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ONTOGENIC CHANGES IN LEAF PIGMENTS, TOTAL SOLUBLE PROTEIN AND RUBISCO IN TWO BARLEY VARIETIES IN RELATION TO YIELD

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Summary. Two barley (*Hordeum vulgare* L.) forage varieties – *Hemus* and *Karnobat*, with high and low Rubisco content in the fully expanded first leaves (Metodiev and Demirevska-Kepova, 1992), were compared in pot vegetation experiments in respect of yield. Changes in the content of leaf pigments, the levels of total soluble and Rubisco immunoreactive protein were followed during six phenophases: 3rd leaf, tillering, stem extension, heading, anthesis and milk ripeness. The results show maintenance of high level of photosynthetic components in the phases critical for grain filling regardless of the differences observed in the primary leaves. Thus, the use of Rubisco as an early selection criterion for improved yield is dubious. The question, whether Rubisco content in leaves during the grain-filling period might be a useful marker for productivity, remains open and needs further investigations.

Key words: *Hordeum vulgare* L., leaf pigments, phenology, Rubisco, varieties, yield

Abbreviations: cv – cultivar; DW – dry weight; EDTA - ethylene diamine tetraacetic acid; ELISA – enzyme-linked immunosorbent assay; FW – fresh weight; PMSF – phenyl-methyl sulphonyl fluoride; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39; Rubisco) is the first and key enzyme in the Calvin-Benson cycle of photosynthetic fixation of CO_2. The

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rate of photosynthesis and biomass accumulation depend largely on the quantity and activity of Rubisco (Lorimer, 1981). Under natural environmental conditions the level of this protein could be a limiting factor in photosynthesis throughout the life span of the leaf (Makino et al., 1983). In addition to its key enzymatic function, Rubisco is a remarkably abundant protein (up to 65% of total leaf protein), storing considerable amount of nitrogen (Ellis, 1979). There is some evidence that a part of Rubisco is not available for carboxylation and plays the role of storage protein (Eichelmann and Laisk, 1999). Rapid selective proteolysis of this enzyme was observed at the onset of natural and experimentally induced senescence, providing aminoacids for redistribution to reproductive structures (Crafts-Brandner et al., 1990). The dual role of Rubisco as a key photosynthetic and leaf storage protein predetermines its importance for plant productivity. Correlation has been observed between Rubisco activity and high yield in several species (Frey and Moss, 1976; Murthy and Singh, 1979; Martinez-Barajas et al., 1992). The activity of Rubisco has been more or less correlated with its amount (Friedrich and Huffaker, 1980). Genetic variation in Rubisco content in cereal varieties has been reported (Tingey and Andersson, 1986; Metodiev and Demirevska-Kepova, 1992). It has been suggested that Rubisco could be used as a selection criterion for high yield (Frey and Moss, 1976; Murthy and Singh, 1979; Osaki et al., 1993).

Data about the role of photosynthesis in plant productivity are controversial. Duration and rate in leaf photosynthesis were in a close relationship with crop yield for soybean, wheat, barley, sorghum, maize and tobacco (Zelitch, 1982; Sarquis et al., 1998). In many cases, chlorophyll quantity was maximal in the leaves serving as a major source of photoassimilates for developing sinks during the grain-filling period (Herzog, 1982; Zhdanova and Karyagina, 1997). It was established that the amounts of Rubisco and chlorophyll in the leaves of high-yield cultivars of rice, wheat, maize, soybean and potato at successive growth stages were larger than in the standard-yield cultivars (Osaki et al., 1993). The same tendency could be expected for barley cultivars. On the other hand, breeding for high rates of leaf net photosynthetic CO_2 exchange and its components has not yet brought any success in improving crop yield (Gifford and Jenkins, 1982; Richards, 2000).

In a comparative study on Rubisco quantity in the fully expanded first leaves of ten Bulgarian barley cultivars it was established, that the amount of Rubisco varied from 15 to 50 per cent of the total soluble protein and from 2.5 to 10 $mg.g^{-1}$ FW. Differences in total soluble protein were not significant (Metodiev and Demirevska-Kepova, 1992). *Hemus* has been recognized as a standard barley forage variety for several years (Stephanov and Peev eds, 1986). To evaluate the usefulness of Rubisco as an early selection criterion, it was necessary to follow the time course of its accumulation in ontogenesis.

The aim of the present investigation was to compare the developmental changes in leaf pigments, leaf soluble protein and Rubisco quantities in two barley cultivars

– *Hemus* with high Rubisco content and *Karnobat* with low Rubisco content in the first leaves, in relation to some yield characteristics.

Material and Methods

Plant material. Barley (*Hordeum vulgare* L.) cultivars were obtained from the Barley Institute of Karnobat. Vegetation experiments were carried out in the greenhouse of the Institute of Plant Physiology during 1996-1997 and 1999-2000. Seeds were sowed in pots with 5 kg of dry soil, 10 pots per variety, 40 seeds per pot, in the third decade of October. The soil was from the experimental field of the village Gorny Lozen near Sofia and contained 4.06 mg N, 45 mg P_2O_5 and 18 mg K_2O per kg absolutely dry weight. Additional fertilization with N, P and K was applied in order to obtain a planned yield of 40 g grain per pot (from 5 main plants per pot). N was imported four times over the whole vegetation period – at sowing, at the stage of the third leaf, at the end of tillering and at the stage of stem extension. Tap water was added periodically to maintain about 60% of the maximal field water capacity throughout plant vegetation. The plants were grown under ambient temperature and solar radiation and were regularly weeded. During the day pots were transferred outdoors. In winter the pots were periodically covered with snow. At the phase of the 3rd leaf the number of plants per pot was reduced to 20 and at the tillering phase – to 5 in two consecutive steps. Leaf material for analyses was sampled during the following stages of development: 3rd leaf, tillering, stem extension (average leaf sample), heading, anthesis and seed ripening (flag leaf sample). Under the experimental conditions plants developed normally. Grain yield parameters were determined at the stage of full ripening, when the plant were with completely yellow leaves and well developed ears and grains.

Leaf extracts. Two grams of leaf material, stored under liquid nitrogen until extraction, were homogenized with 8 ml of ice-cold 100 mM Tris-HCl, pH 8, containing 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM EDTA, 2 mM PMSF, 12.5% glycerol (v/v), 20 mM β-mercaptoethanol, 120 mg Polyclar. After centrifugation at 12 000×g for 30 min the supernatant was used for estimation of total soluble protein and Rubisco content.

Determination of protein quantity was done spectrophotometrically according to Bradford (1976).

Determination of leaf pigment quantity was according to Arnon (1949) and calculated using the formula of McKinney (1941).

Quantitative determination of Rubisco in leaf extracts from barley was carried out immunochemically by sandwich ELISA using rabbit polyclonal antibodies specific against barley Rubisco and goat anti-rabbit IgG-peroxidase conjugate (Metodiev and Demirevska-Kepova, 1992).

Results

Climatic conditions during the experiments and general observations on plants. During 1996–1997 the winter was severely cold (day temperature drop till -15 °C in December and January led to frostbite of separate leaves), whereas in 1999–2000 the autumn and winter were mild. Night temperatures never dropped below 0 °C in December and below -10 °C in January. The unusually high temperatures in the late spring and the beginning of the summer of 2000 exerted certain unfavourable influence and accelerated the vegetation cycle about 2–3 weeks. With these exceptions, as a whole, the climatic conditions were favourable for the development of the barley plants. During both years the plants of cv. *Hemus* developed slightly faster and accumulated more biomass in the overground organs during the tillering phase in comparison with cv. *Karnobat* (Table 1).

Table 1. Biomass accumulation in the overground plant organs during the vegetative growth stage of barley varieties

Phase	*Hemus*		*Karnobat*	
	FW per plant (g)	DW per g FW	FW per plant (g)	DW per g FW
3rd leaf	0.41±0.054	0.1089	0.415±0.049	0.1041
tillering	1.90±0.330	0.1529	1.210±0.210	0.1467
stem extension	23.94±6.215	0.2030	23.020±7.342	0.2150

During the reproductive stage, DW per g FW in flag leaves was more or less constant and without significant differences between varieties (0.2089±0.0147 for *Hemus* and 0.2110±0.0106 for *Karnobat*). The cold-resistance and recovery from frostbite was better in cv. *Hemus*. No differences between the two varieties were observed in the time of entering on and the duration of the phenophases. Differences in leaf areas were more markedly expressed among plants than between cultivars.

Developmental changes in leaf pigment content. The amounts of chlorophyll *a*, chlorophyll *b* and carotenoids for the six phenophases of 99/00-vegetation period are presented in Fig. 1. Average leaf samples were collected during the vegetation growth stages (I – 3rd leaf, II – tillering, III – stem extension), whereas during the reproductive stages (IV – heading, V – anthesis, VI – milky ripeness) analyses were performed with flag leaf samples. The pigment content of both cvs followed characteristic time-course changes with no significant differences between varieties. The same dynamic changes in chlorophylls were observed in 96/97 but the maximal values were lower. Generally, chlorophyll *a* quantity in the flag leaves increased remarkably during the reproductive stage and remained at a high level till the milky ripeness phase. Changes in chlorophyll *b* content were smaller. Carotenoids tended to accumulate after the tillering

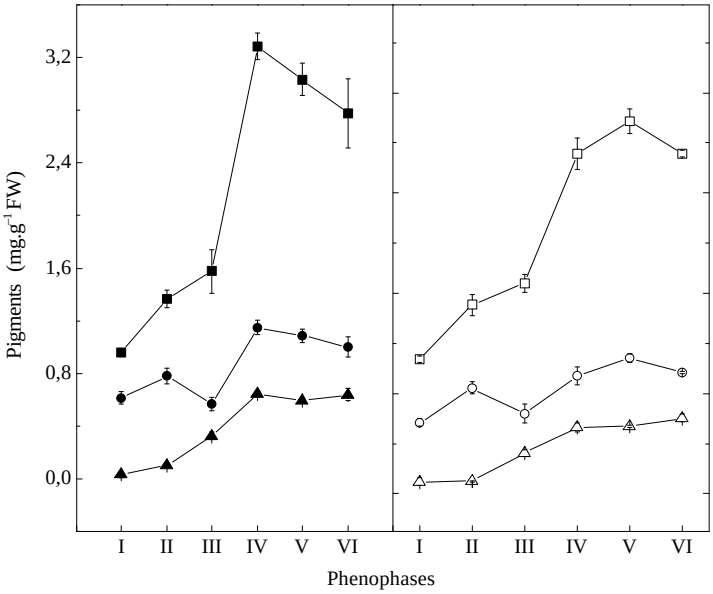

Fig. 1. Content of leaf pigments in the leaves of barley cultivars *Hemus* (solid symbols) and *Karnobat* (open symbols). Squares - chlorophyll *a*, circles - chlorophyll *b* and up triangles - carotenoids. Vertical bars - standard deviations. On the abscissa – phenophases: I – 3rd leaf; II – tillering; III – stem extension; IV – heading; V – anthesis and VI – milk ripeness.

phase in 99/00. Differences in carotenoids were more expressed comparing the two vegetation years than between the cultivars. In 96/97 carotenoids accumulated as early as the 3rd leaf stage with no significant difference between varieties (data not shown). A possible explanation may be the climatic conditions in 96/97 with severe winter.

Total soluble protein and Rubisco content in the leaves in different phenophases. Data on total soluble leaf protein for the vegetation period 99/00 are presented in Fig. 2. A similar increase in leaf protein content with maximal accumulation at anthesis and a slight reduction at the milky ripeness phase were observed during the 96/97-vegetation period. However, significant differences between the cvs were obtained only at anthesis in 99/00, whereas in 96/97 (data not shown) there was not such a difference. The increase in the soluble protein during phases III–VI is most probably due to greater dry biomass of the leaves (Table 1 and the text bellow). The quantity of Rubisco immunoreactive protein on a fresh weight basis (Fig. 3) was higher in cv. *Hemus* at tillering and stem extension phases. At heading and anthesis the differences in Rubisco amount between the cvs became insignificant. After anthesis, Rubisco quantity in cv. *Hemus* decreased to a greater extent in comparison with cv. *Karnobat*. Hence, the differences in Rubisco quantity between cvs. *Hemus* and *Karnobat*, observed in 10-day-old barley seedlings, tended to diminish during ontogenesis. In

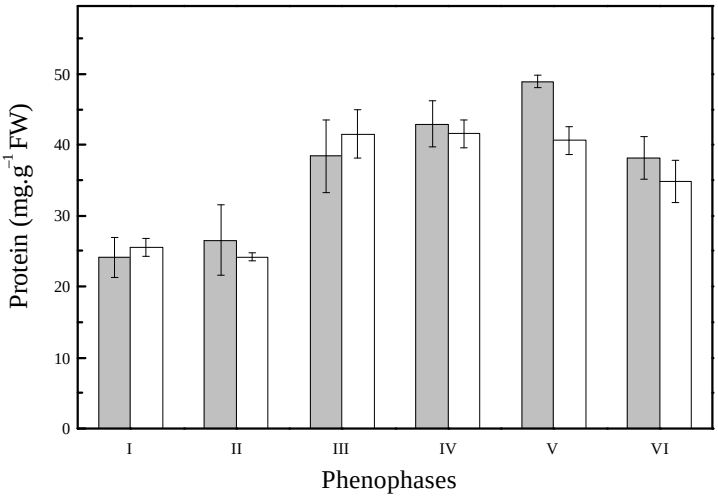

Fig. 2. Total soluble protein content on fresh weight basis in the leaves of the varieties *Hemus* (dark columns) and *Karnobat* (light columns). Vertical bars represent standard deviations. On the abscissa – phenophases: I – 3rd leaf; II – tillering; III – stem extension; IV – heading; V – anthesis and VI – milk ripeness.

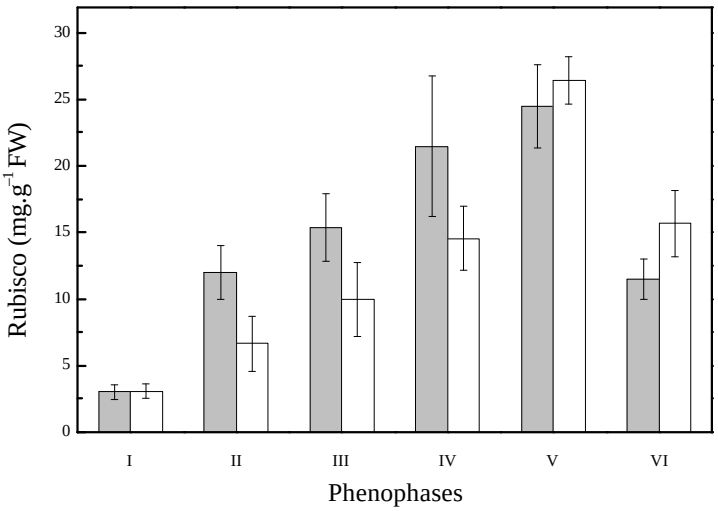

Fig. 3. Rubisco content on fresh weight basis in the leaves of the varieties *Hemus* (dark columns) and *Karnobat* (light columns). Vertical bars represent standard deviations. On the abscissa – phenophases: I – 3rd leaf; II – tillering; III – stem extension; IV – heading; V – anthesis and VI – milk ripeness.

addition, during the vegetative growth stage (for example, tillering) Rubisco was 27.5% from the total soluble protein in cv. *Karnobat* and 45% in cv. *Hemus*. At anthesis Rubisco was 65% of the total soluble protein in cv. *Karnobat* and 50% in cv. *Hemus*. In contrast to lower initial quantity of Rubisco, cv. *Karnobat* maintained high level of this enzyme during the reproductive stage.

Yield characteristics of the two cultivars. Pot experiments could be useful for preliminary characteristics of yield potential and comparative purposes. Spoor and Simmonds (1993) found good correlation between yields of the same varieties in pot and field trials for small-grain cereals like barley, wheat and oats. Some yield characteristics for the two cvs under investigation are compared in Table 2.

Table 2. Yield characteristics (average of 3 pots, 5 plants per pot, data from 2 years)

Cultivar	Number of productive spikelets per plant	Grain weight per pot (g)	Weight of 1000 grains (g)	hectoliter mass (kg)
Hemus	9 ±1.75	44.22±11.82	38.01±0.69	56.46±2.67
Karnobat	10.5±1.95	53.15± 5.01	37.07±0.47	58.7 ±2.91

The cvs *Hemus* and *Karnobat* did not differ substantially in productivity. Cv. *Hemus* showed a tendency to form slightly larger grain mass (probably due to a faster Rubisco degradation) but cv. *Karnobat* compensated with somewhat higher tillering capacity and total grain quantity. The lack of differences in yield between *Hemus* and *Karnobat* was in concert with the equally high Rubisco quantity at anthesis for both varieties.

Discussion

The process of photosynthesis is of prime importance for biomass production. The amounts of chlorophyll and Rubisco have often been considered as indices of light-harvesting capacity and Calvin-cycle capacity of leaves. In the present investigation we observed distinct differences in these indices comparing vegetative and reproductive stages in the life cycle of both barley varieties. There was a tendency of excess-investment in the photosynthetic apparatus and maintenance of high chlorophyll, carotenoid and Rubisco levels during the reproductive stage. Thus, the reliability of the photosynthetic apparatus was ensured, resulting in maximum possible productivity of photosynthesis under the given environmental conditions.

Generally, in natural conditions the normal chlorophyll content of leaves is adequate to absorb the available photon flux and chlorophyll contents are adjusted to the flux available. At low limiting irradiance leaf photosynthesis is saturated with respect to chlorophyll above 20 mg chlorophyll per cm^{-2} leaf area (Gifford and Jenkins, 1982).

In our experimental conditions the chlorophyll $a+b$ content was above this value even at the stage of third leaf (with lowest chlorophyll content) for both varieties. The photoprotective and stress protective function of carotenoids has been well-established (Eskling et al., 1997). In this study the carotenoids were maintained at high level throughout the reproductive phase. It is considered that the high pigment level is determined by the sink-source relations between grains as developing sinks and leaves as principal source of photoassimilates (Herzog, 1982; Zhdanova and Karyagina, 1997). The flag and the two penultimate leaves have been regarded as the most important source of assimilates supply to the ears in barley (Kisseleva and Nekrasova, 1993). The *Hemus* variety differed from *Karnobat* in the rate of Rubisco accumulation, but at the stages critical for grain filling both cultivars accumulated high levels of Rubisco. Two functions have been attributed to Rubisco as a key photosynthetic and nitrogen storage protein. The nitrogen storage function of Rubisco is often discussed but not yet elucidated (Makino et al., 2000). It seems that the rate of Rubisco degradation may be in relation to grain size. The *Hemus* cultivar formed larger grains compared with *Karnobat* both in our pot experiments and in field conditions (Stephanov and Peev, 1986).

Our results confirm the opinion that by anthesis the capacity of photosynthesis for the major grain crops is high and that photosynthesis is not limiting during grain filling period (Richards, 2000). This kind of excess investment and safety of photosynthesis may be of value in adverse environmental conditions (various stresses, especially drought, unfavourable temperature, salinity, etc) often observed in the field. In our experiments plants received adequate water and fertilizer supply in order to avoid any drought and nutritional stress. Differences in cold-resistance between the compared varieties were observed, whereas the initial difference in Rubisco levels was effaced in successive developmental stages. Thus, the use of Rubisco as an early selection criterion for improved yield is dubious. During the grain-filling period the flag and penultimate leaves in barley serve as a major source of photoassimilates (Kisseleva and Nekrasova, 1993). However, the question whether Rubisco content in these leaves might be an useful marker for productivity, remains open and needs further investigation.

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References

Arnon, D. I., 1949. Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris.* Plant Physiol., 24, 1–15.

Bradford, M. M., 1976. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248–254.

Crafts-Brandner, S. J., M. E. Salvucci, D. E. Egli, 1990. Changes in ribulosebisphosphate carboxylase oxygenase and ribulose 5-phosphate kinase abundances and photosynthetic capacity during leaf senescence. Photosynth. Res., 23, 223–230.

Eichelmann, H., A. Laisk, 1999. Ribulose-1,5-bisphosphate carboxylase/oxygenase content, assimilatory charge, and mesophyll conductance in leaves. Plant Physiol., 119, 179–189.

Ellis, R. J., 1979. The most abundant protein in the world. Trends Biochem. Sci., 4, 241–244.

Eskling, M., P-O. Arvidsson, H-E. Akerlund, 1997. The xantophyll cycle, its regulation and components. Physiol. Plant., 100, 806–816.

Frey, N. M., D. N. Moss, 1976. Variation in RuDPCase activity in barley. Crop Sci., 16, 209–213.

Friedrich, J. W., R. C. Huffaker, 1980. Photosynthesis, leaf resistance and ribulose 1,5-bisphosphate carboxylase degradation in senescing barley leaves. Plant Physiol., 65 (6), 1103–1107.

Gifford, R. M., C. L. D. Jenkins, 1982. Prospects of applying knowledge of photosynthesis toward improving crop production. In: Photosynthesis, Vol. II. Development, Carbon Metabolism and Plant Productivity, ed. Govindjee, Acad. Press Inc., pp. 420–457.

Herzog, H., 1982. Relation of source and sink during grain filling period in wheat and some aspects of its regulation. Physiol. Plant., 56 (2), 155–160.

Kisseleva, I. S., G. F. Nekrasova, 1993. Photosynthetic carboxylation in leaves and nonfoliar organs of barley. Russ. J. Plant Physiol., 40 (3), 324–328 (in Russ.).

Lorimer, G. H., 1981. The carboxylation and oxygenation of ribulose-1,5-bisphosphate: the primary event in photosynthesis and photorespiration. Ann. Rev. Plant Physiol., 32, 349–383.

Makino, A., T. Mae, K. Ohira, 1983. Photosynthesis and ribulose 1,5-bisphosphate carboxylase in rice leaves. Plant Physiol., 73 (4), 1002-1007.

Makino, A., H. Nakano, T. Mae, T. Shimada, N. Yamamoto, 2000. Photosynthesis, plant growth and nitrogen allocation in transgenic rice plants with decreased Rubisco under CO_2 enrichment. J. Exp. Bot., 51, 383–389.

Martinez-Barajas, E., V. C. Villanueva, J. Molina Galan, H. Loza Tavera, E. Sanches de Jimenez, 1992. Relation of Rubisco to maize grain-yield improvement – effect of water restriction. Crop Sci., 32 (3), 718–722.

Metodiev, M., K. Demirevska-Kepova, 1992. Rubisco quantitation in leaves of different barley varieties by enzyme-linked immunosorbent assay. J. Exp. Bot., 43, 247, 155–158.

Murthy, K. K., M. Singh, 1979. Photosynthesis, chlorophyll content and ribulose diphosphate carboxylase activity in relation to yield in wheat genotypes. J. Agric. Sci., 93 (1), 7–11.

Richards, R. A., 2000. Selectable traits to increase crop photosynthesis and yield of grain crops. J. Exp. Bot., 51, Special issue: Genetic Manipulation of Photosynthesis, 447–458.

Sarquis, J. I., H. Gonzalez, E. S. de Jimenes, J. R. Dunlap, 1998. Physiological traits associated with mass selection for improved yield in a maize population. Field Crops Res., 56 (3), 239–246.

Spoor, W., N. W. Simmonds, 1993. Pot trials as an adjunct to cereal breeding and evaluation of genetic resources. Fields Crop Res., 35 (3), 205–214.

Stephanov, T. G., H. V. Peev, eds., 1986. Barley in Bulgaria. Zemizdat, Sofia, (In Bulg.).

Tingey, S. V., W. R. Andersson, 1986. Variation in concentration of ribulose diphosphate carboxylase in leaves of barley, wheat, and hybrids of crested wheatgrasses. J. Exp. Bot., 37, 625–632.

Zelitch, J., 1982. The close relationship between net photosynthesis and crop yield. Bioscience, 32, 796-802.

Zhdanova, L. P., T. B. Karyagina, 1997. Chlorophyll content in sunflower leaves as related to seed development and plant senescence. Russ. J. Plant Physiol., 44 (2), 242–248 (In Russ.).