Arsenate toxicity: effects on oxidative stress response molecules and enzymes in red clover plants

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Abstract

The effects of different soil concentrations of arsenate on red clover plants (Trifolium pratense L. cv. Reno) were investigated. Red clover plants were grown in a greenhouse on a sandy soil amended with different concentrations of Na₂HAsO₄ (5, 10 and 50 mgAs kg⁻¹ soil) or a heavy metal mixture (5 mg Cd, 300 mg Zn and 10 mgAs kg⁻¹ soil), respectively. We studied the accumulation of arsenic as well as macro- and micronutrients and investigated biochemical stress responses in clover shoots caused by arsenic incorporation in clover shoots. Increases in superoxide dismutase (SOD) activity, peroxidase activity as well as decreases in chlorophyll (chl) and carotenoid concentrations correlated with increasing arsenic content in plants. The analyses of native PAGE SOD activity staining indicated one Mn-SOD and two major Cu/Zn-SOD isoenzymes in clover shoots, whose capacity increased in response to arsenate treatment. Glutathione content was reduced at the highest As concentration applied. Antioxidative polyamines (PAs) accumulated at higher doses of arsenate in the soil. Increase in SOD activity and accumulation of PAs as well as chl loss could be prevented by application of Zn and Cd together with As. The results indicate that higher doses of arsenate produce oxidative damage in clover shoots. The protective role of applying a mixture of Zn and Cd together with As is discussed.

Keywords: Trifolium pratense; Arsenate; Oxidative stress; Superoxide dismutase; Peroxidase; Glutathione; Chlorophyll

1. Introduction

Arsenic (As) is ubiquitous to the environment. Major anthropogenic sources of As distribution are metal processing, burning of coal, and the application of arsenic-based pesticides or herbicides [1]. An important natural origin of As contamination is volcanism [2]. Arsenate (AsO₄³⁻) and arsenite (AsO₃³⁻) are the primary chemical forms occurring in soils. Soil microorganisms form these compounds by oxidation/reduction or methylation/de-methylation reactions [3].

Bioavailability, uptake and phytotoxicity of arsenic to plants are influenced by factors such as arsenic concentration in soil, As species, plant species [4] and soil properties, like redox potential, drainage conditions [5], pH and soil phosphorus content [6,7]. The concentration tolerated by plants varies from 1 to 50 mgAs kg⁻¹ soil. Arsenite is more toxic than arsenate, and both of them are more toxic than organic arsenical compounds [8,9].

Arsenate is chemically similar to phosphate. It uncouples the oxidative phosphorylation by displacing phosphate in ATP synthesis [7,10]. Arsenate competes with phosphate for the uptake system, but the affinity to phosphate is much stronger than to arsenate. An increased phosphate level leads to reduced arsenate uptake in plants and vice versa [11]. Arsenate has been reported to reduce chlorophyll (chl) biosynthesis in maize [40]. The chl content in leaves of Pisum sativum L. increased, but the ratio of chl a/b decreased by treatment with arsenate [13]. Simola [14] described a breakage and swelling of thylakoid membranes and accumulation of starch in chloroplasts under arsenate stress.

Arsenic also can trigger the formation of phytochelatins (PCs) which are thiol (SH)-rich peptides induced by
heavy metals including Cd, Cu and Zn [41]. Rapid induction of metal-binding PCs in response to inorganic arsenic has been shown to occur in cell suspension cultures of Rauwolfia serpentina, in Arabidopsis seedlings and enzyme preparations of Silene vulgaris [12].

The scavenging system controlling reactive oxygen species (ROS) comprises non–enzymatic antioxidants (e.g. glutathione (GSH), ascorbate, carotenoids) and enzymatic antioxidative systems (e.g. superoxide dismutase (SOD), peroxidase (POD), catalase, GSH reductase) [15]. In the past years, a role of polyamines as antioxidants has been postulated [16,17,39]. Polyamines have been found within the vacuole and in isolated mitochondria and chloroplasts [18] and are involved in the structural and functional stabilization of cell membranes and organelles under stress conditions.

ROSs may be generated through the conversion of arsenate to arsenite. This may result in damage to DNA, proteins and lipids. In arsenate treated Holcus lanatus L., increases in lipid peroxidation, SOD activity and PC production correlated with increasing arsenate concentrations [19]. Antioxidative enzymes, like catalase, GSH-S-transferase and SOD have been shown to increase after exposure of maize to arsenate and arsenite [20]. Rapid induction of metal-binding PCs in response to inorganic arsenic has been shown to occur in cell suspension cultures of Rauwolfia serpentina, in Arabidopsis seedlings and enzyme preparations of Silene vulgaris [12].

In the present study we investigated the toxicity of arsenic to greenhouse grown red clover plants (Trifolium pratense cv. Renova) cultivated in soil. The influence of increased concentrations of arsenate on biomass production, chl content, antioxidative capacity (as SOD, POD and GSH), SOD isoenzyme pattern and the content of antioxidative polyamine (PAs) in the shoots was investigated in order to achieve a better understanding of the biochemical mechanisms of defence against arsenic stress in plants. Since in many cases of soil pollution with arsenic the soil sites are contaminated with more than one heavy metal, a combination of arsenic with zinc and cadmium was included in the studies. From our investigations we suggest an increased oxidative stress by exposure of red clover to arsenate.

2. Materials and methods

2.1. Plant material and growth conditions

Trifolium pratense L. cv. Renova plants were grown in pots with 2.5 kg sandy soil containing different concentrations of arsenate (nutrient concentrations in the soil (mg kg⁻¹): P 89; K 90; Mg 113; Na 72; Cu 7.3; Mn 64; Zn 14 // CaCO₃ 3.6%). The experiment was carried out under greenhouse conditions. The arsenate was applied to the soil before sowing of the clover. Arsenate was applied as Na₂HAsO₄·7H₂O. Three concentrations (5, 10 and 50 mgAs kg⁻¹ soil) were tested. In addition an arsenate application (10 mgAs kg⁻¹ soil) together with cadmium chloride (5 mgCd kg⁻¹ soil) and zinc chloride (300 mg Zn kg⁻¹ soil) was also included in the experiment, designed as ‘heavy metal mix’. Control plants were cultured in the absence of arsenate treatments. Six replicate pots were cultivated of each experimental variant.

2.2. Harvest procedure

Plants were grown for 10 weeks and then harvested. The shoots and roots were separated. Roots were washed with distilled water, and the fresh weight was determined. The plant material was then frozen and lyophilized for the determination of the dry weight, ground in a stainless steel mill, and stored at −20 °C.

2.3. Element analysis

Element content was determined for freeze dried shoot samples. The samples (200 mg) were digested with 2 ml HNO₃ (conc.) and diluted to 10 ml with bideionized water. Arsenic in the solution was determined by hydride generation atomic absorption spectrometry. Macro- and micro-nutrients were determined by ICP-AES (Liberty 150, VARIAN).

2.4. Preparation of extracts for enzyme assays and protein determination

The lyophilized and ground shoot samples (500 mg) were homogenized on ice with Ultraturrax (Jahnke & Kunkel, Stauffen) for 2 min in 3 ml of homogenizing solution containing 100 mM potassium phosphate buffer, 2% (w/v) polyvinylpyrrolidone and 2 mM dithioerytritol (pH 7.2) and extracted for 2 h at 4 °C under gentle stirring. The homogenate was filtered and centrifuged at 14000 × g and 4 °C for 30 min. The supernatant was dialyzed against 50 mM potassium phosphate buffer at 4 °C for 8 h. The dialyzed extracts were cleared by centrifugation (14000 × g, 15 min) and used for further analyses.

The protein concentration of homogenates and of freeze dried samples was determined after precipitation with TCA according to the method of Lowry et al. [21] using bovine serum albumin as standard.

2.5. Enzyme assays

2.5.1. Superoxide dismutase

Total SOD activity was determined according to Mc Cord and Fridovich [22]. The reaction in a 1-ml cuvette containig 50 mM potassium phosphate buffer (pH 7.5)
was followed spectrophotometrically at 550 nm and 27 °C. In this assay, one unit of SOD activity was defined as the amount of enzyme required to inhibit the ferricytochrome c reduction by 50%.

2.5.2. Peroxidase

The POD activity was determined at 25 °C with guaiacol [25]. In the presence of H$_2$O$_2$, POD catalyzes the transformation of guaiacol to tetraguaiacol (brown product). This reaction can be recorded at 470 nm. The reaction mixture contained 100 mM citric acid/potassium phosphate buffer (pH 5.0), 33 mM guaiacol and 0.3 mM H$_2$O$_2$. Horse-radish POD (Sigma) was used as standard enzyme.

2.6. SOD activities in non-denaturing polyacrylamide gels

Non-denaturing PAGE of the crude protein extracts was carried out on 12% polyacrylamide gels (120 × 110 × 1 mm) using a Biometra electrophoresis system (Biometra, Göttingen) according to the manufacturer’s specifications. Protein solutions (20 μg) were loaded on to the gel and separated for 3 h at 4 °C, 120 V and 30 mA.

Immediately after electrophoresis, the activity of SOD isoenzymes was visualized using the nitroblue tetrazoliumchloride (NBT) staining procedure [23]. The gel was incubated in 2.5 mM NBT (Roth) for 20 min at 25 °C and then soaked for 30 min in the dark in 50 mM potassium phosphate buffer at pH 7.5 containing 0.028 mM riboflavin and 0.3% (v/v) tetramethylethylenediamine (Roth). The gel was then illuminated to promote the photoreactive staining by SOD. The stained gels were finally scanned and analyzed with a raytest system (scanning software AIDA 2.0).

In order to identify the nature of SOD enzymes, activity staining gels were incubated for 30 min in 50 mM potassium phosphate buffer at pH 7.5 containing 2 mM KCN or 5 mM H$_2$O$_2$. Cu/Zn-SODs are inhibited by KCN and H$_2$O$_2$; Fe-SODs are inactivated by H$_2$O$_2$ but resistant to KCN and Mn-SODs are resistant to both inhibitors [24].

2.7. Extraction and assay of GSH

Extraction was carried out by grinding 100 mg of freeze dried plant material (using a Ultraturrax) in 2 ml of 5% (w/v) sulfosalicylic acid at 0 °C. After centrifugation at 14000 × g for 15 min (4 °C) the supernatants were immediately assayed. The total GSH (GSH plus GSSG) content was determined spectrophotometrically at 412 nm, using GSH reductase, 5,5'-dithiobis-2-nitrobenzoic acid and NADPH [26].

2.8. Determination of chl and carotenoid content

Chlorophyll and carotenoids were extracted by homogenizing 100 mg dry weight of ground shoot tissue in 10 ml acetone solution (80 acetone: 15 water: 5 conc. NH$_3$-solution [25%]; v/v). After centrifugation for 10 min at 4000 × g, chl and carotenoid content were analyzed spectrophotometrically in the supernatant at 480, 645, 647, 652, 663, 664 and 750 nm, as described by Schopfer [27].

2.9. Analyses of free polyamines

Shoot samples were extracted with 5% perchloric acid. HPLC, in combination with UV detection, was used to separate and quantify di- and polyamines (spermidine (Spd), spermine (Spm) and putrescine (Put)) prepared as their benzoyl derivates from plant tissues [28]. The PA levels were expressed as μmol g$^{-1}$ dry weight.

2.10. Statistical analysis

The data represent means calculated from three replicated pots. A least significant difference test (LSD-test) was employed for comparison of changes at $P < 0.05$.

3. Results

3.1. Effect of arsenate on the element content of red clover shoots

Arsenic levels in the shoot tissues of red clover increased with increasing arsenate concentrations in the soil (Table 1). Zinc concentrations in the shoots decreased with increasing arsenate application. At the highest arsenate application rate Zn content in the shoots was only about half that of the control. Whereas Zn concentrations decreased due to arsenic application the concentrations of copper increased with increasing arsenic supply: a concentration of 50 mg kg$^{-1}$ As in the soil significantly increased the Cu content of red clover shoot tissue to 20 mg kg$^{-1}$ d.w., whereas the concentration of Zn was minimal. As a consequence, the Cu/Zn ratio decreased to 25% of that of the control (Table 2). The application of zinc and cadmium (400/20 mg kg$^{-1}$ soil) with arsenate (‘heavy metal mixture’) did not influence uptake of arsenic. The application of the heavy metal mixture, however, resulted in the highest shoot concentrations of iron and manganese of all variants (Table 1). The resulting ratios of Fe/Mn, Fe/Zn, and Fe/(Mn + Cu + Zn) therefore reached highest values (Table 2). Phosphorus concentrations in the shoots of red clover were significantly reduced by the
lowest As treatment and the application of the heavy metal mixture.

3.2. Effect of arsenate on the growth of red clover

A significant growth reduction of red clover shoots by arsenate was only observed at the highest arsenate level applied corresponding to a shoot content of 1.3 μg g⁻¹ d.w. As (Table 3). The treatments with 5 and 10 mg kg⁻¹ As resulted in higher biomass productions than in the control plants. Root growth was not affected by arsenic treatment also in the variant with the highest arsenate application. Adding zinc and cadmium together with arsenate further stimulated the growth of roots and shoots beyond the stimulation of arsenate alone. At higher concentrations of arsenate the root/shoot dry weight ratio increased, indicating a higher toxicity of arsenate to shoots than to roots.

3.3. Effect of arsenate on the content of chl and carotenoids

The soil application of 5 mg kg⁻¹ As did not alter the content of chl a and b and of carotenoids in red clover shoots, the higher concentrations of 10 and 50 mg kg⁻¹ As in the soil reduced the chl and carotenoid contents in clover plants significantly by about 14 and 12%, respectively (Table 4).

This decline in the chl and carotenoid content indicated the poisoning effect of high arsenic concentrations to green leaves. The addition of zinc and cadmium together with arsenate in the heavy metal mixture variant reduced the toxic effects of arsenic, as indicated by the highest chl content of all variants tested.

3.4. Effect of arsenate on the concentration of free polyamines

Polyamines are involved in the structural and functional stabilization of cell membranes and organelles under stress conditions and protect plant structures from damage by ROS. Both free and conjugated polyamines are involved in this protective role of polyamines. Because of methodological limitations only free polyamines were determined in the experiment. The concentrations of the polyamines Spm/Spd and the diamine Put in the shoots of red clover plants were increased at the 10 mg As application. At the 50 mg soil application the concentrations were further increased.

Table 1
Element content in the shoots of red clover plants as affected by different arsenate concentrations in soil

<table>
<thead>
<tr>
<th>Element content in the shoots (mg kg⁻¹ (d.w.))</th>
<th>Untreated</th>
<th>Arsenic</th>
<th>5 mg kg⁻¹</th>
<th>10 mg kg⁻¹</th>
<th>50 mg kg⁻¹</th>
<th>hm-mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenic</td>
<td>0.2 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>1.6 ± 0.8</td>
<td>6.5 ± 2.3*</td>
<td>1.3 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>9.4 ± 0.7</td>
<td>8.4 ± 1.1</td>
<td>9.1 ± 0.9</td>
<td>20.0 ± 2.7*</td>
<td>6.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>42.9 ± 3.5</td>
<td>38.8 ± 0.7</td>
<td>34.9 ± 5.6</td>
<td>23.0 ± 2.6*</td>
<td>330.3 ± 33.9*</td>
<td></td>
</tr>
<tr>
<td>Manganese</td>
<td>62.0 ± 2.8</td>
<td>47.0 ± 3.6</td>
<td>80.5 ± 2.6</td>
<td>73.6 ± 5.6</td>
<td>151.7 ± 13.2*</td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>72.6 ± 19.4</td>
<td>98.8 ± 4.4</td>
<td>88.5 ± 3.1</td>
<td>76.1 ± 12.9</td>
<td>146.6 ± 29.9*</td>
<td></td>
</tr>
<tr>
<td>Cadmium</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3.08 ± 0.27*</td>
<td></td>
</tr>
<tr>
<td>Aluminium</td>
<td>90.3 ± 34.2*</td>
<td>49.3 ± 10.0</td>
<td>48.2 ± 7.2</td>
<td>41.6 ± 10.5</td>
<td>51.7 ± 8.0</td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td>4576 ± 72</td>
<td>4100 ± 32</td>
<td>4330 ± 532</td>
<td>3921 ± 296*</td>
<td>4112 ± 325</td>
<td></td>
</tr>
<tr>
<td>Phosphorus</td>
<td>1578 ± 46</td>
<td>1139 ± 32*</td>
<td>1438 ± 28</td>
<td>1692 ± 12</td>
<td>1253 ± 10*</td>
<td></td>
</tr>
</tbody>
</table>

* Significant differences (P < 0.05) as assessed by LSD-test.

Table 2
Selected element ratios in the shoots of red clover plants as affected by different arsenate concentrations in soil

<table>
<thead>
<tr>
<th>Ratio of element content in shoots</th>
<th>Soil treatment</th>
<th>Untreated</th>
<th>Arsenic</th>
<th>5 mg kg⁻¹</th>
<th>10 mg kg⁻¹</th>
<th>50 mg kg⁻¹</th>
<th>hm-mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe/Mn</td>
<td>1.17</td>
<td>2.10</td>
<td>1.10</td>
<td>1.03</td>
<td>0.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe/Zn</td>
<td>1.7</td>
<td>2.5</td>
<td>2.5</td>
<td>3.3</td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe/Cu</td>
<td>7.7</td>
<td>11.7</td>
<td>9.7</td>
<td>3.8</td>
<td>22.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe/Mn + Cu + Zn</td>
<td>0.64</td>
<td>1.05</td>
<td>0.71</td>
<td>0.65</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn/Cu</td>
<td>4.56</td>
<td>4.62</td>
<td>3.84</td>
<td>1.15</td>
<td>50.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P/As</td>
<td>7890</td>
<td>1423.8</td>
<td>898.8</td>
<td>260.3</td>
<td>963.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
treatment, the concentrations of Spm and Spd decreased and those of the diamine Put further increased. Thus we detected a significantly increased Put content at 10 and 50 mg kg$^{-1}$ concentration in soil, Put increasing by about 84% at the highest arsenate soil content. Fifty milligram per kilogram of Arsenic in soil induced the highest Spm accumulation in the shoots (raising to about 263%) Fig. 1.

In the presence of zinc and cadmium together with arsenate the amine concentrations in the shoots were lowest.

3.5. Effect of arsenate on antioxidative enzymes

3.5.1. Superoxide dismutase

To test the hypothesis that arsenate can induce oxidative stress, SOD activity was measured as indicator of oxidative stress. At 10 mg kg$^{-1}$ of arsenate in soil, SOD activity was highest (Table 5); it increased by about 41%. The highest concentration of arsenate in soil reduced SOD activity to the level of untreated plants. An application of 5 mg kg$^{-1}$ As as well as the mixture of heavy metals resulted in the lowest SOD activities (reduction of about 30 and 21%, respectively).

When arsenate (0.1 mM) in vitro was added to an SOD assay containing protein extract of red clover shoots or roots significant and progressive inhibition of the activity of SOD was found (Fig. 2).

The ratio between SOD activity and the carotenoid content in shoots increased strongly at 10 and 50 mg kg$^{-1}$ As in soil. The soil containing the lowest As treatment or the mixture of heavy metals induced no changes in comparison to the control (Table 5).

3.5.2. Activity of SOD isoenzymes

Plant extracts were stained for SOD activity following native PAGE. Three distinct bands of SOD were detected, which were identified as a Mn-SOD and two Cu/Zn-SODs on the basis of their sensitivity to KCN and H$_2$O$_2$ (Fig. 3B). Cu/Zn-SODs were designated as Cu/Zn-SOD I and Cu/Zn-SOD II, according to their increasing mobility in gels. Cu/Zn-SOD II was the major isoenzyme in shoots. The major SOD isoform in shoots is the Cu/Zn-SOD which occurs in chloroplasts. The

Table 3
Dry matter production of red clover plants as affected by different arsenate concentrations in soil

<table>
<thead>
<tr>
<th>Soil treatment</th>
<th>Arsenic</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mg kg$^{-1}$</td>
<td>10 mg kg$^{-1}$</td>
<td>50 mg kg$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>Shoot</td>
<td>0.0293 ± 0.0006*</td>
<td>0.0385 ± 0.0015</td>
<td>0.0333 ± 0.0040</td>
<td>0.0243 ± 0.0040*</td>
</tr>
<tr>
<td>(100%)</td>
<td>(131.4%)</td>
<td>(113.7%)</td>
<td>(82.9%)</td>
<td>(195.6%)</td>
</tr>
<tr>
<td>Root</td>
<td>0.0170 ± 0.0010</td>
<td>0.0233 ± 0.0031</td>
<td>0.0223 ± 0.0075</td>
<td>0.0170 ± 0.0020</td>
</tr>
<tr>
<td>(100%)</td>
<td>(137.1%)</td>
<td>(131.1%)</td>
<td>(100.0%)</td>
<td>(342.9%)</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.5802</td>
<td>0.6052</td>
<td>0.6697</td>
<td>0.6996*</td>
</tr>
</tbody>
</table>

* Significant differences ($P < 0.05$) as assessed by LSD-test.

Table 4
Effect of arsenate supply on the concentration of chlorophylls and carotenoids in the shoots of red clover plants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pigment concentration (mg g$^{-1}$ dry weight ± S.D.)</th>
<th>Ratio chlorophyll/carotenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>chl a</td>
<td>chl b</td>
</tr>
<tr>
<td>Untreated</td>
<td>5.99 ± 0.04 (100%)</td>
<td>1.86 ± 0.01 (100%)</td>
</tr>
<tr>
<td>5 mg kg$^{-1}$ As</td>
<td>6.04 ± 0.12 (100.8%)</td>
<td>1.95 ± 0.05* (104.8%)</td>
</tr>
<tr>
<td>10 mg kg$^{-1}$ As</td>
<td>5.26 ± 0.06* (87.8%)</td>
<td>1.63 ± 0.03* (87.6%)</td>
</tr>
<tr>
<td>50 mg kg$^{-1}$ As</td>
<td>5.17 ± 0.02* (86.3%)</td>
<td>1.64 ± 0.01* (88.2%)</td>
</tr>
<tr>
<td>hm-mixture</td>
<td>6.09 ± 0.08 (101.7%)</td>
<td>1.95 ± 0.02* (104.8%)</td>
</tr>
</tbody>
</table>

* Significant differences ($P < 0.05$) as assessed by LSD-test.
SOD isoenzyme pattern in *Trifolium pratense* cv. Renova shown in Fig. 3B was similar to that reported previously in Ref. [29] for *Trifolium pratense* L.

We could not detect a new isoenzyme in red clover plants treated with arsenate or the mixture of heavy metals (Fig. 3A), but the pattern of isoenzymes was altered. The soil concentrations of 10 and 50 mg kg\(^{-1}\) As induced a strong increase in the activity of Cu/Zn-SODs. Plants receiving a heavy metal mixture treatment, however, showed increased Cu/Zn-SOD II activity.

3.5.3. Peroxidase

The specific activity of POD increased with a higher arsenate content in soil (Table 5). Expressed on a protein basis, increases to 250\% in comparison to the control occurred in shoots of plants treated with 10 mg kg\(^{-1}\) of As. The highest arsenate concentration reduced POD activity.

3.6. Effect of arsenate on protein and GSH content

The concentration of protein significantly decreased in the shoot tissues with increasing arsenate supply (Table 6). This effect, however, was not found with the heavy metal mixture treatment where no reduction in protein concentration occurred.

Arsenate treatments of 5 and 10 mg As kg\(^{-1}\) did not reduce the concentration of GSH in clover shoots (Table 6). An application of 50 mg and the mixture of heavy metals reduced the content of GSH significantly.

4. Discussion

Though arsenic has not been shown to be an essential plant nutrient [9], increased dry matter production was observed at low arsenate treatment in the experiment with red clover presented here. Similar increases in dry weight were observed in tomato plants by Burló et al. [30], when plants were grown in soilless culture in the presence of arsenate (1–2 mg l\(^{-1}\)). Carbonell-Barra-china et al. [31] reported that the supply of arsenate at concentrations of 0.2–0.8 mg l\(^{-1}\) in hydroponic culture increased total dry matter production of *Spartina alterniflora* and *Spartina patens* compared to control plants. Several reports ascribed this phenomenon to
higher phosphorus content in the plants [30,31]. This speculation cannot be responsible for the increased dry matter production in our study because *Trifolium pratense* treated with low arsenate concentrations did not display higher concentrations of phosphorus in the shoots. In plants treated with the lowest arsenate

![Fig. 2](image-url) Inhibition of SOD activity in root and shoot protein extracts from red clover plants by 0.1 mM arsenate; (± S.D.)

![Fig. 3](image-url) A. SOD isoenzymes present in shoot extracts of *Trifolium pratense* cv. Renoa as affected by arsenate in soil. Protein extracts were resolved on non-denaturing polyacrylamide gels and activity staining was carried out as described in Section 2. In panels A and B, 20 µg protein were applied per lane, respectively. Plants were cultivated at untreated soil (lane 1, zero arsenic) or at arsenate treated soil (lane 2, 5 mgAs kg⁻¹ soil; lane 3, 10 mgAs kg⁻¹ soil; lane 4, 50 mgAs kg⁻¹ soil) or at heavy metal mixture treated soil (lane 5, 10 mgAs kg⁻¹ soil, 5 mgCd kg⁻¹ soil, 300 mgZn kg⁻¹ soil). Extracts analyzed in panel B were obtained from plants grown at untreated soil. When indicated, SOD activity staining was carried out in the presence of 2 mM KCN or 5 mM H₂O₂.

![Fig. 3](image-url) B. Control, CN⁻, H₂O₂

![Fig. 3](image-url) Mn-SOD

![Fig. 3](image-url) Cu/Zn-SOD I

![Fig. 3](image-url) Cu/Zn-SOD II

![Fig. 3](image-url) Mn-SOD
concentration we found a higher iron content of the shoots than in the untreated clover plants. Bergmann [32] and Marschner [33] reported positive effects of iron on chloroplast development and chl synthesis. Maybe this is the reason for better growth of red clover plants at this soil treatment.

Under our experimental conditions red clover plants grown with 50 mg kg\(^{-1}\) As showed visible symptoms of phytotoxicity like chlorosis and growth reduction after a 10 week growth period. At the highest arsenate soil treatment we observed 20 mg Cu kg\(^{-1}\) shoot (d.w.). This exceeds the copper level of about 17 mg kg\(^{-1}\) (d.w.), which is phytotoxic to red clover shoots [34]. Chlorosis symptoms at the highest arsenic supply thus could be a direct result of the action of increased copper concentrations on membranes [33].

When antioxidant zinc was used simultaneously with arsenate and cadmium, the decrease in chl concentration was completely prevented. Bietger and O’Dell [35] described effects of zinc on biomembranes. Zinc interferes with the movement of ions across membranes, affects the activities of membrane bound enzymes, affects the function of permeability channels and of carrier/transport proteins in the membrane.

In our experiment by the application of 10 mgAs kg\(^{-1}\) both Spm, Spd and Put concentrations and SOD- as well as POD activity in clover shoot tissue were significantly increased in comparison to the control. The concentrations in photosynthetic pigments are also significantly reduced. These findings indicate oxidative stress conditions caused by the arsenate application to the soil. Tamaki and Frankenberger [38] reported, that arsenate is reduced rapidly to arsenite via cytochrome/cytochrome oxidase, using oxygen as a final electron acceptor. However, superoxide radicals can be generated during the reaction of cytochrome oxidase with oxygen [15]. Thus exposure to arsenate leads to a stress dependent production of ROS and to lipid peroxidation of membranes [19].

The protection afforded by polyamines against oxidative stress has been proposed to involve scavenging free radicals [36] and the reduction of lipid peroxidation [16,37,39]. Benavides et al. [16] reported that Spm is the most effective inhibitor of lipid peroxidation in sunflower leaf discs. Exogenous diaminopropane, Spd and Spm inhibited loss of chl and reduced the level of malondialdehyde in oat leaves [39]. In our experiments in arsenate treated red clover plants, the Spm content in the shoots increased by about 263% at 10 mg kg\(^{-1}\) arsenic in soil. This could be seen as a protective mechanism against the destruction of membranes caused by arsenate stress.

The increased activities of antioxidative enzymes in response to arsenate application in our experiment with red clover are suggestive of potent feeding of oxidative stress. Three major bands of SOD activity were detectable following the staining of native PAGE gels for enzyme activity, inhibitor studies indicating two Cu/Zn-SODs and one Mn-SOD. Supply of arsenate (10 and 50 mg kg\(^{-1}\)) induced a strong enhancement in the activity of Cu/Zn-SOD II which was localized in chloroplasts of *Trifolium pratense*.

Interestingly, at the highest arsenate application tested in our pot experiment both SOD activities and Spm/Spd concentrations are reduced and reach values comparable to those of the control. The pigment concentrations also do not decrease further beyond the values of the 10 mg application. The GSH concentrations, however, while not influenced in the 10 mg application variant decreased at the 50 mg kg\(^{-1}\) As application. GSH is consumed by the ascorbate-GSH pathway, by which H\(_2\)O\(_2\) is scavenged and PCs are catalyzed by phytochelatin synthetase. Since GSH is depleted during PC synthesis the decrease in the GSH concentrations may indicate strong PC synthesis in clover shoots at the arsenate application rate of 50 mgAs kg\(^{-1}\) soil. This view is also supported by the very low GSH values observed in the hm-mix variant: Cadmium and zinc are strong inducers of PC synthesis [41] and may have initiated PC synthesis more than arsenic alone which is a poorer inducer. Since also shoot copper concentrations were drastically increased at the highest arsenate application this metal could have contributed to PC induction, too. PC production was not determined in the experiment. The results, however, suggest that the investigation of PC synthesis upon

### Table 6 Effect of arsenate supply on protein and GSH concentrations and changes in ratio of glutathione/POD activity in shoots of red clover plants

<table>
<thead>
<tr>
<th>Soil treatment</th>
<th>Arsenic</th>
<th>Protein (mg g(^{-1}) d.w.)</th>
<th>GSH (μ moles g(^{-1}) d.w.)</th>
<th>Protein (d.w.)</th>
<th>GSH (μ moles d.w.)</th>
<th>Protein (d.w.)</th>
<th>GSH (μ moles d.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>181.40 ± 1.27</td>
<td>154.65 ± 4.31*</td>
<td>5.02 ± 0.54</td>
<td>100%</td>
<td>107%</td>
<td>80.8%</td>
<td>107%</td>
</tr>
<tr>
<td>5 mg kg(^{-1})</td>
<td>154.65 ± 4.31*</td>
<td>162.75 ± 3.89*</td>
<td>4.06 ± 0.54</td>
<td>85.3%</td>
<td>89.7%</td>
<td>80.8%</td>
<td>89.7%</td>
</tr>
<tr>
<td>10 mg kg(^{-1})</td>
<td>162.75 ± 3.89*</td>
<td>159.20 ± 2.12*</td>
<td>4.06 ± 0.54</td>
<td>89.7%</td>
<td>87.8%</td>
<td>80.8%</td>
<td>87.8%</td>
</tr>
<tr>
<td>50 mg kg(^{-1})</td>
<td>159.20 ± 2.12*</td>
<td>180.20 ± 11.74</td>
<td>4.06 ± 0.54</td>
<td>99.3%</td>
<td>99.3%</td>
<td>80.8%</td>
<td>99.3%</td>
</tr>
<tr>
<td>hm-mixture</td>
<td>162.75 ± 3.89*</td>
<td>159.20 ± 2.12*</td>
<td>4.06 ± 0.54</td>
<td>89.7%</td>
<td>87.8%</td>
<td>80.8%</td>
<td>87.8%</td>
</tr>
</tbody>
</table>

* Significant differences (P < 0.05) as assessed by LSD-test.
arsenate treatment will be necessary to clarify the protecting role of Cd and Zn to arsenate stress in clover shoots.

References


