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Growth regulators – a biological means for stimulating photosynthetic activity, nitrogen fixation and the increase of the biological value of protein in forage peas (*Pisum arvense L.*)

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Abstract. *The aim of the study was to determine the effect of foliar application of growth regulators on photosynthetic activity, nitrogen fixation and the biological value of protein in two cultivars of winter forage peas – Mir and Vesela. For achieving the aim set, a three-year field trial was carried out in the Training and Experimental Fields of the Agricultural University of Plovdiv, on a plot untreated with commercial fertilizers. The following growth regulators were used: RENI, RENI D, Bormax, Manganese Chelate and Molybdenite, applied at the phenological stage of pea plant budding. The effect of those products on the studied characteristics was compared to the untreated control. The reported three-year results show that foliar applied growth regulators had a positive effect on photosynthetic and nitrogenase activity, as well as on the characteristics of the nitrogen fixation apparatus (total number of nodules, number of active nodules and weight of the nodules) and on the biological value of the obtained protein in both cultivars of winter forage peas.*

Keywords: growth regulators, organic farming, protein crops, symbiotic nitrogen fixation

Introduction

Protein crops occupy an important place in the structure of agricultural production. Their inclusion in crop rotation schemes as predecessors of other crops greatly reduces nitrogen fertilization, which is especially important in the introduction of organic farming and maintaining the soil free of contaminants.

Biological nitrogen fixation from the air under natural conditions is the major way to include atmospheric nitrogen in the biosphere. It is carried out by organisms (nitrogen fixers) that contain the enzyme nitrogenase, which catalyzes the conversion of molecular atmospheric nitrogen (N_2) into an ammonium ion (NH_4^+). That process consumes energy which is supplied by adenosine triphosphate (ATP) molecules. The nitrogenase enzyme complex is composed of two protein molecules, one of which is combined with an iron-containing and the other with an iron-molybdenum cofactor. The properties of the enzyme complex are almost the same in the different nitrogen-fixing microorganisms and the two proteins that are involved in nitrogenase usually form an active enzyme, even when they come from two different microbial genera. That is an opportunity to regulate nitrogen fixation in different nitrogen-fixing plants with one agent. It is assumed that magnesium ions play a role in the conformational protection of two-component nitrogenase (Tsvetkova and Georgiev, 2004).

Some of the bacteria that contain nitrogenase live freely in soil. Those which need to have another organism as a partner, usually a higher plant, in order to carry out nitrogen fixation, are of particular interest. That type of nitrogen fixation is known to be symbiotic when the two organisms are in close contact with each other during vegetation and associative if the contact is not complete. In that case the organisms grow close to each other in the rhizosphere of the higher plant roots. Legume-rhizobia symbiosis is the infection of the legumes with bacteria of the genus *Rhizobium*. Root nodule bacteria living in soil, penetrate through the root hairs into the cells of the growing root and begin to multiply. The root cells of the host plant begin to divide intensively, forming swellings filled with nodule bacteria. Symbiotic nitrogen fixation begins with a process of colonization of the higher plant roots by the bacterium (microsymbiont), then the bacterium attaches to the roots and after a process of penetration, which can be described as endocytosis, a specific root formation is observed, called ‘a symbiotic nodule’. That process of nodule formation in the different types of symbiosis takes different time, but generally lasts 20-30 days, during which time the symbiont plant is developing (Tsvetkova and Georgiev, 2004).

Bacteria receive all the necessary nutrients from the host plant, mainly carbohydrates, which are necessary not only for the growth and reproduction of bacteria, but also as a source of energy for the nitrogen fixation from the air. Nitrogenase activity and nitrogen fixation are highly dependent on photosynthesis and those two processes are directly related. Nitrogen fixation

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is most active in the flowering stage and after it, when the nitrogen needs of the developing seeds and fruits increase (Kimenov, 1994). Plants consume 10.3 mg of carbohydrates for every milligram of fixed nitrogen.

RENI is a product that regulates nitrogen metabolism in plants and finds usage in crop production. Its action is expressed in increasing the activity of the major enzyme systems associated with nitrogen metabolism. Its application at certain rates of the components and the combinations between them reduces the content of nitrates in vegetables and other crops (Popov, 1999), improves the antioxidant properties of grapes and its quality (Masheva and Popov, 2005; Roychev et al., 2005), increases symbiotic nitrogen fixation in legumes (Popov and Dzimotudis, 2007). Directly influencing the activity of various enzyme systems related to nitrogen metabolism (Popov and Georgieva, 2009), RENI products affect nitrate reduction and nitrogen fixation and indirectly they have a positive effect on a number of physiological processes such as photosynthesis, water exchange, etc. (Popov, 1999; Popov et al., 2010a,b).

Molybdenum is involved in many metabolic processes in higher plants. Its main function is to regulate the redox reactions in some steps of the metabolism of nitrogen, phosphorus and iron. In addition, it is involved, although indirectly, in the biosynthesis of chlorophyll, carbohydrates and ascorbic acid (Gorbanov et al., 2005). The introduction of manganese in the form of $MnSO_4$ is also an effective measure of the pea cultivation technology. Its application increases grain yields by an average of 250 to 300 kg/ha (Gómez et al., 2006). As an element having variable valence, Mn actively participates in the regulation of redox reactions in the processes of respiration, photosynthesis, nitrogen and carbohydrate metabolism, etc. What is more, it participates in the active center of hydroxyl reductase and the so-called assimilation enzyme (Gorbanov et al., 2005).

Boron plays a key role in the cell membrane structure, seed yield, root elongation and sugar metabolism in the plant, while Molybdenum stimulates mainly nitrogen fixation in legumes and the reduction of NO_3^-, associated with the higher protein content (Gupta et al., 2011). According to Reguera et al. (2010), boron is a particularly important element for nitrogen fixation and bean-rhizobial symbiosis. Boron deficiency reduces the stability of macromolecules in the nodules, which are responsible for the infection with nodule bacteria and for regulating the oxygen concentration. Boron can be attributed to the group of trace elements, the need for which is well established. However, very little is known about its role and the mechanism of its participation in plant cell metabolism.

Increasing the efficiency of symbiotic nitrogen fixation is a good approach to obtain higher yields and better grain quality in legumes. A number of authors (Streeter, 1988; Layzell et al., 1990; Waterer and Vessey, 1993; Kaiser et al., 1997) believe that legumes can fully meet their nitrogen needs from the absorbed atmospheric nitrogen. At the same time, favorable factors enhancing symbiotic nitrogen fixation are also required to produce high yields. Such factors could be some synthetic substances with regulatory functions, stimulators or inhibitors of certain metabolic units, repressors or depressors of protein biosynthesis, including cofactors of enzymes from nitrogen metabolism. These arguments motivated the conduct of the present study, the purpose of which is to establish the effect of foliar growth regulators on photosynthetic activity, nitrogen fixation and biological value of protein in two varieties of winter pea cultivars - Mir and Vesela.

Material and methods

Object of study

To achieve the aim of the present study, a field trial was carried out to establish the effect of RENI, applied separately and in combination with boron, as well as the commercial products Bormax, Manganese chelate and Molybdenite on photosynthetic and nitrogenase activity and on the biological value of protein in winter pea cultivars Mir and Vesela. The experiment was based on the split-plot design method in four replications and the size of the experimental plot was 10 m^2. During the budding stage the two cultivars were treated with the following concentrations: RENI – 0.5%; RENI D – 0.5%; Manganese chelate – 0.4%; Molybdenite – 0.2%; Bormax – 0.4%.

Plant analyses

Photosynthetic activity was determined using a portable measuring system LCA-4, England - FAR (µmol m^{-2} s^{-1}). The measurements were performed one week after the foliar treatment of the pea plants with growth regulators.

Total and essential amino acids (%) were determined in the grain of peas by a Knauer type aminoanalyzer after hydrolysis of the plant material with 6n HCl.

Essential amino acid index (EAAI) was calculated by the formula of Oser (1951) and by the logarithm of Prof. D. Pavlov (Khanam et al., 2013), reflecting the ratio of essential amino acids from essential amino acids of egg white.

Nitrogenase activity was assessed by the method of Hardy et al. (1968), modified by Popov et al. (1985). Whole roots with the nodules were analyzed.

The tested sample was placed in a glass syringe with a capacity of 50 cm^3 fitted at the outlet with a short silicone hose. In another syringe, a mixture of acetylene and air was prepared. The two syringes were connected, achieving a 10% concentration of acetylene. The broad substrate specificity of nitrogenase was used, which in addition to nitrogen, is also able to reduce acetylene to ethylene. In that case, the affinity for acetylene was higher than the affinity for nitrogen. The presence of nitrogen as an air component has practically no effect on the formation of ethylene.

Incubation of the samples was performed at a room temperature for 10 minutes. After incubation, part of the gas mixture was transferred to a syringe, after which the content of the formed ethylene was analyzed on a gas chromatograph (Perkin Elmer).

A flame ionization detector with a nitrogen gas carrier was used. The gas mixture was partitioned over silica gel adsorbent (12-foot steel column 1/8 inch in diameter). The results were recalculated based on a standard curve derived from pure ethylene. All the analyses were performed in 3 replications of the variants.

Forage pea cultivars used

Winter forage peas, Mir cultivar – developed at the Institute of Plant Genetic Resources in Sadovo (Allerr Fruheste x Sofia 135 x No. 30). It is characterized by extremely early maturity, vigorous growth and development in the spring. It overwinters successfully throughout the territory of Bulgaria. It has been registered for growing throughout the country since 1984.

Winter forage peas, Vesela – 23 E cultivar – established at the Institute of Plant Genetic Resources in Sadovo (Knezha x Frimas x Final), recognized by the State Variety Commission in 1993. Medium-early cultivar, grown for dry forage grain.

Products used

RENI products are combinations of molybdenum, manganese, magnesium ions in different concentrations and ratios, which are additionally and purposefully combined with agents with a biochemical and physiological action, such as trace elements, synthetic regulators of cytokinin type, basic metabolites, etc.

The effect of the products is expressed in increasing the activity of the major enzyme systems associated with nitrogen metabolism: nitrogenase, nitrate reductase, glutamine synthetase, asparagine synthetase, etc. They are suitable for foliar treatment and have proven their efficiency in reducing the content of nitrates in vegetables and other crops, increasing the antioxidant properties of grapes, its quality and yield, increasing the yield and quality in various legumes. Two of the RENI products were used in the present study, referred to as RENI – a combination of molybdenum, manganese and magnesium ions, and RENI-D – the main composition of RENI with B (boron) added.

Manganese chelate – foliar fertilizer for fertigation, hydroponics and foliar application in manganese deficiency. It is applied in a concentration of 0.2-0.4% solution. The use can be combined with foliar nutrition with an aqueous solution of urea. In the present trial Mn was in a chelated form (EDTA), which is more readily available to plants.

Molybdenite – a foliar fertilizer for fertigation, hydroponics and foliar application in crops with high molybdenum requirements: potatoes, cabbage, broccoli, beans, peas, tomatoes. Leaf application is in a concentration of 0.1-0.2%.

Bormax – a foliar fertilizer for all crops with high requirements for boron – maize, beets, fruits, potatoes, legumes, vegetables and flowers. Foliar application is in a concentration of 0.3-0.4% and the application rate – 1 l/ha.

Statistical analysis

Statistical analysis was done by ANOVA at 5% probability.

Results and discussion

Higher values of photosynthesis were reported after treatment with regulators applied in the budding stage in all the studied variants in both cultivars compared to the control, except for the variants with Mn chelate and Bormax in Mir cultivar (Figure 1).

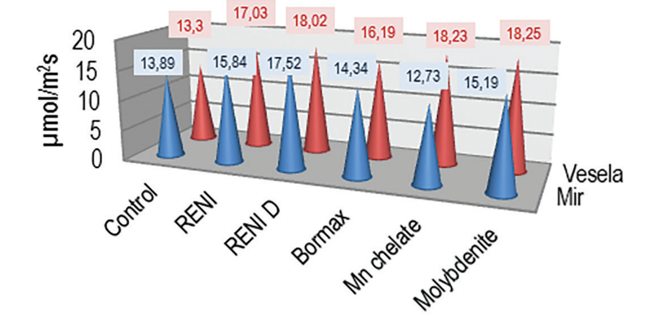

Figure 1. Photosynthetic activity in forage peas, cvs. Mir and Vesela, on average for the three years of study, µmol/m^{2}s

Mir cultivar responded with the highest values of photosynthesis after treatment with RENI D – 17.52 µmol/m^{2}s (26.1%), followed by the variant treated with RENI – 15.84 µmol/m^{2}s (14.0%) versus 13.89 µmol/m^{2}s in the control variant.

Vesela responded with the most active photosynthetic activity after treatment with Molybdenite and Mn chelate – 18.25 (37.2%) and 18.23 (37.1%) µmol/m^{2}s, respectively, followed with an insignificant difference by the variants treated with RENI D and RENI – 18.02 (35.5%) and 17.03 (28.0%) µmol/m^{2}s.

Table 1 presents the values of the elements characterizing the symbionts: root weight with nodules (g), total number of nodules, number of active nodules and weight of nodules (g). The summarized analysis of the average results from the three years of the study clearly shows that Mir cultivar has a better developed nitrogen fixation apparatus. It forms a larger number and heavier nodules, compared to Vesela. At the same time, the applied growth regulators affected to a much lesser degree the components of the nitrogen binder in Mir cultivar compared to Vesela.

The weight of the root with the nodules in Mir cultivar was the highest in the variants treated with RENI – 1.90 g and Molybdenite – 1.86 g, exceeding the control by 8.6% and 6.3%, respectively. An increase was also reported in the weight of the nodules in the variant treated with Molybdenite (0.295 g) – 7.7% higher than the control. The other characteristics (total number of nodules and number of active nodules) did not change significantly after foliar application of that growth regulator. Treatment with RENI D and Bormax led to the formation of the largest number of active (pink) nodules – 31.9 and 31.5, which is 10.4% and 9.1% more than the control. Those data confirm the thesis that boron is a key element that actively influences the structure of the nitrogen fixation apparatus, and hence the activity of nitrogen fixation in peas (Carpena et al., 1999; Zehirov and Georgiev, 2006; Stancheva et al., 2007).

Molybdenum, which is the major component of RENI, is an element that builds the nitrogenase complex and has a direct effect on nitrogen fixation, affecting also the uptake of other nutrients – zinc, cobalt, copper, nickel (Atkins et al., 1984) and phosphorus (Huber et al., 1992). It enters the plants as an anion (MoO_4^{2-}) and is involved in the active centre of nitrogenase. In addition, it is an activator of transamination reactions that produce new amino acids.

Table 1. Characteristics of the nitrogen fixation apparatus, on average for the three years of study

Variants	Elements							
	Weight of roots with nodules		Total number of nodules		Number of active nodules		Weight of nodules	
	g	%	Number	%	Number	%	g	%
Mir cultivar								
Control	1.75	100.0	33.01	100.0	28.90	100.0	0.260	100.0
RENI	1.90	108.6	35.13	106.4	30.90	106.9	0.272	104.6
RENI D	1.47	84.0	34.23	103.7	31.90	110.4	0.204	78.5
Bormax	1.66	94.6	35.50	107.5	31.53	109.1	0.262	100.8
Mn chelate	1.76	100.6	35.40	107.2	28.83	99.8	0.278	106.9
Molybdenite	1.86	106.3	32.07	97.2	27.20	94,1	0.295	113.5
LSD 5%	*0.32*	-	*7.23*	-	*6.84*	-	*0.112*	-
Vesela cultivar								
Control	1.41	100.0	26.13	100.0	17.53	100.0	0.217	100.0
RENI	1.46	104.3	28.00	107.2	20.10	114.7	0.276	127.2
RENI D	1.47	104.3	29.27	112.0	24.87	141.9	0.207	95.4
Bormax	1.38	97.9	29.63	113.4	24.63	140.5	0.240	110.6
Mn chelate	1.20	85.1	30.07	115.1	21.90	124.9	0.227	104.6
Molybdenite	1.42	100.7	29.90	114.4	24.50	139.7	0.222	102.3
LSD 5%	*0.48*	-	*10.71*	-	*10.11*	-	*0.118*	-

*No significance at LSD<5%

The results in Figures 2 and 3 show an obvious change in the activity of the nitrogenase enzyme complex in both cultivars after treatment with the studied growth regulators. The results of the chromatographic analysis of the different variants in Mir cultivar were much higher than those of the control (Figure 2). The largest amount of ethylene was established (which shows higher nitrogenase activity in the selected method): in the first place after treatment with Bormax, second – with Mn chelate and third – with RENI D. The results obtained corresponded to the larger number and the higher weight of the active (pink) nodules reported in those variants of the study (Table 1). Vesela cultivar responded with an increase in the nitrogenase activity in all the variants, the largest increase being established in the pea plants treated with Bormax, RENI D and Mn chelate (Figure 3).

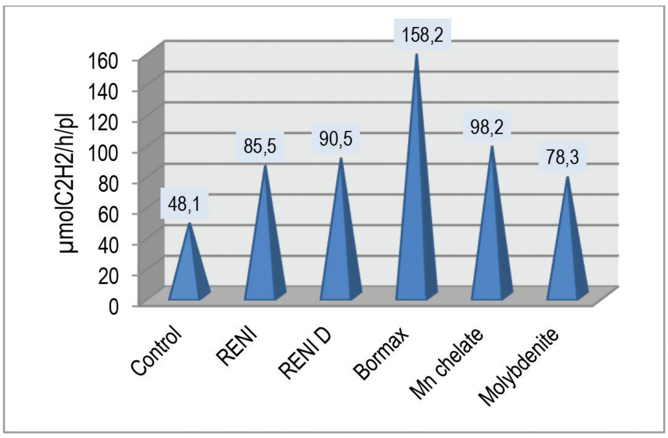

Figure 2. Changes in nitrogenase activity (µmol C_2H_2/h/pl) in forage pea roots, Mir cultivar

The higher activity of nitrogenase was due to the larger number of active (pink) nodules, with a higher weight, found on pea plant roots in those variants (Table 1).

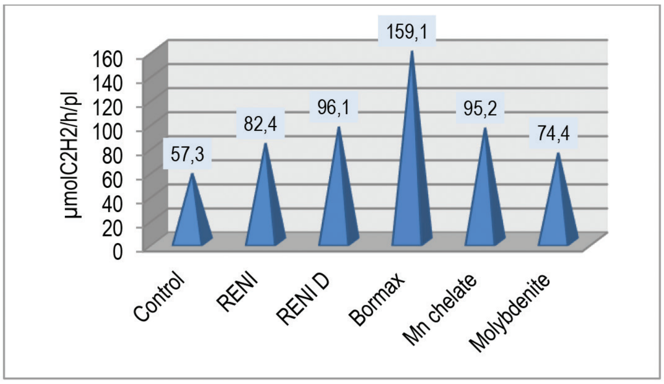

Figure 3. Changes in nitrogenase activity (µmol C_2H_2/h/pl) in forage pea roots, Vesela cultivar

The data from the gas chromatographic analysis of both pea cultivars obviously show the stimulating effect of boron on the activity of the enzyme nitrogenase. This is in support of the thesis of Reguera et al. (2010) that boron is a particularly important element for nitrogen fixation and bean-rhizobial symbiosis. The exact mechanism of its stimulating effect will be studied in future research experiments.

The analysis of the data presented in Table 2 shows that in Mir cultivar, the methionine content in the grain protein increased in the variants treated with Bormax, RENI D and RENI. Methionine is a key amino acid and its increase in pea protein is a valuable criterion for determining the biological value of feed. Referring to the essential amino acids, the isoleucine content in the grain protein also changed significantly after treatment with Molybdenite, Mn chelate and RENI D – by 9.3, 8.1 and 7.2% on average. An increase in the EAAI index was reported in the variants treated with Bormax (0.67) and RENI D (0.65), which reflects the increase in the biological value of the protein due to the application of those two products.

Table. 2. Essential amino acid content in the grain of winter pea cultivars Mir and Vesela (% to protein) and Essential amino acid index (EAAI) on average for the period 2008-2009

Amino acids	Treatment					
	Control	RENI	RENI D	Bormax	Mn chelate	Molybdenite
				Mir cultivar		
Lysine	7.91	7.71	7.82	7.89	7.62	7.94
Phenylalanine	5.76	5.76	5.72	5.75	5.08	5.34
Leucine	7.52	7.75	7.63	6.92	7.62	7.53
Isoleucine	4.30	4.29	4.61	4.25	4.65	4.70
Methionine	0.182	0.217	0.334	0.514	0.195	0.163
Valine	5.36	5.43	5.27	5.45	5.51	5.35
Threonine	3.38	3.27	3.40	3.28	3.50	3.39
Sum total	34.41	34.43	34.78	34.05	34.18	34.41
EAAI	0.60	0.61	**0.65**	**0.67**	0.61	0.60
				Vesela cultivar		
Lysine	8.12	7.78	7.75	7.80	8.04	7.78
Phenylalanine	5.05	5.52	5.36	5.85	5.11	4.84
Leucine	7.90	7.38	7.28	7.24	7.75	7.54
Isoleucine	4.73	4.57	4.64	4.59	4.68	4.46
Methionine	0.295	0.419	0.431	0.281	0.216	0.238
Valine	5.47	5.33	5.34	5.36	5.45	5.46
Threonine	3.43	3.29	3.38	3.38	3.35	3.42
Sum total	35.00	34.29	34.18	37.50	34.60	33.74
EAAI	0.64	**0.66**	**0.67**	**0.68**	0.63	0.63

In Vesela cultivar, an increase in the values was reported in only two of the studied essential proteinogenic amino acids in the grain protein – phenylalanine and methionine, obtained in the variants treated with Bormax, RENI D and RENI.

The phenylalanine content increased in the protein of the pea plants treated with Bormax, RENI and RENI D by 15.8, 9.3 and 6.1%, respectively. The content of the sulfur-containing amino acid methionine also increased significantly after the application of RENI D and RENI – by 46.1 and 42.0% compared to the control. Consequently, the application of Bormax, RENI D and RENI increased the biological value of the protein in the forage pea of Vesela cultivar, thanks to the increase in the methionine content.

EAAI index values increased in the variants treated with Bormax (0.68), RENI D (0.67) and RENI (0.66), which confirmed the increase in the biological value of the protein in those three variants.

What is more, the studied growth regulators, introduced at the stage of pea budding, stimulated the nitrogen fixation process in a biological way, which is of particular value in terms of the accumulation of nitrogen, which is deficient in living matter, and, in its turn would be available in larger amounts in soil to be used by the successor crop after forage peas. Larger amounts of organic and mineral nitrogen in soil reduce the amounts of the applied mineral nitrogen fertilizers, and hence, the risk of air, soil and water pollution as a result of washing or evaporation.

Conclusion

From the obtained results the following conclusions can be made: (i) The regulators applied at the budding stage of the pea crop increased the photosynthetic activity, Mir cultivar responding with the highest values after treatment with RENI D – 17.52 μmol/m^{2}s (26.1%), followed by the variant treated with RENI – 15.84 μmol/m^{2}s (14.0%) versus 13.89 μmol/m^{2}s in the control. In Vesela cultivar the highest photosynthetic activity was established after treatment with Molybdenite and Mn chelate – 18.25 (37.2%) and 18.23 (37.1%) μmol/m^{2}s, respectively, followed, with an insignificant difference, by the variants treated with RENI D and RENI – 18.02 (35.5%) and 17.03 (28.0%) μmol/m^{2}s. (ii) The weight of the roots with the nodules in Mir cultivar was the highest in the variants with application of RENI – 1.90 g and Molybdenite – 1.86 g, exceeding the control by 8.6% and 6.3%, respectively. Treatment with RENI D and Bormax resulted in the formation of the largest number of active (pink) nodules – 31.9 and 31.5, which is 10.4% and 9.1% more than the control. (iii) In Mir cultivar the highest nitrogenase activity was reported in the pea plants treated with Bormax, Mn chelate and RENI D. The results obtained corresponded to the larger number and the higher weight of the active (pink) nodules, reported in those variants of the study. Vesela cultivar responded with an increased nitrogenase activity in all the tested variants and again the largest increase was established in the plants treated with Bormax, RENI D and Mn chelate. (iv) The application of Bormax and RENI D products led to an increase in the content of the limiting sulfur-containing amino acid methionine in the grain of the winter peas of Mir cultivar and to an increase in the values of the EAAI index, which is related to an increase in its biological value. Treatment with Bormax, RENI D and RENI resulted in an increase in the amount of the essential amino acids phenylalanine and methionine, increasing the biological value (EAAI index) of the grain protein in the winter forage peas of Vesela cultivar.

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