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Molybdoenzymes and molybdenum cofactor in dormant and developing wheat seeds

The distribution of the activities of molybdenum cofactor (MoCo) and molybdenum containing enzymes – nitrate reductase (NR), aldehyde oxidase (AO) and xanthine dehydrogenase (XDH) in different parts of dormant and developing wheat seeds are studied. The activities of MoCo, AO and XDH were highest in the embryo. The aleurone layer of the seed also showed these activities; however, activity levels in the aleurone were approximately three times lower than those in the embryo. The activities of these molybdoenzymes were not detected in the endosperm portion of the seed. There was no detectable NR activity in any part of the seed. The XDH activity remained at a steady level in both the embryo and endosperm throughout the course of seed development and maturation, whereas AO activity was extremely low in these same seed parts. However, AO activity increased at a high rate when seeds entered desiccation phase. At this stage, the content of ureides also increased by two and a half times. It is known that AO is involved in abscisic acid (ABA) biosynthesis, converting abscisic aldehyde to ABA, and the by-product of this reaction is superoxide. Therefore, we propose that increased ABA synthesis results in the increase of reactive oxygen species (ROS), which are scavenged by uric acid forming ureides.

Key words: Aldehyde oxidase, dormancy, molybdoenzymes, molybdenum cofactor, nitrate reductase, wheat seeds, xanthine dehydrogenase.

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Бидай дәнінің тыныштық күйі мен даму сатысындағы молибдоферменттер және молибденді кофактор

Тыныштық күй мен даму сатысындағы бидай дәндерінің түрлі бөліктерінде молибденді кофакторлар (Mo-co) мен молибденді ферменттер: нитрат редуктаза (НР), альдегидоксидаза(АО) мен ксантиндегидрогенезаның(КДГ) таралуы зерттелді. MoCo, АО мен КДГ жоғары белсенділікте ұрықта анықталды. Ұрық бөліміне қарағанда алейронды қабатта осы ферменттер мен MoCo белсенділігі үш есе төмен болды. Дәннің эндоспермдік бөлімінде бұл ферменттердің белсенділігі тіркелмеді. НР белсенділігі дәннің ешқандай бөлігінде анықталмады. КДГ белсенділігі даму және жетілу кезеңдерінде ұрық бөлімінде де, эндосперм бөлігінде де бірдей болып сақталса, ал АО белсенділігі аталған бөліктерде экстрималды төмен деңгейде анықталды. Алайда, АО дән дегидратация фазасы кезінде көптеген ылғалды жоғалтқан жағдайда жоғары жылдамдықпен артты. Осы фазада уреидтер де 2,5 есе артты. АО абсциз альдегидін АБҚ-на дейін өзгертіп, АБҚ биосинтезіне қатысатындығы және осы реакция өнімі супероксид екендігі белгілі.

Сондықтан да, біз АБҚ-ның жоғары биосинтезі несеп қышқылы арқылы бейтараптанып, уреид түзетін оттегінің реактивті радикалдарының пайда болуына алып келеді деп болжаймыз.

Түйін сөздер: альдегид оксидаза, бидай дәндері, тыныштық, молибдоферменттер,молибденді кофактор, нитрат редуктаза, ксантин дегидрогеназа.

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Молибдоферменты и молибденовый кофактор в состоянии покоя и развития семени пшеницы

Изучены распределение молибденового кофактора (Мо-со) и активностей молибденсодержащих ферментов – нитрат редуктазы (НР), альдегид оксидазы (АО) и ксантиндегидрогеназы (КДГ) в различных частях покоящегося и созревающегося зерна пшеницы. Были показаны наиболее высокие активности МоСо, АО и КДГ – в зародыше. В три раза меньше уровень активности этих же ферментов и Мосо наблюдался в алейроновом слое, чем в зародыше. Активности этих ферментов не обнаруживались в эндоспермальной части семени. Активность НР не обнаруживалась ни в одной части зерна. Активность КДГ оставалась равномерной как в зародыше, так и в эндосперме в течение периода развития и созревания, тогда как активность АО была экстремально низкой в этих частях зерна. Однако активность АО увеличилась с высокой скоростью, когда зерновка теряла влажность во время фазы дегидратации. В этой фазе содержание уреидов также увеличивалось в 2,5 раза. Известно, что АО вовлечен в биосинтез АБК, превращая абсцизовый альдегид до АБК, и супероксид становится продуктом этой реакции. Поэтому мы предполагаем, что повышенный биосинтез АБК приводит к увеличению реактивных радикалов кислорода, которые нейтрализуются с помощью мочевой кислоты образуя уреиды.

Ключевые слова: альдегид оксидаза, семена пшеницы, покой, молибдоферменты, молибденовый кофактор, нитрат редуктаза, ксантин дегидрогеназа.

Introduction

Pre-harvest sprouting (PHS) in wheat, germination of the grain on the plant before harvest, is a major source of loss for the grain industry in Kazakhstan. PHS affects the economic value of the harvest since germinated seeds are sold at a much lower price for use as feed than the grain for flour .The phenomenon is due to lack of ABA in young seeds and/or their insensitivity to the dormancy-inducing hormone. ABA is a plant growth regulator involved in various processes, including response to environmental stressand seed maturation and dormancy **[1]**. Dormancy is a mechanism to prevent germination during unfavorable ecological conditions, when the probability of seedling survival is low [2]. A dormant seed is one that is unable to germinate for a specific period of time under a combination of environmental factors that are normally suitable for the germination of the non-dormant seed [3].In cereal crops, an optimum balance between dormancy and non-dormancy is desirable. Dormancy at harvest is desired because it prevents the germination of the physiologically mature grain (i.e. PHS)in the head prior to harvest, a phenomen on that considerably lowers grain quality and is especially common in cool, moist environments. ABA regulates a number of key events during seed development, such as the deposition of storage reserves, prevention of precocious germination, acquisition of desiccation tolerance, and induction of primary dormancy [4].

In higher plants, ABA is derived from an epoxy-carotenoid precursor that is oxidatively cleaved to produce xanthoxin [5,6]. Following the cleavage, xanthoxin is converted to ABA by a series of ring modifications to yield abscisic aldehyde, which is oxidized to ABA by AO (EC 1.2.3.14), a molybdenum-containing enzyme [7]. In addition to AO, plant MoCo-containing enzymes include nitrate reductase (NR; EC 1.7.1.1) and XDH (EC 1.17.1.4) [9] XDH and AO have been characterized from various organisms and shown to be homodimers of 150-kDa subunits that have a high similarity in their amino acid sequence and motifs, among which are the binding sites for two iron-sulfur centers and a MoCo binding region[9,10, 11]. Whereas, NR requires a dioxo-molybdenum, center XDH and AO contain mono-oxoMoCo in which the second oxygen was replaced by a sulfur ligand. AO belongs to a multigene family [9] and appears to display a broad range of substrate specificities [8,10,12,13] one of which is the oxidation of indole-3-acetaldehyde to indole-3-acetic acid (IAA) [12].

Much attention has recently been focused on plant AOs because of their involvement in the biosynthesis of ABA, a phytohormone that plays important roles in the prevention of pre-harvest sprouting of seeds. AO is localized almost exclusively in the embryo of dry, dormant wheat seeds, and this is due to the production of ABA in the dormant embryo [13]. However, until the present study the activity of AO has never been investigated during the entire

course of seed development and maturation.Alikulov and Schieman [14]showed the existence of active MoCo in dormant dry seeds of wheat and barley. In contrast, the activity of NR was not detected in barley and wheat seeds, and the cell-free extract of dormant seeds did not react with polyclonal antibodies obtained against barley, squash and tobacco NRs [14]. In this paper, we report the results of our study on the distribution of molybdenum containing enzymes and Moco in different parts of the wheat seed, and their alteration during seed development and maturation. The data on the synthesis of these enzymes in developing and maturing wheat seeds that are obtained from greenhouse-grown plants are presented. We also discuss the possible role of molybdenum-containing enzymes in the biochemical processes of seed development and maturation.

Materials and Methods

Plant material. In our investigations used the seeds of the spring wheat (*Triticumaestivum L.*) cultivar *Lutescence 70*. The seeds were obtained from the A.I. Baraev Kazakh Scientific-Research Institute of Grain Farming (Republic of Kazakhstan, Akmola region, town of Shortandy). The cultivar was grown in fields at the A.I. Baraev Kazakh Scientific-Research Institute of Grain Farming near the town of Shortandy (Republic of Kazakhstan, Akmola region) and in Kurgalzhin lands near the city of Astana (Republic of Kazakhstan, Akmola region) in 2011 and 2012.

Experimental set-up. For growth chamber experiments, wheat seeds were pre-germinated in an incubator at 21^0C and planted in free-draining, 11 cm-diameter pots filled with acid-washed quartz sand. Plants were watered with a modified Hoagland's solution and grown in a growth chamber at 22^0C /18^0C day/night temperature under a 12-hour photoperiod. For green house experiments, seeds were germinated and grown in 10 L plastic pots filled with a soil substratum containing light high sphagnum peat, dark high peat, upper sphagnum peat moss, PG-Mix (14+16+18), chalk and sand (the group of OJSC Peat Enterprise «Nesterovskoie» and «Torfo» Ltd., Russia; www.torfo.ru). Plants were grown in a greenhouse at 22^0C/18^0C day/night temperature under a 12-hour photoperiod. In order to harvest samples of the same development stage, approximately 150 spikes were tagged in the pots. Developing spikes were removed from the mother plant at five-day intervals, starting at day 10 after pollination (DAP) to day 40 and also at the stage of complete seed maturity. After the removal, spikes were frozen immediately in liquid nitrogen and kept in a freezer at -80^0C. MoCo and molybdoenzyme activities were assayed in extracts obtained from the frozen kernels on the same day.. In the case of all samples, kernels were removed from spikes immediately before homogenization,

Separation of seed parts. The surface of the seeds was sterilized in NaClO solution (1% active chlorine)for 5 minutes, washed and imbibed in distilled water. Mature and dormant seeds were soaked at room temperature for 48 hours. Since after such imbibition seeds became very soft, it was easy to dissect and separate their parts. Moreover, experiments showed that the MoCo content and the activities of XDH, AO and NR did not change in the seeds after 48 hours of imbibition. The embryos were removed manually, and the endosperm was removed from the seed coat by gentle squeezing. Seed coat was used as aleurone layer. Although the embryo and endosperm obtained by this procedure were clean, the seed coat was contaminated with endosperm fragments. However, the above-mentioned molybdoenzymes did not have any activity in the endosperm (see Results, Table 1), and the separation procedure provided satisfactory analysis of molybdoproteins in the seed parts isolated. In the case of developing seeds, it was not possible to excise embryos cleanly from seeds younger than 25 DAP. Therefore, at early stages of seed development, seeds were divided to the embryo and endosperm at an approximate ratio of 1:4, respectively, along the length of seeds. The youngest age at which relatively clean embryos could be obtained for satisfactory analysis was 35 DAP.

Preparation of tissue extracts. Structural parts of the wheat seed were macerated with acid washed sand in an ice-cold extraction buffer containing 50 mM Tris-HCl (pH = 7.5), 1 mM ethylene diaminetetraacetic acid (EDTA), 1 mM L-glutathione (GSH), 10 μM leupeptin, 0.01 mM Na_2MoO_4 and 0.01 mM flavin adenine dinucleotide disodium salt dihydrate (FAD). All extractions were done at a ratio of 100 mg fresh weight/ 1 ml buffer. The homogenate was centrifuged at 15,000 rpm at 50C for 20 minutes and the supernatant part was saved for analysis.

Determination of MoCo activity. Cofactor activity in seed tissue was estimated using a NR-mutant nit-1 of Neurosporacrassa that is unable to synthesize endogenous MoCo. MoCo activity was determined by the method of Savidov et al. [15]. Protein fractions (40μl) of the wheat seed, obtained

by S-300 gel chromatography, were used as MoCo sources. MoCo was dissociated from its binding proteins by heating at 800C in the presence of 4 mM GSHfor 2 min. The MoCo preparations (50µl) were added to a reaction mixture containing crude extract (50µl)of nit-1 of N. crassa, 10µM FAD, 5 mM Na_2MoO_4, 5 mM KNO_3, 0.2 mM nicotinamid-eadenosine dinucleotide phosphate (NADPH) and 5 mM Glucose-6-Phosphate (G6P) in a final volume of 150µl. NADPH-NR complementation was carried out at 100C for 24 hours. Nitrite accumulation took place during complementation period because of the activity of the newly reconstituted NR. Nitrite was determined immediately after the complementation and prior to the assay of the reconstituted enzyme. The reconstituted enzyme was assayed with 0.4 mM NADPH and 5 mM KNO_3 at 300C for 30 min. The MoCo activity was estimated on the basis of the amount of nitrite produced by restored NADPH-NR (µmol ml-1h-1).

Determination of enzyme activities. NR activity was determined in vitro according to Savidov et.al.[15] .The reaction medium contained 50 mM Na-phosphate buffer (pH = 7.7), 5 mM KNO_3 and 10 µM FAD. The reaction was started by the addition of the enzyme (i.e., seed part extract). After 10 min of incubation at 280C, the reaction was terminated by adding 50 µl of a mixture of 0.3 mM phenazinemethosulfate (PMS) and 1 M Zn acetate (1:1) to remove residual NADPH from the assay medium. This step was followed by the addition of 0.1 ml of a mixture of 0.02% (NED) and 1% (SA) (1:1) for nitrite determination. Reaction mixtures were centrifuged at 10000 rpm for 5 min, and their absorbance was read at 540 nm after 20 min.

XDH and AO activities were detected in 7.5% polyacrylamide gels after protein fractionation by native gel electrophoresis using hypoxanthine and xanthine as substrates for XDH and vanillin or indole-3-aldehyde as substrates for AO [11], respectively. The samples, which contained approximately 20-25 µg of soluble protein, were loaded onto wells and electrophoresed at 40C for 1.5-2 hours. Then, the gels were equilibrated and washed in 100 mM sodium phosphate buffer (pH = 7.5) by gentle shaking for 10 min, which was followed by incubation in a reaction mixture containing 0.1 mM PMS, 1 mM 3(4,5-dimethylthiazolyl-2)2,5-diphenyltetrazolium-bromide (MTT) and 1 mM specific XDH/AO substrate. The activities of XDH and AO were estimated based on the amount of MTT reduction that resulted in the development of specific formazan bands. The intensity of formazan bands was directly proportional to enzyme activity in the presence of excess substrate and tetrazolium salt. Quantitative analyses were made by scanning the formazan bands in the gel with a computing laser densitometer (Molecular Dynamics) using the software provided (Image Quant version 3.19.4). Standard curves for estimation of XDH activity were based on formazan band density in the gels loaded with increasing amounts of commercial xanthine oxidase (1 unit per 1.3 mg protein, Sigma). XDH activity in gels was expressed as enzyme unit's mg-l protein h-l.

XDH activity was also assayed spectrophotometrically according to Triplett et al. [20].by following the production of reduced nicotinamide adenine dinucleotide (NADH) at 340 nm. AO activity also was assayed monitoring the change of absorbance at 600 nm of the electron donor 2,6-dichloroindophenol (DCIP) [14].The absorbance of the enzyme products were measured in a spectrophotometer (Jenway).

Ureide and soluble protein determination. Ureides were extracted with 80% ethanol and measured according to Vogels and Van der Drift [16] using allantoin (Sigma) as reference. The colorimetric assay was carried out essentially as described by Vogels and Van der Drift to measure ureides, by adding phenylhydrazine .The reaction mixture contained the following: 50 mM TES-NaOH (pH 7.8), 0.5 mM MnSO4, 10.4 mM phenylhydrazine-HCl, 2.5 mM sodium ureidoglycolate, and an adequate amount of enzyme. The reaction was started by the addition of substrate, and the incubation was carried out at 300C. Convenient aliquots were drawn from the reaction mixture at different incubation times and transferred to tubes kept on ice. After 5 min on ice, 1 ml of 12 M HCl and 0.2 ml of 46.6 mM (1.6% w/v) potassium ferricyanide were added to each tube. The absorbance was measured at 535 nm after 15 min of incubation at room temperature. Controls were always included to determine the non-enzymatic formation of ureides.Water-soluble proteins in the assays were measured according to Bradford [17] using bovine serum albumin (BSA) as reference.

Results

MoCo content and molybdoenzymes activities in the structural parts of dormantwheat seeds.

Table 1 shows the distribution of MoCo, AO, XDH and NR activities in different structural parts of the dormant wheat seeds. According to these data, MoCo activity was highest in the embryo and aleurone layer, while it was negligible in the endo-

sperm portion of dormant seeds. The embryo and aleurone layer extracts exhibited also XDH and AO activities (Table 1). However, activities of these molybdoenzymes in the aleurone layer were lower than that in the embryo. There was no XDH and AO activity that is detectable in endosperm extracts. Similarly, no NR activity was detected in extracts of any dormant wheat seed part (Table 1)

Table 1 – Activities of MoCo, AO, XDH and NR in different parts of dormant wheat seeds

Seed Parts	MoCo*	AO*	XDH*	NR*
Embryo	19.7±	85±	1.8±	0.0
Aleurone layer	12.5±	65±	1.3±	0.0
Endosperm	0.1±	0.0	0.0	0.0

*μmol NO^-_2 formed by nit^{-1} NR mg^{-1} protein $hour^{-1}$.
**□□nmol DCIP mg^{-1} protein min^{-1}.
***μmol NADH mg^{-1} $hour^{-1}$.
****μmol NO^-_2 mg^{-1} protein $hour^{-1}$.

The distribution of AO activity in the different structural parts of dormant wheat seeds after native gel electrophoresis (Figure 1). AO bands were visualized in the embryo and aleurone layer. The AO activity was highest (up to 61%) in embryo extracts, followed by aleurone layer extracts (31%).

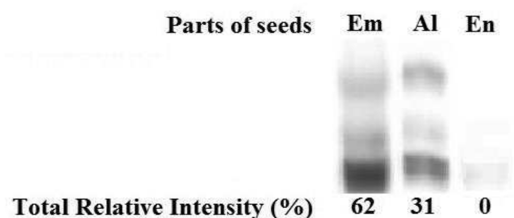

Figure 1 – The activity of AO in different parts of dormant seeds from the wheat cultivar Lutescens 70 (Em – embryo, Al – aleurone layer, En – endosperm) MoCo content and molybdoenzymes activities in the structural parts of developing and maturing wheat seeds

MoCo, NR, XDH and AO activities in developing seeds were measured at 5-day intervals starting at 10 DAP. The time course of the changes in activities of MoCo and molybdoenzymes in the embryo during development and maturation are shown in Table 2.The MoCo level in the embryo and aleurone layer gradually increased from10 DAP and reached a maximum at 40 DAP. The activity of MoCo in both the embryo and aleurone layer was low at 10 DAP. However, a slow but steady increase of MoCo activity occurred in both parts of the developing seeds after 15 DAP, and the synthesis of the cofactor was high at 30 DAP, reaching a maximum at 35-40 DAP and then plateauing and showing no change from 35-40 DAP to maturity. In the case of AO, extremely low activity was detected in the embryo and aleurone layer of developing seeds until 30 DAP (Table 2, Figure 2).

After 30 DAP, the activity increased rapidly in the embryo reaching a maximum at 40 DAP and showing a slight decrease at maturity. In contrast, there was only slight increase in enzyme activity in the aleurone layer, and the activity remained at a steady level thereafter (Table 2, Figure 2).

XDH activity was present in the embryo and aleurone layer of seeds throughout their development although the activity in the aleurone layer was significantly lower than that in the embryo. Since such ureides as allantoin and allantoic acid are a product of purine catabolism, XDH activity and ureides were assayed in tandem throughout the seed development. XDH activity remained at a constant level until full maturity, but the content of ureides increased 4.5 fold around 35-40 DAP (Figure 5), corresponding to a sharp increase in AO activity (Table 2). Ureides reached a maximum at and around 35-40 DAP,and then decreaseduntil full maturation. Throughout seed development, the amount of ureides was much higher in the embryo than that in the aleurone layer. For example,theureides content in the embryo was 5 times higher than that in the aleurone layer during seed dehydration(data not shown).

There was no NR activity detectable in vivo and in vitro in the embryo, endosperm and aleurone layer of developing seeds. In contrast, when seeds were removed from the spikes that were at different stages of maturation and incubated in 50 mM KNO3 solution for 30 hours, NR activity was detected in embryos starting at 40 DAP (Table 2). The maximum level of NR activity was observed in the embryo portion of mature seeds.

Table 2 – Changes in MoCo, AO, XDH and NR activities in the embryo of wheat seeds during development and maturation (Em – embryo, Al – aleurone layer)

DAP	MoCo*			AO**			XDH***			NR****#		
	Em	Al	En	Em	Al	En	Em	Al	En	Em	Al	En
10	5.3±	1.4±	0.0	10.2±	2.3 ± 1.4	0.0	1.6±	0.4±	0.0	0.0	0.0	0.0
15	8.5±	1.6±	0.0	10.8±	2.5 ± 1.1	0.0	1.7±	0.5±	0.0	0.0	0.0	0.0
20	9.2±	1.7±	0.0	11.5±	3.4 ± 1.4	0.0	1.5±	0.3±	0.0	0.0	0.0	0.0
25	10.7±	1.8±	0.0	13.6±	4.1 ± 1.8	0.0	1.9±	0.7±	0.0	0.0	0.0	0.0
30	13.5±	2.2±	0.0	20.3±	4.8 ± 1.7	0.0	1.7±	0.5±	0.0	0.0	0.0	0.0
35	17.3±	2.5±	0.0	38.8±	5.5 ± 2.4	0.0	1.8±	0.4±	0.0	0.0	0.0	0.0
40	17.5±	2.7±	0.0	69.4±	9.0 ± 2.6	0.0	1.8±	0.6±	0.0	0.9±	0.0	0.0
Full maturity	17.6±	3.6±	0.0	66.5±	11.8 ± 3.3	0.0	2.0±	0.6±	0.0	4.8±	0.0	0.0

*µmol NO^-_2 formed by nit^{-1} NR mg^{-1} protein hour^{-1}.

**nmol DCIP mg^{-1} protein min^{-1}.

***µmol NADH mg^{-1} hour^{-1}.

****µmol NO^-_2 mg^{-1} protein hour^{-1}. Developing seeds were removed from spikes and incubated in 50 mM KNO_3 for 30 hours and NADH-NR activity was determinedin their extracts.

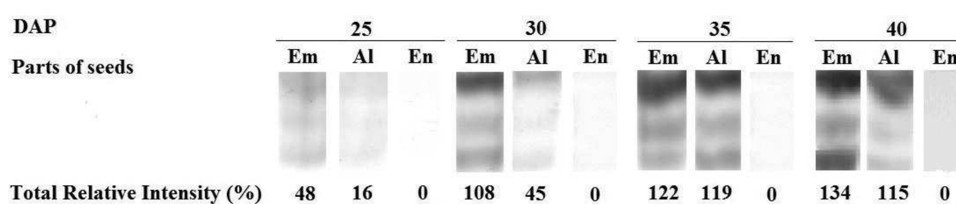

Figure 2 – The activity of AO in different parts of developing and maturing seeds of the wheat cultivar Lutescens 70 (Em – embryo, Al – aleurone layer, En – endosperm)

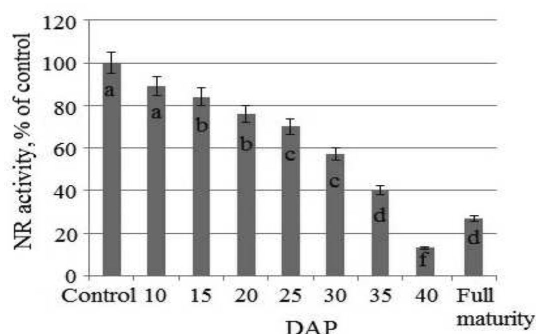

Figure 3 – Effect of cell-free extracts of developing seeds on the induction of NR activity in the embryos of dormant wheat seeds*

*100 mg of freshly excised seeds were rigorously homogenized in 1 ml distilled water and then centrifuged. Then, the supernatant was heated in boiling water for 5 minutes and centrifuged. The second supernatant was mixed with nitrate solution to a final concentration of 50 mM (this concentrations of nitrate was found to be optimal for the induction of NR in the embryo). Then, this solution was used as incubation medium to test its inhibitory effect on the NR induction in the embryo.

Furthermore, we studied the effects of cell-free extracts obtained from seeds at different stages of development on the induction of NR activity in the embryos excised from dormant seeds (Figure 3).The results showed a correlation between the inhibitory effects of endosperm extracts of developing seeds and the AO activity, as shown in Table 2. The effect of ABA on NR activity was also investigated by

treating excised embryos in that they were incubated in the presence of different ABA concentrations and 50 mM KNO3for 14 hours. As shown in Figure 4, the increasing concentrations of ABA indeed increased the inhibition of NR activity in excised wheat embryos because decreases in enzyme activity were proportional to the concentration of ABA used. However, NR activity was not inhibited by ABA in cell-free extracts when 100μM ABA was added to the reaction mixture.

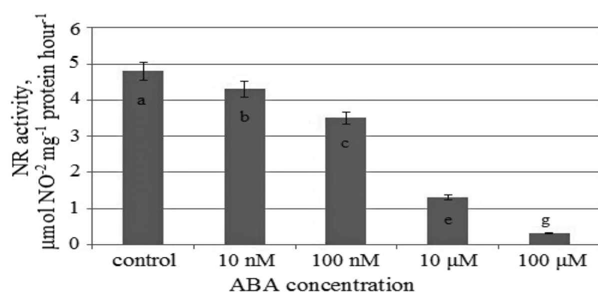

Figure 4 – Effect of increasing ABA concentrations on the induction of NR activity in excised wheat embryos.

Discussion

Although NR is widespread among plants and the enzyme was first isolated and characterized more than 40 years ago, its role in developing and maturing seeds has not been investigated. This enzyme is considered to be a limiting factor for growth, development, protein production and yield in plants.In this study, we were unable to detect NR activity in extractsof dormant wheat seed parts when NADPH, reduced flavin adenine dinucleotide (FADH) or Methyl viologenhydrogenase (MVH) was used as electron donors (Table 1). Indeed, our previous study showed that antibodies to NR did not detect a cross-reacting antigen among proteins extracted from embryos, endosperm and aleurone layer (seed coats) of the wheat seed. Consistent with the previous results, our current study did not also detect any NR activity in vivo and in vitro in the embryo, endosperm and aleurone layer. However, the incubation of wheat seeds in nitrate solution for 24-30 hours resulted in the appearance of NR activity in the embryo but not in the endosperm and aleurone layer (Table 2). Gupta et al. [18]detected NR activity in excised embryos of barley seeds after 4 hour of incubation in nitrate solution. Furthermore, these authors demonstrated that endosperm extracts inhibit the induction of NR in excised embryos. In our experiments, embryos of 35-40 DAP were first excised and then incubated in nitrate solution, which led to the induction of NR activity. Specifically, a significant level of NR activity was observed, detectable first after 6 hour of incubation in 50 mM nitrate and reaching a peak after 14-16 hours of incubation (data not shown).These data suggest that nitrates are taken up and assimilated in wheat roots and leaves but not in developing and maturing seeds.

Furthermore, we studied the effects of cell-free seed extracts made at different stages of development on the induction of NR activity in embryos excised from dormant seeds (Figure 3). These results show that endosperm extracts inhibit the activity of AO. It is also known that the induction of NR activity is enhanced by cytokinins and suppressed by ABA. Since AO is involved in ABA biosynthesis, we hypothesized that ABA regulates the expression of NR in wheat embryos. Thus, we studied the effect of ABA on NR activity by subjecting the excised embryos to ABA treatment. Our results indicated that the induction of NR activity was responsive to ABA. It should be emphasized that the actual ABA uptake rate by the embryo is not measured and can be lower than that shown in Figure 4. Because we do not know how much ABA was taken up by the embryo, the actual ABA concentrations at the target site may be much lower than the concentrations that were used in the experiment. Nevertheless, our dose-response study of ABA clearly indicates that the expression of NR activity is regulated by ABA. In fact, the endosperm ABA seems to play a role in the regulation of NR expression in developing wheat embryos. Previously showed that the regulation of NR gene expression by ABA in barley leaves is at the level of transcription. Thus, NR activity in wheat embryosis induced by nitrate at later stages of seed development when the ABA level is low. It is conceivable that, nitrates taken up from the soil do not reach developing seeds because of their assimilation in roots and then in leaves.

As mentioned before, the embryo and aleurone layer extracts of dormant wheat seeds contained both AO and XDH activities; however, AO and XDH activity in the aleurone layer was lower than that in the embryo. The activities of these enzymes were not detected in endosperm extractsof dormant seeds; this is not surprising since there was little no MoCo observed in this tissue (Table 1). It is known that AO catalyzes the conversion of abscisic aldehyde to ABA, and AO is a molybdenum contain-

ing enzyme. This conclusion was confirmed by the experiments of Omarov et al. [13] performed on roots of plants fed with tungstate. Our results show that AO activity in both the embryo and aleurone layer of the developing wheat seed begins to increase when the water content of seeds begins to decrease. Indeed, the water content of the seed attained a peak at 25 DAP and then reached its lowest level at maturity, whereas a linear increase in fresh and dry matter of the seed was observed between 10 and 30 DAP (data not shown). After this age, the activity of AO in the embryo showed a sharp increase, reaching a maximum at 40 DAP (Table 2). As for the increase of AO activity in the embryo, it followed the pattern of MoCo activity observed in the embryo. Thus, MoCo and AO activities reached their maximum level at 40 DPA, whereas AO activity in developing seed parts increased sharply at 30 DAP. The increase of MoCo activity in the embryo and aleurone layer accompanied their AO activity during seed development and maturation. Although we did not measure the ABA content in the embryo and aleurone layer of developing and maturing seeds, Kawakami et al. [19] showed that the ABA content in embryos of wheat resistant to pre-harvest sprouting began to increase at 30 DAP and reached maximum at 40 DAP. Thus, our results are in accordance with the data obtained by Kawakami et al [19]. In fact, when seeds enter the stage of maturity the activity of AO remains almost at steady levels, whereas the ABA content decreases two times .

XDH plays a key role in catabolic pathways of purines from which ureides such as allantoin and allantoic acid are produced [11, 20] XDH activity data from embryos and aleurone layers of both dormant and developing seeds are presented in Tables 1 and 2. Our results show that XDH activity remains at a steady level throughout seed development and maturation, while ureides increase in a correlated fashion with the increase in AO activity. This observation raises interesting questions concerning the role of AO in ureide biosynthesis.

It should be pointed out that such environmental stresses as drought, salinity and chilling cause dehydration of plant cells. Similarly, natural seed maturation process also leads to dehydration of cells in seeds. It has also been well established that there is correlation between ABA content and plant response to dehydration. Furthermore, plant cells respond to environmental stresses by producing reactive oxygen species (ROS). There is also substantial data indicating that molybdo enzymes, xanthine oxidase and AO are the main cytosolic producers of ROS during oxidative stress in animal cells . Both superoxide anion and hydrogen peroxide are produced via either a one-electron or two-electron transfer in the reaction of animal AO with oxygen . Based on the similarity of animal and plant AOs, we propose that the plant enzyme also produces free oxygen radicals during the conversion of abscisic aldehyde to ABA. Furthermore, lignostilbene dioxygenases, the key enzymes in ABA biosynthesis, which use oxygen in the conversion of violoxanthin and neoxanthin to xanthoxin, may also produce superoxide anion . Thus, increased ABA synthesis, which in plant cells can rise from 10- to 50-fold during environmental stresses, results in the increase of ROS.

Uric acid, a product of XDH reaction, in living cells is oxidized to allantoin by the enzyme uricase, and also non-enzymatically as potential antioxidant by ROS. Uric acid is shown to be a powerful,water-soluble ROS scavenger in animal cells. It is an effective inhibitor of ROS at levels found in human plasma, whereas ascorbate and glutathione are protective at concentrations exceeding those of uric acid . Uric acid may also play an effective role in the protection from ROS attack and removing the oxidative stress in plants. For example, catabolism of hypoxanthine in maize root tips was accelerated more than 3.5-fold by salt stress or such stress resulted in the accumulation of allantoin in tissue culture leading to increased conversion of uric acid to allantoin. Thus, we propose that increased synthesis of ABA in seeds leads to the increase of ROS content during their maturation. ROS chemically oxidize uric acid to allantoin, thus, the amount of ureides increaseas result of seed dehydration during maturation. It is well known that glutamine and asparagine are important sources of ammonia during seed germination. Increased synthesis and accumulation of ureides during seed maturation may also play important roles during seed germination later because they are an important reserve and transport forms of nitrogen in plants. Based on these results we propose that the main function of plant XDH is in the production of uric acid, which plays an important role in the production of ROS scavengers, uric acid and ureides. Thus, XDH activity remained at a steady and considerably high level during seed development, while the increased allantoin synthesis followed the pattern of AO activity increase (Table 2, Fig.5).

Calculation of DW: at each stage of seed development, 20 of 40 freshly excised embryos from the spikes and seeds were homogenized in buffer and the extracts were used for allantoin and total antioxidant determinations. Another 20 of 40 seeds were dried and weighed.

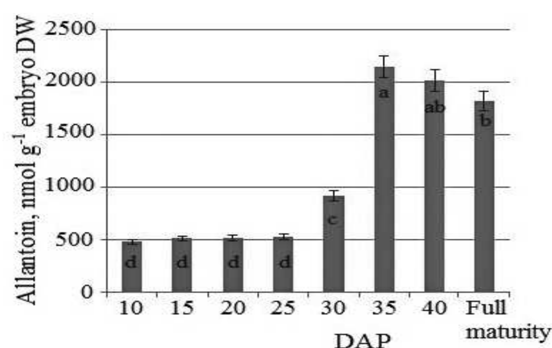

Figure 5 – Total ureides (allantoin) content in the embryo of developing and maturing wheat seeds.

Conclusions

In developing wheat seeds two molybdenum-containing enzymes, AO and XDH, are synthesized constitutively. Whereas, NR, the third molybdoenzyme, is induced by nitrate. Seed molybdoenzymes may play an important role in developing cereal seeds. It has been known that molybdenum-deficiency decreases the activity of plant molybdoenzymes, and in molybdenum-enriched soybean seeds N accumulation, seed yield, and seed protein content increase(Campo et al., 2009). However, biochemical relationships among molybdoenzymes in the maturing wheat seed is very much in need of further investigations. This would help understand the biochemical mechanism of pre-harvest sprouting of grains, which remains to be a major problem in agriculture.

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