ORGANIC ACID CHANGES IN BERMUDAGRASS ROOT EXUDATES UNDER ALUMINUM STRESS

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CHAPTER I

INTRODUCTION

Specific aspects of plant rhizosphere have been poorly understood for centuries due to the complexity and involvement of the many biotic and abiotic factors. The concept of a rhizosphere was first introduced by Lorenz Hiltner (1904) and has been used extensively since then. After a century of research and development, the rhizosphere (Hartmann, 2005; Hinsinger and Marschner, 2006) is now described as a complex interface where soil, plant roots, and microbes interact with each other and where numerous molecular processes take place. Among those molecular processes, plant roots release a great variety of compounds into the rhizosphere and interact with beneficial and pathogenic microorganisms.

Almost all of the compounds contained in plant tissues can be released into the rhizosphere as root products except for chlorophyll and some other compounds involved in photosynthesis. These root products provide a driving force in the rhizosphere, not only for the plant itself, but also for the rhizosphere and the various microbial populations living in it. For soil, root products such as mucilage perform as glue between soil particles in the rhizosphere and lead to the formation of a soil aggregation structure. Other substances released from plant roots such as organic acids, proteins, enzymes, lipids, and carbohydrates are critical regarding to the adjustment of rhizosphere pH, chemical compositions, and act as indicators of plant physiological status.

According to literature, root products have been investigated for decades and plant rhizosphere research is becoming more prevalent (Hinsinger and Marschner, 2006). Along with the development of high-technology equipment, scientists have already analyzed and identified most of the components in root products under various conditions. Consequently, the functions and chemical characteristics of most of these detected root products are known due to their chemical characteristics. In addition, research has shown that factors such as plant age, nutrient condition, heavy metal toxicity, water supply, and light intensity can change the quantity and quality of root exudates. There are also theories about root exudation mechanisms explained at the physiological level including diffusion and ion-channels. However, the results of previous studies are not adequate to reveal all of the mysteries still present in the underground rhizosphere. This is due to the fact that root products are highly plant specific and environment dependent and in some cases questionable methodologies have been applied. There is a need to develop more applicable and functional experimental techniques to obtain a better understanding of root exudates in the plant rhizosphere.

Literature Review

Root Exudates

Root exudates are defined as the organic and inorganic substances released into surrounding medium by healthy and intact plant roots (Rovira, 1969; Uren, 2000; Jones et al., 2009; Whipps, 1990). Rovira et al. (1979) proposed the first nomenclature system of root exudates and classified five groups of all the organic materials released from plant roots. This nomenclature system has since been used extensively. According to this system, root exudates contain five groups according to their modality of release: 1. Exudates, 2. Secretions, 3. Plant

mucilages (including four sub-categories depending on different secreted locations), 4. Mucigels, and 5. Lysates.

Whipps (1990) provided an additional summary of root exudates according to their mode of expulsion which included: 1. Water-soluble exudates which are not involved with metabolic energy (e.g. sugars, amino acids, organic acids), 2. Secretions whose release depends on metabolic processes (e.g. polymeric carbohydrates and enzymes), 3. Lysates such as cell walls and sloughed cells, and 4. Gases such as ethylene and CO_2.

Besides the two systems listed above, there are additional types of root exudate classifications provided by researchers. For example, organic compounds in root exudates can be categorized by their chemical properties (e.g. stability, volatility, and molecular weight); or by the way they are used by microorganisms (Pinton et al., 2000). The classification based on their utilization by microorganisms is more relevant to the microbial ecology of the rhizosphere. The system based on the modality of release is more difficult because some mechanisms involved are still not clear. Even though, the original nomenclature system is still the most widely cited, recently, Jones et al. (2009) updated Rovira's nomenclature system and provided more complete information than the original system. According to Jones et al. (2009), exudates are the only group of materials which are passively excreted. Other than that, secretion, mucilage, border cells and senescence-derived compounds are all actively secreted into the rhizosphere. Jones's nomenclature system was adopted as the standard classification in this study, and organic acids which belong to "exudate" group were investigated as target components. Furthermore, when referring to "root exudates", the term always indicates all the compounds the plant root releases into the rhizosphere.

Factors Affecting Root Exudates

Numerous factors can affect the release of root exudates (Rovira, 1969; Neumann and Römheld, 2000; Curl and Truelove, 1986; Koo, 2001). They can be summarized into three groups: internal factors (plant species/cultivars, plant age); abiotic factors (plant physiology status, growth conditions) and biotic factors (microbe-plant root interactions).

To further explain each of the three groups, first of all, different plant species/cultivars and plant age exhibit large variations on the quantity and composition of root exudates. Kamilova et al. (2006) compared organic acid and sugar exudations among tomato (*Lycopersicon esculentum* L.), cucumber (*Cucumis sativus* L.) and sweet pepper (*Capsicum annuum* L.) during three different growth stages termed the seed stage (2 d after planting), seedling stage (4 d after planting), and root exudates stage (14 d after planting). Among the three vegetables, the total amount of organic acids and sugars in root exudates increased along with plant growth; and the organic acid fractions were all higher than the sugar fractions. However, dominant organic acids varied among plant species and along with plant development. Tomato roots exude citric acid in all three growth stages with a dramatic decrease in malic acid and increased exudation of succinic acid. Succinic acid was the dominant acid in cucumber during the seedling stage and citric acid was dominant during the seed and root exudate stages. Succinic acid was dominant during the seed stage in sweet pepper whereas citric acid was dominant during the seedling and root exudate stages.

Aulakh et al. (2001) studied ten rice cultivars (*Oryza sativa* L.) including three traditional cultivars, four high-yielding dwarf cultivars, two new-type cultivars and one hybrid cultivar during four growth stages (seedling, panicle initiation, flowering, and maturity). They found significant differences in individual organic acid exudation among different cultivars and during different plant growth stages. They demonstrated that the amount of malic acid decreased

significantly in six cultivars at maturity but remained at the same exudation level in the other four cultivars. Citric acid only appeared in five cultivars at the seedling stage, in eight cultivars at panicle initiation, and was not detected in the other two stages. The proportion of organic acids in total organic carbon (TOC) in the ten cultivars increased from 39 % at seedling stage to 67 % at maturity.

Plant physiological status (mostly when affected by stress) was concluded as the second most important factor influencing organic acid expulsion because it significantly affects the composition and amount of root exudates (Neumann and Römheld, 2000). For example, nutrient availability (e.g. P deficiency, Fe deficiency), soil physical and chemical properties (e.g. soil pH, texture, moisture), light intensity, water supplies (drought/flood), heavy metals (e.g. Cu, Zn), aluminum toxicity, and CO_2 concentration (Johnson et al., 1996; Hodge et al., 1997; Phillips et al., 2006) can affect root exudation. Curl and Truelove (1986) provided a summary about the effects of foliar sprays on root exudates, indicating that chemical compounds applied to shoot tissues can be transferred and to plant roots and affect the composition of root exudates.

Aside from the factors listed above, rhizosphere microbial activities also contribute to the variation in root exudate composition and quantity under natural environments. Rovira and Davey (1974) summarized four ways in which microorganisms may affect root exudates: (1) by affecting permeability of root cells; (2) by affecting metabolism of roots; (3) absorption of certain exuded compounds; and (4) by altering nutrient availability to the plant. However, due to the complex relationship between the plant root and rhizosphere microorganisms, there are no general rules for quantification. Microorganisms in the rhizosphere may degrade certain organic compounds in root exudates and result in inaccurate quantification. Jones and Darrah (1994) indicated that the rapid mineralization rate of citric acid was affected by soil microbial biomass and had an average half-life of 11.7 hr in soil solution. On the other hand, microbial colonization and metabolites can also stimulate root exudation. Meharg and Killham (1991) have shown

increased assimilation of ^{14}C by inoculated plant roots compared to plants growing under sterile conditions. More importantly, the increased carbon assimilation compensated the loss of carbon exuded from plant roots. The presence of microorganisms may result in carbon degradation and can also stimulate or metabolize root exudates or alter the amount of root exudates in the soil profile. In summary, because of the complex nature of the rhizosphere and the highly variable composition of root exudates, research should be conducted under well-controlled growth environments to better understand plant root exudate factors without the direct interference of soil microorganisms.

Major Functions of Organic Acids in Rhizosphere

Root exudates are known to be involved and play very important roles in many molecular processes in the rhizosphere. For example, they facilitate nutrient uptake (e.g. P and Fe), detoxify rhizosphere heavy metal stress (e.g. Mn and Cu); alleviate drought/flood stress, attract beneficial pathogens and regulate rhizosphere pH. Although the exudation/secretion rate varies for different plant species, the function of root exudates in plant self-defense and their influence on interactions among underground communities are critical.

Nutrient acquisition. When a plant suffers from nutrient deficiency, it may release various root exudates as chelators to facilitate nutrient uptake. Graminaceous species secreted Fe-chelator [phytosiderophores, (PS) to solubilize Fe^{3+} and increase the uptake of Fe to grass plants (Takagi, 1976; Ueno et al., 2007; Cesco et al., 2006). Similar strategy to facilitate nutrient availability is also applied to P deficiency, except different chelators are involved. When subjected to P deficiency, plants develop proteoid roots (dauciform roots) that exude large amounts of organic acids (e.g. citric acid and malic acid) that combine with insoluble P and increase P availability (Playsted et al., 2006; Johnson et al., 1996; Hoffland, 1992).

Metal detoxification. Root exudates are also important as metal chelators in the rhizosphere for metal detoxification. Among the different classes of root exudate components, amino acids and sugars appear to have little or no effect in complexation or mobilization of metal ions (Jones et al., 1994; Jones and Darrah, 1994). On the other hand, organic acids have the ability to form metal-organic acid complexes which are non-toxic or less toxic for plant uptake. Exuded organic acids are considered to be the most abundant and chemically active component of root exudates in the rhizosphere. They appear to have a major role in complexing metal ions and reducing their toxicity to (Jones and Darrah, 1994; Jones et al., 1996; Haydon and Cobbett, 2007).

In addition, mucilage (contains mainly polysaccharides) is one of the high molecular-weight compounds in root exudates, which can stabilize rhizosphere soil particles and help develop soil aggregation and soil structure (Watanabe et al., 2008; Jones et al., 2009). Mucilage is also believed to be a lubricant during root elongation reducing soil impedance (Iijima et al., 2003). Yet, the mechanisms and physiological functions of some root exudates are still a mystery and need to be investigated.

Aluminum Toxicity

Aluminum Toxicity in Soils

Aluminum toxicity is the term used when plants are growing in acid soils with a relatively high concentration of soluble aluminum, which prohibits plant growth. There are two major factors that contribute to aluminum toxicity in acid soils, aluminum concentration and soil acidity. Aluminum makes up about 8 % by weight of the Earth's solid surface and is the most abundant metal in the earth's crust. However, its toxicity to plant is low under alkaline conditions. Therefore, there is little concern of aluminum toxicity in alkaline soils even when the soil aluminum content is high. The dominant aluminum ion presents in soil pH > 6.3 is $Al(OH)_4^-$, which is non-toxic to plant growth. It widely believed that the ionic form of aluminum,

$Al(H_2O)_6^{3+}$ often expressed as Al^{3+} for is the most dominant phytotoxic aluminum ion rather than the hydroxyl aluminum ion, e.g. $Al(OH)^{2+}$, $Al(OH)_2^{+}$ or $Al(OH)_3^{0}$, when pH drops below 5.0 (Kinraide, 1990; Kinraide, 1991). Therefore, in acid soils (pH < 5.0) with high mineral contents, more solid-phase aluminum dissolves into soil solution as the soil pH decreases which results in a very high concentration of soluble Al^{3+}. This is how the aluminum toxicity occurs. Kinraide (1991) provided more detailed information about the composition of aluminum ion in soil solution as a function of pH. According to Kinraide (1991), the solubility of Al^{3+} decreases with increasing soil pH. About 90 % of total aluminum ions are present as soluble Al^{3+} in pH < 4.0 and there is no Al^{3+} activity in pH > 6.3.

Aluminum toxicity in soils has been well studied because the subject is of considerable importance to several scientific disciplines, especially crop science, environmental chemistry, and soil science. Aluminum phytotoxicity in acid soils has gained more and more attention because it can result in a significant reduction of crop production. According to Johnson et al. (1997), aluminum toxicity has become a major limiting factor for large wheat-producing areas in Oklahoma, Texas, and Kansas.

Aluminum toxicity targets the plant root apex. The exposure to even a micro concentration of aluminum can cause rapid inhibition of root growth (minutes to hours), with an inhibition of root cell elongation and expansion 6-24 hr after aluminum application (Samac and Tesfaye, 2003; Delhaize and Ryan, 1995; Čiamporová, 2002). For sensitive plants in general, it takes approximately 30 min to 2 hr for a statistically significant inhibition of root elongation to occur (Barceló and Poschenrieder, 2002). Although cell elongation and expansion are inhibited quickly, cell division is also inhibited by aluminum over the long term (Kochian et al., 2004). Detailed discussion about the mechanisms of aluminum toxicity can be found in Kochian (1995).

Ryan et al. (1993) concluded that only the apical 2-3 mm of maize roots exposed to 2 mM $AlCl_3$ solution inhibited root growth (shortening of meristem). Application of aluminum to all other root regions did not significantly affect root growth. As a result of aluminum toxicity, water and nutrient uptake by plants from soil is affected by an impaired root system that leads to a more drought sensitive plant (Samac and Tesfaye, 2003).

Besides the aluminum toxicity itself, there are always other nutrient deficiencies (e.g. P, K, Ca, Mg) along with aluminum stress (Foy, 1988; Shamsi et al., 2007; Rout et al., 2001) that confuse symptoms with essential nutrient deficiencies. On plant leaf tissues, aluminum stress is observed as yellowish leaves and dead leaf tips. Stunting and small plants are typical because excessive free aluminum in the soil combines with P and Fe making them unavailable for plant uptake resulting in P and Fe deficiency. Overall, it is much easier to characterize aluminum toxicity in plant root tissues than nutrient deficiencies because Al^{3+} binds with the cellular components of roots and mostly is not translocated to the upper parts of the plants.

Aluminum Tolerance Mechanisms

Over past decades, researchers have been trying to reveal the secrets about how some plants can tolerate toxic levels of aluminum. There have been many different hypotheses proposed but most of them have little support. Although there have been rigorous tests of these hypotheses, it has been widely accepted that there are two classes of mechanisms related to aluminum tolerance (Talyor, 1991; Kochian, 1995; Kochian et al., 2002; Ma, 2000; Barceló and Poschenrieder, 2002). The first one includes mechanisms that allow plants to contain aluminum in the symplast (internal tolerance). The other suggests mechanisms that prevent aluminum from entering the root apex (exclusion). Among both, specific organic acids are involved and play important roles (Fig. 1.1.).

The distinction between the two classes of mechanisms is the aluminum detoxification site. Internal tolerance mechanisms indicate the cases where aluminum enters the symplast while in the external tolerance mechanism category aluminum is prevented from entering the symplast.

As for the internal tolerance mechanisms, organic acid chelating with aluminum is of great importance according to the limited research results provided so far. Although the whole process of internal aluminum detoxification is still unclear until now, there is evidence showing the presence of aluminum-accumulating plant species and possible explanations for internal tolerance mechanisms. Aluminum concentration in buckwheat leaves reached about 450 mg kg^{-1} (dry wt.) aluminum treatment. Watanabe et al. (1998) investigated the aluminum accumulator plant *Melastoma malabathricum* L. and found aluminum concentrations of 31600 mg kg^{-1} in leaf tissues, accounting for 76.1 % of the total aluminum detected in the whole plant after two months of 1 mM aluminum treatments. These results are remarkably high when compared to wheat (*Triticum aestivum* L.) or rape (*Brassica napus* L.) species, which accumulate aluminum at concentrations less than 50 mg kg^{-1} (Ma et al., 1997b). It's been shown that some of these plant species accumulate aluminum by forming aluminum-organic acid complexes, which is one internal tolerance mechanism.

One of the speculations of how plants use this mechanism is based on studies with buckwheat (Ma et al., 1998; Ma and Hiradate, 2000). Aluminum was taken up by plant roots, although the mechanisms of aluminum uptake were unknown, and chelated with organic acid (in this case with oxalate). The aluminum-oxalate complex was then transferred into the xylem and by ligand exchange reaction formed an aluminum-citrate complex. Once the aluminum-citrate complex was unloaded from the xylem to the leaf cells, aluminum was chelated with oxalic acid again by another ligand exchange reaction; the aluminum-oxalate was then stored in the vacuole. Results from another study support those just described. Ma et al. (1997b) used nuclear magnetic

resonance (NMR) to assess aluminum-oxalate in buckwheat leaves, in a 1:3 molar ratio, the most stable form of the aluminum-oxalate complex (stability constant of 15.12) (Table 1.1).

Another example of aluminum internal tolerance was found in hydrangea plants (*Hydrangea macrophylla* Thunb.). After aluminum exposure, hydrangea plants accumulated as much as 5 mg g^{-1} (dry wt.) in its leaves (Ma et al., 1997a). Aluminum was combined with citric acid in a molar ratio of 1:1 in hydrangea leaf tissues. Although the standard constant of aluminum-citrate shown in Table. 1 is 8.32; it increases to 11.7 at pH of 7.0 which is the typical pH inside the cytosol. This strong complex ability could effectively reduce the combination between aluminum and other cellular components such as ATP (aluminum-ATP stability constant: 10.9), and resulting in less aluminum phytotoxicity.

The current hypothesis for this mechanism is aluminum uptake by roots of aluminum-accumulating plants and complexed with organic acid to prevent the combination between aluminum and other cellular components in the cytosol. Then, the aluminum-organic acid complex is transferred into upper parts of the plant and stored in the vacuole. However, there are still a lot of unknowns with this mechanism that need to be elucidated.

As for the external tolerance mechanisms, it is well established and believed that aluminum-tolerant wheat cultivars can exclude aluminum from their root apex. Rincón and Gonzales (1992) have shown that aluminum-sensitive genotypes accumulate about 9 times more aluminum in the root tips (0-2 mm) than those in aluminum-tolerant genotypes after 19 hrs exposure to 50 μM of aluminum. This indicates that aluminum-tolerant genotypes are able to encode a mechanism to prevent aluminum from accumulating in root tips. Delhaize et al. (1993) found similar and more complete evidence to support these exclusion mechanisms. The aluminum-sensitive wheat used in their study accumulated 4.6 times more aluminum in root apices (terminal 2-3 mm of root) during a 2-16 hr exposure in 100 μM of aluminum than the

aluminum-tolerant genotype. Also, root growth of the aluminum-sensitive genotype had a rate of 0.05 mm hr^{-1} less than the aluminum-tolerant over the initial 4 hrs of aluminum exposure. Tice et al. (1992) claimed similar results supported by their apoplastic and symplastic compartmentation study. Their results not only supported possible exclusion mechanisms, but also provided evidence that wheat may not have the internal tolerance mechanisms because the total aluminum accumulation and compartmental distribution in apoplastic and symplastic among the two genotypes were similar.

Besides wheat species, many other plant species were also investigated for their ability to release organic acids that detoxify aluminum. Citric acid was found to response aluminum toxicity in root exudates of aluminum-tolerant barley (*Hordeum vulgare* L.) cultivars (Zhao et al., 2003; Gallardo et al., 1999), rice cultivars (Ma et al., 2002; Yang et al., 2008), and snapbean (*Phaseolus vulgaris* L.) cultivars (Miyasaka et al., 1991). Citric and malic acids were found in response of aluminum toxicity in root exudates of rye (*Secale cereal* L.) cultivars (Li et al., 2000), and maize cultivars (*Zea mays* L.) (Piñeros et al., 2002; Mariano and Keltjens, 2003; Jorge and Arruda, 1997; Kollmeier et al., 2001). Oxalic acid was also found in root exudates of taro (*Colocasia esculenta* L.) cultivars (Ma and Miyasaka, 1998). Detailed information about organic acid exudation rate is listed on Table 1.2.

Specific mechanisms involved in aluminum exclusion tolerance are listed in Fig. 1.1. Details about the mechanisms of organic acid exudation will be discussed here. Under normal conditions with no aluminum stress, organic acids flow passively across the lipid bi-layer at a slow rate in response to the electric charge gradient that exists across plasma membrane (Jones, 1998). However, when plant roots are stressed with toxic aluminum in soil solutions, an efflux of certain organic acids can be increased by another transport system in root cells. This allows organic acids to be released rapidly into the rhizosphere to achieve aluminum detoxification. After numerous physiological studies with wheat and maize cultivars, an anion channel is

believed to be the aluminum transport system that exists in the plasma membrane (Ryan et al., 1997; Kollmeier et al., 2001; Zhang et al., 2001).

Organic acid exudation is involved in both of the two patterns. Pattern one indicates the rapid exudation of organic acid (no delay) while pattern two shows the possible pattern of delayed exudation of organic acid, which may be involved with gene induction and protein synthesis. Although it is believed that aluminum can activate an anion channel that leads to organic acid exudation into the rhizosphere and combine with mobile aluminum to facilitate detoxification; how the anion channel is activated is still unclear. However, three possibilities for aluminum triggers the anion channel were proposed by Delhaize and Ryan (1995): 1. Aluminum interacts directly with an anion channel protein. 2. Aluminum interacts with a specific receptor on the membrane surface or with the membrane itself and activates channel activity through a series of secondary messages in the cytoplasm. 3. Aluminum enters the cytoplasm and alters channel activity either directly or indirectly via secondary messengers. Although there is still no evidence to support the existence of each of the components in pattern two, the theory of pattern two is still under investigation.

Organic Acid Changes under Aluminum Stress

Organic acids have the ability to complex with metals mainly because they include carboxyl groups. The intensity and stability of the metal ion-organic acid complex depend on organic acid species (e.g. numbers of carboxyl groups), chemical properties of the metal (e.g. trivalent or divalent), and soil pH. Organic acids with one carboxyl group (e.g. lactate, formate) have very little metal-complexing ability compared to those with multiple carboxyl groups (e.g. citric acid). Hue et al. (1986) suggested that the aluminum detoxifying abilities of organic acids are highly correlated with the relative positions of OH and COOH groups on the main carbon

chain of organic acids. It was established that citrate has the highest binding activity for aluminum followed by oxalate, malate, and succinate (Hue et al., 1986).

Detailed information about organic acid exudation rate and organic acid content in root tissues of some important monocot plants are listed in Table 1.2. and Table 1.3.

Objectives

The objectives of these studies were 1) to develop a plant growth system for turfgrass root exudate studies; 2) to evaluate the quantity of five organic acids (oxalate, citrate, malate, malonate, and succinate) in bermudagrass root exudates under aluminum stress; 3) to determine the relationship between organic acid exudation and external aluminum concentration; and 4) to determine bermudagrass physiological status in response to aluminum treatments.

Hypotheses

The hypotheses of these studies are 1) the plant growth system is able to provide a healthy growth condition for turfgrass rhizosphere studies with easily access of root exudates collection. 2) aluminum stress has significant effects on organic acid exudation in bermudagrass root exudates; 3) organic acid exudation (quantity and quality changes) in bermudagrass root exudates positively correlate with external aluminum concentrations; 4) aluminum stress significantly reduces bermudagrass biomass and affects bermudagrass visual quality.

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Table 1.1. Stability constants of Al^{3+} and Fe^{3+} complexes of two organic ligands†.

Organic ligand	Ligand per metal ion	Stability constant	
		Al^{3+}	Fe^{3+}
Oxalic acid	1	6.10 (1.0)‡	7.53 (0.1)
	2	11.09 (1.0)	13.64 (0.1)
	3	15.12 (1.0)	18.49 (0.1)
Citric acid	1	8.32 (0.25)§	11.20 (0.1)
	2	NA	NA

† Data taken from Martell and Smith (1974) unless otherwise noted.

‡ Numbers in parentheses represent ionic strength (in mol/L) at which the stability constant was determined.

§ Data taken from Sillén and Martell (1971).

Table. 1.2. Organic acid exuded in response to aluminum stress by several monocots.

Plant species/Cultivar	Organic acid	Al treatment (μM)	Organic acid concentration†	Reference
Barley (*Hordeum vulgare* L.)				
Sigurdkorn (Al-tol.) ‡	citrate	0	0.00 nmol root apex^{-1} 24 hr^{-1}	Zhao et al., 2003
Sigurdkorn (Al-tol.)	citrate	5	2.07 nmol root apex^{-1} 24 hr^{-1}	Zhao et al., 2003
Sigurdkorn (Al-tol.)	citrate	10	2.44 nmol root apex^{-1} 24 hr^{-1}	Zhao et al., 2003
Sigurdkorn (Al-tol.)	citrate	20	2.40 nmol root apex^{-1} 24 hr^{-1}	Zhao et al., 2003
Kearney (Al-sen.)	citrate	0	0.00 nmol root apex^{-1} 24 hr^{-1}	Zhao et al., 2003
Kearney (Al-sen.)	citrate	5	0.29 nmol root apex^{-1} 24 hr^{-1}	Zhao et al., 2003
Kearney (Al-sen.)	citrate	10	0.29 nmol root apex^{-1} 24 hr^{-1}	Zhao et al., 2003
Kearney (Al-sen.)	citrate	20	0.33 nmol root apex^{-1} 24 hr^{-1}	Zhao et al., 2003
Carmen (Al-tol.)	citrate	0	520.00 nmol (g FW)$^{-1}$ 24 hr^{-1}	Gallardo et al., 1999
Carmen (Al-tol.)	citrate	200	940.00 nmol (g FW)$^{-1}$ 24 hr^{-1}	Gallardo et al., 1999
Steffi (Al-sen.)	citrate	0	560.00 nmol (g FW)$^{-1}$ 24 hr^{-1}	Gallardo et al., 1999
Steffi (Al-sen.)	citrate	200	480.00 nmol (g FW)$^{-1}$ 24 hr^{-1}	Gallardo et al., 1999
Cheri (Al-sen.)	citrate	0	490.00 nmol (g FW)$^{-1}$ 24 hr^{-1}	Gallardo et al., 1999
Cheri (Al-sen.)	citrate	200	430.00 nmol (g FW)$^{-1}$ 24 hr^{-1}	Gallardo et al., 1999
Carmen (Al-tol.)	malate	0	180.00 nmol (g FW)$^{-1}$ 24 hr^{-1}	Gallardo et al., 1999
Carmen (Al-tol.)	malate	200	100.00 nmol (g FW)$^{-1}$ 24 hr^{-1}	Gallardo et al., 1999

Plant species/Cultivar	Organic acid	Al treatment (μM)	Organic acid concentration	Reference
Rye (*Secale cereale* L.)				
King	citrate	0	1000.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000
King	citrate	10	3333.33 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000
King	citrate	30	6416.67 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000
King	citrate	50	9583.33 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000
King	malate	0	0.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000
King	malate	10	1167.67 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000
King	malate	30	4000.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000
King	malate	50	6250.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000
Maize (*Zea mays* L.)				
Cateto-Colombia	malate	0	3.60 nmol root apex^{-1} 24 hr^{-1}	Piñeros et al., 2002
Cateto-Colombia	malate	5	0.96 nmol root apex^{-1} 24 hr^{-1}	Piñeros et al., 2002
Cateto-Colombia	malate	10	3.60 nmol root apex^{-1} 24 hr^{-1}	Piñeros et al., 2002
Cateto-Colombia	malate	20	6.72 nmol root apex^{-1} 24 hr^{-1}	Piñeros et al., 2002
Cateto-Colombia	malate	40	6.48 nmol root apex^{-1} 24 hr^{-1}	Piñeros et al., 2002
Cateto-Colombia	malate	80	1.91 nmol root apex^{-1} 24 hr^{-1}	Piñeros et al., 2002
Cateto-Colombia	citrate	0	0.00 nmol root apex^{-1} 24 hr^{-1}	Piñeros et al., 2002
Cateto-Colombia	citrate	5	0.77 nmol root apex^{-1} 24 hr^{-1}	Piñeros et al., 2002
Cateto-Colombia	citrate	10	2.86 nmol root apex^{-1} 24 hr^{-1}	Piñeros et al., 2002

Plant species/Cultivar	Organic acid	Al treatment (μM)	Organic acid concentration	Reference
Maize (*Zea mays* L.) cont.				
Cateto-Colombia	citrate	20	5.57 nmol root apex^{-1} 24 hr^{-1}	Piñeros et al., 2002
Cateto-Colombia	citrate	40	10.37 nmol root apex^{-1} 24 hr^{-1}	Piñeros et al., 2002
Cateto-Colombia	citrate	80	11.46 nmol root apex^{-1} 24 hr^{-1}	Piñeros et al., 2002
CMS36 (Al-tol.)	citrate	0	0.71 nmol root apex^{-1} 24 hr^{-1}	Mariano and Keltjens, 2003
CMS36 (Al-tol.)	citrate	20	3.21 nmol root apex^{-1} 24 hr^{-1}	Mariano and Keltjens, 2003
CMS36 (Al-tol.)	citrate	40	4.93 nmol root apex^{-1} 24 hr^{-1}	Mariano and Keltjens, 2003
CMS36 (Al-tol.)	citrate	100	3.30 nmol root apex^{-1} 24 hr^{-1}	Mariano and Keltjens, 2003
CMS36 (Al-tol.)	malate	40	2.64 nmol root apex^{-1} 24 hr^{-1}	Mariano and Keltjens, 2003
CMS36 (Al-tol.)	fumarate	40	0.04 nmol root apex^{-1} 24 hr^{-1}	Mariano and Keltjens, 2003
BR106 (Al-sen.)	citrate	0	0.69 nmol root apex^{-1} 24 hr^{-1}	Mariano and Keltjens, 2003
BR106 (Al-sen.)	citrate	20	1.89 nmol root apex^{-1} 24 hr^{-1}	Mariano and Keltjens, 2003
BR106 (Al-sen.)	citrate	40	1.63 nmol root apex^{-1} 24 hr^{-1}	Mariano and Keltjens, 2003
BR106 (Al-sen.)	citrate	100	1.89 nmol root apex^{-1} 24 hr^{-1}	Mariano and Keltjens, 2003
Al-tol.	citrate	0	0.00 nmol root^{-1} 24 hr^{-1}	Jorge and Arruda, 1997
Al-tol.	citrate	10.3	2.53 nmol root^{-1} 24 hr^{-1}	Jorge and Arruda, 1997
Al-tol.	citrate	17.5	3.03 nmol root^{-1} 24 hr^{-1}	Jorge and Arruda, 1997
Al-tol.	citrate	26.5	3.38 nmol root^{-1} 24 hr^{-1}	Jorge and Arruda, 1997
Al-tol.	citrate	50.0	5.27 nmol root^{-1} 24 hr^{-1}	Jorge and Arruda, 1997

Plant species/Cultivar	Organic acid	Al treatment (μM)	Organic acid concentration	Reference
Maize (*Zea mays* L.) cont.				
Al-sen.	citrate	0	0.00 nmol root^{-1} 24 hr^{-1}	Jorge and Arruda, 1997
Al-sen.	citrate	10.3	1.20 nmol root^{-1} 24 hr^{-1}	Jorge and Arruda, 1997
Al-sen.	citrate	17.5	0.57 nmol root^{-1} 24 hr^{-1}	Jorge and Arruda, 1997
Al-tol.	malate	0	0.00 nmol root^{-1} 24 hr^{-1}	Jorge and Arruda, 1997
Al-tol.	malate	26.5	2.97 nmol root^{-1} 24 hr^{-1}	Jorge and Arruda, 1997
Al-tol.	malate	50.0	4.99 nmol root^{-1} 24 hr^{-1}	Jorge and Arruda, 1997
Al-sen.	malate	0	0.00 nmol root^{-1} 24 hr^{-1}	Jorge and Arruda, 1997
Al-sen.	malate	10.3	0.85 nmol root^{-1} 24 hr^{-1}	Jorge and Arruda, 1997
Al-sen.	malate	17.5	1.23 nmol root^{-1} 24 hr^{-1}	Jorge and Arruda, 1997
ATP-Y (Al-tol.)	citrate	0	6.55 nmol root apex^{-1} 24 hr^{-1}	Kollmeier et al., 2001
ATP-Y (Al-tol.)	citrate	100	30.00 nmol root apex^{-1} 24 hr^{-1}	Kollmeier et al., 2001
ATP-Y (Al-tol.)	citrate	200	19.92 nmol root apex^{-1} 24 hr^{-1}	Kollmeier et al., 2001
Lixis (Al-sen.)	citrate	0	6.11 nmol root apex^{-1} 24 hr^{-1}	Kollmeier et al., 2001
Lixis (Al-sen.)	citrate	100	16.37 nmol root apex^{-1} 24 hr^{-1}	Kollmeier et al., 2001
Lixis (Al-sen.)	citrate	200	14.84 nmol root apex^{-1} 24 hr^{-1}	Kollmeier et al., 2001
ATP-Y (Al-tol.)	malate	0	12.22 nmol root apex^{-1} 24 hr^{-1}	Kollmeier et al., 2001
ATP-Y (Al-tol.)	malate	100	25.75 nmol root apex^{-1} 24 hr^{-1}	Kollmeier et al., 2001
ATP-Y (Al-tol.)	malate	200	27.05 nmol root apex^{-1} 24 hr^{-1}	Kollmeier et al., 2001

Plant species/Cultivar	Organic acid	Al treatment (μM)	Organic acid concentration	Reference
Maize (*Zea mays* L.) cont.				
Lixis (Al-sen.)	malate	0	8.95 nmol root apex^{-1} 24 hr^{-1}	Kollmeier et al., 2001
Lixis (Al-sen.)	malate	100	16.15 nmol root apex^{-1} 24 hr^{-1}	Kollmeier et al., 2001
Lixis (Al-sen.)	malate	200	17.45 nmol root apex^{-1} 24 hr^{-1}	Kollmeier et al., 2001
Rice (*Oryza sativa* L.)				
Koshihikari (Al-tol.)	citrate	0	0.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Ma et al., 2002
Koshihikari (Al-tol.)	citrate	20	742.86 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Ma et al., 2002
Koshihikari (Al-tol.)	citrate	50	1157.14 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Ma et al., 2002
Koshihikari (Al-tol.)	citrate	100	1342.86 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Ma et al., 2002
Kasalath (Al-sen.)	citrate	0	0.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Ma et al., 2002
Kasalath (Al-sen.)	citrate	20	685.71 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Ma et al., 2002
Kasalath (Al-sen.)	citrate	50	800.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Ma et al., 2002
Kasalath (Al-sen.)	citrate	100	1085.71 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Ma et al., 2002
Nipponbare (Al-tol.)	citrate	0	0.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Yang et al., 2008
Nipponbare (Al-tol.)	citrate	25	0.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Yang et al., 2008
Nipponbare (Al-tol.)	citrate	50	173.33 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Yang et al., 2008
Nipponbare (Al-tol.)	citrate	100	148.33 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Yang et al., 2008
Zhefu802 (Al-sen.)	citrate	0	0.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Yang et al., 2008
Zhefu802 (Al-sen.)	citrate	25	140.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Yang et al., 2008

Plant species/Cultivar	Organic acid	Al treatment (μM)	Organic acid concentration	Reference
Rice (*Oryza sativa* L.)				
Zhefu802 (Al-sen.)	citrate	50	130.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Yang et al., 2008
Zhefu802 (Al-sen.)	citrate	100	246.67 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Yang et al., 2008
Wheat (*Triticum aestivum* L.)				
Atlas (Al-tol.)	malate	0	15.12 nmol plant^{-1} 24 hr^{-1}	Pellet et al., 1997
Atlas (Al-tol.)	malate	5	41.52 nmol plant^{-1} 24 hr^{-1}	Pellet et al., 1997
Atlas (Al-tol.)	malate	20	112.32 nmol plant^{-1} 24 hr^{-1}	Pellet et al., 1997
ET3 (Al-tol.)	malate	0	6.00 nmol plant^{-1} 24 hr^{-1}	Pellet et al., 1997
ET3 (Al-tol.)	malate	5	31.92 nmol plant^{-1} 24 hr^{-1}	Pellet et al., 1997
ET3 (Al-tol.)	malate	20	82.08 nmol plant^{-1} 24 hr^{-1}	Pellet et al., 1997
Scout (Al-sen.)	malate	0	8.64 nmol plant^{-1} 24 hr^{-1}	Pellet et al., 1997
Scout (Al-sen.)	malate	5	7.20 nmol plant^{-1} 24 hr^{-1}	Pellet et al., 1997
Scout (Al-sen.)	malate	20	8.88 nmol plant^{-1} 24 hr^{-1}	Pellet et al., 1997
ES3 (Al-sen.)	malate	0	10.08 nmol plant^{-1} 24 hr^{-1}	Pellet et al., 1997
ES3 (Al-sen.)	malate	5	9.60 nmol plant^{-1} 24 hr^{-1}	Pellet et al., 1997
ES3 (Al-sen.)	malate	20	9.84 nmol plant^{-1} 24 hr^{-1}	Pellet et al., 1997
Al-tol.	citrate	0	1.92 nmol plant^{-1} 24 hr^{-1}	Delhaize et al., 1993
Al-tol.	citrate	50	4.08 nmol plant^{-1} 24 hr^{-1}	Delhaize et al., 1993
Al-sen.	citrate	0	4.08 nmol plant^{-1} 24 hr^{-1}	Delhaize et al., 1993
Al-sen.	citrate	50	1.92 nmol plant^{-1} 24 hr^{-1}	Delhaize et al., 1993

Plant species/Cultivar	Organic acid	Al treatment (μM)	Organic acid concentration	Reference
Wheat (*Triticum aestivum* L.)				
Al-tol.	malate	0	< 1.92 nmol plant^{-1} 24 hr^{-1}	Delhaize et al., 1993
Al-tol.	malate	50	85.68 nmol plant^{-1} 24 hr^{-1}	Delhaize et al., 1993
Al-sen.	malate	0	1.92 nmol plant^{-1} 24 hr^{-1}	Delhaize et al., 1993
Al-sen.	malate	50	7.92 nmol plant^{-1} 24 hr^{-1}	Delhaize et al., 1993
Al-tol.	succinate	0	1.92 nmol plant^{-1} 24 hr^{-1}	Delhaize et al., 1993
Al-tol.	succinate	50	13.92 nmol plant^{-1} 24 hr^{-1}	Delhaize et al., 1993
Al-sen.	succinate	0	1.92 nmol plant^{-1} 24 hr^{-1}	Delhaize et al., 1993
Al-sen.	succinate	50	1.92 nmol plant^{-1} 24 hr^{-1}	Delhaize et al., 1993
Atlas 66 (Al-tol.)	malate	0	0.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000
Atlas 66 (Al-tol.)	malate	10	5000.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000
Atlas 66 (Al-tol.)	malate	30	6875.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000
Atlas 66 (Al-tol.)	malate	50	8250.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000
Atlas 66 (Al-tol.)	citrate	0	437.50 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000
Atlas 66 (Al-tol.)	citrate	10	437.50 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000
Atlas 66 (Al-tol.)	citrate	30	812.50 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000
Atlas 66 (Al-tol.)	citrate	50	1500.00 nmol (g root DW)$^{-1}$ 24 hr^{-1}	Li et al., 2000

†DW indicates root tissue dry weight and FW indicates root tissue fresh weight.

‡ Al-tol. indicates aluminum tolerant cultivars and Al-sen. indicates aluminum sensitive cultivars.

Table 1.3. Organic acid content in plant roots as affected by aluminum stress.

Plant Species/Cultivar	Al^{3+} (µM)	Location on root	Organic acid concentration†				Reference
			Citrate	Fumarate nmol g^{-1} FW	Malate	Succinate µmol g^{-1} DW	
Barley (*Hordeum vulgare* L.)							
Daton (Al-tol.) ‡	0	whole root	17.70 µmol g^{-1} DW	NA§	26.85 µmol g^{-1} DW	243.88	1
Daton (Al-tol.)	55	whole root	16.66 µmol g^{-1} DW	NA	43.25 µmol g^{-1} DW	297.23	1
Daton (Al-tol.)	110	whole root	15.61 µmol g^{-1} DW	NA	83.53 µmol g^{-1} DW	224.41	1
Kearney (Al-sen.)	0	whole root	24.46 µmol g^{-1} DW	NA	49.97 µmol g^{-1} DW	324.33	1
Kearney (Al-sen.)	55	whole root	15.61 µmol g^{-1} DW	NA	74.58 µmol g^{-1} DW	218.48	1
Kearney (Al-sen.)	110	whole root	11.97 µmol g^{-1} DW	NA	62.64 µmol g^{-1} DW	130.41	1
Sigurdkorn (Al-tol.)	0	root apex	0.60 nmol root $apex^{-1}$	NA	NA	NA	2
Sigurdkorn (Al-tol.)	10	root apex	0.85 nmol root $apex^{-1}$	NA	NA	NA	2
Kearney (Al-sen.)	0	root apex	0.50 nmol root $apex^{-1}$	NA	NA	NA	2
Kearney (Al-sen.)	10	root apex	0.53 nmol root $apex^{-1}$	NA	NA	NA	2
Maize (*Zea mays* L.)							
SA 3 (Al-tol.)	0	whole root	15.9 nmol g^{-1} FW	17	130.1 nmol g^{-1} FW	NA	3
SA 3 (Al-tol.)	6	whole root	22.0 nmol g^{-1} FW	15.9	1154.6 nmol g^{-1} FW	NA	3
Tuxpeño (Al-sen.)	0	whole root	15.0 nmol g^{-1} FW	12.2	145.4 nmol g^{-1} FW	NA	3
Tuxpeño (Al-sen.)	6	whole root	24.8 nmol g^{-1} FW	17.9	1043.6 nmol g^{-1} FW	NA	3

Plant Species/Cultivar	Al^{3+} (μM)	Location on root	Organic acid concentration				Reference
			Citrate	Fumarate nmol g^{-1} FW	Malate	Succinate μmol g^{-1} DW	
Maize (*Zea mays* L.) cont.							
Cateto-Colombia (Al-tol.)	0	0-2 cm	88.57 nmol g^{-1}	NA	NA	NA	4
Cateto-Colombia (Al-tol.)	5	0-2 cm	111.43 nmol g^{-1}	NA	NA	NA	4
Cateto-Colombia (Al-tol.)	10	0-2 cm	134.29 nmol g^{-1}	NA	NA	NA	4
Cateto-Colombia (Al-tol.)	20	0-2 cm	214.29 nmol g^{-1}	NA	NA	NA	4
Cateto-Colombia (Al-tol.)	30	0-2 cm	325.71 nmol g^{-1}	NA	NA	NA	4
Cateto-Colombia (Al-tol.)	40	0-2 cm	328.57 nmol g^{-1}	NA	NA	NA	4
Wheat (*Triticum aestivum* L.)							
Al-tol.	0	root apices	NA	NA	1.24 nmol root $apex^{-1}$	NA	5
Al-tol.	200	root apices	NA	NA	1.32 nmol root $apex^{-1}$	NA	5
Al-sen.	0	root apices	NA	NA	1.52 nmol root $apex^{-1}$	NA	5
Al-sen.	200	root apices	NA	NA	1.20 nmol root $apex^{-1}$	NA	5

†DW indicates root dry weight and FW indicates root fresh weight.

‡ Al-tol. indicates Al tolerant cultivars and Al-sen. indicates aluminum sensitive cultivars.

§ NA, organic acid was detected in samples or data was not availabe.

[1]Foy et al., 1987.

[2]Zhao et al., 2003.

[3]Pellet et al., 1995.

[4]Pineros et al., 2002.

[5]Delhaize et al., 1993.

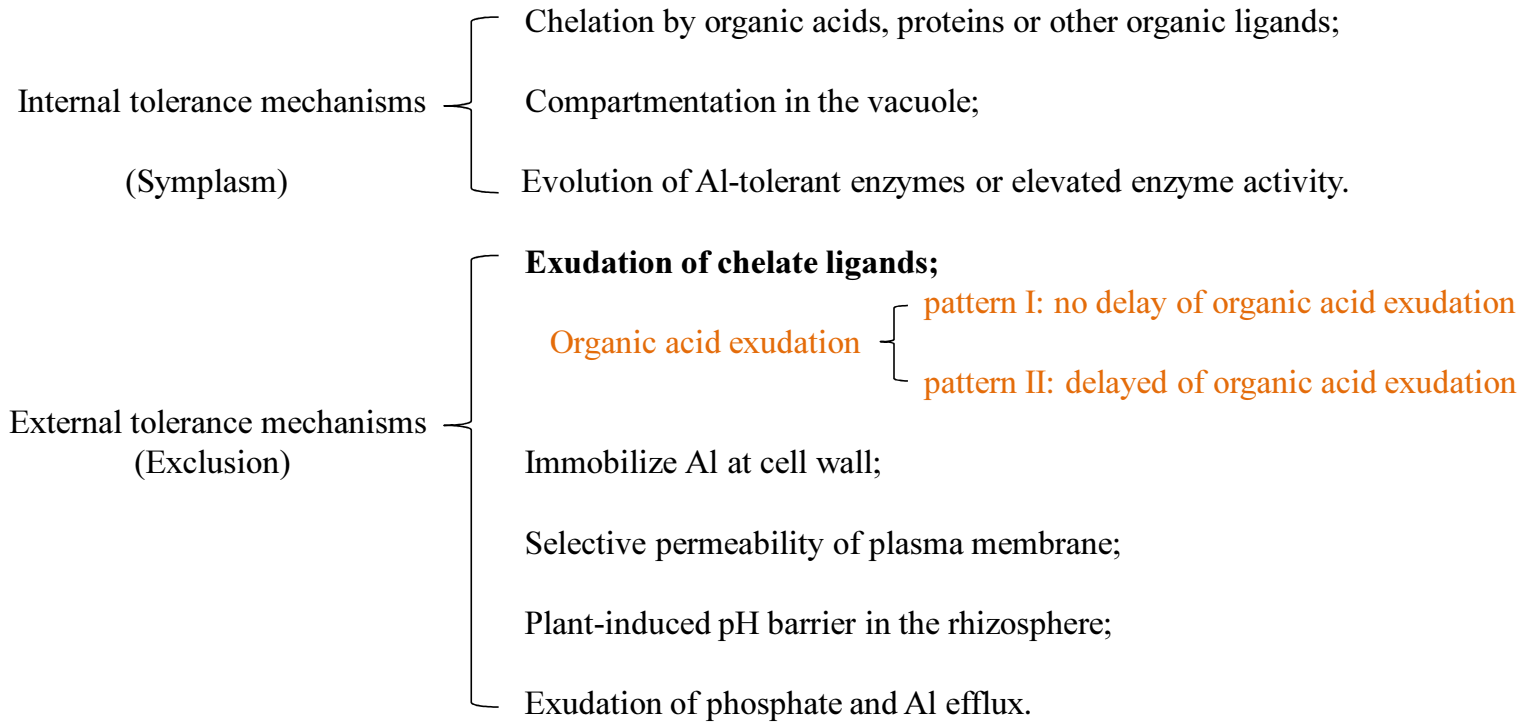

Fig. 1.1. Aluminum tolerance mechanisms (Adapted from: Taylor, 1991; Ma, 2000; Ma et al., 2001).

CHAPTER II

A PLANT GROWTH SYSTEM DESIGNED FOR TURF RHIZOSPHERE STUDIES

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ABSTRACT. The plant rhizosphere is poorly understood, in part due to the lack of proper methodologies. The objective of this work was to develop a plant growth system which allows for easy collection of plant root exudates for further evaluations. The developed system would permit optimized plant growth under a controlled laboratory environment. The deep and adjustable root zone, sufficient light for growth, and the capability of cutting shoot tissues were designed especially for turfgrass studies. The key advantage of this design is that no complicated or high-maintenance equipment is needed and it can be easily constructed with simple, non-expensive materials. 'U-3' bermudagrass was planted for four weeks in growth units and exhibited healthy growth conditions were confirmed throughout the study. We conclude that this growth system can be used in a broad range of turfgrass root exudate studies.

Keywords: root exudates, rhizosphere, plant growth system, bermudagrass.

INTRODUCTION

Identification and quantification of components in root exudates have been widely investigated for a century. However, due to the complex nature of the rhizosphere the study of root exudates are with difficulties. Factors affecting root exudates were briefly described in the first chapter (Rovira, 1969; Neumann and Römheld, 2000; Curl and Truelove, 1986; Koo, 2001), and according to Lambers (1987) abiotic and biotic factors together had a greater impact on root exudates than internal factors (e.g. plant species/cultivars). Hence, most of the previous studies have used controlled environments for plant growth and root exudate collection.

Two major culture methods were widely used for collecting root exudates in solution cultures (Miyasaka et al., 1991; Jones and Darrah, 1993; Pellet et al., 1995; Jorge and Arruda, 1997; Li et al., 2000; Jones et al., 1995) and soil culture (Shane et al., 2008; Henry et al., 2006; Hodge et al., 1996; Lipton et al., 1987; Aulakh et al., 2001; Johnson et al., 1996; Hodge et al., 1999). The major advantages of solution culture are the convenience of quantification of root exudates at certain points and the plants are easily maintained under sterile conditions, especially for short term studies (Ma et al., 1998). However, the drawbacks of solution culture are considerable and should be taken into account when making decisions of experimental methodologies. The most serious problem with solution culture is the lack of mechanical impedance of plant roots, which result in physiological and morphological changes of root growth and further change exudation (Jones, 1998; Neumann and Römheld, 2000; Barber and Gunn, 1974; Hodge et al., 2009). Other concerns about plant growing in solution culture include possible damage to root cell membranes due to the use of distilled water (Jones et al., 1995) and lack of aeration results in the formation of aerechyma (Hodge et al., 2009). Trofymow et al (1987) reported 2-4 times more carbon release from plants grown in soil than those grown in hydroponic

culture. Foy and Murray (1998) reported 18-fold less Al tolerance of Kentucky bluegrass cultivars in nutrient solution than in the soil. Although no certain reasons were confirmed to explain the differences, it still indicated the significant changes of the root zone growing in solution culture. On the other hand, sand culture is able to provide a better natural growth condition (Hodge et al., 2009). However, it is challenging to be kept aseptic during the course of the experiment, especially when large amounts of sand are needed.

In addition to the choice of growth medium, whether or not to include microbial activity in root exudates studies is also under a series of debates. There are three groups of methodologies when dealing with microbial activities. The first is to use absolute sterile conditions to grow the plants and collect root exudates samples under the same conditions. Such studies mostly adopted nutrient solution as the growth medium due to the larger chance of maintaining the sterile environment (Miyasaka et al., 1991; Jones and Darrah, 1993; Pellet et al., 1995). The second is to include microbial activities in the results; studies like that were mostly conducted under field conditions and the results more reflected real world conditions (Phillips et al., 2008; Polomski and Kuhn, 2002). The last and the most widely used method is in between the first two, because techniques such as plant surface sterilization and medium cultivation to exclude microbial growth at a certain stage of the study (e.g. seed germination), but the growth condition was not necessarily sterile during the entire study period (Wang et al., 2006; Zeng et al., 2008).

Various arguments with supporting evidence about whether or not microbial activities in the rhizosphere should be included in root exudates studies have been discussed. On one hand, rhizosphere microorganisms consume certain compounds in root exudates and in that way make the quantification results underestimated. For example, Kraffczyk et al. (1984) reported significant reduction of monosaccharides in maize root exudates under non-sterile condition compared to those detected under sterile condition. van Hees et al. (2005) also indicated the rapid removal of low molecular weight compounds in root exudates by soil microorganisms. However,

it appeared different species of microorganisms have certain consumption preferences of root exudates. Somers et al. (2004) indicated the stimulation of growth of plant growth-promoting bacteria (PGPB) with carbohydrates and amino acids in root exudates. Inskeep and Comfort (1986) reported organic acids and amino acids as the prevalent chemical substances in soluble root exudates, indicating carbohydrates, in that case, are more preferred by microorganisms than organic acids and amino acids. It is concluded in most cases carbohydrates are the primary compound in root exudates consumed by microorganisms as their growth substrates (Jones and Darrah, 1994; Jones et al., 1996; van Hees et al., 2005).

On the other hand, rhizosphere microbial activities also increase root exudation making the quantification overestimated (Meharg and Killham, 1991). Exudation of low molecular weight organic acids can be produced by rhizosphere bacteria and fungi during the plant decaying process (Stevenson, 1989). Leyval and Berthelin (1993) reported the increase of total C compounds with the inoculation of ectomycorrhizal fungus or rhizobacteria. Schwab et al. (1984) also reported significantly increased exudation of carbohydrates and amino acids in plants that formed symbiotic associations with certain soil-inhabiting fungi.

To summarize, there is no specific rules on how to choose the growth condition and collection apparatus in root exudate studies. The decisions should be made mainly based on target compounds, treatment effects, required facility supports, and experiment budgets should be taken into considerations.

OBJECTIVES

The objectives of this study were to develop a plant growth system for turfgrass root exudate studies, and specifically, to 1) grow bermudagrass under healthy and well controlled conditions; and 2) develop procedures to collect root exudate samples and to measure bermudagrass physiological changes.

MATERIALS AND METHODS

Design of Plant Growth Unit

The construction of the sterile plant growth unit (Fig. 2.1.) was modified from Da and Deng (2003), and Henry et al. (2006). Each set of plant growth unit consists of five Magenta boxes (3×3×4 inch for the box on top, and 3×3×3 inch for the rest) obtained from Bio-World (Dublin, OH, USA). Five boxes were stacked together to make one growth unit, and boxes were numbered 1 to 5 starting from the top down. Four holes (1.5 cm diameter) were drilled on each side of box 1 for air circulation purpose. Each of the four holes was covered with a micro air filter. This autoclavable air filter was cut from a Sun bag (Sigma-Aldrich, St. Louis, Missouri, USA) with a pore size of 0.02 μm which can provide a sufficient air circulation for bermudagrass growth while screening most of the airborne microbes from entering the growth unit. For box 2 and 3 stacked in the middle, the bottoms were completely removed. A hole (1 cm diameter) was drilled in the center bottom of box 4 to place a filter paper roll. The filter paper roll was made by a 12.5 cm diameter Whatman No. 1 filter paper (90 mm) to prevent sand from entering the collection bottle located in box 5, and to increase leachate conductivity. A 30 ml brown bottle (Glass, Amber, Fisher Scientific) was placed in box 5 to collect leachate samples. The bottom side of the filter paper roll was inserted into the brown bottle, so there was no sample leached out. GE silicone II sealant (Huntersville, NY) was used to seal the connection parts for the top four boxes to prevent leakage in the system. To increase the transmittance of light into the growth unit, a special cap instead of the regular magenta box cap was used to cover the top. The special cap was made by cutting the bottom of another magenta box; each growth unit was covered by one of the specially made transparent cap.

The top box was for grass shoot growth; the three in the middle were used as grass root zone containing 650 cm^3 washed sea sand (Fisher Scientific, New Jersey) as the growth medium.

Chemical and physical properties of the sea sand were recorded in Table 2.1. Before sand was added into the growth unit, sealing condition was checked by filling each growth unit with deionized water and all the connection parts were checked for leakage. GE silicone sealant was used to seal leaking spots.

Seed Surface Sterilization

'U-3' bermudagrass (*Cynodon dactylon* (L.) Pers.) was used in this study. Seeds were purchased from Eckroat seed company (Oklahoma City, OK).

'U-3' bermudagrass seeds were stirred in a 500 ml beaker with a mixed solution of 20 % (v/v) commercial bleach solution and 0.1 % (v/v) Tween-20 (Fisher Scientific, US) as a surfactant (Henry, 2003) for 30 min. Seeds were then rinsed with deionized water eight times (Delhaize et al., 1993), for 10 min each time to rinse off all the chemicals and seed coats left on seed surfaces. Floating seeds were discarded during the rinsing process.

After surface sterilization, seeds were transferred from the beaker into several prepared glass Petri dishes 100×10 mm (Pyrex, USA) under an EdgeGARD Laminar-flow hood (Model EG 5252, Baker Co. Sanford, Maine). To prepare the Petri dish, a filter paper was rinsed with deionized water and placed in the bottom part; the top part was covered and was autoclaved at 21 psi and 121°C for 20 min.

After all the sterilized seeds were transferred into Petri dishes, each Petri dish was sealed at the edge with Parafilm (Chicago, IL) to preserve moisture. All the sealed Petri dishes were then incubated in a growth chamber (Percival Model I-35 LL, Percival Mfg. Co., Boone, IA) with a setup of day/night temperature of 24/22°C and a photoperiod of 10 hr for three days for germination. According to preliminary data, it took 24 to 48 hr for bermudagrass seeds to germinate with the condition described above. Most seedlings developed at least one leaf after three days and were ready to be transferred into test tubes for tissue culture cultivation.

Tissue Culture Cultivation

Tissue culture cultivation was used in this study with the purpose of providing a sterile, nutritionally sufficient condition for bermudagrass seedling growth. The recipe of tissue culture medium used in this study was shown in Table 2.2.

After three days of germination, seedlings with at least one leaf were transferred from the Petri dish into test tubes with tissue culture medium in them. Each test tube contained one seedling. All the test tubes with seedlings were placed on a tissue culture rack with a 45° slant (Fig. 2.2.) and then put in growth chamber. The day/night temperature in the growth chamber was set at 24/22°C with a photoperiod of 12 hr. Seedlings were checked every day, and all test tubes and racks were relocated every day to even environmental effects.

Sea Sand Preparation

Washed sea sand (650 cm^3 sea sand for each growth unit) was used as the growth medium. Sand was washed with deionized water several times until all the dirts were washed out, and then dried in oven at 100°C for 24 hr. The dry sand was autoclaved for 90 min at 21 psi and 121°C for three consecutive days. On the fourth day, 645 cm^3 dry sand was poured into each growth unit; 5 cm^3 sand left for each growth unit was put into test tubes and autoclaved separately. All growth units and test tubes containing sand were then autoclaved for 90 min at 21 psi and 121°C. After autoclave, sand was allowed to cool down for 24 hr to room temperature before seedlings could be transferred into growth units.

Nutrient Solution

Nutrient solution (Table 2.3.) used in this study was modified from Hoagland nutrient solution (Hoagland and Arnon, 1950). The pH of the nutrient solution was adjusted to 4.5. The needed volume of nutrient solution was measured with a graduate cylinder and prepared in

individual beakers for each growth unit every time before watering. Beakers with nutrient solutions were covered with aluminum foil and autoclaved at 21 psi and 121°C for 30 min. Deionized water was added into the autoclave pan to reduce nutrient solution evaporation and minimize pH change during autoclaving. After autoclaving, at least 12 hr was allowed for nutrient solutions to cool down to room temperature before watering. Extra nutrient solutions were prepared to measure the pH after autoclave. The pH change within 0.1-0.2 unit was accepted to be used to water plants. All the pH measurements were conducted on a pH meter (Cole-Parmer model 5943. Chicago, IL). The pH meter was calibrated using standard buffer solutions (Oakton standard buffers; pH 4.01 and 7.00, certified by NIST) every time before use.

Bermudagrass Cultivation in Growth Units

After two weeks growing in the tissue culture medium, bermudagrass seedlings with six were transferred into four growth units; 14 seedlings for each growth unit. After all the seedlings were transferred, 5 cm^3 of prepared sea sand was poured into each growth unit to cover the seedlings. After seedlings were covered by sand, 100 ml of prepared nutrient solution was carefully pipetted into each growth unit until excessive liquid leached out from the bottom. All growth units were covered on top by the specially made transparent cap and sealed with Parafilm. The root zone was covered by aluminum foil wrapped cardboard, to protect roots from exposing to light. This process was entirely conducted under the laminar flow hood.

After all the seedlings were transferred into growth units, they were placed on a laboratory bench top with two high-power growth lights above (Fig. 2.4.). The average light intensity was 1000 μmol m^{-2} s^{-1}. The reason to choose to use growth lights on a bench is because the growth chamber could not provide a preferred light intensity for bermudagrass growth. Light intensity in the growth chamber was about 350 μmol m^{-2} s^{-1} at its highest and bermudagrass experienced shade stress. The room temperature was set at 25°C with a photoperiod of 12 hr. The

height of the growth light above the growth unit was 20 cm to avoid heat damage while providing sufficient light.

Each growth unit was watered with 30 ml nutrient solution every three days by slowly pipetting nutrient solution onto plant shoot bases to avoid sand from washing off. Leachate containing nutrient solution and root exudates were collected into brown bottles placed at the bottom of the growth units. Leachate samples were stored in a -20°C freezer before further process. Brown bottles were replaced with new autoclaved bottles after each watering.

Due to the fast growth of bermudagrass, shoots reached the top of the growth unit within a short time and blocked light at the canopy. Therefore, shoot tissues were cut once every seven days to maintain a healthy plant growth while minimizing the disturbance of the system. Cutting height was maintained at about 1.5 inch and the 1/3 rule was applied (cut no more than one third of the grass blade at one mowing). Clippings from each growth unit were collected into plastic bags filled with deionized water and stored in 4°C refrigerator and were scanned with WinRhizo (Regents Instruments Inc., Québec, Canada) software within 24 hr. Shoot tissue samples were then dried in an oven at 80˚C for 48 hr (Baldwin et al., 2005), then dry weight was recorded.

Harvest

Each growth unit was carefully cut into two parts on the vertical side and the root zone was gently taken out. Each plant was gently shaken by hand. Sand that immediately fell from the root zone was defined as “bulk sand”, and sand attached to the roots after shaking was collected as “rhizosphere sand”. Rhizosphere sand was then washed off by immerging root parts into a beaker containing 10 ml deionized water. Shoot parts were cut and sealed in plastic bags with deionized water, and stored at 4˚C refrigerator until analyze.

Bulk sand was further washed on a screen with deionized water to obtain fine roots left in the sand; all the roots were collected from the bulk sand and were put into the same beaker with

rhizosphere sand. Sonic cleaning method (Barber and Gunn, 1974; Miyasaka et al., 1991) was used to extract root exudates from the root surface and rhizosphere sand without damaging root tissues. The mixed solution was sonicated for 3 min and filtered with a 0.45 μm membrane into a 25 ml volumetric flask. Another 10 ml deionized water was added and sonicated for 2 min. Sonicated solution was filtered and collected into the same volumetric flask, volume was added to 25 ml and the solution was mixed well. The sonicated solution samples were finally transferred into 30 ml brown bottles and stored in a -20˚C freezer until further processed. Root tissue fresh weight was measured and samples were frozen in liquid nitrogen and stored in -80˚C freezer. Rhizosphere sand from each growth unit was dried in an oven at 100˚C for 48 hr and dry weight was recorded.

Shoot tissue samples were scanned with WinRhizo software and then dried in oven at 80˚C for 48 hr. The dry weight was then recorded. The accumulated shoot tissue samples were gathered and ground by a cyclone mill (UDY Corp., Boulder, CO). Ground samples were then analyzed for P, K, Ca, Mg, S, Zn, Cu, Mn, Fe, B using an Inductively Coupled Plasma (ICP) by the soil, water and forage analytical laboratory at Oklahoma State University. 0.5 g of ground sample was digested using 10 ml concentrated nitric acid (HNO_3) and was allowed to stand overnight. The sample was then heated at 115°C for 2.5 hr, and cooled to room temperature. 50 ml of distilled deionized water was added and mineral concentrations were determined by running the sample on ICP.

RESULTS AND DISCUSSION

Tissue Culture Cultivation

In order to encourage bermudagrass root growth while maintaining regular shoot growth during tissue culture cultivation process, the ratio of Naphthaleneacetic acid (NAA) and 6-Benzylaminopurine (BA) (Gamborg et al., 1976) was adjusted and results were compared. The

regular ratio for NAA: BA is 1:1 (v/v) with a concentration of 5.37 μM of NAA and 4.44 μM of BA. The different NAA: BA ratios of 6.65:0 (v/v) and 0.65:0 (v/v) were tested, and records from preliminary studies indicated that the NAA: BA ratio of 0.65:0 has the best result of encouraging bermudagrass root growth while maintaining satisfactory shoot growth. Fig. 2.5. shows the effects of the three different ratios of NAA: BA on bermudagrass growth. Plant preservative mixture (PPM) was added at a concentration of 1ml/L for the purpose of preventing airborne microbial contamination. PPM is heat stable and can be autoclaved with tissue culture medium, which makes it easily operated.

Microbial contamination in each test tube was checked visually, seedlings with contamination were discarded. Two types of microbial contaminations were observed in this study: external contamination which was caused mostly by airborne microbes and internal contamination which was from the inside of the seed. The first type of contamination can be greatly reduced by surface sterilization and careful operation under the clean hood during transfer. The internal contamination can only be detected with seedling growth. It took no more than three days to eliminate external contamination while up to ten days for internal contamination. The surface sterilization method described above can effectively sterilize bermudagrass seeds. However, the percentage of internal contamination could be very high and up to 20 % (e.g. 40 out of 200 seedlings), which indicated extra seedlings should be prepared in case of high rate of internal contamination.

It usually took about two weeks for all the seedlings to grow six leaves before they can be transferred into growth units. According to preliminary studies, seedlings with six leaves were more easily established after transferred into growth units than those had less than six leaves. On the other hand, seedlings that grew in test tubes for longer than two weeks started to experience nutrient deficiency and could not be used in the experiment.

Plant Health

Mineral nutrient contents in bermudagrass shoot tissues were analyzed and recorded. The average concentrations of macronutrients of P, Ca, K, Mg, Na, and S were 0.53 %, 0.81 %, 3.48 %, 0.24 %, 0.04 %, and 0.56 % respectively. The average concentrations micronutrients of Fe, Zn, Cu, Mn, Ni, B, and Al were 224.51 ppm, 46.47 ppm, 18.55 ppm, 162.29 ppm, 10.17 ppm, 35.85 ppm and 52.l4 ppm respectively. According to McCrimmon (2001), mineral contents of major macronutrients were all within the sufficiency range of 0.2-0.5 % for P, Mg, S, 0.5-1.5 % for Ca, and 2.0-5.0 % for K. This indicated that no macronutrient deficiencies occurred during the experimental period, further indicated the effectiveness of the growth unit and the experimental procedures for maintaining healthy bermudagrass growth. It is critical to avoid nutrient deficiency in root exudate studies because nutrient deficiency could result in composition and concentration changes in root exudates and lead to unreliable results (Rovira, 1969; Neumann and Römheld, 2000; Curl and Truelove, 1986).

Bermudagrass biomass and rhizosphere sand weights were recorded. The averages of root fresh weight and accumulated shoot dry mass were 1.05 g and 0.37 g respectively. There was no significant difference observed among the four growth units on both root fresh weight and shoot dry mass, indicating the success of the controlled growth environment.

Bermudagrass visual quality ratings throughtout the study were taken every three days based on a scale of one to nine with one of being dead grass and nine of being green and vigorously growing grass. The average of the visual quality ratings of the four growth units during the 30-day study was 8.0, and there was no significant difference among the four growth units throughout the study. Bermudagrass seedlings completely established in the growth units after two weeks of transfer (Fig. 2.4.). Most of the seedlings developed new tillers during the second week in growth units, and had three tillers at the end of the 30-day study. Leaf tissues

appeared green and whole plants showed vigorously growth throughtout the study, indicating the success of the growth unit design on providing a healthy growth condition. Most bermudagrass roots were observed reaching the bottom of root zone on the third week, however, no roots were growing out of the bottom hole and interfering with the lechate sample.

Changes of Leachate pH

Leachate pH was measured after each collection. The initial sand pH was 7.0 at the beginning of the study. Nutrient solution pH was adjusted to 4.5 before each watering. The averages of leachate pH collected from the four growth units increased slightly from 5.3-5.8 during the 30-day experiment. However, the result did not indicate significant difference among the four growth units based on different collection dates. The slight change in pH could be cation exchange activity occurring between the large quantity of Ca^{2+} and Mg^{2+} absorbed on the sand particle surfaces and positively charged ions in nutrient solution.

CONCLUSION

The growth unit system designed in this study is suitable for turfgrass rhizosphere studies especially for root exudate evaluation. However, it could also be applied to other plant species. This system provided a controlled and optimized growth condition with sand as the growth medium, which ensures mechanical impedance for plant roots growth. What is more important is the convenience of leachate sample collection, without disturbance or damaging of plant roots. The requirement of high light intensity for specific plant species such as bermudagrass was achieved with the transparent cap covered on top. Meanwhile, aeration through micro-pore filter is critical in either sand or hydroponic culture (Hewitt, 1952). The length of shoot zone and root zone can be easily adjusted separately based on specific needs by adding more cutting more magenta boxes. The growth unit system is also applicable with many other rhizosphere studies such as specific strains of rhizobium inoculation, nutrient stress (e.g. P and Fe deficiencies), and

heavy metal stress. The system supports long term growth of plants and collection of leachate samples, making observations of root exudate changes practical.

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Table 2.1. Chemical and physical properties of the sea sand used in this study.

pH†	Density (g/cm^3)	Mineral Nutrient (ppm)									
		N‡	P	K	Ca	Mg	S§	Fe	Zn	B	Cu
7.0	1.59	14	2	40	720	138	18	3.6	0.1	0.5	0.1

† 1:1 w/v sand and H_2O (Zhang et al., 1998).

‡ NO_3-N.

§ SO_4-S.

Table 2.2. The modified tissue culture medium recipe used to culture bermudagrass.

Compound	Concentration (/L)
MS†	4.32 g
Sucrose	87.64 mmol
Thiamine	1.33 µmol
i- Inositol	5.55 µmol
NAA‡	0.17 µmol
BA§	0.00 µmol
PPM¶	1.00 mL
Plant growth agar#	9.00 g

† Murashige & Skoog Basal salt mixture (M571, Phyto Technology Laboratories, Shawnee Mission, KS).

‡ Naphthaleneacetic acid (Sigma Co., St. Louis, MO).

§ 6-Benzylaminopurine (Sigma Co., St. Louis, MO).

¶ Plant preservative mixture (Plant Cell Technology, Inc. Washington, DC).

Plant tissue culture agar (Phyto Technology Laboratories, Shawnee Mission, KS).

Table 2.3. The modified Hoagland nutrient solution used to fertilize plants in growth units.

Compound	Amount for 1L	Final Concentration
Macronutrient	(g)	(mM)
$Ca(NO_3)_2 \cdot 4H_2O$	236.10	7.00
KNO_3	101.10	5.00
KH_2PO_4	136.10	2.00
$MgSO_4$	120.37	2.00
Micronutrient		
H_3BO_3	0.0280	0.45
$MnSO_4 \cdot H_2O$	1.5372	9.09
$ZnSO_4 \cdot 7H_2O$	0.2000	0.70
$CuSO_4 \cdot 5H_2O$	0.1000	0.40
$NaMoO_4$	0.0250	0.14
$FeCl_3$	1.6220	10.00

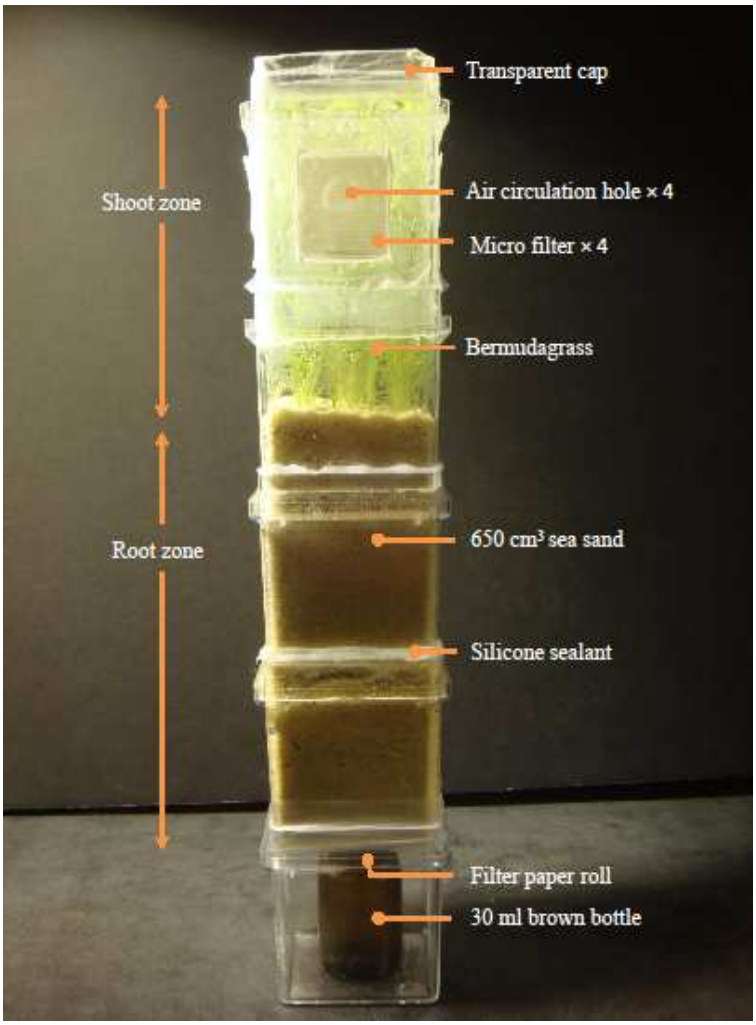


Fig. 2.1. Construction of the plant growth unit.

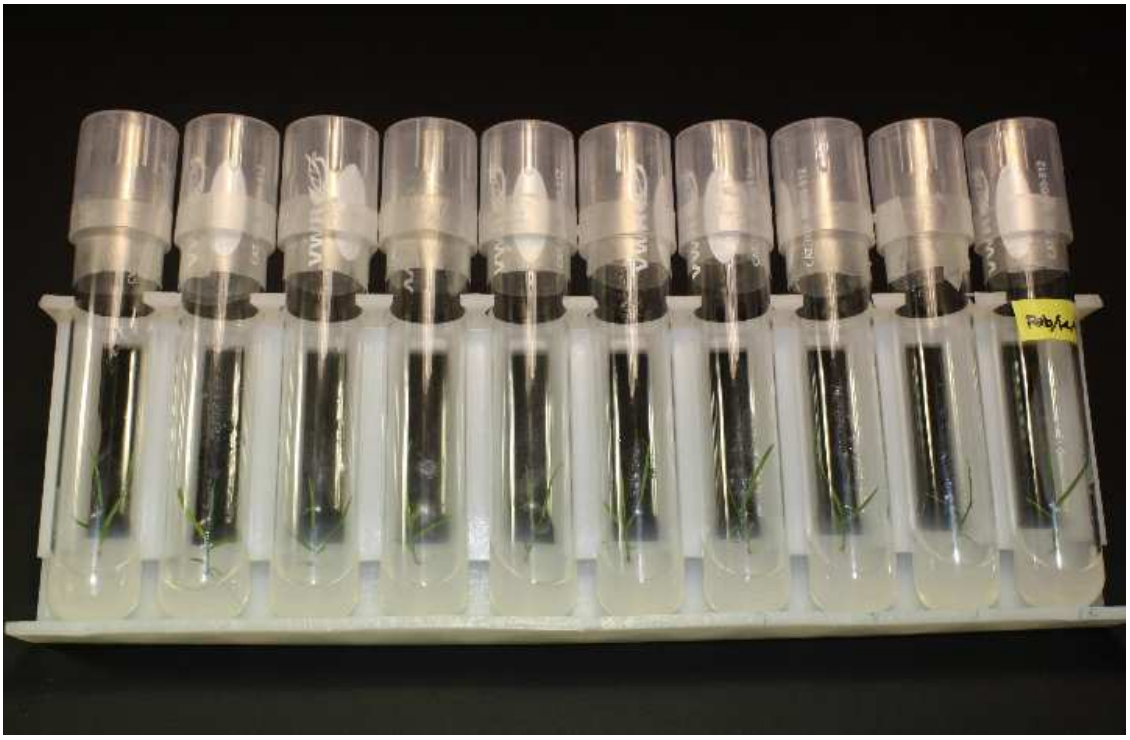


Fig. 2.2. Test tubes with germinated bermudagrass seedlings placed on tissue culture racks with a 45° slant.

Fig. 2.3. Bermudagrass growing in growth units under growth light.

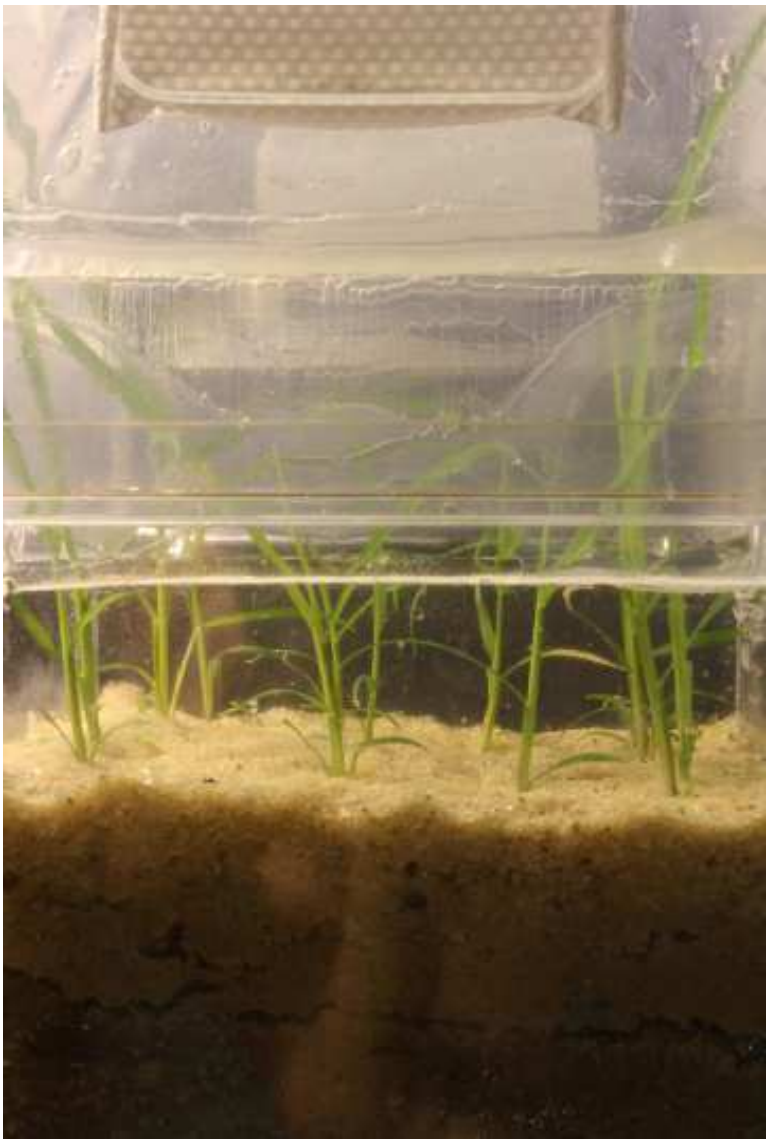
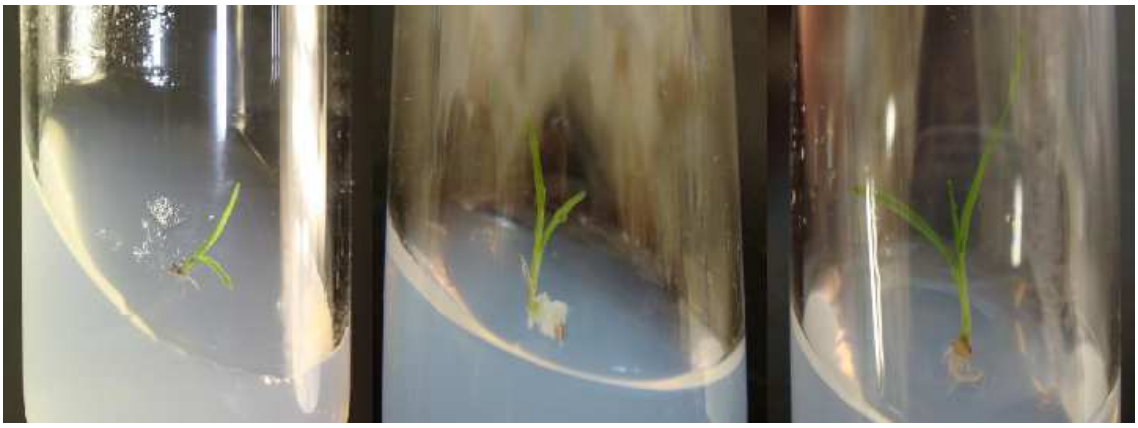


Fig. 2.4. ‘U-3’ bermudagrass growing in a growth unit 7 days after transfer.



a b c

Fig. 2.5. Effects of three different ratios of Naphthaleneacetic (NAA) and 6-Benzylaminopurine (BA) on ‘U-3’ bermudagrass root development. a. NAA:BA = 1:1 (v/v) with a concentration of 5.37 µM for NAA and 4.44 µM for BA; b. NAA:BA = 6.65:0 (v/v) with a concentration of 1.79 µM for NAA and 0.00 µM for BA, callus was accumulated instead of plant roots; and c. NAA:BA = 0.65:0 (v/v) with a concentration of 0.17 µM for NAA and 0.00 µM for BA. Fast root development without visually detected leaf tissue growth inhibition.

CHAPTER III

VARIABILITY OF ORGANIC ACID CONCENTRATIONS IN BERMUDAGRASS ROOT EXUDATES UNDER ALUMINUM STRESS

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ABSTRACT. Root exudates can act as root zone chelators to reduce harmful effects when plants are under heavy metal stress. Aluminum toxicity in acidic soils is a common problem in bermudagrass areas across the United States. Therefore, a controlled environment study was developed to evaluate organic acid changes of 'Princess-77'. Aluminum ($AlK(SO_4)_2 \cdot 12H_2O$) treatments were applied at 100 ppm and 200 ppm to bermudagrass growing in a 4.5 pH sand profile. Treatments also included a 4.5 pH treatment with no Al and a 7.0 pH control with no Al. Each treatment was replicated three times in a randomized complete block design. Five organic acids (oxalate, citrate, malate, malonate, and succinate) were found in bermudagrass root tissues. Citric and malic acid were the dominant acids compared to oxalic acid. Oxalic acid only accounted

for 11 % of the total organic acid concentration in root tissues compared that in root exudate samples (63 %), which may indicate the specific response of oxalic acid to external Al stress. Al was accumulated in shoot tissues at concentrations of 1214.41 mg/kg and 2261.13 mg/kg dry wt. under 100 ppm and 200 ppm Al treatment respectively. There was a significant (P = 0.01) negative linear relationship (r^2 = 0.61) between Al leaf accumulation and bermudagrass root mass where root mass decreased significantly (P < 0.05) as Al accumulation increased. Citric, malic, oxalic, and succinic acid were found in bermudagrass root exudates. Oxalic acid was the dominant acid accounting for 51-81 % of total organic acids and the exudation rate had a significant (P = 0.01) positive linear relationship (r^2 = 0.78) with external Al concentrations. Citric acid was induced by Al stress and higher exudation rate was detected in 100 ppm Al stress, the average exudation rate of malic acid also increased under Al treatment compared to the control treatments. The results indicated potential Al tolerance mechanisms presented in bermudagrass cultivar 'Princess-77'.

Keywords: Bermudagrass (*Cynodon dactylon* var. *dactylon*), aluminum stress, root exudate, organic acid.

INTRODUCTION

Aluminum toxicity has been recognized as the major factor limiting agricultural production on acid soils worldwide. About 30 % of the world total land consists of acid soil (Kochian et al., 2004). It's been estimated that about 50 % of total arable land is impacted by aluminum toxicity including southern and transitional zones in the United States, where warm-season turfgrass are grown (von Uexküll and Mutert, 1995; Baldwin et al., 2005). In acid soil (pH < 5.0) with a high mineral content, aluminum is soluble in the soil solution and forms various aluminum hydroxides depending on the pH (Kinraid, 1991). Hue et al. (1986) and Kinraide (1991)

have indentified Al^{3+} as the most phytotoxic aluminum ions to plant growth (Al^{3+} will be represented as Al for the rest of the text). Al toxicity inhibits plant root growth and further impacts water and nutrient uptake systems, therefore resulting in a weak and sensitive plant. The exposure to even micro concentration of Al causes inhibition of primary root elongation and lateral root formation (Samac and Tesfaye, 2003), moreover, the inhibition of root elongation occurs within a very short time period (30-120 min) (Delhaize and Ryan, 1995; Barceló and Poschenrieder, 2002; Doncheva et al., 2005).

It has been found that some plants growing on Al toxic soils evolved various tolerant mechanisms to cope with Al toxicity. One of the well proved mechanisms is by secreting specific organic acid anions into rhizosphere that combine with the mobile Al and form less or non-toxic Al-organic acid complex, therefore reducing Al toxicity externally (Ma, 2000; Ma et al., 2001; Ryan et al., 2001; Samac and Tesfaye, 2003).

Vast studies of Al accumulating plants and species regarding Al detoxification have been investigated during the past century. However, most of the plant species have been investigated are crop plants (e.g. maize, wheat, barley, rice), (Table 1.2.). Although there are several published studies about Al tolerance in turfgrass (Baldwin et al., 2005; Liu et al, 1997; Liu et al., 1998; Liu, 2005; Wu et al., 1981; Rengel and Robinson, 1989a; Rengel and Robinson, 1989b; Foy and Murray, 1998), most emphasized nutrient uptake and genetic variations under Al stress. There is no data reported on organic acid exudation from turfgrass related to Al tolerance. This study was developed to investigate changes in organic acid secretions of bermudagrass under aluminum stress and the potential tolerant mechanisms involved.

Objectives

The objectives of these studies were 1) to evaluate the quantity of five organic acids (oxalate, citrate, malate, malonate, and succinate) in bermudagrass root exudates under aluminum

stress; 3) to determine the relationship between organic acid exudation and external aluminum concentration; and 4) to determine bermudagrass physiological status in response to aluminum treatments.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

'Princess-77' bermudagrass (*Cynodon dactylon* L.) was used in this study (Baldwin et al., 2005; Wu et al., 1981; Liu, 2005). Bermudagrass was grown in the growth unit apparatus modified from Da and Deng (2003), and Henry et al. (2006). The design of growth unit has been illustrated in chapter 2.

Bermudagrass seeds were surface sterilized by soaking in a mixed solution of 20 % commercial bleach solution and 0.1 % Tween-20 for 30 min, followed by rinsing with deionized water. Seeds were then geminated in a Petri dish filled with wet filter paper for three days and transferred into plant growth agar (Phyto Technology Laboratories M571) until each seedling grew out six leaves. Fourteen seedlings of similar size with no evidence of microbial contamination were transferred into each growth unit with 650 cm^3 sea sand (Fisher Scientific, New Jersey) as the growth medium.

Due to the need of lowering sand pH for effective Al stress application and low pH control treatment, sea sand buffer index was measured by auto titrated and pH was adjusted to 4.5. Buffer index for pH of 4.5 was 1.97 equivalent/g. Sand was soaked in the required volume of 0.1M HCl solution until dried out under room temperature, the wet and dry cycle was repeated twice before autoclaving. After autoclaving, 24 hr was allowed for everything to cool down to room temperature before seedlings could be transferred into growth units.

After transfer, the root zones were all wrapped with a black cardboard covered with aluminum foil to protect roots from exposing to light. All growth units were placed on a bench top under a controlled lab environment maintained at an average light intensity of 1000 μmol m^{-2} s^{-1} with a 14 hr photoperiod, constant temperature of 25°C, and relative humidity (RH) of 64 %.

Plants were allowed to establish in growth units for two weeks before Al treatments were started. Each growth unit was watered with 30 ml of modified Hoagland nutrient solution (Hoagland and Arnon, 1950) every three days and shoot tissues were cut once a week. The concentrations of mineral nutrients in the solution were (in mM): 7.0 mM $Ca(NO_3)_2$, 5.0 mM KNO_3, 2.0 mM KH_2PO_4, 2.0 mM $MgSO_4$, 45.3 μM H_3BO_3, 9.1 μM $MnSO_4 \cdot H_2O$, 0.7 μM $ZnSO_4 \cdot 7H_2O$, 0.4 μM $CuSO_4 \cdot 5H_2O$, 0.1 μM $NaMoO_4$, 0.0 μM FeEDTA. Nutrient solution pH was adjusted to 6.5 for neutral pH control and 4.5 for the rest. All the nutrient solutions were autoclaved before watering, extra solutions were prepared to check the pH after autoclave.

Application of Aluminum Treatment

Al treatments started after two weeks plants grown in the growth units and last for another two weeks before plants were harvested. For the Al treatment, Al was added into 30 ml of nutrient solution and filtered by a 0.45 μm membrane (Millipore Corp.), then applied to each growth unit. According to the sand dry weight in each growth unit (333 g sand/growth unit), 0.59 g and 1.17 g $AlK(SO_4)_2 \cdot 12H_2O$ was added to provide 100 ppm Al and 200 ppm Al separately. To ensure a thorough Al treatment, 300 ml of 100 ppm and 200 ppm $AlK(SO_4)_2$ solution was flushed through each Al treated growth unit and nutrient solution were used to flush all growth units under two control treatments at the last day of the first two weeks. Al solutions were made by filtering rather than autoclaving in order to avoid precipitation and/or pH change during autoclaving (Miyasaka et al., 1991). Also, Al solution pH could not be adjusted to 4.5 due to the precipitation of Al and OH^- during pH adjustment.

Root Exudates Collection

Root exudates were collected at each watering. After carefully pipetting 30 ml of nutrient solution into each growth unit, and waiting until dripping of leachate in the collection bottle stopped, a new collection bottle was placed in the growth unit. The leachate sample was filtered through a 0.45 μm membrane into a 50 ml volumetric flask, mixed well and separated into five subsamples: 15 ml was used to measure pH immediately after watering; 25 ml was for organic acid analyses; two 5 ml were stored as extra samples. All other leachate samples were all stored in -20˚C freezer until further preparation processed.

Harvest

After two weeks of Al treatment, growth units were carefully cut along the side and whole plants were taken out. For each individual plant, bulk sand was carefully shaken off leaving only the rhizosphere sand (sand attached on roots). Rhizosphere sand was separated with roots by immerging root parts in a beaker with 10 ml deionized water. Shoot tissues were collected into paper bags and dried in an oven at 80˚C for 48 hours (Baldwin et al., 2005).

Bulk sand from each growth unit was washed on a screen with deionized water to collected roots left in the sand. Root tissues collected from each growth unit were sonicated for 3 min, the solution was filtered through a 0.45 μm membrane into a 25 ml volumetric flask, then another 10 ml deionized water was added and sonicated for 2 min (5 min in total) (Barber and Gunn, 1974; Miyasaka et al., 1991). The solution was filtered and collected into the same volumetric flask, and then the volume was brought up to 25 ml and mixed well. The sonicated solution samples were finally transferred into 30 ml brown bottles and stored in -20˚C freezer until further processing.

After sonication, root sample fresh weight was measured and root tissues were then frozen in liquid nitrogen and stored in a -80˚C freezer until analysis. Rhizosphere sand from each growth unit was dried in an oven at 100˚C for 48 hrs and dry weight was recorded.

Data Collection

Shoot Tissue Nutrient Analysis

Shoot tissue dry samples were ground to powders by a cyclone mill (UDY Corp., Boulder, CO). Ground samples were then analyzed using an Inductively Coupled Plasma (ICP) by the soil, water and forage analytical laboratory at Oklahoma State University. A 0.5 g ground sample was digested using 10 ml concentrated nitric acid (HNO_3) and was allowed to stand overnight. Each sample was then heated to 115°C for 2.5 hr, and then cooled to room temperature. 50 ml of distilled deionized water was added and mineral concentrations were determined by running the sample on ICP. Nutrient content of P, K, Ca, Mg, S, Zn, Cu, Mn, Fe, B, and Al was reported.

Organic Acid Extracted from Root Exudate Samples

Organic acids in root exudate samples were extracted using an ion-exchange column chromatography procedure (Shen, et al., 2002; Wang et al., 2006; Ma et al., 1997c; Zeng et al., 2008). A 25 ml of root exudate subsample was defrosted and passed through a Kontes glass column (1 cm ID, 30 cm length) packed with 5.0 g of cation-exchange resin (Amberlite IR-120B H^+ form, Sigma-Aldrich Co., St. Louis, MO). The eluate was collected and applied to another Kontes glass column packed with 2.0 g of anion-exchange resin (Dowex 1*8-200 formate form, Sigma-Aldrich Co., St. Louis, MO). The organic acids retained on the anion-exchange resin were eluted with 1 M HCl, and then the eluate was collected and concentrated in a speed-vac. (Savant Instruments, Holbrook, NY) to dryness. After the residue was redissolved in 1 ml 0.05 M H_2SO_4, the concentration of organic acids in the sample was determined by high performance liquid chromatography.

Organic Acid Extracted from Rhizosphere Samples

A 25 ml sonicated solution including organic acids extracted from the root surface and rhizosphere sand was processed following the same column chromatography procedure described above for root exudate samples.

Organic Acid Extracted from Root Tissue Samples

Frozen roots were ground to fineness in mortar and pestle with 1 ml deionized water, the resulting mixture was centrifuged at 10000 RCF and 20°C for 15 min using a Beckman GS-15R centrifuge (Beckman, Palo Alto, CA). The supernatant was saved and the pellet was extracted with 1 ml deionized water and spun again. The extraction procedure was repeated for three more times and the total supernatant was filtered through a 0.45 μm membrane. The samples were then processed using the same ion-exchange column chromatography procedure for the organic acid purification.

Chromatographic Procedure for Organic Acid

The high-performance liquid chromatography (HPLC, Dionex 4500i; Sunnyvale, CA, USA) for organic acid quantifications was equipped with VDM-2 variable wavelength detector, GPM-2 gradient pump, and AS3500 autosampler. Separation was performed on an Aminex HPX-87H column (Bio-Rad, Hercules, CA) with a guard column (Bio-Rad, Hercules, CA) and a column heater (Eppendorf CH500, Hamburg, Germany). The column temperature was set at 60°C, 0.005 M H_2SO_4 was used as the mobile phase at a flow rate of 0.6 ml min^{-1}. Peak area of target organic acids were detected under the wavelength of 214 nm, and compared to reference standards. All data acquisition and instrument control were accomplished using PeakNet software (Version 5.21; Dionex), on a COMPAQ TFT 5010 computer.

Data Analyses

Data analysis was completed using SAS 9.1 (SAS Institute Inc., Cary, NC) for a randomized complete block with three replications. The GLM procedure was used to complete analysis of variance testing. When the criteria were met for ANOVA at the 0.05 probability level, mean separation tests were performed using the least significant difference (LSD) test at the 0.05 probability level. Organic acid, Al concentration, root weight, and rhizosphere data were subjected to simple linear regression analysis and coefficient of determination calculation using the REG procedure at the 0.05 significance level.

Experimental Design

The experimental design was a randomized complete block design with two control treatments (pH 4.5 control and pH 7.0 control), two levels of aluminum treatments (100 ppm Al and 200 ppm Al), and each treatment replicates three times. All the growth units within same treatment were relocated and reoriented everyday to reduce environmental effects.

RESULTS AND DISCUSSION

Al Accumulation in Bermudagrass Shoot Tissues

Al concentrations in bermudagrass shoot tissues were shown in Table 3.1. The 100 ppm Al treatment resulted in an Al concentration of 45.01 mmol/kg dry wt. equals to 1214.41 mg/kg dry wt.; while Al concentration increased to 2261.13 mg/kg dry wt. under 200 ppm Al treatment after 16 days of treatment.

According to Ma et al. (1997b) and Kochian et al. (2004), Al was absorbed through root tissues and was translocated slowly to the above ground parts. Most plants accumulate no more than 200 mg Al/kg dry weight, while the Al-accumulators were found to accumulate 10 times or

more of Al (Ma et al., 1997a). Matsumoto et al. (1976) reported more than 30000 mg Al/kg in old tea tree (*Melaleuca alternifolia* L.) leaves (dry wt.).

The ability of plants to absorb and transfer Al from soil into the upper part is attributed to the internal Al tolerance mechanisms, which were briefly described in the first chapter. Current explanations with supporting evidence of a few plants (Watanabe et al., 1998; Ma et al., 1998; Ma and Hiradate, 2000; Cuenca et al., 1990) are Al is transferred within the xylem as the form of Al-organic acid complex (there could be more than one organic acids involved) and is finally stored in leaf cell vacuole as the form of Al-organic acid complex, which is non-phytotoxic. Although this study was not designed to investigate internal Al tolerance mechanisms of bermudagrass cultivar, and there is no data collected to prove that the internal Al tolerance mechanisms are the explanations for the high concentrations of Al accumulated in shoot tissues during this relatively short period; we still speculate one or more of the internal mechanisms was involved (e.g. Al was complexed with one or more organic acid ligand and was isolated in the vacuoles). Comparing to the other "Al-accumulators", bermudagrass is more widely cultivated (in warm climates all over the world between 30° south and 30° north latitude) and is fast growing, which could be one of the explanations for fast Al accumulation. More important, as a turfgrass, the upper ground tissues can be mowed to a very low height and the biomass with accumulated Al can be removed and recycled with clippings. However, bermudagrass (as in this case cultivar 'Princess-77') under both the two levels of aluminum stress showed upper ground tissue damage. Due to the fact only one cultivar has been tested in this study, there is no comparison to make the conclusion that all other bermudagrass cultivars would show the same tissue damage when experiencing same stress.

Bermudagrass Growth as Affected by Aluminum

It is known that the presence of phytotoxic Al in soil usually resulted in deficiencies of P, Ca, and Mg for plant growth, and particularly depresses Ca and Mg uptake more than other

mineral nutrients (Rengel and Robinson, 1989b). It is assumed that these deficiencies are due to the inhibited uptake of these mineral nutrients by Al. Mineral nutrient contents in bermudagrass shoot tissues are listed in Table 3.2. According to McCrimmon (2001), P, K, and S concentrations in bermudagrass shoot tissues under all treatments were within the sufficiency range (0.2-0.5 %, 2-5 %, and 0.2-0.5 % respectively), which will further confirm that the organic acids detected in root exudates were not induced by P deficiency (Tawaraya et al., 2009; Playsted, et al., 2006); while Ca and Mg shows deficiencies (< 0.5 %, and < 0.2 % respectively) under Al stress treatments. Huang and Bachelard (1993) reported consistent results with pine trees (*Pinus radiate* D. Don). Baldwin et al. (2005) also reported decreased relative Ca concentration in ten warm season grass with increased Al concentrations. Thornton et al. (1986) reported reduction of P, K, Mg, and Ca in sugar maples (*Acer saccharum* Marsh.) grown in Al nutrient solution.

The mechanisms about how Al inhibited mineral nutrients uptakes have been thoroughly studied since last centry and it was well accepted that Al inhibits minereal nutrient uptake by directly blocking root-cell ion transport proteins (Kochian et al., 2005). MacDiarmid and Gardner (1998) reported Alr genes (isolated from yeast strains that increased Al^{3+} tolerance of yeast species of *Saccharomyces cerevisiae* when overexpressed) as Mg^{2+} transporters localized on a plasma membrane. The Al toxicity reduced Mg^{2+} influx through Alr proteins and therefore resulted in magnesium uptake inhibition. Evidence was also provided that Al interacts with plasma-membrane channel proteins and reduces the uptake of ions such as K^{+} and Ca^{2+} (Piñeros and Kochian, 2001). On the other hand, there are contrary results indicating Al toxicity might not be the direct cause of specific nutrient deficiency. For example, P concentrations in both Al-tolerant and Al-sensitive Kentucky bluegrass (*Poa pratensis* L.) cultivars increased with increased Al concentrations (Foy and Murray, 1998); they also concluded the greater Al sensitivity of Al-sensitive bluegrass cultivar was not related to reduced uptake of P. Other studies about mineral nutrient uptake inhibitions affected by Al can be found in literatures and will not be

discussed in details in this study (Huang and Bachelard, 1993; Ryan et al., 1994; Rengel and Robinson, 1989a; Malkanthi et al., 1995).

Al toxicity symptoms on both root and shoot tissues can be visually observed, however, root tissue damage is known to be an Al toxicity characteristic (Kochian, 1995). For sensitive plants, it takes approximately 30 min to 2 hr for statistically significant inhibition of root elongation to occur (Barceló and Poschenrieder 2002), which can be observed as stunted, brittle, and swollen root tips. Reduced water and nutrient absorbing efficiency of plant roots finally results in root biomass reduction. Root fresh weight measured at harvest was confirmed with this observation (Fig. 3.1.), and bermudagrass root fresh weight decreased significantly with increased Al stress.

Bermudagrass shoot tissues were observed as relatively small plants with yellowish leaves with dead leaf tips, and had less biomass coverage under Al stress. Visual quality rating through out the study was shown in Fig. 3.2. The rating values reached their highest after a week of transfer, the point where bermudagrass seedlings completely established in growth units. Al stress started to show visual damage on bermudagrass shoot tissues after three days of Al application, while siginificant visual difference between Al treatments and control treatments started on day 21 and was last to the end of the study. The Al toxicity was observed as leaf chlorosis which may due to reduced photosynthetic activities. Bermudagrass leaves also showed reduction in sizes and numbers after two weeks of Al stress application (Fig. 3.3.). However, accumulated shoot dry mass collected from Al treatment (both Al-100 ppm and Al-200 ppm) samples did not show a significant reduction comparing to low pH and neutral pH controls, while there is a significant (P = 0.01) negative linear relationship (r^2 = 0.61) between shoot tissue Al accumulation and root mass.

Rhizosphere sand was also collected at harvest and the dry weight measurements were recorded in Fig. 3.4. This indicates decreased root biomass resulted in less rhizosphere sand and less capacity of providing a microbial favorable environment. It was noted that neutral pH also decreased root mass significantly, yet maintained shoot tissue dry weight (Fig. 3.1.) when comparing with low pH control. The possible reason for root tissue reduction in the neutral pH control could be because of the interference of biocarbonate in the sand or nutrient solution with root metabolism and therefore inhibit root expansion (Lee and Woolhouse, 1969). On the other hand, P deficiency or Ca over-accumulation was not observed in shoot tissues of neutral pH control compared with low pH control; indicating no alkaline affect on bermudagrass upper ground growth.

Organic Acid Changes in Root Exudate

Five organic acids including citric, malic, malonic, oxalic, and succinic acid were tested by chromatography and only malonic acid was not detected in bermudagrass root exudate samples (Table 3.3.). Oxalic acid is the dominant organic acid in bermudagrass root exudates among all the treatments and accounts for 51-81 % of all the organic acids detected in root exudates over the 30-day study. Citric acid responded mainly to Al stress at 6-15 % and was not detected in the low pH control treatment. Malic acid at 12-23 % was found among all treatments except the neutral pH control. Succinic acid was detected mainly in low pH and neutral pH control treatments and a relatively small percentage (1-3 %) under Al stress treatment.

The exudation rate of all the four organic acids throughout the study was shown in Fig. 3.5. It is observed that organic acid exudation of low pH control and both Al stress treatments had a trend of exudation which is: decline from about 70 nmol/plant at the beginning of the study and reached the lowest exudation point then started to increase. For the neutral pH control, oxalic acid as the dominant organic acid exuded at a variable rate from less than 10 nmol/plant to a more than

20 nmol/plant, also quite amount of succinic acid detected on day 24 and day 27. The reduced trend of exudation before Al stress was applied can be explained by the aging of plant materials (Rovira, 1969; Curl and Truelove, 1986). Furthermore, the increased trend of organic acid exudation in Al stress treated samples could be explained as the response to Al stress; due to following reasons: a) the increased exudation responded after Al stress application (within three days), and b) the increased amount is considerable (26-fold more oxalic acid exuded under 200 ppm Al stress than low pH control on day 21, Fig. 3.7.); c) specific acid such as citric and malic acid showed up only in Al stress treated samples after the exudation declined before treatment started (Fig. 3.6.).

Vast studies have investigated exudation of certain organic acids in regards to Al detoxification as one of the external Al tolerance mechanisms (Fig. 1.1.). It has been proven that citric and malic acid are the major organic acids in root exudates involved with Al detoxification (Ma et al., 1997c; Miyasaka et al., 1991; Kollmeier et al., 2001; Mariano and Keltjens, 2003; Piñeros et al., 2002; Delhaize et al., 1993; Zhang et al., 2001). Oxalic acid exudation is also been reported in response of Al stress in taro (*Colocasia esculenta* L.) and buckwheat (*Fagopyrum esculentum* Moench.) root exudates (Ma and Miyasaka, 1998; Zheng et al., 1998; Ma et al., 1997b).

According to the results of this study, oxalic acid is the dominant organic acid in bermudagrass root exudates and could be one of the specific organic acids induced by Al. The response of oxalic acid to Al stress was detected within three days (Fig. 3.7.) confirming the findings of Ma and Miyasaka (1998), the exudation rate was also increased with increased Al stress (5.55 -16.46 nmol/plant, Table 3.3.) which was consistent with the result reported by Ma et al. (1997b). However, the exudation of oxalic acid was not completely linear along with treatment duration.

The two levels of Al treatments in this study also induced exudation of citric and malic acid in bermudagrass root exudates. However, malic acid was detected in response of Al within three days after stress started (Fig. 3.5.); while citric acid was found on day 6 after Al application. Moreover, both malic and citric acid had higher average exudation rate in Al-100 ppm treatment than Al-200 ppm treatment (Table 3.3.). The average exudation rate of citric and malic acid was shown in Fig. 3.8. There was a significant (P = 0.001) positive linear relationship (r^2 = 0.86) between total exudation rate of oxalate, citrate, and malate with increased external Al concentrations.

Organic Acids in Bermudagrass Root Tissues

Organic acids extracted from bermudagrass root tissues were shown in Table 3.4. and Fig. 3.9. seperately. Five organic acids (oxalate, citrate, malate, malonate, and succinate) were found in bermudagrass root tissues. The concentrations of oxalate, citrate, and malate are shown in Fig. 3.9., and due to the relatively low concentrations of malonate and succinate, results for those two acids were shown in Table 3.4. Among all the five organic acids, citric acid and malic acid were the dominant acids in all treatments taking accout for averages of 47 % and 40 % of the total organic acid concentration separately (Fig. 3. 10.). This result is consistent with the results reported from Zhao et al. (2003). Unlike the proportion results obtained from root exudate samples, oxalic acid concentration in root tissues (11 %) is much lower than that in bermudagrss root exudate (63 %). Malonic acid was only found in the roots of neutral pH control and 100 ppm Al treatment with relatively lower concentration of 0.05 and 2.31 nmol/g of fresh root tissue. On the other hand, the concentrations of succinic acid were found to be much higher in both Al treated root tissues than those in the two control treatments. However, there is only one rhizosphere sample found with succinic acid at the day of harvest. This indicates that even though succinic acid was contained in relatively higher concentrations inside root tissues with Al treatments, it is unlikely to be released by plant roots into the rhizosphere in reponse to Al stress.

This result was reasonable since succinic acid was not known to form a stable Al-organic acid complex with Al due to its chemical nature.

Organic Acids Detected on Surface of Roots and Rhizosphere Sand

Organic acids quantified on the surface of bermudagrass roots and rhizosphere sand are listed in Table 3.5. Oxalic acid was shown in all treatments with the highest concentration in 200 ppm Al stress and lowest concentration in neutral pH control. Citric and malic acid both had significantly higher concentrations under Al stress treatments than in the two controls (Fig. 3.11.). Note that succinic acid was detected in leachate samples among all treatments but 200 ppm Al stress on day 27 (Fig. 3.5.), however, it was found in only one of 200 ppm Al stress rhizosphere samples after harvest. It is under expectation that succinic acid was not shown in rhizosphere samples, because it's known that not a strong chelator as citric acid or malic acid, and was not reported to be involved with Al detoxification. Another observation is citric and malic acid were both detected in 200 ppm Al stress rhizosphere samples at a higher concentration than those in leachate samples, 2-8 fold more citric acid and 2-4 fold more malic acid. The fact that citric and malic acid were not detected in leachate samples of 200 ppm Al stress on day 27 but were found at much higher concentrations in the rhizosphere samples indicates that bermudagrass under 200 ppm Al stress did not stop releasing these two organic acids after 15 days of Al treatment. The speculated cause to explain this change may be due to the sample collection procedure. Leachate samples were collected from the root zone grown in 650 cm^3 sand medium and better represent the average exudation trend in the entire root zone, while rhizosphere samples were extracted from root surface and rhizosphere sand which are more emphasized on the active and sensitive part of root zone.

Changes of Leachate pH

Leachate pH was measured after each collection and results were shown in Fig. 3.12. The initial pH of sand used for Al treatments and low pH control was adjusted to 4.5 and 7.0 for neutral pH control respectively at the beginning of the study. The pH of nutrient solution used to water plants was adjusted to 4.5 for Al treatments and low pH control and 6.5 for neutral pH control. Before the Al treatment started leachate pH of Al stress and low pH control treatments were maintained at about 4.0; while leachate pH of neutral pH control increased from 6.0 to 6.7. After Al treatments started, leachate pH of low pH control decreased on day 15 and then started increasing to 6.7 on the last day of collection. For the two levels of Al stress, leachate pH decreased to around 2.6 because of the low pH of Al solution applied. However, both leachate pH of 100 ppm Al and 200 ppm Al treatment increased slightly by 0.71 and 0.72 units respectively. According to Wang et al. (2006), Al induced rhizosphere alkalinization which contributed to the Al resistance in wheat cultivar 'Atlas66', because the rhizosphere alkalinization changed the rhizosphere Al speciation and increased plant Al resistance. Due to the method of leachate sample collection and the growth medium (sand) used in this study, the leachate pH changes discussed may be biased. However, it can be concluded that 'Princess-77' bermudagrass has the ability to mediate rhizosphere pH when experiencing Al stress.

CONCLUSION

According to the results of this study, citrate, malate, oxalate, and succinate were found in bermudagrass root exudates. Oxalic acid is the dominant acid accounting for 51-81 % of total organic acids. The exudation rate was increased with increased Al stress. Citric acid was induced by Al stress, while the average exudation rate of malic acid decreased under Al treatment.

Five organic acids (oxalate, citrate, malate, malonate, and succinate) were found in bermudagrass root tissues with citric and malic acids as the dominant acids (accounting for 47 %

and 40% of total organic acid concentrations in root tissues separately). Oxalic acid, not like in root exudate samples, only accounts for 11 % of the total organic acid concentration, which may indicate the specific response of oxalic acid to external Al stress.

'Princess-77' bermudagrass accumulated Al in shoot tissues at considerable concentrations of 1214.41 mg/kg and 2261.13 mg/kg dry wt. under 100 ppm and 200 ppm Al treatment respectively compared to 75 and 34 ppm in low pH and neutral pH control treatments. This work is not able to provide evidence of internal Al tolerance mechanisms in bermudagrass shoot tissues; further work is needed to test the speculation.

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Table 3.1. Accumulated Al concentration† in bermudagrass shoot tissues under control and Al stress.

Values are means of n = 3.

Treatment	Shoot tissue Al	Shoot dry weight
	mmol/kg dry wt.	g
Control (pH 4.5)§	2.80 c‡	0.59 ab
Control (pH 7.0)¶	1.25 c	0.61 a
Al-100 ppm#	45.01 b	0.57 b
Al-200 ppm††	83.80 a	0.59 ab
LSD (0.05)	18.80	0.03

† Al concentrations were measured using inductively coupled plasma analyses.

‡ Values within a column followed by the same letter are not significantly different at $P < 0.05$.

§ Sand pH was adjusted to 4.5 for control and Al treatments at the beginning of the study.

¶ Sand pH was 7.0 for neutral pH control at the beginning of the study.

33.32 mg Al was applied as AlK(SO4)2 ·12H2O into 333.2 g sand (for each growth unit).

†† 66.64 mg Al was applied as AlK(SO4)2 ·12H2O into 333.2 g sand (for each growth unit).

Table 3.2. Mineral nutrients† in bermudagrass shoot tissues under control and aluminum treatments.

Values are means of n = 3.

Treatment	P	Ca	K	Mg	Na	S	Fe	Zn	Cu	Mn	Ni	B
	%						ppm					
Control (pH 4.5)	0.76a‡	0.98a	5.11a	0.33a	0.05ab	0.63b	154.15a	21.37a	14.50a	92.74a	11.22a	20.69a
Control (pH 7.0)	0.76a	0.97a	5.12a	0.31a	0.07a	0.66b	122.92ab	27.16a	9.74b	91.66a	5.26b	20.01a
Al-100 ppm	0.41c	0.35b	3.13b	0.10b	0.03b	0.65b	120.11b	26.87a	9.02b	36.69b	12.14a	14.22a
Al-200 ppm	0.50b	0.41b	3.33b	0.12b	0.04b	0.85a	133.05ab	33.67a	10.33b	32.96b	7.97ab	39.23a
LSD (0.05)	0.09	0.09	0.53	0.03	0.03	0.14	32.86	ns	3.72	9.22	4.96	ns

† Mineral nutrient concentrations were measured using inductively coupled plasma analyses.

‡ Values within the same column followed by the same letter are not significantly different at $P < 0.05$.

Table 3.3. Organic acid exudation rate and percentage of exudation during the study.

Values are means of n = 3.

Day†	Organic Acid	Control (pH 4.5)		Control (pH 7.0)		Al-100 ppm		Al-200 ppm	
		nmol/plant·day‡	%§	nmol/plant·day	%	nmol/plant·day	%	nmol/plant·day	%
3-12	Oxalate	10.06 ± 5.29	71	2.85 ± 1.03	100	10.66 ± 4.86	72	11.55 ± 5.56	77
	Citrate	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0
	Malate	4.02 ± 4.76	29	0.00 ± 0.00	0	4.06 ± 2.79	28	3.48 ± 4.03	23
	Succinate	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0
15-30	Oxalate	2.53 ± 1.98	46	3.33 ± 2.44	40	5.55 ± 4.82	48	16.46 ± 11.92	83
	Citrate	0.00 ± 0.00	0	0.11 ± 0.27	1	3.16 ± 3.20	27	1.71 ± 2.64	9
	Malate	0.00 ± 0.00	0	0.00 ± 0.00	0	2.33 ± 1.36	20	1.29 ± 1.48	6
	Succinate	3.00 ± 7.36	54	4.87 ± 9.24	59	0.63 ± 1.53	5	0.44 ± 1.09	2
3-30	Oxalate	5.54 ± 5.16	62	3.14 ± 1.93	51	7.60 ± 5.27	59	14.50 ± 9.78	81
	Citrate	0.00 ± 0.00	0	0.07 ± 0.21	1	1.90 ± 2.89	15	1.03 ± 2.16	6
	Malate	1.61 ± 3.44	18	0.00 ± 0.00	0	3.03 ± 2.10	23	2.16 ± 2.81	12
	Succinate	1.80 ± 5.70	20	2.92 ± 7.33	48	0.38 ± 1.19	3	0.27 ± 0.84	1

† Collection of leachate samples started on day 3; Al stress started on day 15. The study was last for 30 day from the beginning to the end.

‡ Exudation rate of each individual organic acid was expressed as nmol/plant·day and the values are means ± SD.

§ % was calculated as the amount of an individual organic acid exuded over a time period (e.g. 3-12) divided by the total amount of organic acids exuded during the same time period under the same treatment.

Table 3.4. Malonic and succinic acid (nmol/g fresh wt.) extracted from bermudagrass root tissues after harvest.

Values are means of n = 3.

Treatment	Malonate	Succinate
Control (pH 4.5)	0.00 a†	3.88 c
Control (pH 7.0)	0.05 a	15.60 c
Al-100 ppm	2.31 a	105.82 b
Al-200 ppm	0.00 a	147.89 a
LSD (0.05)	NS‡	37.51

† Values within the same column followed by the same letter are not significantly different at $P < 0.05$.

‡ NS indicates no significant difference.

Table 3.5. Organic acids (nmol/plant) extracted from rhizosphere sand and root surface at harvest.

Values are means of n = 3.

Treatment	Oxalate	Citrate	Malate	Succinate
Control (pH 4.5)	6.82 ab†	0.00 b	0.00 b	0.00 a
Control (pH 7.0)	3.96 b	2.01 b	0.00 b	0.00 a
Al-100 ppm	5.30 ab	15.85 a	8.19 a	0.00 a
Al-200 ppm	10.15 a	19.84 a	11.88 a	8.01 a
LSD (0.05)	5.66	6.92	4.05	NS‡

† Values within the same column followed by the same letter are not significantly different at $P < 0.05$.

‡ NS indicates no significant difference.

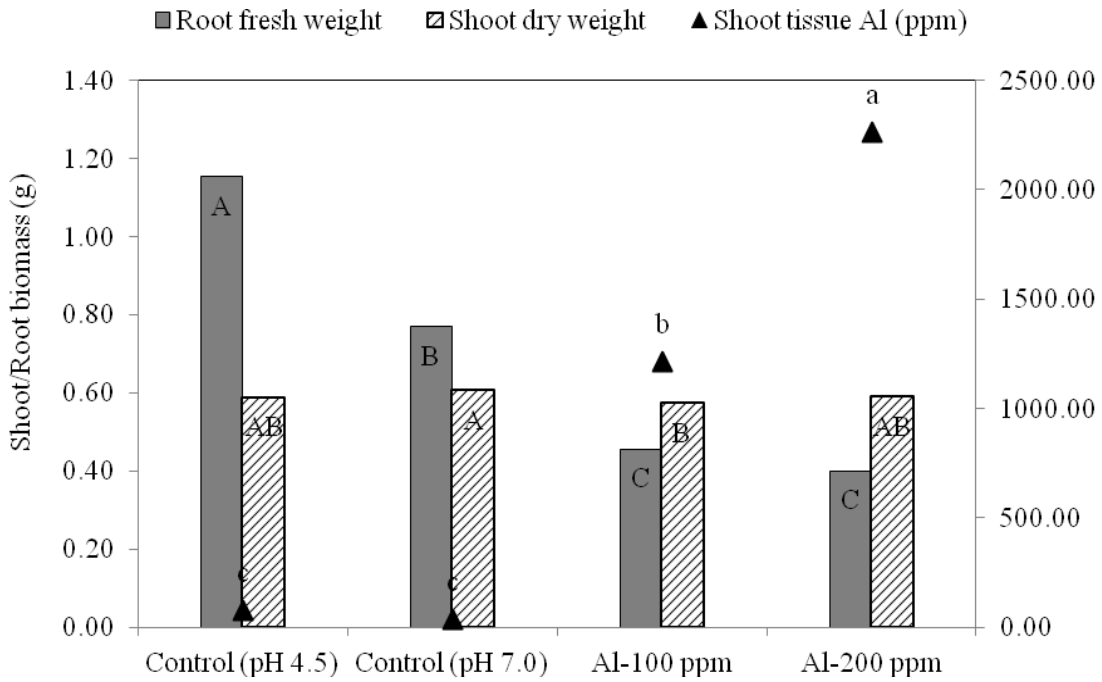

Fig. 3.1. Bermudagrass biomass and leaf tissue accumulated Al affected by two levels of Al stress treatments. Means followed by the same letter within same parameter are not significantly different at $P < 0.05$.

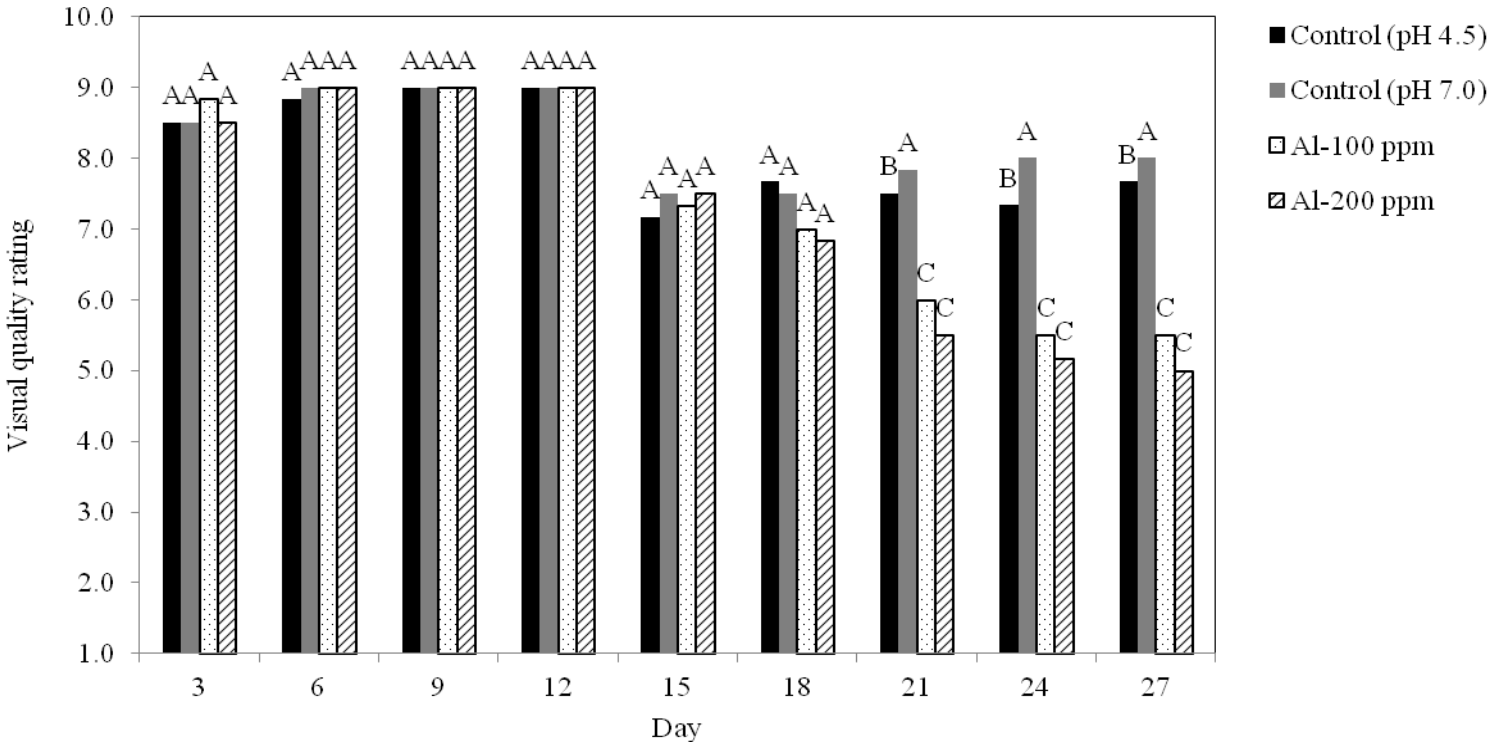

Fig. 3.2. Visual quality ratings of bermudagrass growing under control and Al stress treatments over a 27-day duration. Ratings were given on a visual rating scale from 1-9 with 1 being severely Al damaged and 9 being healthy tissue appearance. Al treatment started on day 15. Same letters within the same day are not significantly different at $P < 0.05$.

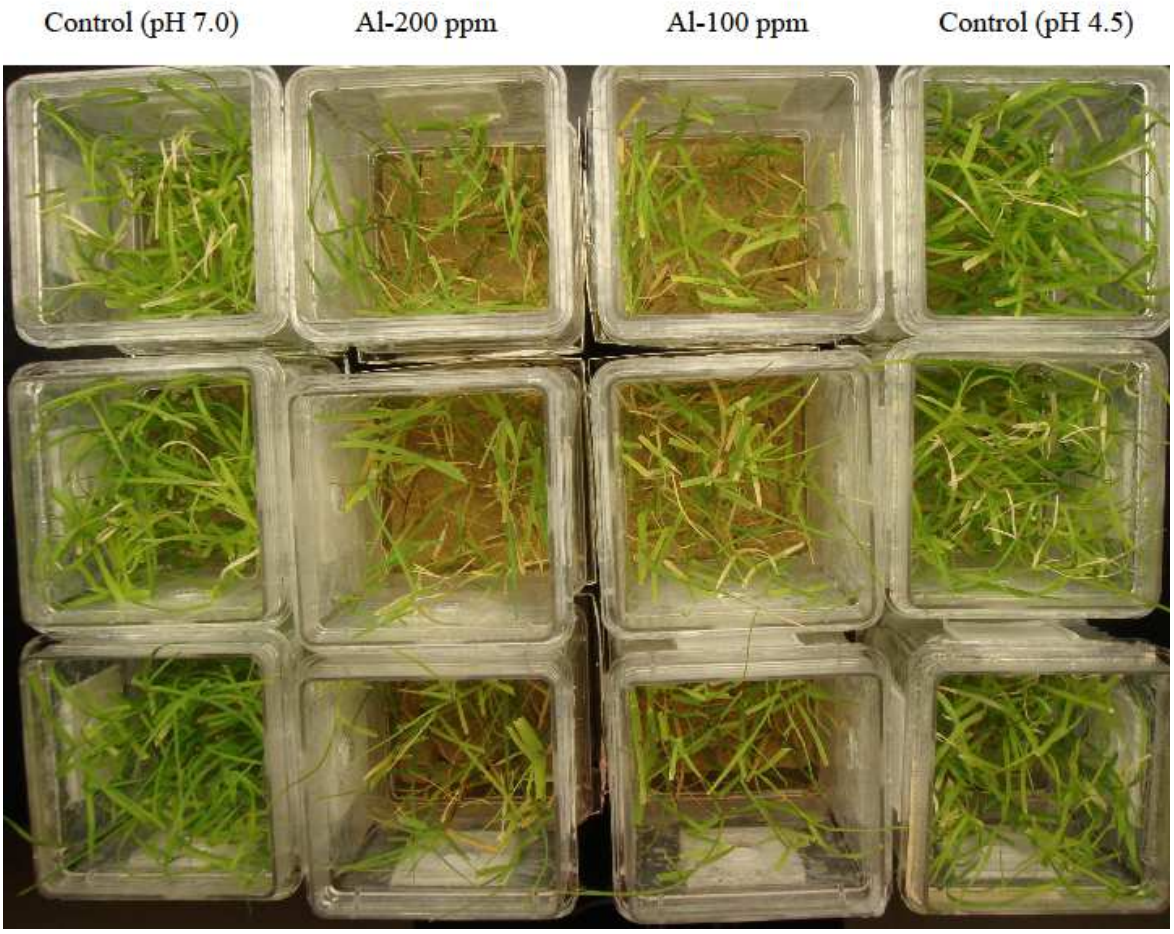

Fig. 3.3. Bermudagrass growing under control and Al treatments after 15 days.

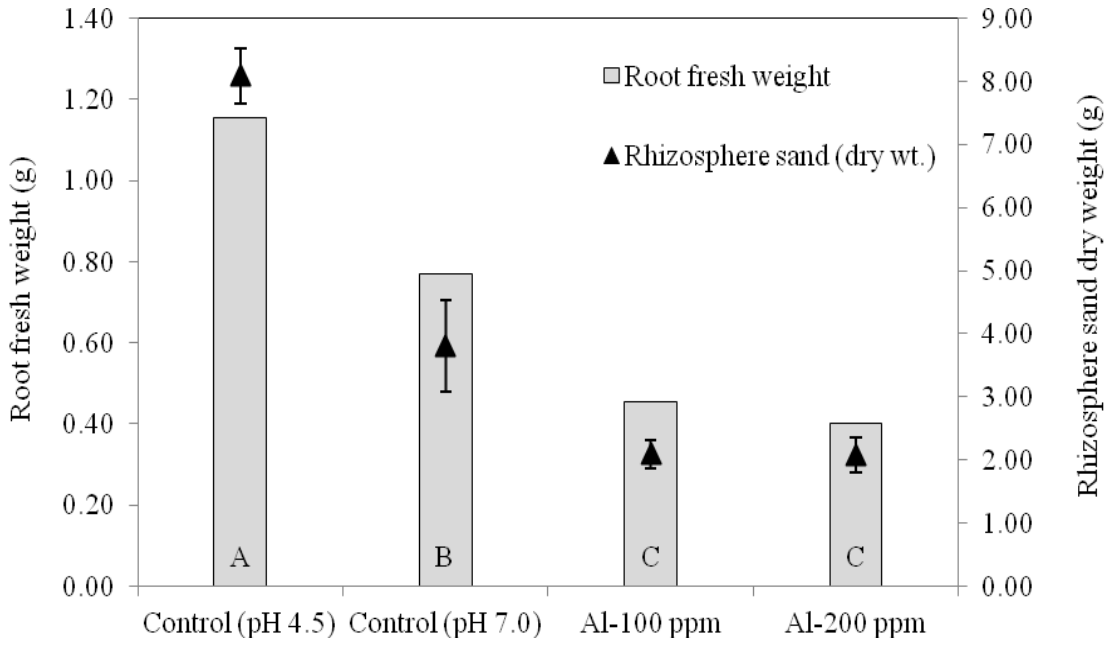

Fig. 3.4. Root fresh weight and rhizosphere sand dry weight collected from control and Al stress treatments. Means followed by the same letter within same parameter are not significantly different at $P < 0.05$. Error bars indicate standard errors of the mean (n = 3).

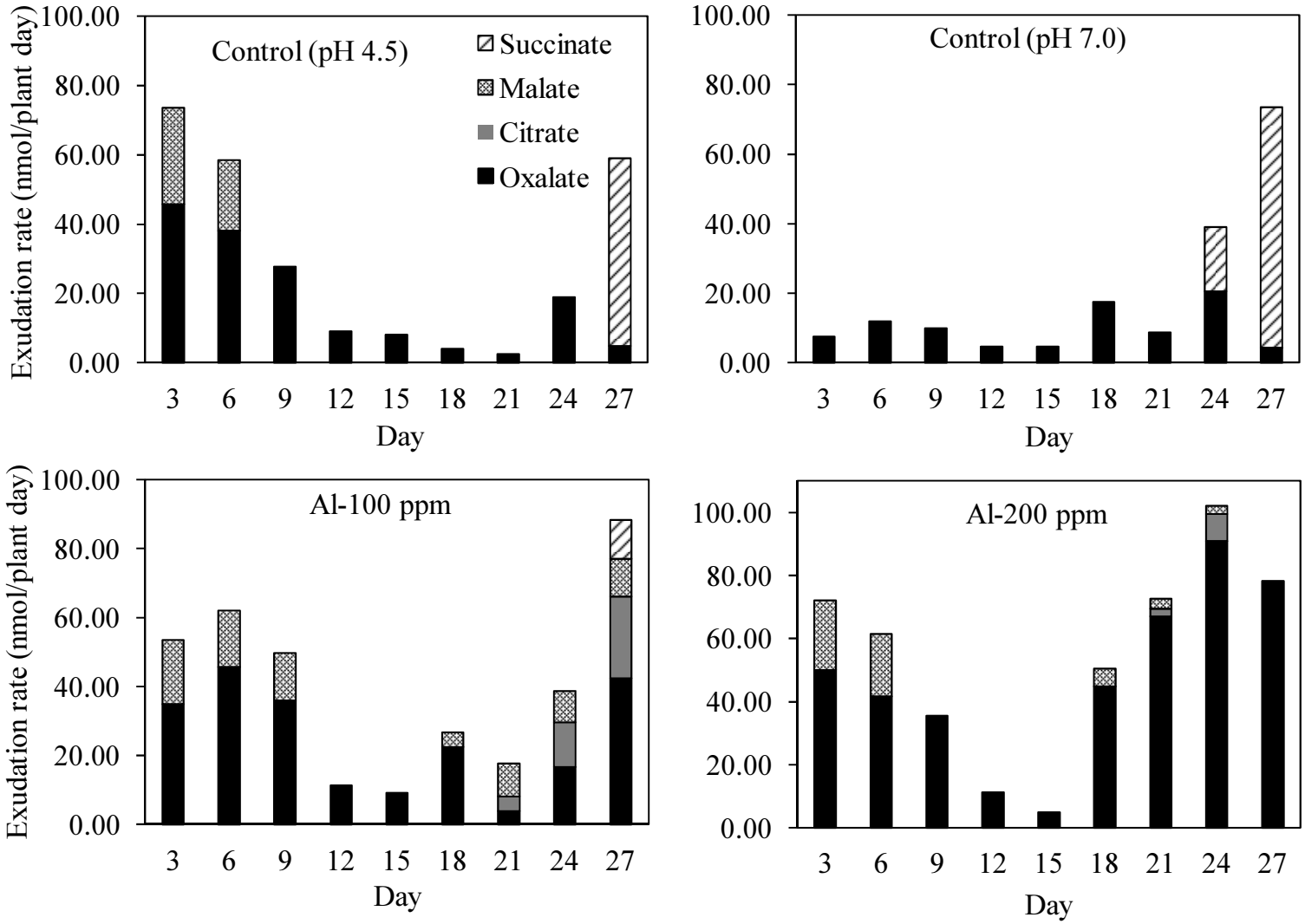

Fig. 3.5. Four organic acid exudation rates under control and Al stress treatments over 27 days.

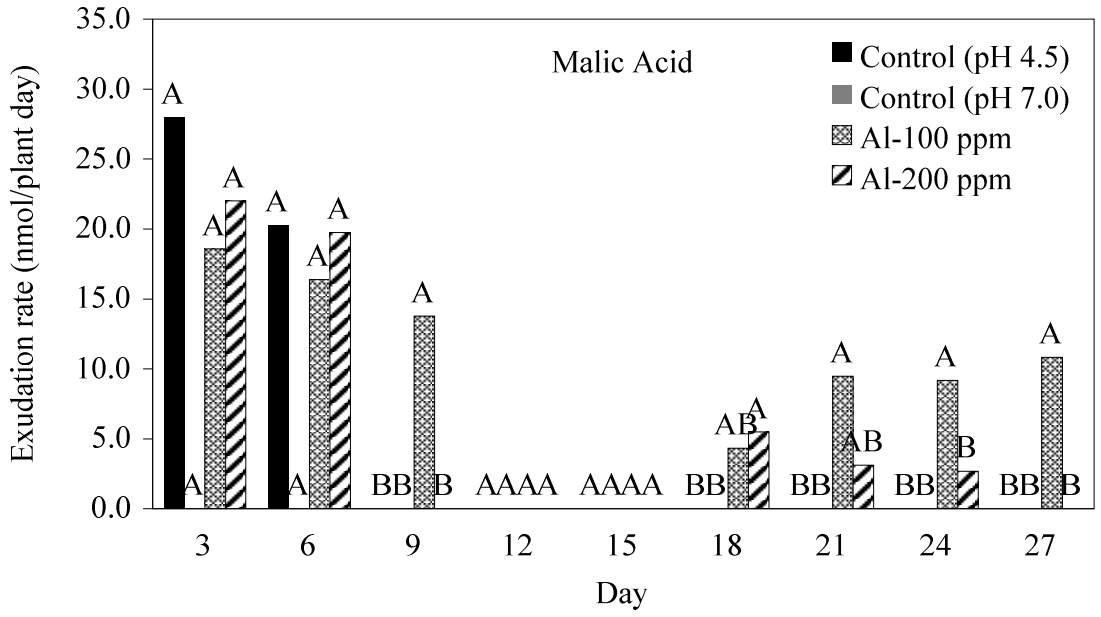

Fig. 3.6. Exudation rates of malic acid detected in bermudagrass leachate samples over 27 days. Note that leachate samples were not collected on day 0 and day 30, Al treatment started on day 15. Means (n = 3) followed by the same letter with same collection date are not significantly different at $P < 0.05$.

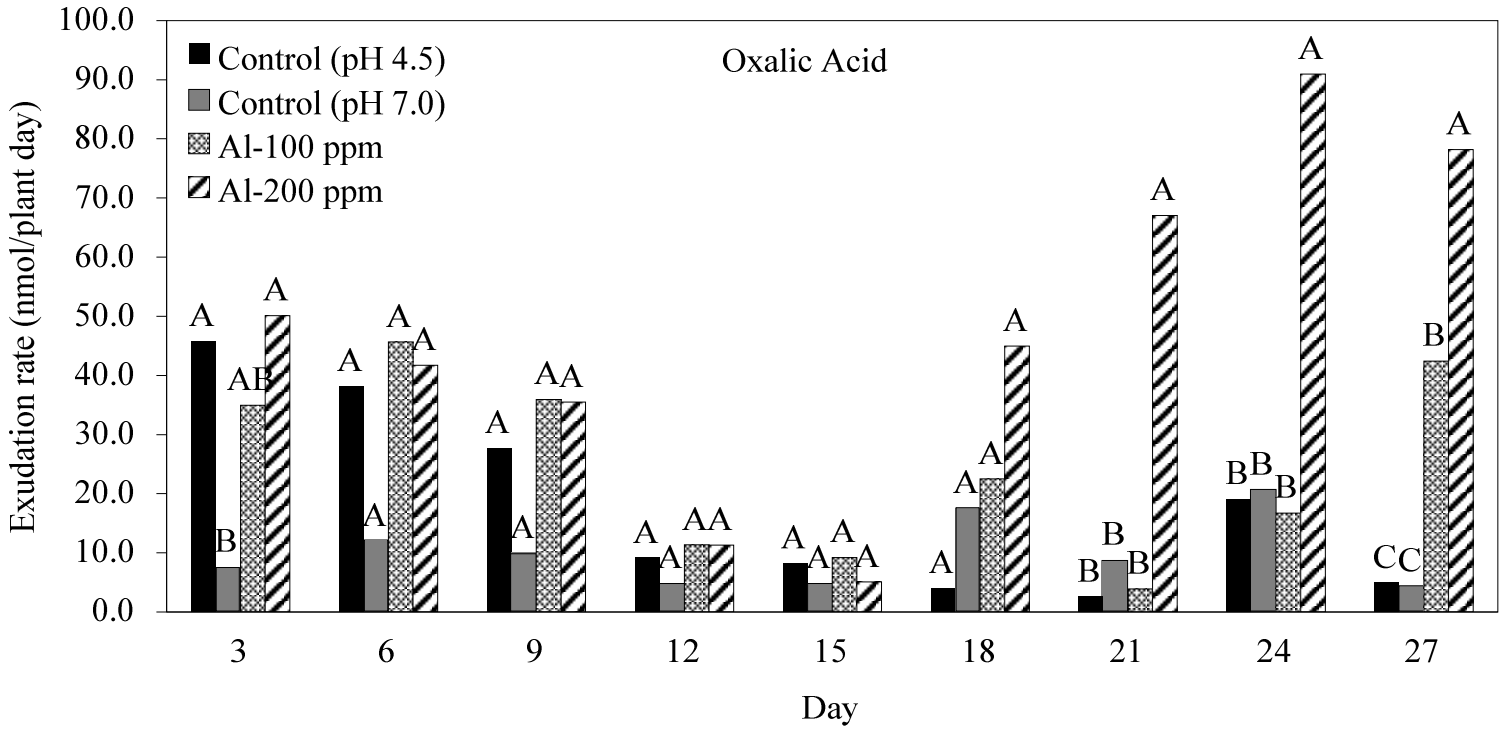

Fig. 3.7. Exudation rates of oxalic acid detected in bermudagrass leachate samples over 27 days. Note that leachate samples were not collected on day 0 and day 30, Al treatment started on day 15. Means (n = 3) followed by the same letter with same collection date are not significantly different at $P < 0.05$.

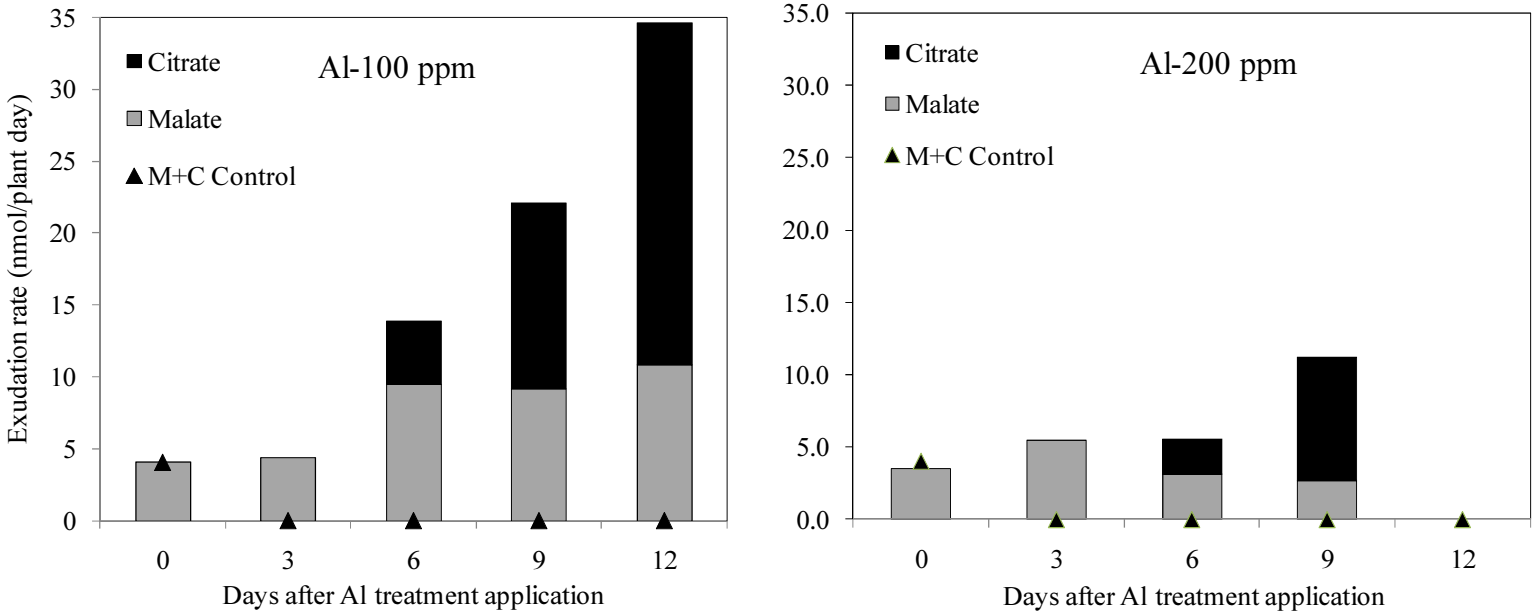

Fig. 3.8. Citric acid and malic acid detected from bermudagrass leachate samples after Al treatments began. "M+C Control" indicates the total concentration of malic and citric acids detected in low pH control samples. Note that there is no malic nor citric acid detected from neutral pH control samples over the same duration, comparison was only made between Al stress treatments and low pH control.

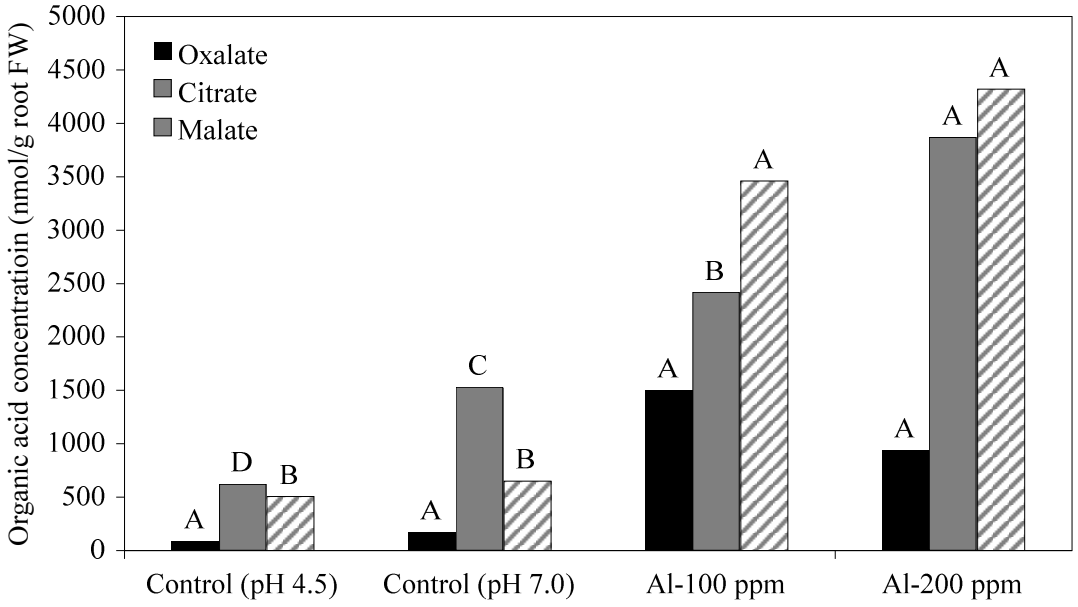

Fig. 3.9. Oxalic, citric, and malic acids extracted from bermudagrass root tissues after harvest. Means (n = 3) followed by the same letter of the same organic acid species are not significantly different at $P < 0.05$.

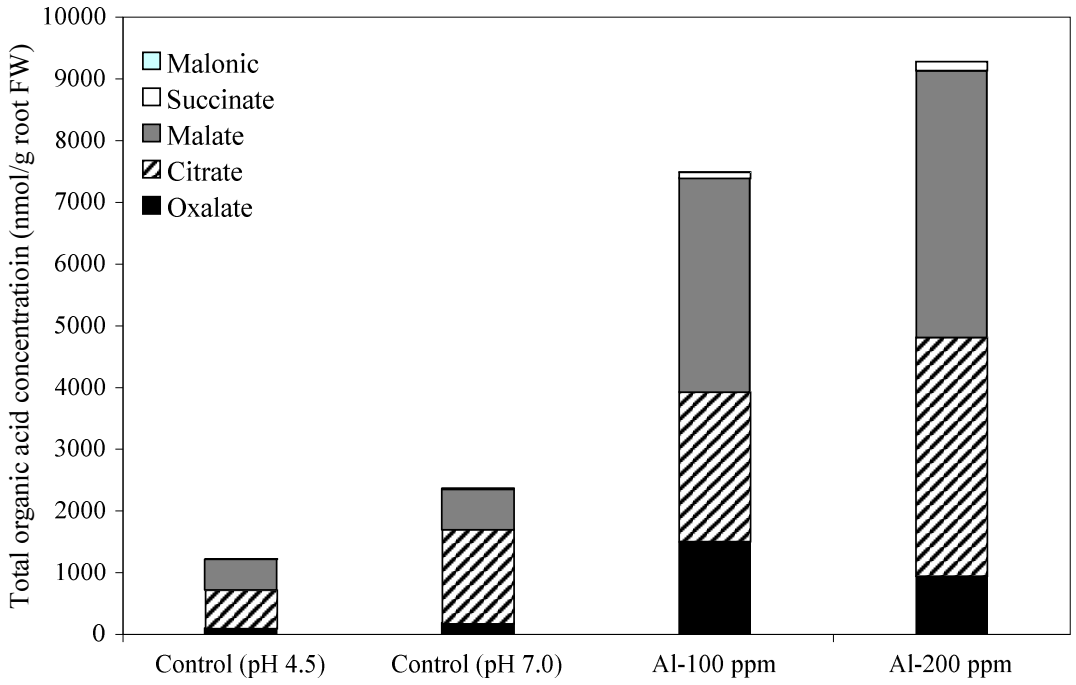

Fig. 3.10. Five organic acid concentrations in bermudagrass root tissues after harvest. Averages were calculated with means of n = 3.

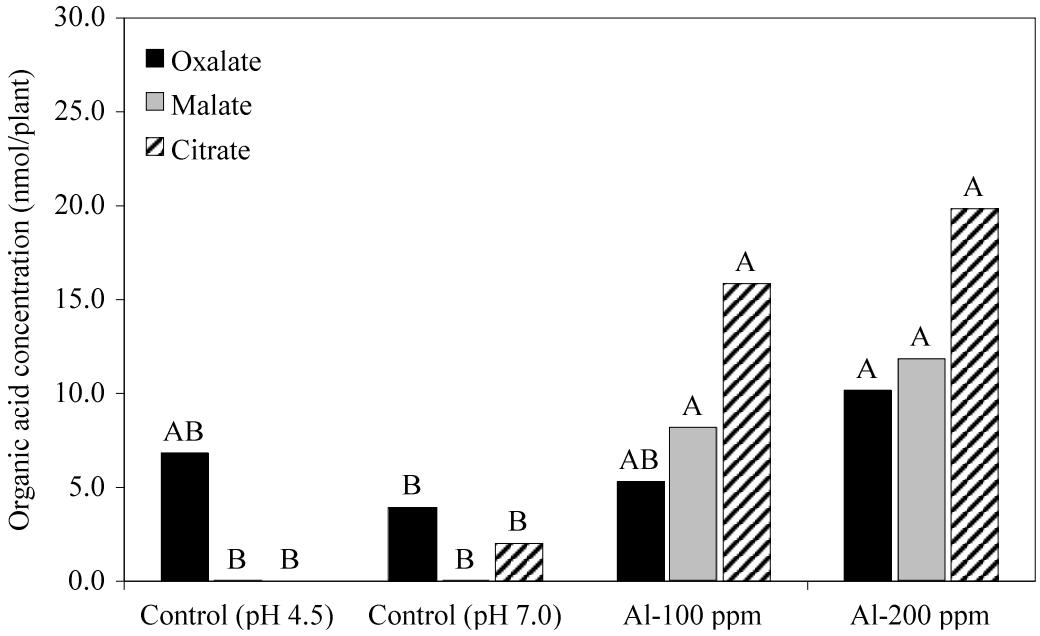

Fig. 3.11. Organic acids extracted from root surface and rhizosphere sand at harvest. Means (n = 3) followed by the same letter of the same organic acid species are not significantly different at $P < 0.05$.

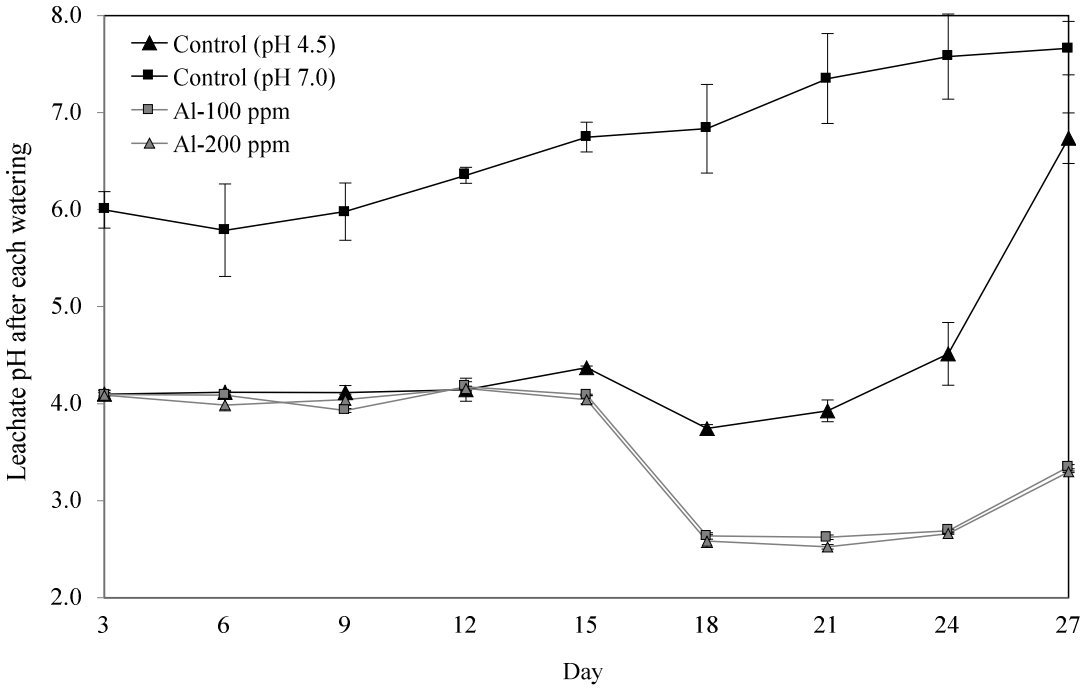

Fig. 3.12. The pH of leachates collected from control and aluminum stress treatments after each watering. Leachate samples were not collected on day 0 and Al treatment started on day 15. Error bars indicate standard errors of the mean (n = 3).

VITA

YANG TIAN

Candidate for the Degree of

Master of Science

Thesis: ORGANIC ACID CHANGES IN BERMUDAGRASS ROOT EXUDATES UNDER ALUMINUM STRESS

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Name: Yang Tian Date of Degree: December, 2011

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: ORGANIC ACID CHANGES IN BERMUDAGRASS ROOT EXUDATES UNDER ALUMINUM STRESS

Pages in Study: 103 Candidate for the Degree of Master of Science

Major Field: Horticulture

Scope and Method of Study:

Aluminum toxicity in acidic soils is a common problem in bermudagrass areas across Oklahoma. Certain organic acids in root exudates can act as root zone chelators to reduce harmful effects when plants are under Al stress. Therefore, a growth unit study was developed to evaluate organic acid changes in 'Princess-77' bermudagrass root exudates. Bermudagrass was grown in sand profile treated with 100 ppm and 200 ppm of Al stress. Two control treatments with no Al at sand pH of 4.5 and 7.0 were also included. Each treatment was replicated three times in a randomized complete block design. The objectives of the study were to identify and quantify organic acids in bermudagrass root exudates affected by Al stress and the correlation between organic acid exudation and external Al concentration.

Findings and Conclusions:

Al was accumulated in shoot tissues at concentrations of 1214.41 mg/kg and 2261.13 mg/kg dry wt. under 100 ppm and 200 ppm Al treatment respectively. There was a significant (P = 0.01) negative linear relationship (r^2 = 0.61) between Al leaf accumulation and bermudagrass root mass where root mass decreased significantly ($P < 0.05$) as Al accumulation increased. Citric, malic, oxalic and succinic acid were found in bermudagrass root exudates. Oxalic acid is the dominant acid accounts for 51-81 % of total organic acids and the exudation rate had a significant (P = 0.01) positive relationship (r^2 = 0.78) with external Al concentrations. Citric acid was induced by Al stress and higher exudation rate was detected in 100 ppm Al stress, the average exudation rate of malic acid also increased under Al treatment comparing to control treatments. The results inidicate potential Al tolerance mechanisms presented in bermudagrass cultivar 'Princess-77'.

ADVISER'S APPROVAL: Dr. Justin Q. Moss