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The effect of presowing saturation with molybdatenum and presence of nitrate on the allantoin content in sprouted wheat grain

Abstract: It is generally known that when the grain is primed, saturated with a solution of biologically important elements and subsequently dried, the aleurone layer selectively permits ions of various metals. It is also known that in the case of a deficiency of molybdenum in the soil, a non-molybdenum inactive population of molecules of xanthine dehydrogenase and nitrate reductase is synthesized in plant cells. Presowing priming of wheat grain in molybdate solution of optimal concentration allows grain to germinate more actively. Upon germination of the wheat grain, in the process of embryo cells division, exogenous molybdenum is incorporated into the molecule of the non-molybdenum forms of the newly synthesized molybdoenzymes – nitrate reductase and xanthine dehydrogenase. This leads to the complete activation of the non-molybdenum forms of nitrate reductase and xanthine dehydrogenase in sprouted wheat grain. Since nitrate assimilation induces nitrate reductase synthesis, which uses reduced nicotinamide adenine dinucleotide as donor of electrons, nicotinamide adenine dinucleotide oxidized pool is formed in the cells, what is specifically required for the oxidation of xanthine to allantoin (via uric acid) by xanthine dehydrogenase. Our results show that prior to sowing priming of wheat grain of the "Baiterek" cultivar in molybdate solution and further growth in a nitrate containing medium abruptly increases the content of the antioxidant allantoin in germinated grain.

Key words: allantoin, xanthine dehydrogenase, nitrate reductase, priming, wheat, molybdenum.

Introduction

Allantoin is an important reserve and transport form of nitrogen in plants, that is to say allantoin is the source of the amino group for the biosynthesis of amino acids and is easily transported by plant conducting pathways [1; 2]. Numerous studies show that purine catabolism plays a role not only in the metabolism of nitrogen, but also affects plant resistance to adverse abiotic factors (soil salinity and drought) [3; 4]. Exogenous allantoin increased the viability and the growth rate of *Arabidopsis thaliana* sprouts grown in a medium containing sodium chloride (NaCl) [3]. A direct correlation was observed between the level of allantoin in seeds and the resistance of seedlings to low temperature and drought [4].

Molybdenum (Mo) is an essential element for higher plants and plays a vital role in many physiological and biochemical processes. Four of molybdoenzymes have been found in plants, namely nitrate reductase (NR),aldehyde oxidase (AO), xanthine dehydrogenase (XDH) and sulfite oxidase (SO) [5]. These molybdoenzymes participate in diverse metabolic processes, such as nitrate assimilation, phytohormone synthesis, purine catabolism and sulfite detoxification in plants [6]. Among them, AO has been shown to catalyse the final steps in the conversion of indole-3-acetaldehyde to indole-3-abscisic acid (IAA), and the oxidation of abscisic aldehyde to ABA [7]. Mutations in either AO apoprotein or enzymes involved in Mo-cofactor (Moco) biosynthesis and Moco activation (sulfuration) disrupt ABA synthesis [5; 8]. A low ABA level results in a wilty appearance of plants as a result of excessive transpiration, loss of stomatal control, altered seed dormancy and impaired defense responses to environmental stress [6; 7]

Stress activates AO as well as Moco-hydroxylase-sulfurylase (MHS) enzyme, which in turn activates AO and XDH, changing one of Mo's atoms of oxygen for sulfide [9; 10].

The two inorganic N-forms readily available for uptake by plants, NO_3^- and NH_4^+, switch on N-assimilation specifity pathways to synthesize fundamental

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cell components such as amino acids, nucleic acids, proteins and photosynthetic pigments [11].

The key and rate-limiting enzyme for NO_3^-assimilation is nitrate reductase (NR, EC 1.6.6.1). This enzyme catalyzes the first step in the NO_3^- reduction pathway to yield NO_2^-, which is then further reduced to NH_4^+. The latter ion is incorporated into organic N-compounds by the activity of the enzymes glutamine synthetase and glutamate synthase (the GS/GOGAT pathway). The resulting amino acids, glutamine and glutamate then serve as substrates to produce additional amino acids [12]. While NO_3^- transport and assimilation occur practically throughout the entire plant, NH_4^+ is taken up directly into the roots and the resulting assimilation products are transported to the growing plant parts [13; 14].

Generally, plants may transport N in the form of amides (glutamine and asparagine) and/or ureides (allantoin and allantoate). Ureides are more rich in N (4N:4C) in comparison to amides and amino acids and, therefore, their involvement in the transport of assimilated ammonia from the root to the shoot minimizes loss of carbon originating from photosynthesis [15].

Xanthine dehydrogenase (XDH, EC 1.2.1.37) is an important enzyme in purine catabolism where it catalyzes the oxidative hydroxylation of hypoxanthine to xanthine and xanthine to uric acid that ultimately yields the purine degradation products, ureides (allantoin and allantoate) [16; 17]. Recently, AtXDH1 transcript was shown to be induced by drought, salinity and abscisic acid treatments [18-20], indicating a role for XDH in purine catabolism in plants subjected to environmental stresses.

A common consequence of the influence of abiotic factors on plants is an increase in the intracellular concentration of reactive oxygen species (ROS) [21]. One of the least active forms of oxygen, superoxide, spontaneously or in the presence of transition metals is converted into more aggressive forms, for instance, hydroxyl radical, which can cause damage to many cellular biomolecules – lipids, proteins and DNA. Oxidation of lipids causes lipid peroxidation, i.e. destruction of plasma membranes, oxidation of proteins leads to loss of their function, formation of reactive oxygen species, DNA oxidation might directly induce mutations [21].

Therefore, one of the possible measures, reducing the negative effect of these stresses on plants, can be realized through a system of natural antioxidants. Antioxidants are substances that have the ability to suppress free radical oxidation, are capable of reacting with ROS to form either molecular products or radicals with lower reactivity [21]. One of these potential antioxidants is allantoin. Stability of *Arabidopsis thaliana* to NaCl stress caused by exogenous allantoin was accompanied by a decrease in the formation of ROS in seedlings. Exogenous allantoin stimulated growth and decreased the content of malondialdehyde (the product of membrane peroxidation) in rice seedlings [22], increased the expression of two antioxidant genes in *Arabidopsis thaliana* seedlings under saline conditions [3]. It was shown that allantoin induces ABA adaptive phytohormone synthesis under high salt concentrations [23; 24]. Thus, allantoin is an effective environmental protector against the effects of environmental stressors mediated by oxidative stress.

Allantoin is also used for medicinal purposes. It has been proved that allantoin possesses a keratolytic effect, which promotes cellular regeneration of the skin, rapid healing of abrasions and cracks, and the removal of irritations. Therefore, allantoin is a part of more than 1300 different cosmetic products. When administered orally, it increases the leukocyte count and improves lymph flow, protects the tissues of the stomach and intestines, and facilitates the repair of the gastrointestinal tract [25]. Allantoin is non-toxic and does not cause inflammatory reactions, is effective even in small concentrations.

Allantoin of plant origin is derived from the roots of the comfrey (*Symphytum officinale*), sprouted wheat, soybean and rice hulls [25]. It is well known that wheat germination is a functionally rich stage of wheat growth and germinated grain extract contains a large number of biologically active compounds and antioxidants, including allantoin [26; 27].

One promising approach to improve salt tolerance during germination is seed priming, which implies controlled seed inhibition followed by drying and facilitates the improvement of later development of seedlings [28]. The process of seed priming involves prior exposure to an abiotic stress, making a seed more resistant to future exposure. Seed priming stimulates the pre-germination metabolic processes and makes the seed ready for radicle protrusion. Priming of tomato seeds with 1M NaCl is recommended prior to sowing directly in saline soils [29]. Priming of seeds results in increased activities of the antioxidant enzymes superoxide dismutase, catalase and glutathione reductase. After priming, plant seeds reach higher level of proteins and nucleic acidssynthesis.Priming increases the antioxidant system activity and the process of membranesrepair, promotes seed vigor during germination, and further growth under salinity stress [28].

Based on the above-mentioned information it is of great importance and actuality to study the application of seed priming with relatively high concentrations of essential mineral elements as a fertilization technology to minimize soil and foliar fertilization, thus reducing environmental contamination. Thus, the improvement seed germination and seedling stand under high salinity may enhance not only forestation processes in the Aral Sea region but also reclamation of salt-affected land for crop production in the region.

This paper presents the results of the influence of presowing processing of wheat grain in molybdate solution by priming and germination in the presence of nitrate on the activity of XDH and on the content of allantoin in the germinated grains of this plant.

Materials and methods

Grain and 5-day old seedlings of "Baiterek" soft spring wheat cultivar were used as objects of the study.

The effects of so-called presowing seed priming are now widely studied. Priming means complete saturation of seeds with water and subsequent drying under controlled conditions [28]. The results of numerous studies have shown that simple saturation of plant seeds with water (or solutions of various salts) and subsequent drying leads to an increase in the percentage of their germination, growth and development of seedlings [29]. In our studies, used wheat grain was sterilized for 5 min in 1% potassium permanganate and then thoroughly washed with distilled water.

Priming of wheat seeds was carried out according to the method of Koehler K.H et al. [28]. The activity of NR and XDH was determined without priming on the whole grain and grain parts, endosperm and embryo, and in seeds after priming in water and with various concentrations of sodium molybdate. Dry wheat grain was completely saturated with water for 15 h at the room temperature and dried for 20 h [29]. Untreated and primed seeds were divided into endosperm part and embryo. These parts of the grain were mixed with 10 mM sodium phosphate buffer (pH 6.5 containing 10 μM EDTA) in a ratio of 1:10 (g/ml) and homogenized in a porcelain mortar. The homogenate was centrifuged at room temperature for 15 min at 15,000 g.

In the following experiments, we conducted presowing priming of the seeds in water, in a solution of molybdate and in a solution of tungstate. These seeds were then germinated in a medium containing potassium nitrate. As a control, wheat 5-day old seedlings with emerged radicals were homogenized in sodium phosphate buffer, and the activity of NR and XDH molybdoenzymes was determined without treatment and after heating at 80 °C for 5 min in the presence of 10 mM glutathione (GSH) and 10 mM sodium molybdate. After heat treatment, NR activity was determined by the reduced diaphorasive active benzylviologen by appeared NADH. It is very sensitive to high temperature (the diaphorase domain of NR attaches NADH and transfers electrons to the Moco, and the reduced benzylviologen directly transfers electrons to it).

The activity of NR was determined by a well-known method, where the enzyme was isolated from the wheat grain by chilled buffer containing 25 mM Tris-HCl, pH 8.4, 1 mM DTT, 5 mM cysteine, 3 mM EDTA, 10 μM FAD, 5 μM Na_2MoO_4, and 0.1 mM phenylmethyl sulfofluoride in a ratio of 1:3 [8]. The homogenated plant material was centrifuged at 15,000 g in Sorvall RC-5 centrifuge with cooling at 3-5 °C for 10 min. The nitrate reductase activity was determined in a reaction mixture containing 30 mM K-phosphate buffer (pH 7.5) of 25 mM KNO_3 and 0.25 mM NADN. The enzymatic reaction was carried out at 28°C for 15 min. The reaction was stopped by adding 50 μl of the mixture in a 1:1 ratio (v/v) of 0.3 mM phenazine methosulfate and 1 M Zn acetate followed by precipitation to remove residual NADH. Nitrite was determined in 1 ml of the mixture in a ratio of 1:1 (v/v) 1% sulfanilamide in 3.0 M HCl and 0.02% N-naphthyl (1)-dihydrochloride. Absorption was measured at 540 nm after 20 min. The NR activity was expressed as μmol NO_2^- g^{-1} wet weight per h^{-1}

A well-known method for enzyme extract ion using a buffer containing 10 mM Tris-HCl or 10 mM phosphate buffer (pH 6.5) was used for determination of XDH activity: 0.1 mM EDTA, 1 mM DTT, 10 mM GSH, 5 μM FAD. 1 g of plant material was extracted in the buffer in a ratio of 1:10 [20]. Plant material was centrifuged in a Sorvall RC-centrifuge at 15,000 g and 4°C for 15 min. Obtained supernatant was studied by spectrophotometric measurement of NADH at 340 nm. The reaction mixture contained 1 mM hypoxanthine, 2.5 mM NAD, 1 mM DTT, and 100-200 ml desalted extract 300-500 μg/mg^{-1} protein in 1.5 ml 50 mM phosphate buffer (pH 7.8) at 25 °C.

Extraction of enzyme with buffer containing 10 mM Tris-HCl or 10 mM phosphate buffer (pH 6.5): 0.1 mM EDTA, 1 mM DTT, 10 mM GSH, 5 μM FAD was used to determine the XDH activity [20].1 g of plant material was extracted in the buffer in a ratio of 1:10. The homogenized plant material was

centrifuged in a Sorvall RC-centrifuge at 15,000 g and 4 °C for 15 min, the supernatant obtained was used followed by spectrophotometric measurement of NADH at 340 nm. The reaction mixture contained 1mM hypoxanthine, 2.5 mM NAD, 1 mM DTT, and 100-200 ml desalted extract 300-500 μg/mg^{-1} protein in 1.5 ml 50 mM phosphate buffer (pH 7.8) at 25 °C.

The total amount of allantoin in the germinated grains was determined by the method of Vogels et al. [31]. Ureides were extracted with 80% ethanol in a ratio of 1:4 (v/v). Following extraction, allantoin and allantoic acid were determined using the appropriate standards as described by Vogels and van der Drift [31]. The resulting color formation was estimated spectrophotometrically at 535 nm.

Results and discussion

The precursor of allantoin is uric acid, which is formed during the oxidation of the purine base of xanthine, catalyzed by XDH Mo-containing enzyme [32].

Therefore, we conducted estimation of XDH and allantoin activity in "Baiterek" soft spring wheat cultivar, which were at rest in wheat, and after treatment in distilled water by priming, the activity of XDH and the content of allantoin were determined in the supernatants obtained. The results are shown in Table 1.

Table 1 – XDH activity and allantoin content (mg/g) obtained from wheat grains treated with priming

Variants	XDH activity, nmol UA/mg/min	Allantoin content, mg/g of grain dry weight
Control (whole grain)	>0.3	~0.2
Control (endosperm)	5.4 ± 0.7	>0.1
Control (embryo)	>0.3	1.3 ± 0.2
Priming H_2O (whole grain)	~0.5	~0.2
Priming H_2O (endosperm)	>0.3	>0.1
Priming H_2O (embryo)	7.2 ± 1.3	1.4 ± 0.1

As can be seen from the Table 1, the main content of allantoin is found in the embryo of both forms of wheat grain. Processing of grain using the priming method hardly increased the content of allantoin in the embryo and the endosperm part of the grain. Earlier, we found that the aleurone layer in the endosperm, the germ-free part, in the wheat grain contains XDH [29].

It is well known that the deficiency of molybdenum in the soil leads to a low activity of molybdoenzymes in plants [32].

According to the data of the Institute of Soil Science of the Academy of Sciences, KazSSR, Kazakhstani soils, although alkaline, contain molybdenum 3-5 times less than the critical concentration (0.1 mg Mo/kg for temperate soils) necessary for normal plant growth and development [33]. Earlier, we showed that a non-molybdenum population of XDH is synthesized in the germ of wheat grains, which is activated in vivo by heating (80°C) in the presence of GSH and sodium molybdate [35].

Apparently, the existence of the non-molybdenum forms of XDH is associated with a lack of molybdenum in the soils.

It is supposed that the formation of uric acid and allantoin depends on the level of assimilation of nitrate by plants. Assimilation of nitrates is a fundamental process in the plant kingdom, and therefore the NR enzyme restoring nitrate is regarded as a limiting factor in the growth, development, protein formation and final crop yield. NR is an inducible enzyme, i.e. it is synthesized only in the presence of a medium containing nitrate. In the catabolism of purines, XDH oxidizes xanthine to uric acid. Complete degradation of purines includes the stages of transformation: purine nucleotides → hypoxanthine → xanthine → uric acid (UA) → allantoin [34]. For the oxidation of xanthine, XDH uses oxidized NAD^+ as an electron acceptor. As mentioned above, the product of enzymatic oxidation of xanthine is UA, which is further oxidized with the participation of uricase, or non-enzymatically in the presence of ROS, forming allantoin. Thus, the formation of UA and allantoin directly depends on the concentration of oxidized NAD^+ in the plant cell.

When assimilating nitrate, NR restores it to nitrite (NO_2^-). In the reduction of nitrate, NR as an electron donor uses NADN, thus forming NAD^+. The more nitrate is reduced in the plant cell, the more NAD^+ is formed, required for XDH catalytic reaction. In the oxidation of xanthine, its electrons restore the NAD^+ again, turning it into NADH. The more active is XDH, the more allantoin is formed via UA.

Allantoin, as a potential antioxidant, neutralizing ROS, increases the resistance of plants to oxidative stress caused by unfavorable environmental conditions. It has been established that nitrate nutrition increases plant tolerance to salinity, which might be explained as follows. Since the reduced pyridine

nucleotide NADH is the only physiological electron donor for the catalytic reaction of NR, a high level of nitrate reduction under stress conditions maintains a high level of NAD^+. For the enzymatic reduction of one nitrate molecule, 4 molecules of NADH (8 electrons) of 6 protons are required, thuswise, the assimilation of nitrate is when set beside with of other molecules, the most effective NAD^+–generating reaction [36; 37].

Thus, an important antioxidant role of NR is the constant maintenance of the high level of NAD^+, required for the synthesis of allantoin. Therefore, in our next experiments, we studied the effect of presowing priming of wheat grains in a solution of sodium molybdate on the activity of NR, XDH, and the content of allantoin in sprouted wheat grains. Wheat grains after Mo-priming were grown on a medium containing potassium nitrate.

Our preliminary results showed that the priming of wheat grains in solutions of molybdate sodium to 75 mM concentration does not affect the process of their germination [34]. It was also established that 0.45 mM potassium nitrate concentration is sufficient to induce NR, and therefore, the germination medium contained this concentration of KNO_3 [37]. On the 5th day after the appearance of the radicals in germinated grain, the activity of XDH, NR and allantoin was determined.

Table 2 shows the results of wheat grain priming with molybdenum and tungsten, it can compete with molybdenum for incorporation into the enzyme complex and results in enzyme inactivation [38], their subsequent germination in a medium with nitrate on the activity of NR, XDH, and the content of allantoin in 5-day wheat seedlings.

Table 2 – Influence of presowing priming of wheat grain on the activity of NR, XDH and on the content of allantoin in 5-day seedlings of wheat grain

Variants	NR activity, nmol NO_2^-/ mg protein/min	XDH activity, nmol UA/мmg/min	Allantoin, mg/g grain dry weight
Control	0.0	6.5 ± 0.7	13.2 ± 1.4
Priming in H_2O	0.0	7.2 ± 1.2	13.8 ± 1.6
Priming in 75 мМ MoO_4^- solution	0.0	8.3 ± 1.3	19.4 ± 2.5
Priming in 0.45 мМ NO_3^- solution	15.7 ± 2.3	7.5 ± 0.9	14.0 ± 2.1
Priming in 75 мМ MoO_4^-+0.45 мМ NO_3^-	20.3 ± 2.7	11.3 ± 1.9	21.2 ± 1.7
Priming in 75 мМ WO_4^- + 0.45 мМ NO_3^-	0.0	~1.3	~1.2

The results presented in Table 2 show that the presupposition of accumulation of exogenous molybdenum in the grain increases the content of allantoin in the germinated grains. And with germination of wheat grain, saturated with molybdenum, in a medium with nitrate sharply increases the level of allantoin in sprouted grains. These results confirm the relationship between the assimilation of nitrate and the formation of allantoin. A presowing saturation of grain with tungsten, a chemical analogue of molybdenum, somewhat reduced the content of allantoin in such grains. As is known, in the absence of molybdenum in a growth medium, its chemically analogous tungsten is easily included in the active center (instead of molybdenum) of molybdoenzymes. Since unlike molybdenum, tungsten atoms do not possess the ability to transfer electrons in the active center of NR and XDH, they become inactive, no regeneration of NAD^+ and new syntheses of urate, the precursor of allantoin is seen.

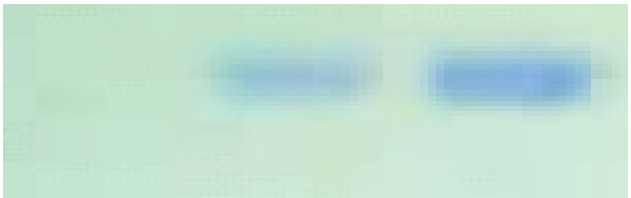

Figure 1 – Zymogramma of sprouted seeds after priming in a solution of tungsten (1), in water (2) and in molybdate solution (3)

As mentioned above, we previously found that in the embryo of dormant wheat seeds there is a non-molybdenum population of XDH, which is activated

by exogenous molybdate after heating at 80 °C in the presence of GSH. This indicated the existence of a non-molybdenum population of this enzyme intact wheat seeds. Therefore, in order to clarify the effect of presowing priming in molybdate solution in the following experiments, we conducted priming of the seeds in water, in molybdate solution and in a solution of tungstate. These seeds were then germinated in a medium containing potassium nitrate. The results are shown in Table 3.

Table 3 – Effect of heat treatment on nitrate reductase and xanthine dehydrogenase activity of wheat seedlings after priming under various conditions

Variants	Seeds treatments	NR activity	XDH activity
Control (without priming)	Without treatment	0.0	6.7 ± 1.2
	heat treatment + MoO_4^- + GSH	0.0	9.2 ± 1.4
Priming in H_2O	Without treatment	15.4 ± 25	7.5 ± 0.9
	heat treatment + MoO_4^- + GSH	22.1 ± 3.8	11.3 ± 1.9
Priming in MoO_4^- solution	Without treatment	20.3 ± 3.7	9.8 ± 1.4
	heat treatment + MoO_4^- + GSH	22.4 ± 2.8	12.7 ± 1.9
Priming in WO_4^- solution	Without treatment	0.0	1.2 ± 0.2
	heat treatment + MoO_4^- + GSH	19.5 ± 3.2	10.9 ± 1.5

As can be seen from the Table 3, in order to restore the lack of molybdenum in the non-molybdenum XDH and NR forms in germinating seeds, it is sufficient to presow them in the molybdate solution. Molybdoenzymes in such sprouted seeds are activated only by slightly exogenous molybdenum, i.e. molybdenum accumulated during priming of seeds germinated is a part of the newly synthesized NR and XDH molecules. Inactive molecules after priming in tungstate solution are fully activated after heat treatment in the presence of exogenous molybdenum and GSH. Such in vitro activation of non-molybdenum forms of molybdoenzymes is explained as follows.

As is known, in the active center of molybdoenzymes, the molybdenum atom is directly bound to the Moco, in the non-molybdenum forms of enzymes this cofactor is contained. During heat treatment, the molecules of molybdoenzymes are partially denatured and the access of molybdenum and GSH to the active center of enzymes is opened. Since the sulfhydryl cofactor groups binding the molybdenum atom in the presence of oxygen rapidly oxidize, the presence of GSH protects their oxidation. In such a situation, exogenous molybdenum is easily bound by these co-factor thiols. Moreover, with denaturation, the active molybdenum-containing molecules of NR and XDH are formed.

Conclusion

It is generally known that in the cells of various organisms (plants and animals), the non-molybdenum forms of molybdoenzymes are synthesized. One of the ways to activate them in plants is presowing seed priming. It is also known that molybdenum accumulated in plant seeds by presowing priming during their germination is transported to roots and ground parts. At the same time, all molecules of molybdoenzymes are provided with this metal, i.e. they all become active. Presowing priming of wheat seeds in solution increases the activity of nitrate reductase and xanthine dehydrogenase, and accordingly the formation of allantoin. It is known that different types of wheat differ in the level of activity of molybdoenzymes. It is known that different types of wheat differ in the level of activity of molybdoenzymes. Therefore, the identification of wheat with high nitrate reductase and xanthine dehydrogenase activity in the seed embryo will allow them to be used as a source of allantoin after presowing priming in a molybdate solution.

Thus, the obtained results convincingly show the important role of the interrelation of molybdoenzymes of xanthine dehydrogenase and nitrate reductase in the formation of allantoin in the germ of

wheat grain during its germination. In addition, the existence of the molybdenum-free forms of not only xanthine dehydrogenase in the embryo of wheat grain, but also of another nitrate reductase molybdoenzyme is shown again. Based on the analysis of the results of studies, it can be concluded that the biosynthesis of the natural antioxidant – allantoin increases in the embryo of wheat grain when it is saturated with the molybdate solution by presowing priming and when germinating in the presence of nitrate. Thus, our data suggest that wheat seeds of varieties with a high content of molybdoenzymes xanthine dehydrogenase and nitrate reductase can be one of the sources of an important biologically active substance – allantoin.

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