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The prevention of pre-harvest sprouting

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B. Table of Contents

C. Executive Summary 3

D. Research Objectives 4

E. Methods and Results 4

F. Impact, Relevance, and Technology Transfer 20

G. Project Activities/Outputs 21

H. Project Productivity 21

I. Future Work 21

J. Literature Cited 22

C. Executive Summary

Pre-harvest sprouting (PHS), the germination of cereal grain on the mother plants before harvest, is a major source of losses for the grain/flour-producing industry in Kazakhstan. It would therefore be of use to be able to rapidly select cereal varieties that are tolerant to PHS. We found that the activity of the enzyme nitrate reductase (which is expected to rise during germination) was inhibited during the early stages of germination in seeds of a PHS-tolerant but not a PHS-sensitive wheat cultivar. This inhibition was specific to nitrate reductase activity and could be a trait that could be used as a marker of PHS-tolerant cultivars.

The plant growth regulator abscisic acid (ABA) has been demonstrated to have an important role in regulating seed embryonic maturation and germination and we set out to determine whether differing levels of seed embryo ABA and/or sensitivity of the embryo to ABA can determine the susceptibility of wheat seeds to PHS in PHS-sensitive and PHS-tolerant cultivars. Our results showed a clear correlation between seed ABA content and dormancy. Furthermore, we found that the PHS-tolerant cultivar had a greater embryonic sensitivity to ABA.

A key enzyme in the ABA biosynthetic pathway, aldehyde oxidase, requires molybdenum (Mo) as a cofactor and recent findings suggest that Mo influences wheat seed dormancy. We therefore attempted to improve ABA biosynthesis (and thus reduce PHS) via foliar applications of Mo to wheat plants. Our results showed that such foliar Mo treatments led to increased levels of seed dormancy, particularly in the PHS-sensitive cultivar and this could be correlated with increased levels of seed ABA. Thus, agro-management practices such as better Mo fertilization can be employed to further reduce PHS. We also attempted to reduce PHS via seed priming (the controlled imbibition of seeds followed by drying), with a solution of Mo. Our results showed that such a treatment reduced seed germination in PHS-tolerant cultivars. This was correlated with accumulation of Mo in the seed and incorporation of infiltrated Mo into the protein fraction of primed seed extracts. Furthermore our results showed that Mo-priming led to an increase in seed ABA levels, which could be correlated with a rise in aldehyde oxidase.

During the course of the project, the Kazakhi PI and trainees learned advanced biochemical and physiological techniques during their visits to the Israeli lab. Furthermore, they became acquainted with the use and maintenance of modern laboratory equipment and computers and were able to interact with a large number of scientists with expertise in fields related to their work. The purchase of equipment from Israel and the discussions regarding scientific and research organization, contributed both to the scientific and administrative skills acquired during this project and to increasing the technological base of the Kazakhi laboratory.

D. Research Objectives

Pre-harvest sprouting (PHS) is a process by which cereal grain with weak dormancy sprouts on the mother plant before harvest or immediately after harvesting under storage conditions. Cereal grain requires water to break dormancy and germinate but rain does not only fall at desired times and can fall during final grain ripening. The rain plus rain-induced humidity can trigger a sequence of genetic and physiological processes leading to PHS.

Cereals are of extreme importance in Central Asia where they constitute a vital component of the staple diet. PHS was one of the factors that led to the collapse of the Soviet economy due to its massive damaging effect on the grain/flour producing capacity of the country. Indeed, Kazakhstan, as a large grain producer still suffers large-scale crop losses due to PHS.

Overall Aim

To prevent pre-harvest sprouting (PHS) of wheat seeds by increasing the synthesis of the plant hormone ABA and to accelerate wheat growth rate, thus allowing early grain maturation before the onset of the rainy season in Kazakhstan.

Specific Objectives

(i) To identify early metabolic markers of PHS sensitivity

(ii) To determine physiological mechanisms which determine the lack of dormancy and the propensity to germinate before harvesting.

(iii) To identify agrotechniques that can minimize PHS in the field

(iv) To determine the effect of PHS-minimizing agrotechniques on physiological mechanisms of PHS.

E. Methods and Results

Methods

Plant material. Pre-harvest sprouting resistant cultivar Lutescence-70 and susceptible cultivar Novosibirskaia-67 were obtained from the Kazakh Research Institute for Agriculture in Almalibakh (Almaty). Both cultivars were grown in the field at the AgroBioStation of the Kazakh National University near the city of Almaty (South Kazakhstan) and in the Kurgalzhin region near Astana city (North Kazakhstan) in 2001 and 2002.

Plant growth conditions. For growth chamber trials, wheat seeds were pre-germinated in an incubator at 20°C and planted in free-draining, 11 cm-diameter pots filled with acid-washed quartz

sand. All treatments were replicated four times. In greenhouse experiments, plants were irrigated with a modified Hoagland's solution (Trivedi and Erdei, 1992) and grown in a growth room at 20°C/15°C day/night temperature under a 12 hr photoperiod. Field trials were carried out at the two sites mentioned above.

Inception of, and release from dormancy during grain development. In order to harvest at the same development stage for all the samples, approximately 250 spikes were tagged in the field plots at ear emergence. Starting 2 weeks after tagging, and twice a week during grain filling, 50 spikes were harvested and hand-threshed. Two kernels per spike were collected for determination of dry weight and moisture content. 50 uniform seeds were placed in a forced-ventilation oven at 30°C for 48 hrs at which time they reached a constant weight. These artificially dried seeds were kept at 5°C until the last sample was taken at the end of grain ripening. The seeds were then transferred to room temperature and incubated for 24 hrs. Germination tests for all samples were immediately conducted in the dark at 25°C in 10 cm petri dishes lined with three sheets of Whatman No. 1 filter paper, saturated with 3 mls distilled water. Three replicates of 50 seeds were used per cultivar-sampling date and the remaining seeds were kept at room temperature to enable germination tests to be repeated after 3 d. Germinated seeds (those whose coleoptile had emerged through the husk) were counted and expressed as a percentage of germinated seeds out of the total number of imbibed seeds. By analyzing the results of the initial test and the one repeated after the 3-day storage period, it was possible to distinguish dormancy from lack of viability in artificially dried immature seed (dormancy is defined as the inability of viable seeds to germinate under conditions otherwise adequate for germination). Embryos or seeds that germinated were counted daily for 12 days. Germination index was calculated with maximum weight given to the embryos or seeds that germinated first and less weight to those that germinated later (Reddy and Metzger, 1985).

Foliar treatments with Mo. Seeds of PHS-susceptible wheat cultivar *Novosibirskaia-67* and PHS-resistant *Lutescence-70* were germinated and cold-treated for four weeks at 5°C. The cold-treated seedlings were planted in 11 cm-diameter pots filled with acid-washed swimming pool filter sand. The wheat cultivars were grown in a growth room at 20°C/15°C day/night temperature with a 12-hour photoperiod. All pots were irrigated twice weekly with Mo-deficient Hoagland's solution. Enough nutrient solution was applied to ensure a sufficiently high leaching fraction to prevent accumulation of salts. Pots were otherwise watered daily with distilled water. At the 6-leaf stage, half of the plants were sprayed with a foliar application of 10, 50 and 100mM Mo as sodium molybdate, or tungsten as sodium tungstate. The remaining plants were not sprayed and served as

the control. The Mo (or tungsten) application was repeated at the flag leaf stage. 0.5% Tween 20 was added as a surfactant.

Individual ears were tagged at anthesis and harvested 60 days thereafter. The moisture contents at this stage were approximately 16% and 20% for *Lutescence-70* and *Novosibirskaia-67*, respectively. The seeds were subsequently stored at 5°C for 7 days which brought the moisture content down to approximately 13% and 15%, respectively. The seeds were then stored at -30°C until further use.

Seed Priming. Seed priming was carried out according to a modified method of Rowse (1996) Dormant dry seeds of both PHS-resistant and PHS-sensitive wheat cultivars were soaked in distilled water or Mo solution and incubated until full hydration of the seeds. Imbibition and incubation of wheat seeds were carried out under different temperatures (5°C – 30°C), and for different time periods. All seed treatments were carried out under a layer of water/Mo solution of about 1.5-2.0 cm to minimize oxygen diffusion in order to prevent seed germination. Full hydration of wheat seeds occurred between 28-32 hrs after imbibition depending on the temperature. Seeds were then dried and germinated. Plants were grown and seed collected from mature plants for germination and other tests.

Sensitivity to ABA during germination. Germination tests on post-harvest seed stored at room temperature were conducted by adding ABA into the germination medium.

Germination analysis. Germination tests were carried out in Petri dishes lined with three Whatman No. 1 filter papers moistened with distilled water. Four replicates of 25 wheat seeds were used per treatment. In the case of different temperature treatments, seeds were germinated in a thermostat-chamber. Germination was recorded daily for 7 days after the first seeds of a particular cultivar had germinated. Radical protrusion was used as a criterion for germination. Germination was presented in the form of accumulated percentages or in the form of a germination index (G.I.) (Walker-Simmons, 1988). The G.I. was calculated as follows:

$$G.I. = \frac{7.n1 + 6.n2 + \ldots\ldots\ldots + 1.n7}{\text{total days (7) x total seed no. (100).}}$$

where:

7, 6,.....1 = weightings given to seeds germinating on the first, second up to the seventh day of the germination test, respectively.

n1, n2,..n7= the number of seeds that germinate on the first, second up to seventh day of the germination test respectively.

The germination index was modified in that the first four days of the germination test during which no germination took place in any of the treatments was disregarded in terms of the germination index. Day 5 of the germination test would thus be day 1 for the purpose of determining the germination index.

Quantification of ABA during grain development. Samples of 10 spikes were harvested at different stages of maturation from each cultivar throughout the grain development period. They were frozen in liquid nitrogen and stored at 70°C for determination of endogenous ABA content. The samples were then thawed and the grains from the central part of each spike lyophilized. The analyses were carried out on the embryo-half of each grain in order to avoid the dilution effect on ABA caused by the endosperm. Samples were milled and 50 mg of dry flour was extracted with water. ABA determination was performed according to Kawakami *et al.* (1998) and the results were expressed as ng ABA per g of dry weight (DW).

ABA Determination (Immunoassay). The embryo and endosperm of wheat seeds were ground to a fine powder with liquid nitrogen and suspended in solution consisting of 80% methanol, 2% glacial acetic acid and butylated hydrotoluene (20 mg l^{-1}). The extracts were shaken in the dark for 24 hours and then centrifuged at 12000 rpm in a microfuge at 4°C for 20 minutes. Supernatants were diluted 10 times in TBS (150mM NaCl; 25 mM Tris-HCl, pH 7.5). ABA was quantified by the Phytodetek – ABA immunoassay (Sigma, USA) according to Mertens *et al.* (1983).

Nitrate analysis. Nitrate determination was carried out according to Cataldo *et al.*, (1975). Total protein content of the milled kernel was carried out using the standard Kjeldahl technique.

Seed Mo content. Seeds primed in Mo solutions were washed several times with double distilled water prior to Mo determinations. Mo content infiltrated into the seed during priming was determined using both direct and indirect methods. For the direct method, Mo content in extracts of embryo and endosperm was determined using an atomic absorption spectrophotometer (AAS-IN,

Karl-Zeiss, FRG). For the indirect method, extracts of the MoCo-deficient mutant *nit-1* of *Neurospora crassa* were used to measure MoCo activity of seed extracts which reflects Mo concentration (Savidov *et al.*, 1998).

Nitrogen assimilation enzyme activities

Nitrate reductase activity was determined according to Sagi *et al.* (1998). Nitrite reductase activity was measured according to Aslam and Huffaker (1989).

AO activity

The embryo and endosperm of wheat seeds were homogenized (1:3 w/v) with acid-washed sand and ice-cold extraction buffer (250mM Tris-HCl (pH. 8.5); 1mM EDTA; 10mM reduced glutathione (GSH) and 2mM DTT). Extracts were centrifuged at 27000g for 10 minutes at 4°C. 100µg supernatant was subjected to native PAGE in a Laemmli buffer system (Laemmli, 1970) at 4°C. After electrophoresis, AO activity was visualized by staining at room temperature with 0.1M Tris-HCl (pH 7.5); 0.1mM phenazine methosulphate; 1mM MTT; (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide) and 1mM indol-3-aldehyde. Activity of AO is estimated on the basis of MTT reduction, which results in the development of specific formazan bands (Zdunek-Zastocka *et al.*, 2004).

Results

(i) Identification of an early marker of PHS-sensitivity

In order to select possible metabolic markers of PHS-sensitivity, we took note of the fact that the presence of highly active molybdenum co-factor (MoCo) has been previously shown in dormant seeds of wheat and barley (Alikulov and Schieman, 1985). MoCo is a common cofactor for all plant Mo-enzymes such as nitrate reductase (NR), xanthine dehydrogenase (XDH) and aldehyde oxidase (AO) (Mendel, 1998; Sagi *et al.*, 1998; Omarov *et al.*, 1998). The activity of AO (an enzyme of the plant hormone, ABA, biosynthetic pathway) steeply increases when seeds enter into the desiccation stage at approximately 40 days after anthesis (DAA) and is correlated with an increase in ABA content during seed maturation (Kawakami *et al.*, 1998). ABA plays a pivotal role in the establishment of primary seed dormancy, the rest period that normally lasts until after harvest, thereby ensuring against sprouting on the ear (Derera, 1989; King, 1989; Hilhorst and Karssen, 1992; Hilhorst, 1997). We therefore elected to measure the activity of NR, an enzyme that would be expected to rise during the germination process. Furthermore, we employed the PHS-tolerant wheat cultivar, Lutescence-70 and the PHS-sensitive cultivar, Novosibirskaia-67 to

determine whether NR is more active in the PHS-sensitive cultivar and could therefore act as a marker of PHS-sensitive cultivars.

Table 1 shows the percent germination of seeds from Lutescence-70 and Novosibirskaia-67, at various periods during seed development. PHS-sensitive Novosibirskaia-67 showed strongly reduced dormancy compared with PHS-tolerant Lutescence-70 (Table 1). Both Lutescence-70 and Novosibirskaia-67 seemed to follow a similar pattern of release of dormancy during development, with relative differences in the stage at which the seed was able to complete germination. No precocious germination occurred prior to the onset of dormancy during seed development, as both cultivars were fully dormant at the beginning of the germination test 2 weeks after anthesis. After the onset of dormancy, however, Novosibirskaia-67 exhibited precocious germination, starting to lose dormancy progressively from 25 days after anthesis (DAA) while Lutescence-70 remained largely dormant until full maturity. Furthermore, Novosibirskaia-67 showed a higher germination percentage than Lutescence-70 at the end of the grain ripening process, reaching 65% germination at seed maturity.

In order to determine NR activity in seeds of PHS-tolerant and sensitive cultivars, seeds were imbibed in KNO_3 solution (nitrate activates the NR enzyme) and embryos excised at the appropriate times. Alternatively, embryos were first excised (detached) from dry seeds and then imbibed in nitrate. Detached embryos of both cultivars showed significant levels of NR activity as early as 4 hours after imbibition (data not shown). Enzyme activity increased thereafter to peak activity at 12 hours after imbibition (Table II) followed by a decrease to a minimum activity after 36 hours.

Table I. Germination capacity of the grains of PHS-tolerant and PHS-sensitive wheat cultivars harvested at different stages of development.

Days after anthesis (DAA)	Lutescence-70		Novosibirskaia-67	
	Germination (%)	Dry weight (%)	Germination (%)	Dry weight (%)
10	0	23	0	21
15	0	30	0	30
20	0	35	0	37
25	3-5	52	6-8	50
30	7-10	67	10-13	56
35	11-12	85	13-16	75
40	10-11	94	15-19	90
45	14-16	96	22-30	93
50	20	98	32-45	93
55	22-25	97	40-60	92
Full maturity	25	100*	55-65	93

In the attached embryos (i.e. within the seeds along with the endosperm) from PHS-tolerant Lutescence-70, induction of NR activity was inhibited for 24 hours after imbibition (Table II). In contrast, NR activity was detected in detached PHS-sensitive Novosibirskaia-67 embryos in the early hours after imbibition. The inhibition of NR activity in PHS-tolerant attached embryos may be due to an inhibitory factor produced by the endosperm. Other enzymes of nitrate assimilation pathway, such as nitrite reductase, NiR, (Table II), glutamine synthase and glutamate dehydrogenase were found to be present in the attached embryo right from the initiation of germination in wheat (Gilmanov *et al.*, 1988). **Thus, the inhibitory effect of PHS-tolerant seed endosperm seems to be specific for NR induction, a trait that could be used as a marker of PHS-tolerant cultivars..**

Table II. Nitrate reductase (NR) and nitrite reductase (NiR) activities in attached and detached embryos of PHS-tolerant and PHS-sensitive wheat seeds.

	Lutescence 70				**Novosibirskaia 67**			
Incubation time (h)	**Attached embryos**		**Detached embryos**		**Attached embryos**		**Detached embryos**	
	NR	**NiR**	**NR**	**NiR**	**NR**	**NiR**	**NR**	**NiR**
12	0.0	8.5	4.3	22.5	0.7	17.6	5.2	23.7
16	0.0	10.3	3.5	22.0	1.2	18.7	5.0	21.4
20	0.0	15.5	2.7	18.6	2.4	21.8	4.3	18.0

NR activity is expressed as μmol NO^-_2 mg^{-1} protein $hour^{-1}$.
NiR activity is expressed as nmol NO^-_2 mg^{-1} protein $hour^{-1}$.

In order to confirm that the endosperm of PHS-tolerant seeds was indeed responsible for inhibiting NR activity in the embryo, we incubated detached, imbibed embryos from the PHS-tolerant and sensitive cultivars with endosperm extracts from each of the cultivars. Endosperm extracts of both cultivars led to decreased NR activity of detached embryos from both cultivars with the PHS-tolerant endosperm extracts having a greater effect on PHS-sensitive embryos than PHS-sensitive extracts (Table III). However, PHS-sensitive endosperm extracts led to a similar large decrease in NR activity in PHS-tolerant embryos as did PHS-tolerant endosperm extracts. These results suggest that (i) Seed endosperm is the source of the NR inhibitory factor. (ii) PHS-tolerant endosperm contains more (or more active) NR inhibitory factor than PHS-sensitive endosperm. (iii) PHS-tolerant embryos are more sensitive to the NR inhibitory factor.

It is expected that the inhibitory factor from the endosperm should disappear as the seed germinates. Table IV shows that PHS-tolerant endosperm extracts gradually lost their ability to

inhibit NR activity of both PHS-tolerant and sensitive embryos, the longer the period between seed imbibition and endosperm excision.

Table III. Effect of PHS-tolerant and PHS-sensitive endosperm extracts on the induction of NR in their detached embryos after 12 h incubation with 60 mM KNO_3 (every 4 hrs detached embryos were transfered to fresh endosperm extracts + KNO_3).

Endosperm extracts and detached embryos combinations	**NR activity in detached embryos**
PHS-tolerant detached embryo	4.5
PHS-tolerant seed endosperm + its detached embryo	0.5
PHS-sensitive detached embryo	5.5
PHS-sensitive seed endosperm + its detached embryo	2.9
PHS-tolerant seed endosperm + detached embryo of PHS-sensitive seeds	1.9
PHS-sensitive seed endosperm + detached embryo of PHS-tolerant seeds	0.9

NR activity is expressed as $\mu mol\ NO^-_2\ mg^{-1}$ protein $hour^{-1}$.

Table IV. Time course of NR inhibition in PHS-tolerant and sensitive embryos by PHS-tolerant endosperm extracts.

Time after seed imbibition that endosperm was extracted (hrs)	**6**	**12**	**18**	**24**	**30**
NR activity in PHS-tolerant embryos	0.7	1.8	3.2	4.3	4.7
NR activity in PHS-sensitive embryos	1.9	2.9	4.7	5.6	5.7

NR activity is expressed as $\mu mol\ NO^-_2\ mg^{-1}$ protein $hour^{-1}$.

(ii) Determination of physiological mechanisms that determine the lack of dormancy and the propensity to germinate before harvesting.

ABA has been demonstrated to have an important role in regulating seed embryonic maturation and germination (Black, 1991). Exogenously applied ABA promotes embryonic development and is an effective inhibitor of embryonic germination in immature wheat grains (Dunwell, 1981). Grains removed from the maturing plant of a sprouting-susceptible cultivar and placed in water will easily germinate (Black *et al.*, 1987). In contrast, grains removed from a maturing sprouting-resistant cultivar will not germinate easily. We therefore tested whether ABA might be the endosperm factor responsible for NR inhibition by incubating detached PHS-tolerant

embryos in the presence of different ABA concentrations plus KNO_3 for 12 hours. Increasing concentrations of ABA increased inhibition of NR activity in the detached embryos (Table V).

Table V. Effect of ABA on NR activity in PHS-tolerant embryos

ABA concentration (nM)	**0**	**10**	**100**	**10**
NR activity	4.8	4.3	3.5	1.3

NR activity is expressed as $\mu mol\ NO^-_2\ mg^{-1}$ protein $hour^{-1}$.

If ABA indeed has a role in PHS, then it would be expected that PHS-sensitive seeds would contain less ABA and therefore would be prone to pre-harvest germination. Therefore, the ABA content of seeds from PHS-resistant and PHS-sensitive cultivars was determined during seed development. The amount of endogenous ABA decreased through grain ripening (from DAA 40 onwards) in both cultivars (Table VI). This decrease in both cultivars may be necessary to trigger the germination process since ABA content was lowest at seed maturity, when the germination percent was highest. Furthermore, PHS-resistant Lutescence-70 seeds exhibited a higher ABA content than PHS-sensitive Novosibirskaia-67 throughout development, reaching 70% higher by full maturity. This suggests that differences in ABA content likely play a role in the pre-harvest resistance of Lutescence-70.

Table VI. ABA content of whole grains of PHS-tolerant and PHS-sensitive wheat cultivars at different stages of development.

Days after anthesis (DAA)	***Lutescence-70***			***Novosibirskaia-67***		
	ABA content ($ng\ mg^{-1}$ DW)	Germination (%)	Dry weight (%)	ABA content ($ng\ mg^{-1}$ DW)	Germination, (%)	Dry weight (%)
10	0.65	0	23	0.40	0	21
15	0.70	0	30	0.40	0	30
20	0.70	0	35	0.45	0	37
25	0.65	3-5	50	0.45	6-8	40
30	0.75	7-10	65	0.45	10-13	56
35	0.80	11-12	75	0.55	13-16	75
40**	0.95	10-11	89	0.60	15-19	86
45	0.85	14-16	92	0.60	22-30	88
50	0.60	20	95	0.40	32-45	92
55	0.60	22-25	98	0.35	40-60	95
Full maturity	0.50	25	100*	0.30	55-65	95

*100% - dry weight of 50 seeds of Lutescence-70. **-time of maximum fresh weight.

ABA contents were also measured in excised embryos and endosperms from both cultivars. Embryonic ABA in both cultivars peaked as the grain reached maximum fresh weight (40 DAA) (data not shown). After that time the grains dried rapidly and embryonic ABA in both cultivars declined to a low level. Embryonic ABA levels in the sprouting-susceptible *Novosibirskaia-67* grains were consistently about 20 to 40 % lower that in the sprouting-resistant *Lutescence-70* grains and averaged 70-75% of the levels *Lutescence-70* at the time of maximum fresh weight. Generally, ABA levels of both cultivars were 2-fold higher in the embryo than in the endosperm with highest endosperm ABA levels at maximum seed fresh weight. Thereafter, ABA decreased rapidly as the grain began to desiccate and decrease in fresh weight

The experiment above measuring inhibition of NR activity of PHS-tolerant and sensitive embryo by endosperm extracts (Table III) suggested that PHS-tolerant embryos are more sensitive to the NR inhibitory factor. If this factor is ABA then PHS-tolerant embryos should show greater inhibition of germination by the lower concentrations of ABA than PHS-sensitive seeds. Table VII shows the results of incubating PHS-tolerant and PHS-sensitive embryos with different concentrations of ABA in the germination medium. Throughout seed maturation, the germination index for embryos from both cultivars germinated in water was at or near the same levels A comparison of embryonic sensitivities to ABA showed that during grain maturation ABA inhibited embryonic germination more in the PHS-resistant cultivar *Lutescence-70* that in the PHS-susceptible cultivar *Novosibirskaia-67*. Embryos from both cultivars were more sensitive to ABA during the earlier stages of development. Embryonic sensitivity to ABA was first measured at about 25 DAA when isolated embryos can germinate. At that time a 10-fold higher concentration of ABA was required to reduce the germination index in *Novosibirskaia-67* embryos compared to *Lutescence-70*. Similarly, large cultivar differences in sensitivity to ABA continued from 25 to 40 DAA as the grains steadily increased in fresh weight. Near 40 DAA, grains of each cultivar reached maximum fresh weight, the desiccation stage of grain maturation commencing thereafter. The grains rapidly dried after 40 DAA and embryonic sensitivity to ABA declined. After the grain reached the maximum percentage dry weight at 45 to 50 DAA, sensitivity to ABA was lost, and *Novosibirskaia-67* embryonic germination was no longer blocked by ABA at any concentration. However, the highest concentration of 50 μm ABA still reduced the *Lutescence-70* embryonic germination index by 50% even 10 days after the final 90% dry weight was achieved at 45 DAA.

Table VII. Embryonic sensitivity (the germination index) of PHS-tolerant and PHS-sensitive wheat grains to ABA at different stages of development.

	Lutescence-70				*Novosibirskaia-67*			
	ABA (μM)							
DAA	0 (H_2O)	0.5	5.0	50	0 (H_2O)	0.5	5.0	50
25	110	55	0,0	0,0	117	105	70	40
30	112	80	15	0,0	120	105	65	45
35	115	65	10	0,0	120	105	80	45
40	112	55	45	15	120	120	95	45
45	110	90	65	35	120	120	115	100
50	115	110	80	60	120	120	115	110
55	120	120	100	50	120	120	120	110
Full maturity	120	120	110	20	120	120	115	110

Thus, there is considerable circumstantial evidence that ABA is involved in regulating the onset of dormancy and in maintaining the dormant state, i.e. a clear correlation between ABA content of the grains and dormancy. Furthermore, we have found that the cultivar with more resistance to sprouting has a greater embryonic sensitivity to ABA suggesting that ABA has a role in preventing pre-harvest sprouting.

(iii) Identification of agrotechniques that can minimize PHS in the field

a) Foliar applications of Mo

Several researchers working on pre-harvest sprouting in maize have reported that molybdenum (Mo) deficiency can lead to pre-harvest sprouting (Tanner, 1978; Farwell *et al.*, 1991). It is well known that the germination of wheat seeds is largely dependent on cultivar. We surmised that one possible factor responsible for the difference in germination resistance of different wheat cultivars is the presence or deficiency of Mo in wheat seeds which may affect Mo-enzymes such as AO which is part of the ABA biosynthetic pathway (Koshiba *et al.*, 1996; Marin and Marion-Poll, 1997; Schwartz *et al.*, 1997). Furthermore, it has been shown that the presence of exogenous Mo enhances the activity of aldehyde oxidase in barley roots, while replacement of Mo with tungsten (its chemical analog) results in total inactivation of aldehyde oxidase in barley roots (Omarov *et al.*, 1998). According to data at the Institute for Soil Research of the Kazakh Academy of Sciences, most soils of Kazakhstan contain 8-12 times less Mo than the critical concentrations required for vigorous plant development. Therefore, foliar fertilization of wheat plants with Mo represents a very localized targeted fertilization that might have a beneficial effect on PHS.

We applied Mo solution at the flag leaf stage, collected mature seeds and examined their germination ability. We used germination index as a measure of ability to germinate since this

parameter also takes into account time to germination. Table VIII shows that a 100mM Mo application resulted in a large reduction in the germination index of PHS-resistant Lutescence-70, germinating over a 7-day period. In fact, seed from the 100mM Mo-treated plants of the very dormant Lutescence-70 did not germinate at all and had to be treated with 100μM gibberellic acid (GA) before germination occurred. Even with this GA treatment there was a significant difference in the dormancy between the seed from Mo- and tungsten-treated and Mo-deficient plants. Tungsate is an inhibitor of Mo-enzymes. These results suggest that Mo has a significant effect on the dormancy of Lutescence-70. In the case of PHS-susceptible Novosibirskaia-67 seeds, significant differences were also observed between the three treatments but only during the initial stages (2-3 days), of the germination test (data not shown). However, the final germination percentages (at 7 days) did not differ significantly.

Table VIII. Effect of foliar applications of molybdenum and tungsten on the seed dormancy of PHS-tolerant Lutescence-70 and PHS-sensitive Novosibirskaia-67.

Treatment	Germination Index
Lutescence-70	
Molybdenum-deficient	115
+ 100 mM molybdate	25
+ 100 mM tungstate	118
Novosibirskaia-67	
Molybdenum-deficient	110
+ 100 mM molybdate	100
+ 100 mM tungstate	105

PHS-resistant Lutescence-70 seeds contain higher levels of ABA and embryos are more sensitive to ABA than PHS-susceptible Novosibirskaia-67 (Tables VI and VII). Since Mo is an essential cofactor for the ABA biosynthetic enzyme aldehyde oxidase we examined whether differences in seed ABA levels could account for the contrasting responses of the two cultivars to foliar application of Mo. To answer this question, seed Mo and ABA levels from Mo-sprayed plants and controls were monitored in the seed from both cultivars (Table IX).

Table IX. Effect of foliar molybdenum application on the molybdenum and ABA content in seeds of PHS-tolerant Lutescence-70 and PHS-sensitive Novosibirskaia-67.

Treatment	Molybdenum content (µg/g FW)	ABA content (ng/mg)
	Lutescence-70	
Mo-deficient	0.02	0.30 ± 0.07
+10mM molybdate	0.05	0.45 ± 0.12
+50mM molybdate	0.18	0.53 ± 0.17
+100mM molybdate	0.56	0.70 ± 0.18
+10mM tungstate	0.02	0.20 ± 0.10
+50mM tungstate	0.03	0.20 ± 0.07
+100mM tungstate	0.03	0.15 ± 0.05
	Novosibirskaia-67	
Mo-deficient	0.02	0.14 ± 0.02
+10mM molybdate	0.03	0.15 ± 0.02
+50mM molybdate	0.15	0.23 ± 0.04
+100mM molybdate	0.50	0.36 ± 0.07
+10mM tungstate	0.03	0.10 ± 0.02
+ 50 mM tungstate	0.02	0.08 ± 0.02
+100 mM tungstate	0.01	0.10 ± 0.03

Increasing concentrations of Mo in the foliar spray led to an increase in Mo content of both Lutescence-70 and Novosibirskaia-67 seeds. This increase in Mo content could be correlated with a rise in seed ABA levels. ABA content of PHS-resistant Lutescence-70 seeds was approximately twice that of Novosibirskaia-67 in all Mo treatments. Thus, the large reduction in the germination index of seeds from Mo-sprayed Lutescence-70 plants compared to Novosibirskaia-67 (Table VIII), is likely a result of Mo-induced increases in seed ABA content.

b) Seed priming with Mo

Although our study shows that Mo applied as a foliar application increases the dormancy of both PHS-resistant and PHS-susceptible wheat cultivars (Table VIII and data not shown), treating young seedlings with high ion concentrations could be deleterious. An alternative method of delivering Mo is seed priming, the controlled imbibition of seeds followed by drying. Seeds are then germinated, plants grown to maturity and the seed produced may be more resistant to PHS. This technique has evoked great interest in recent years as a technique to improve crop establishment and control PHS. Seed priming also improves seed vigor, speed and synchronization of emergence, increased tolerance to sub-optimal environmental conditions, improved stand size and greater yield (Basu, 1994). In addition, the accelerated growth and development of wheat seedlings would allow early grain maturation of wheat well before the onset of the rainy season in

Kazakhstan. Furthermore, seed priming would decrease the need for wide spread soil and foliar fertilizations thereby presenting a cheaper and more effective alternative.

Table X shows the effect of seed priming with various concentrations of Mo on the germination of seeds taken from plants grown from primed seed. Priming with 25 or 50mM Mo had little effect on % germination of seeds from either PHS-tolerant or sensitive cultivars. However, 100mM Mo led to a 25% reduction in germination of PHS-tolerant seeds and only a small reduction in germination of PHS-sensitive seeds. As expected, PHS-sensitive seeds always showed greater % germination than PHS-tolerant seeds at all Mo concentrations.

Table X. Effects of seed priming in the presence of different concentrations of molybdenum and tungsten on the germination of wheat seeds of PHS-tolerant Lutescence-70 and PHS-sensitive Novosibirskaia-67.

Metal	Exogenous Mo Concentration (mM)	Percent germination	*Biomass of seedlings (% of control)
Lutescence-70			
Molybdenum	Water	22-24	100
	25	22-24	100
	50	22-25	108
	75	23-26	114
	100	16-19	115
Novosibirskaia-67			
Molybdenum	Water	56-65	100
	25	56-67	100
	50	57-59	100
	75	57-58	110
	100	50-52	100

*Total weight of 50 seedlings, e.g. shoots + roots

Our hypothesis is that priming of wheat seeds with high concentrations of Mo results in the accumulation of the metal in the seeds. Upon germination, Mo is transported initially to new roots and leaves of the seedlings. Later Mo is transported from the roots and leaves of adult plants to newly developing seeds that require Mo for ABA-synthesis. Thus, increased content of ABA in the seeds may result in the establishment of seed dormancy. In order to support this hypothesis, we next checked whether seed priming indeed led to increased accumulation of the metal in the primed seed.

Table XI. Seed molybdenum content in response to priming with the metal

Exogenous Mo Concentration (mM)	Seed molybdenum content (μg L^{-1}) ± S.D
Lutescence-70	
Water	235 ± 37
25	475 ± 96
50	655 ± 175
75	815 ± 155
Novosibirskaia-67	
Water	175 ± 40
25	365 ± 60
50	535 ± 150
75	720 ± 165

Measurement of Mo content of primed seeds by atomic absorption spectrophotometry clearly showed increasing accumulation of Mo in seeds of both cultivars with increasing Mo concentration in the priming solution. No significant difference in Mo levels between the wheat cultivars at each Mo concentration was found suggesting that seed coats of both cultivars have a similar permeability. Similar results were obtained using the indirect method of Mo measurement (see methods).

We next asked whether the Mo accumulated in the seeds during priming remained as a free metal or whether it was incorporated into Mo-containing macromolecules such as proteins. To this end, extracts from either water- or Mo-primed seeds from both cultivars were separated by gel-filtration on a Sephadex G-25. Two fractions were eluted, fraction A containing high molecular weight molecules such as proteins and fraction B containing low molecular weight molecules such as salts. Mo content of each fraction was then measured by the direct method.

Table XII. Distribution of molybdenum in different fractions of seed extracts of PHS-tolerant Lutescence-70 and PHS-sensitive Novosibirskaia-67.

Cultivar	Seed Priming Treatment	Mo content (μg L^{-1}) Fraction A	Mo content (μg L^{-1}) Fraction B
Lutescence-70	Water	20	21
	Mo (100mM)	42	135
Novosibirskaia -67	Water	17	22
	Mo (100mM)	32	138

Table XII shows that the seed Mo content of both cultivars was higher in fraction B from Mo-primed seeds than in fraction A. This result indicates that the majority of the infiltrated Mo accumulates as the free metal. However, the Mo content of fraction A was significantly increased by Mo priming. In the PHS-resistant Lutescence-70 seeds, Mo priming led to a 2-fold increase in the Mo content of fraction A, while in PHS-sensitive Novosibirskaia-67 seeds, there was a 1.5-fold increase. These results suggest that Mo infiltrated into wheat seeds during priming not only accumulated as free metal but was also incorporated into Mo-containing enzymes or proteins of fraction A.

In order to test whether priming with Mo leads to increased seed ABA content, seeds of PHS-resistant Lutescense-70 and PHS-sensitive Novosibirskaya-67 were primed with either 50mM Na_2MoO_4 or 50mM Na_2WO_4. Tungsate (WO_4) is an inhibitor of Mo-enzymes. Priming with water alone or untreated dormant seeds were used as controls.

Table XIII. Effect of seed priming with Mo on seed ABA levels (Relative Units)

Treatments	Lutescence-70			Novosibirskaya-67		
	Embryo	Endosperm	Total	Embryo	Endosperm	Total
Dormant seeds	1.75	1.41	3.16	1.39	0.37	1.76
H_2O	2.30	1.10	3.40	2.02	1.17	3.19
Na_2MoO_4	3.52	1.52	5.04	2.80	1.50	4.30
Na_2WO_4	1.68	0.34	2.02	0.98	0.55	1.53

The results showed that dormant seeds of Lutescence-70 contained more total ABA than Novosibirskaya-67, consistent with the PHS-resistance of Lutescence-70. Priming with H_2O alone led to a rise in ABA content of both varieties. Priming with Mo caused a greater rise in ABA levels than water alone in both varieties. However, PHS-resistant Lutescence-70 seed exhibited an approximately 15% greater rise in ABA levels compared to the H_2O treatment than PHS-sensitive Novosibirskaya-67. Priming with tungsten led to inhibition of both the H_2O-mediated and Mo-mediated rise in ABA level in both species and in fact reduced ABA content to below that of dormant seeds. This suggests that the rise in ABA levels when seeds are primed with H_2O and Mo is due to an increase in Mo-enzyme activity.

Since AO is a Mo-enzyme, we tested whether seed priming led to an increase in AO activity in both wheat varieties.

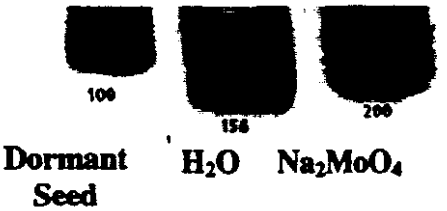

Figure 1. The effect of seed priming on the AO activity of PHS-resistant Lutescence-70 seeds. Activity gels were used to measure AO activity contained in 100μg protein. Values under the formazan-stained bands are relative density of the bands normalized to the value obtained with dormant seeds, which was set as 100%.

Figure 1 shows a 56% rise in AO activity when PHS-resistant Lutescence-70 seeds were primed with water alone. Although this treatment did not lead to an overall increase in seed ABA levels it did cause an increase in embryo ABA content (Table XIII). Priming with Mo led to twice the level of AO activity observed in dormant seeds consistent with an overall rise in seed ABA levels (Table 1).

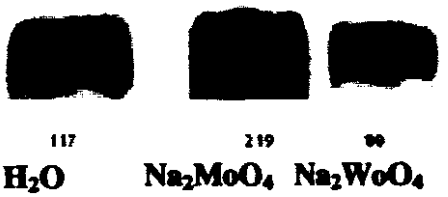

Figure 2. The effect of seed priming on the AO activity of PHS-sensitive Novosibirskaya-67 seeds. Activity gels were used to measure AO activity contained in 100μg protein. Values under the formazan-stained bands are relative density of the bands normalized to the value obtained with dormant seeds, which was set as 100%.

Mo priming also led to an increase in AO activity in PHS-sensitive Novosibirskaya-67 seeds and this increase was slightly less (13%) than that observed with PHS-resistant Lutescence-70 seeds. Although this is consistent with a higher rise in ABA levels in the PHS-resistant variety, it is not clear at this stage whether the small difference in AO activity rise with Mo priming between the two species, can account for differences in ABA levels. Priming with tungstate reduced AO activity to below control levels.

F. Impact, Relevance and Technology Transfer

The Kazakhi PIs and trainees have learned biochemical and physiological techniques through characterization of mechanisms of PHS in sensitive and tolerant wheat varieties. During their visits to the Israeli lab, they learned many useful skills including the use and maintenance of modern laboratory equipment, the use of modern computers with fast access to online literature searches and other resources. Another important impact on the Kazakhi PI's and trainees was the interaction with a large number of scientists with expertise in fields related to their work. Their participation in

regular lab meetings including powerpoint presentations of their results contributed to their development as scientists. Due to administrative difficulties in obtaining equipment in Kazakhstan, the purchase of equipment from Israel and the discussions regarding scientific and research organization, contributed both to the scientific and administrative skills acquired during this project and to increasing the technological base of the Kazakhi laboratory.

In practical terms, the impact of this project was two-fold: (i) An easy-to-measure marker of PHS-tolerant cultivars was identified, namely inhibition of nitrate reductase activity in imbibed PHS-tolerant seeds. This marker has the potential for rapid selection of PHS-tolerant wheat (and perhaps other cereal) cultivars. (ii) The finding that seed ABA levels and embryo sensitivity to ABA were lower in the PHS-sensitive cultivar led us to test foliar applications and seed priming with Mo, an cofactor of an enzyme of the ABA biosynthetic pathway. The major effect of Mo application was found on the PHS-tolerant cultivar possibly due its increased sensitivity to ABA. However, this could still be useful in reducing PHS that can still occur even in the tolerant cultivar particularly in Mo-deficient soils in Kazakhstan.

G. Project Activities/Outputs

Nov. 2002 – Feb. 2003: Dr Alikulov visited the Israeli partner's laboratory. The purpose of the visit was to discuss results, plan further experiments and to write a new US-AID proposal.

Sept. 2003 – Feb. 2005: Dr. Galia Shalakhmetova was a trainee in the Israeli partner's laboratory. The purpose of her visit was to characterize the effect of seed Mo priming on PHS-tolerant and sensitive varieties.

Publication of some of the results is in preparation.

H. Project Productivity

The project achieved the goals that were to be fulfilled during the allocated time span.

I. Future Work

No future work is anticipated except for preparation of results for publication.

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