Stress and Activity of Molybdenum-Containing Complex
(Molybdenum Cofactor) in Winter Wheat Seeds

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ABSTRACT

Molybdenum, applied in vivo, restored the damage from low temperature with winter wheat (Triticum aestivum, var “Sadovo I”) grown on acid soil and, in addition, sharply increased productivity (G Salcheva, D Georgieva, 1982; G Salcheva et al., 1977, 1979). Two fractions with molybdenum-cofactor activity in seeds were detected. One of them has a molecular weight of about 230 kilodaltons corresponding to xanthine oxidase activity and leaf nitrate reductase activity. The other has a molecular weight of about 60 kilodaltons. The ratio between the molybdenum-cofactor activity of these fractions was different in ‘mother’ seeds used in the experiment, in seeds obtained from the damaged plants, and in seeds obtained from the damaged plants restored by in vivo molybdenum addition. Every one of these fractions consisted of several components in which molybdenum-cofactor activity and stability in vitro was different. We suggest that plants store molybdenum as molybdenum carriers in these low molecular weight fractions.

During the past 10 years, it has been shown that addition of Mo to acid soils will protect wheat plants against damage caused by low temperature or water logging (2, 19, 20). If the soil is not acid, then Mo does not protect the wheat against low temperature (17, 18). In wheat where addition of Mo was beneficial, photochemical and photosynthetic activity of chloroplast and ribulose bisphosphate carboxylase was restored (15, 19, 21); however, the most rapid recovery was found in nitrogen metabolism, especially NR activity (18). A similar effect was found by L’vov et al. (8, 16), when legumes were grown on saline soil; the addition of Mo to the soil appeared to have an effect on the restoration of NR activity.

Molybdenum is found in the Mo-containing complex, (5, 7, 10). Mo-Co is found in a number of Mo-containing enzymes such as NR, XO, SO, and aldehyde oxidase and has both a structural and functional role in these enzymes (5, 7, 10). Biologically active Mo-Co has been found in dry seeds (1), leaf extracts (6, 14), roots (1; R Vunkova, unpublished observations) and cells of higher plant tissue cultures (12, 13). However, data on the influence of external stresses on Mo-Co activity have not been reported. In our previous investigations of wheat plants grown on acid soil, it was shown that Mo, which was applied prior to prolonged low temperature stress, caused a strong increase in both in vivo and in vitro NR activity, a decrease of the nitrate content, as well as an increase in Mo-Co activity in leaves and tiller nodes of winter wheat (3). Under these conditions, the diaphorase activity of NR remained unchanged (18).

The aim of the present work was to study the influence of low winter temperature upon the activity of Mo-Co in seeds obtained from wheat which had been grown on acid soil and either treated with Mo or not. From these seeds, we were able to separate Mo-protein carriers which differed in mol wt, Mo-Co activity, and in vitro stability.

MATERIALS AND METHODS

Plant Material and Growth Conditions. The experiments with winter wheat (Triticum aestivum, var “Sadovo I”) were carried out in 1984/1985 and 1985/1986. The plants were grown on cinnamon forest soil with pH 4.19 (KCI) at 60% soil moisture and fertilization with nitrogen (NH₄NO₃), phosphorous (superphosphate), and potassium (K₂SO₄). The single dose of nitrogen was 0.331g, of phosphate 0.372g, and of potassium 0.248g K₂O per kg of absolutely dry soil. In the autumn, in the second leaf stage, some pots were treated with Mo at a concentration of 1 mg Mo per kg absolute dry soil. The plants were grown in the open air and, during rainfalls and snowfalls, were moved under polyethylene sheds. The minimum and maximum diurnal temperatures were monitored with the minimum temperature during vegetative growth reaching −9°C to −10°C.

Seeds obtained from the plants grown on a soil with neutral pH 6.8 (‘mother’ seeds, type I), seeds obtained from plants grown on acid soil (type II), and seeds obtained from plants grown on acid soil treated with Mo (type III) were analyzed according to the schemes:

<table>
<thead>
<tr>
<th>type</th>
<th>soil pH</th>
<th>plants</th>
<th>non-Mo-treated</th>
<th>type II seeds</th>
<th>soil pH</th>
<th>plants</th>
<th>Mo-treated</th>
<th>type III seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>6.8</td>
<td>→</td>
<td>4.19</td>
<td>→</td>
<td>non-Mo-treated</td>
<td>→</td>
<td>type II seeds</td>
<td>plants</td>
</tr>
</tbody>
</table>

Chemicals. Bovine serum albumin, xanthine oxidase, and GSH were obtained from Sigma Chemical Company, St. Louis, MO; Ultragel AcA-34 from LKB (Sweden).

Mo-Co Extraction and Assay Procedure. Seeds were ground in a mortar and pestle for 1 min with 0.1 M K/Na phosphate buffer (pH 7.6) containing 0.5 mM GSH and 25 mM Na₂MoO₄ (tissue to extraction buffer, 1:10). The extraction was carried out at 4°C for 15 min. The homogenate was centrifuged at 14,000g for 20 min at 4°C. Mo-Co activity was determined in the supernatant without heating (‘free’ Mo-Co) and after heat release for 90 s at 80°C in the presence of GSH under N₂ (‘bound’ Mo-Co).

1 Abbreviations: NR, nitrate reductase; Mo-Co, molybdenum cofactor; PAAG, polyacrylamide gel; XO, xanthine oxidase; XDH, xanthine dehydrogenase; SO, sulfite oxidase.
**In Vitro Complementation.** *Neurospora crassa* mutant strain nit-1 was grown and the extract was obtained according to Mendel and Müller (12). *In vitro* restoration of nit-1 NADPH-NR was performed for 40 min at 25°C anaerobically with Mo-Co preparations. One unit of Mo-Co activity was defined as the ability of the reconstituted nit-1 NR to form 1 nmol nitrite per min.

**Ultratgel AcA-34 Gel Filtration.** Seed extracts (tissue to buffer, 1:4) were applied to a column 2.5 × 147 cm. The column was equilibrated with 0.1 M K/Na phosphate buffer (pH 7.6) containing 0.5 mM EDTA and 25 mM Na₃MoO₄. Fractions were collected at 4°C and bound Mo-Co activity was assayed.

**Determination of XDH activity by Polyacrylamide Electrophoresis.** Polyacrylamide disc electrophoresis under non-denaturing conditions was carried out according to Mendel and Müller (11).

**Molybdenum Determination.** Mo content was determined in two repetitions by instrumental neutron activation analysis with a standard kale powder Bowen by multichannel analyzer (Intertektechnique IN 96, France) and high purity germanium detector (ORTEC, USA) with resolution FWHM 1.9 KeV and efficiency 40% (60Co 1332.5 KeV).

**RESULTS**

**Comparison of Mo-Co Activity in Dry Seeds.** When dry wheat seeds were ground and extracted, higher bound Mo-Co activity was found than free Mo-Co (Table I). In Mo-treated seeds (types I and III), Mo-Co activities were higher than in nontreated seeds (types II). The free Mo-Co content was about 25% of bound in types I and II seeds, while free Mo-Co was 50% of bound in type III seeds. Using neutron activation analysis, Mo could not be detected in type II seeds and was present at 0.240 ppm in type I seeds and 0.300 ppm in type III seeds.

**Separation of Mo-Co Containing Proteins.** Extracts of the three types of wheat seeds were separated by gel filtration on Ultratgel AcA-34 (Fig. 1). The column was calibrated using barley leaf NR, bovine XO, and BSA, which we designated fraction A for the higher mol wt portion where NR and XO eluted and fraction B for the lower mol wt region were BSA eluted. For all three seed types, fraction B had a higher Mo-Co content than fraction A. Mo-Co activity demonstrable without heating or free Mo-Co could only be shown for fraction B for types I and II seed, while it could be shown for both fractions for type III (data not shown). The distribution between fractions A and B was different for types I and III seed. For example, the ratio between the Mo-Co activity of fraction B and A was 1.5 to 1.8 in type I seed, while it was 2.2 to 3 for types II and III seed.

**Identification of Mo-Co Carriers.** Fraction A contained XDH activity as shown in Figure 2 and also co-eluted with barley leaf NR activity. Thus, fraction A was designated the 'enzyme' Mo-Co fraction. Fraction B, which co-eluted with BSA, contained no XDH activity (Fig. 2).

**Table I. Mo-Co Factor Activity in Winter Wheat Seeds**

All measurements were made in three repetitions from the extracts of 0.1 g seed powder in 2 ml buffer. All other factors were as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Variants</th>
<th>Reconstituted nit-1 N. crassa NR Activity</th>
<th>Bound</th>
<th>Free</th>
<th>% Free</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mo-co</td>
<td>Mo-co</td>
<td>Mo-co</td>
<td>Mo-co</td>
</tr>
<tr>
<td></td>
<td>nmoles NO₃⁻ min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I seeds</td>
<td></td>
<td>6.52</td>
<td>1.68</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Type II seeds</td>
<td></td>
<td>1.33</td>
<td>0.30</td>
<td>20.4</td>
<td>18.5</td>
</tr>
<tr>
<td>Type III seeds</td>
<td></td>
<td>8.79</td>
<td>4.39</td>
<td>139.9</td>
<td>270.9</td>
</tr>
</tbody>
</table>

* Mother seeds.  
** Seeds from next generation of plants not treated with Mo.  
* Seeds from next generation of plants treated with Mo.

**FIG. 1.** Ultratgel AcA-34 gel filtration of wheat seed Mo-co extracts. Fractions (3.6 ml) were collected at 4°C from a column 2.5 × 147 cm, and bound Mo-cofactor was determined. NR, XO, and BSA denote the positions of which the reference proteins milk xanthine oxidase, barley leaf NR (crude extract), and bovine serum albumin as marker proteins are eluted.

**Subfractions of Mo-Co Fractions A and B.** Several subfractions of major fractions A and B could be detected in the gel filtration elution pattern (Fig. 1). While there was little qualitative difference in the elution patterns of the three seed extracts, the contents of various subfractions were significantly different. For example, some components of fraction A in type II seed appeared to be completely absent. In type III seed, the Mo-Co activity of several subfractions was several times higher than similar elution positions from extracts of either seed type I or II. These results are in general agreement with those of Table I. In addition, the stability of Mo-Co subfractions were different, with those of fraction A of type III seeds being the most stable (data not shown).

**DISCUSSION**

Molybdenum is not free in the cell but is associated with stabilizing macromolecules (7). The presence of Mo-storage proteins in *Escherichia coli* and *C. pasteuriannum* as well as a 60 kDa protein in *C. thermoacetaticum* has been postulated (9). Our investigations showed that Mo in winter wheat seeds is associated with protein carriers with different mol wt (Fig. 1). These results...
confirm those of Alikulov and Schemann (1). The high mol wt fraction of Mo-containing proteins from wheat seeds, which corresponded in size to elution position of NR and XDH, consisted of several distinct species with differing Mo-Co activity. The lower mol wt fraction, which corresponded to the elution position of BSA, was also composed of several different Mo-Co-containing proteins, which might be storage Mo-carrier proteins or breakdown products of the high mol wt fraction. A metabolic relationship may exist between the type or molecular size of Mo-Co carriers that is different depending on plant age or stress. For example, the ratio of fraction B to A was different in the three types of seeds studied here. The increased fraction B in type III seeds may be a means for the seeds to store Mo in lower mol wt proteins rather than in enzyme proteins.

Low temperature treatments have been observed to alter activity of several different enzyme systems in plants (4). Prolonged exposure of wheat plants grown on acid soil to low temperatures influenced the total Mo-Co activity in the seeds produced by these plants (Table 1) as well as the ratio between the two fractions of Mo-Co carrier proteins in the seeds (Fig. 1). Low soil pH is a limiting factor for the normal supply of Mo to plants. Prolonged low temperature stress probably causes the breakdown of Mo-containing enzymes and the release of Mo-Co or Mo. The damaged plants formed about 90% sterile ears (19) and the grains formed (type II seeds) were small and deformed. The Mo content of these seeds is below detection even by neutron activation analysis. Exogenously applied Mo in low levels (1 mg Mo/kg dry soil) was rapidly assimilated and prevented the Mo deficiency during vegetative growth of treated plants. We suggest that plants and seeds store Mo mainly in the low mol wt Mo carriers of fraction B. This Mo store could serve as a source of Mo in the biosynthesis of active Mo-containing enzymes such as the NR and XDH found in fraction A.

High nitrogen fertilization alters soil characteristics resulting in some decrease in soil pH. Application of Mo to acid soils could overcome the decreased assimilation of Mo in these soil-plant systems. Studies of the cellular forms of Mo and its incorporation into important enzyme systems which are influenced by external stresses, is, one hopes, a means for gaining understanding of plant mechanisms associated with resistance to stress.

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