5-2003

Effect of Drought, Flooding, and Potassium Stress on the Quantity and Composition of Root Exudates in Axenic Culture

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EFFECT OF DROUGHT, FLOODING, AND POTASSIUM STRESS ON THE QUANTITY AND COMPOSITION OF ROOT EXUDATES IN AXENIC CULTURE

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Plant Science
(Crop Physiology)

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UTAH STATE UNIVERSITY
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2003

ABSTRACT

Effect of Drought, Flooding, and Potassium Stress on the Quantity and Composition of Root Exudates in Axenic Culture

by

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Root exudates include important chelating compounds and can change the rhizosphere pH by several units. These changes are essential for nutrient uptake and can also alter solubility of soil contaminants and increase plant uptake. Mild root-zone stress may increase exudation and more severe stress can damage membranes and increase root turnover, all of which increase root-zone carbon. Increased carbon from this rhizodeposition can increase microbial activity, which might help degrade contaminants. We studied the effect of three types of stress on root exudation of crested wheatgrass (*Agropyron cristatum*): low K^+, drought, and flooding. These stresses were compared to two types of controls: 100% NO_3^- and high NH_4^+: NO_3^- ratio. We developed an improved axenic system to keep plants microbe-free for 70 days while analyzing exudates for total organic carbon (TOC) and organic acids. Axenic conditions were confirmed by plate counts of the leachate and microscopic observations of the leachate

and roots. Optimal conditions for plant growth were maintained by monitoring temperature, light, humidity, water, O_2, CO_2, nutrient availability, and root-zone pH. Plants were grown in Ottawa sand that was layered by size to optimize water availability. Total organic carbon released over the 70-day growth period in mg per gram dry plant was 2.6 in the control, 2.3 in the NH_4^+ treatment, 3.7 in the flood and K^+ stress treatments, and 4.4 in the drought treatment, which was the only treatment significantly higher than controls ($p = 0.05$). TOC and organic acid levels in the exudates peaked before the end of the study. The peak TOC levels, expressed as mg TOC per gram new dry plant mass, were 1.9 in the control, 3.0 in the NH_4^+ treatment, 2.9 in the flood, and 5.8 in the drought and K^+ stress treatments. Organic acids were measured by gas chromatography-mass spectrometry (GC-MS). Malic acid was the predominant organic acid, and accounted for the majority of the TOC in the drought treatment. Oxalic, succinic, fumaric, and malonic acids accounted for less than 10% of the TOC. These data indicate that stress may enhance phytoremediation by changing root-zone exudate composition.

(184 pages)

ACKNOWLEDGMENTS

My time at the Crop Physiology Lab at Utah State University has been enjoyable and educational. I would like to thank Dr. Bruce Bugbee for being my major advisor and for always being willing to meet and discuss my project. Thank you to my committee members, Dr. Jenny Norton and Dr. Bill Doucette, for their support and expertise on the microbial and chemical aspects of this research, and for their knowledgeable perspectives that contributed to this research.

Thank you to Dr. Melinda Hamilton, Dr. Carl Palmer, and Dr. Richard Hess at INEEL for funding this research, for their suggestions, and for their support at my presentations at national meetings.

Thanks to my mother, Nancy, for the continuous supply of cookies during my graduate work and for all the encouragement. Steve Klassen helped with much of my thesis by providing comments on scientific writing and providing many ski and mountain bike tours. Thank you to Julie Chard for the help with my research and for being a wonderful office mate.

Further assistance for my research also came from Dr. Mike Petersen for the GC-MS analysis, Dr. Paul Grossl for the use of the TOC analyzer, Rita Nelson, Randilynn Manning, Priscila Palmer, Dr. Jonathan Frantz, Derek Pinnock, Glen Ritchie, Heidi Fabrizius, Coreen Crouch, and Richard Logsdon.

Amelia Henry

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INTRODUCTION

OVERVIEW

Root Exudates in Phytoremediation

Phytoremediation is the cleanup of contaminated soils using plants. Root exudates play a role in phytoremediation due to their potential to assist in plant uptake of contaminants by mechanisms similar to nutrient uptake, to act as a substrate for microbes that could break down contaminants, or to change soil properties and affect the mobility of contaminants.

Definition of "Root Exudate"

The release of all forms of carbon from roots has been termed rhizodeposition (Marschner, 1995). Rhizodeposition products, which are available for microbial metabolism in the rhizosphere (zone adjacent to the root) and on the rhizoplane (root surface), can be categorized as exudates, lysates, secretions, and gasses. The difference between exudates and secretions is that exudates are passively released and secretions are actively released. Secretions include polymeric carbohydrates and enzymes (Whipps, 1990). The products of extensive cell degeneration have been termed "root lysate," for example: sloughed-off root hairs or root cap, epidermal, and cortical cells (Liljeroth et al., 1990). The most common definition of the term "root exudate" has been "substances which are released into the surrounding medium by healthy and intact plant roots" (Rovira, 1969) and is the definition used in this discussion.

Root exudates include high and low molecular weight compounds. High molecular weight compounds in root exudates include the mucilage, gelatinous material covering root surfaces, and ectoenzymes. Phosphatase is an ectoenzyme that mobilizes organic P in the soil for plant use. Low molecular weight root exudates are released in larger quantities and include organic acids, sugars, phenolics, amino acids, phytosiderophores, flavonoids (Marschner, 1995), and vitamins (Whipps, 1990). Phytosiderophores are natural chelating agents known to be important for plant iron nutrition. The term "root exudate" is used in the literature to describe all organic compounds released from roots.

An inclusive list of root exudate components found in the literature (Uren, 2001), which includes over 100 different compounds, is also representative of a list of potential cell chemical constituents. The major source of the addition of cell contents to the rhizosphere is root border cells, formerly known as sloughed off root cells. These cells are living when released from the root and act as an interface between the soil and root through protection of the root as it grows though the soil and interacts with soil microbes (Hawes et al., 1998).

Quantity and Composition of Root Exudates in the Literature

Although quantitative comparisons of exudates vary widely, average estimates have been reported in the literature (Tables 1, 2, 3, and 4).

Using axenic wheat, Prikryl and Vancura (1980) expressed root exudates as 50% root dry weight, or 12% whole plant dry weight over a growing season. Based on a

Table 1. Organic Acids detected in root exudates (expressed in μg g dry root^{-1} day^{-1} or % (w/w) of exudate).

	Species				
	Triticum tugidum var. durum (wheat)[5]	*Linum usitatissiumum* (flax)[6]	*Diplachne fusca* (brown beetle grass)	*Lepidium. sativum* (cress)[1]	*Zea mays* (corn)[6]
Compound					
2-Oxoglutarate					3.89
Glyoxalate					0.0365
Glycolate					0.0944
Succinic Acid	0.964	0.032	110		
Fumaric Acid	0.18	0.05	140		0.008
Malic Acid			7240		
Citric Acid			450		
Uronic acid				47.8%	
Malonic	0.260	0.097			
Oxalic	0.410				
Time	14 d	14 d	14 d	3d	12 d
Growth Conditions	Sterile, in solution	Sterile, in solution	Sterile, in solution	germination box	in solution
Reference	Szmigielska et al., 1995	Szmigielska et al., 1995	Kloss et al., 1984	Ray et al., 1988	Petersen and Böttger, 1991

Table 2. Miscellaneous compounds detected in root exudates (expressed in μg $g_{dry\ root}^{-1}$ day^{-1} or % (w/w) of exudate)

Compound		**Time**	**Species**	**Growth Conditions**	**Reference**
inositol	54.1	16 d	*Medicago sativa* (alfalfa)[2]	sterile, in sand	Hamlen et al., 1972
total protein	24.6%	3d	*Ledpidium sativum* (cress)[1]	germination box	Ray et al., 1988
phenolic cmpds	26	5d	*Glycine max* (soybean)[7]	sterile, in solution	D'Arcy, 1982
phenolic cmpds	92	5d	*Lens culinaris* (D'Arcy, 1982)[7]	sterile, in solution	D'Arcy, 1982
phenolic cmpds	261	4d	*Glycine max* (soybean)[7]	sterile, in sand	D'Arcy, 1982

Table 3. Carbohydrates detected in root exudates (expressed in μg $g_{dry\ root}^{-1}$ day^{-1} or % (w/w) of exudate).

	Species	
	Lepidium. sativum (cress)[1]	*Medicago sativa* (alfalfa)[2]
Compound		
sucrose		45.6
ribose		0.76
fructose		25.69
maltose		202.8
rhamnose	5.8%	
fucose	4%	
arabinose	21.8%	52.8
xylose	13.8%	4.86
mannose	5.7%	36.8
galactose	33.3%	
glucose	15.1%	482.6
Time	3d	16 d
Growth Conditions	germination box	sterile, in sand
Reference	(Ray et al., 1988)	(Hamlen et al., 1972)

Table 4. Amino Acids detected in root exudates (expressed in μg $g_{dry\ root}^{-1}$ day^{-1} or % (moles/mole) of exudate).

	Species	
	Spinaceae oleraceae (spinach)[3]	***Lepidium sativum*** (cress)[4]
Compound		
Asp	0.75	10.5%
Thr	0.25	5.7%
Ser	0.5	7.3%
Glu	0.75	15.3%
Pro	0.3	5.3%
Gly	1.4	8.9%
Ala	3.5	7.2%
Val	0.3	6.3%
Met	0.5	1.7%
Ile	0.25	4.2%
Leu	0.5	7.2%
Tyr	0.25	2.6%
Phe	0.2	4.2%
His	0.4	2.4%
Lys	0.3	7.2%
Arg	0.2	38%
Trp	0.25	
Asn	0.2	
Cys	0.15	
Hyp	0.25	Trace
Time	21 d	3d
Growth Conditions	in soil	germination box
Reference	Futamata et al., 1998	Ray et al., 1988

1. expressed as percent of total carbohydrates, protein, and uronic acid detected
2. assuming an average root dry weight of 0.9 g (over the 16 day growth period)
3. assuming 0.1 g dry root per gram fresh root
4. expressed as percent of total amino acids detected
5. assuming average dry root weight of 0.75 g (over 14d)
6. assuming average dry root weight of 0.6 g (over 12d)
7. assuming average dry root weight of 0.15 g (over 4-5d)

compilation from the literature, Lynch and Whipps (1990) described rhizodeposition as 4-70% of carbon allocated to the roots, which is 30-60% of net photosynthetic carbon. Many other authors have summarized their results in terms of amount carbon exudates per gram plant, or as a percentage of net photosynthetic productivity (NPP) (Table 5).

Table 5. Quanitative estimates of root exudation.

Percentage fixed carbon released as exudate	**Notes**	**Reference**
9	Hydroponic wheat	Minchin and McNaughton, 1984
8-15	Crested wheatgrass, grown in clay	Biondini et al., 1988
7-13		Barber and Martin, 1976
12	Maize	Jones and Darrah, 1993
0.9		Barber and Gunn, 1974
2		Helal and Sauerbeck, 1989
5-10	Wheat and barley grown in sterile soil	Barber and Martin, 1976
10	Includes root debris	Uren, 2001
19		Helal and Sauerbeck, 1989
7	Corn	Haller and Stolp, 1985
5	Rape	Shepherd and Davies, 1993
mg organic C released plant^{-1} day^{-1}		
0.2-1.2	Hydroponic maize, dry mass <1g	Groleau-Renaud et al., 1998
0.04-0.1	Includes only sloughed-off tissue, from peanut	Griffin et al., 1975
Miscellaneous		
7.3 mg/g root/ day	36 day old plants	Kraffczyk et al., 1984
94.2 mg/g dry root	5 day old plants	Schönwitz and Ziegler, 1982
1.8% plant dry mass	Cereals in solution	Barber and Gunn, 1974
3.5% plant dry mass	Cereals in solution with glass beads	Barber and Gunn, 1974
<600 mg/ g root dry mass		Lynch and Whipps, 1990

ROOT EXUDATE STUDY SYSTEMS

The type of system used in root exudate studies has differed depending on the goal of the study. To quantify total amounts of carbon released by plant roots, ^{14}C labeling (Barber and Martin, 1976; Whipps and Lynch, 1983; Minchin and McNaughton, 1984; Norton et al., 1990; Shepherd and Davies, 1993), estimations based on microbial activity (Barber and Martin, 1976; Haller and Stolp, 1985; Biondini et al., 1988), or both (Helal and Sauerbeck, 1989), have been used in both soil and hydroponic systems. To identify specific compounds in the root exudate, mist chambers (Smucker and Erickson, 1976; Timotiwu and Sakurai, 2002) and solution culture (Szmigielska et al., 1995; Fan et al., 1997) have most commonly been used. Modifications have been made to these systems to observe the effects of certain treatments, such as addition of glass beads to solution culture to study the effects of mechanical impedance of roots on root exudates (Barber and Gunn, 1974; Groleau-Renaud et al., 1998), root-zone membranes to study the effects of microbial metabolites on axenic roots (Meharg and Killham, 1995), and changes in the nutrient solution (Ratnayake et al., 1978; Zhang et al., 1991), or soil type (Ström, 1997) to observe effects of nutrient stresses. Most recently, bioluminescence genes have been inserted into bacteria which fluoresce in the presence of a certain exudate, for example galactose (Bringhurst et al., 2001). This allows for identification of timing and specific site along the root of release of that compound.

MICROBIAL EFFECTS OF PLANT GROWTH AND ROOT EXUDATES

Axenic (sterile) plant culture is required to study the quantity and composition of root exudates without their alteration by microbes. The presence of microbes in the rhizosphere can change the quantity of root exudates through metabolism and the composition through partial degradation.

Plant growth in the absence of rhizosphere microbes may be altered compared to non-axenic (normal) conditions, which may also affect the production and release of root exudates. Though these factors may raise questions about the applicability axenic plant culture, the purpose of this study is to be able to identify root exudates of the plant with as little environmental and biotic influence as possible. Most whole-plant exudate studies suggest that root exudates increase with microbial inoculation. Many of these studies compare root exudates of plants grown under axenic and non-axenic conditions but lack specific detail on the materials and methods. Microbe-based mechanisms that might increase carbon released by plant roots include consumption of exudates by microbes (thereby increasing the concentration gradient between the inner root and the soil), competition for nutrients (plants have been shown to respond to nutrient limitations by increasing the release of certain root exudates), and release of specific compounds that elicit a response by the root including phytohormones (Arshad and Frankenberger, 1998), siderophores (Mozafar et al., 1992), and compounds that reduce the integrity of root cells and membranes (Collmer and Keen, 1986).

It is difficult to compare plants grown under axenic and non-axenic conditions without affecting other aspects besides the presence or absence of microbes. Sterilization procedures may change the growth medium and affect plant health. The microbes present in the non-axenic treatment may be contaminants that do not represent soil microbes. Many other papers have also reported changes in root exudation when rhizospheric bacteria are present, but all have conclusions that are similarly uncertain (Prikryl and Vancura, 1980; Barber and Lynch, 1977; Lee and Gaskins, 1982; Biondini et al., 1988; Kraffczyk et al., 1984).

Although microbes can affect root exudate production, it is not clear whether axenic culture will result in more, less, or even changed levels of exudates released by plant roots.

In a 1977 study, Barber and Lynch grew hydroponic barley under axenic and non-axenic (inoculated) conditions. The exudates from the axenic trial were analyzed for carbohydrate content. A direct count was performed on the rhizosphere organisms from the non-axenic trial, and microbial biomass was estimated. The observed number of bacteria was greater than the expected number of bacteria based on the carbohydrates collected under axenic conditions. They concluded that microorganisms increased the release of root exudates.

Their study makes several assumptions, particularly the amount of carbohydrate (0.35 mg bacteria per mg glucose consumed) used by the microbes on which the major conclusion is based. Also, the inoculum was not necessarily rhizospheric microbes from soil, only microbes that grew in “non-axenic” treatments. The biggest assumption is that

the plants in the axenic treatment actually remained free of microbes throughout the 16-day study. No mention was made that any check on the sterility was performed. Converting the data in this study from total mg carbohydrate over several periods of varying length to mg released per day reveals a steep drop-off in exudation rates following an exponential increase, indicating microbial metabolism of exudates due to contamination. A similar decrease was not observed in the direct counts of microbes in the non-axenic trial, which leveled-off with time. Contamination would have resulted in an underestimation in carbohydrate production in the non-axenic trial and therefore an overestimation of increased exudation due to inoculation.

A brief communication by Merbach and Ruppel (1992) was written with the intention to help elucidate the effects of microbes on root exudation, but again the materials and methods section renders the conclusions questionable. Plants were exposed to $^{14}CO_2$ and grown in both axenic and non-axenic soil culture. Increased exudation--3 to 12 times as much ^{14}C--was observed under non-axenic (and inoculated with *Serratia rubidea*) treatments. Higher carbon use efficiency was also observed under non-axenic conditions, along with higher CO_2 uptake. A preliminary check for sterility was performed by plating the surface-sterilized seeds. The soil growth medium used in the axenic treatment, however, had been autoclaved four times. An unintentional artifact in this experiment could have been reduced plant health in the axenic treatment since autoclaving affects much more than the viability of microbes in a soil, including soil structure and nutrient availability. The increase in root exudation that was concluded as a microbial effect may have actually been a plant-health issue: healthier

plants grow faster, and therefore have more carbon to release as root exudates. Increased carbon use efficiency in the non-axenic treatment of this study also may have been due to plant health, not microbes as suggested by the authors.

The conflicting data in the papers presented above is likely due to several factors:

1. metabolism of specific compounds would depend on the composition of the microbial populations
2. whether inoculated or just the "non-axenic" treatments (presumably due to unintentional contamination) were used
3. growth conditions (temperature, growth medium, etc.), which influence the composition of microbial populations.

VERIFICATION OF AXENIC CONDITIONS

Many microbial species require specific conditions for growth and cannot be cultured under laboratory conditions. Most studies use agar plates to detect microbial contamination. Dilute nutrient concentrations, rather than standard media recipes, should be used with the agar to create hospitable growth conditions for contaminants. Since less than 1% of all soil microbes are capable of growing on agar plates, additional checks are required because contamination by certain species may go undetected (Brock, 1987). The most direct method for identifying the presence of microbes would be microscopic observations of the root surface at sites of highest exudation (i.e. the zone of elongation). Analysis for the presence of signature compounds, such as phospholipid fatty acids, has also been used to detect and quantify microbes.

THE IMPORTANCE OF PLANT HEALTH IN ROOT EXUDATE STUDIES

Plant health is an important consideration in root exudate studies as plant stress can alter root exudates. Factors that have been documented to affect root exudates include nutrient stress (Ström et al., 1994; Hoffland et al., 1989; Römheld, 1991, drought (Whipps and Lynch, 1983), hypoxia (Grineva, 1961), light levels (Hodge et al., 1997), root-zone CO_2 concentrations (Zhao et al., 2000), defoliation (Paterson and Sim, 2000), and root damage (Ayers and Thornton, 1968). These factors should be taken into consideration when designing an axenic plant culture system and determining the methods for maintenance.

OBJECTIVES

The overall objective of this research was to characterize the quantity and composition of root exudates from crested wheatgrass. Specifically, to: 1. Develop procedures for growing axenic plants; 2. Grow healthy, unstressed axenic plants; 3. Determine the effects of nutrient stress on crested wheatgrass root exudates; 4. Determine the effects of drought and flooding stress on crested wheatgrass root exudates; 5. Quantify the total organic carbon (TOC) of exudates and the organic acid composition by gas chromatography-mass spectrometry (GC-MS); 6. Predict plant growth from transpired water.

HYPOTHESES

Hypothesis 1: High NH_4^+ and low K^+ stress will increase TOC exuded from the roots.

Hypothesis 2: Low K^+ will induce exudation of nutrient-sequestering compounds.

Hypothesis 3: Periods of drought will result in a higher quantity of exudates.

Hypothesis 4: Periods of flood-induced hypoxia will result in a higher quantity of exudates.

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CHAPTER 2

DESIGN AND MAINTAINANCE OF AN AXENIC PLANT CULTURE SYSTEM TO FACILITATE OPTIMAL GROWTH IN LONG-TERM STUDIES[1]

Abstract

We developed a unique axenic system to grow crested wheatgrass (*Agropyron cristatum*) that promotes plant health and can be kept sterile for 70 days. Individual plants were grown in small, flow-through glass chambers that were positively pressured with filtered air. These chambers were kept in a laminar flow hood modified to include high-pressure sodium lamps. Plants were grown in Ottawa sand and watered with a sterile nutrient solution. Excess nutrient solution leached from the system and was collected regularly for analysis. System components were selected for their inert properties and treated to minimize TOC contamination of exudate samples. Particular attention was paid to plant health by regulating temperature, light levels, CO_2, humidity, and nutrients. Planting and watering manipulations were adapted to maintain sterile conditions in the chambers. Microbial contamination was monitored during the study by plating out aliquots of the leachate onto dilute nutrient agar and with microscopic observations of stained leachate. These results were confirmed at the end of the study by direct microscopic observations of root samples. Plants remained free of microbial contamination throughout a 70-day growth period.

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Introduction

Axenic plant culture is the growth of plants in a controlled environment in the absence of microbes. The term "axenic" refers to a system in which all biological populations are defined. Other terms used to describe these types of systems include sterile, aseptic, and gnotobiotic.

Most axenic plant culture systems have been designed for short-term plant growth. Applications of nutrient stress or microbial inoculation have also been included in design of these systems. The main challenge associated with long-term axenic plant culture is to maintain optimal plant growth conditions in a microbe-free environment. Optimal growth in the controls is imperative in order to apply stresses and assure that responses are due to those stresses alone, not artifacts from the growth conditions.

Requirements for long-term axenic plant culture

An ideal axenic plant culture system should continuously provide the following:

1) CO_2, temperature, humidity, and light control in the shoot zone

These factors are necessary for optimum photosynthesis and growth rates.

2) Nutrients, water, oxygen and absence of light in the root zone

Adequate aeration of the root zone is necessary for aerobic metabolism. Dry conditions in the root zone can lead to stomatal closure and decreased cell water potential. Root growth is inhibited in the presence of light.

3) Mechanical impedance to the roots

Since plant morphology differs between solution culture and solid-substrate culture, a solid substrate is necessary for growth comparable to the field.

4) Inert components

It is important that system components do not adsorb or desorb compounds to or from the system, including organic substances and ions, which could interfere with analytical procedures or plant growth.

5) Maintenance of sterility

Proper sterile techniques, such as autoclaving system components, enclosing the system to minimize exposure to the surrounding environment, and watering without introduction contaminants, are necessary to maintain axenic conditions.

6) Access to verify sterility

Since microbes are strongly associated with plants and ubiquitous in the environment, it is likely that axenic plant culture systems will become contaminated even with significant efforts to minimize contamination. It is therefore necessary to monitor for contamination throughout the study.

7) Access to apply treatments such as nutrient stress or inoculation

Axenic plant culture can be used to determine responses to certain treatments of the plant alone, without any microbial influence. Therefore it is of use to include a method in the design of the system for application of the treatments that allow for maintenance of sterility (i.e. a port for addition of certain compounds or design of nutrient and watering regimes).

8) Access for periodic collection of root-zone solution for analysis

Collection and analysis of the root-zone solution is a non-destructive means of monitoring plant responses to treatments, for example changes in root exudates, nutrient

uptake, or pH. A method for extraction of the root-zone solution must also prevent introduction of microbial contaminants to the system or the root-zone solution sample for accurate analytical results.

Previous studies

Agar media

Axenic plant cultures on agar plates (Heist et al., 2002) or with agar and Millipore membranes (Meharg and Killham, 1991) have been used to study the effects of specific microbes on plants. Agar media allow continuous monitoring of sterility but do not facilitate long-term studies. They do not allow for continuous root-zone solution analysis, or allow for uniform nutrient, water, or oxygen delivery to the root surfaces. Agar plates also provide growth conditions that make treatment responses difficult to extrapolate to the field.

Solution culture

Solution culture allows for periodic analysis of root-zone solution and checking for contamination but provide growth conditions quite different from the field and are difficult to keep sterile due to the necessity of frequent watering. Axenic solution cultures have sealed root zone with cotton wool or a viscous material like Vaseline or wax (Mench and Martin, 1991; Groleau-Renaud et al., 1998). Growth is significantly altered compared to the field due to increased nutrient availability and absence of root hairs when grown hydroponically. The viability of specific microbes inoculated into a hydroponic system is limited without surfaces to grow on, as in soil.

Solid substrates

Soil has been used as a growth medium for axenic plant culture in some early systems (Whipps and Lynch, 1983). Due to the difficulties with sterilization and the structural and geochemical changes that occur from autoclaving or gamma irradiation, soil is an unsuitable growth medium for axenic plant culture. Biondini et al. (1988) enclosed plant roots in pots of sterilized fritted clay with ports for solution input and output. Microcosms containing sand as a growth medium with separate collection containers for nutrient solution leachate have been used for axenic plant culture (Ayers and Thornton, 1968; Lipton et al., 1987; Hodge et al., 1996).

Glass beads provide optimal growth conditions and possibilities for an ideal axenic plant culture system. Sand is similar to glass beads but less expensive, and has fewer reactive surfaces than soil or fritted clay. Sand as a growth medium allows for complete removal of root-zone solution without sorption of any components. Ottawa sand is 99.9% pure quartz sand originating from Ottawa, IL, that is inert and available in multiple grain sizes, which can be layered to form a size gradient for root growth. Use of a single sand size leads to dry conditions at the top and waterlogged conditions at the bottom. A more uniform water potential for optimal root growth can be achieved by having a finer grain size at the top of the column to retain water against the gravitational potential.

Sterile techniques and verification of sterility

Most past studies have used a preliminary check for contamination by germinating surface-sterilized seeds on agar plates. Many studies performed a

secondary check for contamination using only the plate count method by plating out samples of solution from the root zone, usually once at the end of the study (Mench and Martin, 1991; Basu et al., 1994; Groleau-Renaud et al., 1998). Other studies did not indicate how axenic conditions were verified after planting (Barber and Lynch 1977; Hodge et al., 1996).

Development of improved methods to facilitate long-term axenic plant culture was necessary, particularly techniques that maintained sterility while allowing for optimal growth. Dilute nutrient concentrations in the agar are more similar to growth conditions in the rhizosphere. Since less than 1% of all soil microbes are capable of growing on agar plates, additional checks such as microscopy are required because contamination by certain species may go undetected (Brock, 1987).

Sterilization methods most commonly used include autoclaving of the system components, gamma irradiation or autoclaving of the growth medium, and surface sterilization of seeds by soaking in diluted solutions such as H_2O_2, NaOCl, or $HgCl_2$.

Long-term studies

Most axenic plant studies have not been conducted over a long-term (more than 40 days) due to difficulties keeping the systems microbe-free. Microbial contamination is minimized in systems in which the entire plant is enclosed. Hodge et al. (1996) developed a completely enclosed system that allowed for inoculation of microorganisms and the use of radio-labeled CO_2 to track flow of assimilated carbon, with modifications made to these systems (Paterson and Sim, 1999 and 2000) to reduce carbon contamination by the system components and reinforce seals. The system by Hodge et

al. (1996) consisted of 2 Kilner jars, the mouths of which were sealed together, surrounding an inverted reagent bottle (base removed) that contained sand as a growth medium. Holes were drilled in the cap of the reagent bottle for drainage and a section of a syringe barrel was used to support the plant. Four ports were drilled into Kilner jars that allowed for air and fluid exchange to and from the system.

Our intent was to design a simpler system than those already developed with fewer joints, ports, and transfers of solution to be less prone to contamination over time so that longer studies could be maintained. In this paper, specific sterile techniques for axenic plant culture are identified. The plant-growth system discussed here is simple, reproducible, and relatively inexpensive.

Materials and methods

Plant growth container: root zone

The root-zone container was a 22-cm long glass column (38-mm outside diameter, 35-mm inside diameter) with two ports at the top for addition of nutrient solution and air (Figure 1). Five layers of Ottawa sand with different grain sizes were used to maintain a relatively uniform water potential from the bottom to the top of the container. Grain size and layer thickness were determined using the van Genuchten (1980) water retention model (Figure 2). The sand depths were, from top to bottom: 4.5 cm fine (0.315-0.425 mm); 3.5 cm fine/medium mix (0.315-0.630 mm); 2.5 cm medium (0.425-0.630 mm); 2.5 cm medium/coarse mix (0.425-0.850 mm); 3 cm coarse (0.630-0.850 mm), see Table 1 for size conversions. The column was closed on the bottom with

a one-holed silicone stopper with a 9.5-cm long glass tube lined with a silanized glass wool wick to aid water flow out of the growth container. The glass wool wick and surrounding tube were attached to a leachate collection vial via a two-holed silicone stopper (one hole filled with glass wool to allow air displacement while preventing contamination).

The sand in the columns was pre-loaded with iron to increase plant iron availability during the trial since iron chelates would interfere with TOC analysis and were not used. A 200-μM solution of $FeCl_3$ was poured through the columns. When the pH of the solution leaching from the sand decreased to below 4, nutrient solution (pH=5.5) was poured through the sand to increase the pH within the column and precipitate the iron on the surface of the sand.

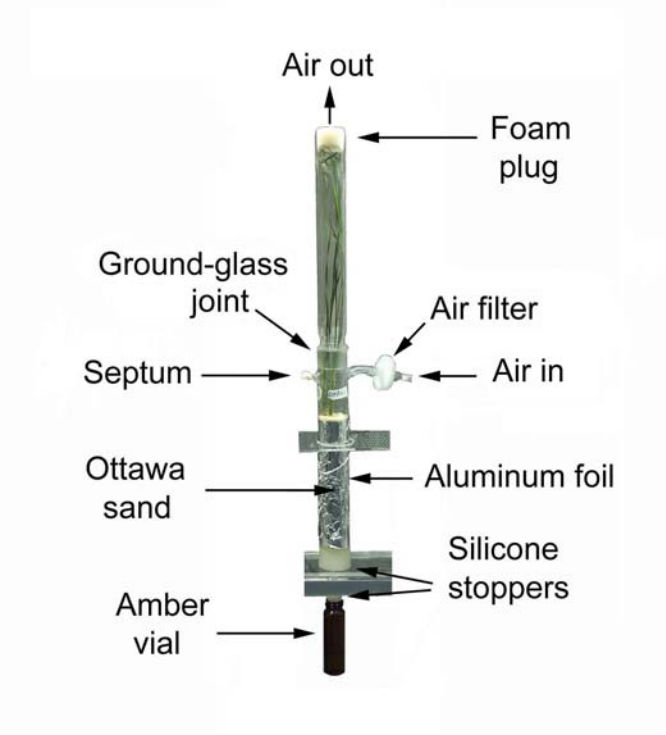

Fig. 1. Glass-column system used for axenic plant culture and study of root exudates.

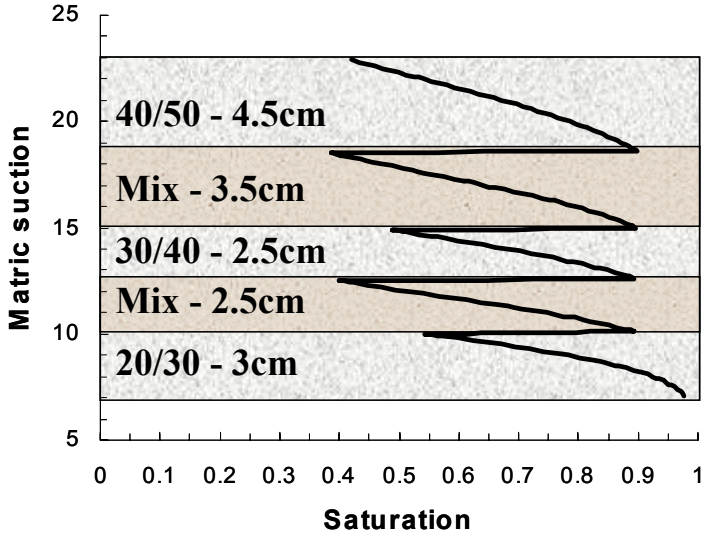

Fig. 2. Relationship between volumetric water fraction and matric potential in each of the 5 sand layers of the column as predicted by the van Genuchten water retention model. The volumetric water content of each layer is at least 40% but not more than 90%.

Plant growth container: shoot zone

The top section of the container was made from a second 22-cm long glass column connected to the root-zone column by a ground glass joint. The column was sealed at the top with an open-cell foam plug to prevent contamination.

Plant culture

One pre-germinated crested wheatgrass seed was planted in each root-zone

column, except the unplanted columns. All columns were maintained for 70 d in a laminar flow hood (Contamination Control, Inc.). The hood was modified for plant growth by fitting with two high-pressure sodium lamps that supplied a PPF of 550 μmol m^{-2} s^{-1} during a 16-h photoperiod (Figure 3). The air temperature was maintained at 25°C. Plants were watered to excess every 1 to 4 d with filtered and autoclaved nutrient solution, the composition of which was predetermined under non-axenic conditions to obtain optimal growth (Appendix A) to obtain at least 25 ml of leachate and maintain healthy plants. Airflow through the upper columns was supplied during the light period and maintained at 65 ml/min to 1 liter/min based on plant size and the air flow necessary to eliminate condensation inside the upper columns. Increasing the airflow successfully controlled condensation.

Columns were supported in racks made of Plexiglas and angled aluminum edging, which could be easily cleaned and surface sterilized with 70% ethanol. These racks reduced possible contamination because they could easily be cleaned and allowed laminar flow of sterile air. Root zones were protected from light by wrapping columns with aluminum foil.

Collection of leachate

Amber vials containing nutrient solution leachate and root exudates were replaced with sterilized, empty vials after each watering. Leachate samples were capped and stored at 4°C until analysis within 24 h.

Fig. 3. Plants growing in the laminar flow hood.

Procedures to minimize microbial contamination

1. Surface sterilization of seed

Crested wheatgrass seeds were agitated on a shaker for 60 min in a solution with 20% Clorox and 0.1% Tween 80 as a wetting agent. After 60 min, seeds were rinsed with sterilized deionized water and placed on petri plates with 1/10 strength Difco nutrient broth and Bacto agar (1.5%) in a 25^{o}C incubator for 3 d to test for residual microbial activity on the seed surface. After 3 d the radicles were about 15 mm long. On average, one seed out of 50 had residual contamination. Seed vigor was not significantly reduced by the treatment (see Appendix B).

2. Sand cleaning and sterilization

It is necessary to remove organic carbon from the sand to minimize background carbon levels. Sand was washed with deionized water, dried at 90°C, washed with 30% H_2O_2, and baked overnight at 90°C to increase the reaction of organic carbon with H_2O_2. The H_2O_2 treatment was repeated and the sand was given a final rinse with filtered (0.45 μm) deionized water. The sand was then poured into the glass columns and autoclaved twice, one day apart, for one hour at 21 psi, 121°C to allow any remaining endospores to germinate between autoclavings.

3. Cleaning and sterilization of components

All components were rinsed with methanol, dried at 80°C to remove trace organic carbon, and autoclaved for 45 min at 21 psi, 121°C.

4. Transfer of germinated seeds from petri dishes to root-zone columns

Plant-growth containers were assembled and seedlings were transplanted to the sand in a laminar flow hood. Forceps and column lips were flamed before use. All manipulations were done using sterile gloves.

5. Filtration of column air

Air was pumped through the upper part of the glass plant-growth columns to supply CO_2 and remove water vapor. This air was filtered through glass wool and foam plugs before and after the pump, and through a sterilized, bacterial air filter (Pall Gelman®, aerosol retention = 0.3 μm) before entering each column.

6. Sterilization of the nutrient solution

Nutrient solution was filtered through a 0.45 μm membrane, then autoclaved at 21 psi and 121^{o}C for at least 45 min. After autoclaving, the nutrient solution was allowed to cool completely before using it to water the plants.

7. Syringe injection of nutrient solution to replace transpired water

Separate flasks of nutrient solution and sterile syringes were used for each group of 5 plants. Septa were cleaned with 70% ethanol before each injection. A new sterile needle was used for each plant. A syringe was discarded immediately if it touched anything other than the nutrient solution.

8. HEPA filter in laminar flow hood

The High Efficiency Particulate Air (HEPA) filter in the laminar flow hood was replaced at the start of the first trial and was tested and certified.

9. Autoclaving

A calibrated pressure gauge was used to monitor the autoclave and ensure that appropriate pressure (at least 15 psi) was maintained during the entire cycle.

Checking for contamination

Aliquots of leachate (20 μl) from each plant were pipetted onto plates of 1/10 strength Difco nutrient broth and 1.5% Bacto agar. Plates were incubated for at least 3 d at 25^{o}C before visual examination for microbial growth (Figure 4). Repeated sampling of the solution in the leachate vials provided the ability to distinguish between a truly contaminated plant and contamination that was introduced after a sample had

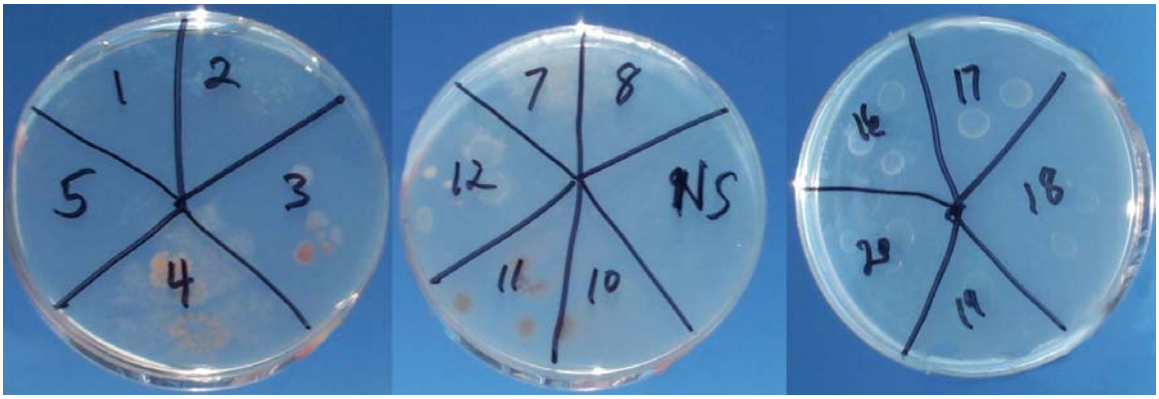

Fig. 4. Microbial growth from the leachate on day 47 of a preliminary trial. Numbers refer to individual plants. NS refers to autoclaved nutrient solution. Plants 1, 8, 10, and the nutrient solution have no microbial growth on these plates, thus suggesting no contamination. The microbial growth from plants 2, 3, 4, 7, 11, 12, 16, 17, 20 is apparent, but microbial growth from plants 5, 18, 19 is not visible in this picture.

been collected (a false positive). Identification of false positives occurred when subsequent platings of leachate from the same column were clean.

In addition to plating, a direct total microbial count was used to double-check sterility. The Epifluorescent Microscopic Method (Clesceri et al., 1998) was used: 5 ml of leachate was stained with acridine orange, filtered through a 0.2-µm non-fluorescent membrane, mounted on a microscope slide, and observed at 100x under a UV light. Microbes could be seen in the leachate of plants that were identified as contaminated on the plates (Figure 5). A rhizoplane stain using phenolic aniline blue (Rovira et al., 1974) was also performed on root samples at the end of the study (see Appendix F). Small

sections of root were excised and soaked in phenolic aniline blue solution (Schmidt and Paul, 1982) for 3 min. Roots were then rinsed in sterile filtered water, mounted in water on a microscope slide and observed under 100x (Figure 6). This confirmed the visual observations in the petri dishes.

Harvest

Plants were harvested in a laminar flow hood using sterile techniques. The plant growth columns were disconnected from the airflow and removed from the storage rack. After the upper glass column was removed, the plant was pulled from the root-zone column, which removed much of the sand as it was attached to the roots. Sand that immediately fell off the root or out of the column was labeled "bulk sand" and saved in a sterile container for analysis. The roots were then separated to remove the remaining sand, which was labeled "rhizosphere sand" and stored in a sterile container. A small segment of root was then excised for microscopic observation. The shoot was cut off and dried in an 80^{o}C oven. The root and remaining attached sand was stored in a sterile container. All root and sand samples were stored at –20^{o}C.

Plant nutrient analysis

Inductively coupled plasma emission spectrophotometry (ICP-ES) was performed to determine nutrient content of shoots at the Utah State University Plant and Soil Analysis Laboratory. The digest was performed using 1 gram of dried, ground shoots with 8 ml HNO_3 and heated to 95.1 ^{o}C for 2 h. Two ml H_2O_2 was then added, and the sample was heated again four times for 30 min. Four ml H_2O_2 was added, the sample

heated for 30 min, and the volume brought up to 50 ml with deionized water. Samples were then run on the ICP, in which the sample as an aerosol was put into a plasma. The heat of the plasma excites the elements in the sample and detects the elements present and their concentrations based on the wavelength and intensity of light emitted as the elements drop down from their excited states. Detection limits ranged from 1.5 - 25 mg/kg and 0.001 - 0.005 % plant dry mass.

Trials

Axenic plant culture was conducted through six separate trials during which the percent of non-contaminated plants was recorded. After each successive trial, changes were made in the materials and methods of the next to decrease contamination. The most successful was Trial 6, which is described in the materials and methods.

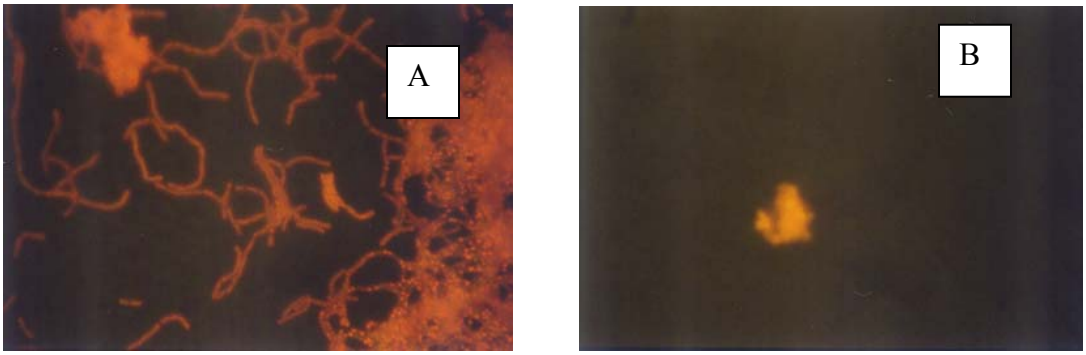

Fig. 5. (A) Acridine orange stain of bacteria in the leachate from a contaminated column. (B) Leachate from a non-contaminated column. Only root debris is visible.

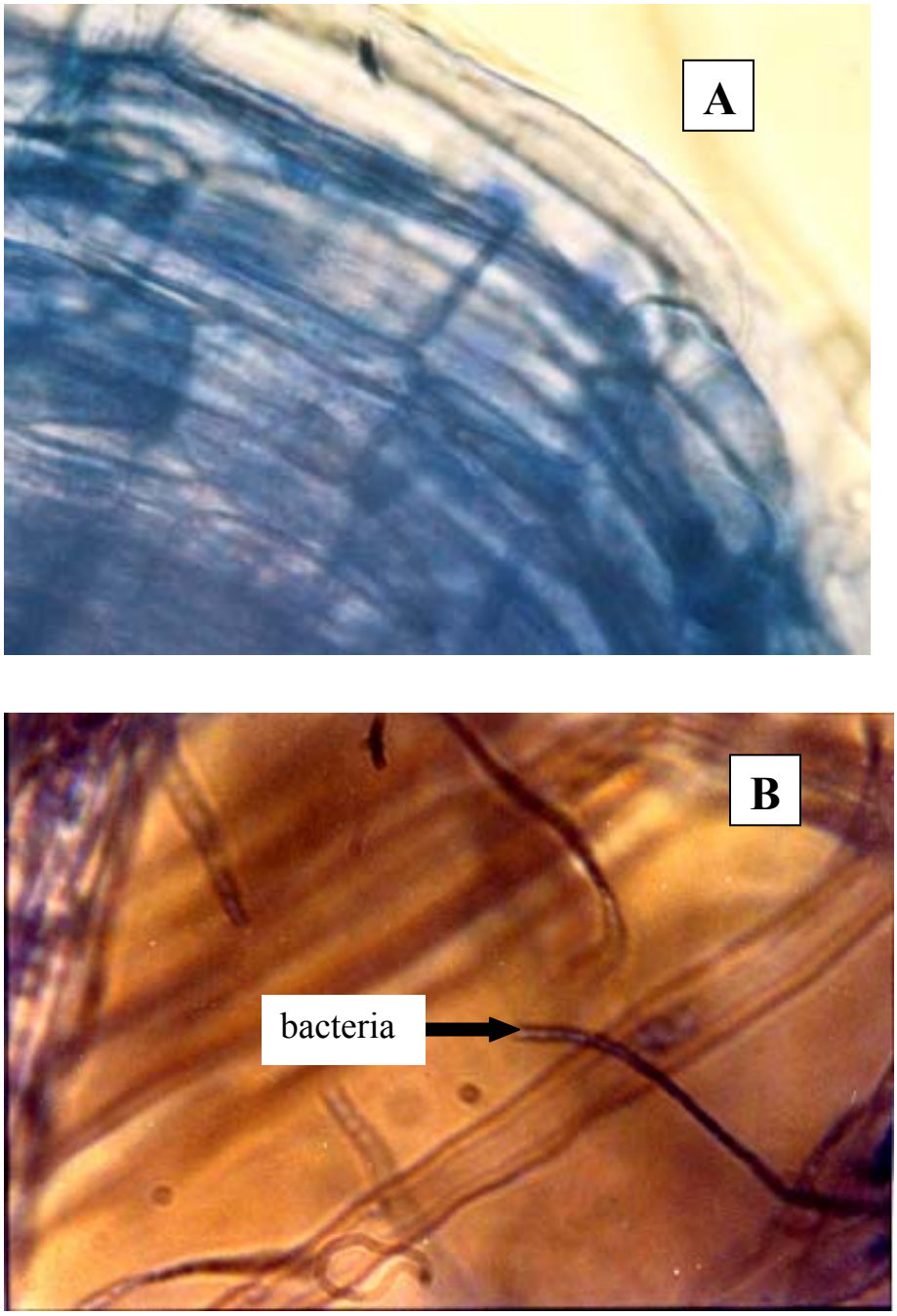

Fig. 6. Root segments with root hairs stained with phenolic aniline blue. There was no evidence of microbial contamination around the top segment (A), but bacterial cells were observed around the roots of root segment B.

Results

Plant health

Plants appeared green and healthy throughout the trial. The tops of leaves touching the foam plug curled when leaf length exceeded the length of the upper column. This eventually occurred in all planted columns.

ICP analysis of plant shoots showed all nutrients to be present in normal amounts (Table 6).

Table 6. *Average nutrient content of plant shoots in Trial 6 detected using ICP.*

P	K	S	Ca	Mg	Fe	B	Mn	Cu	Zn	Ni	Al
----------%----------					----------mg/kg----------						
0.3	3.04	0.2	0.23	0.09	50.4	51.8	49.4	11.2	51.2	--	0.57

Maintenance of axenic conditions

Contamination at the end of the study was reduced by up to 85% compared to previous trials by growing the plants in a laminar flow hood and enclosing the shoot in a glass column (Figure 7). Several improvements in sterile technique were made in Trial 6:

1. Exposure time of the growth columns and germinated seeds to the surrounding air during planting was minimized by preassembling components before sterilizing.
2. Sterile gloves were used for all manipulations.

3. Extra sand was autoclaved separately to pour on top of seedlings during planting. This eliminated the need for burying the seedling with a spatula, thereby reducing the number of manipulations and length of time the column was exposed to the surrounding air. The appropriate amount of sand needed to adequately cover the seedling was predetermined under nonsterile conditions.
4. Airflow manifolds were mounted directly to the racks holding the columns, which improved laminar flow of sterile air through the workspace.

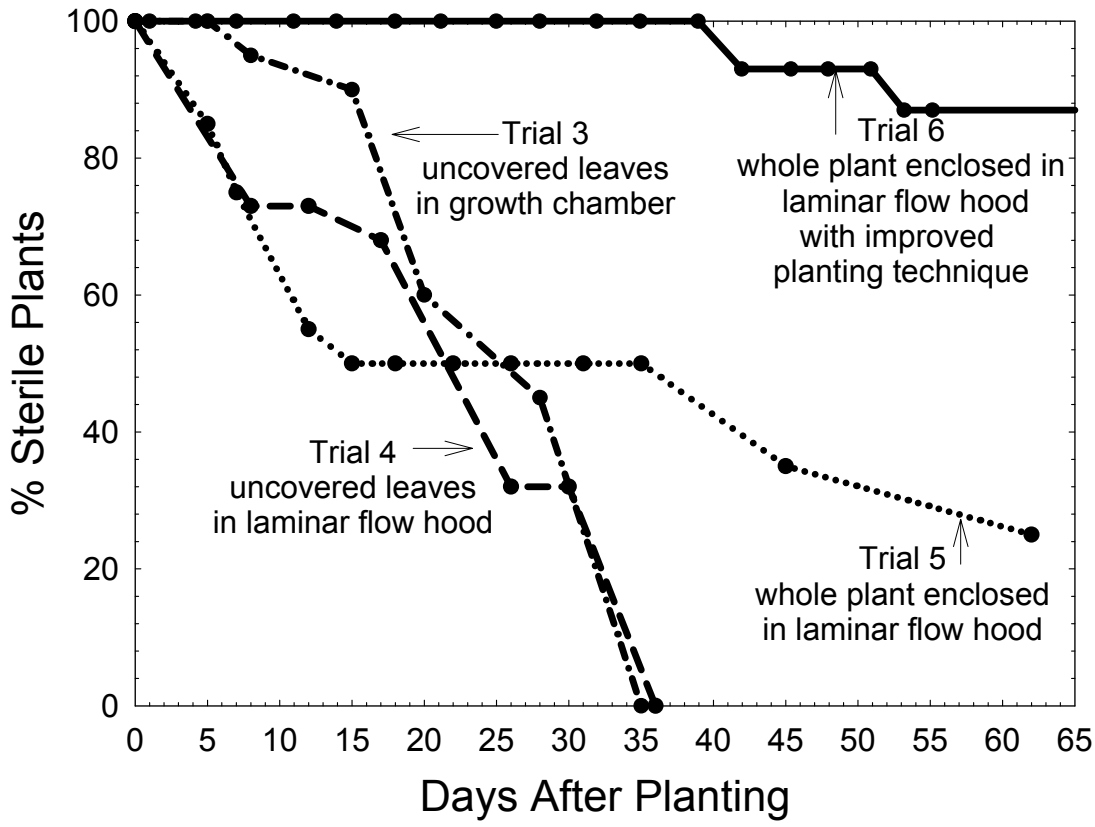

Fig. 7. Effect of time on contamination in four cultural conditions. At the end of Trial 6, 2 out of 15 columns were contaminated.

Discussion

Axenic plant culture requires much preliminary practice of the sterile techniques. The manipulations associated with planting result in a significant potential for contaminations. Planting is also the stage at which plant survival rates are lowest due to desiccation or root damage. Rapid transfer of the germinated seed from the agar plate to the moistened sand is necessary. It is not necessary that the entire seedling be covered by sand, just the seed coat. Additional time spent burying the seedling during planting results in more exposure time and higher possibility of microbial contamination.

In the most successful trial, 85% or 12 out of 14 plants remained free of microbes for 70 days, after which the trial was terminated due to plant size. In both of the two contaminated plants, roots grew through the glass wool and out the bottom of the drain tube. Because a rhizoplane stain of roots from within the system was free of microbial growth, it is likely that only the emerging root segment was contaminated. Although plants were kept in the laminar flow hood and sterile gloves were always worn when watering and replacing vials, direct exposure of the plant to the surrounding air frequently resulted in contamination. This implies that attempts at long-term axenic plant culture would be less successful in a growth chamber that was not enclosed in a laminar flow hood.

The leachate and rhizoplane stain methods had limitations. Since acridine orange stains DNA, any living material in the leachate, including root cells, was stained. Little material, however, was seen fluorescing in the microscopic observations of leachate stained with acridine orange. This may be due to low levels of contamination since no

microbial multiplication occurs using this method and leachate stain often captures single cells at a time, compared to agar plates in which growth makes the microbes more visible. The sand and glass wool could have filtered the microbial cells in the leachate. Contaminating microbes would be also most likely attached or in close association to the root and resistant to being washed into the leachate. The acridine orange method did work well for visualizing the leachate from plants with emerging roots, probably because the contaminating microbes were protruding from the system and more free to be washed into the leachate, not filtered by sand and glass wool.

Young root segments and root tips, where microbial symbiosis or infection is most likely to occur (Curl and Truelove, 1986), were excised for staining with phenolic aniline blue and mounted on slides. One slide was made for each plant, which may have limited the contamination check since only a small part of the root zone was represented on each slide.

Procedures for cleaning the system components probably reduced microbial contamination. The 20% H_2O_2 treatment used to reduce residual TOC on the sand was powerful enough to kill many microbial cells, and the deionized water washes of all components also removed microbial cells and spores. All water used for cleaning was filtered since any debris would interfere with microscopic observations.

The exudates in this study were mixed in with leached nutrient solution upon collection. This presented some difficulty since the salts from the nutrient solution interfered with certain qualitative analyses of the exudates (e.g. HPLC, anthrone test for sugar content). Therefore it is necessary to select or develop the appropriate qualitative

analysis that will not be affected by salt content of the exudate sample.

Some short-term solution culture studies have removed the roots from the nutrient solution and soaked them in deionized water to collect root exudates (Fan et al., 1997; Ratnayake et al., 1978; Ström et al., 1994). This procedure would reduce salt interference with analyses but may bias results since the amount and composition of root exudates can reflect the nutritional status of the plant and deionized water contains no mineral nutrients. Nutrient deficiencies may change the composition or induce increased amounts of exudates, for example, phosphatases, phytosiderophores, or other nutrient chelating compounds such as organic acids (Kraffczyk et al., 1984; Römheld, 1991; Ratnayake et al., 1978). Wang et al. (2002) have shown that genes associated with P, K, and Fe deficiencies can be induced within 1 h after withholding these nutrients from the plant. Since this response was localized to the root, a rapid response in root exudate composition to nutrient deficiency is also likely. In the root exudate studies using deionized water as the exudate collection medium, plant roots were immersed in deionized water for exudate collection from 1 h up to 24 h. The high water potential of deionized water compared to nutrient solution may also cause cell lysis, releasing the root-cell contents that would then be counted as exudate. Aulakh et al. (2001) found 0.01M $CaSO_4$ to be a good leachate collection medium that did not interfere with TOC or HPLC analyses and did not increase TOC or the proportion of sugars exuded, as observed with deionized water.

To improve the system, taller and wider upper columns for the plant-growth container could allow longer studies provided that contamination or space in the laminar

flow hood was not a limiting factor. Many plants had grown to the top of the container by day 70. The crowed leaves resulted in pockets of reduced airflow and therefore higher humidity and a reduced gradient for transpiration. The size of the root zone was less of a concern since nutrients and water were being replenished regularly and the sand medium provided ample pore space for oxygen to reach the roots.

The system for long-term axenic plant culture for the study of root exudates described here is suitable for many other types of root exudate studies, including the effects of inoculation with pure microbial cultures on root exudates and the effects of abiotic stresses such as heavy metals. Regardless of the purpose of the study, this system provides a method for healthy plant culture that can provide interesting insight to the dynamics of root exudation.

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CHAPTER 3

CHANGES IN ROOT EXUDATION UNDER FLOOD, DROUGHT, AND NUTRIENT STRESS[2]

ABSTRACT

Root-zone stress may increase the production of root exudates and enhance phytoremediation. Increased TOC in the rhizosphere can support higher levels of organic contaminant-metabolizing microorganisms. Root exudates can also chelate inorganic contaminants and change soil pH, which can alter solubility and plant uptake. We studied the effects of low K+, high NH_4^+/NO_3^- ratio, drought, and flooding on root exudates. Crested wheatgrass was grown under sterile conditions for 70 days. Treatments were induced beginning on day 35 by altering the composition and frequency of application of the nutrient solution. Exudates were quantified using a Total Organic Carbon analyzer. Drought stress significantly increased TOC exuded per g dry plant by 70% compared to the control ($p = 0.05$) The K^+ stress and flood treatments increased by 44% and 45%, respectively, although these changes were not statistically significant based on the two replicates used in this study. Malic acid was the most predominant of the organic acids, which were identified and quantified using a GC-MS. These results indicate that cultural manipulations may enhance phytoremediation.

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INTRODUCTION

The characterization of compounds exuded by plant roots may be useful for phytoremediation. Root exudates are substrates for microbial growth and co-metabolism of organic soil contaminants. Exudates can influence inorganic contaminant mobility and bioavailability in soil. Nutrient, water, and oxygen status of a plant can be altered to influence both exudate composition and quantities. In addition to soil remediation, root exudates have also been studied for allelopathy, carbon partitioning within a plant, and rhizosphere microbial signaling to soil-borne pathogens or nitrogen-fixing bacteria. The information obtained from this study is intended for use by the Idaho National Engineering and Environmental Laboratories (INEEL) and the Department of Energy (DOE) to improve phytoremediation of metals and organics-contaminated soil. Because of their chelating properties that can be useful for phytoremediation and their ubiquitous presence in previous root exudate studies, organic acids were selected as the class of compounds that were characterized in this study.

Factors That Affect Root Exudates

Factors that can influence root exudation include the presence of microbes, plant nutrient status, plant water status, oxygen availability, species, growth medium, and other environmental conditions.

Microbes

Microorganisms metabolize root exudates, thereby modifying exudate composition and quantity. Many types of compounds released by microorganisms have the potential to affect the release of root exudates, including pectic enzymes (Collmer and Keen, 1986), phytohormones (Arshad and Frankenberger, 1998), and siderophores (Mozafar et al., 1992). Axenic (sterile) conditions are necessary for analyzing the unmodified composition of exudates. Some studies have concluded that microorganisms can increase the efflux of exudates, up to double the amount (Prikryl and Vancura, 1980; Merbach and Ruppel, 1992). Mechanisms proposed to cause this increase include an increased concentration gradient of exudates between the root surface and the rhizosphere with microbial degradation, or increased permeability of root cell membranes and stimulation of exudate release by microbial metabolites. Effects of microbes on root exudates is dependent on microbe species (Lee and Gaskins, 1982; Meharg and Killham, 1995).

Microorganisms can change the composition of exudates that have been released into the soil, including C:N ratio and relative concentrations of compounds in the exudates (Kraffczyk et al., 1984; Biondini et al., 1988). Microbial metabolism of exudates can affect the nutritional status of a plant by metabolizing exudates. Inoculated plants have shown decreased amounts of phytosiderophores in exudates and displayed more iron deficiency (Von Wirén et al., 1993). Plants may reduce microbial phytosiderophore degradation by spatially separating phytosiderophore release along the root from microorganisms (Römheld, 1991), or by timing the release of

phytosiderophores (Marschner et al., 1986). Since not all microbes affect root exudates in the same way, the least confounded mode of studying root exudates is in the complete absence of microbes.

Plant Nutrient Status

Iron deficiency is known to increase phytosiderophore release, but the availability of other plant nutrients can also affect root exudates. Phosphorous deficiency is well characterized to increase exudation of phosphatase (Ratnayake et al., 1978; Hoffland et al., 1989; Gilbert et al., 1999). In our study, nitrogen and potassium treatments were used since they could be easily applied in the field for phytoremediation, and because of the similarities between potassium and cesium, a contaminant of INEEL soil: root exudates released to solubilize K^+ could solubilize Cs^+ and increase plant uptake of Cs^+. Kraffczyk et al. (1984) reported an increase in exudation of sugars, organic acids, and amino acids when grown in K^+-deficient conditions, as well as higher levels of glutamic and aspartic acids when NO_3^- was used as a nitrogen source as opposed to NH_4^+. Several studies have observed increased exudates with higher N (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 by N.

Drought

Plant water status can be an important component when manipulating root exudates since it is easily controlled in the field. 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 an excess amount of carbon within the plant that was subsequently released by the root, or that the water stress caused root death that was detected as exudate.

Since water stress reduces growth, inducing drought stress to increase exudation can be challenging. Crested wheatgrass in the field shows a growth pattern of rapid increase in dry matter accumulation, then utilization of all available soil water, and finally avoids drought by going dormant. Frank and Bauer (1991) reported that crested wheatgrass evapotranspiration is more a function of plant development stage than calendar date. When exposed to treatments of 50, 100, and 150% of rainfall, crested wheatgrass water use efficiency decreased with increasing water treatment, indicating that crested wheatgrass did not use high amounts of water efficiently. Crested wheatgrass was also observed to extract soil water to lower soil water potentials than western wheatgrass (*Pascopyrum smithii*), another rangeland species.

Flooding

Excess water in the root zone 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.

Mechanical Impedance

Mechanical impedance within the root zone has been shown to increase root exudation, change exudate composition, and change root morphology (Groleau-Renaud et al., 1998; Barber and Gunn, 1974; Schönwitz and Ziegler, 1982). These studies compared plants grown in solution culture with and without glass beads. Soil controls were not used. Hypothesized mechanisms for changes in root exudates from mechanical impedance include changes in root structure since different types of roots have different exudate patterns, and increased abrasion to damage root cells.

Adsorption and Re-absorption

Adsorption of root exudates to the growth medium can occur. In studies using glass beads, rinsing the beads was included in methods to collect root exudates (Barber and Gunn, 1974; Groleau-Renaud et al., 1998).

Re-absorption of exudates by the plant roots has also been reported. Jones and Darrah (1993) observed that plants grown in solution that was replaced daily accumulated about 9 times more carbon exuded from the roots than plants in static and non-sterile solutions. Jones and Darrah (1993) also claimed that the greater the volume of solution, the greater amount of carbon collected, and that re-absorption in static cultures will always occur irrespective of solution volume. They attributed the lower amounts of exudation in static solution to re-absorption of the exudate. However, the change in osmotic potential that might have been induced by changing the nutrient

solution may have caused more exudates to be released from the plants, thereby creating the difference between the two treatments.

Jones and Darrah (1993) hypothesized that re-absorption in the roots occurs via a concentration-dependent active-uptake mechanism energized by the plasmalemma membrane, where the H+ gradient is the driving force for solute transport. The authors also suggest that the increased exudation under K^+ deficiency observed by Kraffczyk et al. (1984) is actually decreased absorption since K^+ stimulates H-ATPase.

Organic Acids in Root Exudates

The release of organic acids to the rhizosphere contributes to plant health in several ways, including aluminum immobilization and solubilization of inorganic phosphorous. 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. Therefore a slow release of organic acids from roots is likely to be always occurring (Jones, 1998). Increased levels of dicarboxilic and tricarboxilic acids are released from the roots of plants that are able to grow in calcareous soils compared to those that cannot, possibly because of the ability of these acids to solubilize certain nutrients from the soil, such as Fe, Mn and P (Ström, 1997). The acidification of the rhizosphere, however, is due more to proton secretion, not the presence of organic acids (Petersen and Böttger, 1991).

In a 28-day test tube study, Kloss et al. (1984) compared axenic plants with those inoculated with a N-fixing bacterium and showed that bacteria metabolized 95% of the

organic acids exuded, assuming the amount exuded was the same in the axenic plants and the inoculated plants. These results also supported the observation that oxalic acid has not been detected in the root exudate of C-4 plants, whereas C-3 plants do exude oxalic acid.

Root exudates in phytoremediation

Studies of in vitro addition of root exudates to organic compounds suggest increased degradation in the presence of root exudates, including phenanthrene by oat exudates (Miya and Firestone, 2001), pyrene by corn exudates (Yoshitomi and Shann, 2001), atrazine by poplar exudates (Burken and Schnoor, 1996), and 2-chlorobenzoic acid by wild rye exudates (Siciliano et al., 1998).

Root exudates can contribute to phytoremediation of inorganic compounds by mobilizing the contaminants in the soil (Mench et al., 1988) and increasing plant uptake of metals in the rhizosphere, i.e. root exudates from *Nicotiana* spp. have increased the bioavailability and uptake of cadmium and other cations (Mench and Martin, 1991). However, Zhao et al. (2001) reported that root exudates are not associated with hyperaccumulation of Zn, Cd, or Cu in *Thlaspi caerulescens*, a well known Zn hyperaccumulator.

Organic and inorganic compounds are often present in the soil. Root exudates could enhance degradation of organic contaminants by providing nutrients that increase microbial activity, increasing the bioavailability of contaminants by promoting desorption from soil surfaces, and by providing substrates for co-metabolism, which is

the microbial breakdown of organic soil contaminants when supplied with a more accessible carbon source (Miya and Firestone, 2001). Root exudates may also have enzymatic properties that can break down contaminants (Siciliano et al., 1998). Increasing the proportion of organic acids in the rhizosphere could increase the amount of chelation and plant uptake of inorganic contaminants. Other changes in exudate composition could change the rhizosphere pH, thereby immobilizing or mobilizing contaminants in the soil.

This study sought to determine if stress can alter the quantity and composition of root exudates, which has the potential to increase the effectiveness of phytoremediation of both organic and inorganic contaminants.

MATERIALS AND METHODS

Crested wheatgrass (*Agropyron cristatum*), a rangeland species, was chosen for this study because of its ability to grow in regions with low rainfall. Cultivar CD-II, which was developed at the USDA-ARS facility on the Utah State University campus, was chosen for its increased vigor and stress resistance.

Plant Growth

Plants were grown under axenic conditions. All maintenance and manipulations were carried out in a laminar flow hood using sterile technique to avoid contamination. Sterile technique includes the use of 70% ethanol and flaming for tools and surfaces, and autoclaving to sterilize all sand, solutions, and growth containers.

Ottawa sand was used as a growth medium to provide mechanical impedance. The impedance to root growth was similar to that of a sandy soil since the columns were close to their maximum packing density. Ottawa sand is a relatively inert medium that does not significantly bind negatively-charged compounds (see Appendix C). A 22-cm long glass column (38-mm outside diameter, 35-mm inside diameter) was used as the growth container (Figure 1) for axenic culture. A range of sand sizes was used to maintain uniform water content throughout the growth container. Grain size and layer thickness were determined using the van Genuchten (1980) water retention model. The sand layers were, from top to bottom: 4.5 cm fine (40-50 grit); 3.5 cm fine/medium mix (30-50 grit); 2.5 cm medium (30-40 grit); 2.5 cm medium/coarse mix (20-40 grit); 3 cm coarse (20-30 grit). The top of the container was closed with a second 22-cm long glass column (connected by a ground glass joint) and an open-cell foam plug to prevent contamination and reduce water loss by the system. The tube was closed on the bottom with a one-holed silicone stopper lined with a silanized glass wool wick to aid water flow out of the growth container. This glass wool wick was enclosed in a small glass tube attached to a leachate collection vial by a two-holed silicone stopper (one hole filled with glass wool to allow air displacement while preventing contamination).

Glass columns were washed with methanol and heated at 175^{o}C to remove residual carbon. Sand was washed with 30% H_2O_2 and rinsed with deionized water. All components were given a final rinse with filtered deionized water (0.45 μm Pall Gelman membrane). After partial assembly, columns were autoclaved twice on two separate days and fully assembled in a laminar flow hood (see Chapter 2 for more details).

Seeds from recently grown crested wheat grass plants (cv. CD-II) were soaked in tap water for 30 minutes. About 40% of the seeds sunk in the water and 60% floated. The germination of the seeds that sunk was 90% (Appendix B). Germination of those that floated was 65%. The denser, sinking seeds were selected and used in all studies. Seeds were surface sterilized in a sterile beaker with a 20% Clorox and 0.1% Tween 80 solution for 60 minutes on a shaker at 100 rpm. Seeds were then rinsed with deionized water and placed on petri plates containing dilute (0.8 g/L) nutrient broth (Difco) and Bacto agar (1.5%) in an incubator. This aggressive treatment did not significantly reduce seedling vigor (germination rate = 75%, see Appendix B).

One pre-germinated seed was removed from the petri plate in a laminar flow hood and planted in each column, except the unplanted column. The sterilized upper columns and foam plugs were then connected to the planted lower columns. All columns were saturated with sterile filtered nutrient solution and collection vials were attached.

All columns were maintained in a laminar flow hood (Contamination Control, Inc.). The hood was modified for plant growth by fitting with two high-pressure sodium lamps to supply light (500 μmols m^{-2} s^{-1}, 16 hr photoperiod) and a ductwork system to maintain temperature at 25^{o}C.

Plants were watered periodically with filtered nutrient solution (for composition see Appendix A) to obtain 25 ml of leachate and maintain healthy plants. Double-strength nutrient solution was used for the first 35 days of the trial to help the seedlings become established. Single strength nutrient solution was used in all treatments after

day 35, when the induction of treatments began. No iron chelate was used in the nutrient solution to minimize non-plant sources of carbon, which would interfere with the TOC analysis. A new syringe needle was used for each column, and septa were surface sterilized with 70% ethanol before watering. Leachate bottles were replaced at each watering. Plant water use was monitored from volumes of nutrient solution added and recovered. It was assumed that the contents of each leachate vial contained a representative sample of the root exudates in the rhizosphere.

Plants in four columns did not become established after planting and were replanted during the first week. These columns were assigned to the control treatment so as to avoid biasing the treatments, which had only two reps each.

Root-zone Stress Treatments

Nutrient solutions and watering volumes were manipulated to induce treatments of low K^+, increased NH_4^+, drought and flooding. Two plants were assigned to each treatment beginning on day 35, with 6 control plants watered with the same frequency and nutrient solution as previous, with the exception of reducing the strength of the nutrient solution to reduce the buildup of salts. K^+ stress was induced by decreasing the concentration of K^+ in the nutrient solution from 5.5 mM to 0.5 mM. For the NH_4^+ treatment, the NH_4^+: NO_3^- ratio was changed from 0:7 to 2:6. This ratio was increased to 4:3 on day 57. Drought was induced by watering with 75% less volume than the controls, with waterings every 2 to 4 days of an adequate volume of nutrient solution to

obtain leachates. A stopper was placed in the drain tube of the flood treatment to induce flooding.

Detection of Microbial Contamination

Microbial contamination was assessed weekly by pipetting 20-µl samples of leachate onto petri dishes containing 1/10 strength nutrient agar (1.5% agar). Plates were stored in a 26^{o}C incubator for two weeks and assessed for microbial growth. Leachate samples were further examined for contamination at the end of each study by direct count using acridine orange , which stains DNA (Clesceri et al., 1998). A rhizoplane stain was performed on select root samples using phenolic aniline blue, a compound that stains the carbohydrate callose (Schmidt and Paul, 1982).

Harvest

Plants were harvested on Day 70. Roots and sand were stored in sterile containers at -20^{o}C until extraction and analysis by TOC or GC-MS. Plant shoots were rinsed with deionized water, then dried and analyzed for mineral nutrient content by digestion and ICP at the Utah State University Plant Analysis Lab (see Chapter 2). TOC content of the sand was determined by analyzing a 0.1N NaOH extract of a 5-g subsample that was agitated for 45 minutes at 100 rpm.

TOC Analysis

A sub-sample of each leachate was diluted and used to determine the Total Organic Carbon (TOC) in the sample. This analysis was performed on a Tekmar-Dohrman, Pheonix-8000 TOC analyzer that operates using a UV-persulfate reaction (see Appendix F). Leachate analyses were performed when collected and re-run together at the end of the study using the same standard curve. The values obtained at the end of the study were slightly lower and were reported here.

To determine the percent soluble TOC in each leachate sample, the TOC of a 5-ml subsample filtered aseptically with a low-retention syringe tip filter (Pall Gelman acrodisc, Supor membrane) and diluted with sterile filtered water was determined. 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.

GC-MS Analysis

The analyses of dicarboxylic acids were performed by: 1) extracting the acids from the media (roots or sand), 2) esterifying the acids to increase their volatility, and 3) analyzing the methyl esters using gas chromatography with mass spectrometry (GC-MS).

The roots and attached sand were separated and sub-sampled into approximate 5-gram and 1-gram samples, respectively. Three ml of 0.1 N NaOH solution was added to each subsample. The sample solutions were intermittently mixed for one hour at room

temperature and then separated from the sand or root matrix. The sodium hydroxide extraction was not performed on leachate samples, which were treated directly. Samples were then esterified; 1-ml aliquots of each extract or leachate were added to 3 ml of methanol and acidified with 0.6 ml of 50% sulfuric acid. The acidic methanol mixture was heated for one hour at 50^{o}C, then cooled and diluted with an additional 3 ml of water. One ml of chloroform was added, the sample vigorously shaken then incubated about 30 minutes. The chloroform layer was removed and its components in the chloroform were quantitated using GC-MS (Agilent models 6890N/5973). Chromatography conditions were: 1 microliter injection, column flow rate, 0.6 ml/min, split/ratio = 3.6, column – DB-624, 30m x 0.25mm x 1.4µm, temperature program, start at 50^{o}C, 5 degrees per minute to 200^{o}C and hold 5 minutes. Methyl esters of the dicarboxylic acids were quantitated using pure compounds (Aldrich, Milwaukee, WI). A concentration range of 0.5 ppm to 10 ppm showed good linearity for calibration of each organic acid (r^2 = 0.997 to 0.999).

Treatments were compared on SigmaStat using one-way ANOVA and a Tukey Test or Dunn's Method as follow-up analyses when applicable (Appendix H). Exudates were expressed in terms of µg TOC per gram new plant by dividing µg TOC per gram plant per day by the relative growth rate throughout the study. ANOVA was performed on the 4 dates after the treatments were applied (days 42, 51, 57, and 63) in which a full data set was present; exudates were not collected at intermediate dates for the drought and flood plants because the mode of treatment application involved intervals with no leachate collection.

RESULTS

Plant Health

Plants were green and healthy throughout the trial. Some yellowing was observed on the shoots of the flooded treatment, which was likely caused by the induction of 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 by the end of the study.

The ammonium treatment was effective in lowering the rhizosphere pH. The pH of the leachates on day 68 ranged from pH = 4.2 in the NH_4^+ treatment to pH = 8 in the control and water stress treatments (Figure 8).

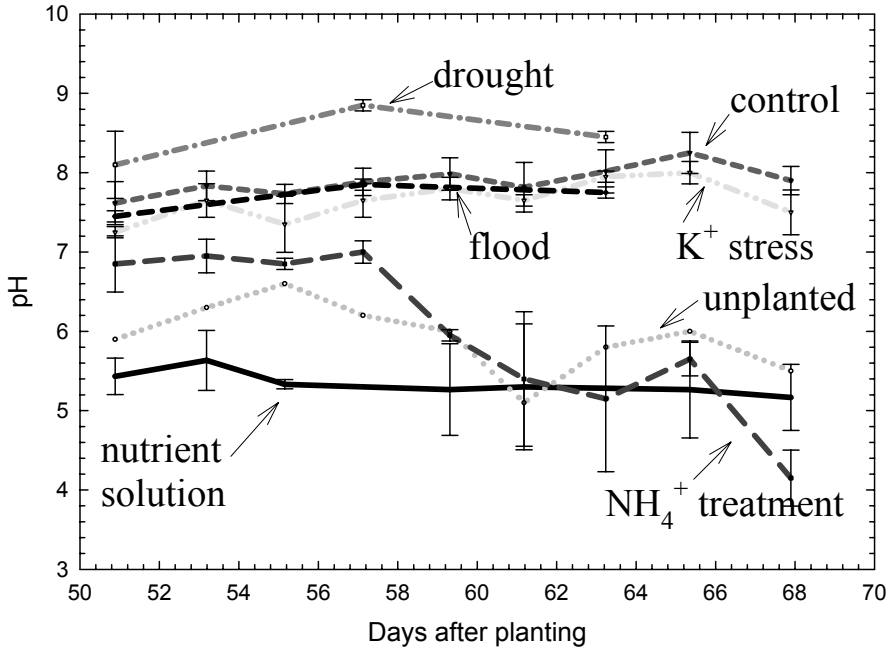

Fig. 8. pH of leachate from all treatments. Nutrient solution used for watering is also shown. Cation exchange resulted in the low pH of the NH_4^+ treatment.

Plants were watered every 2-3 days so that no more than 35 of the 49 ml field capacity volume were transpired. This maintained a minimum gravimetric water content of 0.047. Transpiration was determined by subtracting 25% of the water lost by the unplanted column from the water lost by each column (Figure 9); the water lost due to evaporation from the sand was expected to be greater in the unplanted column due to a higher vapor pressure deficit between the sand and the air, whereas the air in the planted columns was more humid due to transpiration. The 25% unplanted water loss was not subtracted from plants in the drought treatment, in which the air contained a low relative

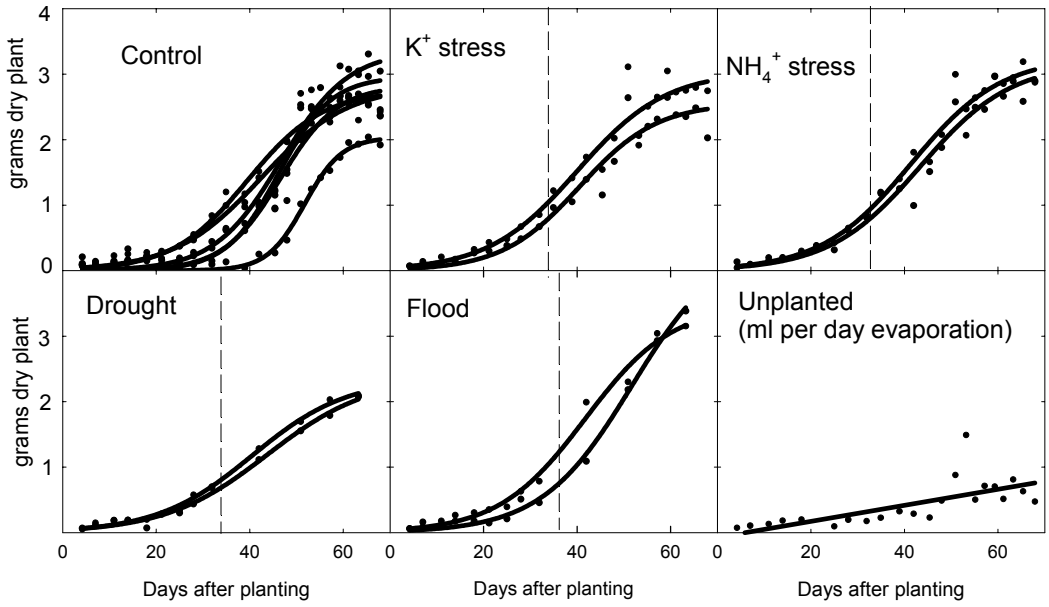

Fig. 9. Plant mass over the study estimated from transpiration rate. Each curve represents one plant. Dashed lines indicate initiation of treatments. Transpiration curves are identical but on a scale of 0 to 20 ml day^{-1}. Evaporation was subtracted from all treatments.

humidity, or from the first 15 days of plant 35, after which a blocked air filter was replaced. Average shoot dry mass ranged from 1.50 g from the drought treatment to 2.50 g in the NH_4^+ treatment (Table 7). The average shoot mass of the 4 replanted controls was 1.42 ± 0.25 grams. Root mass was not determined since a GC-MS analysis was performed on direct extract of the roots, requiring the roots to remain sterile, and because of the difficulty of completely separating roots and sand. A preliminary axenic trial in which the plants eventually became contaminated showed between 14% roots on average in the flood treatment and 35% in the NH_4^+ treatment (see Appendix D). These root percentages were not significantly different (P = 0.211) and a root mass of 25% total plant mass was assumed for all calculations.

Plant mass is highly correlated with transpiration rate ($r^2 = 0.96$, see Appendix E). The transpiration rate at the end of the study and final dry plant mass were used to calculate a proportion to convert transpiration rates to approximate plant mass throughout the study. Fitting a sigmoidal curve to the data points allowed for

Table 7. Shoot dry mass at harvest for each treatment. Plants were not identical sizes when treatments were started.

Treatment	n	Average (g)	Standard Deviation
Control without small plants	2	2.51	± 0.28
NH_4^+ stress	2	2.50	± 0.06
K^+ stress	2	2.00	± 0.19
Drought	2	1.50	± 0.17
Flood	2	2.42	± 0.81

approximate plant mass to be determined at any point in time for each plant. Since transpiration data wasn't available for the flood treatment, points were estimated based on final plant size and initial transpiration before treatments.

Plant digest and ICP was performed on plant shoots to determine mineral nutrient content (Table 8). The potassium content of the plants in the K^+ stress treatment was about 50% of K^+ content of the controls. All other nutrients were present in normal amounts.

Total Organic Carbon

TOC in the leachates was monitored throughout the trial (Figure 10). Amounts of TOC released were increased compared to the control in the K^+ stress, drought, and flooding treatments, but the high NH_4^+ TOC decreased compared to the control. When

Table 8. Nutrient content of all treatments (n = 2) using ICP. Nutrients are present in normal amounts, except low K^+ levels in the K^+ stress treatment. See Appendix D for standard deviations.

	P	K	Ca	Mg	S	Fe	B	Zn	Mn	Cu
	----------%----------					----------mg/kg----------				
Control	0.30	3.19	0.22	0.09	0.17	53.70	47.03	48.80	44.37	9.09
High NH_4^+	0.34	3.07	0.22	0.09	0.34	41.70	52.35	73.35	72.10	18.00
K^+ stress	0.25	1.26	0.48	0.15	0.13	193.25	51.15	39.95	35.10	8.78
Drought	0.33	3.45	0.24	0.12	0.21	47.40	81.80	50.85	53.65	12.22
Flood	0.23	2.17	0.23	0.07	0.13	52.20	35.60	36.60	37.55	9.59

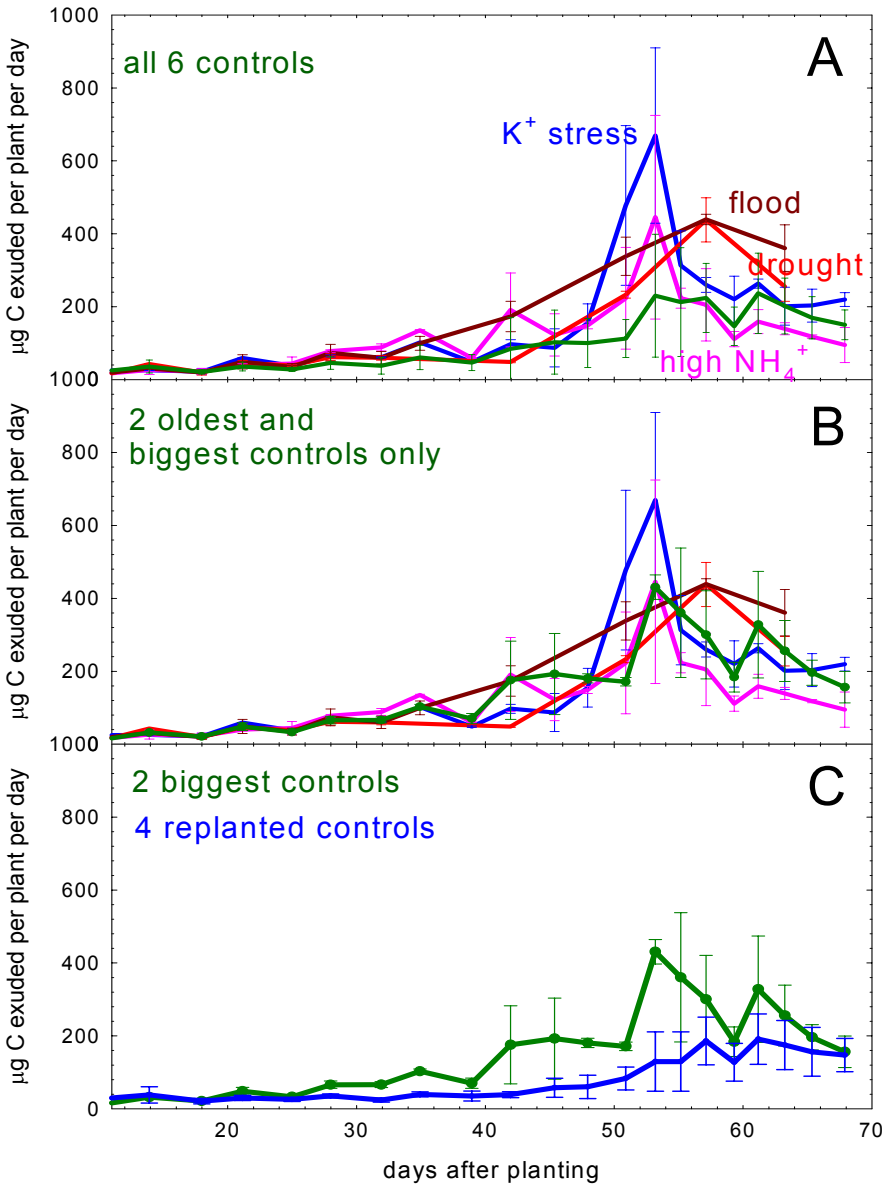

Fig. 10. Exudation rates per plant per day over the duration of the study. Four of the six control plants were smaller because they were replanted. A) Treatments compared to average of all 6 control plants. B) Treatments compared to the 2 oldest control plants only. C) Comparison of the biggest controls are the smaller replanted controls.

analyzed over four time intervals during the treatments (days 35-42, 45-51, 53-57, and 59-63), the flood plants released significantly greater amounts of TOC than the control from days 45-51. Shoot dry mass was measured and used to express exudates as cumulative TOC exuded per gram dry shoot (Table 9). Exudation rates observed here were lower than those reported in the literature (Table 10).

Only the amount of TOC released per gram dry plant by the drought treatment was significantly higher than the control and high NH_4^+ based on a one-way analysis of variance (ANOVA) and Tukey test ($P = 0.013$), but the K^+ stress and flood treatments also exuded higher amounts of TOC than the control. The high NH_4^+ treatment on an average per gram dry plant basis released less TOC then the control plants. The amount

Table 9. Cumulative TOC (since 11 days after planting) collected as exudate on a per gram dry plant basis (estimating 25% roots). Two control plants and all treatments were grown from the start of the study, and four additional control plants were planted on day 10 ('replanted controls').

	mg C exuded per g dry plant ± std. Average	Percent of control
Control: all 6	2.5 ± 0.41	100
Controls: 2 biggest	2.7 ± 0.35	108
Controls: 4 replanted	2.4 ± 0.46	96
High NH_4^+	2.2 ± 0.10	90
K^+ stress	3.6 ± 0.56	144
drought	4.2 ± 0.50	171
flood	3.6 ± 0.90	145

Table 10. Seven ways to express exudation, based on measured shoot dry mass and assuming 25% roots based on preliminary trials (see Appendix C), and that C accounts for 40% of exudate mass, based on the assumption that exudate composition is CH2O. Data are from the 6 control plants.

Unit	Average value	% of Published value
g C in exudate per total plant dry mass x 100	0.25 ± 0.04	
g exudate per g dry mass x 100	0.62 ± 0.1	36[1]
μg C per g dry root mass * day	185 ± 31	
mg C per plant * day	0.10 ± .04	50[2]
mg exudate per g dry root * day	0.57 ± 0.09	8[3]
mg exudate per g dry plant	6.44 ± 1.1	
mg exudate per g dry root	33 ± 5.0	35[4]

1. compared to Barber and Gunn, 1974.
2. compared to Groleau-Renaud et al., 1998.
3. compared to Kraffczyk et al., 1984.
4. compared to Schönwitz and Ziegler, 1982.

of TOC in the exudates was also highest in the drought and K^+ stress treatments when expressed as μg TOC per gram dry plant per day, calculated by estimating plant mass from transpiration rates (Figure 11).

The percent soluble TOC in the leachates ranged from 75 to 100%. A comparison of the percent soluble TOC over time revealed no treatment effect (Figure 12). TOC remaining on the sand at the end of the study was minimal (Table 11). The average plant-derived TOC distribution in the rhizosphere was: rhizosphere sand 17%; bulk sand 9%; soluble in leachate 69%; insoluble in leachate 11%.

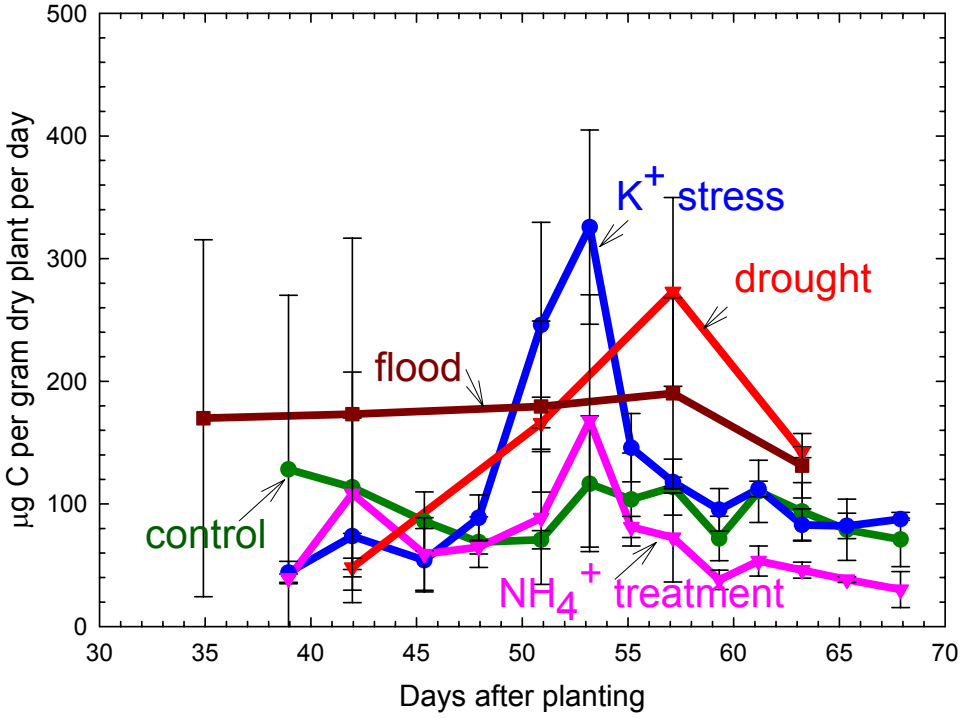

Fig. 11. TOC exuded per gram dry plant mass per day. These values were calculated with the TOC release rates from Fig. 10 divided by plant mass estimated from transpiration in Fig. 9. A graph for µg C per gram dry root per day would be identical but on a scale of 0 to 2500.

Relative growth rate (RGR: grams new plant per gram plant per day) was determined before and after the application of treatments using final plant mass, mass at day 35, and initial (seed) mass (see Appendix D). The RGRs between treatments were not significantly different before ($P = 0.414$) or after ($P = 0.113$) treatments were applied, although drought plants were lowest during the treatment period. Dry plant mass at day 35 was determined by calculating the area under a quadratic curve fitted to transpiration

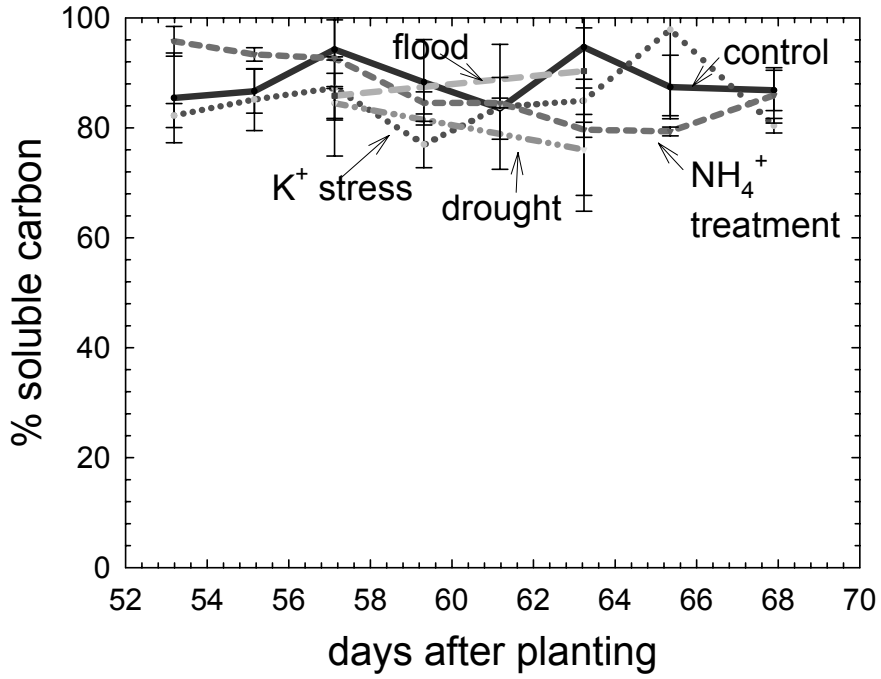

Fig. 12. Percent soluble TOC in leachate samples measured by comparing filtered (0.45 μm) and unfiltered samples of leachate. The majority of TOC was soluble, and no difference between treatments was observed.

Table 11. Percentages of plant-derived TOC remaining in sand. The majority of TOC was in the leachate.

	Bulk sand	Rhizosphere sand
Plant 31	7.84	18.4
Plant 32	2.83	24.8
Plant 33	20.4	17.5
Plant 34	6.63	8.07
Plant 35	5.34	19.5

data of each plant up to day 35, which was a better estimate for initial transpiration before the treatments than the sigmoidal curve, and water use requirement (WUR: ml transpired per gram dry plant, see Appendix D). WUR was determined for each plant over the entire study by dividing cumulative transpiration by the calculated total plant mass and ranged from 148 ml/g in the drought treatment to 183 ml/g in the K^+ stress.

The K^+ stress treatment had the highest amounts of TOC exuded per gram new plant on Day 51 (P=0.047), although this was not isolated by Dunn's Method of all pairwise multiple comparisons (Figure 13). On Day 63 the drought treatment

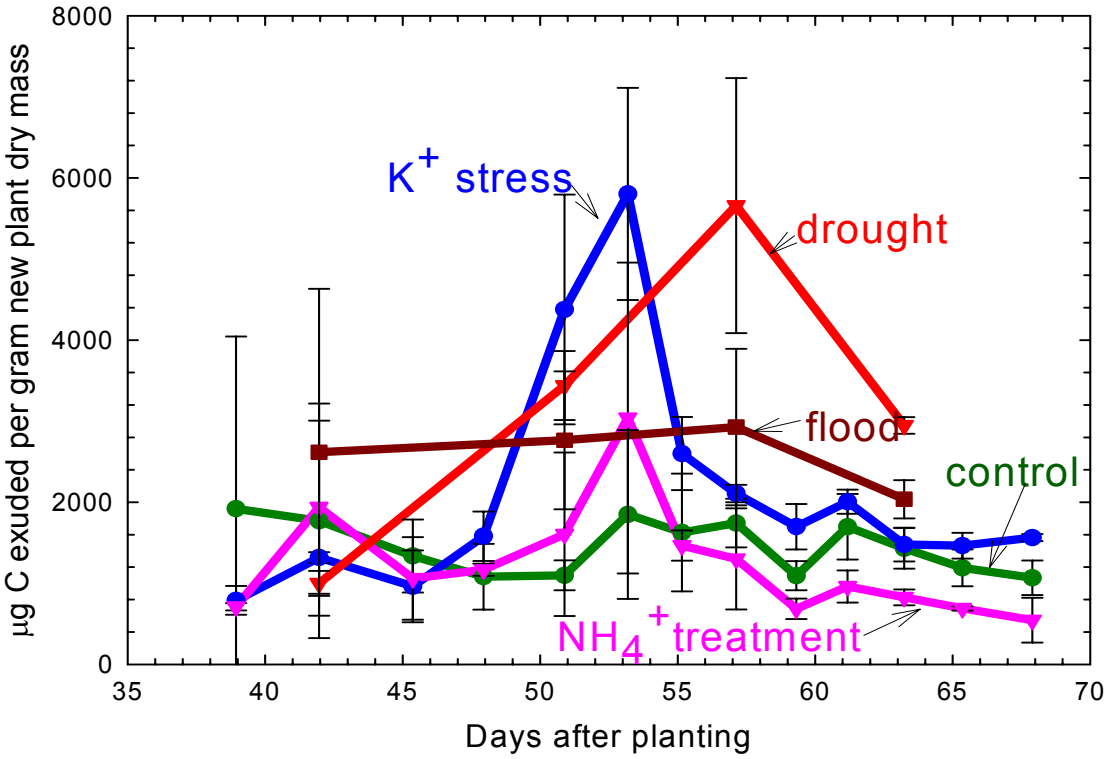

Fig. 13. TOC in exudates per gram new dry plant. New plant dry mass was estimated from transpiration rates and the calculated relative growth rates.

exuded significantly more TOC per gram new plant than the K^+ stress, NH_4^+, and control treatments and the flood treatment exuded significantly more TOC than the control and NH_4^+ treatments as defined by a Tukey Test (p <0.001).

GC-MS

The dicarboxylic acid concentrations in the leachates of preliminary trials was very dilute (less than 1 ppm). The methyl esters of oxalic acid and succinic acid were detected in some samples while those of the other acids were below the detection limit. Due to the low concentrations of dicarboxylic acid in the leachates, root tissue and sand from the rhizosphere were examined. As expected, more acids were detected in the root extracts (Table 12) than in the leachate samples. The cumulative amounts (mg/kg 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 minimal, ranging from 0 to ~20% of concentrations in roots, with the highest sand concentrations in the flooded treatment.

Detection limits of the dissolved acids was about 0.4 mg/L for the leachates using 5 ml samples, about 0.6 mg/kg for the sand using about 5 grams, and about 5 mg/kg of root using about 0.5-gram samples.

Table 12. Derivatized compounds in root samples at harvest(mg/kg) detected using GC-MS.

	control		K^+ stress		NH_4^+		drought		flood	
Compound	ave	s.d.	ave	s.d.	ave	s.d.	ave	s.d.	ave	s.d.
methyl 2-methyl-propanoate*	26	14	8	11	9	13	48	8	9	1
3,3-dimethyl-2-butanone*	30	18	14	6	16	8	55	2	11	1
methyl 2-methyl-butanoate*	24	11	10	14	9	13	55	6	12	4
dimethyl oxalate	31	13	8	1	14	0	65	13	13	7
methyl caproate*	6	10	13	6	5	6	0	0	11	1
dimethyl malonate	29	11	16	6	20	4	55	0	14	3
dimethyl fumarate	128	78	80	71	46	52	625	446	100	64
dimethyl succinate	71	45	23	11	19	14	209	71	50	17
dimethyl malate*	177	96	62	26	60	58	617	301	125	111

*estimates only, no standard

The esterification procedure worked poorly for hydroxy-diacids since it was not designed for these compounds. It is possible that other hydroxyacids than those detected were present in the exudates. Organic acids were corrected for recovery rates, which were: oxalic acid 22% ± 3% n=10, malonic acid 50% ± 6% n=10, fumaric acid 51% ± 6% n=10, succinic acid 56 % ± 4% n=10 malic acid 15% ± 7 n=8. Similar to the rates of TOC release, the rates of organic acid release in the exudates also peaked before the end of the study (Figure 14). 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 was represented in highest amounts in all treatments, and the drought treatment contained the highest percentages of organic acids (Figure 15).

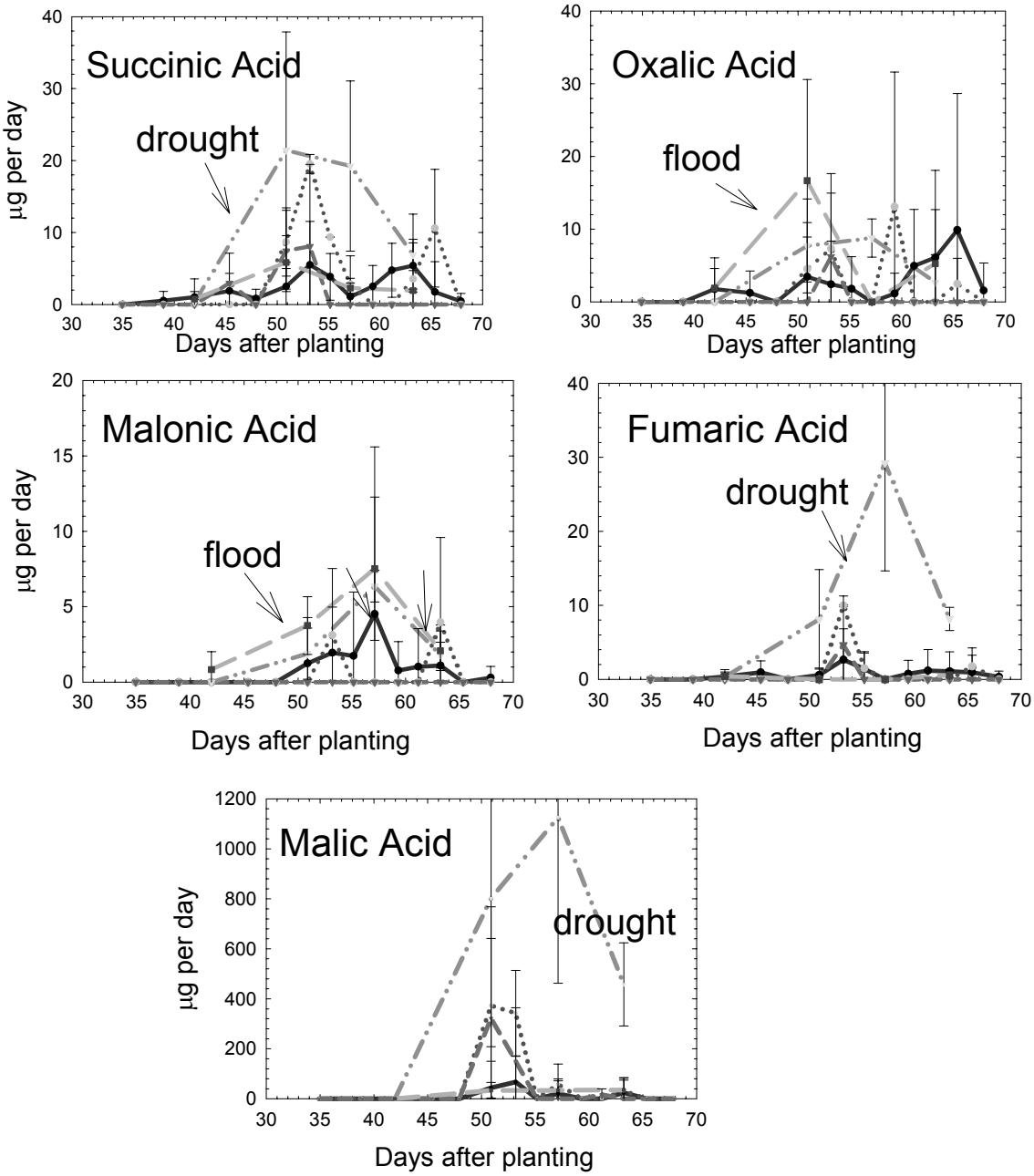

Fig. 14. Release rates of four organic acids detected in leachate samples. All values are corrected for recovery eficiencies. Note the different scales of the y-axes.

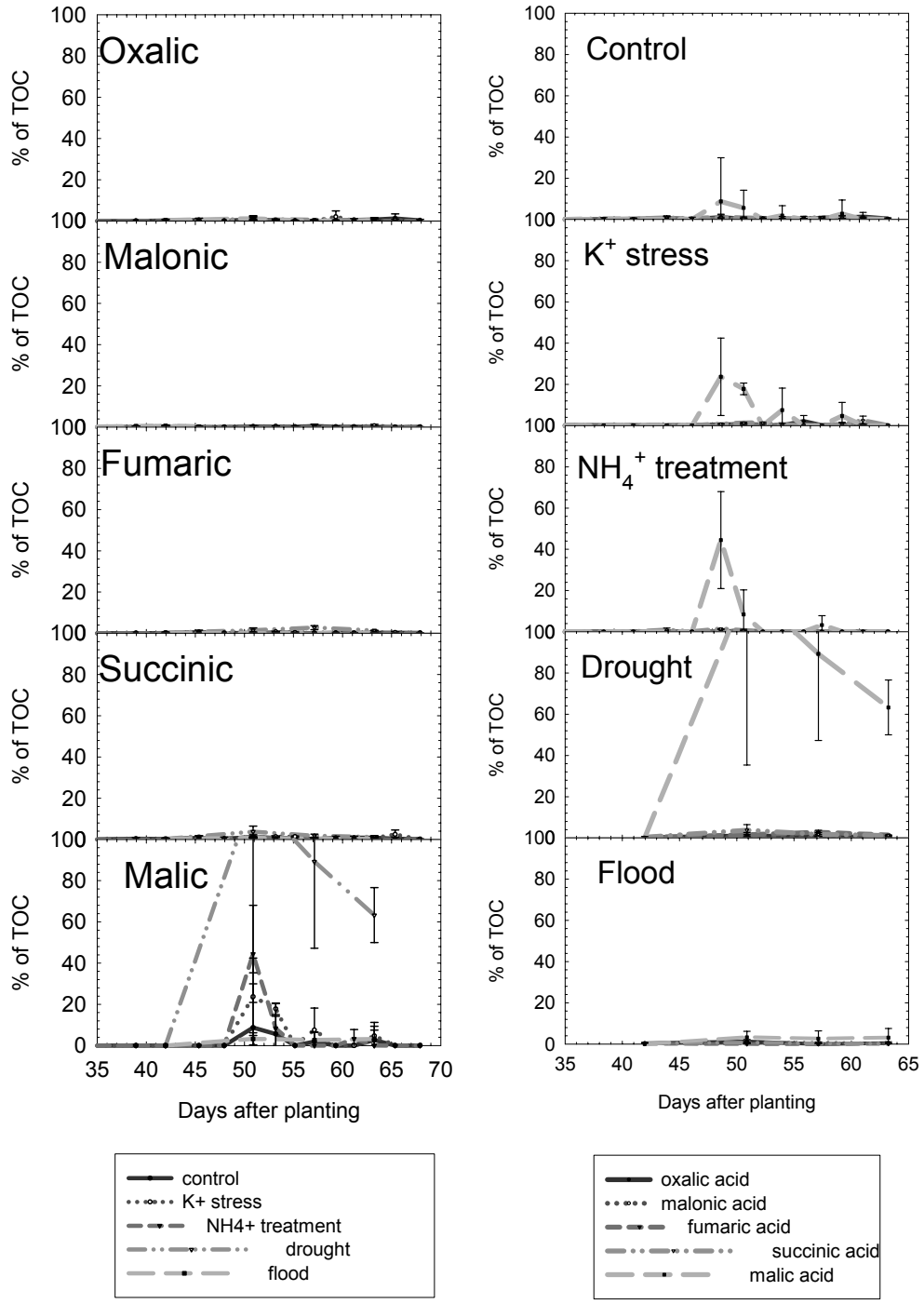

Fig. 15. Organic acids as a percent of total organic TOC, shown by acid and by treatment.

Organic acids ranged on average from < 1% of the TOC for malonic acid, <2% oxalic acid, <4% fumaric and succinic acids, and up to 100% malic acid. Malic acid showed the largest peaks in the chromatograms (Figure 16), which has two carboxyl groups and one hydroxyl group (Figure 17). 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. Compared to the concentrations in the root extracts, the cumulative amounts organic acid collected in the exudate were significantly correlated for fumaric and succinic acid, but not for malonic or oxalic acid (Figure 18).

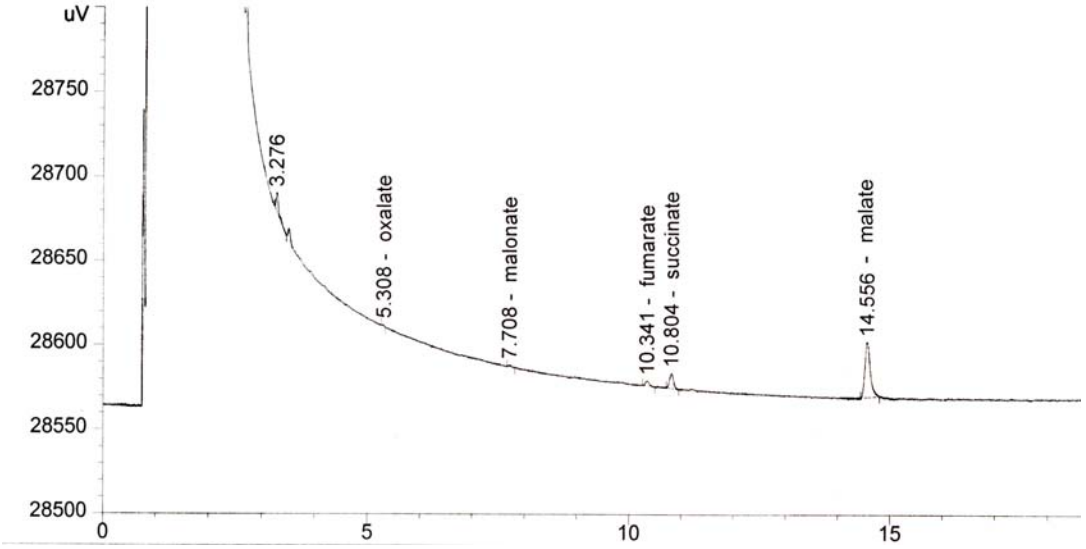

Fig. 16. GC chromatogram from exudate analysis. The sample shown is from Plant 28 (drought), day 51. The largest peak was for malic acid.

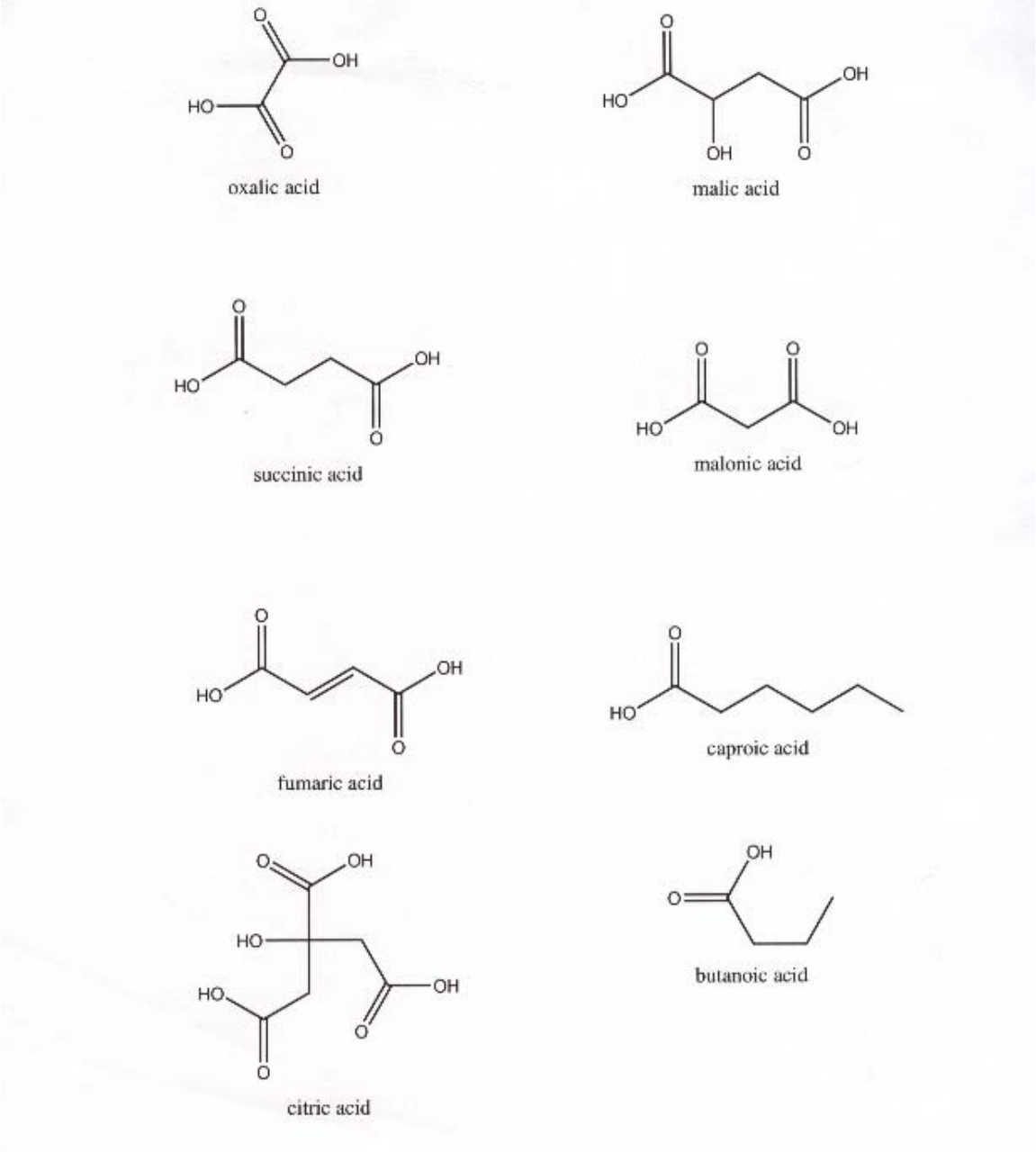

Fig. 17. Structures of organic acids in root exudates. All acids shown were detected in the root exudates of this study, except citric acid.

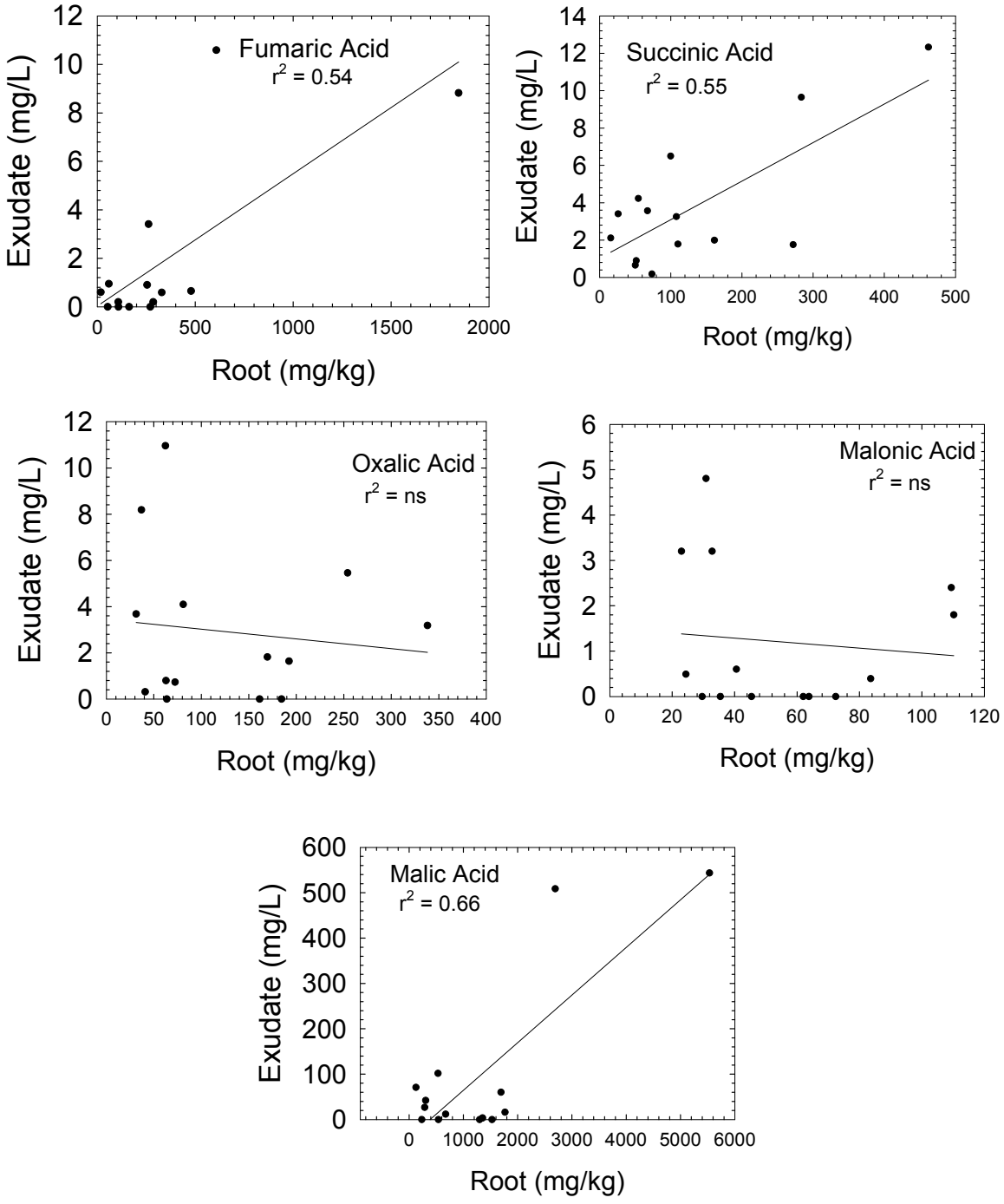

Fig. 18. Comparison of organic acids concentrations of roots with cumulative amounts of organic acid detected from the leachate (exudate).

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 is one of the major factors in determining root exudate composition.

Plants in the high NH_4^+ treatment decreased rhizosphere pH but were similar to the controls, as evidenced by the high RGR and low amounts of root exudate. Therefore the high NH_4^+ treatment can be considered a second control. The reduced plant size (60-95% of the controls) in all stressed plants shows that the treatments affected plant growth.

Whipps and Lynch (1983) hypothesized that drought stress reduces growth more than it impairs photosynthesis. This may apply if growth is defined as root and shoot expansion, but our results indicate that growth, when defined as gain in dry mass, was reduced equally compared to photosynthesis: transpiration rates of the drought-stressed plants were 22 % less than the control, indicating proportionally reduced photosynthesis rates, and shoot mass was decreased 22% on average compared to the controls. The average RGR was similarly reduced by 26% compared to the control. The lower average WUR of the drought plants indicates the ability to continue photosynthesis while adapting to drier conditions. Whipps and Lynch concluded that the discrepancy in impairment between growth and photosynthesis may have resulted in excess carbon within the plant that is subsequently released as root exudates. Since it would be

extremely inefficient for a stressed plant to dispose of fixed carbon through exudation, increased TOC released from the roots of drought-stressed plants is more likely due to cell damage induced by the treatment.

Total Organic Carbon

Total organic carbon (TOC), expressed as μg TOC released per day, peaked at about day 55 of the 70-day trial. Since this peak was seen in 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 growth rate. The decrease in TOC could be a response due to volume limitation in the growth containers. A decrease in growth rate that results in less TOC released as exudate could also be a response to the imposed nutrient treatments, but is probably not the case in this study since the ICP analysis revealed adequate plant nutrition aside from the imposed stresses. Other possibilities of low-level stress include drought between waterings and salt buildup, although plants were continuously monitored for transpiration and watered frequently enough to maintain a volumetric water content above 28% of field capacity, and a preliminary monitoring of the EC of leachates indicated that there was no significant salt buildup (average nutrient solution in EC = 1.14 ± 0.3 mS cm^{-1}, average leachate EC = 1.43 ± 0.4 mS cm^{-1}, see Appendix C). Plant age and stage in the life cycle could influence growth rate and root exudation patterns. The peaking trend was least evident in the smallest controls, but was observed in the largest controls. Therefore the restricting size of the column was most likely the cause of reduced growth and exudation rate.

Plant roots in the K^+ stress, drought, and flooding treatments showed increased trends in the amounts of TOC per gram plant compared to the controls and the NH_4^+ treatment. This was expected for the K^+ stress conditions. Plant roots release compounds to sequester nutrients (Marschner, 1995), and decreasing the availability of those nutrients (for example by modifying the nutrient solution or decreasing the amount of nutrient solution added as in the K^+ stress and drought treatments) can increase the production and release of nutrient sequestering compounds (Hoffland et al., 1989; Ström et al., 1994; Ström, 1997). The potassium stress induced in this study may have increased the release of K^+-sequestering compounds.

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 due to decreased water availability in the drought treatment could explain the increased amounts of TOC released into the sand.

Flooding the columns decreased oxygen availability to the roots. Without adequate oxygen, roots would be no longer able to perform normal metabolic functions. The increased TOC trends in the flooding treatment may have been caused by root death, which was detected in the leachate as increased exudate TOC. The low root: shoot ratios observed in the flooding treatment during preliminary trials (controls = 27 ± 9% roots, flood = 13 ± 4% roots, see Appendix C) supports this. The ponding of water and subsequent soaking of lower parts of the shoots as well as the roots may also have contributed to TOC in the leachate by increasing contact time and solubility of plant-derived TOC in the flooding treatment.

The slightly lower amounts of TOC released by the NH_4^+ treatment compared to the control may be due to the decreased need of these plants to produce NO_3^--reducing compounds (see discussion on GC-MS data).

The drought and flooding treatments, which had the least frequent replacement of exudate-containing root-zone solution, show an increased amount of TOC released by roots. This is in contrast to the conclusions of Jones and Darrah (1993), who reported that re-absorption occurs at the highest levels when the exudates are exposed to the roots for longer periods of time. Re-absorption is likely occurring in all treatments, but it is possible that some TOC counted as root exudates in this study was not re-absorbable, like border cells or remains of root die-off, and that the re-absorption concluded by Jones and Darrah was of compounds that are more readily absorbed by the roots. Exudates would not be reabsorbed in damaged tissue, which may explain lower re-absorption in the drought and flooding treatments.

Site of Microbial Contamination

TOC released as exudate continued to show exponential increases despite the presence of microbes in the leachate of contaminated columns. A spatial separation between the microbes and the site of exudate production is probable. Microbes were observed on the roots protruding from the system, but not within the column. Although microbes were detected in the leachate of these columns, the plants were included in the results as non-contaminated plants since all leachate samples were analyzed immediately

after collection and would be minimally affected by the brief contact with microbes before analysis.

Expression of Results

The unit used to describe exudate production in the literature varies among studies. Some early studies reported results in amount of exudate per plant. Radiolabel studies commonly express exudates as percent carbon fixed by the plant. Other studies expressed exudates per unit mass, e.g. per gram plant or per gram root.

The lower amounts of exudate in this study compared to the literature may be due to species difference, age (plants in the literature were all younger than those in our study), or growth conditions (most plants in the literature were grown hydroponically). Average exudation rates in this study would be higher if the study was terminated earlier since exudation rates decreased with time. This study was continued as long as possible due to the success with keeping the plants free of microbes. It should also be noted that it has been suggested that roots of non-sterile plants release more carbon than those of sterile plants (Barber and Lynch, 1977; Prikryl and Vancura, 1980). Studies in which complete axenic conditions were not accomplished could overestimate the quantity of root exudates released by plants grown under axenic conditions.

For purposes of scaling these results to the field to be applied to phytoremediation, a more specific mode of expressing results than “g C per plant” or “g C per g root” is necessary since growth conditions are so different between the axenic plant culture tube and the field and because the treatments affected plant mass as well as

exudate release. A more scale-able mode of expressing quantitative exudate results is in micrograms TOC exuded per gram new growth. This was accomplished in this study by calculating plant relative growth rate based on changes in transpiration rate. Plant mass could also be determined throughout the study by destructive harvests. Besides the difference in growth conditions, expressing exudates on a RGR basis is important since the majority of both exudation and new root growth occurs at the root tip.

Combining the RGR data with root:shoot ratios to obtain root growth could allow exudates to be expressed as per gram new root. We chose to express exudates on a whole plant basis since the shoot is equally important as the root in exudation as the source of carbon to be exuded (through photosynthesis). A treatment that reduces root growth is likely to reduce shoot growth as well. Plants were not equal sizes when treatments were started despite being planted on the same day. The difference in time to establishment for each plant is another reason why exudates are more accurately compared based on relative growth rate.

The unbalanced design (6 control plants, 2 plants of each treatment) and the low number of reps, which made the standard deviation of the average exudate amounts equal to the range, limited the statistically significant conclusions made in this study. The conclusions made on these results were from the most conservative statistical analyses available.

Soluble Total Organic Carbon

The points above 100% in Figure 12 represent trace variability due to small

amounts of TOC added by filter and its magnification in dilution calculations. Since most of the TOC released by the roots was in the form of soluble TOC in the leachate, the majority of root exudates in this study was in the form of compounds released directly by the root or from damaged cells, not whole entire cells from which the cellulose cell walls would contribute high amounts of insoluble TOC. The 17% of TOC found in the rhizosphere (sand immediately adjacent to the root) is likely an overestimate due to small root pieces that were contaminating sources of TOC in the sand samples.

Since preliminary results indicate a slightly low percentage of roots in the flooding treatment, root die-off may have contributed to the increases in TOC released by the flood roots compared to the control. The percent soluble TOC was not measured during the period when the flood treatment had the highest amount of TOC released per plant. A lower percent soluble TOC would be expected during this period from the increased leaching of cells walls due to root die-off. Microscopic observations at the end of the study revealed few root hairs on plants in the flood treatment. Loss of root hairs upon induction of flooding could also have contributed to increased release of TOC by this treatment.

Organic Acid Composition

Several of the organic acids identified from the root extractions play important roles in plant function. Malate is used as a counter-ion for cations 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 another organic acid used for charge compensation in nitrate reduction. Oxalic acid is also used in the precipitation of excess solutes within a plant as calcium oxalate.

The drought treatment was the only treatment that varied significantly in concentrations of compounds detected from the GC-MS, indicating that there was an increased need for production of organic acids in the drought-stressed plants. This may be due to an excess of ions within the plant as a result of roots dying off and the need for organic acids to precipitate with those ions to maintain adequate soluble concentrations within the plant. A very low water potential of the root cells induced by the drought treatment in order to survive the low water potential of the root zone could have resulted in cell lysis upon watering and subsequent detection of cell contents as exudates.

Other non-significant differences in concentrations detected with the GC-MS were decreased levels in most organic acids compared to the control in treatments other than drought and flood stress. Despite an increase in TOC in the exudates of the K^+-stressed plants, no increase in organic acids was observed, indicating that potential K^+ sequestering compounds are not among the organic acids detected by the GC-MS. Since some of the compounds detected are important for nitrate reduction, these compounds were, as observed, expected to decrease in the NH_4^+ treatment due to decreased need to reduce nitrate.

There were several difficulties associated with the organic acid analysis. Acids were present in low concentrations in the leachate, and sample volumes were too low to be concentrated, for example by lyophilization. The most difficulty was in derivatizing

the acids to methyl esters for analysis, especially acids that contain a hydroxy group in their structure, like malic acid. The presence of the hydroxy group on a neighboring carbon atom changes the reactivity of the carboxyl group making the esterfication reaction less likely to go to completion and leaving of some acid in the sample unreacted. Low concentrations coupled with low derivatization rates may have reduced the number of acids detected if they were present below detection limits. For example, citric acid has been reported as a common acid found in root exudates and serves in phosphate mobilization as well as chelating Fe and Al in the rhizosphere, but was not detected in this study. Citric acid is also a hydroxy acid, thus its decreased ability to be esterified and presence in low concentrations may have contributed to it not being detected in this study. The absence of citric acid may also be due to adequate Fe supplied to the plants, thus reducing the need to exude Fe-sequestering compounds. Kraffczyk et al. (1984) also saw an increase in exudation of organic acids under K^+ stress. A large reduction in plant mass with K^+ stress was seen in the Kraffczyk study that was not seen in our study. This reduction may be associated with the different growth medium (solution culture with no mechanical impedance) and the fact that the solution was not aerated throughout the 23-28 day studies, which even though tested as aerobic throughout the study may have contained anaerobic microsites that reduced growth and nutrient uptake more strongly under K^+ stress.

The percentages of organic acids detected out of the total carbon released by the roots indicates 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 reached the highest percent of TOC in the drought and flood treatments, with peaks also in the K^+ stress plants. The peaking trend in the proportion of organic acids in the exudates indicates the changing composition of the exudates with time. This suggests an exudate response to age or increased stress levels.

Malic acid was present in higher concentrations than any other acid detected in the exudates. Malic acid has also been observed as 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 4-6 day old wheat grown in solution can reach up to 82% of organic acids in Al-tolerant cultivars exposed to Al (Delhaize et al., 1993). Phosphorous deficiency can increase exudation of malic acid in *Brassica napus* L., which helps the plants solublize rock phosphate (Hoffland et al., 1992). Based on ICP analyses of shoots and nutrient solution, neither P-deficiency nor exposure to Al were factors in this study (see Appendix D).

Aside from the organic acids identified here, the remainder of the exudate composition is unknown. Other compounds in the exudates, such as sugars and amino acids, may have different properties but could serve as a substrate for microbial co-metabolism of soil contaminants and be useful for phytoremediation.

The analytical methods used in this study were the same as the methods used by Kloss et al. (1984): the form of the acids was changed (derivatized) for analysis, with capillary gas chromatography used to obtain quantitative information about the organic acids and gas chromatography-mass spectrometry used to identify them.

Other studies have also used gas chromatography for exudate analysis, which is reportedly more sensitivity than HPLC or ion chromatography, in which it is more difficult to separate the different acids (Szmigielska et al., 1995). A more detailed method was used by (Fan et al., 1997) with NMR and GC-MS for de novo identification of exudate components, which is currently the best method for characterizing root exudates that include unknown compounds.

Correlation of Organic Acid Concentrations in Roots and Exudates

The concentrations of organic acids found in living root tissue correlates somewhat with concentration in the leachate. The existence of some correlation implies that with refined detection methods it may be possible to know the concentration of certain exudate components by extracting from the root, which is a less difficult process than analyzing leachates due to the higher concentrations found in the root. Organic acids detected in the rhizosphere would 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 structure of some root exudates, such as phytosiderophores and exoenzymes, is complex and energetically expensive for the plant to manufacture. These compounds are produced for purposes outside the plant, such as 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 is therefore unrepresentative of the plant status earlier in the study. Furthermore, not all root cell constituents are actively exuded; there may be many compounds in the root that are not detected in the exudates and therefore have no correlation to the exudate. Conversely, since our quantification of exudates was based on compounds that were identified in the root due to the low detectability of dilute compounds in the exudates, some compounds present only in the exudates may be inadvertently omitted if they were produced more as exudates than stored in the root.

This low correlation also emphasizes the importance of axenic plant culture for root exudates studies. Simpler methods such as analysis of non-sterile root extracts or C^{14} labeling reveal only certain aspects of root exudation. Axenic plant culture studies simultaneously portray the total amounts of exudates released, patterns of exudation with time, and patterns of specific compounds with time and change in plant status. With refinement of the qualitative analysis methods, the study of root exudates through axenic plant culture can continue to increase in importance for providing an unbiased view of basic plant function.

Organic Acid Function in Phytoremediation

Besides serving as a substrate for microbial metabolism of organic contaminants, organic acids in root exudates may chelate and thus increase plant uptake of inorganic contaminants. The chelating power of an organic acid depends on the number of

carboxyl groups and other charged groups as well as their position and the size of the molecule in relation to the contaminant (Figure 17).

Of all common organic acids detected in root exudates, citric and malic acids have strong chelating abilities of inorganic contaminants. Citric acid, which contains three carboxyl groups and one hydroxyl group, was not detected in this study but has been observed to increase the solubility of Cd, Pb, U (Wu et al., 2003; Chen et al., 2003; Shahandeh and Hossner, 2002).

Wu et al. (2003) found that malic acid, which contains two carboxyl groups and one hydroxyl group, has shown higher chelating abilities than citric acid for Zn and Cd. Malic acid also appears to have higher levels of chelation of Al than succinic acid (Delhaize et al., 1993). Oxalic acid was not observed to increase the solubility of Cu, Zn, Cd, or Pb (Wu et al., 2003), but Shahandeh and Hossner (2002) reported that oxalic acid can increase plant uptake of U.

Extrapolation to the Field

Exudate values reported here must be scaled to the field. Root exudates in this study are expressed on a per gram new growth basis calculated from relative growth rate. These values can apply to the field where growth rates can differ greatly compared to laboratory conditions.

Plant response to stress in terms of exudate release is not proportionally related to phytoremediation effectiveness. Drought, flooding, and nutrient stress have the potential to reduce soil microbe viability. Organic acid levels were increased with the application

of drought stress, which could increase mobility of organic contaminants. Drought stress could also reduce transpiration and therefore decrease mass flow of exudates to root surfaces.

Continuous vs. Phasic Stress

These responses suggest that phasic application of stress may be useful for phytoremediation. Continuous stress reduces plant growth. Stressing the plants periodically during the life cycle allows a recovery period between stresses and promotes continued growth. Furthermore, phasic stress allows for microbial activity during favorable (non-stress) conditions compared to constant stress. Although sudden rewetting can of soil can decrease bacterial biomass, soil bacteria can recover from drought stress (Kieft et al., 1987), and the diversity of bacterial communities in grass soils is relatively resistant to change with drying-rewetting cycles (Fierer et al., 2003).

Estimating Root Exudates in the Field

Controls released about 1 mg TOC per gram new growth. Assuming crested wheatgrass produces 1 kg dry mass of per m^2 per season, 100 mg TOC per m^2 per season would be released as exudates to the soil.

Drought and K^+-stressed plants reached a peak of about 5.5 mg TOC per gram new growth. Flooding stress achieved half the increase in TOC release of these stresses and is less likely to be successful in the field because of the difficulty of flooding a field. Drought or K^+ stresses could be applied 5 times during the season, with half the season

stress phase, the other time recovery. Therefore a baseline of 50 mg TOC per m^2 per season for the recovery periods plus an additional 137.5 mg based on half the potential exudate release if the stresses could sustain a similar exudation peak as in this study would equal 187.5 mg TOC per m^2 per season when K^+ or drought stress was applied. This value could reach as high as 275 mg TOC per m^2 per season if no lag time between peaks occurred.

Applying Plant Stress in the Field

Although the K^+ and drought stressed plants reached the same rate of exudation, K^+ stress reached this rate faster and plant size was not reduced compared to control. This exudation rate decreased rapidly, but it could potentially be repeated with the application of phasic stresses.

A cesium-contaminated soil, however, may not produce plants with the same exudate response to K^+ stress since Cs can be taken up by the plant in place of K^+ (Zhu and Smolders, 2000; see Appendix G). In this case the plant may not detect a nutrient deficiency or release K^+-sequestering exudates to remedy the deficiency.

Soil remediation efforts in dry climates makes drought stressing the most practical mode manipulating crested wheatgrass plants for phytoremediation. Recovery periods could be induced through irrigation, which would lengthen the current growth period for crested wheatgrass and result in increasing levels of TOC released as root exudates.

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CHAPTER 4

CONCLUSIONS

A simple system was designed for the long-term axenic culture of crested wheatgrass. Microbial contamination occurred in only two out of 15 plants and was isolated to sites outside the root zone. Contamination of these two columns did not affect exudate concentration compared to the other replications, so sterility was essentially maintained at 100% throughout the 70-day study. The system designed here can be applied to many other types of plant studies, including the effects of inoculation or abiotic stresses.

The quantity of root exudates appears to reflect the plant's health status. Drought stress significantly increased TOC exuded per g dry plant by 70 % compared to the control ($p = 0.05$). TOC in the exudate of the high NH_4^+ plants was slightly lower than the control (10% lower mg C per g dry plant) and increased 44% and 45% in the K^+ stress and flood treatments, respectively, although these changes were not statistically significant based on the two replicates used in this study.

The exact source of the exudates is unknown. Although the percent soluble TOC in the exudates was consistently 80-95 % of the total, it is not clear whether the compounds were released directly from the root, from whole cells, or from dying roots. For future research, it would be useful to distinguish among compounds released directly from the root, whole cells released from the root, or dying roots.

Exudate composition was also affected by stress. Organic acid content was highest in the exudates of the drought-stressed plants, and malic acid was present in

highest levels in all treatments. The organic acids accounted for changing percentages of exudate TOC with time. Future research should emphasize identification and quantification of other components of the exudates besides organic acids.

Stress could be used to enhance phytoremediation. The stresses applied in this study could all be applied in the field. Increased levels of TOC in the exudates could support increased levels of microbes, which could increase co-metabolism of organic contaminants. Ammonium as the form of N supplied to the plants can decrease rhizosphere pH, which could solublize inorganic contaminants. Increased levels of organic acids in root exudates during stress can increase the chelation of inorganic soil contaminants and potentially increase plant uptake. Results from this study could be scaled to the field using plant relative growth rate to estimate the contribution of stress-induced root exudates to phytoremediation.

APPENDICES

APPENDIX A. Nutrient Solutions

Nutrient Solution for sand culture of Crested Wheatgrass

		Double Strength		Single Strength	
SALT	**STOCK CONC.**	**ml per 20 L**	**FINAL CONC.**	**ml per 20 L**	**FINAL CONC.**
$Ca(NO_3)_2$	1M	40	2 mM	20	1 mM
$K(NO_3)$	2M	80	8 mM	40	4 mM
KH_2PO_4	0.5M	40	1 mM	20	0.5 mM
$MgSO_4$	1M	20	1 mM	10	0.5 mM
$FeCl_3$	50 mM	8	20 μM	4	10 μM
Fe-HEDTA & $FeCl_3$	100 mM	0	0 μM	0	0 μM
$MnCl_2$	60 mM	4	12 μM	2	6 μM
$ZnCl_2$	20 mM	8	8 μM	4	4 μM
H_3BO_3	40 mM	1	2 μM	0.5	1 μM
$CuCl_2$	20 mM	4	4 μM	2	2 μM
Na_2MoO_4	1 mM	2	1 μM	1	0.5 μM
HNO_3	1M	1	50μM	1	50μM

Nutrient Solution for K^+ stress test of Crested Wheatgrass

		Double Strength		Single Strength	
SALT	**STOCK CONC.**	**ml per 20 L**	**FINAL CONC.**	**ml per 20 L**	**FINAL CONC.**
$Ca(NO_3)_2$	1M	100	5 mM	50	2.5 mM
$K(NO_3)$	2M	0	0 mM	0	0 mM
KH_2PO_4	0.5M	40	1 mM	20	0.5 mM
$MgSO_4$	1M	20	1 mM	10	0.5 mM
$FeCl_3$	50 mM	8	20 μM	4	10 μM
Fe-HEDTA & $FeCl_3$	100 mM	0	0 μM	0	0 μM
$MnCl_2$	60 mM	4	12 μM	2	6 μM
$ZnCl_2$	20 mM	8	8 μM	4	4 μM
H_3BO_3	40 mM	1	2 μM	0.5	1 μM
$CuCl_2$	20 mM	4	4 μM	2	2 μM
Na_2MoO_4	1 mM	2	1 μM	1	0.5 μM
HNO_3	1M	1	50μM	1	50μM

Nutrient Solution for high NH_4^+ treatment of Crested Wheatgrass

		Double Strength		Single Strength	
SALT	**STOCK CONC.**	**ml per 20 L**	**FINAL CONC.**	**ml per 20 L**	**FINAL CONC.**
$Ca(NO_3)_2$	1M	40	2 mM	20	1 mM
$(NH_4)_2SO_4$	1M	40	2 mM	20	1 mM
$K(NO_3)$	2M	80	8 mM	40	4 mM
KH_2PO_4	0.5M	40	1 mM	20	0.5 mM
$MgSO_4$	1M	20	1 mM	10	0.5 mM
$FeCl_3$	50 mM	8	20 μM	4	10 μM
Fe-HEDTA & $FeCl_3$	100 mM	0	0 μM	0	0 μM
$MnCl_2$	60 mM	4	12 μM	2	6 μM
$ZnCl_2$	20 mM	8	8 μM	4	4 μM
H_3BO_3	40 mM	1	2 μM	0.5	1 μM
$CuCl_2$	20 mM	4	4 μM	2	2 μM
Na_2MoO_4	1 mM	2	1 μM	1	0.5 μM
HNO_3	1M	1	50μM	1	50μM

APPENDIX B. Germination Tests

Germination tests were conducted using seeds of crested wheatgrass cv. CD-II in germination boxes. The germination box was a transparent plastic box containing two layers of germination (blotter) paper and was watered with tap water. In the first test, a control was kept on a lab bench at 23^{o}C, while another control and water stress treatments were kept in a 25^{o}C incubator. All treatments reached a germination rate above 80% (Figure 19). The lab bench control showed the highest germination rates. Over and under-watering treatments had similarly low germination rates as compared to the controls. It was determined that seed water status has a much greater effect on germination rate than small (<5^{o}C) temperature changes.

Another germination test was conducted to compare germination rates of seeds that sink and seeds that float in solution. Seeds were soaked in tap water for 30 minutes. About 40% of the seeds sunk in the water and 60% floated. The germination of the seeds that sunk was 90% and germination of the floaters was 65% (Fig. 20). The denser, sinking seeds will be selected and sterilized for the exudate studies.

Seeds were immersed in solutions of 1, 5, and 10% Clorox for 10 and 30 minutes (Figure 20). Increasing Clorox concentration and/ or duration of soak did not significantly decrease germination (Fig. 21). Large amounts of these seeds (>50%) still showed bacterial and fungal contamination so a more aggressive treatment was tested. Approximately 75% of seeds soaked in 20% Clorox for one hour germinated within 6 days (Fig. 22). Sinking and floating seeds had not been separated for this test, suggesting a higher germination rate for this treatment may be possible. Biondini, Klein,

and Redente (1988) found that high Clorox concentrations and long treatment periods were necessary to obtain sterile seedlings of crested wheatgrass.

Seeds were sterilized using Clorox as in the germination tests mentioned above, with the addition of 0.1% Tween 80 in the solution to act as a dispersing agent. No reduction in germination was noticed with the Tween 80. Additionally, deionized water was determined to be as effective as dilute acid or deionized water with Tween 80 as a seed rinse. Following sterilization, seeds were placed on petri plates with dilute (10%) nutrient broth (Difco) and Bacto agar (Difco) in an incubator. Agar concentrations of 1.5 and 2% were tested. The seedlings germinated faster on the 1.5% agar plates due to the higher water potential of these plates. Germination and any contamination were observed

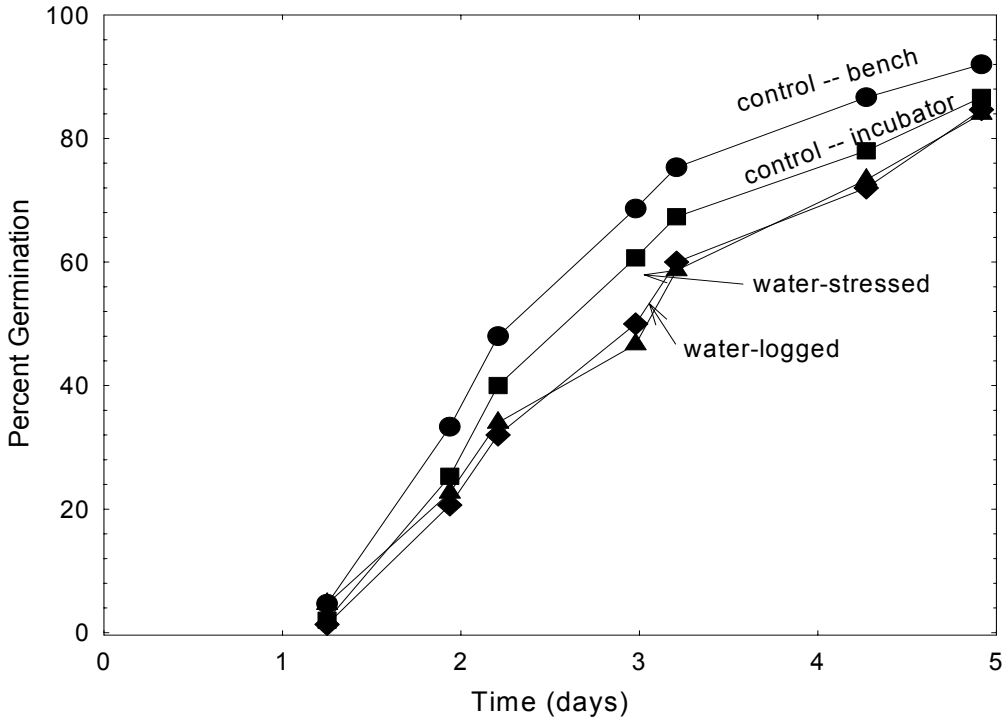

Fig. 19. Germination rates of crested wheatgrass at different water levels.

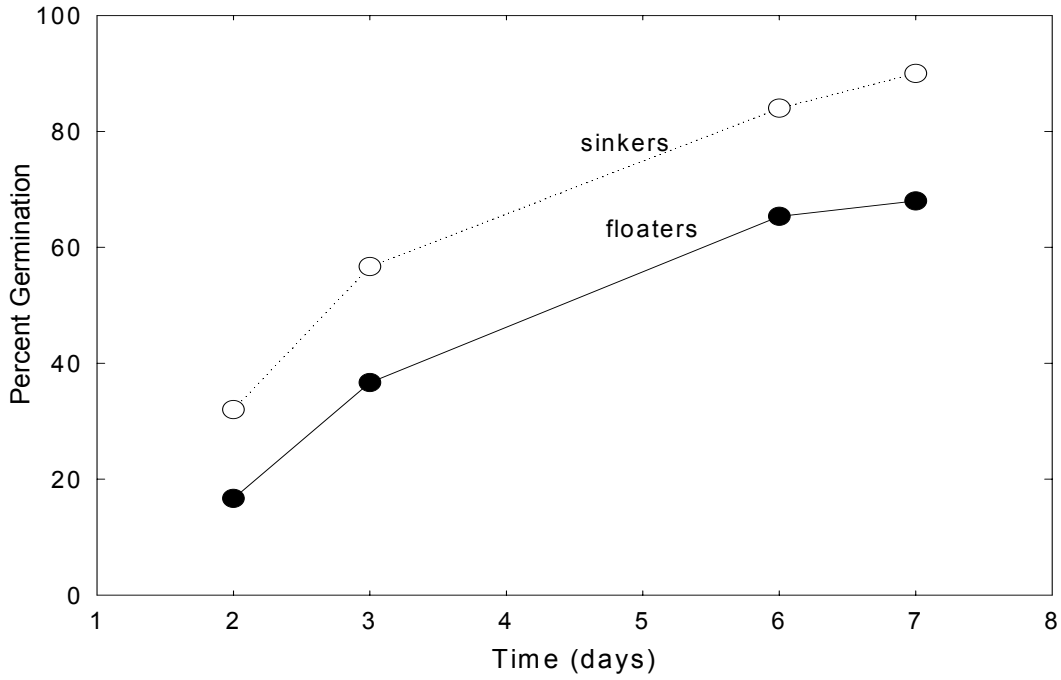

Fig. 20. Germination rates of crested wheatgrass seeds that sink or float in water.

within four days at 26^{o}C. Bacterial or fungal growth on the agar plates indicated residual microbial contamination. The only Clorox treatment in which plates were not significantly contaminated within four days was 20% Clorox for one hour.

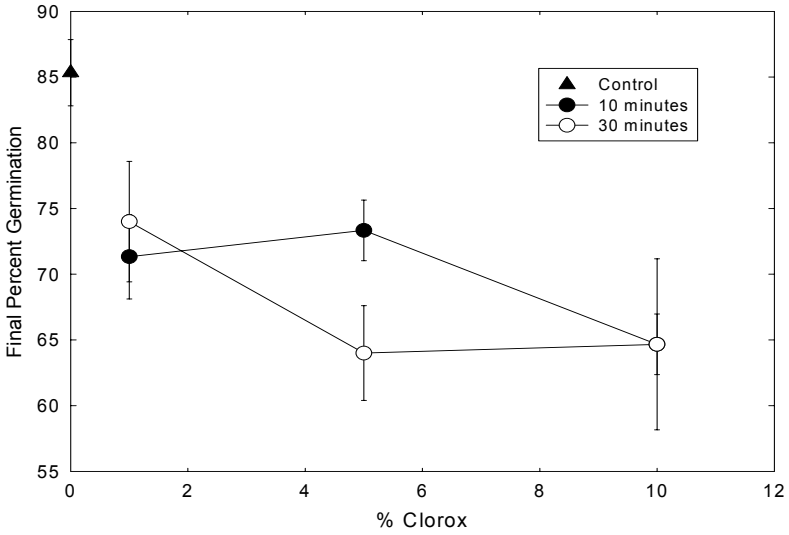

Fig. 21. Germination rates of crested wheatgrass seeds soaked in increasing amounts of Clorox for 10 and 30 minutes.

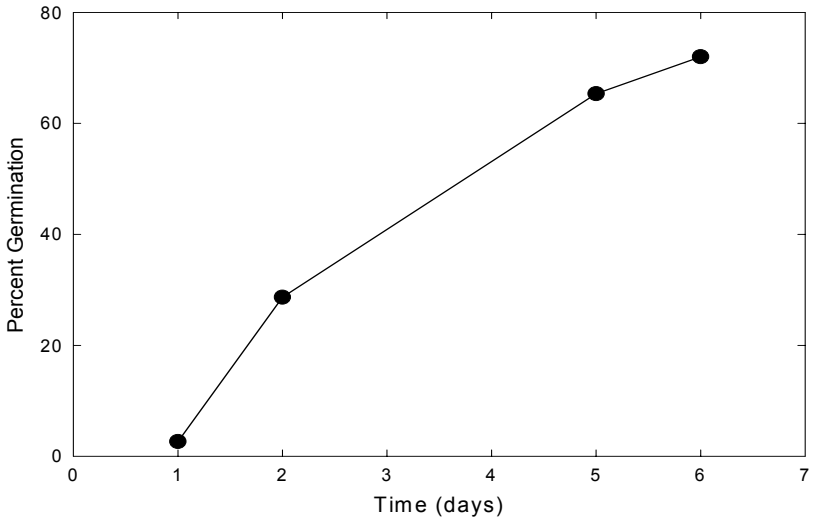

Fig. 22. Germination rates of crested wheatgrass seeds soaked in 20% Clorox and 0.1% Tween 80 for 1 hour.

APPENDIX C. Preliminary Trials

Stresses in non-sterile systems

Trial 1: Four types of root-zone stress: low potassium (K^+), high ammonium (NH_4^+), low water (drought) and high water (hypoxia) were induced. The stresses were applied continuously to established plants growing in the sand columns by reducing potassium in the nutrient solution, increasing ammonium in the nutrient solution, watering less frequently, and replacing the stopper in the bottom of the column with a solid stopper to induce water logging. The most prominent stress symptom in all treatments was browning from drought due to under-watering. Other symptoms included reddish, wide leaves of the hypoxic plants. Once the plants were put on a daily watering regimen, drought symptoms disappeared.

Trial 2: Nutrient solutions were adjusted for lower K in the low potassium treatment and a higher proportion of NH_4^+ in the high ammonium treatment. The water stress treatment was induced by watering on a schedule of reduced water (~50% less than control plants) for 3 days, then 100% of the control for one day. Placing a solid stopper at the bottom of the column and watering equal amounts to the control induced flooding. The control and high NH_4^+ treatments showed the highest amounts of transpiration and healthiest plant appearance (Figs. 23 and 24).

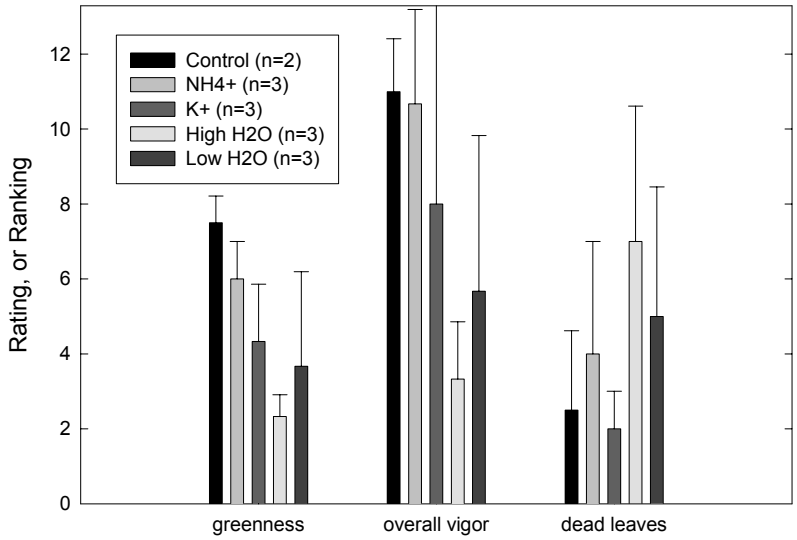

Fig. 23. Stressed plant ratings. Greenness rating: 10 is dark, 1 is yellow. Overall vigor: plants are ranked from 1 to 14, 14 is most vigorous. Error bars represent one standard deviation from the mean for each treatment.

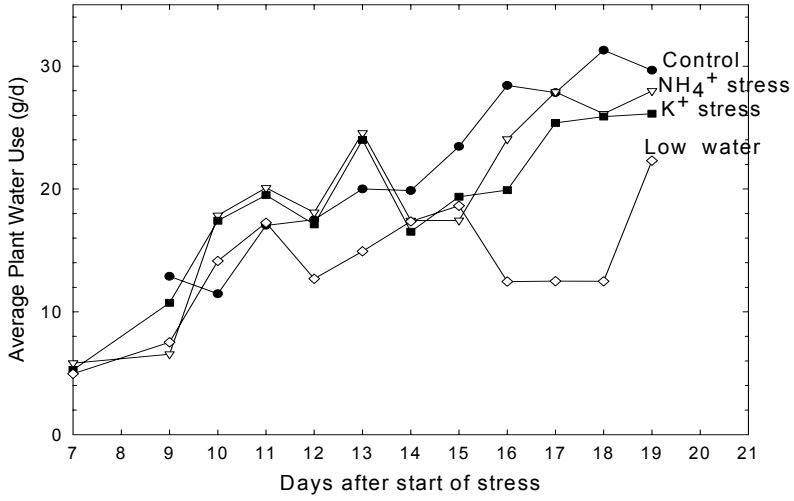

Fig. 24. Average daily water use by plants exposed to low potassium (K^+), high ammonium (NH_4^+), low water (drought) stresses.

Preliminary Axenic Trials

Trial 1

Plant growth and leachate collection in this trial was affected by poor water flow due to scotch brite pads being used as the interface between the sand and the drain tube. Three sand sizes, fine medium and coarse, were used. Samples of leachate were streaked onto agar plates to occasionally monitor microbial contamination which occurred in all plants within the first 20 days. TOC was monitored (Figure 25), which leveled-off with time due to microbial contamination. Plants were grown in a growth chamber, where watering and other manipulations were also conducted.

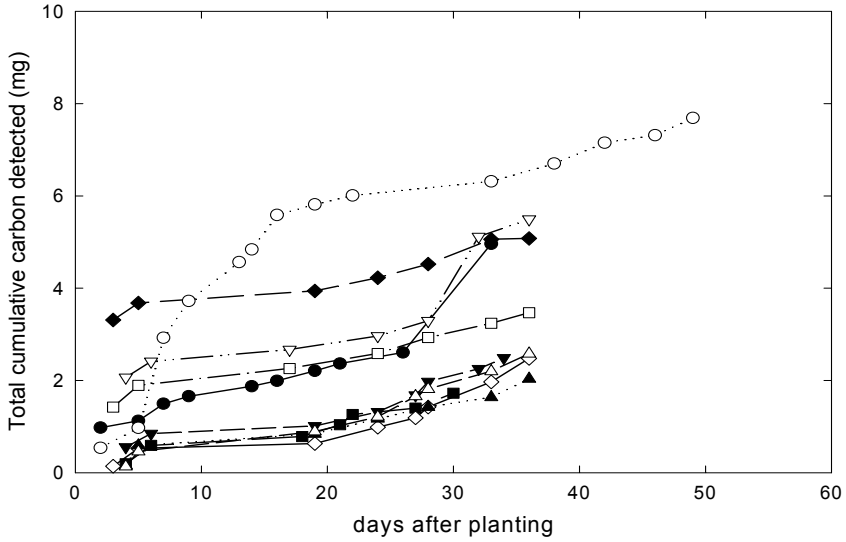

Fig. 25. Cumulative TOC detected from plants 1-18 (Trial 1).

Trial 2

The water flow in Trial 2 was improved due to the use of sand layers, the sizes and depths calculated by Dr. Scott Jones. A very high background TOC was discovered in the unplanted columns of this trial (Figure 26). Further investigation revealed that components contributed TOC in the unplanted columns (Figure 27). Plants were transported from the growth chamber for watering.

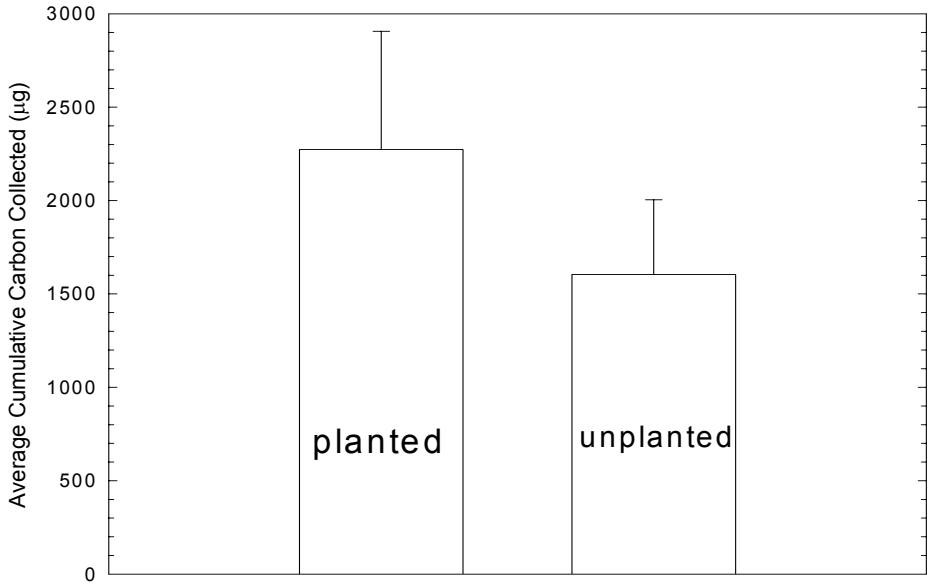

Fig. 26. Trial 2: Average amounts of TOC collected from planted and unplanted columns

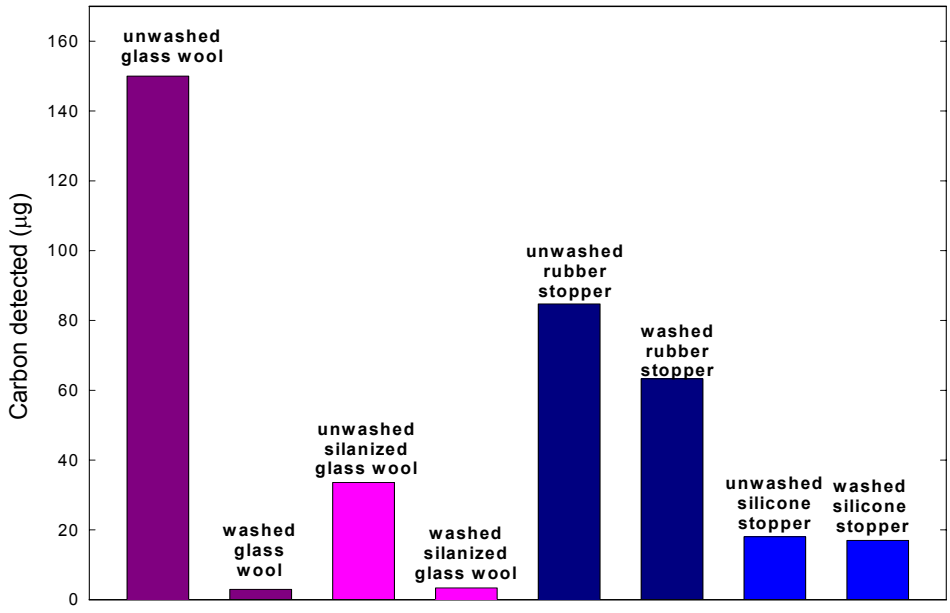

Fig. 27. TOC associated with column components. 0.3 grams glass wool was used. Deionized rinse water was analyzed for TOC content.

Trial 3

The background TOC was significantly reduced in Trial 3 (Figure 28). This was accomplished by washing all components with DI water and methanol, in which any residual TOC would be soluble. The rubber stoppers used in previous trials were changed to silicon. Silanized glass wool was used, which is more inert than regular glass wool has less possibility of binding charged exudates. Sand was muffled at 600 C for 10 hours. After muffling, the sand turned pink from oxidizing trace amounts of hematite in the sand. It was decided that muffling was impractical for large volumes of sand and another method to rid the sand of residual carbon should be used. All columns in trial 3 tested as contaminated immediately. This was hypothesized to be due incomplete

sealing of the containers that were exposed during transport and storage after autoclaving. Both leachate and sand from the top of the column were plated to test for microbial contamination.

Trial 4

This trial was conducted in the laminar flow hood. Residual TOC was low in trial 4 and finally a distinction in TOC levels of the exudate could be seen between the sterile plants and the non-sterile and unplanted columns (Figure 29). Only the leachate was plated out to test for contamination since it was hypothesized that testing the sand was an additional manipulation that exposed the system to potential microbial contamination. The root zone sealed from contamination by a foam plug with the shoots uncovered.

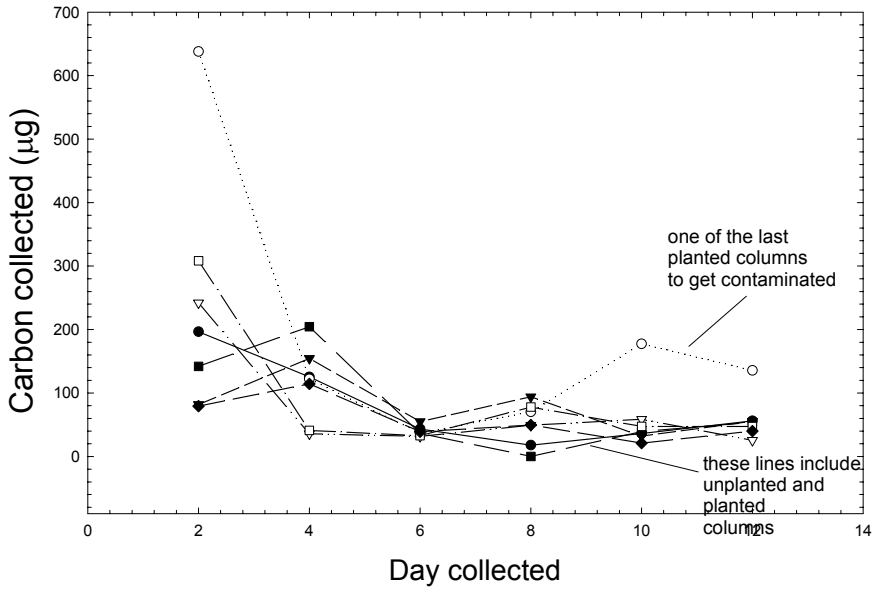

Fig. 28. Trial 3: TOC collected from planted and unplanted columns.

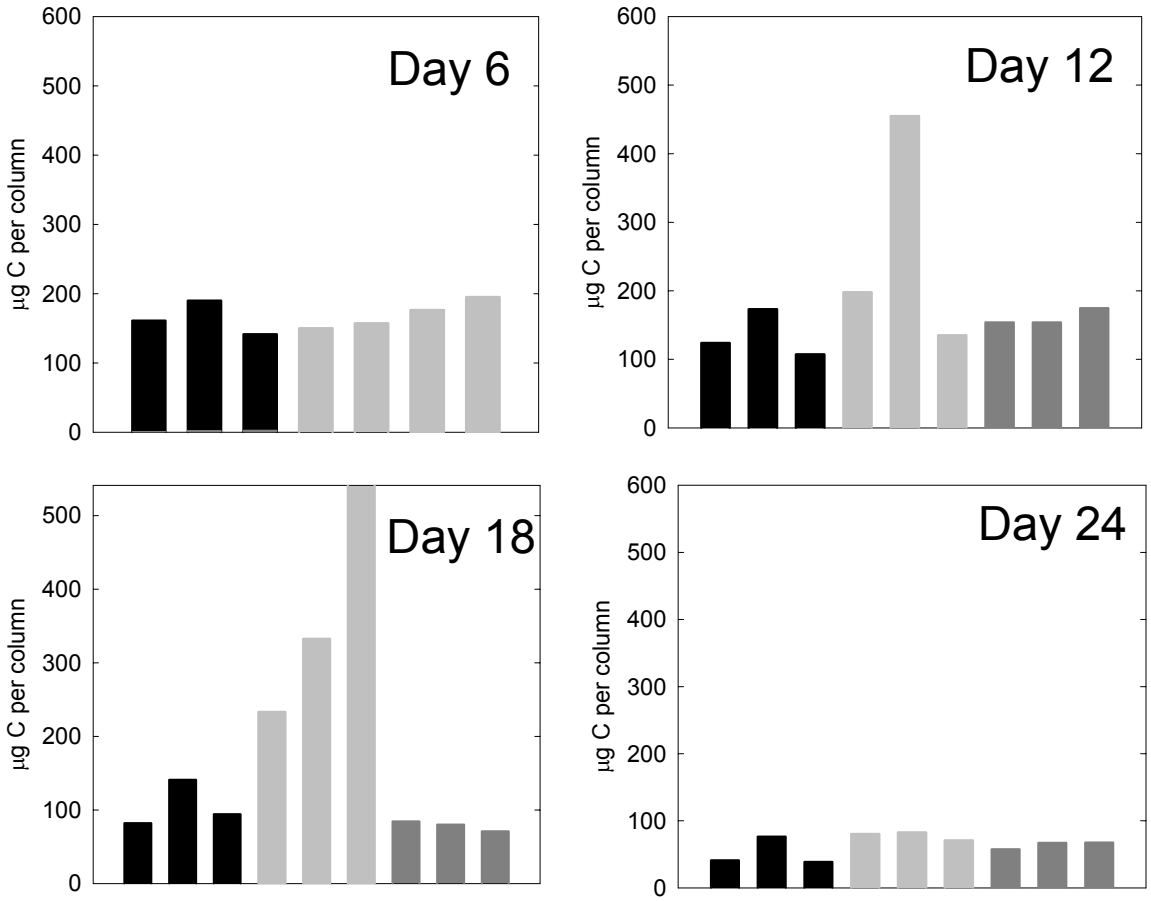

Fig. 29. TOC collected on 4 dates from unplanted (black), large plants (light gray), and contaminated plants (dark gray) during Trial 4.

Trial 5

New columns were developed to enclose the entire plant within two glass tubes connected by a ground-glass joint. An additional port on the lower column was added for airflow through a filter to each plant, which ranged up to 1 SCFH per plant by the end of the study. Nutrient solution concentration was doubled since the covered plants were watered less often. Plants were grown for 70 days. Two plants were harvested early because of their large size.

Increased amounts of TOC were collected in the exudates of Trial 5, even from contaminated plants. Different types of microbial growth were detected from each contaminated column. Our results indicate that microbes in the contaminated columns did not metabolize all of the exudates. This may be due to spatial separation of the contaminant and the exudate within the column, making the exudate inaccessible. Based on the Ca levels in this analysis (Table 14), it was decided to reduce the amount of K^+ in the nutrient solution from 11 mM to 9 mM.

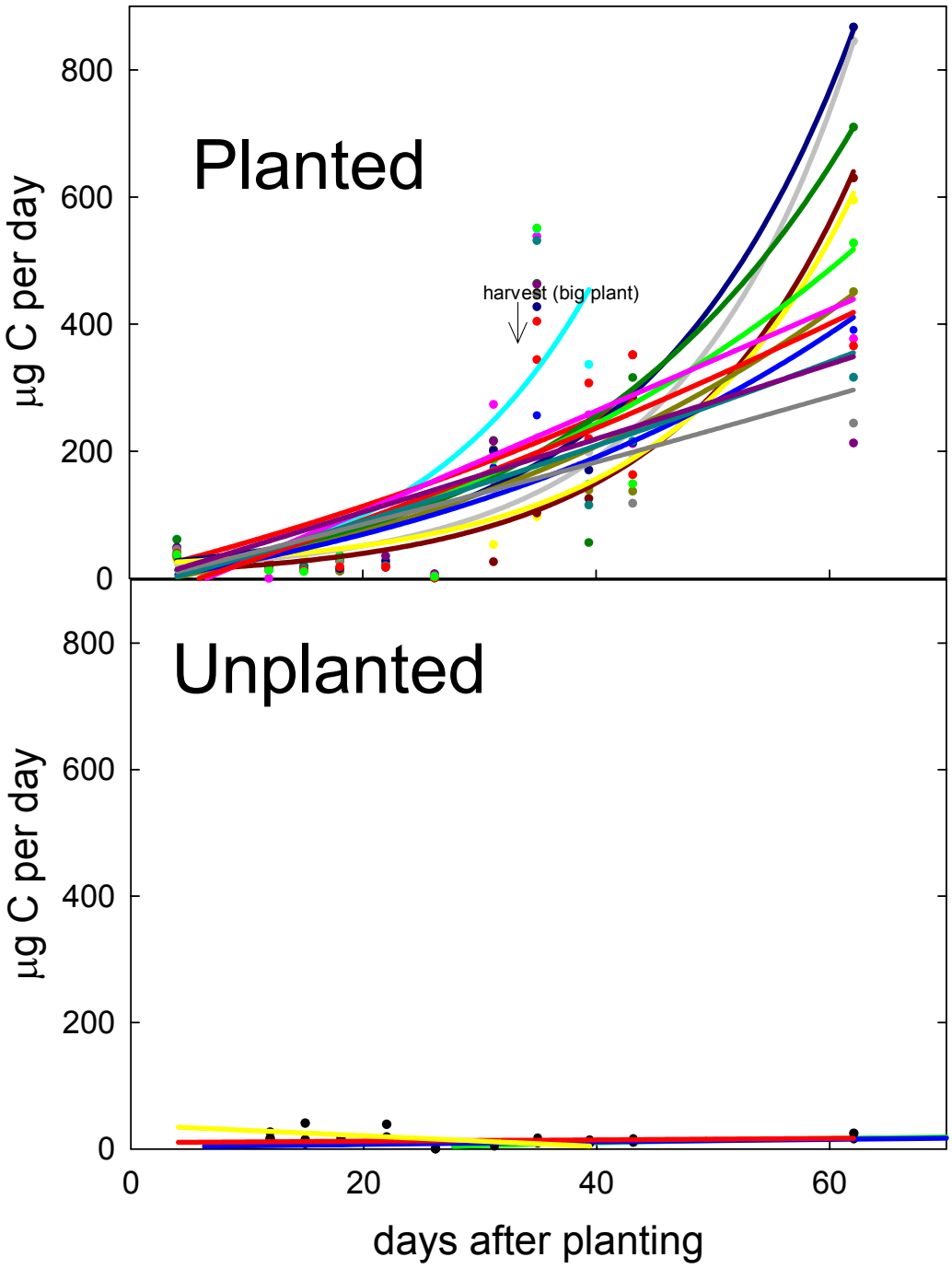

Fig. 30. TOC data from trial 5.

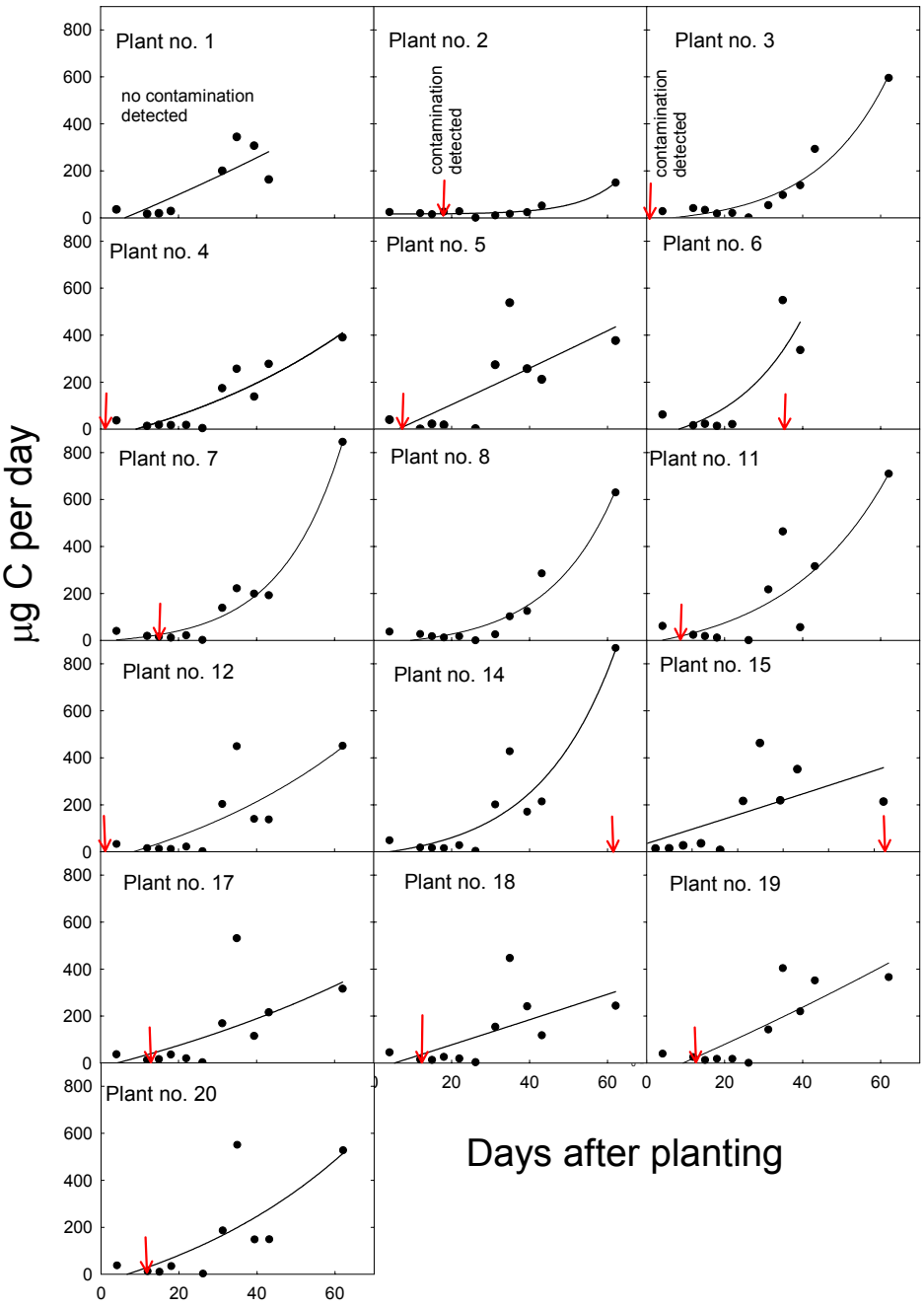

Fig. 31. TOC results from trial 5. Arrows indicate date microbial contamination was detected on agar plates.

Table 13. Shoot dry mass of plants from trial 5.

Plant Number	Shoot dry mass (grams)
1	1.61
2	0.81
5	1.39
6	1.29
7	1.22
8	1.02
11	1.5
12	1.38
14	1.43
15	1.12
17	1.54
18	1.2
19	1.17
20	1.62

Table 14. Nutrient analysis of the shoots of four plants from trial 5.

Plant number	Mo	Na	Ni	P	Pb	S	Se	Sr	Zn
	mg/kg	%	mg/kg	%	----------mg/kg----------				
1	<	47.1	<	0.26	<	0.21	<	<	83.2
2	<	146	<	0.37	6.76	0.24	<	<	137
6	<	94.5	<	0.29	<	0.21	<	<	86.2
11	<	44.9	<	0.22	2.52	0.20	<	<	53.3

Plant number	Al	As	B	Ba	Ca	Cd	Co	Cr	Cu	Fe	K	Mg	Mn
	----------mg/kg----------				%	----------mg/kg----------					%	%	mg/kg
1	30.1	<	9.12	<	0.24	<	<	<	8.66	88.7	3.56	0.13	56.6
2	55.2	<	14.6	<	0.23	<	<	<	12.5	101	4.33	0.12	63.3
6	27.6	<	7.27	<	0.21	<	<	<	12.9	102	4.12	0.12	48.1
11	25.2	<	18.7	<	0.26	<	<	<	11.0	61.1	4.61	0.12	46.9

Trial 7:

Plants were grown under a 24-hour photoperiod for 40 days, at which time all planted columns had become contaminated. The plants had experienced two days of high temperatures at the beginning of the study. By day 40 the leaves were tall and thin unlike the broader leaves of previous trials. The TOC in the exudates of Trial 7 increased at first and then leveled off in most plants. No exponential increases were seen as in previous trials. Percent soluble TOC values were very erratic.

Since the onset of contamination was delayed in this trial, the source of most contamination was probably from transporting the nutrient solution flasks from the sterilizer to the freezer to cool. This was not done in Trial 6 because of better planning that allowed time for the nutrient solution to cool in the sterilizer. The contamination that occurred in this trial and the subsequent lack of exponential increase in exudate TOC confirmed the sterility of Trial 6.

Root mass was determined by the following process:

1) Remove sand from roots by rinsing roots in water. Smaller root pieces float in water.
2) Filter the rinse water to capture small root pieces on a pre-weighed, oven-dried filter.
3) Dry all root parts and filter in a pre-weighed, oven-dried bag.
4) Weigh after 2 days in 80^{o}C oven to determine root dry mass.

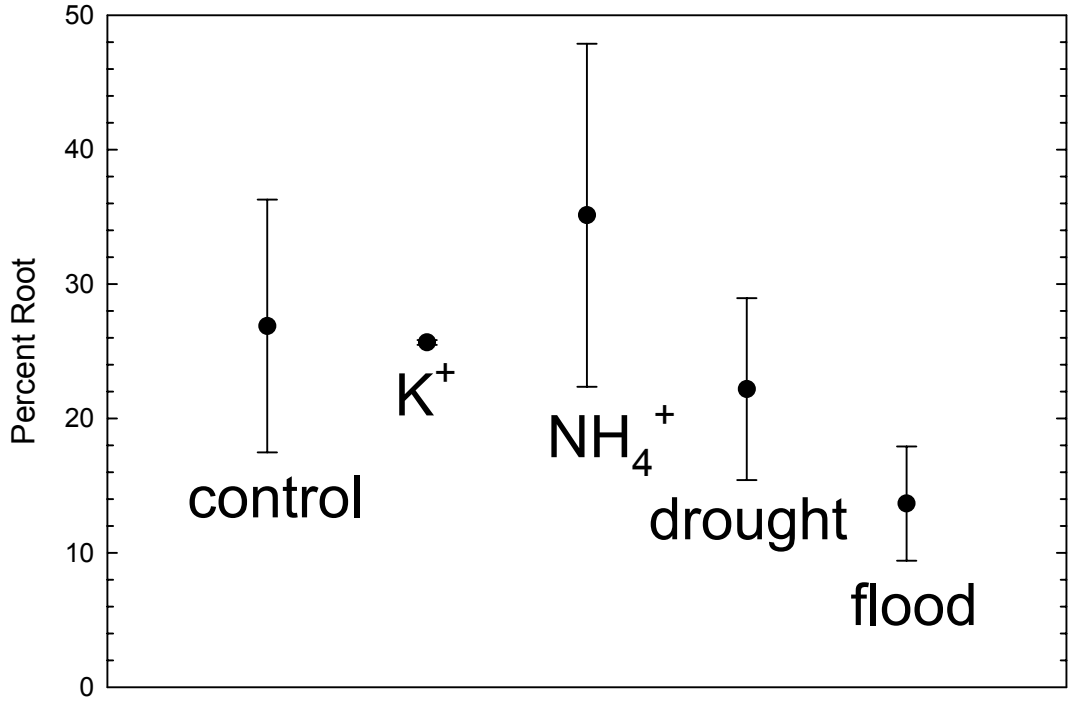

Fig. 32. Trial 7 root percentages of total plant mass

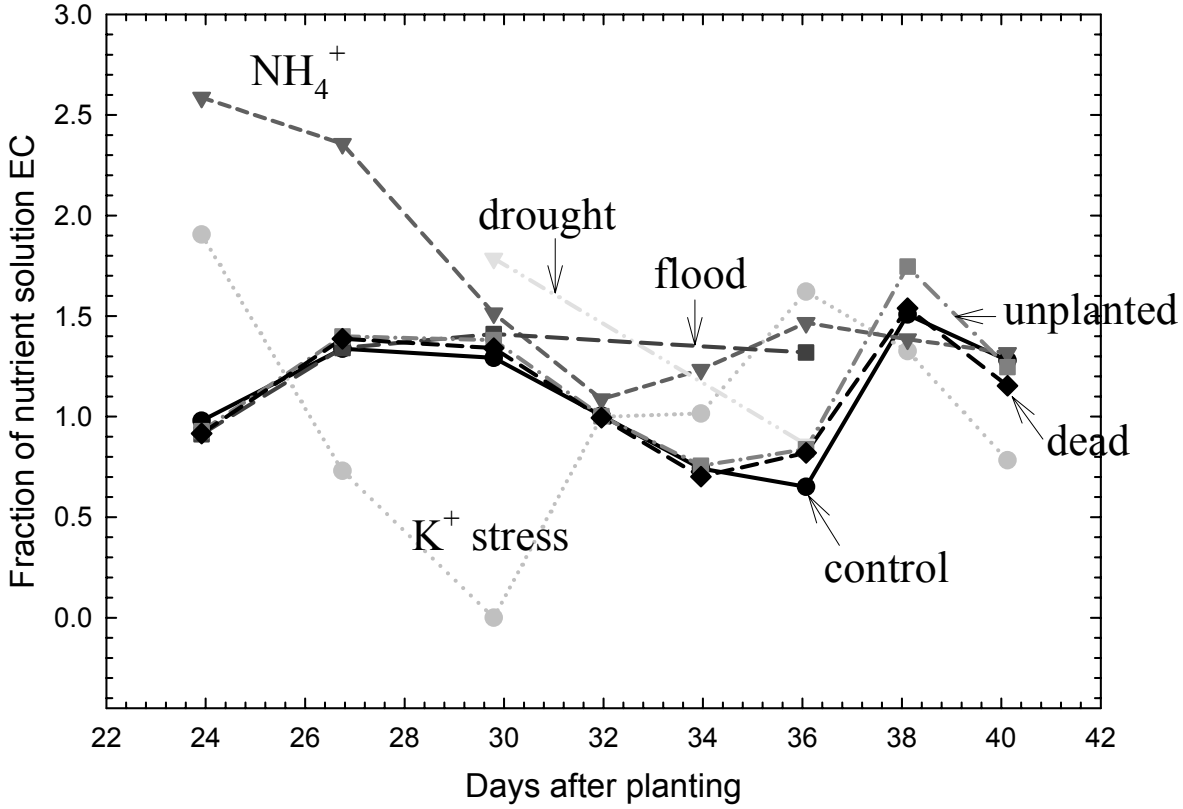

Fig. 33. Electrical Conductivity of leachates – fraction of nutrient solution added.

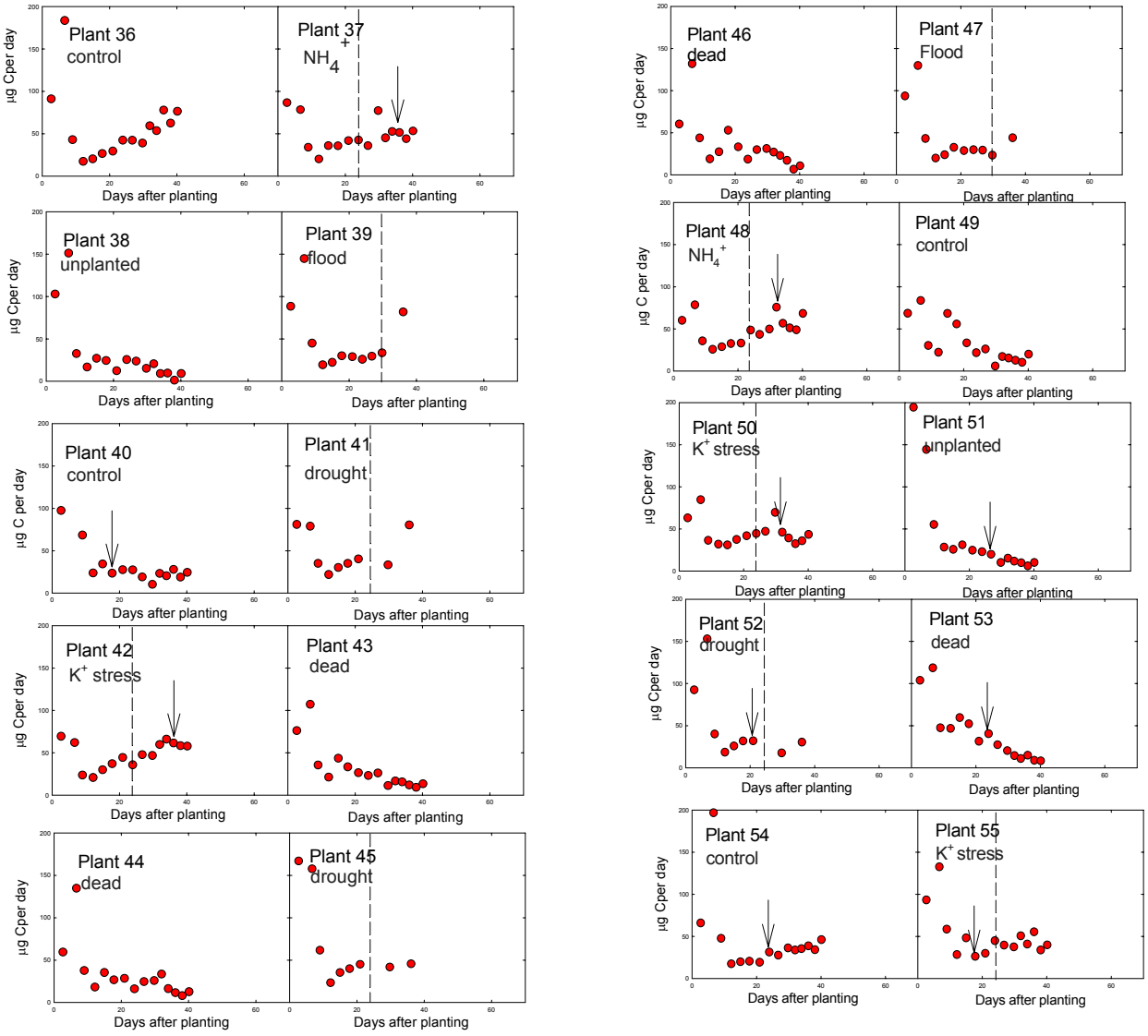

Fig. 34. Trial 7: TOC collected per day. Dashed lines indicated when treatments began. Arrows indicate when contamination was first detected.

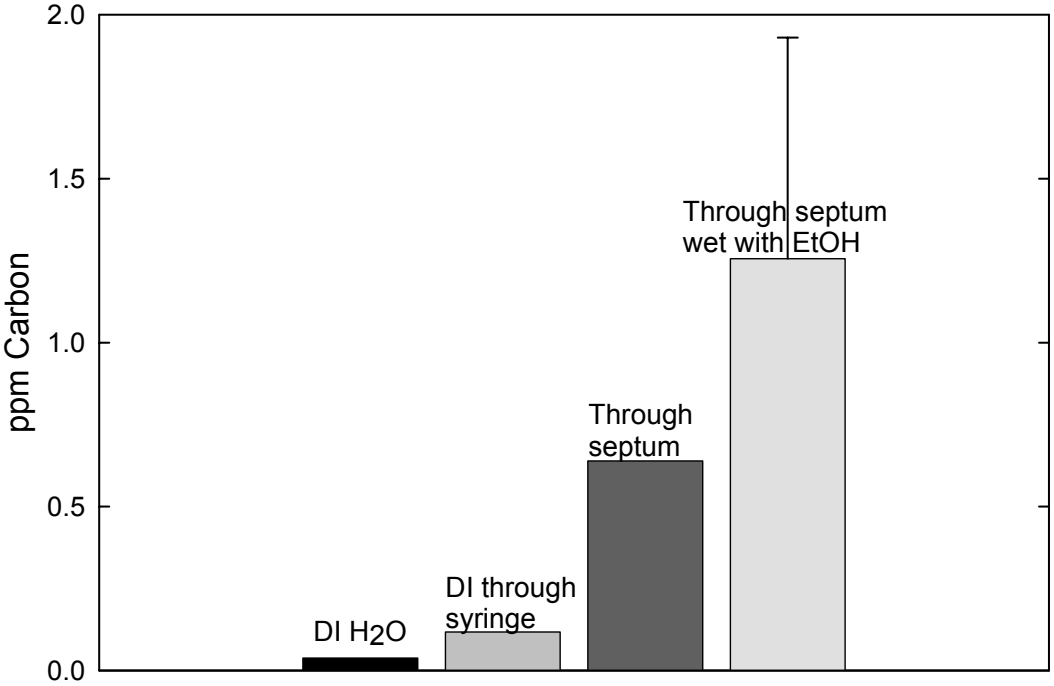

Fig. 35. TOC added by various steps in the watering process.

It was decided based on this study to allow ethanol to dry fully from the septum before inserting the needle to water.

Iron Loading of Sand in Columns

Beginning with Trial 5, columns were loaded with 200μM $FeCl_3$ after packing with sand (see Materials and Methods of Chapter 2). Based on tests of pH and electrical conductivity leaching from the columns (Figure 36), the treatment used was effective in getting the sand to retain iron. Plants grown in non-sterile columns loaded with $FeCl_3$ showed slightly higher SPAD-meter readings than those loaded with only nutrient solution, indicating that the Fe-loading treatment was beneficial to plant health.

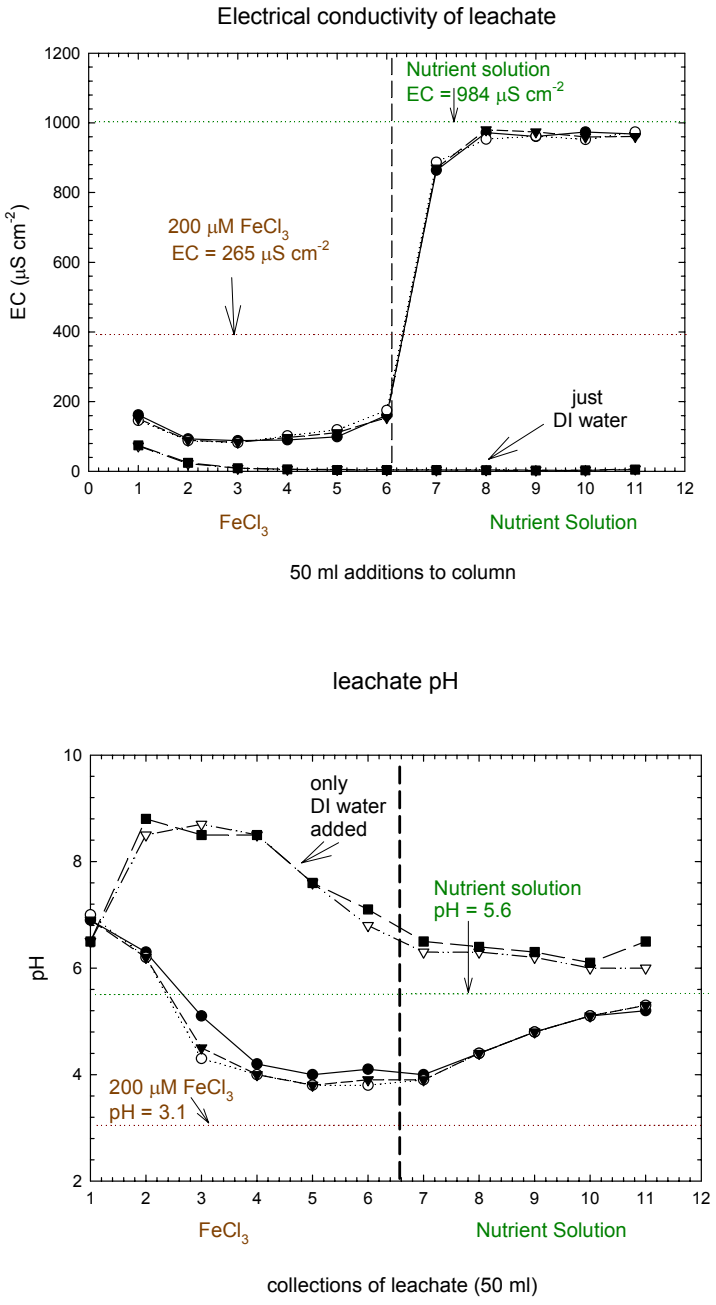

Fig. 36. EC and pH of leachate during Fe-loading of columns.

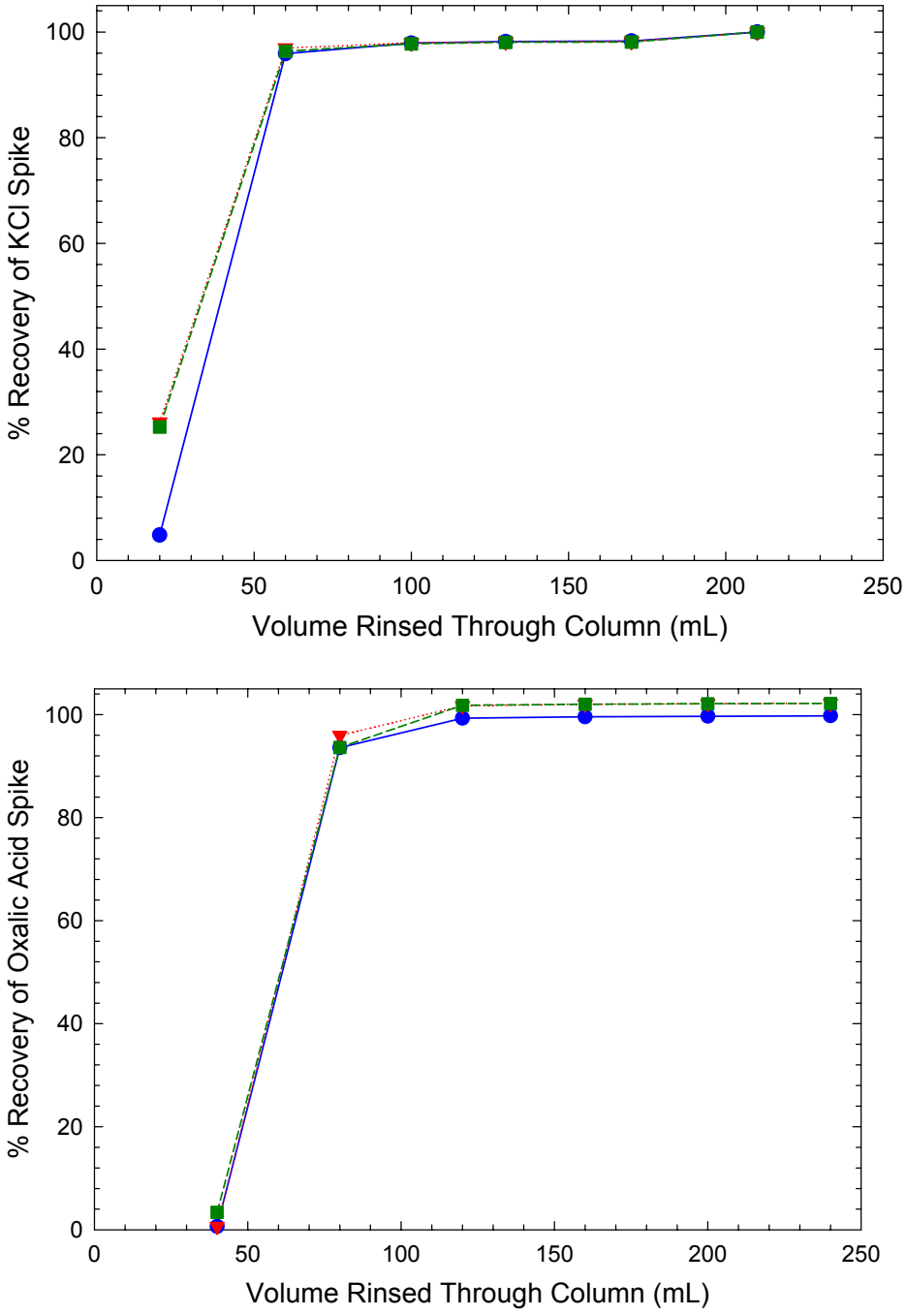

Fig. 37. Recoveries of inorganic (KCl) and organic (oxalic acid) compounds through sand columns, based on electrical conductivities.

APPENDIX D. Additional data from Trial 6

Table 15. Nutrient content of shoots including standard deviations.

	P	K	Ca	Mg	S	Fe	B	Zn	Mn	Cu
	----------%----------					----------mg/kg----------				
Control										
Average	0.30	3.19	0.22	0.09	0.17	53.70	47.03	48.80	44.37	9.09
Std. Dev.	0.02	0.36	0.05	0.01	0.02	8.74	5.38	10.98	9.10	0.64
K+ stress										
Average	0.25	1.26	0.48	0.15	0.13	33.5	51.15	39.95	35.10	8.78
Std. Dev.	0.04	0.11	0.13	0.05	0.02		19.02	2.05	7.35	2.30
NH4+ treatment										
Average	0.34	3.07	0.22	0.09	0.34	41.70	52.35	73.35	72.10	18.00
Std. Dev.	0.06	0.51	0.06	0.02	0.10	9.33	20.15	9.26	10.18	8.20
Drought										
Average	0.33	3.45	0.24	0.12	0.21	47.40	81.80	50.85	53.65	12.22
Std. Dev.	0.08	0.64	0.04	0.02	0.03	3.96	19.37	8.70	15.77	3.23
Flood										
Average	0.23	2.17	0.23	0.07	0.13	52.20	35.60	36.60	37.55	9.59
Std. Dev.	0.01	0.08	0.04	0.01	0.02	8.63	2.83	1.70	3.75	0.58

Table 16. Trial 6 shoot dry mass.

Plant	dry mass
21	2.14
22	
23	1.62
24	2.54
25	2.99
26	2.45
27	2.31
28	1.37
29	2.7
30	1.41
31	1.85
32	1.65
33	1.08
34	1.87
35	1.54

Table 17. Average nutrient content of nutrient solution (mg/L).

Al	0.07	Mn	11
B	0	Mo	0.26
Ca	35	Ni	0.43
Co	0	P	0
Cu	0.07	S	0
Fe	0	Se	15
K	127	Si	0
Mg	15	Zn	0.18

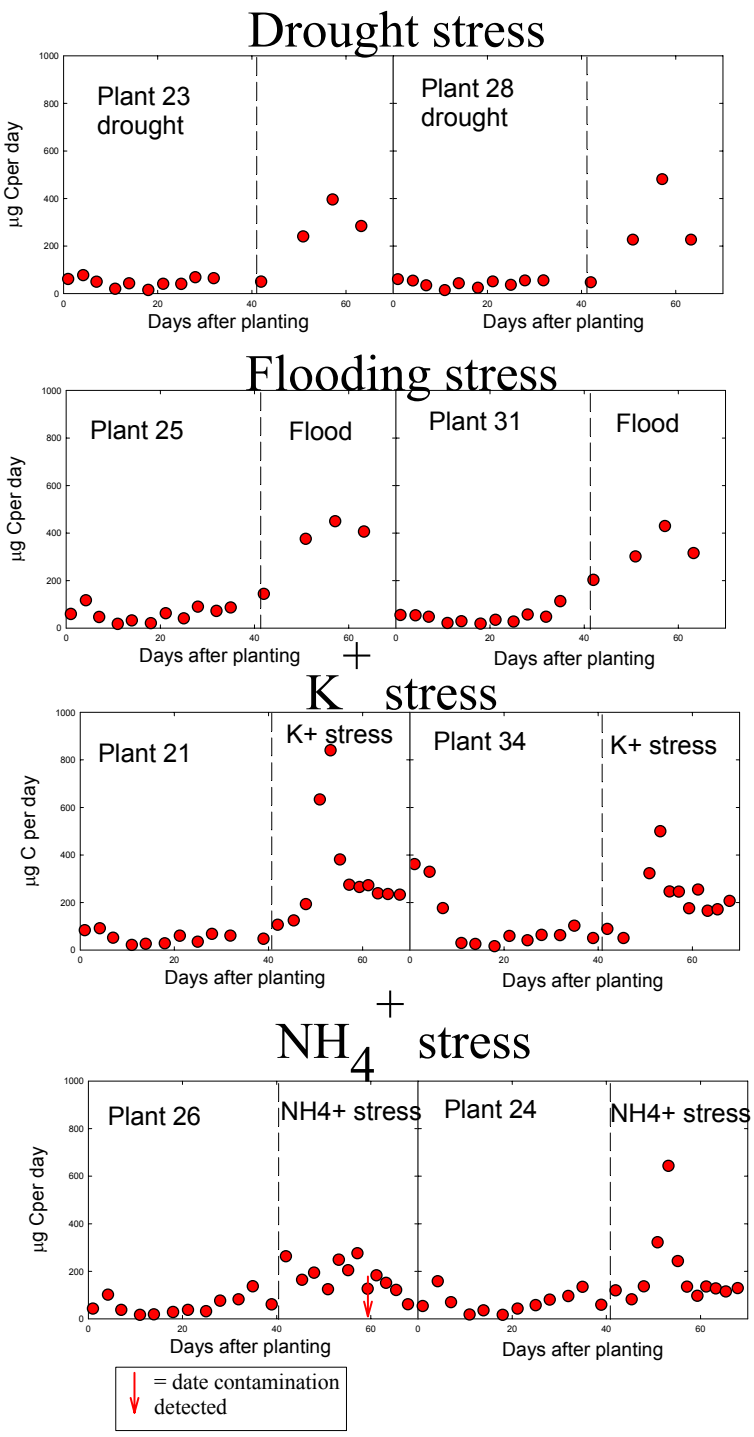

Fig. 38. TOC release rates for each plant in all treatments.

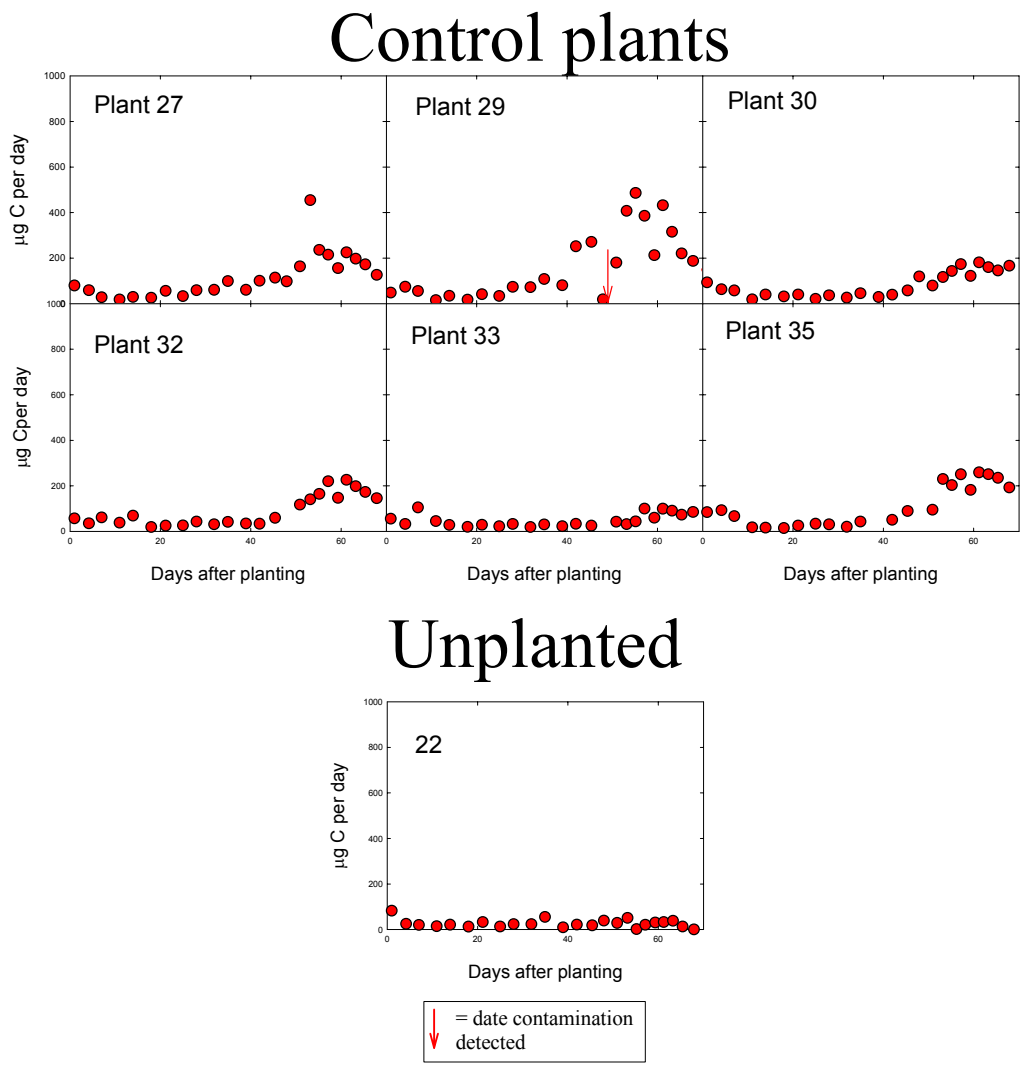

Fig. 39. TOC release rates for control plants and collected from the unplanted column.

Table 18. Relative Growth Rates before treatments (days 0-35), after treatments (days 35-70) and Water Use Requirements.

	RGR before treatments	RGR after treatments	WUR (ml/g)
Control			
Average	0.118	0.066	164
Std. Dev.	0.017	0.010	30
Control without small plant			
Average	0.139	0.055	133
Std. Dev.	0.003	0.0003	8
K^+ stress			
Average	0.132	0.056	183
Std. Dev.	0.001	0.001	6
NH_4^+ treatment			
Average	0.139	0.056	137
Std. Dev.	0.002	0.001	15
Drought			
Average	0.132	0.048	148
Std. Dev.	0.004	0.000	6
Flood			
Average	0.129	0.064	162
Std. Dev.	0.015	0.006	40

An attempt was made to classify chemical compounds exuded based on their retention on strong acid or strong base ion exchange resins and subtracting the remaining TOC from the measured TOC of the sample. These resins, however, added TOC to the sample and in some cases resulted in final TOC concentrations higher than measured TOC. Therefore, no results were obtained on classification of the exudates using this method.

APPENDIX E. A Hydroponic Study of Relative Growth Rate and Water Use Efficiency of Crested Wheatgrass (*Agropyron cristatum*)

Crop Physiology Lab Project

Plant Science 6210

Abstract

Plant water use varies among species and with stage of life cycle. This study was conducted to correlate plant growth with water use in crested wheatgrass (*Agropyron cristatum*). A hydroponic study was conducted in a growth chamber on the relative growth rate and water use efficiency of crested wheatgrass. A relative growth rate of 0.2 g fresh mass gained per gram plant per day was observed, along with a water use efficiency of 0.1 g fresh mass gained per gram water transpired per day. Plant mass and transpiration correlated linearly ($R^2 = 0.96$). Plant mass grew exponentially with plant height ($R^2 = 0.75$). It was therefore concluded that water use could be visually estimated by plant height.

Introduction

Hydroponic plant culture provides a non-destructive means of monitoring plant mass and water use; plants and bottles can be periodically weighed throughout the study and plants can continue to grow after weighing and nutrient solution is refilled.

The information obtained from a hydroponic study can be applied to other types of plant culture and can be very useful in knowing how much water a plant needs

depending on its size and how big a plant is. For example, the Root Exudate Project at the Crop Physiology Lab requires sterile sand culture of plants with nutrient solution lacking iron chelate for exudate analysis purposes. The plants are required to be watered as little as possible due to risk of bacterial contamination while watering. Over-watering also leads to the leaching of important nutrient-sequestering compounds exuded from the roots.

Relative growth rate (RGR) is the amount of new plant tissue per gram existing plant per day. Water use efficiency (WUE) is the amount of new plant growth (Pnet) per gram water transpired. These two parameters can be determined from the hydroponic study because plant weight can be monitored throughout the plant's lifetime, and they offer the opportunity to compare the effects of different treatments on RGR and WUE.

Materials and Methods

Seeds of crested wheatgrass (*Agropyron cristatum*) were germinated either on a slant board or in a germination box. The germination box was a transparent plastic box containing two layers of germination (blotter) paper and was watered with tap water in a 26^{o}C incubator. The slant board was made of Plexiglas lined with paper towels, immersed in tap water, and covered on a lab bench. Seeds were germinated on a slant board only after the seedlings from the germination box failed to grow well (see below).

The contents of all experimental bottles are shown in Table 19. Five 2-liter hydroponic bottles were planted; 2 with germination box seedlings and 3 with slant board seedlings. Germination box seedlings were started 2 weeks before planting, and

the nutrient solution used included all essential nutrients, plus Si, 100mM Fe-EDTA (a chelating agent), and 1 mM MES buffer according to the nutrient solution criteria discussed by Bugbee (1995). Plants were supported in the bottles with foam plugs that were cut in half with a cross-sectional wedge removed. Foam plugs were dried in a drying oven for at least 24 hours before use. An air pump was used to aerate the root zones via tubes of uniform length running to the bottom of the hydroponic bottles at 0.1 L/min. All joints on the manifold were sealed with silicon caulk to prevent leaks and assure uniform airflow.

Unplanted controls included 2 2-liter bottles filled with tap water and bubbled with the same flow of air as the planted bottles, 1 2-liter bottle of tap water that was not bubbled, and 2 1-liter bottles of tap water that were not bubbled. All control bottles were plugged with the same foam plugs used in the planted bottles. All bottles were kept in a growth chamber (24^{o}C, 24% RH) and periodically rotated in terms of position and bubbling tube.

Table 19. Contents of the bottles used in the RGR experiment.

Bottle	Contents
1	plant germinated on slant board
2	plant germinated in a germination box
3	plant germinated in a germination box
4	plant germinated on a slant board
5	plant germinated on a slant board
a*	2L tap water, bubbled
b	2L tap water, bubbled
c	1L tap water, not bubbled
d	1L tap water, not bubbled
f*	2L tap water, not bubbled

*treatments for bottles a and f were switched during the last 2 days of the study to verify evaporation data

At the beginning of the study, all seedlings were from the germination box. However, the combination of low light (due to inadequate lighting), inadequate bubbling (due to leaks in the manifold), and the small roots caused the plants to become stressed. Therefore, on days 20 and 24, three plants were replaced with seedlings that were germinated on a slant board. On day 28, FeEDTA and NH_4Cl added to all planted bottles to reduce chlorosis. Plants became fully green again within two days.

Once the seedlings had been transplanted into the hydroponic bottles and appeared to be well established, plant fresh weights were measured periodically. Before weighing, roots were blotted on an absorbent pad to remove as much excess water as possible. Each entire hydroponic bottle was also weighed, and nutrient solution lost through evaporation and transpiration was replaced accordingly.

Transpiration was obtained by subtracting the average water lost by the control bottles from the amount of water lost by each planted bottle.

Results

Water loss due to bubbling was predicted to be 0.288 g/day using the following calculations:

absolute humidity of chamber: 5 g per L air
absolute humidity of bubble (100% RH): 23 g per L air
gradient: 18 g/L
flow rate per bottle: (0.1 L/min)/ 9 tubes = .01 L/min

$$\frac{18\text{g}}{\text{L}} \times \frac{.01\text{ L}}{\text{min}} \times \frac{1440\text{ min}}{\text{day}} = \frac{0.288\text{ g}}{\text{day}}$$

Water loss by the planted and unplanted bottles were plotted against time and are shown in Figure 1. The unplanted bottles averaged about 1.8 g/day, whereas the planted bottles lost increasing amounts of water through time.

Relative growth rate is shown plotted against plant fresh mass in Figure 40 and was calculated using the formula $(\ln (M_1/M_2))/\Delta t$ where M is fresh mass and t is time. Plant 2, which was the biggest plant, showed a slightly decreasing RGR with time, but had an average RGR of 0.2 g fresh mass gained per gram plant per day.

Water use efficiency is plotted against plant fresh mass and is shown in Figure 41. To obtain WUE, the daily gain in fresh mass was divided by daily transpiration. The WUE values for plant 2 were relatively constant, averaging about 0.1 g fresh mass gained per gram water transpired per day.

To be able to visually estimate plant water use, correlations were made between transpiration and plant mass and between plant mass and plant height. These graphs are

shown in Figures 42 and 43. Plant mass and transpiration correlated completely linearly ($r^2 = 0.96$). Plant mass grew exponentially with plant height ($r^2 = 0.75$).

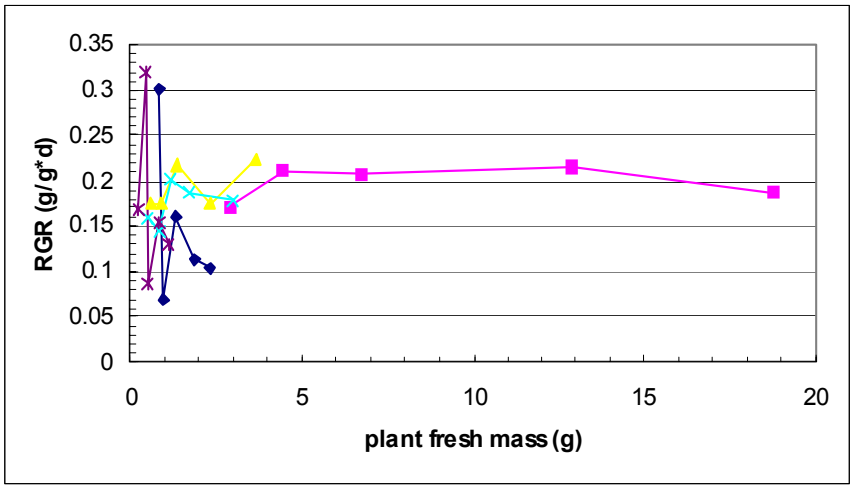

Fig. 40. Relative growth rate (RGR) of hydroponic crested wheatgrass.

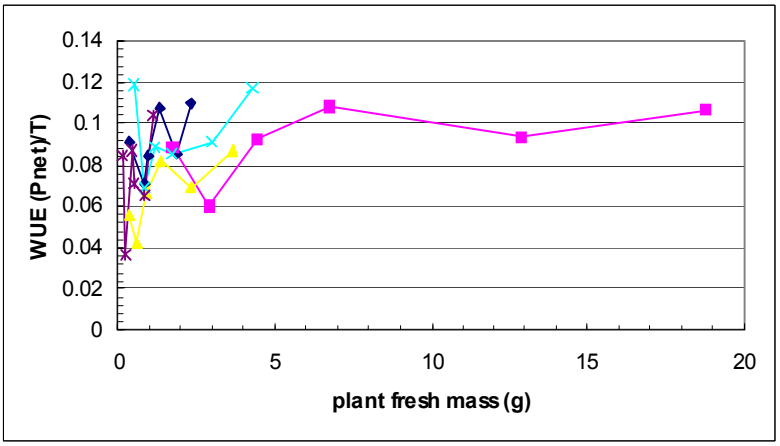

Fig. 41. Water use efficiency (WUE) of hydroponic crested wheatgrass.

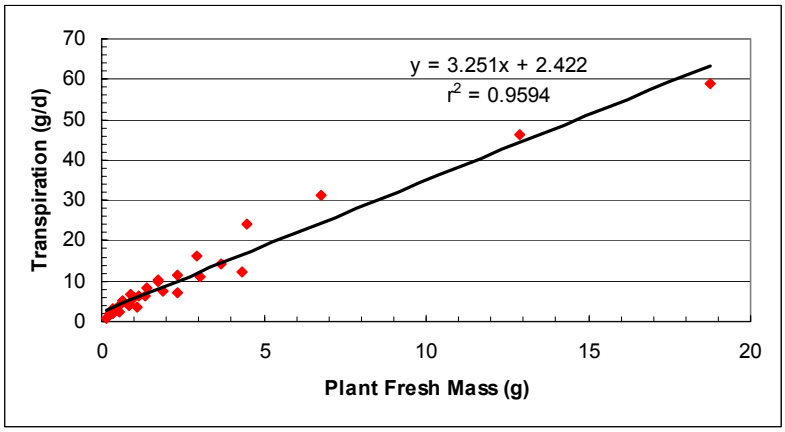

Fig. 42. Plant fresh mass is highly correlated with transpiration rate.

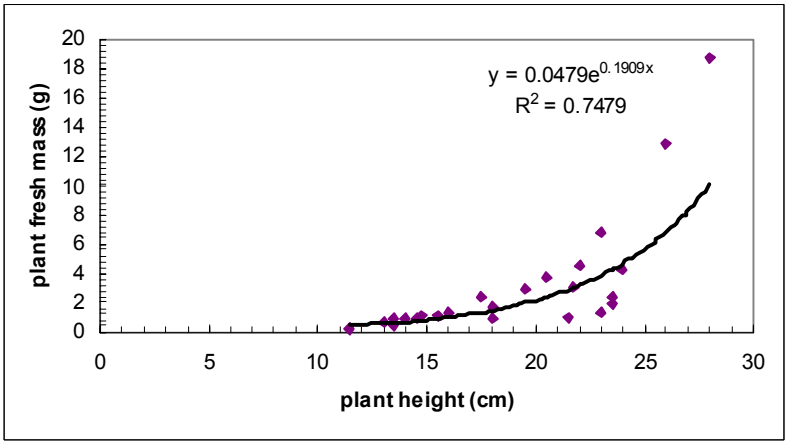

Fig. 43. Plant fresh mass increased exponentially with plant height.

Discussion

The slant board provided much better germination than the germination box because the roots and leaves were straight, making its shape more conducive to growing in the hydroponic setup. Consequently, the seedlings germinated on the slant board adapted well to growing hydroponically and quickly caught up with the size of the earlier planted seedlings.

One problem encountered early in the study was wetting of the foam plugs supporting the plants. Since the plants were very small at first, the plugs were pushed down into the neck of the bottle to ensure root hydration. However, this caused water to be easily wicked by the plug and evaporated, giving unreliable evaporation data. When the plants grew to an adequate size, plugs were dried and then placed further up the bottle neck. Data taken from when the plugs were wet was not included in the growth and water use analysis.

Another problem was that the initial lighting in the growth chamber was quite low (about 300 μmoles/m^2*s). Therefore, the lights were kept on for 24 hours a day. On day 24, lights were replaced to give a PPF of 615 μmoles/m^2*s and the photoperiod was subsequently set at 16 hours.

It was expected that the bubbled controls would exhibit some water loss and that water lost from the unbubbled controls would be negligible. A side experiment with bottles left on the lab bench showed that evaporation does occur through the foam plugs, and that splashing the foam plugs, as in when the bottles are transported to be weighed, increases evaporation (data not shown). This could also be due to bottles heating up (they were dark brown), and a temperature (and therefore humidity) gradient forming between the nutrient solution and the surrounding air. Water loss from the unplanted bottles was fairly consistent, regardless of the bottle size or the presence of aeration. One exception was control bottle (a), which exhibited consistently high evaporation rates, even when treatments were switched with bottle (f) to being unbubbled. This was

probably due to some holes in the bottle that were inadequately sealed, and therefore the data from bottle (a) was omitted from the growth and water use analysis.

In Figures 40 and 41, the data from plant 2 is the most indicative of any trends since it was by far the largest plant. Figure 40 indicated that RGR would decrease slightly as the plant gets bigger. This is what was expected because as the plant gets older, it becomes less efficient in photosynthesis. It is expected that the other plants will show a similar trend when they get bigger. During the last few measurements, some symptoms of *Pythium*, a fungal disease, were observed in plants 1 and 2 (sparse roots at the base of the plant and root browning). Therefore, growth rate may have been compromised due to this disease. For future studies, Jenkins et al. (2000) recommend inoculating the hydroponic system with a highly complex microbial community to out-compete the *Pythium.*

Figure 41 indicates that water use efficiency stays the same throughout the plant life. This is because water use efficiency changes more with diffusion rates of water and CO_2 due to gradients and stomatal aperture, as opposed to RGR which changes due to less efficient biological functioning with age. Frank and Bauer (1991) observed a decrease in water use efficiency with increased water available to crested wheatgrass. Therefore, watering crested wheatgrass as little as possible will not only minimize chances of contamination and nutrient leaching, but also maximize water use efficiency.

The largest source of error seemed to occur when the smallest plants were weighed on the microgram balance. Since it was impossible to blot all of the water off the roots before weighing, a larger proportion of the total weight of the smallest roots is

residual water. This overestimation of plant weight could cause RGR and WUE to be underestimated.

Another possible error is the underestimation of transpiration; evaporation could be higher in the planted bottles since the stems open the wedge in the foam plug, allowing more water vapor to escape from the bottle than in the unplanted controls.

Figure 42 shows a linear relationship between plant fresh mass and transpiration. It was expected that this would be a nonlinear relationship because as the plant gets bigger, more shading occurs and therefore the plant cannot maintain as steep an increase in transpiration with mass. A more non-linear relationship would probably have been observed if the plants were allowed enough time to grow to a larger size. Figure 43 shows that a relationship exists between plant height and plant fresh mass. Therefore, this data and the data presented in Figure 39 can be used to visually estimate plant water use based on plant height, can therefore be useful in studies like the Root Exudate Project. It is not yet clear, however, that the amount of water used by plants grown hydroponically is the same as water use by plants grown in sand or soil media.

Suggestions for future studies include correlating information obtained from this study with transpiration of plants grown in sand and soil, and comparing RGR and WUE values with plants grown under different treatments (i.e. nutrient stress).

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Jenkins, D.G., K.L. Cook, J.L. Garland, and K.F. Board. 2000. *Pythium* invasion of plant-based life support systems: biological control and sources. Life Support and Biosphere Science 7: 207-218.

APPENDIX F. Cesium uptake by crested wheatgrass (*Agropyron cristatum*)

December 11, 2001

Abstract

Cesium is not an essential plant nutrient but is taken up by plants due to its similarity to K. Potassium is taken up rapidly by a K^+ transporter or more slowly by a K^+ channel. Crested wheatgrass (*Agropyron cristatum*) was grown in 1) an equimolar K and Cs solution [250 μM], 2) high Cs [1000 μM], 3) low K [100 μM] and 4) a control [4.7 mM K, 0 Cs]. Cesium uptake reduced the relative growth rate (RGR) but did not affect K uptake in shoots. The levels of Cs taken up among treatments suggested a rapid turnover of ion uptake mechanisms.

Introduction

Radiocesium contamination in soil is of concern due to the mutagenetic effects of Cs in the human body and the long half-life of Cs in soil (Nishita et al. 1962). Crested wheatgrass is found growing in Cs contaminated soils. Cesium uptake by plants can be beneficial for the phytoremediation of Cs-contaminated soil.

Cesium uptake into plants has been shown to be similar to K uptake. Both elements have a +1 charge as alkali metals, and have similar atomic radii (237.6 pm for K and 237.1 pm for Cs). Disturbance of K uptake will deleteriously affect many plant functions: K plays a major role in plant function as a macronutrient, including enzyme activation, stomatal movement, and osmoregulation (Marschner, 1995).

Understanding K uptake by the plant began with interpreting the shapes of kinetic curves (Kochian and Lucas, 1982). Today, the field has progressed to the stage of cloning specific K^+ channels. Two major mechanisms have been attributed to K uptake by the plant: a K^+ transporter and a K^+ channel. The K^+ transporter, or 'high affinity' uptake mechanism, is used by the plant at low rhizospheric K concentrations. This mechanism has shown little discrimination against Cs uptake. The K^+ channel, or 'low affinity' mechanism, is used at high K^+ concentration and shows high discrimination against Cs uptake (Zhu and Smolders, 2000).

Although Cs is taken up at much lower rates than K, Smolders and Kiebooms (1996) observed that highest ^{137}Cs uptake occurs at lowest concentrations of K. Since K^+ strongly suppresses Cs uptake, it has been concluded that Cs must be absorbed by the K^+ uptake system of the root. The largest effects of K concentration on Cs uptake have been observed at concentrations of 10 μM to 250 μM K, above which no further effects are seen. This suggests a separate and as yet unidentified means of Cs uptake, since Cs uptake at K concentrations above 250 μM remains constant but greater than 0.

Cs has been observed to accumulate more in plant roots than in shoots (Zhu and Smolders, 2000). Genotypic differences have also been observed. Buysse et al. (1996) reported greatest genotypic differences in Cs uptake at lowest concentrations of K in solution. A Cs-K distribution factor (DF = Cs:K in plant/ Cs:K in substrate) has been observed at values of 0.01-0.8 in most species tested, verifying the lower efficiency of Cs uptake compared to K.

In this study, the effects of Cs added to low concentration of K in the nutrient solution are determined in terms of relative growth rate (RGR) and shoot Cs and K concentrations.

Materials and Methods

Seeds of crested wheatgrass (*Agropyron cristatum*) were germinated on a slant board made of Plexiglas lined with paper towels, immersed in tap water, and covered on a lab bench. Eight 2-liter hydroponic bottles were planted; the nutrient solution used included all essential nutrients, plus Si, 100mM HEDTA (a chelating agent), and 1 mM MES buffer according to the nutrient solution criteria discussed by Bugbee (1995). Plants were supported in the bottles with foam plugs that were cut in half with a cross-sectional wedge removed. An air pump was used to aerate the root zones via tubes of uniform length running to the bottom of the hydroponic bottles. Tubes were tightly fitted to the manifold to prevent leaks. All bottles were kept in a growth chamber (25^{o}C) and periodically rotated in terms of position and bubbling tube. Once the seedlings had been transplanted into the hydroponic bottles and were well established (after 25 days), plant fresh mass was measured on a microgram balance. Before weighing, roots were blotted on an absorbent pad to remove as much excess water as possible.

Three Cs treatments were induced on 2 replicate plants immediately following plant fresh mass measurements:

1. Treatment 1 (equimolar Cs and K): 250 μM K^+ and 250 μM Cs^+;
2. Treatment 2 (high Cs): 250 μM K^+ and 1 mM Cs;

3. Treatment 3 (low K): 100 μM K^+ and 250 μM Cs^+;

4. Control: 4.7 μM K^+ and 0 Cs^+

Cesium was added as CsCl. Treatment nutrient solutions were replaced daily to maintain the low K^+ concentrations in the 3 treatments. Plant fresh mass was measured again after 4 days immediately before harvest. The average of the relative growth rates for each treatment was calculated using the formula $(\ln (M_1/M_2))/\Delta t$ where M is fresh mass and t is time. After treatment for 4 days, the shoots for all plants were harvested, dried, and analyzed for Cs and K concentrations using an ICP-MS.

Results

No symptoms of K stress, such as wilting and yellowing, were observed and shoots appeared healthy (Fig. 44). The appearance of brown roots was attributed to

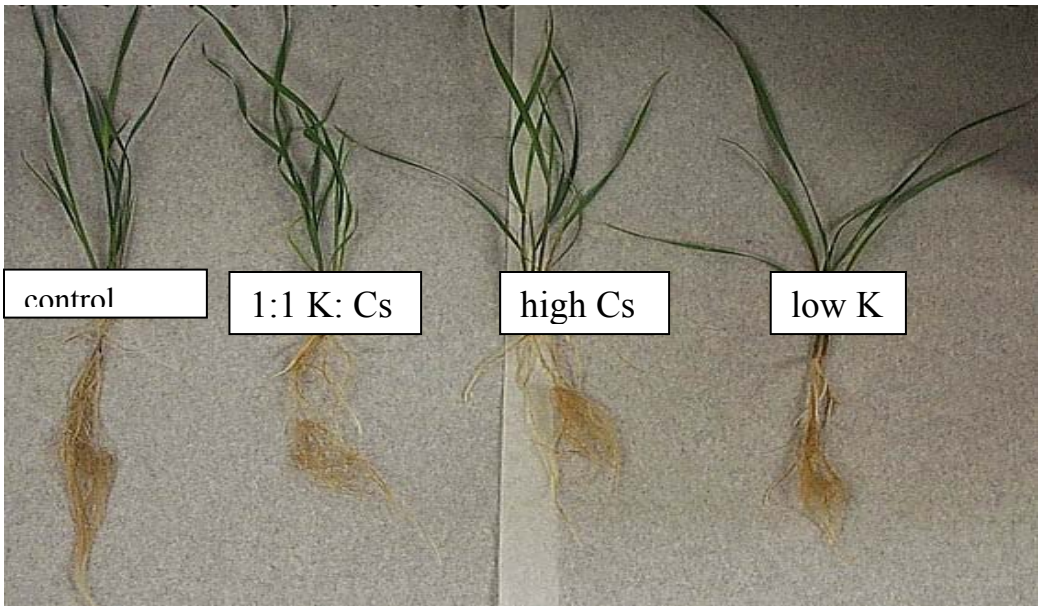

Fig. 44. One replicate of each treatment immediately before harvest.

stress experienced before treatment as the newest roots were growing well. Figure 45 shows the relative growth rate for each treatment. RGR was significantly lower than control for plants grown in the high Cs (Treatment 2) and low K (Treatment 3) solutions.

Concentrations of K and Cs in μg/g dry weight of the plant shoots were determined for each treatment (Figures 46 and 47). The K content was lower than the control in all treatments. Cs content was highest in 1 mM Cs solution (Treatment 2) and second highest in the low K^+ solution (Treatment 3).

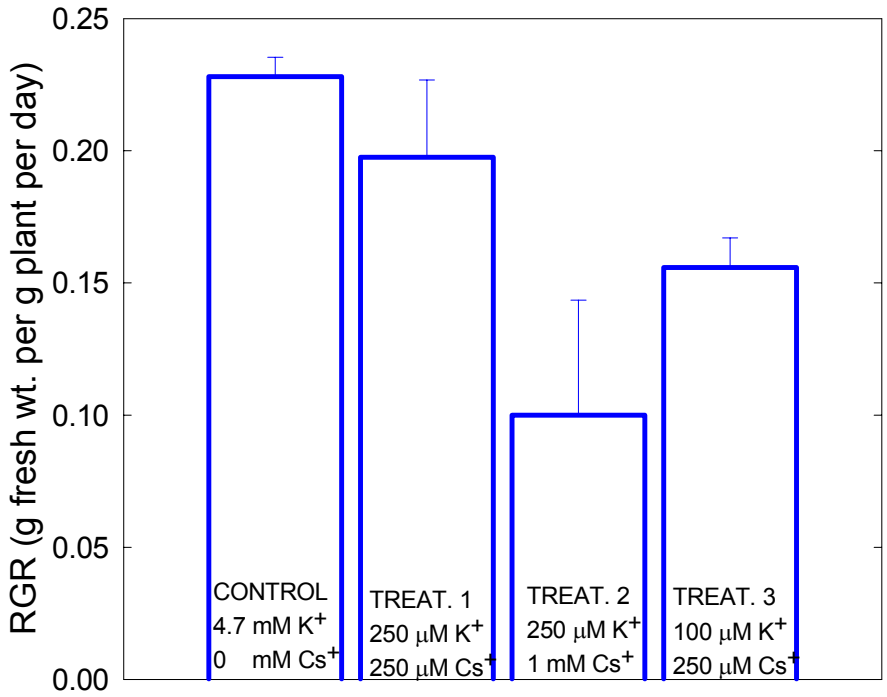

Fig. 45. Relative growth rate (RGR) for each treatment over the 4-day treatment period.

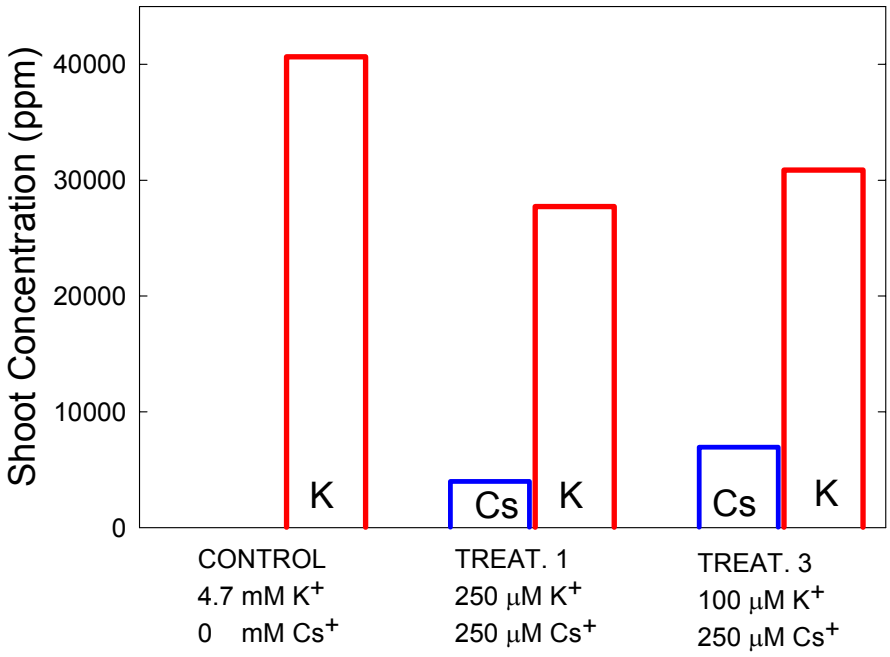

Fig. 46. Effect of decreasing nutrient solution K on shoot K and Cs concentrations.

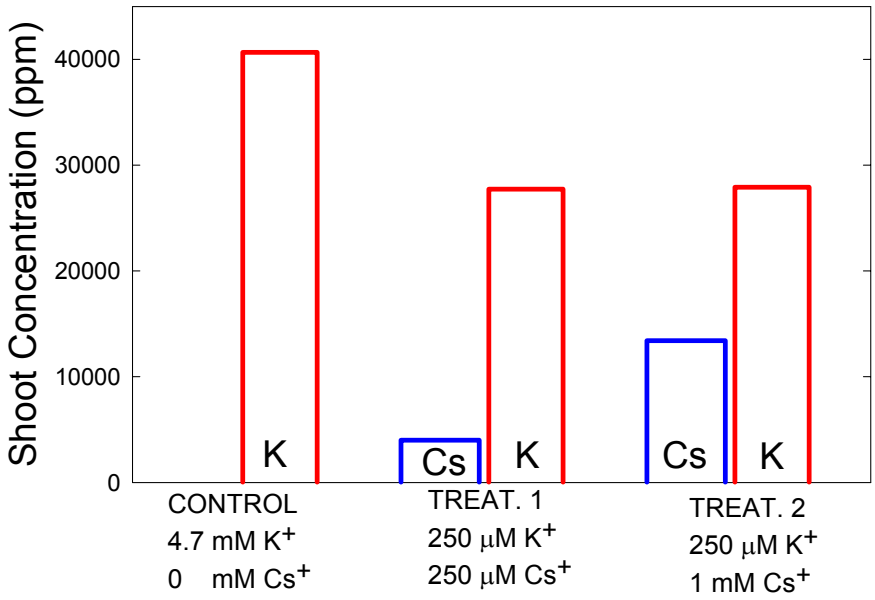

Fig. 47. Effects of increasing nutrient solution Cs on shoot K and Cs concentrations.

Discussion

Plants were supplied with excess K for the first 25 days. Thus, the primary uptake mechanism would have been K^+ channels. Cs uptake was higher than expected for a plant relying on channels for K uptake which discriminate against Cs. The high Cs uptake observed suggested that the plants were relying on high affinity transporters which discriminate less against Cs. Since the treatments were only applied for 4 days, the uptake of Cs suggests a rapid turnover of K uptake mechanisms from low affinity to high affinity, depending on solution K concentration.

Based on the shoot concentrations, Cs uptake did not inhibit growth by reducing K uptake. All shoot treatments contained about the same K concentration but varying Cs concentrations. Although shoot K concentrations in all treatments were lower than the control, this appears to be more of a function of lower K in solution (250 μM vs. 4.7 mM) than increased Cs uptake. Whole plant concentrations of Cs and K may reveal a different interaction between the two elements, particularly if K concentrations in roots were affected by increased Cs uptake. Higher Cs in the shoots corresponded with lower RGRs (Figs. 45-47). It is possible that Cs interrupts plant function after being taken up rather than by interfering with K uptake. This interference could be competition with K inside the plant, for example insufficient enzyme activation by a Cs replacement. Pfeffer et al. (1992) used NMR spectroscopy to show that transport of Cs into the vacuole through the tonoplast is slower than transport into the cytoplasm through the plasma membrane. Furthermore, this study reported that vacuolar Cs does not exchange readily with K, whereas cytoplasmic Cs does, and concluded that Cs is prevented from moving

back into the cytoplasm. This reduced mobility of Cs as compared to K could affect cation-anion exchange within a plant and, in turn, functions like osmoregulation.

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APPENDIX G. Standard Operating Procedures

Sterile Plant Trial Setup

Wear gloves (sterile gloves for planting)
Sand

- For 20 columns: 1200 ml fine, 800 ml medium, 800 ml coarse
- Wash sand with DI water (in a beaker until water is no longer cloudy)
- Filter DI water
- Rinse sand with concentrated H_2O_2 twice (leave overnight in 90 C drying oven)
- Rinse sand with filtered DI water

Glassware (columns, vials, nutrient solution flasks, dilution water bottles, drain tubes)

- Wash all glassware with DI water and methanol (see protocol)
- Rinse with filtered DI
- Bake in 170 C drying oven overnight

Non-glassware (caps, stoppers, glass wool)

- Rinse with DI water and methanol
- Rinse with filtered water
- Bake 45 minutes in 80 C oven

Column packing

- Insert glass wool into drain tubes
- Insert stoppers into columns
  Measure and mix sand (through sieve) for corresponding layers
- Fill columns with sand
- Load with 250 μM Fe solution until solution coming out is same color (make solution with filtered DI water)
- Raise pH by rinsing column with nutrient solution
- Attach air hose

Sterilizing components

- Columns: 2 x 1 hour, 24 hours apart. Dry cycle. } on campus
- Column tops (with foam plugs in top): 45 min, dry cycle } on campus
- Vials: do first set in sterilizer with 2-holed stoppers, next overnight in 170 C oven
- Vials of planting sand: 2 x overnight in 170 C oven, 24 hours apart
- Caps, nutrient solution, dilution water: 1 hour in sterilizer at pressure
- Air filters: with middle hose attached, in sterilizer 45 min at pressure

Seed sterilization

- Soak seeds in tap water 30 min, discard floaters

- Soak 1 hour in 20% Clorox, .01% Tween 80 solution. On shaker.
- Plate ~30 seeds on 1/10th strength nutrient agar (1.5% agar), 3 plates
- Germinate in 25 C incubator

Laminar Flow Hood
- Clean interior with 70% ethanol
- Test airflow with anemometer (should be about 29 meters/min)
- Clean racks with ethanol, store in hood

Sterilizer
- Clean out interior
- Re-apply vacuum grease to lip

Nutrient solution
- Prepare according to recipe (control and treatments)
- Filter
- Store in clean amber jugs

Planting
- Turn on airflow (very low, <5 ml/min per column)
- Take column out of sterilizing container
- Wrap foil around column, put in sterilized rack
- Attach filter
- Saturate with nutrient solution though flamed lip
- Add seed with flamed forceps
- Bury with vial of planting sand
- Attach upper column

TOC analyzer
- Remake standards
- Calibrate
- Needle, new range

Buy Supplies
- Needles
- Syringes
- Gloves
- Air & syringe-tip filters
- 70% ethanol
- methanol

Phoenix 8000 Total Organic Carbon Analyzer Instructions for Use

Prepared by Julie Chard
Before Starting, do the following:

1. Empty waste bucket.
2. Fill DI bottle at rear of instrument with fresh DI.
3. Make new persulfate reagent (blue line tube - if more than one week old).
4. Make new phosphoric acid solution (red line tube - if more than one month old).
5. Make new standards (if more than one month old). Store in refridgerator.
6. restart the computer after every 100 samples

STARTUP

1. Open Phoenix 8000 software from desktop.
2. Under "Setup" select "Instrument".
3. Click on the circle next to "Ready" to take instrument out of standby mode.
4. Exit to main screen.
5. Check to make sure that the UV lamp turned on.
6. Under "Results" select "Sample Blanks Review".
7. Highlight the row entitled "TC Blanks Range 2" and click on the "Clear" button.
8. Exit to main screen.
9. Click on "Run" to open the Run Screen. This screen has 3 windows:
    a. Sample Analysis – shows what sample is running and has Start, Exit, etc. buttons.
    b. Strip Chart – shows instrument response graphically.
    c. Analysis Results – shows results from the most recent analyses.
10. Click on the "Sample Setup" button.
11. Under "File" select "New". You should get a blank Sample Setup screen.
12. Begin filling in your sample setup:
    a. ALWAYS start with 6 Cleans followed by 5 or 6 Blanks.
    ** NEVER run more than six reps of a single Clean (sample gets too hot)**
    b. Run a plain DI water sample at the beginning and end of your samples.
    c. Run a sample of one of your standards at least at the beginning and end of your samples.
    d. Use the down arrow key to get a new row. Use the Insert and Delete buttons to insert and delete rows.

Example sample setup:

Pos	Sample ID	Sample Type	Method ID	Reps
1001	Clean	Sample	Cleaning Procedure	6
1002	Blank	Blank TC Range 2	Blank TC Range 2	3
1	DI-1	Sample	Simult TOC Range 0.1 – 20 ppm C	2
2	5 ppm standard	Sample	Simult TOC Range 0.1 – 20 ppm C	2
3	20 7/8 1:4	Sample	Simult TOC Range 0.1 – 20 ppm C	1
4	20 7/10 1:3	Sample	Simult TOC Range 0.1 – 20 ppm C	1
5	20 7/12 1:3	Sample	Simult TOC Range 0.1 – 20 ppm C	1
6	DI-2	Sample	Simult TOC Range 0.1 – 20 ppm C	2
7	5 ppm standard	Sample	Simult TOC Range 0.1 – 20 ppm C	2
1001	Clean	Sample	Cleaning Procedure	6
1003	No sample	Sample	Simult TOC Range 0.1 – 20 ppm C	3

NOTE: 25 ml is the minimum sample volume for 1 rep. 37 ml needed for 3 reps.

13. Check that your samples in the autosampler match the position number in the setup file. Position #1 is the back left-hand corner. Position #7 is the front left-hand corner. Position #8 is in the back, to the right of Position #1.
14. When done filling in the sample setup, click on the "Save/Use" button. Save the file as your initials and the date (for example, ah073101 for Amelia Henry, July 31, 2001).
15. You should be back to the Run Screen now. Press the "Start" button to begin running.
16. Stay and watch the first few Cleans to be sure the instrument is working properly.
17. When the run is complete, the results will print out automatically.

REAGENT PREPARATION

Prepare fresh acid reagent *once per month*:

- 188 mL DI water + 37 mL concentrated (85%) phosphoric acid

Prepare fresh persulfate reagent *once per week*:

- 213 mL DI water + 25 g sodium persulfate ($Na_2S_2O_8$) + 9 mL concentrated (85%) phosphoric acid

STANDARD PREPARATION
Make 1, 5, 10, and 20 ppm standards from 1000 ppm stock solution in amber vial with green cap.
Use your DI water for the 0 ppm standard.

	Standard Concentration			
Reagent to add	*1 ppm*	*5 ppm*	*10 ppm*	*20 ppm*
1000 ppm stock	0 mL	1.25 mL	2.5 mL	5 mL
20 ppm standard	12.5 mL	0 mL	0 mL	0 mL
water	237.5 mL	248.75 mL	247.5 mL	245 mL

CREATING A NEW STANDARD CURVE

1. Under "Setup" select "Calibration ►". Select "Standards...".
2. In the Standards screen, click on "Save As" and enter the name of the new calibration curve you are about to create (for example, ah073101). Click on the "OK" button.
3. Under "Setup" select "Calibration ►". Select "Set Active". Set your new curve as the active curve for all analytical ranges.
4. Click on "Run".
5. Click on the "Sample Setup" button.
6. Under "File" select "New". You should get a blank Sample Setup screen.
7. Fill in your sample setup for the calibration curve:
    a. Run 6 cleans at the beginning and at the end of the run.
    b. Under "Sample Type" when you select "TOC Standard" another screen will come up. Select the "0.1 - 20 ppm C" range. Another screen will come up. Select the standard from the list (highlight the row) that corresponds to the concentration of the standard you are running. Click the "Exit" button. You will have to repeat this for each standard concentration.
    c. Once you have completed the previous step, "TOC Range 0.1 – 20 ppm C" should automatically appear in the "Method ID" column of that row.
    d. Fill each vial up to the shoulder so that you can run two reps of each standard.

Sample standard curve sample setup:

Pos	Sample ID	Sample Type	Method ID	Reps
1001	Clean	Sample	Cleaning Procedure	6
1	0 ppm	TOC Standard	TOC Range 0.1 – 20 ppm C	2
2	1 ppm	TOC Standard	TOC Range 0.1 – 20 ppm C	2
3	5 ppm	TOC Standard	TOC Range 0.1 – 20 ppm C	2
4	10 ppm	TOC Standard	TOC Range 0.1 – 20 ppm C	2
5	20 ppm	TOC Standard	TOC Range 0.1 – 20 ppm C	2
1001	Clean	Sample	Cleaning Procedure	6

8. When the run is complete, under “Results” click on “Calibration”. You will now see a screen that shows a graph in the upper part of the screen and lists results in the lower part of the screen.
9. Verify that the Calibration Curve ID is the same as the one you entered in step 2.
10. Mark the samples you want to use in your calibration curve by clicking on the boxes next to them in the “Use” column (left-hand side of the results table).
11. Click on the “Recalc” button.
12. Verify that the r^2 value for your curve is 0.998 or greater. If your r^2 value is too low, make new standards and start over.
13. Click on the ”OK” button.
14. You should now be ready to run samples using your new curve.

MISCELLANEOUS

To print out the results of a run that is currently running:

1. Under “Results” select “Multiple Analyses”.
2. Under “File” select “Print Detailed Report”.

To print out the results of a previous run:

1. Under “Results” select “Multiple Analyses”.
2. Under “File” select “Open”
3. Scroll down until you reach the desired .prn file. The filenames begin with the date of the run and end with the time the run was completed (for example: 08131043.prn is the run started August 18 at 10:43 am). Select the desired file and open it.
4. Under “File” select “Print Detailed Report”.

To adjust the baseline:

1. Remove the front panel on the TOC analyzer. This will require a flat-head screwdriver and the removal of four screws.
2. Adjust the baseline up or down by turning the Zero screw. DO NOT adjust the Span screw.
3. Replace the front panel.

Washing Glassware and Plastics for use in Total Organic Carbon Analysis

Prepared by Julie Chard

For glass items (e.g. vials or columns):

1. Remove labeling.
2. Empty contents of glassware.
3. Fill glassware to 1/3 volume with deionized water (DI).
4. Shake glassware with DI to remove organic matter adhered to glassware walls.
5. Dump out DI.
6. Fill glassware to 1/3 volume with methanol (MeOH).
7. Shake glassware with MeOH.
8. Dump MeOH into an approved waste container.
9. Fill glassware to 1/3 volume with deionized water (DI).
10. Shake glassware with DI to remove MeOH.
11. Dump out DI and re-rinse with DI.
12. Bake glassware in 170^{o}C oven for a minimum of two hours.

For non-glass items (e.g. caps or lids):

1. Rinse with DI water.
2. Rinse with MeOH.
3. Rinse twice more with DI water.
4. Place rinsed plasticware in a paper bag.
5. Heat at 80^{o}C for 30 minutes or until dry (DO NOT heat longer than a few hours).

Phenolic Aniline Blue for Rhizoplane Stain

1. Prepare 5% aqueous phenol: add 11.5 ml of 100% phenol to 188.5 ml DI H_2O. Store bottle in room 133.

2. Prepare Phenolic Aniline Blue: In fume hood, mix 1 ml 6% aqueous aniline blue with 15 ml aqueous phenol. Add 4 ml glacial acetic acid. Store bottle in room 133.

3. Stain root: excise a small root segment (near root tip) with a sterile razor in the laminar flow hood. Place root segment in a covered beaker containing ~50 ml phenolic aniline blue for 3 minutes. Remove root segment with sterile forceps and rinse with sterile filtered water.

4. Prepare microscope slide: mount root segment in sterile filtered water and cover with #1 cover slip. Seal edges with clear nail polish.

5. Waste: pour used phenolic aniline blue in the labeled waste container in fume hood (room 144).

APPENDIX H. Statistical Analyses

Shoot dry mass (g)

One Way Analysis of Variance
Normality Test: Passed (P > 0.200)
Equal Variance Test: Passed (P = 0.346)

Group Name	N	Missing	Mean	Std Dev	SEM
control	6	0	1.782	0.605	0.247
K^+	2	0	2.005	0.191	0.135
NH_4+	2	0	2.495	0.0636	0.0450
drought	2	0	1.495	0.177	0.125
flood	2	0	2.420	0.806	0.570

Source of Variation	DF	SS	MS	F	P
Between Groups	4	1.623	0.406	1.431	0.300
Residual	9	2.550	0.283		
Total		13	4.173		

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.300).
Power of performed test with alpha = 0.050: 0.110
The power of the performed test (0.110) is below the desired power of 0.800.
You should interpret the negative findings cautiously.

Cumulative TOC exuded from day 11 - day 32 (before treatments applied)

One Way Analysis of Variance
Normality Test: Passed (P > 0.200)
Equal Variance Test: Passed (P = 0.036)

Group Name	N	Missing	Mean	Std Dev	SEM
control	6	0	795.899	173.030	70.639
K^+	2	0	1030.730	2.827	1.999
NH_4^+	2	0	1112.621	135.730	95.976
drought	2	0	984.028	27.866	19.705
flood	2	0	978.494	246.257	174.130

Source of Variation	DF	SS	MS	F	P
Between Groups	4	205356.067	51339.017	2.013	0.176
Residual	9	229546.803	25505.200		
Total	13	434902.870			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.176).

Power of performed test with alpha = 0.050: 0.206
The power of the performed test (0.206) is below the desired power of 0.800.
You should interpret the negative findings cautiously.

Cumulative TOC from day 35-day 68 (after treatments applied)

One Way Analysis of Variance

Normality Test: Passed (P = 0.131)

Equal Variance Test: Passed (P = 0.255)

Group Name	N	Missing	Mean	Std Dev	SEM
control	6	0	4868.705	2404.471	981.621
K^+	2	0	7992.298	2261.047	1598.802
NH_4^+	2	0	5862.814	346.102	244.731
drought	2	0	6853.750	25.645	18.134
flood	2	0	9481.087	597.284	422.344

Source of Variation	DF	SS	MS	F	P
Between Groups	4	39115111.255	9778777.814	2.551	0.112
Residual	9	34496922.499	3832991.389		
Total	13	73612033.754			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.112).

Power of performed test with alpha = 0.050: 0.304

The power of the performed test (0.304) is below the desired power of 0.800.
You should interpret the negative findings cautiously.

Cumulative mg C exuded per gram dry shoot

One Way Analysis of Variance

Normality Test: Passed (P > 0.200)

Equal Variance Test: Passed (P = 0.280)

Group Name	N	Missing	Mean	Std Dev	SEM
control	6	0	2.624	0.620	0.253
K^+	2	0	3.950	0.752	0.531
NH_4^+	2	0	2.349	0.0788	0.0557
drought	2	0	4.618	0.563	0.398
flood	2	0	4.104	1.120	0.792

Source of Variation	DF	SS	MS	F	P
Between Groups	4	10.151	2.538	5.616	0.015
Residual	9	4.067	0.452		
Total		13	14.217		

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.015).

Power of performed test with alpha = 0.050: 0.766

The power of the performed test (0.766) is below the desired power of 0.800.
You should interpret the negative findings cautiously.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
drought vs. NH_4^+	2.269	5	4.773	0.049	Yes
drought vs. control	1.994	5	5.137	0.034	Yes
drought vs. K^+	0.667	5	1.404	0.852	No
drought vs. flood	0.513	5	1.080	0.935	Do Not Test
flood vs. NH_4^+	1.756	5	3.693	0.149	No
flood vs. control	1.480	5	3.814	0.132	Do Not Test
flood vs. K^+	0.154	5	0.324	0.999	Do Not Test
K^+ vs. NH_4^+	1.602	5	3.369	0.205	Do Not Test
K^+ vs. control	1.326	5	3.417	0.195	Do Not Test
control vs. NH_4^+	0.275	5	0.710	0.985	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

Cumulative TOC days 35-42

One Way Analysis of Variance

Normality Test: Passed (P > 0.200)

Equal Variance Test: Passed (P = 0.715)

Group Name	N	Missing	Mean	Std Dev	SEM
Control	6	0	624.428	429.700	175.424
K^+	2	0	848.137	107.487	76.005
NH_4^+	2	0	1221.136	317.199	224.294
drought	2	0	483.716	19.191	13.570
flood	2	0	1518.176	349.935	247.441

Source of Variation	DF	SS	MS	F	P
Between Groups	4	1742022.963	435505.741	3.384	0.060
Residual	9	1158201.512	128689.057		
Total	13	2900224.476			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.060).

Power of performed test with alpha = 0.050: 0.454

The power of the performed test (0.454) is below the desired power of 0.800.
You should interpret the negative findings cautiously.

Cumulative TOC days 45-51

One Way Analysis of Variance

Normality Test: Passed (P > 0.200)

Equal Variance Test: Passed (P = 0.381)

Group Name	N	Missing	Mean	Std Dev	SEM
control	6	0	938.757	587.228	239.735
K^+	2	0	2101.923	959.907	678.757
NH_4^+	2	0	1456.631	189.662	134.111
drought	2	0	2079.419	85.660	60.571
flood	2	0	3019.052	467.258	330.402

Source of Variation	DF	SS	MS	F	P
Between Groups	4	7634177.916	1908544.479	5.908	0.013
Residual	9	2907243.798	323027.089		
Total	13	10541421.713			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.013).

Power of performed test with alpha = 0.050: 0.794

The power of the performed test (0.794) is below the desired power of 0.800.
You should interpret the negative findings cautiously.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
flood vs. control	2080.295	5	6.340	0.010	Yes
flood vs. NH_4^+	1562.421	5	3.888	0.122	No
flood vs. drought	939.633	5	2.338	0.504	Do Not Test
flood vs. K^+	917.129	5	2.282	0.525	Do Not Test
K^+ vs. control	1163.166	5	3.545	0.172	No
K^+ vs. NH_4^+	645.292	5	1.606	0.785	Do Not Test
K^+ vs. drought	22.504	5	0.0560	1.000	Do Not Test
drought vs. control	1140.662	5	3.476	0.184	Do Not Test
drought vs. NH_4^+	622.788	5	1.550	0.805	Do Not Test
NH_4^+ vs. control	517.874	5	1.578	0.795	Do Not Test

Cumulative TOC days 53-57

One Way Analysis of Variance
Normality Test: Passed (P > 0.200)
Equal Variance Test: Passed (P = 0.417)

Group Name	N	Missing	Mean	Std Dev	SEM
Control	6	0	1388.098	813.269	332.016
K^+	2	0	2668.283	781.371	552.513
NH_4^+	2	0	1868.612	501.685	354.745
drought	2	0	2733.357	377.902	267.217
flood	2	0	2740.067	90.243	63.811

Source of Variation	DF	SS	MS	F	P
Between Groups	4	5336584.342	1334146.086	2.779	0.093
Residual	9	4320217.919	480024.213		
Total	13	9656802.262			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.093).
Power of performed test with alpha = 0.050: 0.346
The power of the performed test (0.346) is below the desired power of 0.800.
You should interpret the negative findings cautiously.

Cumulative TOC days 59-63

One Way Analysis of Variance
Normality Test: Passed (P > 0.200)
Equal Variance Test: Passed (P = 0.811)

Group Name	N	Missing	Mean	Std Dev	SEM
control	6	0	1176.595	472.035	192.707
K^+	2	0	1386.317	268.574	189.911
NH_4^+	2	0	825.876	141.180	99.829
drought	2	0	1557.259	247.405	174.942
flood	2	0	2203.792	389.718	275.572

Source of Variation	DF	SS	MS	F	P
Between Groups	4	2275494.325	568873.581	3.607	0.051
Residual	9	1419237.894	157693.099		
Total	13	3694732.220			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.051).
Power of performed test with alpha = 0.050: 0.493
The power of the performed test (0.493) is below the desired power of 0.800.
You should interpret the negative findings cautiously.

RGR before stress (days 0-35)

One Way Analysis of Variance

Normality Test: Passed (P = 0.082)

Equal Variance Test: Passed (P = 0.300)

Group Name	N	Missing	Mean	Std Dev	SEM
Control 6	0		0.118	0.0174	0.00711
K^+	2	0		0.132	0.00172 0.00121
NH_4^+	2	0		0.138	0.00195 0.00138
drought 2	0		0.131	0.00358	0.00253
flood	2	0		0.128	0.0152 0.0108

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.000863	0.000216	1.099	0.414
Residual	9	0.00177	0.000196		
Total		13	0.00263		

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.414).

Power of performed test with alpha = 0.050: 0.063

The power of the performed test (0.063) is below the desired power of 0.800.
You should interpret the negative findings cautiously.

RGR after stress (days 35-70)

One Way Analysis of Variance

Normality Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
control	6	0	0.0893	0.0612	0.0999
K^+	2	0	0.0587	0.0576	0.0599
NH_4^+	2	0	0.0590	0.0560	0.0620
drought	2	0	0.0549	0.0542	0.0556
flood	2	0	0.0659	0.0594	0.0725

H = 8.895 with 4 degrees of freedom. (P = 0.064)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.064)

Day 42 μg C per gram new plant

One Way Analysis of Variance

Normality Test: Passed (P > 0.200)

Equal Variance Test: Passed (P = 0.580)

Group Name	N	Missing	Mean	Std Dev	SEM
control	6	0	1770.017	1446.295	590.447
K^+	2	0	1317.723	65.517	46.328
NH_4^+	2	0	1938.460	1068.312	755.411
drought 1	2	0	999.500	154.660	109.361
flood	2	0	2616.160	2014.686	1424.598

Source of Variation	DF	SS	MS	F	P
Between Groups	4	3072808.685	768202.171	0.441	0.777
Residual	9	15687304.142	1743033.794		
Total	13	18760112.827			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.777).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800.
You should interpret the negative findings cautiously.

Day 51 μg C per gram new plant

One Way Analysis of Variance

Normality Test: Passed (P > 0.200)

Equal Variance Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
control	6	0	1099.930	955.751	1237.150
K^+	2	0	4376.509	3374.921	5378.098
NH_4^+	2	0	1604.869	891.510	2318.228
drought	2	0	3439.480	3138.878	3740.082
flood	2	0	2763.765	2162.377	3365.153

H = 9.638 with 4 degrees of freedom. (P = 0.047)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.047)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
K^+ vs control	8.667	2.537	No
K^+ vs NH_4^+	7.500	1.793	Do Not Test
K^+ vs flood	3.500	0.837	Do Not Test
K^+ vs drought	1.500	0.359	Do Not Test
drought vs control	7.167	2.098	Do Not Test
drought vs NH_4^+	6.000	1.434	Do Not Test
drought vs flood	2.000	0.478	Do Not Test
flood vs control	5.167	1.513	Do Not Test
flood vs NH_4^+	4.000	0.956	Do Not Test
NH_4^+ vs control	1.167	0.342	Do Not Test

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Day 57 μg C per gram new plant

One Way Analysis of Variance

Normality Test: Passed (P > 0.200)

Equal Variance Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
control	6	0	1727.917	1523.392	1842.263
K^+	2	0	2106.583	2030.660	2182.505
NH_4^+	2	0	1300.660	859.222	1742.099
drought	2	0	5658.274	4545.963	6770.586
flood	2	0	2926.775	2244.969	3608.580

H = 9.314 with 4 degrees of freedom. (P = 0.054)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.054)

Day 63 μg C per gram new plant

One Way Analysis of Variance

Normality Test: Passed (P > 0.200)

Equal Variance Test: Passed (P = 0.514)

Group Name	N	Missing	Mean	Std Dev	SEM
Control	6	0	1430.436	251.137	102.526
K^+	2	0	1478.409	208.529	147.452
NH_4^+	2	0	827.016	98.212	69.447
drought	2	0	2947.433	101.897	72.052
flood	2	0	2036.566	235.730	166.686

Source of Variation	DF	SS	MS	F	P
Between Groups	4	5368005.066	1342001.267	27.802	<0.001
Residual	9	434431.172	48270.130		
Total	13	5802436.239			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
drought vs. NH_4^+	2120.417	5	13.649	<0.001	Yes
drought vs. control	1516.998	5	11.959	<0.001	Yes
drought vs. K^+	1469.024	5	9.456	<0.001	Yes
drought vs. flood	910.867	5	5.863	0.016	Yes
flood vs. NH_4^+	1209.550	5	7.786	0.003	Yes
flood vs. control	606.130	5	4.778	0.049	Yes
flood vs. K^+	558.157	5	3.593	0.164	No
K^+ vs. NH_4^+	651.393	5	4.193	0.090	No
K^+ vs. control	47.973	5	0.378	0.999	Do Not Test
control vs. NH_4^+	603.419	5	4.757	0.050	Do Not Test

Trial 7 percent root

One Way Analysis of Variance
Normality Test: Passed (P > 0.200)
Equal Variance Test: Passed (P = 0.100)

Group Name	N	Missing	Mean	Std Dev	SEM	
control	4	0	26.870	9.407	4.704	
K^+	2	0	25.643	0.184	0.130	
NH_4^+	2	0	35.116	12.760	9.022	
drought	3	0	22.181	6.769	3.908	
flood	2	0	13.658	4.254	3.008	

Source of Variation	DF	SS	MS	F	P
Between Groups	4	500.229	125.057	1.859	0.211
Residual	8	538.061	67.258		
Total	12	1038.290			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.211).
Power of performed test with alpha = 0.050: 0.172
The power of the performed test (0.172) is below the desired power of 0.800.
You should interpret the negative findings cautiously.

Cumulative μg oxalic acid

One Way Analysis of Variance
Normality Test: Passed (P = 0.032)
Equal Variance Test: Passed (P = 0.946)

Group Name	N	Missing	Mean	Std Dev	SEM	
control	6	0	77.918	131.442	53.661	
K^+	2	0	64.050	75.717	53.540	
NH_4^+	2	0	14.259	20.165	14.259	
drought	2	0	139.930	64.523	45.625	
flood	2	0	194.266	97.912	69.234	

Source of Variation	DF	SS	MS	F	P
Between Groups	4	40349.398	10087.350	0.854	0.526
Residual	9	106274.526	11808.281		
Total	13	146623.925			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.526).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800.
You should interpret the negative findings cautiously.

Cumulative μg malonic acid

One Way Analysis of Variance
Normality Test: Failed (P = <0.001)
Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
control	6	0	0.000	0.000	12.565
K^+	2	0	15.363	14.359	16.368
NH_4^+	2	0	0.000	0.000	0.000
drought	2	0	67.487	55.872	79.102
flood	2	0	99.051	96.380	101.722

H = 7.346 with 4 degrees of freedom. (P = 0.119)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.119)

Cumulative μg fumaric acid

One Way Analysis of Variance
Normality Test: Failed (P = 0.004)
Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
control	6	0	8.676	0.000	21.032
K^+	2	0	29.572	28.581	30.562
NH_4^+	2	0	10.735	0.000	21.470
drought	2	0	307.521	295.404	319.637
flood	2	0	6.351	5.775	6.927

H = 7.157 with 4 degrees of freedom. (P = 0.128)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.128)

Cumulative μg succinic acid

One Way Analysis of Variance

Normality Test: Passed (P > 0.200)

Equal Variance Test: Passed (P = 0.653)

Group Name	N	Missing	Mean	Std Dev	SEM
control	6	0	73.759	69.394	28.330
K^+	2	0	124.290	17.912	12.666
NH_4^+	2	0	50.568	28.678	20.278
drought	2	0	356.308	90.125	63.728
flood	2	0	83.364	43.926	31.061

Source of Variation	DF	SS	MS	F	P
Between Groups	4	136870.031	34217.508	8.731	0.004
Residual	9	35272.867	3919.207		
Total	13	172142.898			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.004).

Power of performed test with alpha = 0.050: 0.947

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
drought vs. NH_4^+	305.740	5	6.907	0.006	Yes
drought vs. control	282.549	5	7.817	0.003	Yes
drought vs. flood	272.944	5	6.166	0.012	Yes
drought vs. K^+	232.018	5	5.241	0.030	Yes
K^+ vs. NH_4^+	73.722	5	1.665	0.764	No
K^+ vs. control	50.531	5	1.398	0.854	Do Not Test
K^+ vs. flood	40.926	5	0.925	0.962	Do Not Test
flood vs. NH_4^+	32.796	5	0.741	0.983	Do Not Test
flood vs. control	9.605	5	0.266	1.000	Do Not Test
control vs. NH_4^+	23.191	5	0.642	0.990	Do Not Test

APPENDIX I

Permission Letter

April 25, 2003

Dear Dr. Scott Jones,

I am writing to ask your permission to include you as a co-author of my paper "Design and maintenance of an axenic plant culture system to facilitate optimal growth in long-term studies," which will be included as chapter 2 in my Master's thesis in the Department of Plants, Soils, and Biometeorology at Utah State University.

Thank you,

Amelia Henry

Amelia Henry

I hereby give permission to Amelia Henry to include me as a co-author of the following chapter of her thesis: Chapter 2. Design and maintenance of an axenic plant culture system to facilitate optimal growth in long-term studies.

Signed Scott B Jones

Dr. Scott Jones

Department of Plants, Soils, and Biometeorology