Changes in Crested Wheatgrass Root Exudation Caused by Flood, Drought, and Nutrient Stress

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ABSTRACT

Root exudates can chelate inorganic soil contaminants, change rhizosphere pH, and may increase degradation of organic contaminants by microbial cometabolism. Root-zone stress may increase exudation and enhance phytoremediation. We studied the effects of low K^+, high NH_4^+/NO_3^- ratio, drought, and flooding on the quantity and composition of exudates. Crested wheatgrass (*Agropyron cristatum*) was grown in Ottawa sand in sealed, flow-through glass columns under axenic conditions for 70 d. Root exudates were collected and analyzed for total organic carbon (TOC) and organic acid content to compare treatment effects. Plants in the low K^+ treatment exuded 60% more TOC per plant per day ($p = 0.01$) than the unstressed control. Drought stress increased cumulative TOC exuded per gram dry plant by 71% ($p = 0.05$). The flooded treatment increased TOC exuded per gram dry plant by 45%, although this was not statistically significant based on the two replicate plants in this treatment. Exudation from the high NH_4^+/NO_3^- ratio treatment was 10% less than the control. Exudation rates in this study ranged from 8 to 50% of rates in four other published studies. Gas chromatography-mass spectrometry (GC–MS) analysis indicated that malic acid was the predominant organic acid exuded. Fumaric, malonic, succinic, and oxalic acids were also detected in the exudates of all treatments. These results demonstrate that nutrient and water stress have significant effects on the quantity and composition of root exudates. Cultural manipulations to induce stress may change the quantity of root exudates and thus increase the effectiveness of phytoremediation.

ROOT exudates could enhance degradation of organic contaminants by providing substrates that increase microbial activity and/or contaminant bioavailability (Miya and Firestone, 2001). Root exudates themselves may have enzymatic properties that can break down contaminants (Siciliano et al., 1998). Exudates also change rhizosphere pH and can chelate inorganic contaminants. A better understanding of the factors that affect exudation by plant roots could enhance phytoremediation.

Factors that can influence root exudation include nutrient status, plant water status, oxygen availability, and species. Iron deficiency is well known to increase phytosiderophore release (Marschner, 1995), and phosphorus deficiency increases exudation of phosphatase (Ratnayake et al., 1978; Hoffland et al., 1989; Gilbert et al., 1999). Other nutrients can also affect root exudation. Kraffczyk et al. (1984) reported an increase in exudation of sugars, organic acids, and amino acids when grown in K^+–deficient conditions, and increases in some amino acids when NO_3^- was used as a nitrogen source as opposed to NH_4^+. Several studies have observed increased exudates with higher N in the soil (Liljeroth et al., 1990; Paterson and Sim, 2000), which could be due to the larger size of the high-N plants rather than stimulation of exudate production per gram of plant.

Plant water status can alter exudation. Increased amounts of water-soluble compounds and mucilaginous material have been observed around drought-stressed roots (Whipps and Lynch, 1983; Barber and Martin, 1976). Whipps and Lynch (1983) hypothesized that water stress resulted in either excess carbon in the roots that was subsequently released or caused root death that was detected as exudate. Excess water in the root zone (flooding) can lead to hypoxia. Grineva (1961) examined hypoxia by growing plants in a solution bubbled with N_2 gas to eliminate O_2. No plant tissue injury was observed, but more exudates were collected from the hypoxic plants than from plants grown in aerated solution.

Organic acids are an important class of exudates. The release of organic acids to the rhizosphere contributes to plant health in several ways, including aluminum immobilization and solubilization of inorganic phosphorus (Delhaize et al., 1993; Gilbert et al., 1999). A slow release of organic acids from roots is likely to be always occurring since a charge gradient is maintained in all healthy cells by H^+ATPase, which pumps out H^+ ions while concurrently drawing anions out of the cells, particularly organic acids in the dissociated form (Jones, 1998). Increased levels of dicarboxylic and tricarboxylic acids are released from the roots of plants that are able to grow in calcareous soils, possibly because of the ability of these acids to solubilize Fe, Mn, and P from soil (Ström, 1997). Acidification of the rhizosphere, however, is due more to proton secretion than the presence of organic acids (Petersen and Böttger, 1991).

The objective of this study was to determine the extent to which stress alters the quantity and composition of root exudates. This information could lead to improved techniques to enhance the phytoremediation of soil contaminants.

MATERIALS AND METHODS

Plant Growth

Crested wheatgrass (*Agropyron cristatum*) plants of cultivar CD-II were grown under axenic conditions for 70 d in

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Abbreviations: ANOVA, analysis of variance; EC, electrical conductivity; GC–MS, gas chromatography-mass spectrometry; GLM, general linear model; HEPA, high efficiency particulate air; HPLC, high performance liquid chromatography; ICP-ES, inductively coupled plasma emission spectrophotometry; PPF, photosynthetic photon flux; RGR, relative growth rate; TOC, total organic carbon; WUR, water use requirement.

flow-through glass columns containing Ottawa sand (Fig. 1). Details of the system, cultural methods, and sterile techniques are described in Henry et al. (2006), where results from the control treatment of this study are also reported.

Root-Zone Stress Treatments

There were 14 planted columns and one unplanted control. Six columns were assigned to the control treatment, which had 100% nitrate N, adequate nutrients, and ample water. Two replicate columns were assigned to each of the other four treatments: drought, flooding, low K^+, and high NH_4^+. Beginning on Day 35 after planting, nutrient solutions and watering volumes were manipulated to induce the treatments. The low K^+ treatment was induced by decreasing the concentration of K^+ in the nutrient solution from 5.5 to 0.5 m*M*. For the high NH_4^+ treatment, the NH_4^+/NO_3^- ratio was changed from 100% nitrate to 40/60% NO_3^-/NH_4^+. This ratio was decreased to 25/75% NO_3^-/NH_4^+ on Day 57. Drought was induced by watering with 25% of the control. A stopper was placed in the drain tube of the flooded treatment to induce flooding.

Leachate Collection

Plants were watered as needed to minimize water stress in all treatments except the drought and flooded treatments. Additional nutrient solution was added on specific days to obtain leachates. Plants in the drought treatment were watered to obtain leachates every six to 10 d. Exudates were not collected on intermediate dates for the drought and flooded plants because treatment application required intervals with no leachate collection. Transpiration was calculated by subtraction of the nutrient solution supplied minus the leachate volume and evaporation (water loss from the unplanted column).

Contamination Detection

Microbial contamination in the leachate was assessed weekly by plating samples and by microscopic examinations. Contamination was also assessed at harvest by microscopic examination of root samples (Henry et al., 2006).

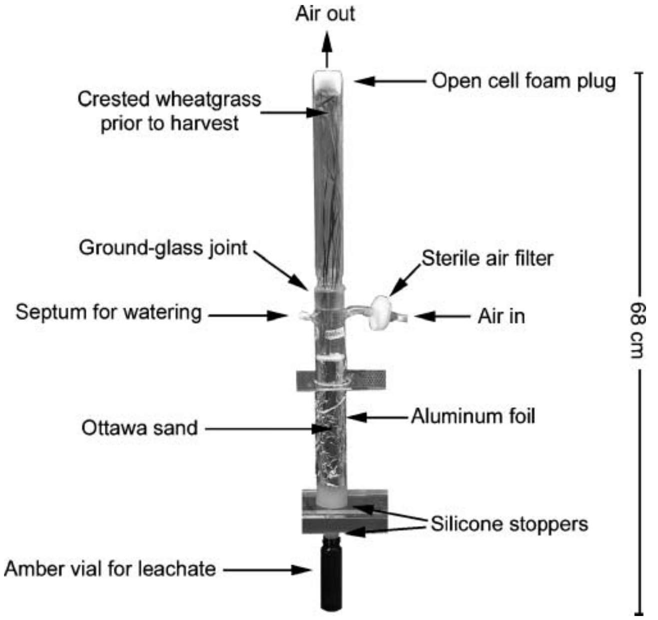

Fig. 1. Glass column system used for axenic plant culture and study of root exudates.

Exudate Analysis: Leachates

Total organic carbon (TOC) in the leachates was determined in each sample with a TOC analyzer (Tekmar-Dohrmann, model Phoenix-8000). The ratio of soluble to insoluble TOC in each leachate sample was determined by aseptically filtering a 5-mL subsample with a low-retention syringe tip filter (Pall Gelman acrodisc, Supor membrane). The TOC that passed through the 0.45-μm filter was assumed to be soluble. A 1-mL aliquot of leachate was also aseptically removed from each sample to measure pH using a microelectrode in a 2-mL vial.

Qualitative analysis of the leachates was based on organic acid content based on the prevalence of organic acids in previous root exudate studies and the importance of root exudates for plant nutrition. To determine organic acid content of the leachates, samples were esterified and organic acid concentrations determined as methyl esters using gas chromatography-mass spectrometry (GC–MS) (Henry et al., 2006). At the end of the study, roots and attached sand were separated and extracted with three mL of 0.1 *M* NaOH. Solutions were periodically mixed for 1 h at room temperature and then separated from the root matrix by filtration. The extracts were analyzed by esterification/GC–MS.

Statistical Analyses

All results with a full data set were analyzed with SigmaStat (SPSS, 2002) using one-way analysis of variance (ANOVA) and a Tukey Test or Dunn's Method. A general linear model (Proc GLM; SAS Institute, 1999) test and mean separation using a Student Newman Keuls test was performed to compare repeated measures of the control, low K^+, and high NH_4^+ treatments.

RESULTS

Plant Health

Plants were green and healthy throughout the trial. Some yellowing was observed in the shoots of the flooded treatment, which was likely caused by the anaerobic conditions in the root zone. Leaves at the top of the tube curled under the foam plug when leaf length exceeded the length of the upper tube. This occurred in all plants and all treatments by the end of the study.

The ammonium treatment effectively lowered the rhizosphere pH. The pH of the leachates in the NH_4^+ treatment steadily dropped from an initial pH of 7.5 to a final pH of 4.2. The leachate pH was 7.5 to 8 in all other treatments.

Nutrient analysis of shoots by inductively coupled plasma-emission spectrophotometry (ICP-ES) showed that the potassium content of the plants in the low K^+ treatment was 40% of the controls (Table 1). Nutrients

Table 1. Average nutrient content of control (n = 6) and treatment (n = 2) shoots using inductively coupled plasma-emission spectrophotometry (ICP-ES). Nutrients were present in adequate amounts, except the low K^+ level in the low K^+ treatment.

Parameter	P	K	Ca	Mg	S	Fe	B	Zn	Mn	Cu
	%					mg kg^{-1}				
Control	0.30	3.2	0.22	0.09	0.17	54	47	49	44	9
High NH_4^+	0.34	3.0	0.22	0.09	0.34	42	52	73	72	18
Low K^+	0.25	1.3	0.48	0.15	0.13	193	51	40	35	9
Drought	0.33	3.5	0.24	0.12	0.21	47	82	51	54	12
Flooded	0.23	2.2	0.23	0.07	0.13	52	36	37	38	10

Table 2. Cumulative total organic carbon (TOC) collected in the leachate over the 70-d study.

	Shoot dry mass at harvest	Cumulative C exuded per plant		C exuded per kg dry plant mass	
Parameter	Average ± SE†	Average ± SE	Percent of control	Average ± SE	Percent of control
	g	mg	%	g	%
Control	**1.78 ± 0.2**	**5.66 ± 1.0**	**100**	**2.5 ± 0.2**	**100**
High NH_4^+	**2.01 ± 0.1**	**6.98 ± 0.3**	**120**	**2.2 ± 0.1**	**90**
Low K^+	**2.50 ± 0.0**	**9.02 ± 1.6**	**160**	**3.6 ± 0.4**	**144**
Drought	**1.50 ± 0.1**	**7.84 ± 0.0**	**140**	**4.2 ± 0.4**	**171**
Flooded	**2.42 ± 0.6**	**10.4 ± 0.6**	**180**	**3.6 ± 0.6**	**145**

† SE, standard error.

in all other treatments were present in adequate amounts. Differences in nutrient contents among treatments were typical for the treatment. Average shoot dry mass ranged from 1.5 g in the drought treatment to 2.5 g in the low K^+ treatment (Table 2). Root mass was not determined since a GC–MS analysis was performed on a direct extract of the roots that required the roots to remain sterile. Root evaluation in a preliminary trial indicated that the average root mass was 25% of the total plant mass at harvest for all treatments. A root mass of 25% was thus assumed for the calculations in this study. Plant dry mass at harvest was highly correlated with cumulative transpiration rate ($r^2 = 0.96$, data not shown), so plant mass throughout the study was back-calculated based on daily transpiration data using the transpiration curve for the whole study and the shoot dry mass at harvest.

Total Organic Carbon

Total organic carbon was monitored in the leachates throughout the trial. The TOC was expressed in four ways to facilitate comparison with other studies and to improve comparisons among treatments: (i) μg carbon per plant per day (Fig. 2); (ii) mg carbon per kg of new growth per day on a dry mass basis (Fig. 3); (iii) cumulative μg carbon per plant (Table 2), and (iv) cumulative g carbon per kg dry plant (Table 2). Rates of exudation expressed as mg carbon per kg plant per day are nearly identical to rates shown in Fig. 3, which were calculated for each sampling date based on TOC values and relative growth rate (RGR) (see below) estimated from final shoot mass. Carbon leached from the unplanted columns ranged from 15 to 60 μg C per day, or about 10% of the planted systems (Fig. 2).

Statistical analysis by SAS using Proc-GLM and mean separation using a Student Newman Keuls test ($\alpha = 0.01$) revealed that the μg C exuded per plant per day (Fig. 2) was significantly higher in the low K^+ treatment than in the control and high NH_4^+ treatments. When all treatments were statistically analyzed over four time intervals (Days 35–42, 45–51, 53–57, and 59–63), TOC released by the flooded treatment was significantly greater than the control from Days 45 to 51 ($p = 0.013$).

Total organic carbon exuded per kg dry plant (Table 2) by the drought treatment was significantly higher than the control and high NH_4^+ treatments based on a one-way ANOVA and Tukey test ($p = 0.013$). The TOC in the low K^+ and flooded treatments was also higher than the control, but these increases were not statistically significant based on the two replicates used in this study. The TOC in the high NH_4^+ treatment on an average per kg dry plant basis was lower than the control plants. The

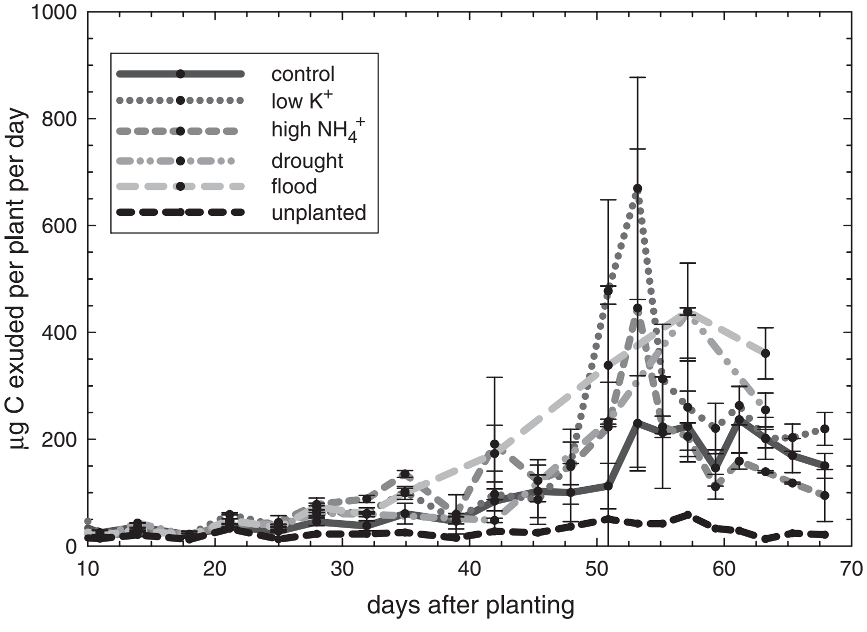

Fig. 2. Exudation rate per plant per day over the duration of the study.

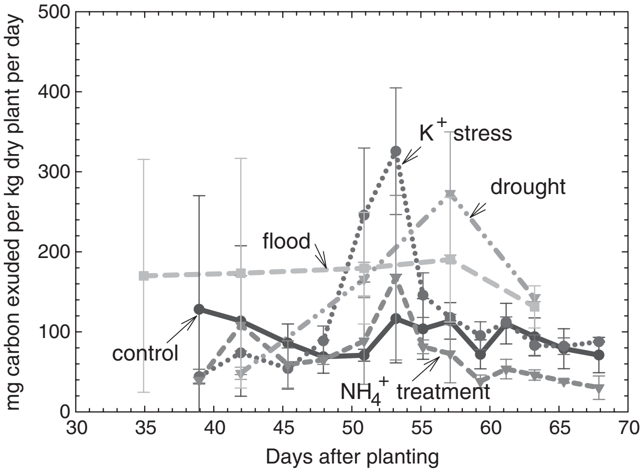

Fig. 3. Total organic carbon (TOC) in exudates per kg new dry plant. Kilograms new dry mass was estimated from transpiration rates and the calculated relative growth rates.

TOC in the exudates also reached the highest levels in the drought and low K$^+$ treatments when expressed as mg TOC per kg dry plant per day.

Relative growth rate (kg new growth per kg plant per day) was determined before and after the application of treatments using final plant mass, estimated mass at Day 35, and initial (seed) mass as described in Henry et al. (2006). The RGRs among treatments were not significantly different before ($p = 0.414$) or after ($p = 0.113$) treatments were applied, although the drought-stressed plants had the lowest average RGRs. Water use requirement (WUR: L transpired per kg dry plant) was determined for each plant by dividing cumulative transpiration over the entire study by the calculated total plant mass. The water use requirement was similar among treatments and ranged from 148 L kg^{-1} in the drought treatment to 183 L kg^{-1} in the low K$^+$ treatment. The WUR of these plants was about 50% less than plants in low humidity field environments, but similar to the WUR of plants in high humidity environments.

Relative growth rate was used to calculate mg carbon exuded per kg new plant (Fig. 3). The low K$^+$ treatment had the highest TOC exuded per kg new plant on Day 51 ($p = 0.047$). On Day 63 the drought treatment exuded significantly more TOC per kg new plant than the low K$^+$, NH$_4^+$, and control treatments, and the flooded treatment exuded significantly more TOC than the control and NH$_4^+$ treatments as defined by a Tukey Test ($p < 0.001$).

Soluble TOC in the leachates ranged from 75 to 100% of the total TOC, with no differences among treatments.

Exudate Composition

Similar to the rates of TOC release, the rates of organic acid release in the exudates also peaked before the end of the study (Fig. 4). The exudates from the drought treatment contained the highest concentrations of fumaric and succinic acids at any point in time. Oxalic, malonic, and malic acids were also quantified. As a percentage of TOC in the leachate samples, malic acid dominated in all treatments, and the drought treatment contained the highest percentages of organic acids (Fig. 5).

Organic acids accounted for <1% of the TOC for malonic acid, <2% for oxalic acid, <4% for fumaric and succinic acids, and up to 100% for malic acid. The drought treatment had significantly higher cumulative amounts of succinic acid in the exudates than the other treatments ($p = 0.004$). The exudates of the high NH$_4^+$ treatment often contained lower concentrations of organic acids than the controls. Cumulative amounts of organic acid collected in the leachates were not correlated to concentrations in the root extracts for malonic or oxalic acid, and only weakly correlated due to remote points for fumaric, malic, and succinic acid (Fig. 6).

Root tissue and sand from the rhizosphere were extracted to identify other compounds that might be present in the leachate and were not detected due to their low concentrations. As expected, more acids were detected in the root extracts (Table 3) than in the leachate samples. The cumulative amounts (mg kg^{-1} root) of malonate, oxalate, succinate, malate, and methyl 2-methyl-butanoate in the root extractions from drought-stressed plants were significantly higher than the controls ($p < 0.05$). Organic acids detected in the rhizosphere sand were low, ranging from 0 to ~20% of concentrations in roots, with the highest concentrations in the flooded treatment.

DISCUSSION

Effects of Stress on Growth

Due to the difficulties in maintaining axenic plants, past root exudate studies have not emphasized plant health even though it significantly affects root exudate composition. The rhizosphere pH decreased in the high NH$_4^+$ treatment, but growth rates and exudation rates were similar to the controls. The reduced size of all stressed plants (60–95% of the controls) indicates the magnitude of stress in these treatments.

Total Organic Carbon

Total organic carbon, when expressed as μg carbon exuded per plant per day, peaked at about Day 55 of the 70-d trial. Since this peak was seen in all treatments including the controls, and since transpiration rates leveled off at the end of the study, it is likely that other factors besides the intended stresses reduced exudation rate. The decrease in TOC could be a response to volume limitation in the shoot zone of the growth containers. Water stress was unlikely since each column was watered to maintain the volumetric water content above 28% of field capacity.

Plant roots in the low K$^+$, drought, and flooding treatments showed increased TOC per kg plant compared with the controls and the NH$_4^+$ treatment. This was expected for the low K$^+$ conditions because decreasing nutrient availability can increase the production and release

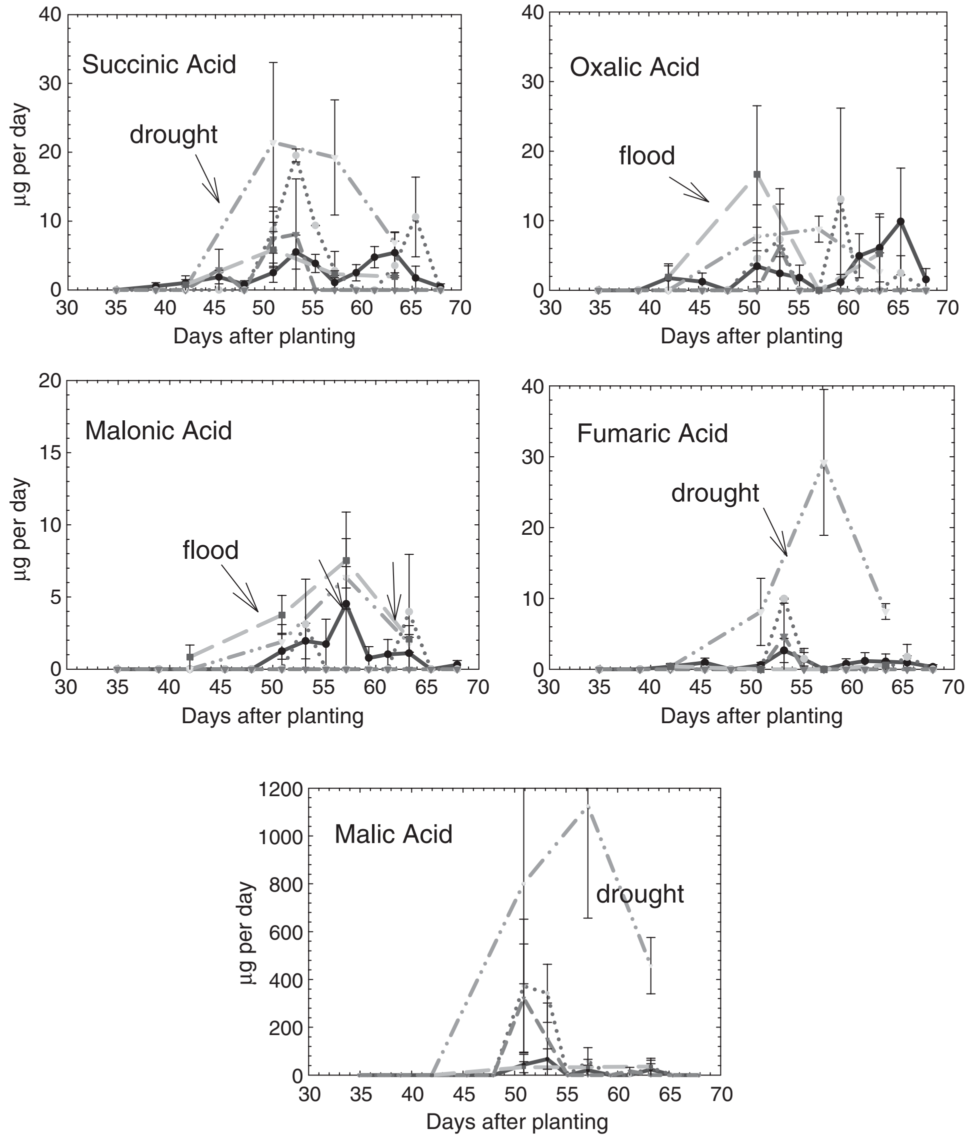

Fig. 4. Exudation rates of five organic acids detected in leachate samples. All values are corrected for recovery efficiencies. Note the different scales of the *y*-axes. Line types for treatments are the same as other graphs.

of nutrient-sequestering compounds (Hoffland et al., 1989; Ström et al., 1994; Ström, 1997).

Roots also release carbon in the form of mucilage and border cells as lubrication and a medium for root growth through the soil. The need for more lubrication in dry soil coupled with the discharge of dying roots and cell leakage due to decreased water availability in the drought treatment could explain the increased amounts of TOC released. In tall fescue, drought has been shown to increase root mortality and reduce root cell membrane integrity, resulting in increased leakage of UV-absorbing organic solutes (Huang and Gao, 2000).

Flooding the columns decreased oxygen availability, which should have reduced root respiration. The increased TOC in the flooding treatment may have been partly caused by root death since root/shoot ratios were reduced in the flooded treatment in preliminary trials (controls = 27 ± 9% roots, flooded = 13 ± 4% roots, data not shown). The ponding of water and subsequent soaking of the lower 1 cm of the shoots may also have contributed to TOC in the leachate.

Jones and Darrah (1993) suggested that re-absorption of exudates was highest when the exudates were adjacent to the roots for longer periods of time. Re-absorption likely occurred in all treatments in this study, but it is possible that some TOC was not re-absorbable, like border cells or remains of root die-off. The drought and flooded treatments had the least frequent replacement of root-zone solution and the greatest potential for re-absorption, but also had the highest leachate TOC. The re-absorption found by Jones and Darrah may have been from compounds that are more readily re-absorbed. Exudates would not be reabsorbed in damaged tissue, which may explain higher leachate TOC in the drought and flooded treatments.

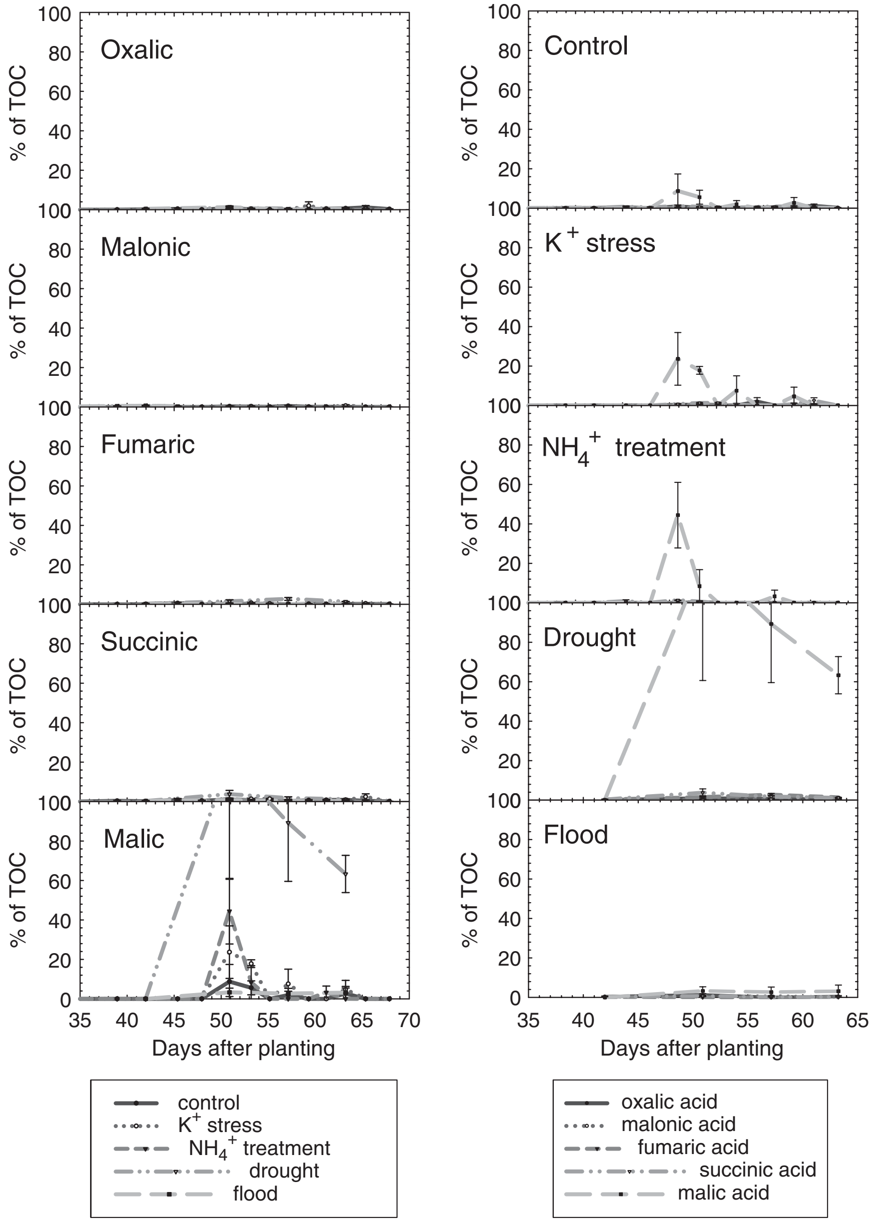

Fig. 5. Organic acids as a percentage of total organic carbon (TOC), shown by acid (left side) and by treatment (right side).

Soluble Total Organic Carbon

Since most of the TOC in the leachate was soluble, the majority of exudates were not whole cells from which the cellulose in cell walls would contribute insoluble organic carbon. Root die-off may have contributed to the TOC from the flooded roots. The percentage of soluble TOC was not measured during the period when the flooded treatment had the highest amount of TOC released per plant. A lower percentage of soluble TOC would be expected during this period if there was increased leaching of cell structural components due to root die-off. Microscopic observations at the end of the study revealed few root hairs on plants in the flooded treatment. Loss of root hairs on induction of flooding could also have contributed to the release of TOC.

Organic Acid Composition

Organic acids play important roles in plant function. Malate is used as a counter-ion for cation uptake to maintain charge balance across membranes. Malate also plays an important role in nitrate reduction in the shoot and is the most common organic acid re-translocated to the root and used for charge balance in nitrate uptake (Marschner, 1995). Oxalate is used for charge compensation in nitrate reduction, and in the precipitation of excess solutes such as calcium oxalate. Kloss et al. (1984) noted that oxalic acid has been detected in the root exudates of C-3 plants, but not C-4 plants.

The drought treatment was the only treatment that had significantly higher concentrations of organic acids. Low water potential in the root cells of the drought treatment

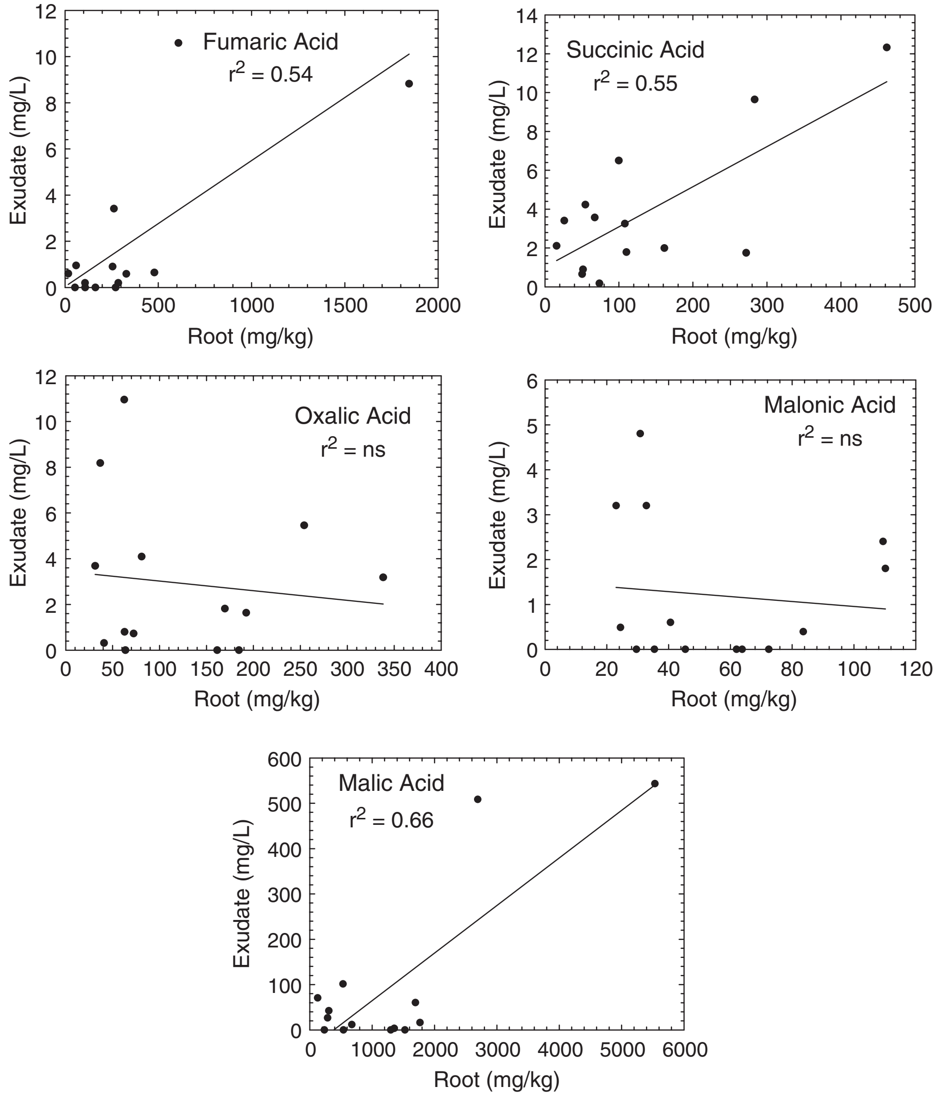

Fig. 6. Comparison of organic acid concentrations in roots with cumulative amounts of organic acid detected from the leachates.

plants could have resulted in cell damage on watering and subsequent detection of cell contents as exudates.

Despite an increase in TOC in the exudates of the low K^+ plants, no increase in organic acids was observed, suggesting that organic compounds other than those measured may have contributed to the TOC. Decreased organic acids in the NH_4^+ treatment may be due to decreased need to reduce nitrate and may account for the slightly lower levels of TOC exuded by the NH_4^+ treatment than the controls. Kraffczyk et al. (1984) saw increased exudation of organic acids under K^+ stress; however, they also measured a large reduction in plant growth in their K^+ stress treatment. Although we had a large reduction in K^+ applied for the low K^+ treat-

Table 3. Derivatized compounds in root samples at harvest (mg kg^{-1}) detected using gas chromatography-mass spectrometry (GC–MS). Citric acid was below detection limits in all studies.

	Control		Low K^+		NH_4^+		Drought		Flooded	
Compound	Mean	SE†	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Methyl 2-methyl-propanoate	26	6	8	8	9	9	48	6	9	1
3,3-dimethyl-2-butanone	30	7	14	5	16	6	55	2	11	1
Methyl 2-methyl-butanoate	24	4	10	10	9	9	55	5	12	3
Dimethyl oxalate	31	5	8	1	14	0	65	9	13	5
Methyl caproate	6	4	13	4	5	5	0	0	11	1
Dimethyl malonate	29	5	16	4	20	3	55	0	14	2
Dimethyl fumarate	128	32	80	50	46	37	625	316	100	46
Dimethyl succinate	71	18	23	8	19	10	209	50	50	12
Dimethyl malate	177	39	62	19	60	41	617	213	125	79

† SE, standard error.

ment, plant growth was not decreased so our plants were probably less stressed.

Organic acids were a low percentage of the total carbon released by the roots, indicating that compounds other than organic acids were present (see Uren, 2001), although not all organic acids detected were quantified and percentages may be an underestimate due to low recovery rates during the GC–MS analysis. Organic acids were the highest percent of TOC in the drought and flooded treatments. Changes in the proportion of organic acids in the exudates suggest a changing composition of the exudates with time, which may be a response to age or increased stress levels.

Malic acid was the predominant organic acid in this study. Malic acid was also the predominant organic acid in hydroponic rice, accounting for up to 87% of organic acids in the exudate depending on cultivar and growth stage (Aulakh et al., 2001). Malic acid in the exudates of 4-to 6-d-old wheat grown in solution reached 82% of organic acids in Al-tolerant cultivars exposed to Al (Delhaize et al., 1993). Phosphorus deficiency can increase exudation of malic acid in *Brassica napus* L., which helps the plants soliblize rock phosphate (Hoffland et al., 1992). However, neither P-deficiency nor exposure to Al were factors in this study.

Correlation of Organic Acid Concentrations in Roots and Exudates

In three out of five organic acids identified in the exudates, the concentrations found in living root tissue were weakly correlated with concentrations in the leachate. Similar results have also been reported from previous studies (see Ryan et al., 2001). Organic acids detected in the rhizosphere should correlate with those found in the root if the source of exudate was whole cells, dying roots, or leaky membranes. Concentrations of certain compounds in the exudates might not correlate with the concentrations found in the root for several reasons. The structures of some root exudates, such as phytosiderophores and exoenzymes, are complex and energetically expensive for the plant to manufacture. These compounds are produced for nutrient sequestration and it would be of little use to the plant to store these compounds inside the root. Exudates are often produced as a response to certain stresses and change with time as the status of the plant changes. Root-extract analysis represents just one point in time, in this case the end of study, and may therefore be unrepresentative of the plant status earlier in the study. Furthermore, not all root cell constituents are actively exuded.

These results indicate that manipulating root exudation through nutrient and water stress may increase the carbon available to microbes and change exudate composition. Cultural manipulations of plant nutrient and water status could be an effective way to increase uptake or breakdown of soil contaminants by plants.

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