The Distribution of Arsenate and Arsenite in Shoots and Roots of *Holcus lanatus* is Influenced by Arsenic Tolerance and Arsenate and Phosphate Supply

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The recent discovery that phytochelatins are important for arsenic (As) detoxification in terrestrial plants results in the necessity to understand As speciation and metabolism in plant material. A hydroponic study was therefore conducted to examine the effects of different levels of phosphate and arsenate [As(V)] on As speciation and distribution in tolerant and non-tolerant clones of *Holcus lanatus*. Speciation of As in tissue (using high-performance liquid chromatography-inductively coupled plasma mass spectrometry) revealed that the predominant species present were the inorganic As species (As(V) and arsenite [As(III)]), although small levels (<1%) of organic As species (dimethylarsinic acid and monomethylarsonic acid) were detected in shoot material. In roots, the proportion of total As present as As(III) generally increased with increasing levels of As(V) in the nutrient solution, whereas in shoots, the proportion of total As present as As(III) generally decreased with increasing levels of As(V). *H. lanatus* plants growing in the high-phosphorus (P) (100 μM) solution contained a higher proportion of As(V) (with regard to total As) in both roots and shoots than plants supplied with low P (10 μM); in addition, tolerant clones generally contained a higher proportion of As(V) with regard to total As than non-tolerant clones. The study further revealed that As(V) can be reduced to As(III) in both roots and shoots. Although the reduction capacity was limited, the reduction was closely regulated by As influx for all treatments. The results therefore provide a new understanding about As metabolism in *H. lanatus*.

Arsenic (As) is widely distributed in the environment, originating either from As in the soil parent material or from discharge of As onto land as a result of human activities. Consequently, people and livestock are being exposed to As via contamination of drinking water and consumption of food grown in As-contaminated soil or irrigated with As-contaminated water. Understanding how As is taken up by plants and subsequently transformed in plant tissue is therefore essential for estimating the risks posed to human and wildlife populations by As-contaminated soils (Meharg and Hartley-Whitaker, 2002).

In aerobic soils, arsenate [As(V)] is the most thermodynamically stable and hence dominant species. The uptake of As(V) by plants has been studied extensively (Hurd-Karrer, 1939; Asher and Reay, 1979; Meharg and Macnair, 1990, 1991; Khattak et al., 1991). It has been shown that As(V) and phosphate are taken up by the same plasma membrane transport system (Ullrich-Eberius et al., 1989; Meharg and Macnair, 1990). However, the transformation of As(V) in the plant tissue and the factors controlling the transformation and the subsequent translocation of As species from roots to shoots are not well understood.

In terrestrial plants, both organic and inorganic As species have been found (Van den Broeck et al., 1998; Koch et al., 2000; Mattusch et al., 2000; Francesconi et al., 2002), with the inorganic species (As(V) and arsenite [As(III)]) being the most dominant. Generally, only small levels of organic As species have been detected in plant tissue; however, it is unclear whether these species are a product of transformation in plants or whether they are simply taken up from the soil as such (Meharg and Hartley-Whitaker, 2002). Although phytoplankton, macroalgae, and mushrooms detoxify As by transformation of inorganic As to less phytotoxic organic As species (dimethylarsinic acid [DMA], monomethylarsonic acid [MMA], and arsenobetaine; Philips, 1990; Byrne et al., 1995), the low levels of organic As species found in terrestrial plants (in comparison with the total As) make it unlikely that this strategy is important for higher plants.

Both inorganic As species, As(V) and As(III), are highly toxic to plants. As(V) is a phosphate analog, and therefore it can compete with phosphate in the cytoplasm, replacing phosphate in ATP to form unstable complex ADP-As, which leads to the disruption of energy flows in cells (Ullrich-Eberius et al., 1989). On the other hand, As(III) is highly toxic to plants because it reacts with sulfhydryl groups (-SH) in enzymes and tissue proteins (Jocelyn, 1972), leading to inhibition of cellular function and death (Ullrich-Eberius et al., 1989).

Recent findings that As(III) is complexed with phytochelatins (PCs; thiol-rich peptides derived from glutathione) in a range of plant tissues (Sneller et al., 1999; Pickering et al., 2000; Schmöger et al., 2000; Hartley-Whitaker et al., 2001b) indicate that PCs play

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an important role in decreasing the toxicity of As in terrestrial plants.

Because As(V) has no affinity for the -SH groups in the PCs, it is believed that once inside the cytoplasm, As(V) is readily reduced to As(III), making it the predominant As species in roots and shoots. Pickering et al. (2000) found only As(III) in roots and shoots of Indian mustard (*Brassica juncea*) by using x-ray absorption near-edge technology. However, in many other studies, both As(V) and As(III) were found in different plant material as determined upon extraction and measurement by HPLC-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS; Van den Broeck et al., 1998; Koch et al., 2000; Mattusch et al., 2000; Vela et al., 2001; Francesconi et al., 2002). Meharg and Hartley-Whitaker (2002) suggested that the As(V) detected in plant material may have originated from PC-complexed As(III). PC-As(III) complexes are unstable in alkaline conditions; therefore, in extraction media with a pH >7.2, the PC-As(III) complexes may dissociate to PC and free As(V), resulting from reduction of PCs and oxidation of As(III) to As(V). However, different ratios of As(V) to As(III) in plant tissues subjected to different amounts of As(V) (Van den Broeck et al., 1998) or sampled in different seasons (Koch et al., 2000) indicate that both As(V) and As(III) do exist in plant tissue. Although the reduction of As(V) to As(III) and subsequent complexation of As(III) to PCs are important in the detoxification of As in higher plants, the factors controlling the speciation are not well understood. In this study, the speciation of As in roots and shoots of tolerant and non-tolerant clones of *Holcus lanatus* as influenced by different supplies of phosphate and As(V) is investigated.

RESULTS

Screening Experiment

The clones from As-contaminated and uncontaminated sites showed a large variation in root growth when exposed to the nutrient solution containing high-As(V) concentration. The root length of tillers originating from plants grown from seed collected at the contaminated site approached a normal distribution with a maximum number of tillers in class 11 (10.1–11 cm root length), whereas distribution of root length of tillers originating from plants grown from seed collected at the uncontaminated site showed a decreasing trend, with a maximum amount of tillers in class 1 (0–1 cm; Fig. 1). Clones tolerant and non-tolerant to As were selected based on the ratio of root growth in the high-As and zero-As solutions after 7 d (Fig. 2). Some plants from the contaminated site had very high ratios due to poor growth in the control solution, whereas they grew well in the high-As solution. Even though these plants may be dependent on As for growth (and thus ultimately very tolerant to As), they were not included in this study.

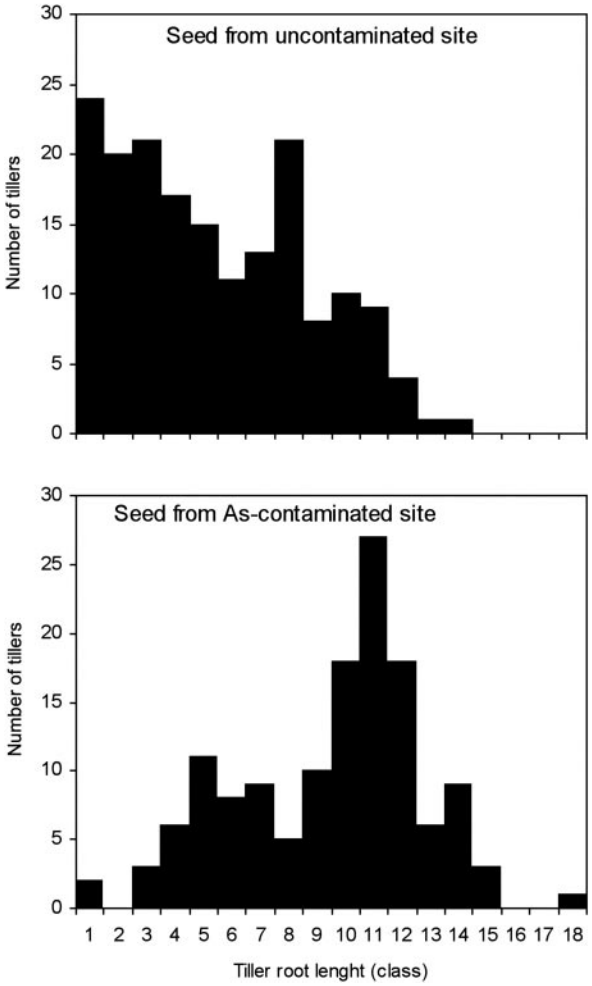

Figure 1. The distribution of root length of *H. lanatus* tillers originating from seed collected at the uncontaminated and the As-contaminated site after 7 d of growth in a nutrient solution supplemented with 133 μM As(V). The root length classes correspond to 0 to 1 cm (class 1), 1.1 to 2 cm (class 2), etc. Seed-grown plants (46 from uncontaminated and 35 from contaminated site) were used to produce tillers (five from each plant).

Plant Growth and Total P Concentrations

The dry weight and P concentration of roots and shoots are presented in Table I, whereas the analysis of variance of these data is presented in Table II. Root dry weight was not influenced significantly by any of the interactions (Table II). Increasing levels of As(V) in the nutrient solution resulted in a decrease in the root dry weight (Table I). Tolerant clones produced more roots than the non-tolerant clones, and the plants supplied with 100 μM phosphate produced significantly ($P < 0.001$) more roots than the plants growing in the 10 μM phosphate solution. The dry weight of shoots was significantly influenced by the As $\times$ P interaction; the shoot biomass decreased with increasing levels of As(V) in the nutrient solution only when the plants were supplied with low phosphate (10 μM). The dry weight of the shoots was

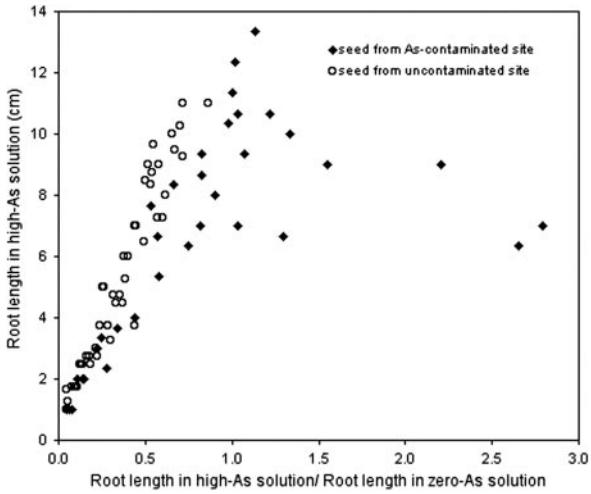

Figure 2. The root length of *H. lanatus* tillers after 7 d in a nutrient solution supplemented with 133 μM As(V) plotted against the ratio of the root length in the high-As (133 μM Na_2HAsO_4) and zero-As solutions after 7 d. Each observation is the average of five tillers isolated from individual plants originating from seed collected at an uncontaminated or an As-contaminated site.

further influenced by the As × clone interaction. Therefore, the shoot biomass of the non-tolerant clones decreased significantly with increasing levels of As(V) in the nutrient solution, whereas for the tolerant clones, the shoot biomass decreased only when the plants were growing in the high-As(V) solution (107 μM). The non-tolerant clones that were growing in the solution with low phosphate (10 μM) and high As(V) (107 μM) died within 4 d of As(V) application.

Phosphorus concentrations in roots and shoots were significantly influenced by the As × P interaction (Table II). Therefore, in plants treated with 100 μM phosphate, the P concentration in roots and shoots decreased with increasing levels of As(V), whereas for plants supplied with 10 μM phosphate, the P concentration increased with higher levels of As(V) in the nutrient solution (Table I). The non-tolerant clones contained significantly ($P < 0.001$) more P in roots and shoots than the tolerant clones.

As Concentrations in Roots and Shoots

As concentration in roots was not influenced by any of the interactions (Table III). The tolerant clones contained significantly ($P < 0.001$) less As in roots than the non-tolerant clones (Fig. 3). The As concentration in the roots increased significantly with increasing levels of As(V) in the nutrient solution, whereas increasing phosphate concentration from 10 to 100 μM decreased the As concentration in the roots by up to 90% in both tolerant and non-tolerant clones and across all levels of As(V) applied.

For the total As concentration in shoots, the clone × P interaction was significant, indicating that the As concentration in shoots was lower in tolerant than in non-tolerant clones at low P (10 μM), but not at high P (100 μM; Fig. 4; Table III). The As × P interaction was also significant, indicating that the As concentration in the shoots increased more with increasing levels of As(V) in the solution at high P (100 μM) than at low P (10 μM). The significance of the As × clone interaction indicated that the non-tolerant clones contained more As than tolerant clones only at 1.33 and 107 μM solution concentration of As(V).

The distribution of As within the plants showed that a greater percentage of As was transported to shoots in tolerant than in non-tolerant clones (20% versus 9%, respectively, averaged across all levels of As(V); see Fig. 5). Increasing the phosphate concentration from 10 to 100 μM enhanced transport of As from roots to shoots, especially in tolerant clones grown at 8 and 107 μM As(V) in the nutrient solution (significant clone × P and As × P interactions).

As Speciation in Roots and Shoots

As species were extracted quantitatively from the freeze-dried plant material (average recovery 110% ± 15%). The recovery was determined as the sum of the

Table I. *Phosphorus concentration and dry weight of roots and shoots of Holcus lanatus plants of As-tolerant (T) and non-tolerant (NT) clones exposed to different concentrations of As(V) (1.33, 8 or 107 μM) and phosphate (10 or 100 μM)*

Data represent the means (SE in parentheses). *, includes dead plant material.

As(V) Concentration	Phosphorus Concentration	*H. lanatus* Clone	Dry Wt		Phosphorus Concentration	
			Roots	Shoots	Roots	Shoots
μM			*mg plant^{-1}*		*mg g^{-1} dry wt*	
1.33	10	T	129 (20)	213 (4)	1.8 (0.03)	1.5 (0.07)
		NT	104 (20)	171 (2)	2.2 (0.2)	1.5 (0.2)
	100	T	423 (80)	234 (20)	3.1 (0.1)	5.4 (0.1)
		NT	415 (30)	173 (8)	3.8 (0.2)	6.1 (0.5)
8	10	T	379 (50)	162 (8)	2.0 (0.06)	1.4 (0.1)
		NT	123 (50)	85 (8)	3.1 (0.6)	2.4 (0.6)
	100	T	430 (80)	238 (4)	3.1 (0.08)	4.9 (0.5)
		NT	380 (80)	172 (6)	3.5 (0.2)	6.3 (0.2)
107	10	T	76 (1)	114 (20)	3.0 (0.004)	2.4 (0.04)
		NT	5 (0.1)*	28 (7)*	10.1 (0.5)*	4.1 (0.2)*
	100	T	414 (30)	198 (3)	2.8 (0.1)	4.0 (0.04)
		NT	227 (20)	142 (3)	3.5 (0.4)	4.5 (0.1)

Table II. *Analysis of variance for dry weight and phosphorus concentrations of shoots and roots of H. lanatus*

Significance was defined as $P \leq 0.05$. d.f., degrees of freedom.

Source	d.f.	*P* Values			
		Dry Wt		Phosphorus Concentration	
		Roots	Shoots	Roots	Shoots
As	2	0.009	<0.001	0.147	0.451
clone	1	0.009	<0.001	0.002	0.001
P	1	<0.001	<0.001	<0.001	<0.001
As.clone	2	0.217	0.036	0.742	0.138
As.P	2	0.168	<0.001	0.008	<0.001
clone.P	1	0.59	0.096	0.826	0.491
As.clone.P	2	0.159	0.093	0.13	0.3
Error	12				

extracted As species (measured by HPLC-ICP-MS) relative to the total As concentrations in plant material (measured in plant digests by hydride generation-ICP-MS). The recovery was slightly better (109% ± 9%) when it was expressed as the total As concentration in the plant extract (independent measurement by flow injection ICP-MS) relative to the total As concentration in plant material (measured in plant digests by hydride generation-ICP-MS). Most of the As species detected in roots and shoots were the inorganic As species As(V) and As(III). Only small amounts of organic As species (MMA and DMA) were detected in shoot material (<1%).

The total As(V) concentration in *H. lanatus* plants increased with increasing As(V) supply (Fig. 4; Table III). The roots of non-tolerant clones generally contained more As(V) than the roots of tolerant clones, especially at 8 and 107 μM As(V) (As × clone interaction was significant). Plants growing in high-P solution (100 μM) contained less As(V) in roots compared with plants supplied with low P (10 μM). However, the significance of the clone × P interaction indicated that the difference was greater for non-tolerant clones compared with tolerant ones. For the As(V) concentration in the shoots, all of the main and the interaction effects were significant, mainly because of the high value of As(V) concentration in dead shoot material of non-tolerant clones treated with 107 μM As(V) and low P (10 μM).

The total As(III) concentration in roots generally increased with increasing levels of As(V) in the nutrient solution, but not for plants grown in the low-P (10 μM) and the high-As(V) (107 μM) nutrient solution (significant As × P interaction; Fig. 5; Table III). The application of P decreased the concentration of As(III) in the roots, but only at 1.33 and 8 μM As(V) in the nutrient solution (significant As × P interaction). The non-tolerant clones contained more As(III) than the tolerant clones, except at low P in one case (significant clone × P interaction).

In the shoots, the total concentration of As(III) increased with increasing levels of As(V) in the nutrient solution (Fig. 5; Table III). The As × P interaction was significant, therefore the application of P generally decreased the concentration of As(III) in shoots, but not at 107 μM As(V) in the nutrient solution.

In roots, the proportion of total As present as As(III) generally increased with increasing levels of As(V) in the nutrient solution but not for plants grown in the low-P (10 μM) solution (significant As × P interaction; Tables IV and V). In the shoots, the proportion of total As present as As(III) generally decreased with increasing levels of As(V) in the nutrient solution. However, the increase between 1.33 and 8 μM As(V) was not significant for plants grown in high-P (100 μM) nutrient solution (significant As × P interaction). Both roots and shoots of tolerant clones generally contained a smaller proportion of

Table III. *Analysis of variance of total As, As(III), and As(V) concentrations in shoots and roots of H. lanatus*

Data were log(10) transformed. Significance was defined as $P \leq 0.05$. d.f., degrees of freedom.

Source	d.f.	*P* Values					
		Total As Concentration		Total As(III) Concentration		Total As(V) Concentration	
		Roots	Shoots	Roots	Shoots	Roots	Shoots
As	2	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Clone	1	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
P	1	<0.001	<0.001	<0.001	<0.001	<0.001	0.031
As.clone	2	0.151	<0.001	0.141	0.176	<0.001	<0.001
As.P	2	0.252	0.002	<0.001	<0.001	0.149	0.019
clone.P	1	0.79	<0.001	0.338	0.019	0.011	<0.001
As.clone.P	2	0.383	<0.001	0.002	0.092	0.002	<0.001
Error	12						

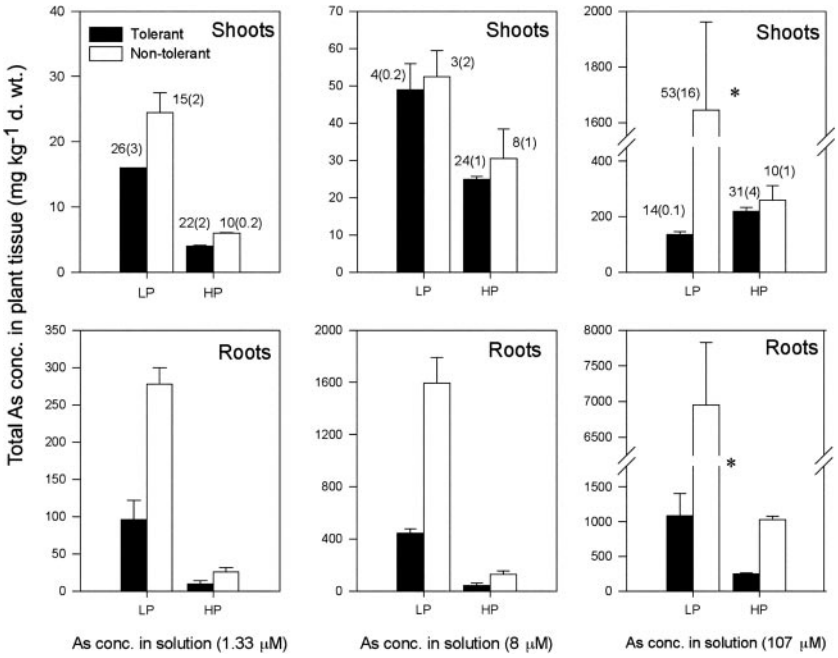

Figure 3. Concentrations of total As in roots and shoots of *H. lanatus* clones tolerant and non-tolerant to As. Plants were exposed to a range of As(V) concentrations in the low-P (10 μM = LP) and the high-P (100 μM = HP) nutrient solutions for 30 d. The fraction (in %) of total shoot As in relation to the total plant As is presented above or next to the bar (SE in parentheses). Data represent the means + SE. * indicates that dead plant material was included.

total As present as As(III) compared with non-tolerant clones. However, the significance of the As × clone and the clone × P interactions indicated that the difference was not significant for clones grown in the low-P solution (10 μM) or clones grown in the high-As solution (107 μM). The application of phosphate significantly ($P < 0.001$) decreased the proportion of total As present as As(III) in roots and increased it in shoots.

For all treatments, the proportion of total As present as As(III) in roots increased with increasing concentrations of As in the roots, until the roots contained mostly As(III) (90%) at total As concentration in tissue greater than 250 mg kg^{-1} (Fig. 6). In contrast to the roots, the proportion of total As present as As(III) in shoots decreased with increasing concentrations of As in shoots regardless of P supply or As tolerance until the shoots contained about 35% of total As present as As(III) at total As concentrations greater than 75 mg As kg^{-1} in shoots (Fig. 6).

In roots, the concentration of As(III) was linearly related to the concentration of total As in roots, re-

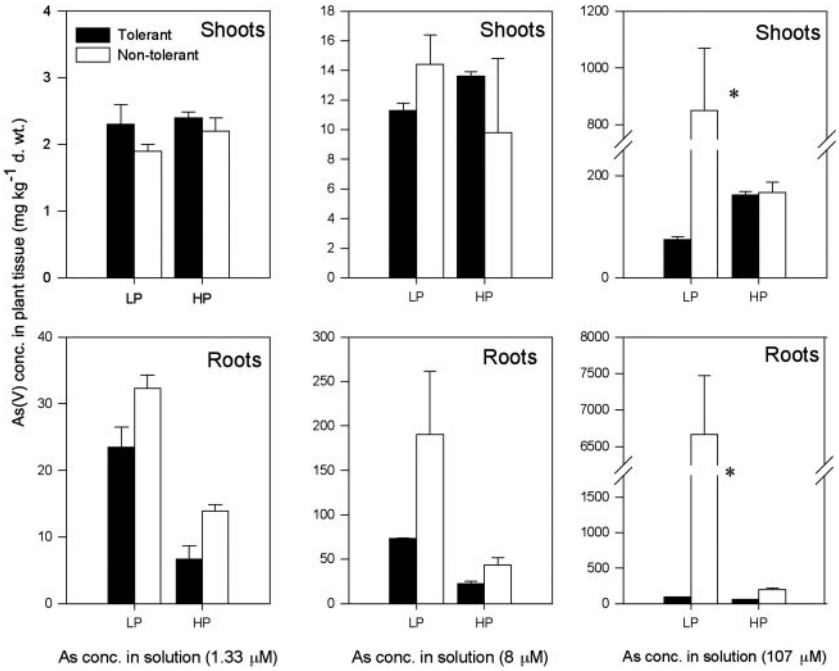

Figure 4. Concentrations of As(V) in roots and shoots of *H. lanatus* clones tolerant or non-tolerant to As. Plants were exposed to a range of As(V) concentrations in the low-P (10 μM = LP) and the high-P (100 μM = HP) nutrient solutions for 30 d. Data represent the mean + SE. * indicates that dead plant material was included.

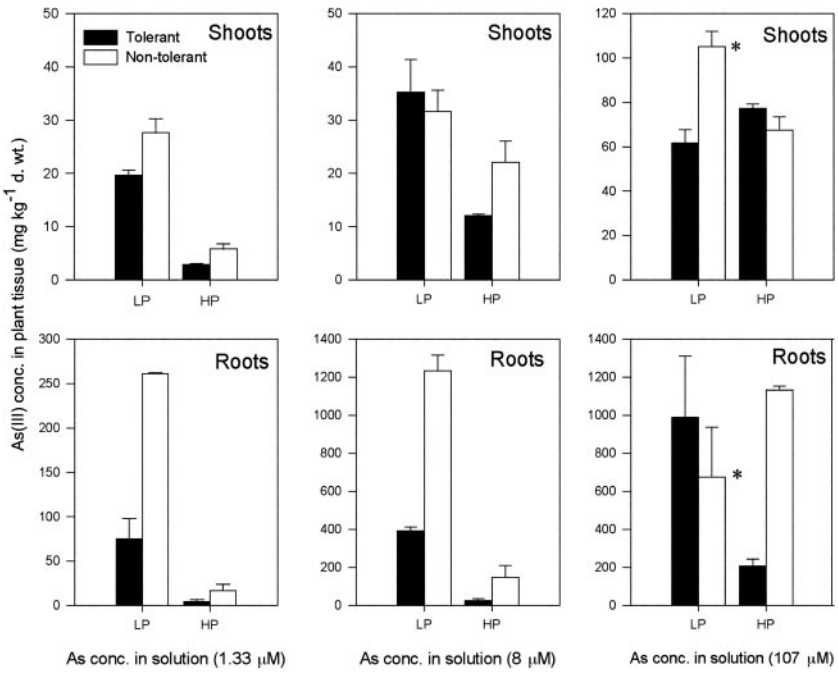

Figure 5. Concentrations of As(III) in roots and shoots of *H. lanatus* clones tolerant or non-tolerant to As. Plants were exposed to a range of As(V) concentrations in the low-P (10 μM = LP) and the high-P (100 μM = HP) nutrient solutions for 30 d. Data represent the mean + SE. * indicates that dead plant material was included.

gardless of As tolerance of clones, P nutrition, and As(V) supply (Fig. 7). In contrast, the concentration of As(V) in roots plotted against the root concentration of total As approached an exponential curve. At low concentrations of total As in roots, the concentration of As(V) increased slightly with increasing total concentration in roots; however, at total As concentrations of more than 1,200 mg kg^{-1}, the concentration of As(V) increased sharply with increasing concentrations of total As in roots, with a slope similar to the As(III) concentration increase (Fig. 7). In contrast to roots, the As(V) concentration in shoots increased linearly with elevated total concentrations of As in shoots. The relationship between shoot concentration of As(III) and total concentration of As, however, was logarithmic, with the As(III) concentration in shoots leveling off at total As concentrations greater than 120 mg kg^{-1}. Dead shoot and root material contained mostly As(V) (>90% of total As).

Relation between Plant Growth and As Speciation

The root dry weight remained constant with increasing proportions of total As present as As(III) until roots contained around 80% of total As as As(III) (Fig. 8). After that, the dry weight of the roots decreased with increasing proportions of total As present as As(III). There was, however, no clear trend apparent between shoot dry weight and proportion of As(III) in shoots (Fig. 8).

Table IV. *The fraction (in %) of As(III) in relation to the total As concentration in roots and shoots of H. lanatus clones tolerant (T) or non-tolerant (NT) to As*

Plants were exposed to a range of As(V) concentrations in the low P (10 μM) and the high P (100 μM) nutrient solutions for 30 d. Data represent the mean (SE in parentheses). *, includes dead plant material.

As(V) Concentration	Phosphorus Concentration	Clone	Proportion of As(III)	
			In Roots	In Shoots
μM			% of total plant As	
1.33	10	T	75 (4)	88 (2)
		NT	89 (0.2)	93 (1)
	100	T	38 (5)	54 (2)
		NT	53 (8)	71 (2)
8	10	T	84 (1)	74 (4)
		NT	87 (4)	67 (0.5)
	100	T	50 (8)	47 (1)
		NT	75 (5)	70 (7)
107	10	T	92 (3)	45 (1)
		NT	9 (0.7)*	7 (0.3)*
	100	T	77 (5)	32 (1)
		NT	85 (1)	28 (1)

Table V. *Analysis of variance of fraction of As(III) as % of total As concentrations in shoots and roots of H. lanatus*

Results from dead plant material were omitted. Main and interaction effects were considered significant when $P \leq 0.05$. Significance was defined as $P \leq 0.05$. d.f., degrees of freedom.

Source	d.f.	*P* Values	
		Proportion of As(III)	
		In Roots	In Shoots
As	2	<0.001	<0.001
Clone	1	0.019	0.001
P	1	<0.001	<0.001
As.clone	2	0.036	0.022
As.P	2	0.001	0.003
Clone.P	1	0.007	0.002
As.clone.P	1 (1)	0.054	<0.001
Error	11 (1)		

DISCUSSION

H. lanatus plants that originated from the As-contaminated site exhibited a higher degree of tolerance than plants growing on an uncontaminated site, a result similar to that reported by Meharg and Macnair (1992; Figs. 1 and 2). Although tolerant clones contain generally less As than non-tolerant ones when exposed to the same concentration of As (Fig. 3), tolerant clones can still have high concentrations in roots and shoots after a period of exposure (Hartley-Whitaker et al., 2001). Therefore, the reduced influx of As(V) alone is not enough to explain As(V) tolerance in *H. lanatus*. The recent finding that tolerant clones, exposed to As(V), contain significantly higher concentrations of As-PC complexes compared with non-tolerant clones, suggested an important role of As-PC complexes in detoxifying high internal As concentrations (Sneller et al., 1999; Pickering et al., 2000; Schmöger et al., 2000; Hartley-Whitaker et al., 2001b, 2002). The current understanding of As(V) metabolism in higher plants indicated that, after being taken up by the plasma membrane phosphate transport system, As(V) is reduced to As(III) in the cytoplasm (possibly by glutathione; Delnomdedieu et al., 1994) and then complexed by PCs. Because the As(III)-PC complexes are unstable at pH >7.2, it is likely that the As(III)-PC complexes are stored in the vacuole (pH 4.5–5.9) rather then in the cytoplasm (pH 7.2–7.4).

In most studies on As-PCs in terrestrial plants, it was assumed that As(III) was the dominant As species tested in both roots and shoots, regardless of the plant species or the As(V) and phosphate concentrations in the growth medium. Pickering et al. (2000) measured only As(III) in roots and shoots of Indian mustard by x-ray absorption near-edge fitting technology. However, both As(V) and As(III) have been determined in several plant species by HPLC-ICP-MS upon extraction of As species from the plant matrix (Van den Broeck et al., 1998; Koch et al., 1999; Francesconi et al., 2002).

When As species are extracted from plant material, however, the PC-As(III) complexes may dissociate into PC and free As(III), with the latter getting oxidized to As(V) if the pH of the extraction media is >7.2 (Meharg and Hartley-Whitaker, 2002). As(III) can also be gradually oxidized to As(V) during aging and drying of plant material (Webb et al., 2003). Therefore, care must be taken when extracting As species from the plant matrix to ensure that the extraction and sample preparation do not modify the in situ As speciation in plant material. However, different ratios of As(III)/As(V) were extracted from the plant material by the same extraction method even though plants had been treated with different amounts of As(V) (Van den Broeck et al., 1998) or the same plants had been harvested in different seasons (Koch et al., 1999), indicating that both As(V) and As(III) do occur in plant material. Moreover, both As(V) and As(III) were recently measured in shoots of *Pteris vittata* using x-ray absorption near-edge structure spectroscopy (Lombi et al., 2002). Because this technique allows for determination of in situ As speciation of plant material without the need for extraction, it is unlikely that the As(V) (25% of total As) reported in *P. vittata* would be an artifact of sample preparation. In the study presented here, different proportions of As(III) and As(V) were measured in tolerant and non-tolerant *H. lanatus* clones, depending on the levels of phosphate and As(V) supplied. A modified protein extracting solution (pH

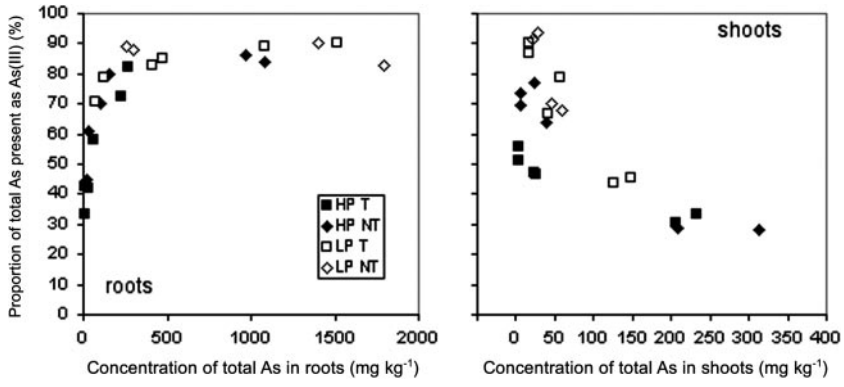

Figure 6. The proportion of As(III) in *H. lanatus* roots or shoots plotted against the concentration of total As in roots or shoots. Tolerant (T) and non-tolerant (NT) clones were grown in nutrient solutions with different levels of As(V) (1.33, 8, or 107 mM) and phosphate (10 μM = LP or 100 μM = HP). Only the data for plant material that was alive at the time of harvest were included.

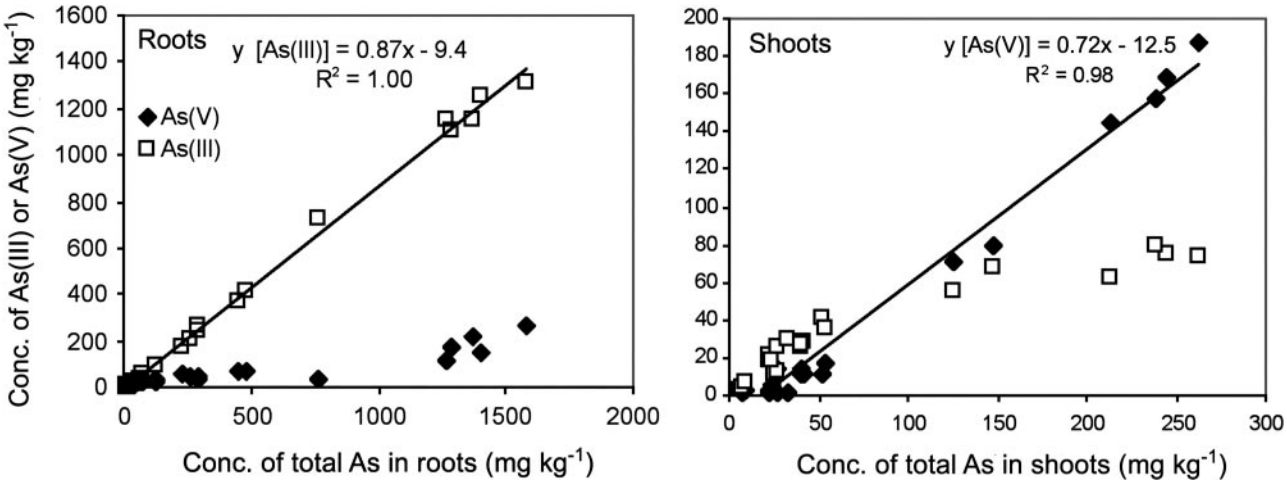

Figure 7. Concentrations of As(III) and As(V) in *H. lanatus* roots and shoots plotted against the concentration of total As in the roots or shoots. Tolerant (T) and non-tolerant (NT) clones were grown in nutrient solutions with different levels of As(V) (1.33, 8, or 107 μM) and phosphate (10 μM or 100 μM). Only the data for plant material that was alive at the time of harvest were included.

buffered at 5.5) was used to extract the As species from the plant matrix. This method was used successfully to extract quantitatively (104% ± 16%) As from freeze-dried root and shoot material of several plant species (oilseed rape [*Brassica napus*], Arabidopsis, and *Senna planitiicola*), while maintaining the integrity of the species extracted (Quaghebeur et al., 2003).

Although the proportion of As(III) varied significantly depending on P nutrition, As(V) supply, and As tolerance of clones, the proportion of As(III) in roots plotted against the concentration of total As in the roots approached a hyperbole (Fig. 6). In vitro studies have shown that both As(V) and As(III) decrease plant cell growth and ultimately lead to plant death; however, As(V) is more toxic than As(III) (Schmöger et al., 2000). Therefore, after As(V) is taken up, plants will protect the roots by reducing As(V) to As(III) (Van den Broeck et al., 1998). As(III) can then be detoxified by complexation to PCs (Sneller et al., 1999; Schmöger et al., 2000). In the study presented here, the proportion of As(III) in the roots increased with increasing concentrations of As in the root material, until the roots contained mostly As(III) (Fig. 6). The fact that at low As concentrations in roots, not all As(V) taken up is reduced to As(III) indicates that the reduction of As(V) to As(III) comes at a cost. Hartley-Whitaker et al. (2001a) reported an increase in reactive oxygen species, resulting in significant lipid peroxidation upon exposure of *H. lanatus* to As(V). The formation of reactive oxygen species could be due to the inhibition of key enzyme systems, together with electron leakage during the conversion of As(V) to As(III) (Halliwell and Gutteridge, 1984). Protection of roots by reducing As(V) to As(III) is obvious from the linear correlation between the As(III) concentration in roots and the concentration of total As in roots for all treatments regardless of whether As(III) was the dominant species (Fig. 7). Differences in As tolerance could therefore be explained, at least partially, by a reduced As(V) influx in tolerant clones.

Hartley-Whitaker et al. (2001b) reported that tolerant clones produced more PCs than non-tolerant clones, each grown at either their respective EC_{50} As(V) concentrations or at equal external concentrations of As(V). Therefore, they concluded that the adaptation of grasses to withstand high levels of As(V) relies on constitutive production of PCs. However, our analysis of their data showed that the PC production in roots was correlated linearly (slope 2.4, $R^2 = 0.95$) to the total As concentration in roots, regardless of the level of As tolerance of clones. Therefore, PCs might provide a constitutive detoxi-

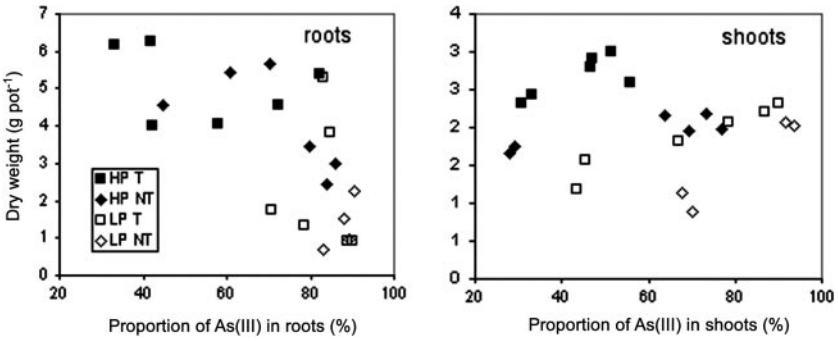

Figure 8. Dry weight of *H. lanatus* roots and shoots plotted against the proportion of As(III) present in roots or shoots. Tolerant (T) and non-tolerant (NT) clones were grown in nutrient solutions with different levels of As(V) (1.33, 8, or 107 μM) and phosphate (10 μM = LP or 100 μM = HP). Only the data for plant material that was alive at the time of harvest were included.

fication mechanism in both tolerant and non-tolerant plants (similar to Cd and Cu tolerance; De Knecht et al., 1992; Schat and Kalff, 1992).

In the present study, the As(III) concentration in the roots correlated linearly (slope 0.87, $R^2 = 1.00$) with the concentration of total As in the roots regardless of As tolerance of clones (and for various proportions of As(III) in roots). The slope of the PC production versus total As concentration in roots (slope = 2.4; data from Hartley-Whitaker et al. [2001b]) is approximately three times (2.4 versus 0.87) the slope of the concentration of As(III) versus total concentration in roots (Fig. 8). Therefore, one As(III) molecule would be complexed with three -SH molecules, a ratio similar to the ratio observed in vitro by Schmöger et al. (2000).

No xylem sap was collected in this study due to inherent difficulties in collecting xylem sap in grasses. In contrast, Pickering et al. (2000) measured As(V) (41% of the total) and As(III) (59%) in xylem sap of Indian mustard plants, with both As species occurring as free ions in the xylem sap (i.e. not complexed to -SH groups) and only As(III) being detected as complexed to -SH groups in roots and shoots. This would therefore indicate that the reduction of As(V) to As(III) can occur in both roots and shoots. In the study presented here, the proportion of As(III) in roots did not necessarily correlate with the proportion of As(III) in shoots (Table IV), providing further evidence that As(V) can be reduced to As(III) in both roots and shoots.

In contrast with roots, the proportion of total As as As(III) in shoots plotted against the concentration of total As in shoots approached a logarithmic curve (Fig. 6). Therefore, it seems that at low-As concentrations in the roots, most of As(V) is reduced to As(III) in the roots, and only a small amount of As(V) is translocated from roots to shoots. At these low concentrations of As(V) in shoots, the shoots are also able to reduce As(V) to As(III) (Fig. 7). However, with increasing concentrations of As in the roots, the maximum capacity to reduce As(V) to As(III) will be reached in roots (Fig. 7), after which more As(V) will be translocated from roots to shoots, eventually overwhelming the shoot capacity to reduce As(V) to As(III). A limited capacity of both roots and shoots to reduce As(V) to As(III) might be due to a requirement for reductants.

CONCLUSIONS

Shoots and roots of *H. lanatus* contain mainly inorganic As species As(V) and As(III), and the speciation of As was closely regulated by As(V) influx in both tolerant and non-tolerant clones. The reduction of As(V) to As(III) is the first step in the detoxification of As(V), followed by the formation of As(III)-PC complexes, illustrating the importance of As speciation in understanding As metabolism in *H. lanatus*.

MATERIALS AND METHODS

Tolerance Test

Seed of *Holcus lanatus* was collected from an uncontaminated site (University of Exeter, Devon, UK) and an As-Cu contaminated site (Gawton United mine, Devon, UK; grid ref. SX452688). Plants of each population were grown from seed in potting mix and maintained in a greenhouse, with tillers screened for As(V) tolerance using a test described by Wilkins (1957) and adapted by Meharg and Macnair (1992). In brief, un-rooted tillers of the genotypes were placed in a nutrient solution containing 0.2 mM $Ca(NO_3)_2$, 0.2 mM KNO_3, 0.2 mM $MgSO_4$, and 0 or 0.133 mM Na_2HAsO_4 for 7 d. The experiment was conducted in a controlled temperature room (25°C/20°C) with a 16-h day. Tolerant and non-tolerant clones were selected from the populations based on the extent of root growth.

Plant Growth

Around 20 un-rooted tillers per clone (12 tolerant and 12 non-tolerant clones used) were placed in a solution containing 0.25 mM $CaCl_2$ and 5 μM H_3BO_3. After 3 d, 12 rooted tillers (one from each tolerant or non-tolerant clone) were transferred to each plastic pot (5.5 L) containing the full-strength nutrient solution (0.2 mM KNO_3, 0.2 mM NH_4NO_3, 0.5 mM $CaCl_2$, 0.25 mM $MgSO_4$, 20 μM FeNa-EDTA, 1 μM $MnCl_2$, 0.1 μM $CuCl_2$, 0.5 μM $ZnCl_2$, 5 μM H_3BO_3, 0.05 μM $Na_2MoO_4 \cdot 2H_2O$, and 1 mM MES). The P treatment was either 10 or 100 μM KH_2PO_4. The pH of the nutrient solution was 6.0 (adjusted with 1 M HCl or 1 M KOH). The As treatments (1.33, 8, or 107 μM As as Na_2HAsO_4) were applied on d 6, after the tillers had 3 d to adjust to the full-strength nutrient solution. The solution was aerated continuously and was changed every 3 d.

The experiment was set up in a completely randomized design with factorial arrangements of treatments. There were two clones (tolerant or non-tolerant), two P levels (10 or 100 μM), and three As(V) levels (1.33, 8, or 107 μM). Each treatment was replicated two times. Plants were grown in a controlled temperature room (25°C/20°C). After 30 d, root and shoot material was harvested. Whereas the shoot material was snap frozen in liquid nitrogen, the roots were first rinsed in ice-cold phosphate buffer (1 mM Na_2HPO_4 + 10 mM MES + 0.5 mM $Ca(NO_3)_2$) to desorb As(V) from the root free space (Asher and Reay, 1979). Both shoots and roots were then freeze-dried, ground, and stored at 4°C in a sealed (air tight) plastic bag containing silica gel (20 g). A preliminary study showed that purging (N_2) of the plastic bag in which the samples were stored did not affect As speciation. Some plant material was dead at the time of harvest. Results presented from dead plant material are identified in tables and figures with an asterisk behind the value.

Determination of Total As and P

Freeze-dried root and shoot material (0.500 g) was digested in concentrated HNO_3 (10 mL) at 130°C until the volume of acid was reduced to around 1 mL. The mixture was then made up to 10 mL with milliQ-water (18 mΩ cm^{-1} resistivity). Total As concentration was measured by mixing 1 mL of digest with 9 mL of a reducing solution containing 1.5% (w/v) potassium iodide, 1.5% (w/v) ascorbic acid, and 10% (v/v) hydrochloric acid. This mixture was heated in a water bath at 50°C for 1 h. Total As was determined by a hydride generation technique using a B050–5540 continuous flow vapor system (PerkinElmer Life Sciences, Boston) interfaced with a PerkinElmer SCEEX ELAN 6000 series ICP-MS (SCIEX, Toronto; for further details, see Quaghebeur et al., 2003).

Phosphorus was determined spectrophotometrically by reaction with malachite green and measurement of A_{630} (Motomizu et al., 1983). Because As(V) interferes with phosphate determination, As(V) was first reduced to As(III) with sodium metabisulphite (Linge and Oldham, 2001). The standard reference material (bush leaves, CBW07603, and GSV-2; Institute of Geophysical and Geochemical Exploration, Langfang, China; certified As concentration, 1.25 ± 0.1 μg g^{-1}; certified P concentration, 1.00 ± 0.03 mg g^{-1}) was analyzed as described above. The total As and total phosphorus recoveries for the certified reference material were 95% ± 10% and 98% ± 3%, respectively ($n = 3$).

Extraction of As Species

Extraction of As species was done according to the method described by Quaghebeur et al. (2003). A portion (150 ± 0.1 mg) of the powdered plant

tissue sample was weighed into a centrifuge tube. An extraction solution (10 mL) containing 0.33 M Suc, 50 mM MES, 5 mM EDTA, and 5 mM L-ascorbate was added. The pH of the solution was adjusted to pH 5.5 with 1 M potassium hydroxide. The samples were heated at 90°C for 20 min in a Mars Microwave extraction system (CEM, Matthews, NC). The temperature was monitored in a control vessel by an armored fiber-optic temperature control probe. Samples were heated to a set temperature, and microwave energy was applied to the sample when its temperature fell below the set temperature. The reaction vessels used were 50-mL polypropylene, non-pyrogenic centrifuge tubes with a breathing hole (0.32-cm i.d.) in the cap. After microwave heating, the samples were allowed to cool down and were filtered (no.1, Whatman, Clifton, NJ; followed by 0.22-μm filter paper) before injection into the HPLC column. Total As in a portion of the filtrate was then measured by flow injection-ICP-MS.

Determination of the As Species by HPLC-ICP-MS

A dual head HPLC pump (600E, Waters, Milford, MA) was used to deliver the mobile phase for the chromatographic work. Samples were injected using a model 7725i injector (Rheodyne, Rohnert Park, CA) equipped with a fixed 20-μL sample loop. Separations were performed on a PRP-X100 anion-exchange column (250- × 4.1-mm i.d., 10 μm) from Hamilton (Reno, NV). The column temperature was 40°C, and the mobile phase consisted of 20 mM $NH_4H_2PO_4$ (pH 5.6 adjusted with aqueous NH_3). The mobile phase flow rate was 1.5 mL min^{-1}. The outlet of the HPLC column was connected via an 800-mm, 0.0625-inch PEEK capillary tube (0.13-mm i.d.) to the cyclonic spray chamber of a PerkinElmer SCEEX ELAN 6000 ICP-MS. The ion intensities at m/z 75 and 77 were monitored. The lens voltage and nebulizer gas flow were optimized daily for maximum counts (m/z 75) using a solution of the mobile phase containing 20 μg As L^{-1}. As compounds were quantified by external calibration with standard solutions of As(III), As(V), DMA, and MMA. For further details on the method, see Quaghebeur et al. (2003).

Statistical Analysis

Data were analyzed using ANOVA (Genstat 5th Edition, 2000, Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, UK).

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