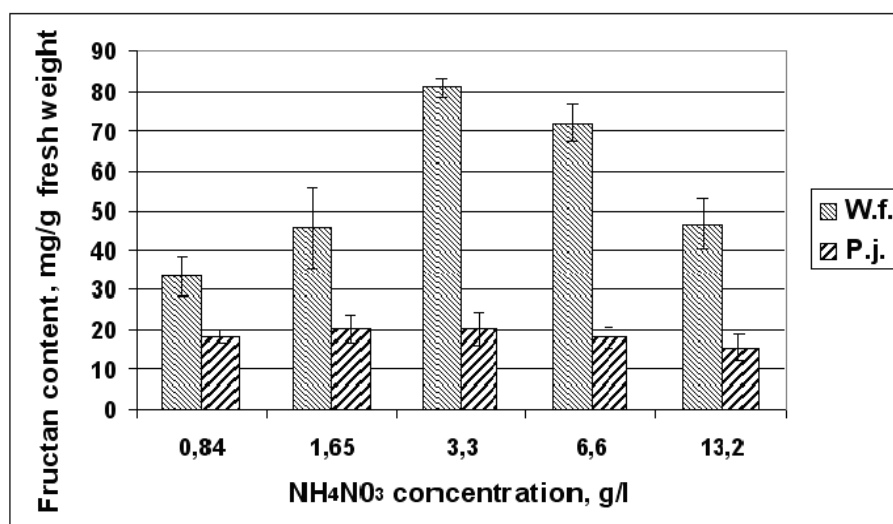


Figure 6 Fructan content in *W. fontinaliopsis* (W.f.) and *P. juniperinum* (P.j.) mosses under the NH₄NO₃ stress conditions

To evaluate whether the moss extracts have UV protective activity the neutral comet assay was performed. The relative amount of DNA in comet tail was used as main parameter that indicates the

level of UV-induced damages (Fig.7). The average DNA rate in tail was 0.125 for positive control, that correlates the results previously obtained for intact lymphocytes (Afanasieva, K, Zazhytska, M & Sivolob, A 2010). In negative control (UV) the average DNA level in tail was 0.139 that shows the accumulation of strand breaks of UV-induced DNA damages in excision sites and, thus, the effectiveness of UV-C irradiation. In UV-irradiated cells which were incubated with extracts (third aliquot) relative amount DNA in tail was 0.052 for 100 μ l and 0.039 for 200 μ l of additive extract. Thus, addition of moss extracts to cell suspensions leads to decreasing of the DNA damages level depending on extract concentration in culture medium.

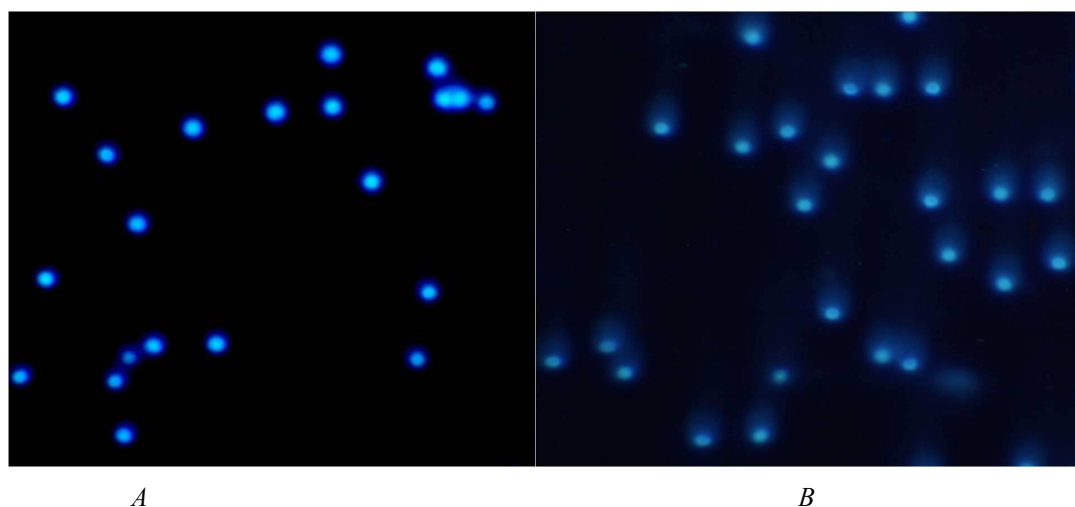


Figure 7 Typical comets after DAPI staining at 20 min of electrophoresis. (a) – after UVC treatment without incubation with moss extracts, (b) – after incubation with extracts and UVC treatment.

Such effect was observed also for the other experiment in non-irradiated cells. In control the average rate of DNA in tail was 0.15. The relative amount DNA in tail was 0.07 -0,09 in cells which were incubated with extracts. The obtained results let us suggest the *W.fontinaliopsis* extract had UV-protecting properties.

The plants (including mosses) are known for various regulatory mechanisms of their protecting of the abiotic stress factors (Valluru, R & den Ende, WV 2008). Fructans are reported able to stabilize membranes and protect plant cells against salt, temperature, drought stresses. (Hendry, GAF 1993, Hinch, DK et al. 2002, Hinch, DK, Hellwege, EM, Heyer, AG & Crowe, JH 2000, Hinrichs, WLJ et al. 2005, Vereyken, IJ et al. 2003).

The salt, nitrogen, cold and high temperature stress in our study has been shown to lead to fructan content increasing for the moss plant species known for their high tolerance to abiotic stress factors. Here the *W.fontinaliopsis* plants appeared prominent as the ones that reacted the stresses by significant fructan level increasing. The greatest effect on fructan accumulation was observed in case of high temperature stress. *W.fontinaliopsis* plants incubation for 24 hours at +37C led to ten-time fructan content increasing comparing to the control variant. The extracts of Antarctic *W.fontinaliopsis* moss known for their high tolerance to abiotic stress effect and high fructan content showed the biological activity in way of decreasing the DNA damage level in case of UV radiation effect. The discovered

effect of fructan level increasing in case of abiotic stress effect may be used for biotechnology for obtaining the biologically active extracts with UV and DNA protective features or enable the high-level fructan production for moss plants.

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