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Evolution of Arsenate Toxicity in Nodulated White Lupine in a Long-Term Culture

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White lupine is an As-resistant legume that is of interest for phytoremediation of As-contaminated soils. To achieve successful phytoremediation, monitoring of the nutritional status of the selected plant species during the entire culture cycle is required to maintain a plant cover with high biomass production. A long-term pot experiment was carried out with nodulated lupine grown on perlite with 10 and 100 μM As concentrations. The reproductive period (from 10 weeks) was the most sensitive phenologic stage of white lupine to long-term As exposure. The 10 μM As treatment increased the uptake and translocation of micronutrients, except for Cu, mainly at flowering with As levels in pods below the statutory limit (1 mg kg^{-1} fresh weight). However, the 100 μM As treatment induced significant differences compared to the control. These findings confirm the relatively high resistance of white lupine to arsenate and support the use of this species in phytoremediation and/or revegetation of As-contaminated sites, with special attention on P and Cu nutrition at flowering.

KEYWORDS: Arsenic; long-term culture; phytotoxicity; *Lupinus albus*

INTRODUCTION

White lupine (*Lupinus albus* L.) is a N_2-fixing legume that is adapted to environments with low pH and low available phosphorus (*1*). In this way, previous short-term experiments that used this species demonstrated its relatively high resistance to heavy metals (*2, 3*) and As. High resistance may be due to several plant characteristics, among which may be glutathione and PCs (*4*). Field experiments have suggested white lupine to be a good candidate for heavy metal and metalloid phytoremediation due to the high resistance of this species to these pollutants; the species has also been considered for revegetation of degraded landfill areas with polluted soils that are slightly acidic or neutral (*1, 5*). Furthermore, only a few papers have addressed the effects of As on legume−rhizobium symbiosis (*6*−*8*), and most of them were based only on short- or medium-term experiments.

The presence of arsenic at high concentrations in soils is mainly due to anthropogenic sources, such as mining, smelting, and application of pesticides (*9*). Elevation of arsenic levels in soils is of considerable concern because of the possibility of plant uptake and subsequent entry into the food chain. In aerobic soils, arsenate is the dominant As species and is thought to be taken up by plants via phosphate uptake systems. Thus, an increased availability of phosphate reduces arsenate uptake through suppression of the high-affinity phosphate/arsenate uptake system (*10*). However, the high-affinity phosphate/arsenate uptake system in white lupine is much less sensitive to down-regulation by phosphate than that in other plants species (*11*). Arsenate sensitivity is intimately linked to phosphate nutrition in plants due to the chemical similarity between arsenate and phosphate. Arsenate inhibits plant growth by disturbing many physiological phosphate functions, such as decoupling phosphorylation in mitochondria (*12*). Therefore, P should play a critical role in the plant's defense against As phytotoxicity. In this way, the P/As molar ratio in plant tissue, defined as the molar concentration of P divided by the molar concentration of As, is considered to be a good indicator of As phytotoxicity (*13*).

In contrast, inside plant cells, arsenate may be detoxified through glutathione-mediated reduction to arsenite. This process probably involves the generation of reactive oxygen species, leading to lipid peroxidation with the synthesis of end-products such as malondialdehyde (MDA) (*14*). This has been used as a biomarker for oxidative stress in plants exposed to arsenate (*14, 15*). Arsenic may also influence nutrient uptake and distribution in plants by competing directly with nutrients and/or by altering metabolic processes (*10*). Most of the studies addressing As interactions with plant nutrients have reported only effects at short exposure times (*7, 16, 17*). One exception is the long-term experiment conducted with the As hyperaccumulator, *Pteris vittata*, reported by Tu and Ma (*13*).

The maintenance of a plant cover with maximum biomass production over the culture cycle is essential for successful phytoremediation (*18*). To achieve this goal, monitoring of As phytotoxicity, arsenic accumulation in edible plant parts and nutritional status in the selected plant species during the entire culture cycle is required. The aim of this research was to study

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the evolution of As toxicity over the entire life cycle of white lupine grown under controlled conditions. In this way, a number of significant parameters, such as plant biomass, nodulation, arsenic and nutrient accumulation, and stress indicators, were examined.

MATERIALS AND METHODS

Experimental Design. White lupine seeds (*L. albus* L.) cv. Marta were surface-sterilized in sodium hypochlorite at 10% v/v for 15 min, rinsed thoroughly with deionized water, and germinated in darkness at 28 °C for 3 days on water-moistened filter paper. Next, seedlings were placed in plastic containers with moistened perlite. After 7 days, the plants were transferred to plastic Riviera pots (each experimental unit consisted of one pot with six plants) containing 7 L of perlite in the upper compartment and 4 L of nutrient solution in the lower compartment of the pot. Plants were inoculated twice (at sowing and 1 week later) with 2 mL of a suspension containing *Bradyrhizobium* sp. (*Lupinus*) strain ISLU-16 in exponential growth phase, at a density of 10^8 cfu mL^{-1}. The greenhouse had the following environmental conditions: temperature, 12−28 °C; relative humidity, 50−80%; photosynthetic photon flux density, 500 μmol m^{-2} s^{-1}. The composition of control nutrient solution was as follows: 0.50 mM $CaCl_2$; 1.50 mM KCl; 0.1 mM NaCl; 1.50 mM KH_2PO_4; 1.00 mM K_2SO_4; and 1.50 mM $MgSO_4$. Micronutrients were supplied as follows: 35.9 μM Fe-EDDHA; 32.8 μM $MnSO_4 \cdot H_2O$; 1.6 μM $ZnSO_4 \cdot 7H_2O$; 1.6 μM $CuSO_4 \cdot 5H_2O$; 46.2 μM H_3BO_3; 1 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; 1 μM $CoCl_2 \cdot 6H_2O$; and 1 μM $Ni(NO_3)_2 \cdot 6H_2O$. The pH of nutrient solutions was adjusted to 6. Nutrient solutions were renewed weekly. Arsenic was added 13 days after plant transfer as ($Na_2HAsO_4 \cdot 0.7H_2O$) at 0, 10, and 100 μM. Three replicates (pots) for each treatment were established in a randomized block design. After 3, 7, 10, and 14 weeks of As treatments, roots, stems, and leaves of each plant were separated and fresh weighed. Nodules were collected after 7 and 10 weeks of As treatments. In the same way pods were collected at 14 weeks.

Analytical Determinations. Plant material was thoroughly washed with tap water and given a final rinse with deionized water. A part of the fresh plant material was frozen in liquid N_2 and stored at −20 °C for subsequent analysis. The remaining plant material was oven-dried at 80 °C for 2 days to determine dry weight. Dried matter was ground and digested with a mixture containing $HNO_3/H_2O_2/H_2O$ 3:2:10 for 30 min in closed containers under pressure (*19*). The filtered acid digestions were analyzed for micronutrient concentrations by atomic adsorption spectrometry (Perkin-Elmer Analyst 800). Analysis of As was carried out by inductively coupled argon plasma mass spectrometry (ICP-MS) (Perkin-Elmer Elan 6000). Total P and N-Kjeldahl were measured by colorimetric determination with a Technicon AutoAnalyzer (*20*).

Frozen samples were homogenized to a fine powder in liquid N_2 with a pestle. This pulverized material was used to determine lipid peroxides as malondialdehyde (MDA) and total thiols, as described by Zornoza et al. (*3*). For the MDA determination, plant material (100 mg of fresh wt) was extracted in 2.0 mL of TCA−TBA−HCl reagent [15% (w/v) TCA; 0.37% (w/v) TBA, and 0.25 mM HCl]. The extract was heated in a sand bath (90 °C, 30 min). After cooling, the flocculent precipitate was removed by centrifugation at 11000*g* for 10 min. Absorbance of the supernatant was measured at 535 nm and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. Total thiols were assayed by shaking 100 mg of fresh wt of plant material with 0.4 mL of $NaBH_4$ (1 mg mL^{-1}) dissolved in 1 M NaOH and 0.2 mL of deionized water. After centrifugation (11000*g*, 10 min), 0.5 mL of supernatant was added to 0.5 mL of 5,5′-dithiobis(2-nitrobenzoic acid) dissolved in neutralizing buffer (0.5 M potassium phosphate, pH 7.2). Absorbance was measured at 412 nm.

Parameters of Nutrient Accumulation in the Plant. Net uptake was defined as the increment of the total amount of one element (M) divided by the increment of the biomass (g) for a given range time (1).

$$\text{net uptake} = (M_t - M_0)/(g_t - g_0) \quad (1)$$

Net uptake was used to study the evolution of micronutrient uptake and the effects of arsenate on the uptake of these elements.

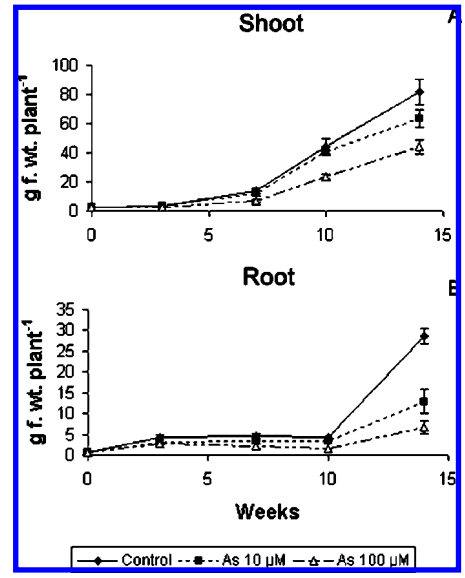

Figure 1. Shoot (**A**) and root (**B**) biomass of white lupine plants grown on perlite with 0, 10, and 100 μM arsenic doses for 3, 7, 10, and 14 weeks. Error bars indicate standard error of three replicates.

The shoot/root ratio of the concentration of micronutrients was used to study the effects of arsenate on the shoot/root translocation of these elements (eq 2).

$$\text{shoot/root ratio} = [M]_{shoot}/[M]_{root} \quad (2)$$

Statistical analyses. Values in the tables and figures indicate mean values ± standard error (SE). Differences among As treatments were analyzed by one-way ANOVA, followed by a posthoc multiple comparison of means using the Tukey test ($P < 0.05$). The statistical program SPSS 15.0 was used.

RESULTS AND DISCUSSION

According to previous studies with cadmium (*3*), a long-term pot experiment with lupine plants was carried out using perlite as substrate because this system better reproduces natural soil conditions, such as low concentrations of contaminants, humidity, and root development (*21*). In this way, the retention of As by perlite with respect to the supplied dose was estimated to be between 30 and 60% (data not shown). Despite this As retention by the perlite, the estimated available concentration of As for the 10 μM As dose (300−525 μg L^{-1}) is 130−300-fold higher than the available concentrations of As usually found in moderately contaminated soils (1−4 μg L^{-1}) and 2−3-fold higher than the maximum concentrations of As reported in soil solutions (147−171 μg L^{-1}) (*9*). Therefore, the 10 μM As treatment might better reflect the supply of arsenate found in contaminated soils. In contrast, the 100 μM As dose was intended to induce notable toxic effects on the plants.

Biomass Evolution, Nodulation, and N Status. The evolution of lupine biomass over the course of the culture cycle exhibited a first stage of slow growth (up to 7−10 weeks) followed by a fast-growth stage wherein the effects of As were more pronounced (**Figure 1**). Exposure to arsenate inhibited plant growth, affecting both shoot and root fresh weights. Growth inhibition was higher in plants grown under 100 μM As than in plants grown under 10 μM As (**Figure 1**). Plants showed significant decreases compared to the control only at 14 weeks for the 10 μM As dose and at 10 and 14 weeks for the 100 μM As treatment (**Figure 1**). The percentages of growth inhibition in 100 μM As-treated roots compared to control at 7, 10, and 14 weeks (54, 64, and 74%) were higher than in

Table 1. Parameters of Nodule Development and Total N Content of 70-Day-Treated White Lupine Plants Grown on Perlite with 0 (Control), 10, and 100 μM As Doses[a]

	control	10 μM As	100 μM As
nodule biomass (mg of fresh wt plant^{-1})	1693 ± 102 a	1637 ± 160 a	1088 ± 193 b
nodule no. plant^{-1}	207 ± 39 a	185 ± 28 ab	135 ± 29 bc
av nodule wt (mg of fresh wt nodule^{-1})	8.32 ± 1.32 a	9.02 ± 2.41 a	8.39 ± 3.30 a
total N content (mmol plant^{-1})	5.60 ± 0.27 a	5.42 ± 0.32 a	4.07 ± 0.14 b

[a] Data are expressed as mean ± SE. Significant differences among treatments are indicated by different characters ($P < 0.05$; $n = 3$).

shoots (50, 47, and 46%). Moreover, the growth inhibition of 100 μM As-treated roots increased over the course of the culture cycle, whereas, in the case of shoots, the percentages were constant. The greatest As-induced reductions in plant biomass occurred in the last weeks of the lupine cycle, coinciding with the flowering and maturation of pods (**Figure 1**). Within this period, flowering (10 weeks) was the highest and fastest vegetative growth stage of lupine plants, where maximum differences in the biomass production between As-treated and control plants were obtained. In contrast, other crops, such as pea (*18*), common bean (*15*), and mung bean (*22*), suffered notable biomass reduction after only a few days of As exposure. The retarding effect of As on lupine growth is similar to the behavior of As-tolerant clones of *Holcus lanatus* (*14*), whereas the growth response in nontolerant clones is closer to the results found in bean and pea. Similarly, for soybean, Reichmann (*8*) reported slight biomass reductions in roots but important decreases in shoot biomass after 34 days of As treatments.

With regard to pod biomass, there were no differences between 0 and 10 μM As doses (26.18 and 25.66 g of fresh wt plant^{-1}, respectively) at 14 weeks, whereas a 30% reduction relative to the control was observed with the 100 μM As supply (17.87 g of fresh wt plant^{-1}). For indeterminate plants such as lupines, vegetative and reproductive growths occur simultaneously and compete for photoassimilates (*23*). Thus, for the higher As dose applied, the parallel biomass reduction that occurred at 14 weeks in both vegetative organs and pods would indicate severe As toxicity. In contrast, for the lower As dose, pod biomass remained unaltered, whereas the biomass of vegetative organs was reduced. This fact indicates that the lower toxicity of the 10 μM As dose allowed the lupine to preserve the reproductive processes, but this occurred at the expense of vegetative growth.

Table 1 shows the parameters of nodule development and the total N content of white lupine after 10 weeks of treatments. The average nodule weight, number of nodules, and total N content in plants treated with 10 μM As did not show significant differences relative to the control plants. However, in 100 μM As-treated plants, both the nodule biomass per plant and the number of nodules were 1.5-fold lower than in the control. Similarly, the total N content decreased significantly (by 27%) in 100 μM As-treated plants relative to the control plants. The observed reductions in nodule biomass and N content at 10 weeks for 100 μM As supply agreed with the plant growth decreases of white lupine at flowering (**Figure 1**). Despite the growth inhibition and other alterations in 100 μM As-treated plants, the nodulation and symbiotic N_2-fixing ability of white lupine were still operative after 10 weeks of As exposure (**Table 1**). A complete inhibition of nodulation with 500 μM As supply and significantly high reductions with 10−250 μM As doses were described by Päivoke (*24*) in 40-day-treated pea plants. Similarly, the N content of lupine plants after 10 weeks of As supply (only 27% reduction for the 100 μM As dose) was less affected by arsenate than were 40-day-treated pea plants treated with 250 μM As (70% reduction) (*24*). Moreover, Porter and Sheridan (*6*) reported a 20% reduction in nitrogenase activity in alfalfa plants treated for 8 weeks with 13.3 μM As. These data suggest that As sensitivity of nodulation and N_2 fixation processes is dependent upon the plant species. In a similar manner, soybeans that were treated for 34 days with As doses equivalent to the low As supplied in this work suffered 20−50% reductions in nodule number; however, the N content of the shoot remained constant. This could be explained by the nitrate supply to the plants and by the increasing N_2-fixing activity of the nodules (*8*). Therefore, the results of the present study seem to indicate the relatively high As resistance of nodulation and symbiotic nitrogen fixation in white lupine.

As and P Distribution in Plant. Over the course of the culture cycle, both the shoot and root concentrations of As were higher for the 100 μM As supply than for the 10 μM As treatment (**Figure 2A**,**B**). Shoot concentrations of As increased with increasing exposure times in As-treated plants, whereas As concentrations in roots from 10 and 100 μM As treatments decreased at 14 weeks and from 7 to 14 weeks, respectively (**Figure 2A**,**B**). Moreover, both the increase of As concentration in shoot and the As decrease in root with increased exposure time were more pronounced in plants treated with the 100 μM As dose than in plants treated with the 10 μM dose. In As-treated plants, the increment of As concentrations in shoot together with the decrease of root concentrations of As in the last weeks of the culture cycle suggests a progressive increase of the root−shoot translocation rate of As (**Figure 2A**,**B**). The increasing accumulation of As in shoots over time coincided with the occurrence of the highest growth inhibition at the end of the lupine life cycle (**Figure 1**). Similar effects, induced by higher As doses and shorter exposure times, were previously reported (*4, 11*) for the growth of lupine plants and other species (*7, 10, 16*).

Regarding the concentrations of As in pods (**Table 2**), the Spanish maximum recommended concentration of As for food crops was established to be 1 mg kg^{-1} on a fresh weight basis (*25*). In the United Kingdom, the statutory limit for arsenic in most foods offered for sale is also 1 mg kg^{-1} of fresh wt (*26*). Assuming an average water content in pods of 90% (data not shown), only in the 100 μM As-treated plant pods did the As concentration rise above the maximum statutory limit (9 mg kg^{-1} dry wt). These results support the potential use of lupine plants in phytostabilization of As-contaminated soils without any risk of As entering the food chain.

Phosphorus concentrations tended to be higher in plants treated with 10 μM As than in control plants, reaching significant increases at 10 weeks (8%) and 7 weeks (27%) in shoot and root, respectively (**Figure 1C**,**D**). In contrast, plants treated with 100 μM As showed significantly lower P concentrations than in the control plants from 7 weeks of As supply. The maximum differences, both in the shoot (40%) and in the root (50%) concentrations of P, between 100 μM As and control plants were obtained at 10 weeks (**Figure 2C**,**D**). Concerning the exposure time, the shoot and root concentrations of P increased up to 10 and 7 weeks, respectively. This was followed by a decrease of P concentration in the last weeks of culture cycle, regardless of As treatment. The evolution of P plant concentration with time (**Figure 2B**,**C**) was directly related to biomass production. Thus, the decrease of P concentration, both in shoot and in root at the end of the culture cycle, was promoted by a

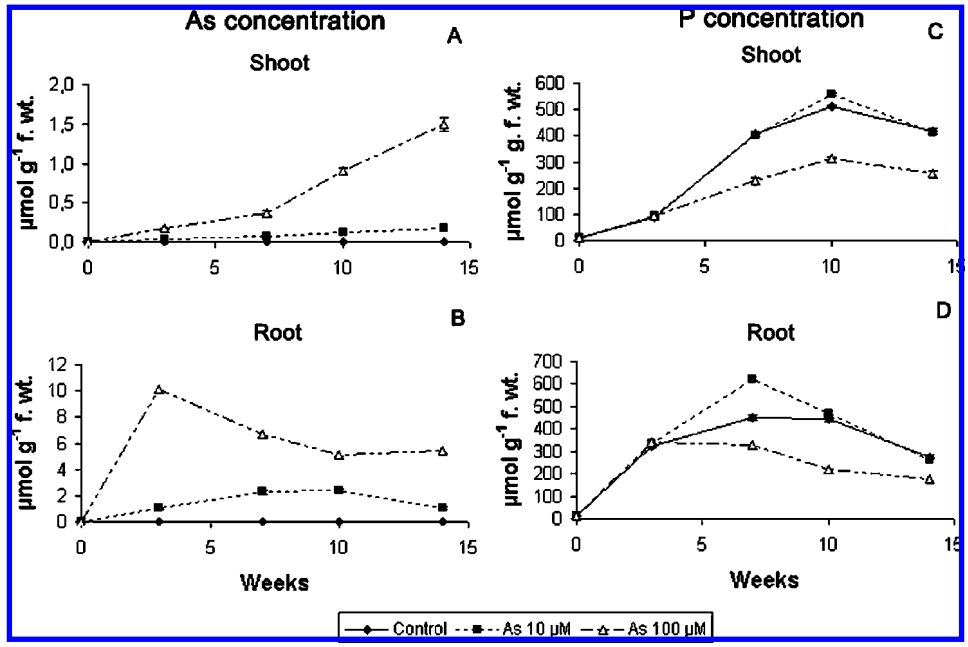

Figure 2. Concentration of As (**A**, **B**) and P (**C**, **D**) in shoot and root, respectively, of white lupine plants grown on perlite with 0, 10, and 100 μM arsenic doses for 3, 7, 10, and 14 weeks. Error bars indicate standard error of three replicates.

Table 2. Fe, Mn, Cu, Zn, P, and As Concentrations (Milligrams per Kilogram of Dry Weight) and Percentages of Elements (in Parentheses) in Pods of Lupine Plants Grown on Perlite for 14 Weeks (Control, 10 μM As, and 100 μM As)[a]

	control	10 μM As	100 μM As
Fe	47.96 ± 6.40 a (4.2)	67.19 ± 0.37 b (6.1)	47.00 ± 4.43 a (10.6)
Mn	314 ± 40 a (12.7)	276 ± 32 a (12.7)	204 ± 29 b (18.1)
Cu	3.39 ± 0.36 a (7.4)	2.53 ± 0.22 a (8.8)	1.32 ± 0.26 b (12.4)
Zn	28.65 ± 4.10 a (12.3)	28.27 ± 0.17 a (15.8)	22.85 ± 3.12 a (26.9)
P	216 ± 12 a (8.4)	223 ± 12 a (9.6)	138 ± 12 b (15.9)
As	nd	4.03 ± 0.92 a (3.7)	9.97 ± 1.53 b (1.6)

[a] Concentrations are expressed as mean ± SE. Significant differences are indicated by different characters ($P < 0.05$; $n = 3$). nd, not detected (detection limit = 0.06 ng of As l^{-1}).

Table 3. P/As Molar Ratios and Pearson Correlations between P/As Molar Ratio and Biomass from White Lupine Plants Grown on Perlite with 10 and 100 μM As Doses[a]

	10 μM As		100 μM As		Pearson coefficient (r)[b]	
weeks	shoot	root	shoot	root	shoot	root
3	2620 ± 342 a	344 ± 28 a	570 ± 29 a	34 ± 0 a	0.4372	0.1892
7	6051 ± 272 b	276 ± 14 ab	642 ± 60 a	50 ± 3 b	0.7580*	0.7226*
10	4466 ± 494 c	193 ± 5 c	350 ± 12 b	43 ± 1 c	0.8810**	0.9988***
14	2343 ± 315 a	268 ± 31 b	172 ± 12 c	33 ± 1 a	0.6166	0.5295

[a] P/As molar ratios are expressed as mean ± SE. Significant differences are indicated by different characters ($P < 0.05$; $n = 3$). [b] ***, **, *, significant at the 0.01, 0.05, and 0.1 level, respectively; $n = 6$.

dilution effect attributable to the fast-growth stage exhibited by lupine plants from 7 to 10 weeks (**Figure 1**). The effects of plant growth on nutrient concentrations have been widely reported (*12*).

The earlier and more intense As-induced reduction of P concentrations in roots compared with shoots (**Figure 2C**,**D**) indicates a progressive translocation of P from roots to shoots over time, as occurs with As. The P nutrition status of a given plant depends on the As concentration present in the growth media. Thus, Tu and Ma (*27*), using the As hyperaccumulator, *Pteris vittata*, concluded that As concentrations below 334 μM benefited plant growth and P uptake. The beneficial effect of low As concentrations (<10 μM) has also been reported for other plants, such as *Brassica napus* (*17*) and *H. lanatus* (*28*). In this study, both the root at 7 weeks and the shoot at 10 weeks of 10 μM As-treated plants showed higher P concentration than the control (**Figure 2C**,**D**), suggesting that the low arsenate dose could enhance P uptake. Because P can be substituted by As, the plant could react as if a P deficiency were in effect, thereby increasing P uptake (*13, 29*). In contrast, both the shoot and root of plants grown under 100 μM As conditions showed lower P concentrations than the control (**Figure 2C**,**D**), reaching a maximum decrease at flowering (10 weeks). This tendency probably resulted from As phytotoxicity, as indicated by the growth inhibition (**Figure 1**).

Arsenic concentration in pods at 14 weeks increased with As dose in the same extent (2.5-fold) that the percentage of As in pods respect to the total As content in the plants decreased (**Table 2**). In contrast, P concentration in pods decreased with the As supply also to the same extent (1.6-fold) that the percentage of P in pods with respect to the total P content in the plants increased. Regarding the percentage of elements in pods with respect to the total content, lupine plants excluded As from pods with increasing As doses in an opposite manner to P. Micronutrient concentrations showed a similar behavior and will be dealt with below.

The P/As molar ratios in As-treated lupine plants over the course of the culture cycle ranged from 33 to 6051 (**Table 3**), indicating a much higher concentration of P than As in the As-treated plants; therefore, the preferential uptake of P could play a protective role against As toxicity (*10, 29*). For doses of both 10 and 100 μM As, the molar ratios of P/As in shoot and root decreased in comparison with the control between 4.6 and 13.6 times and between 4.4 and 10.2 times, respectively. These ratios showed values of the same order as those found in other As

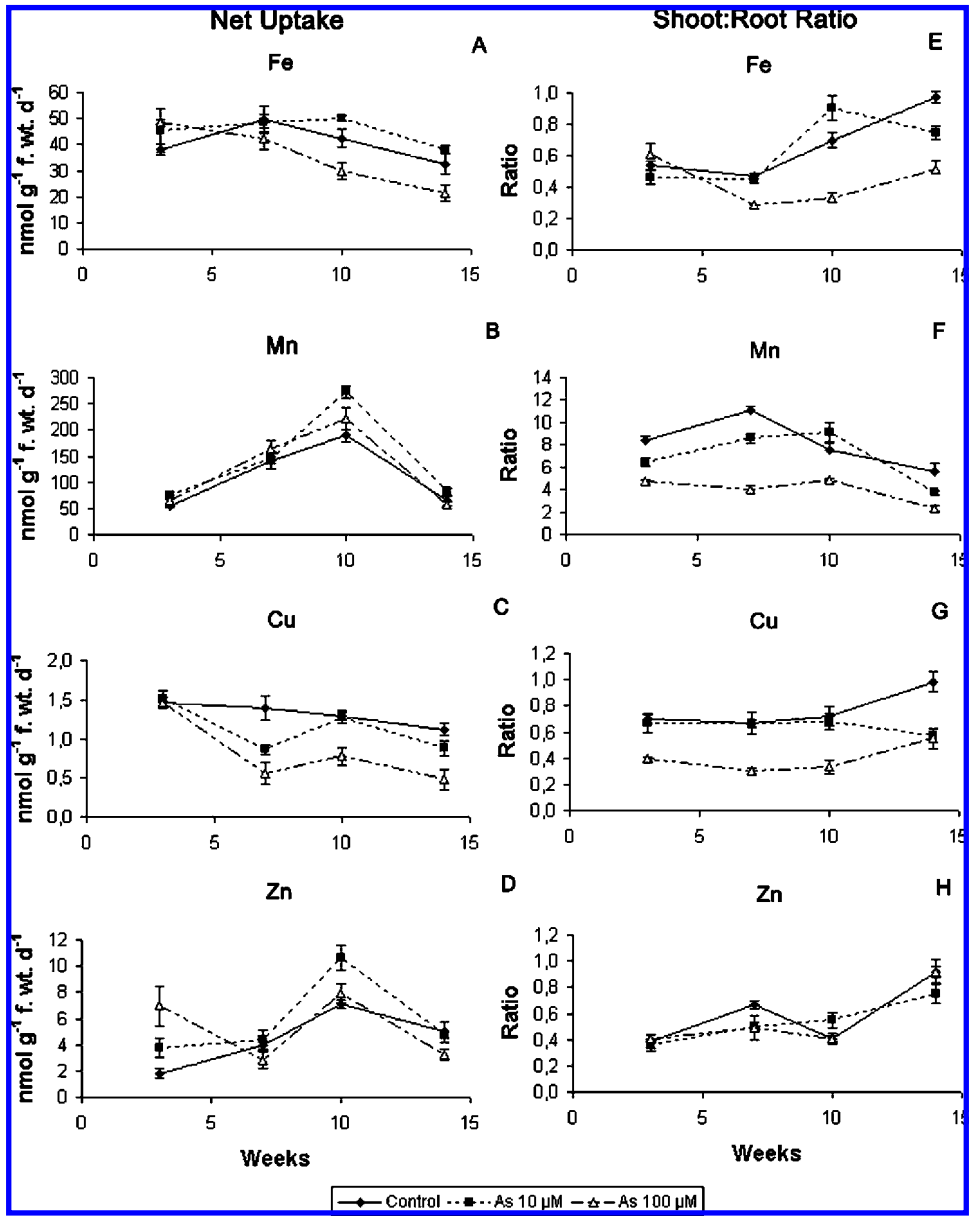

Figure 3. Net uptake (**A**−**D**) and shoot/root ratio (**E**−**H**) of Fe, Mn, Cu, and Zn of white lupine plants grown on perlite with 0, 10, and 100 μM arsenic doses for 3, 7, 10, and 14 weeks. Error bars indicate standard error of three replicates.

nonhyperaccumulator species exposed to arsenate (*7, 16, 30*). Significantly high correlations between P/As molar ratio and biomass at 7 and 10 weeks were observed in both root and shoot (**Table 3**). These correlations limit the use of the P/As molar ratio as an As stress indicator at the lupine flowering stage. Taking into account these results, P fertilization would be critical, especially at flowering, to optimize white lupine growth and for use of this species in As phytoremediation.

Micronutrient Distribution in Plants. The Fe uptake of plants treated with 10 μM As was higher than in the control, at both 10 and 14 weeks. However, the plants treated with 100 μM As showed a significantly lower Fe uptake than the control at the same exposure times (**Figure 3A**). The Mn uptake of As-treated plants was above the levels in control plants. Thus, after 10 weeks, Mn uptakes were 1.2- and 1.4-fold higher in plants treated with 10 and 100 μM As, respectively (**Figure 3B**). In contrast, the Cu uptake in plants treated with As was lower in comparison to control all along the culture cycle, showing 1.3- and 2.3-fold lower values in plants after 14 weeks of treatment with 10 and 100 μM As, respectively (**Figure 3C**). The uptake of Zn did not show a clear trend over time or with As treatments. However, the Zn uptake in 10 μM As-treated plants seemed to be higher than in the control plants, reaching values up to 1.5-fold higher at 10 weeks (**Figure 3D**).

The effect of arsenate on the evolution of micronutrient uptake depended on the element, the As concentration, and the exposure time. Periods of flowering and pod filling (10 and 14 weeks) were the phenologic stages that exhibited higher differences in mineral concentrations between As-treated and control plants. However, there was not a general effect of As on any micronutrient uptake. Despite this, 10 μM As concentration seemed to increase the uptake relative to the control, especially at 10 weeks, except for Cu uptake (**Figure 3**). The beneficial effect of a low As dose on P uptake (mentioned above) could also have involved the uptake of micronutrients in white lupine, although how it occurs is unclear. Cell membranes have been proposed to represent one of the major sites of action of toxic elements, including As (*10*). Alterations in cell membrane may then be expressed as changes in their selectivity, permeability, or other properties, resulting in an altered mineral element balance. This hypothesis could also be applied to the 100 μM As treatment, which, in contrast to the low As dose, promoted

a decrease in the uptake of Fe, Cu, and, to a lesser extent, Zn. Therefore, the main reason for the decrease in the uptake of these micronutrients could be the higher phytotoxicity of 100 μM As treatment compared to 10 μM As supply, as was reflected by the plant growth (**Figure 1**). Decreases in the concentrations of micronutrients in As-treated plants have been reported in pea (*7*), tomato (*16*), and bean (*31*).

Mn uptake was not reduced by As treatments; rather, it was enhanced, especially at 10 weeks (**Figure 3B**). White lupines have a greater Mn requirement than do many other crop plants; moreover, lupines are unable to mobilize Mn once it is deposited in leaf tissue, and lupines need a constant Mn supply to meet pod maturation requirements (*32*). Page et al. (*33*) suggested that Mn is rapidly released into the xylem, that it reaches photosynthetically active leaves via the transpiration stream, and that the element is not later redistributed via the phloem to other leaves. In this way, the As-induced increase of Mn uptake at 10 weeks, shown in this study, could be related to the very high uptake and translocation rate of Mn found in white lupine (**Figure 3B**,**F**) that resulted in Mn accumulation up to 0.2% dry wt in the shoot. This special feature of white lupine has been related to the special protective function of Mn in the photosynthetic system against heavy metals (*3*).

In addition, it is worth mentioning that the micronutrient concentrations, except for Cu, found in white lupine over the course of the culture cycle were within the levels reported to be normal for *L. albus*, according to Reuter and Robinson (*34*), regardless of the As treatment. As-induced decreases in Cu concentration have also been observed in several short-term studies in other crops (*7, 16, 31*), whereas Mascher et al. (*30*) reported Cu increases in red clover shoots after a larger application of As to soil. Thus, the causes of the high sensitivity of Cu content to As supply relative to other micronutrients in lupine plants are still unclear.

The Fe, Mn, and Zn translocation rates in plants with 10 μM As were significantly (1.3-, 1.2-, and 1.3-fold, respectively) higher than in the control plants at 10 weeks, whereas at 14 weeks, the rates were 1.3-, 1.5-, and 1.3-fold lower, respectively (**Figure 3E**,**F**,**H**). No significant differences in the Cu translocation rate between control and 10 μM As-treated plants were observed, except for the 14th week, where the Cu translocation rate in plants treated with 10 μM As decreased up to 1.7-fold compared to control (**Figure 3G**). Significantly lower values of Fe, Mn, and Cu translocation rates in the 100 μM As-treated plants relative to control were observed over the course of the culture cycle (**Figure 3E**,**F**,**G**). However, only at 7 weeks was the Zn translocation rate lower in plants treated with 100 μM As compared with the control (**Figure 3H**).

The translocation ratios of micronutrients along the lupine life cycle were in accordance with their uptake levels, described above. The beneficial effect 10 μM As on P and micronutrient (except for Cu) uptake was also found in the translocation ratios of these micronutrients at 10 weeks (**Figure 3A**,**B**,**D**). Likewise, the phytotoxic effect of 100 μM As concentration was reflected in the translocation of every micronutrient (except Zn) in the last weeks of culture cycle (**Figure 3**). Unloading of ions into xylem vessels within the stele and transpiration rate would be the main potential control points in the regulation of metal translocation from root to shoot (*12*), and therefore they are reasonable processes to be affected by As. The decrease in water content of 100 μM As-treated plants relative to controls (data not shown) seems to indicate that the transpiration rate is the main factor involved in the root−shoot translocation of elements. The insignificant differences in Zn translocation between As-treated and control plants could be due to the rapid retranslocation of Zn from old leaves to fast-growing plant parts of lupine plants (*33*).

Regarding micronutrient levels in pods of lupine plants grown at 14 weeks (**Table 2**), iron concentration in 10 μM As-treated pods was 1.4-fold higher than in the control, whereas no significant differences between 100 μM As and control were observed. The arsenate supply decreased the concentration of the other micronutrients, although significant differences were observed in only Mn and Cu of 100 μM As-treated pods. However, increasing As doses promoted an increase in the percentage of Fe, Mn, Cu, and Zn located in pods with respect to the total plant element concentrations. Elements differ widely in their ability to be remobilized within the phloem pathway from certain organs or tissues to the developing seeds (*12*). With regard to the percentage of elements in pods compared to the total plant element content, lupine plants excluded As from pods with increasing As doses, in contrast to their P and micronutrient uptake (**Table 2**). These findings may suggest a preferential transport of nutrients (at the expense of As) to the pods of As-treated lupine plants. Exclusion of As from fruit or grain has been widely reported in other crop species (*35*).

Stress Indicators. Stress indicators (total thiols and MDA) were measured in lupine plants at 7 and 10 weeks (**Figure 4**) to assess the sensitivity to arsenate. This sensitivity of lupine to arsenate was previously reflected, at these phenological stages, by the correlations between P/As molar ratios and biomass production (**Table 3**). Total thiol concentrations were higher in nodules, followed by leaves, stems, and roots, regardless of treatment. Only in 100 μM As-treated leaves at 10 weeks was thiol accumulation higher than in nodules (**Figure 4A**,**B**). The 10 μM dose tended to increase thiol levels in all organs of both 7- and 10-week-treated plants, although no significant differences compared to controls were obtained. In contrast, 100 μM As treatment significantly increased thiol concentrations in all cases (**Figure 4A**,**B**). Maximum increases of thiols were found at 10 weeks for leaves (1.9-fold) and at 7 weeks for stems, roots, and nodules (2.2-, 3.2-, and 1.6-fold, respectively). The 100 μM As treatment led to significantly higher concentrations of MDA compared with controls (**Figure 4C**,**D**). The highest increases for leaves, stems, roots, and nodules (1.3-, 1.2-, 1.7-, and 1.2-fold) were found at 10 weeks. Finally, the MDA levels were higher in roots, followed by nodules and the remaining plant organs, regardless of the treatment (**Figure 4C**,**D**).

The increases of both total thiols and MDA of the leaves of 100 μM As-treated plants relative to controls were higher at 10 weeks than at 7 weeks. Therefore, the stress indicator data confirmed that the highest sensitivity to As in lupine plants occurred during the flowering period of their culture cycle. Only short-term effects of As on PCs and MDA levels have been previously reported in *H. lanatus* (*14*). Likewise, As-induced increases both of PCs in *L. albus* (*4*) and of MDA in *Phaseolus vulgaris* (*15*) have been found at short exposure times. Moreover, the concomitant increase of MDA with electrolyte leakage in roots of 1-week-As-treated mung bean has recently been reported, confirming the As-induced membrane damage (*22*). Considering the different plant organs, lowest total thiols levels and highest increases in both total thiols and MDA of 100 μM As-treated plants respect to controls were found in roots. Just the opposite, high total thiols levels and low increases of both stress indicators, were obtained in nodules and leaves (**Figure 4**). These results suggested that roots were the plant organ more affected by As exposure compared to nodules and leaves. The higher growth inhibition of roots relative to shoots

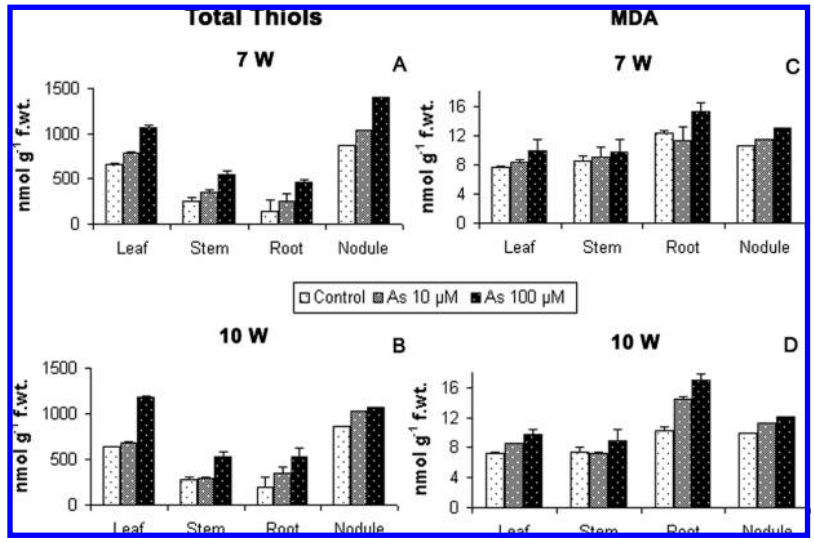

Figure 4. Total thiols (**A**, **B**) and MDA (**C**, **D**) concentrations of white lupine plants grown on perlite with 0, 10, and 100 μM arsenic doses for 7 and 10 weeks. Error bars indicate standard error of three replicates.

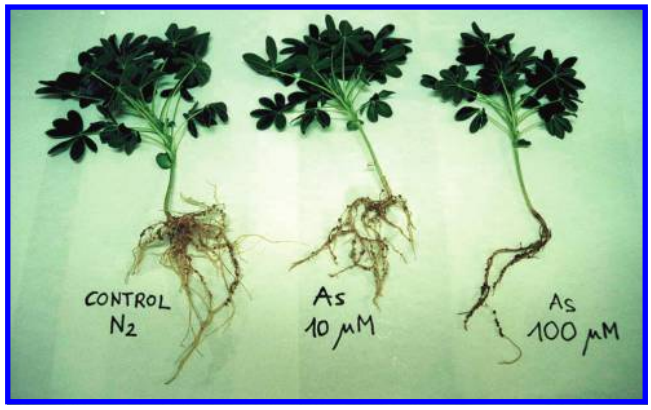

Figure 5. White lupine plants grown on perlite with 0, 10, and 100 μM arsenic doses for 7 weeks.

and the viability of nodules, which showed only a slight decrease in the nitrogen-fixing ability, of the 100 μM As-treated plants supports these findings. This idea can be visualized in **Figure 5**, where the 100 μM As-treated shoot appears green and is almost the same size as the control, whereas the 100 μM As-treated roots appear dark brown and notably smaller than the control. Nodules, although brownish and less abundant, were also present in 100 μM As treated roots.

Summarizing the results reported in this study, we can conclude that over the course of the culture cycle of white lupine, the reproductive period (from 10 weeks) is the most sensitive to long-term As exposure. Within this stage, only the 100 μM As treatment produced significant toxic effects on plant growth, symbiotically fixed N, P, and micronutrient status, especially on Cu, and stress indicators, whereas the 10 μM As dose, which is closer to the As available in As-polluted soils, did not induce important alterations in the plant. Furthermore, with the supply of 10 μM As, levels of the pollutant in pods were maintained below the statutory limit (0.4 mg kg^{-1} fresh wt). Growth reductions induced by 10 μM As supply could be attributed to root oxidative stress rather than to alterations in N_2 fixation. This highlights the higher As-induced damage in roots than in shoots and nodules.

Finally, the relatively high resistance of white lupine to long-term As exposure reported here, together with the preferential As accumulation in roots with minimum presence in pods, could have important implications in the phytoremediation practices with this species in As-contaminated sites (*1*, *5*). P and Cu nutrition in white lupine should be monitored over the course of the culture cycle, particularly at the flowering stage. Further studies focused on the establishment of a permanent vegetation cover with woody plants using white lupine as a colonizer species under field conditions are in progress.

ABBREVIATIONS USED

EDDHA, ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid); MDA, malondialdehyde; ICP-ES, inductively coupled argon plasma emission spectrometry; PCs, phytochelatins; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

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