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Relationship between plant phosphorus status and the kinetics of arsenate influx in clones of *Deschampsia cespitosa* (L.) Beauv. that differ in their tolerance to arsenate

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Abstract

Uptake kinetics of arsenate were determined in arsenate tolerant and non-tolerant clones of the grass *Deschampsia cespitosa* under differing root phosphorus status to investigate the mechanism controlling the suppression of arsenate influx observed in tolerant clones. Influx was always lower in tolerants compared to non-tolerants. Short term influx of arsenate by the high affinity uptake system in both tolerant clones was relatively insensitive to root phosphorus status. This was in contrast to the literature where the regulation of the phosphate (arsenate) uptake system is normally much more responsive to plant phosphorus status. The low affinity uptake system in both tolerant and non-tolerant clones, unlike the high affinity uptake system, was more closely regulated by root phosphate status and was repressed to a much greater degree under increasing root phosphorus levels than the high affinity system.

Introduction

Studies into the physiology and mechanisms of ion transport across the plasmalemma in micro-organisms have been greatly facilitated by the isolation of mutants with altered physiology (Beever and Burns, 1980; Silver and Misra, 1988). Such mutants are normally screened by exposing wild type populations to toxic levels of the ion of interest or an analogue of this ion. A similar approach would be useful in angiosperms, but difficulties arise in screening for mutants as generation time does not allow artificial selection pressures, such of those used in microbial studies, to be applied. An alternative approach is to screen for mutants from environments with elevated levels of toxic ions as a number of species have evolved tolerance to metal contaminated soils and the evolution of plant adaptation to metal contaminated soils must require an alteration to normal physiology. Many contaminating metals may be essential micronutrients such as Cu, Mn, and Zn and other metals behave as analogues of essential nutrients. Arsenate behaves as a phosphate analogue (Asher and Reay, 1979), selenate as a sulphate analogue (Brown and Schrift, 1982) and the divalent cations Cd, Ni and Pb are analogues of essential divalent cations (Clarkson and Lüttge, 1989). Physiological adaptation to contaminating metals may due to adaptation of ion uptake systems (Verkleij and Schat, 1990). Cu tolerance in *Silene vulgaris* is thought to be due to an adaptation of integral plasmalemma proteins (De Vos, Vonk, Vooijs and Schat, 1992) as is Al tolerance in wheat cultivars (Huang, Schaff, Grunes and Kochian, 1992).

In arsenate tolerant clones of the grass *Holcus lanatus* reduction in arsenate influx is achieved through suppression of the high affinity phosphate uptake system leading to reduced influx of both phosphate and arsenate (Meharg and Macnair, 1990, 1991a, 1992a,b). The phosphate uptake system in plant roots is inducible under low plant phosphorus status (Clarkson and Lüttge, 1991) and the mechanism of reduced arsenate influx in tolerant clones of *H. lanatus* appears to be the inhibition of synthesis of the high affinity phosphate carrier under low plant phosphate status, with the high affinity uptake system present at a constitu-

tively suppressed level in tolerant plants (Meharg and Macnair, 1992a).

Deschampsia cespitosa has also evolved tolerance to arsenate and again tolerance is achieved by reduction of arsenate influx in tolerant clones (Meharg and Macnair, 1991b). In the study reported here the relationship between arsenate influx and root phosphorus status was investigated by determining the kinetic parameters of arsenate influx in tolerant and non-tolerant clones of *D. cespitosa* grown at differing levels of phosphate nutrition to investigate the suppression of arsenate influx.

Materials and methods

Plant material

The arsenate tolerant genotype came from a site adjacent to the Coniston smelter, Sudbury, Ontario, Canada. The non-tolerant genotype was collected from a heath population at Sandford, Devon, UK. Prior to the experiments described here, the plants had been maintained for more than 2 y in a glass-house, and grown in pots of John Innes compost. In all experiments, unrooted-tillers of the genotypes were placed in a nutrient solution containing 0.2 mol m^{-3} $Ca(NO_3)_2$, 0.2 mol m^{-3} KNO_3 and 0.1 mol m^{-3} $MgSO_4$ $7H_2O$ for 7d. Nutrient solutions were either phosphate free or amended with K_2HPO_4 depending on the experimental conditions. Tillers were rooted in a glass house in 12 L containers (containing 10 L of nutrient solution) fitted with lids with 28 holes into which the tillers were inserted.

Incubation procedure

To determine arsenate uptake in excised roots, replicate samples of roots (excised at the node) were incubated in 100 mL of aerated test solution for 20 min at room temperature. Stock solutions of arsenate were prepared by dissolving Na_2HAsO_4 $7H_2O$. All test solutions contained 10 mol m^{-3} 2-[N-Morpholino] ethanesulphonic acid (MES) and 0.5 mol m^{-3} $Ca(NO_3)_2$, and the test solutions were titrated to pH 5 with KOH. In all experiments on termination of incubation in test solution the roots were rinsed in 100 mL ice cold solution containing 1 mol m^{-3} K_2HPO_4, 10 mol m^{-3} MES and 0.5 mol m^{-3} $Ca(NO_3)_2$. The roots were then incubated for 10 min in 100 mL ice cold solution of the same composition to ensure desorption of arsenate from the roots' free space (Asher and Reay, 1979). Fresh weights of the roots were determined before analysis.

Analysis

Arsenic was determined by digesting roots in 2 mL concentrated nitric acid (Aristar grade). Samples were digested by heating on a block digester for 1 h at 180 °C and 1 h at 200 °C, to evaporate the samples to dryness. The residue was taken up in 10 mL of 5% HCl (Analar grade) containing 20 mol m^{-3} KI. Arsenic was then determined by a hydride generation technique using a Philips PU9060 continuous flow vapour system which was interfaced with a Philips SP9 series atomic absorption spectrophotometer. Total phosphate was determined on roots dried at 70 °C using auto-analyzer techniques after digestion with hydrogen peroxide and sulphuric acid (Allen, 1974).

Results

Root phosphate status and arsenate influx

D. cespitosa clones were rooted for 7d in phosphate free nutrient solution. Plants were then transferred to nutrient solution containing 0.05 mol m^{-3} phosphate. Influx of 0.05 mol m^{-3} arsenate and root phosphorus status were determined before transfer to the phosphate nutrient solution and at 24h intervals for 5d after transfer. Excised roots were placed for 30 min in the phosphate free aerated MES buffer solution before arsenate influx was determined. Results of root phosphorus status are reported in Figure 1. The phosphorus status of the tolerant roots was relatively constant over the 5d of the experiment and was around 200 $\mu g\ g^{-1}$ root fresh wt. This was in contrast to non-tolerant roots where the phosphorus status increased by about 100% over the course of the experiment, with the phosphorus status increasing at a relatively constant rate. The phosphorus content of non-tolerants was considerably greater than tolerants after 5d in 0.05 mol m^{-3} phosphate. When arsenate influx was determined (Fig. 2) there was little change in the rate of arsenate influx during the experiment in both tolerant and non-tolerant clones. Influx of arsenate was always about 100% greater in non-tolerants compared to tolerants.

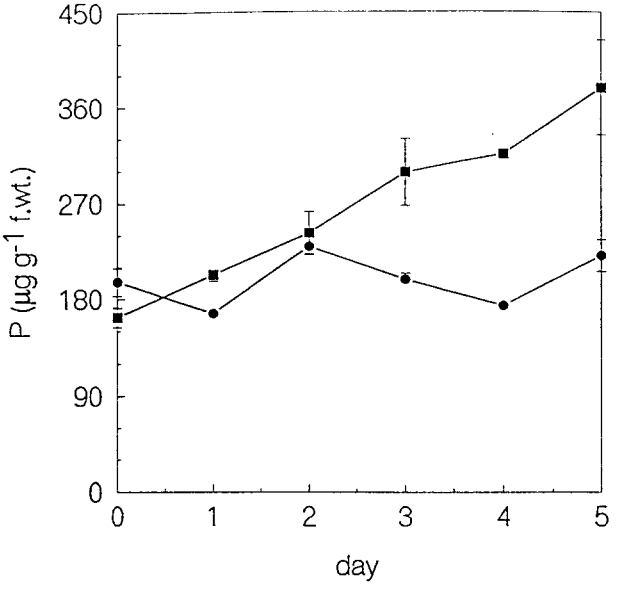

Fig. 1. Phosphorus status of the roots of tolerant and non-tolerant clones on successive days after transfer to nutrient solution containing 0.05 mol m^{-3} phosphate. Circles, tolerant; squares, non-tolerant. Each point was the average of 3 determinations and error *bars* are ± SE of that mean.

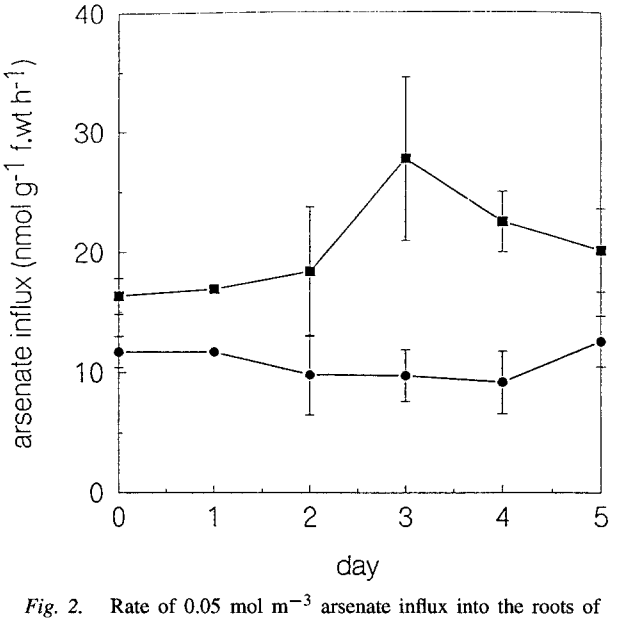

Fig. 2. Rate of 0.05 mol m^{-3} arsenate influx into the roots of tolerant and non-tolerant clones on successive days after transfer to nutrient solution containing 0.05 mol m^{-3} phosphate. Circles, tolerant; squares, non-tolerant. Each point was the average of 3 determinations and error *bars* are ± SE of that mean.

Kinetic parameters of arsenate influx determined at differing plant phosphate status

Concentration dependent influx of arsenate was determined in roots of *D. cespitosa* rooted for 7d in nutrient solutions of differing phosphate concentrations, phosphate levels being 0, 0.05 and 0.5 mol m^{-3}. At the end of the 7d roots were excised and incubated for 30 min in phosphate free MES buffer before influx was determined. Influx was determined at arsenate concentrations ranging from 0.005 - 5 mol m^{-3}. The concentration dependent influx isotherms were analyzed by fitting Michaelis-Menten functions to the data using a computer program based on the Marquardt algorithm (Marquardt, 1963) that iteratively reduces the sums of squares to achieve the best fit. Uptake kinetics are normally described by two additive Michaelis-Menten functions which represent two uptake carriers in the plasmalemma, the model of ion influx that has gained the widest acceptance (Epstein, 1976). One uptake carrier dominates at low substrate concentrations and is termed the high affinity uptake system (substrate concentrations < 0.1 mol m^{-3}), the other is dominant at high substrate concentrations and is termed the low affinity uptake systems (substrate concentrations > 0.1 mol m^{-3}). Both carriers obey saturation kinetics. It was found impossible to fit two additive Michaelis-Menten functions to the data as the low affinity system saturates at very high substrate concentrations, making the model insensitive at low substrate concentrations. As a compromise, data was, therefore, analyzed by splitting the uptake isotherms into their high affinity and low affinity components and fitting single Michaelis-Menten functions to these components. Although this will distort the uptake kinetics of the high affinity uptake system, the distortion will be less than the distortion that would be caused by fitting to the high and low affinity uptake system simultaneously due to the error of incorperated in determination of the kinetics for the low affinity uptake system (Table 2).

High affinity uptake

The phosphorus status of roots grown at 0, 0.05 and 0.5 mol m^{-3} phosphate are reported in Table 1. Phosphorus status increases as expected with increasing phosphate nutrition, although the increase in non-tolerants with increasing phosphate was greater than in tolerants. This is reflected by the highly significant phosphate times genotype interaction term in the analysis of variance (Table 1).

Table 1. Phosphate status of roots grown at different phosphate concentrations

	Total phosphate ($\mu g\ g^{-1}$ fresh wt.)	
P. concentration (mol m^{-3})	Tolerant	Non-tolerant
0	3.45	2.94
0.05	4.41	7.39
0.5	6.32	8.35

Analysis of variance

Source of variation	D.f	Mean squares	F	Prob.
P.	2	26.5	82.5	0.001
Tolerance	1	10.1	31.7	0.001
P. × tolerance	2	4.9	15.3	0.001
Error	12	0.3		

Each treatment was replicated 3 times

For the high affinity uptake system, influx of arsenate in tolerant plants is always lower than in non-tolerant (Fig.3) and is reflected in the kinetic parameters of influx (Table 2). The K_m of influx does vary with the different treatments but there is no discernible pattern, and differences between tolerant and non-tolerant were not great when the SE of the K_m's are considered. The V_{max} was always greater in non-tolerants compared to tolerants at all phosphate treatments. The V_{max} was not very responsive to root phosphorus status except at the highest phosphate level (Table 2). Although, the kinetic parameters show no consistent trend, the influx of arsenate is suppressed with increasing phosphate status. For example, arsenate influx at 0.05 mol m^{-3} arsenate was suppressed by 17 and 30% in non-tolerants and by 30 and 37% in tolerants in plants grown at 0.05 and 0.5 mol m^{-3} respectively compared to plants grown in the absence of phosphate. Analysis of variance of influx data for the high affinity uptake system showed that root phosphate nutrition and genotype had a highly significant effect (at the 0.1% level of significance) on arsenate influx (Table 3).

Low affinity uptake

The kinetic parameters for low affinity influx are much more responsive to root phosphorus status than the parameters for high affinity influx (Table 2). The V_{max} of influx in both tolerant and non-tolerant clones is suppressed with increasing phosphate status. The V_{max} of tolerant plants growing in the absence of phosphate is very large (5250 nmol $g^{-1}\ h^{-1}$) compared to non-tolerants (2356 nmol $g^{-1}\ h^{-1}$). This result is consistent with the results reported by Meharg and Macnair (1991b) for *D. cespitosa* grown under similar conditions where the V_{max} of the low affinity uptake system was 100% greater in the tolerant clone. The greater K_m for tolerants at this level of phosphate nutrition also is in agreement with Meharg and Macnair (1991b) with tolerants having a much lower affinity than non-tolerants. This higher K_m in tolerants leads to reduced influx of arsenate into tolerant plants at lower substrate concentrations (and at physiologically more realistic concentrations) compared to non-tolerants even though the V_{max} is higher (Fig.3). V_{max} decreases by 50% in non-tolerants comparing plants grown at the highest phosphate levels to those grown in the absence of phosphate and in tolerants this decrease was by 90%. Analysis of variance of influx data for the low affinity uptake system showed that there was a highly significant genotype times phosphate nutrition interaction (at the 0.1% level of significance, Table 3). The K_m tends to decrease with increasing phosphate nutrition in both tolerant and non-tolerant plants.

Table 2. Kinetic parameters for arsenate transport in plants grown at different phosphate levels

P conc (mol m^3)		V_{max} (nmol g^{-1} f.wt h^{-1})		K_m (mol m^{-3})	
		High	Low	High	Low
0	Tolerant				
		63.0 ± 22.3	5251 ± 2218	0.037 ± 0.024	8.78 ± 5.08
	Non-tolerant				
		108.0 ± 28.8	2356 ± 514	0.021 ± 0.012	4.15 ± 1.56
0.05	Tolerant				
		70.8 ± 43.7	913 ± 363	0.094 ± 0.082	2.06 ± 1.85
	Non-tolerant				
		111.8 ± 27.5	2481 ± 874	0.040 ± 0.018	6.93 ± 3.78
0.5	Tolerant				
		33.9 ± 8.5	581 ± 83	0.026 ± 0.014	1.23 ± 0.47
	Non-tolerant				
		93.5 ± 40.1	1042 ± 193	0.035 ± 0.028	2.64 ± 1.02

Figures represent kinetic parameters ± standard error estimated by least squares procedure Marquardt (1963).

Table 3. Analysis of variance of influx data reported in Figure 3

Source of variation	D.f	Mean squares	F	Prob.
Analysis of variance: High affinity				
P	2	862.5	10.7	< 0.001
Genotype	1	7984.1	99.3	< 0.001
Arsenate	3	4583.0	57.0	< 0.001
P. × genotype	2	106.4	1.3	0.277
P. × arsenate	6	50.6	0.6	0.706
Genotype × arsenate	3	760.2	9.5	< 0.001
P. × genotype × arsenate	6	6.3	0.1	0.998
Error	45	80.4		
Analysis of variance: Low affinity				
P.	2	811160	54.4	< 0.001
Genotype	1	9230	0.6	0.434
Arsenate	5	1834263	123.1	< 0.001
P. × genotype	2	129190	8.7	< 0.001
P. × arsenate	10	211039	14.2	< 0.001
Genotype × arsenate	5	14183	0.9	0.454
P. × genotype × arsenate	10	55263	3.7	0.001
Error	63	14897		

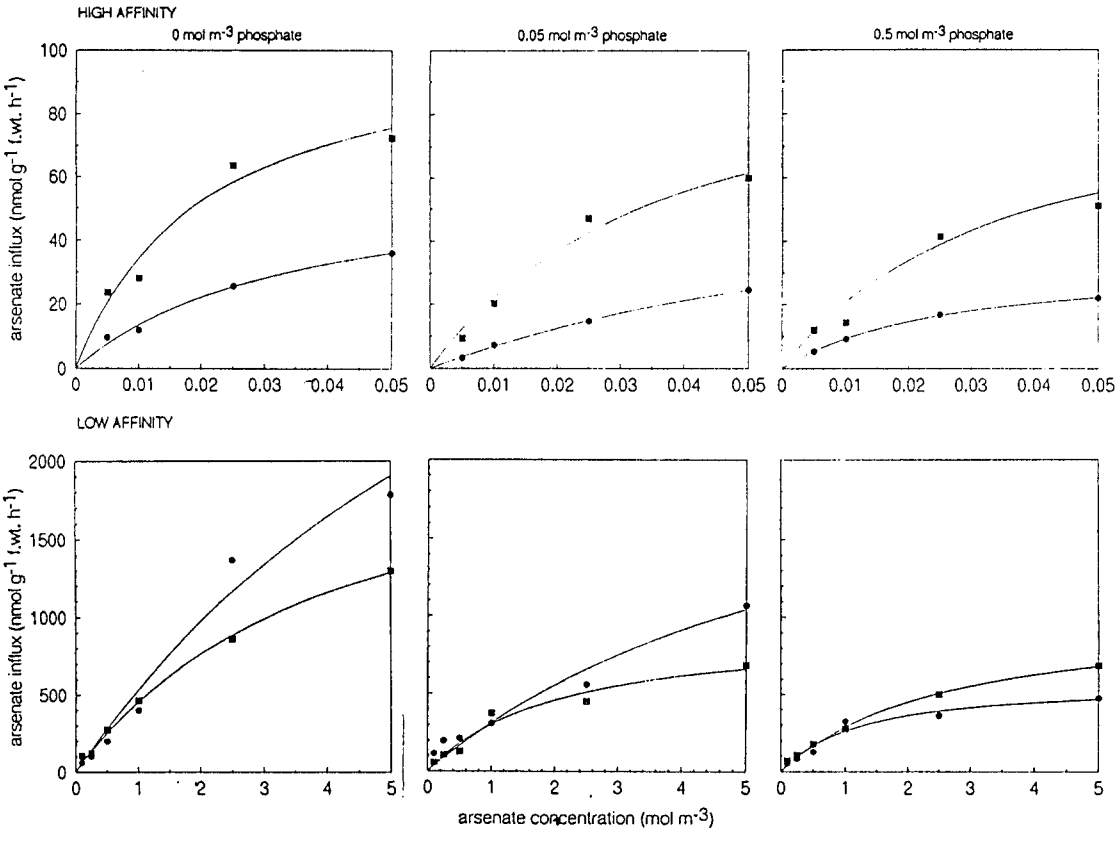

Fig. 3. Rate of arsenate influx at different concentrations of arsenate for plants of differing phosphate status. Circles, tolerant; squares, non-tolerant. Each treatment was replicated 3 times. Functions reported in Table 2 were fitted to the data points.

Discussion

The results reported here are consistent with studies on *H. lanatus* (Meharg and Macnair, 1990, 1991a, 1992a,b) and on *D. cespitosa* and *Agrostis capillaris* (Meharg and Macnair, 1991b) in that adaptation to the phosphate uptake system is involved in arsenate tolerance and that arsenate is taken up by the phosphate uptake system. In *D. cespitosa*, the low affinity uptake system differs greatly between tolerants and non-tolerants and this uptake system is affected differentially by root phosphorus status comparing tolerant and non-tolerants (Tables 2 and 3). The high affinity uptake system in *D. cespitosa* is suppressed by increasing phosphate nutrition in both tolerants and non-tolerants (Fig. 3, Table 2) although, this suppression was not great and only the highest phosphorus status tolerant plants had a suppressed V_{max} of arsenate influx. In *D. cespitosa*, the tolerants always had reduced influx compared to non-tolerants over the range of high affinity influx (Fig. 3).

The mechanism of decreased arsenate influx in *D. cespitosa* was not the same as in *H. lanatus*. In *H. lanatus*, decreased influx of arsenate (and phosphate) in tolerant clones is due to the suppression of the high affinity uptake system, apparently by inhibition of carrier synthesis under low plant phosphate status (Meharg and Macnair, 1992a) with little or no difference in the low affinity uptake systems between tolerants and non-tolerants (Meharg and Macnair, 1990). The high affinity uptake system was not affected by root phosphorus status in tolerant clones of *H. lanatus* but in non-tolerants this uptake system was suppressible under high root phosphorus status, and was suppressed to the level of the tolerant clone by lowering the V_{max} of ion influx (Meharg and Macnair, 1992a). In *D. cespitosa*, the high affinity uptake system in both tolerant and non-tolerant was suppressible under high phosphorus status and the high affinity uptake system in non-tolerant roots of high phosphorus status was not suppressed to the level of tolerants as in *H. lanatus* (Meharg and Macnair, 1992a).

The high affinity uptake system in *D. cespitosa* although, suppressible is not as responsive to changes in root phosphorus status as other phosphate uptake systems that have been investigated in angiosperms. For example, in non-tolerant *D. cespitosa* an increase in root phosphorus content by 2.5 caused no change in the V_{max} of arsenate influx and a 2.9 fold increase in phosphorus only caused a 15% decrease in V_{max} (Tables 1 and 2). The V_{max} in tolerants was more responsive to root phosphorus, but still a 1.35 fold increase in phosphorus had little affect on the V_{max} of arsenate influx. In *H. lanatus* that was not arsenate tolerant grown under similar conditions to those reported here, growing plants at 0.5 mol m^{-3} phosphate caused a 54% decrease in the V_{max} compared to plants grown in the absence of phosphate, under the same conditions there was no change in the V_{max} in tolerants (Meharg and Macnair, 1992a). Clarkson and Lüttge (1991), reviewing the literature on induction of the phosphate uptake system under low plant phosphorus status, found that for a wide range of plants that the high affinity uptake system was enhanced by two to fourfold under phosphorus stress. For example, in barley suppression of arsenate influx occurred under conditions of high root phosphorus status with influx of 0.01 mol m^{-3} arsenate being decreased from 81.6 to 27.7 nmol g^{-1} f.wt. h^{-1} comparing plants grown without and with 0.5 mol m^{-3} phosphate (Lee, 1982). The relative insensitivity of the high affinity uptake system in non-tolerant *D. cespitosa* to increased root phosphorus status is further illustrated by Figures 1 and 2 where influx of 0.05 mol m^{-3} arsenate was determined at 24 h intervals after resupply of phosphate to starved plants. This is in contrast to other species such as barley, tomato and potato in similar experiments where plants were grown for about a week in the absence of phosphate and then phosphate supplied and the rate of phosphate influx determined at a number of intervals (Clarkson and Scattergood, 1982; Cogliatti and Clarkson, 1983; Lefebvre and Glass, 1982). In all these experiments influx of phosphate was rapidly derepressed on provision of phosphate and an increase in root phosphate status, with this derepression occurring within 24h of phosphate provision. In the case of starved barley roots resupplied with 0.015 mol m^{-3} phosphate this derepression occurred within 2 h (Lefebvre and Glass, 1982). The phosphate supplied to these plants was at a lower concentration than phosphate supplied in the experiment reported here (0.015 compared to 0.05 mol m^{-3} respectively). Root phosphorus status was unchanged in arsenate tolerant *D. cespitosa* and arsenate influx was at a constant rate (Figs. 1 and 2).

The low affinity uptake system is much more sensitive in both non-tolerant and tolerant clones with increasing root phosphorus status, achieved by suppression of the V_{max} of arsenate influx (Table 2). This suppression of the low affinity uptake system with high plant phosphate status has also been reported by McPharlin and Bieleski (1987) for *Lemna major* and *Spirodela oligorrhiza.*

The exact role of the interplay between the high and low affinity uptake system in regulating plant phosphorus status is still not clear. Beever and Burns (1980) argue that in fungi the possession of a high and low affinity uptake system allows for close regulation of phosphate influx under a wide range of external phosphate concentrations. In higher plants, regulatory mechanisms may also have evolved to adapt to variation in nutrient supply. Under conditions of nutrient deficiency the high affinity uptake system is normally induced, and in the event of nutrients becoming abundant the high affinity uptake system is suppressed (Clarkson and Lüttge, 1991). In some species that have adapted to environments with severe nutrient deficiencies the ability to regulate the activity of nutrient carriers does not exist and regulation of plant nutrient status is by storing excess nutrients in the vacuoles (Chapin, 1980; Clarkson and Lüttge, 1991). Species from nutrient deficient environments generally have reduced V_{max} and increased K_m compared to species from fertile habitats (Chapin, 1980).

The high affinity uptake system in *D. cespitosa* seems to be constitutive since it is relatively insensitive to root phosphorus status. Influx over the range of the high affinity uptake system is always lower in the arsenate tolerant clone compared to the non-tolerant. This suggests that the high affinity uptake system is present at a lower level in the plasmalemma of the tolerants as the V_{max} is lower. The other explanation of the lower V_{max} is a lower turnover number of the carrier protein for arsenate in tolerants compared to non-tolerants.

Reduction in arsenate influx is the mechanism of arsenate tolerance in both *H. lanatus* and *D. cespitosa* although, the manner in which this reduction is achieved differs. The isolation and characterization of clones with adapted uptake systems may lead to a better understanding of the regulation of nutrient influx in the field environment.

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