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Responses of *Solidago altissima* Gray to Different Salinity Levels During *in vitro* Propagation

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Abstract: Salinity is one of the major abiotic stresses that adversely affect plant productivity and quality in many arid and semi-arid parts of the world. This study investigated the response of *Solidago altissima* tissue cultures under salt stress. *Solidago altissima* shoots were cultured under eleven levels of salt concentrations, derived from a mixture of different salts [sodium chloride (NaCl), calcium chloride ($CaCl_2$) and magnesium sulphate ($MgSO_4$) at ratio of 2:2:1 (w/w/w), respectively] during the proliferation stage. Survival percentage was significantly decreased with increasing salt mixture concentration in culture medium. Salinity at 11000 ppm led to 100% shoot mortality at the 1st subculture. The number of proliferated shoots, shootlet length (cm) and the number of leaves per shootlet were depressed upon increasing of salts mixture in medium. Chlorophyll *a,* chlorophyll *b*, carotenoids and indoles significantly decreased with exposure to higher salt concentration. On the other hand, total soluble phenols and proline contents significantly increased in explants grown on higher salt mixture. SDS-PAGE analyses of extracted proteins revealed that extra polypeptides at 66, 45, 34 and 29 kDa (Kilodaltons) not present in control samples were accumulated in explants grown in different salinity levels.

Key words: Solidago altissima · *In vitro* propagation · Salinity stress

INTRODUCTION

Solidago altissima (Tall Goldenrod), family Asteraceae (Compositeae), is an important perennial ornamental plants that produces golden-yellow flowers in many minute heads. Solidago species recently introduced to Egypt for commercial production, have a wide application as landscape plants and as cut flower for export. Increasing salinity a major problems of agriculture in arid and semi-arid regions. The accumulation of salts in soil may be attributed to high groundwater table accompanied with poor drainage. Soil salinity has, therefore a great impact on decreasing the yield potential of cultivated crops. Crop yields start declining when electroconductivity (EC) of the soil solution goes above 4dS/m [1[. Salinity influences almost every aspect of the physiology and biochemistry of plants and significantly reduces yield [2[. High exogenous salt concentrations cause ion imbalance, leading to ion toxicity and osmotic stress [3]. Most importantly, salinity impacts photosynthesis. Reduced photosynthesis under increasing salinity is not only attributed to stomatal closure and reduction of intercellular CO_2 concentrations, but also to non-stomatal factors [4]. Increased salinity reduces the ability of plants to utilize water and causes a reduction in growth rate, as well as changes in plant metabolic processes [5]. Increased salinity not only reduces biomass, but also other morphological parameters such as plant height, number of leaves, root length and shoot/root ratio [6]. In many plant species, salt stress induces proline accumulation, which may enhance plant salt tolerance [7] and serves as a measure of plant tolerance to salt stress [8]. *In vitro* culture is widely used in the micropropagation of high value ornamental species. The effect of salinity on the micropropagation of several ornamental plants have been studied on the *Acalypha macrophylla* and *Justica gendarus* [9]; *Cupressus sempervirens* [10]; *Lantana camara* [11]; *Cumacrops*

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humillis and *Phoenix canarienses* [12] and *Paulownia imperialis* and *Paulownia fortunei* [13]. Up to now, there are no literatures available on the salinity tolerance on *Salidago altissima* by *in vitro* culture

Therefore, this study aims to investigate the influence of different levels of salinity on growth and biochemical constituents of *Solidago altissima* during proliferation stage and to confirm the salinity tolerance during rooting and acclimatization stages.

MATERIALS AND METHODS

The present study was carried out to investigate the effect of different levels of salts mixture {sodium chloride (NaCl), calcium chloride ($CaCl_2$) and magnesium sulphate ($MgSO_4$) at ratio of 2:2:1 (w/w/w)}, respectively during the proliferation stage on micropropagability and biochemical constituents of *Solidago altissima* var. "Tara".

Preparation of Explants: The *in vitro* shoots (2-3 cm) of *Solidago altissima* var. "Tara", grown on MS medium [14] enriched with 0.5 mg/l benzylaminopurine (BAP), 30 g/l sucrose and 7 g/l Agar, were used as a source material. The medium was adjusted to pH 5.7±0.1, then poured at 25 ml in 200 ml capacity glass jars before autoclaving at 121°C and 1.2 kg/cm^2 for 15 min. The culture were incubated in growth chamber at 24±1° under 16 hrs photoperiod (day light fluorescent tube) at 3 K lux.

Salinity Stress Treatments: The shootlets were aseptically sectioned into shootlets (2-3 cm length) and subcultured in the same previous medium composition supplemented with 0.0, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000 and 11000 ppm of salt mixture. Therefore, in three consecutive shootlet proliferation cycles, twelve components of culture medium were done in 1st subculture and eleven components of culture medium in the 2nd and 3th subcultures, rooting and acclimatization, twenty explants in five replicates, each replicate (jar) containing 4 shootlets, for each treatment were used. After each subculture the following data were recorded: survival percentage of explant and growth parameters [shootlet number per explants, shootlet length (cm) and number of leaves per shootlet].

Determination of Pigments, Total indoles and Phenols: One gram of the fresh shootlets (leaves and stems) was macerated in 10 ml 80% ethanol for 24 h at 0°C. After centrifugation, the residue was re-extracted twice with 10 ml 80% ethanol. The supernatants were pooled and completed to 50 ml using 80% ethanol. Photosynthetic pigments (chlorophyll *a*, *b* and carotenoids), total indoles, total phenols were determined using colorimetric methods described elsewhere [15-17]. The concentration was calculated as mg /100 g fresh weight (fw).

Proline Content: Leaf proline content was determined by a spectrophotometric assay as described by Bates *et al.* [18]. Briefly, 50 mg of lyophilized *in vitro* derived shootlet material was extracted by 5 ml of 3% sulphosalicylic acid. After centrifugation at 4000 × g and 4°C, 1 ml of supernatant was combined with 1 ml of glacial acetic acid and 1 ml of ninhydrin solution. The combined solution was incubated at 80°C in a water bath for 1 h and the resulting mixture was partitioned against 2 ml of toluene after a cooling period. Absorbance at 520 nm was read in the organic layer against a blank. A standard curve of different concentration of proline (Sigma–Aldrich) was used to calculate the concentration proline in leaves.

Analysis of Protein Profile of Leaf by SDS-PAGE: Samples (0.5 g) were homogenized with 2 ml of a buffer containing 50 mm Tris (hydroxymethyl) aminomethane (Tris)-Glycine (pH 8.3), 0.5 m sucrose, 50 mm EDTA, 0.1 m KCl, 2 mm PMSF and 0.1% (v/v) 2-mercaptoethanol in a chilled pestle and mortar at 4 Cº. The homogenate was centrifuged in a cooling centrifuge (Sigma, 2-16PK, Germany) at 14,000×*g* for 10 min. Protein concentration in the supernatant samples was estimated according to the method of Bradford [19]. Supernatant samples (40 µg protein) were mixed with equal volumes of solubilizing buffer [62.5 mm Tris-HCl, pH 6.8, 20% (w/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.01% bromophenol blue] and heated for 4 min at 95°C, cooled on ice before loading on 12.5% polyacrylamide gels. Gels were made according to Laemmli [20].

Rooting Stage: To confirm the resistance for salinity, shootlets (3-4 cm length) resulting from the three subcultures were cultured in half- salt strength MS medium with different levels of salinity treatments (0.0, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000 and 10000 ppm). Twenty-five shootlets in five replications were used for each treatment. At the end of the fourth week of culturing, the root number and root length (cm) were recorded.

Acclimatization Stage: In the acclimatization trail, the rooted shootlets resulting from the different treatments were transferred to plastic pots (0.2 liter) containing peat

moss plus washed sand at 1:1 (v/v) as growing media. The same salinity concentrations were used in irrigated the substrate and covered by transparent polyethylen bags in five replicates for each treatment. The acclimatized *in vitro* plants were kept in acclimatized glasshouse for four weeks after that the survival plants were recorded.

Statistical Analysis: The layout of all experiments was a completely randomized design. The data were statistically analyzed for testing differences between means using L.S.D. according to Steel and Torrie [21].

RESULTS AND DISCUSSIONS

Salt stress is a significant factor that severely affects plant growth and tissue structure. Several parameters have been developed to assess the salt stress tolerance of plants. Growth and survival rates represent the most common parameters so far used [22]. They have been considered as the result of various physiological mechanisms involved to avoid salt effects.

Survival Percentage: *In vitro* explants were grown for three subcultures under salt mixture stress from zero to 11000 ppm. The results suggested that salt concentrations from zero till 6000 ppm had no significant effect in the survival percentages of the explants. Survival percentage of explants grown on salt concentrations up to 6000 ppm was significantly decreased with increasing the salinity levels. The lowest survival percentage (44.4%) was obtained with salt concentration at 10000. Moreover, using 11000 ppm salinity lead to die explants (0.0%) survival (Table 1). The effect of salinity concentrations on relation to the number of subcultures indicated that the lowest survival percentage (71.53%) in the 1st subculture increased gradually in the 2 nd(76.39%) and 3th subculture, which gave the highest percentage (82.64%). This means that the explants become more tolerance by increasing the number of subcultures. The interaction between salinity concentration and the number of subcultures revealed that using zero to 3000 ppm in the 1st subculture had no significant effect on the survival percentages. However, increasing the concentrations more than 3000 ppm caused a highly significant decreased the survival percentages. In case of the 2nd and 3th subcultures up to 4000 and 6000 ppm salinity concentration had no significantly effect on the percentage of survival respectively. In this respect, Chelli-Chaabouni *et al*. [23] cultured two pistachio rootstocks (*Pistacia vera* L. and *P. atlantica* Desf) *in vitro* and they reported that the higher salt tolerance of *P. atlantica* observed seems to be correlated with a higher survival rate.

Growth Parameters: During three subcultures in the proliferation culture media, the growth parameters (shootlets number, shootlet length and number of leaves/shootlet) were significantly affected by the salinity treatments. As shown in Table 2, the growth parameters were decreased with increasing of salinity levels from 1000 to 10000. For levels of salinity stress, the highest number of shootlets was recorded in the control (salt mixture-free)

Table 1: Effect of salinity stress on survival percentage of *Solidago altissima* explants grown *in vitro*.

Treatments		Subculture 1	Subculture 2	Subculture 3	Mean B
0 ppm		100	100	100	100
1000 ppm		100	100	100	100
2000 ppm		100	100	100	100
3000 ppm		100	100	100	100
4000 ppm		91.67	100	100	97.22
5000 ppm		83.33	91.67	100	91.67
6000 ppm		83.33	91.67	100	91.67
7000 ppm		75	75	83.33	77.78
8000 ppm		58.33	66.67	83.33	69.44
9000 ppm		41.67	50	58.33	50
10000 ppm		25	41.67	66.67	44.44
11000 ppm		0	0	0	0
Mean A		71.53	76.39	82.64	--
LSD 0.05	A	5.158			
	B	10.32			
	A×B	17.87			

Table 2: Effect of salinity stress on growth parameters of *Solidago altissima* explants grown *in vitro*.

		Number of shootlets/explant				Length of shootlet (cm)				Number of leaves/shootlet			
Treatments		Sub. 1	Sub. 2	Sub. 3	Mean B	Sub. 1	Sub. 2	Sub. 3	Mean B	Sub. 1	Sub. 2	Sub. 3	Mean B
Control		10.75	10.75	12	11.17	2.18	2.72	3.01	2.64	9.02	9.09	9.85	9.32
1000 ppm		3.42	3.67	4.17	3.75	1.42	2.02	2.23	1.89	7.12	9.19	9.5	8.6
2000 ppm		2.58	3.33	3.83	3.25	1.46	2.27	2.44	2.06	8.85	9.99	10.37	9.74
3000 ppm		2.08	2.58	3.17	2.61	1.37	1.67	1.4	1.48	8.72	8.1	8.45	8.42
4000 ppm		1.33	1.58	2.83	1.92	1.04	1.77	2.04	1.62	8.01	8.92	9.54	8.83
5000 ppm		1.42	1.58	2.58	1.86	1.11	1.43	1.84	1.66	7.96	8.6	8.92	8.49
6000 ppm		1.08	1.33	2.42	1.61	1.15	1.23	1.38	1.26	8.08	8.15	8.42	8.22
7000 ppm		1.08	1.25	2	1.44	0.98	1.11	1.25	1.11	8.11	7.29	7.58	7.66
8000 ppm		1.08	1.17	1.83	1.36	1.14	1.26	1.36	1.25	8.11	7.63	7.5	7.75
9000 ppm		1	1.08	1.42	1.17	0.63	0.81	0.94	0.79	5.33	6.71	6.79	6.28
10000 ppm		1	1	1.25	1.08	0.57	0.71	0.92	0.73	4.67	7.54	7.46	6.56
Mean A		2.439	1.667	3.409	--	1.186	1.546	1.71	--	7.634	8.292	8.58	--
LSD 0.05	A	0.2868				0.193				0.5723			
	B	0.5491				0.386				1.096			
	A×B	0.9511				0.6686				1.898			

Table 3: Effect of salinity stress on pigments, indole, phenol and proline of *Solidago altissima* explants grown *in vitro*.

	Pigments mg/100g fw					
Treatments	Chl.*a*	Chl.*b*	Carotenoids	Indole mg/100g fw	Phenol mg/100g fw	Proline mg/100g fw
Control	99.69	52.4	127	670.2	208.7	0.187
1000 ppm	75.46	36.24	96.44	401	654.1	0.197
2000 ppm	95.68	57.61	111.3	600.1	597.5	0.32
3000 ppm	48.06	49.98	80.7	560	671.5	0.387
4000 ppm	54.44	46.97	62.43	410.7	823.4	0.547
5000 ppm	46.51	32.26	73.96	398.3	470.1	1.678
6000 ppm	89.72	87.93	98.86	407.8	420.2	0.94
7000 ppm	51.47	61.03	57.5	339.7	1031	0.972
8000 ppm	78.54	112.5	84.77	603.9	10.57.0	0.797
9000 ppm	31.99	51.31	72.71	280.3	1065	0.677
10000 ppm	49.26	45.28	61.23	221.3	1754	3.337
LSD 0.05	4.331	10.94	6.862	224.5	99.77	0.9784

(11.17 shootlets/explant) compared to 10000 ppm treatment, which gave the lowest shootlet number (1.08 shootlet/explant). The same observations were showed with shootlet length and the number of leaves/shootlet. The longest shootlet was obtained in the control treatment; however, the shortest one resulted from using 10000 ppm salinity concentrations. Using 2000 ppm salinity produced the greatest number of leaves/shootlet (9.74), while adding 10000 ppm decreased the number of leaves/shootlet to the lowest number (6.56 leaves/shootlet). For the number of subculture revealed that in the 1st subculture significantly decreased the growth parameters compared to the 2nd and 3th subcultures. The highest growth parameters (shootlet number, shootlet length and number of leaves/shootlet) were observed in the 3th subculture. This means that increasing the subcultures caused an increasing in the salinity stress and increasing the plant tissues tolerance. Similar results have been observed in other plants, Dodangeh *et al*. [24] on apple rootstock, El- Sharabasy *et al.* [25] on date palm and Prajuabmon *et al.* [26] on rice seedlings recorded that, high salt concentration decreased all morphological characters. These results could be attributed to the effect of salinity on reducing the synthesis of DNA, RNA and protein in many plants which it might be lead to disturbance in metabolic activities [27, 28].

Pigment Contents: The different salinity concentrations showed a highly significant effect on decreasing chlorophyll *a* content except 2000 ppm concentration which gave the same value as the control. In general, the chlorophyll *a* content varied from 99.69 to 31.99 mg/100g fw. The lowest content of chlorophyll *a* was found by using 9000 ppm salinity concentration (Table 3). In case of chlorophyll *b* content the data showed another trend.

The highest value (112.5 mg/100g fw) was resulted in case of using 8000 ppm salinity concentration. Whereas, the lowest value (36.24 mg/100g fw) was found in 1000 ppm concentration. The carotenoids content showed the same trend as chlorophyll *a* content. The highest value (127.0 mg/100g fw) was obtained in the control treatment whereas, the lowest value (61.23 mg/100g fw) was resulted in case of using the highest salinity concentration (10000 ppm). These results are in agreement with those reported by Turhan and Eris [29] on strawberry, Erturk *et al*. [30] on *Prunus cerasus* x *Prunus canescens* and Stoeva and Kaymakanova [31] on beans which recorded that chlorophyll *a*, chlorophyll *b* and carotenoids were decreased with increasing salinity.

Total Indoles: Data in presented in Table 3 indicated that using salinity concentrations 2000, 3000 or 8000 ppm had no significant effect on total indoles contents compared to the control. Whereas, the other concentrations of salinity had a highly significant effect on decreasing the total indoles contents in the shootlets. The highest value (670.2 mg/100g fw) were resulted from the control treatment whereas, the lowest value (221.3 mg/100g fw) was found in case of using 10000 ppm salinity concentration. The reduction in indoles as a result of salinity stress may be ascribed to the increase of IAA-oxidase under stress conditions. These results are consistent with other workers on grapevine rootstocks and ornamental palm [12, 32-36].

Total Soluble Phenols: Data in Table 3 showed the primitives effect of salinity stress on total soluble phenols accumulation while exhibit favorable increase in harmony with the elevation of salinity levels with significant difference. The immensity of increase of phenols was fulfilled with higher value in shoottlet (1754.0 mg/100g fw) at the highest level of salinity 10000 ppm. The least phenols concentration (208.7 mg/100g fw) took place at control. Increasing salinity levels were accompanied by a gradual increase in the plant concentration of total soluble phenols. Moreover, it is quite clear that there is a reversible relation between phenols compounds accumulation and indoles reduction under salinity stress conditions. Several workers, Van Sumere *et al.* [37], Popovici and Rezink [38], Hanafy [39-41] on *Spinacia oleraceae* L. postulated that phenolic compounds are capable of inhibiting ATP synthesis in mitochondria, uncoupling respiration, affect polar transport of auxins, inhibiting enzyme activity, antagonizing plant hormones biosynthesis and inhibiting ions absorption.

Proline Content: The highest proline value (3.337 mg/100g fw) occurred at the highest salinity level (10000 ppm). While the lowest values (0.1867 and 0.1967 mg/100g fw) were detected at control and 1000 ppm salinity respectively (Table 3). These results may be referred to gene controls proline accumulation, called Osmotic Tolerance Gene, which governs the production of a class of molecules such as betain and proline that protect the cell and its constituents against dehydration. Proline is considered as a cytoplasm protective osmolyte necessary for adaptation to stress [42]. Moreover, Roy *et al.* [43] stated that proline accumulation in response to NaCl could be attributed to both an increase in D-pyrrolin-5carboxylate reductase and a decrease in proline dehydrogenase activity. In addition, Lutts *et al*. [44] and El-Adawe [45] on *Pheonix dactylifera* conducted that salt resistant cultivars accumulate lower amounts of free proline than salt sensitive ones.

Protein Fractions: Total proteins were extracted from leaves of control and salinity treated shoots after the third subculture and analyzed by SDS-PAGE. As visualized from SDS-PAGE, several protein bands of molecular weight 66.0, 45.0, 33.8 and 29.0 kDa appeared as a result of salinity treatment, while disappeared with the control treatment (Fig. 1). The band at 66.0 kDa appeared with treatments were grown under salinity stress from 4000 to 8000 ppm and disappeared with other salinity treatments or control. Bands at 45.0, 33.8 and 29.0 kDa appeared with all salinity treatments and disappeared with the control treatment. These results are in harmony with those obtained by Unni and Rao [46], who reported that certain outer membrane proteins of molecular weight 22, 38, 40, 42, 62 and 68 kDa markedly decrease in the presence of salt in Rhizobium. However, in *Bruguiera gymnorrhiza*, the intensity of a 33 kDa-protein with pI 5.2 increased as a result of NaCl treatment [47]. Jamil *et al.* [48] reported that sugar beet leaf protein content decreased significantly and SDS-PAGE analysis showed significant change in protein profiles in salt treated samples, which suggests that NaCl altered protein pattern.

Rooting Behavior and Acclimatization (Survival percentage): Due to confirm the tolerance of explant to salinity, data in Table 4 showed that salinity stress significantly influenced root number per plantlet and root length (cm). The roots number was increased by increasing salinity levels from control to 10000 ppm, which gave (3.14 to 4.92 roots/plantlet, respectively). While, the highest salinity level 10000 ppm brought the shortest length of roots (1.30 cm) compared to control (3.33 cm).

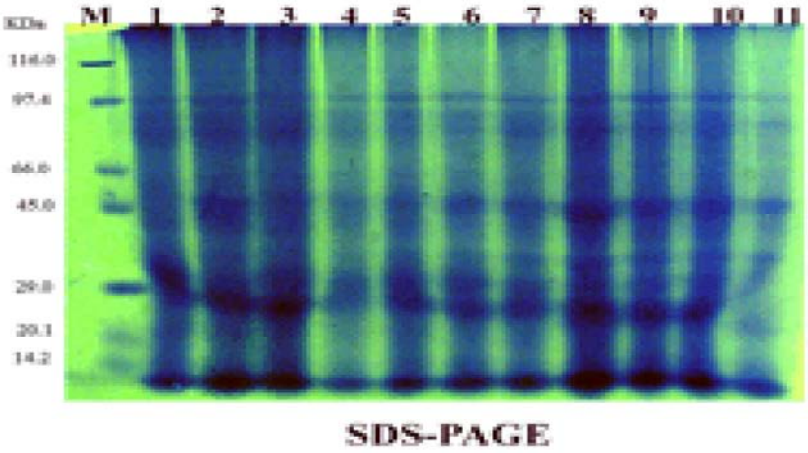

Fig. 1: Patterns of SDS- PAGE electrophoretic protein *Solidago altissima* after salinity stress.
1-control 2- 1000ppm 3- 2000 ppm 4- 3000 ppm 5- 4000 ppm 6- 5000 ppm 7- 6000 ppm 8- 7000 ppm 9- 8000 ppm 10- 9000 ppm 11- 10000 ppm

Table 4: Effect of salinity stress on rooting behavior and acclimatization plantlets of *Solidago altissima*.

	Rooting behaviour		Acclimatization stage
Treatments	Root number	Root length (cm)	Survival %
Control	3.14	3.33	100
1000 ppm	3.14	3	100
2000 ppm	2.75	2.83	97
3000 ppm	2.92	2.83	97
4000 ppm	2.75	2.7	98
5000 ppm	3.83	2.77	97
6000 ppm	3.42	2.17	98
7000 ppm	3.83	1.97	99
8000 ppm	4.67	1.43	97
9000 ppm	4.92	1.57	98
10000 ppm	4.92	1.3	97
LSD 0.05	1.591	0.727	NS

These results are in agreement with those obtained by Ramoliya *et al.* [49] on *Acacia catechu* who described the production of young roots and death of old roots were found to continuous and plant apparently use this processes an avoidance mechanism to remove excess ions and delay onset of ion accumulation in this tissue, this phenomenon, designited (fin root turnover) is of important to the mechanism of salt tolerance.

Regarding to acclimatization, the different salinity stressed levels had no significant effect on the survival percentage in Table 4. This means that all levels of salinity stress on rooting stage produced plantlet successfully in acclimatization and produced high survival percentages ranging from 100-97%. This has already been demonstrated for different plants such as, soybean pretreated for 23 days showed a higher survival rate under severe stress conditions [50], in sorghum pre-treated plants maintained the same growth rate before and after the exposure to high level of salt and they could stand a concentration much higher than non-acclimated plants [51] and in Pea (*Pisum sativum* L.) seedlings were grown in half strength Hoagland solution and exposed to 0, 10, 25 mM NaCl and 2.5% PEG 6000 for 1 week (pre-treatment). There was no negative effect of the pre-treatments on growth (total fresh and dry matter production) and plants pre-treated with 10 mM NaCl had biomass accumulation equal to control plants [52].

CONCLUSION

In conclusion, our results demonstrate that *S. altissima* is tolerant to salinity (up to 10000 ppm), which is indicated by the fact that there were no reductions of root and shoot growth, biomass production, chlorophyll a and b, carotenoids and indoles content between control and salt-treated plants up to 10000 ppm. Thus, our results suggested that *S. altissima* could be successfully cultivated in saline soils without any loss in growth or productivity.

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