ARBUSCULAR MYCORRHIZAL FUNGI ENHANCE TOLERANCE TO BICARBONATE IN *Rosa multiflora* cv. Burr

A Thesis

by

ANDREW DAVID CARTMILL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2004

Major Subject: Horticulture

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Approved as to style and content by:

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ABSTRACT

Arbuscular Mycorrhizal Fungi Enhance Tolerance to Bicarbonate
in *Rosa multiflora* cv. Burr. (August 2004)
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High bicarbonate (HCO_3^-) content and associated high pH of irrigation water is detrimental to plant growth. Sustainable agricultural/horticultural production will increasingly have to rely on economically feasible and environmentally sound solutions to the problems associated with high levels of HCO_3^- in irrigation water. The ability of a mixed *Glomus* Tulasne & Tulasne species inoculum of arbuscular mycorrhizal fungi (AMF), *Glomus* ZAC-19 (containing *Glomus albidum* Walker & Rhodes, *Glomus claroideum* Schenck & Smith, and *Glomus diaphanum* Morton & Walker), to enhance plant tolerance to HCO_3^- was tested on the growth and nutrient uptake of *Rosa multiflora* Thunb. ex J. Murr. cv. Burr (rose). Arbuscular mycorrhizal colonized and non-inoculated (non-AMF) *R. multiflora* cv. Burr were treated with 0, 2.5, 5, and 10 mM HCO_3^-. Increasing HCO_3^- concentration and associated high pH reduced *R. multiflora* cv. Burr growth, nutrient uptake, and acid phosphatase activity (ACP), while increasing alkaline phosphatase activity (ALP). Inoculation with AMF enhanced plant tolerance to HCO_3^- as indicated by greater growth, nutrient uptake, leaf chlorophyll content, higher mycorrhizal inoculation effect (MIE), lower root iron reductase activity, and generally lower soluble and wall-bound ALP activity. While AMF colonization (arbuscules, vesicles, and hyphae formation) was reduced by increasing HCO_3^- concentration, colonization still occurred at high HCO_3^- concentration. At 2.5 mM HCO_3^-, AMF plant growth was comparable to plants at 0 mM HCO_3^-, further indicating the beneficial effect of AMF for alleviation of HCO_3^- plant stress.

DEDICATION

This thesis is dedicated to my parents, family, and friends who have always encouraged me to do my best, and put up with a fair bit of moaning too!

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CHAPTER I
INTRODUCTION

Water is a finite yet renewable resource (Ripl, 2003), which is essential for socioeconomic development and a critical requirement for the maintenance of viable and productive (sustainable) ecosystems (Wallace et al., 2003). Population growth, infrastructure inadequacies, resource mismanagement (massive governmental subsidies and distorting incentives), fragmentation of legislation and regulation, and the inability to adequately quantify the effects of extraction, irrigation, and drainage projects, threaten the long-term sustainability of global water resources (Schultz and De Wrachien, 2002). New sources of water are increasingly expensive to exploit and decreasing in availability and quality (Wolff and Hübener, 1996). Environmental concerns and intensifying competition for municipal and industrial water will reduce the availability of good quality water resources for agricultural/horticultural production (Hamdy et al., 2003; Rosegrant and Ringler, 1998).

Agricultural/horticultural production will increasingly have to preserve and augment good quality water resources through enhanced water efficiency and utilization of non-conventional water resources. This may include utilization of water of marginal quality, high in total dissolved solids (non-volatile solutes) (Oster, 1994; Shalhevert, 1994). The potential bicarbonate (HCO_3^-) content and associated high pH of this irrigation water may be detrimental/limiting to plant growth, due to its adverse effects on availability and solubility of nutrients (P, N, Mg, Fe, Mn, Zn, Cu, and other ions) (Bailey, 1996; Marschner, 1995). Prolonged nutrient deficiency results in significant reductions in growth, yield, and marketability of plant material. Thus sustainable agricultural/horticultural production will increasingly have to rely on economically feasible and environmentally sound solutions to the problems associated with high levels of HCO_3^- in irrigation water.

Arbuscular mycorrhizal fungi [(AMF); Division Zygomycota/Glomeromycota,

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This thesis follows the format and style of the Journal of the American Society for Horticultural Science.

Order Glomales/Glomerales (Morton and Benny, 1990; Schüßler et al., 2001)] exist in symbiotic (mutually beneficial) associations with the fine young roots of higher plants (Smith and Read, 1997). Studies have demonstrated that AMF enhance plant nutrient acquisition (P, N, Zn, Cu, and other ions) (Clark and Zeto, 2000), improve water relations (Augé, 2001), and alleviate cultural and environmental stresses (Jeffries et al., 2003) through greater effective root area and penetration of substrate(s) (direct access to nutrients outside the zone of nutrient depletion that develop close to roots and to nutrients in inaccessible microsites), and activation and excretion of various enzymes by AMF roots and/or hyphae (Marschner, 1995; Smith and Read, 1997).

Therefore, we proposed a model study to determine if inoculation of plant material with AMF would enhance plant growth, nutrient acquisition, and survivability under high levels of HCO_3^- in irrigation water.

This research was conducted with the following objectives:

1. To determine the effects of high levels of HCO_3^- in irrigation water on plant nutrient acquisition and growth.

Hypothesis: High levels of HCO_3^- in irrigation water adversely affect plant nutrient acquisition and reduce growth.

2. To determine if the application of AMF enhance nutrient acquisition and growth of plants irrigated with water high in HCO_3^-.

Hypothesis: Arbuscular mycorrhizal fungi enhance the nutrient acquisition and growth of plants irrigated with water high in HCO_3^-.

CHAPTER II
LITERATURE REVIEW

Water

Population growth, infrastructure inadequacies, resource mismanagement (massive governmental subsidies and distorting incentives), fragmentation of legislation and regulation, and the inability to adequately quantify the effects of extraction, irrigation, and drainage projects, threaten the long-term sustainability of global water resources (Schultz and De Wrachien, 2002). New sources of water are increasingly expensive to exploit and decreasing in availability and quality (Wolff and Hübener, 1996). Environmental concerns and intensifying competition for municipal and industrial water will reduce the availability of good quality water resources for agricultural/horticultural production (Hamdy et al., 2003; Rosegrant and Ringler, 1998).

AGRICULTURAL/HORTICULTURAL SECTOR

The agricultural/horticultural sector currently accounts for approximately 80% (1870 $km^3 \cdot yr^{-1}$) of global water consumption (Postel, 1996). Agricultural/horticultural production does not consume water in the conventional sense, since globally insignificant amounts are bound up in the products produced (Wallace and Batchelor, 1997). Approximately 30% (561 $km^3 \cdot yr^{-1}$) of global irrigation water is lost in storage and conveyance through evaporation, runoff, and seepage/drainage (Boss, 1985), and approximately 63% (825 $km^3 \cdot yr^{-1}$) of irrigation water delivered to the plant material is lost through evaporation, runoff, and seepage/drainage (Postel, 1996).

By 2025 the global demand for water by agricultural/horticultural production is projected to increase by approximately 20% (374 $km^3 \cdot yr^{-1}$) (Dirksen, 2002). The prohibitively high economic, social, and environmental cost of civil engineering works required for large scale extraction, irrigation, and drainage projects limits the scope for increasing the amount of good quality water resources available to

agriculture/horticultural production (Wolff and Hübener, 1996). Thus, the portion of water available to agricultural/horticultural production is projected to decrease by approximately 15% (281 $km^3 \cdot yr^{-1}$) by 2025 (Dirksen, 2002).

Agricultural/horticultural production will increasingly have to preserve and augment good quality water resources through enhanced water efficiency and utilization of non-conventional water resources. This may include utilization of water of marginal quality, high in total dissolved solids (Al^{+3}, HCO_3^-, $H_2BO_3^-$, Ca^{+2}, Cl^-, Cu^{+2}, F^-, Fe^{+3}, Fe^{+2}, Mg^{+2}, $Mn^{+3,4}$, NO_3^-, Na^+, SO_4^{-2}, Zn^{+2}) (Malina, 1996; Oster, 1994; Reed, 1996a; Shalhevert, 1994).

Bicarbonate

BICARBONATE CHEMISTRY

Bicarbonate (HCO_3^-) is one of the major contributors to alkalinity in irrigation water (Petersen, 1996). Carbonate (CO_3^{2-}) is also a contributor to alkalinity; hydroxides, ammonia, borates, organic bases, phosphates, and silicates are minor contributors (Petersen, 1996). Carbonate and HCO_3^- are buffers which impart water with the capacity to resist sudden changes in pH (Bailey, 1996). Therefore, alkalinity is a measurement of the concentration of the carbonates and buffering capacity (Bailey, 1996). Bicarbonate and CO_3^{2-} react with H^+, increasing solution pH (Lindsay, 1979). Slight alkalinity in water may be beneficial in production systems, because it will buffer the substrate solution, and thus permit limited pH variation (Argo and Fisher, 2002). However, high alkalinity may be detrimental to plant growth due to the associated high pH (Bailey, 1996).

Solution pH influences alkalinity by determining the reaction direction of carbonates, and thus the proportion of the carbonate species present in the solution (Lindsay, 1979). The prevalent form of carbonates at pH $\leq$6.36 is H_2CO_3, at pH between 6.36 and 10.33, HCO_3^- is the predominate form, and CO_3^{2-} is predominate at pH >10.33 (Lindsay, 1979).

The origin of alkalinity in irrigation water is from limestone or dolomitic deposits in aquifers (saturated hydrogeologic unit that contains significant quantity of water, with sufficient permeability to transmit that water) (Chaudhry, 1996; Kidder and Hanlon, 1997; Malina, 1996; Oetting et al., 1996). High content of certain types of organic matter in the substrate and standing water (over irrigation) and/or soil compaction may also induce high alkalinity (Argo and Fisher, 2003; Marschner, 1995). Carbon dioxide released from organic matter through microbial respiration accumulates and reacts with water to produce H_2CO_3 (Lindsay, 1979; Lucena, 2000; Marschner, 1995). As previously stated H_2CO_3 may be converted to HCO_3^- depending on solution pH.

EFFECT OF BICARBONATE ON IRON NUTRITION

Irrigation water and substrates high in HCO_3^- have been shown to be a direct or indirect cause for Fe deficiency chlorosis in many plant species, both agronomic and horticultural (Wallace and Wallace, 1986; Welkie, 2000). Iron chlorosis has been defined as the yellowing occurring in young leaves caused by inhibited chloroplast chlorophyll synthesis as a consequence of low iron nutritional status in the plant (Lucena, 2000). It is well known that Fe compounds are very insoluble (Lindsay, 1979). Iron deficiency may be due to low Fe availability as a result of high carbonates and associated high pH (at pH 4 increases of 1 unit result in a 1000-fold decrease in Fe^{3+} solubility) (Lucena, 2000).

Various mechanisms have been suggested to account for HCO_3^- induced Fe chlorosis, for example the inhibition of Fe absorption and translocation to leaves (Alcántara et al., 2000; Bertoni et al., 1992; Romera et al., 1997), and immobilization of Fe in leaves (Bavaresco et al., 1999; Mengel, 1994; Römheld, 2000). It has been demonstrated that HCO_3^- induces a decreased Fe concentration in leaf dry matter, as indicated by a positive relationship between chlorophyll and total Fe concentration in the upper leaves. However, in many cases this correlation can not/has not been observed, and a higher Fe concentration can be found in young chlorotic leaves compared to green

leaves. This phenomena is called chlorosis paradox (Römheld, 2000). This is suggested to be a result of Fe inactivation in leaf apoplast (Römheld, 2000). The higher Fe content in chlorotic leaves may also be a result of a dilution factor (due to diminished leaf expansion compared to non-chlorotic plants) (Bavaresco et al., 1999; Römheld, 2000).

There are at least two root response mechanisms (strategies) to Fe chlorosis in higher plants (Jolley et al., 1996; Marschner and Römheld, 1994). Plant species and cultivars within species exhibiting any response to Fe deficiency are considered Fe efficient (Marschner, 1995). Dicots and non-graminaceous monocots exhibit Strategy I mechanisms characterized by three components; a plasma membrane bound inducible reductase, enhanced net excretion of H^+, and an enhanced release of reductants/chelators (Jolley et al., 1996; Marschner and Römheld, 1994). In addition to HCO_3^- induced decreased Fe solubility, it also impairs the effectivity of H^+ efflux pump by neutralization of H^+ (Jolley et al., 1996; Marschner, 1995). Bicarbonate also lowers the release of phenolics and Fe reduction at the plasma membrane (Moog and Brüggemann, 1994). The relative importance of the three components seems to differ considerably between plant species and genotypes (Jolley et al., 1996; Marschner, 1995).

Strategy II is confined to grasses and is characterized by two components, release of phytosiderophores (Fe deficiency-induced release of non-proteinogenic amino acids), and a high affinity transport system in the plasma membrane of root cells for Fe phytosiderophores (Jolley et al., 1996; Marschner and Römheld, 1994). The release of phytosiderophores is not affected by the external pH.

EFFECT OF BICARBONATE ON PLANT GROWTH

Plant species and cultivars within species vary in their response to HCO_3^- stress. Substrate, irrigation method, and fertilizer type can influence the tolerance of plant species to HCO_3^- (Bailey, 1996). Length of crop period and plant to substrate ratio can also influence plant tolerance to HCO_3^- (Nelson, 1998). Recommended levels of alkalinity in irrigation water range from 0.75 $meq \cdot L^{-1}$ to 1.3 $meq \cdot L^{-1}$ for plug production and 1.25 $meq \cdot L^{-1}$ to 2.6 $meq \cdot L^{-1}$ for greenhouse container production (Bailey, 1996).

Under experimental conditions, significantly inhibited growth and chlorosis has been reported in *Nicotiana tabacum* L. cv. KY-907 (tobacco) in nutrient solution culture with ≥2 mM HCO_3^- (supplied as $NaHCO_3^-$) (Pearce et al., 1999). Zhou et al. (1984) reported HCO_3^- concentrations as low as 1 mM (supplied as $KHCO_3^-$) caused significantly inhibited growth and chlorosis in *Malus domestica* Borkh cv. York Imperial (apple) seedlings grown in nutrient solution culture. Significant growth inhibition and chlorosis has also been reported in pot grown *Viola* x *wittrockiana* Gams. cv. Bingo Yellow (pansy) and *Impatiens wallerana* Hook.f. cv. Dazzler White (impatiens) irrigated with ≥2.38 mM HCO_3^- (supplied as $NaHCO_3^-$) (Kuehny and Morales, 1998), and *Chrysanthemum* x *morifolium* Ramat. cv. Bright Golden Anne irrigated with ≥8.2 mM HCO_3^- (supplied as $NaHCO_3^-$) (Kramer and Peterson, 1990). In general, the effect of HCO_3^- on plant growth appears to be more severe on plants grown in solution culture than in container substrates due to the buffering capacity of the substrates (Kramer and Peterson, 1990; Kuehny and Morales, 1998).

EFFECT OF BICARBONATE ON PLANT NUTRITION

Nutritional responses of plant species and cultivars may differ in response to HCO_3^- stress. Increased concentrations of N, P, K, Mg, and B have been reported in the upper leaves of *M. domestica* cv. York Imperial seedlings with increasing concentrations of HCO_3^- (Zhou et al., 1984). Concentrations of Ca, Mn, Zn, and Cu were unaffected by HCO_3^- concentration. Decreased concentration of Fe was reported with increasing HCO_3^- concentrations. Decreased concentrations of P, Ca, and Mn have been reported with increasing HCO_3^- concentrations in whole plant tissue samples of *C.* x *morifolium* cv. Bright Golden Anne (Kramer and Peterson, 1990). Concentrations of N, K, Mg, Fe, B, Cu, Zn, Al, Mo, and S were unaffected by HCO_3^- concentration. Decreased concentrations of N, P, K, and Zn have been reported with increasing HCO_3^- concentrations in *N. tabacum* cv. KY-907 shoots (Pearce et al., 1999). Concentrations of Ca, Mg, and S were unaffected by HCO_3^- concentration. Shoot Fe concentration increased linearly with increased HCO_3^- concentration; this was attributed to the Fe

source [Fe was supplied as Fe-EDTA (ferric ethylenediaminetetraacetic acid) which is more stable at high pH] which may have made Fe more available to the plant at high HCO_3^- concentrations (Álvarez-Fernández et al., 1996; Lucena, 2003; Reed, 1996b).

Bicarbonate is regarded as a major causal factor for Zn deficiency in *Oryza sativa* L. (rice) grown in calcareous soil. Bicarbonate inhibits root absorption of Zn, immobilizes Zn in roots, and inhibits translocation to shoots (Yang et al., 1993). It is suggested that Zn efficient *O. sativa* cv. IR 8292-31-2 and Shanyou 10 may have enhanced tolerance to HCO_3^-. The effect of HCO_3^- on Zn inefficient *O. sativa* cv. IR 26 and Che 64-7 was shown not to be associated with high pH (Yang et al., 2003).

MITIGATION OF BICARBONATE STRESS AND IRON DEFICIENCY

In nursery and greenhouse production systems it is possible to mitigate low alkalinity levels in irrigation water between 1.5-3.0 meq·L^{-1} by use of acid reacting fertilizers and/or reduced application of limestone to the substrate (Nelson, 1998). The acidifying effect of acid reacting fertilizers is due to the ammonium, ammonia, and urea nitrogen content (Bailey, 1996). Other principal acid reaction nutrients are S, Cl, and P_2O_5. Limestone is added to some commercial substrates to buffer the acidity of substrates such as, peat and pine bark (Bunt, 1988). Less limestone is used to compensate for the carbonates applied in irrigation water (Nelson, 1998).

Alkalinity levels >3.0 meq·L^{-1} can be controlled by the injection of acids into the irrigation water. Injection of sulfuric, phosphoric, or nitric acid to holding tanks and/or flow lines reduces the amount of HCO_3^- and CO_3^{2-} in irrigation water by combining the H^+ from the acid to HCO_3^- or CO_3^{2-} to form H_2CO_3 which is then converted to H_2O and CO_2 (Bailey, 1996; Matkin and Petersen, 1971; Nelson, 1998). Alkalinity levels >8.0 meq·L^{-1} require a large amount of acid to achieve neutralization, which may result in the formation of new salts and increased salinity which is detrimental to plant growth (Nelson, 1998; Tagliavini et al., 2000). Acid injection also poses a potential health and safety risk (Matkin and Petersen, 1971; Nelson, 1998). Therefore, under said conditions reverse osmosis (RO) treatment is necessary, even though this technology is currently

very expensive (Nelson, 1998, Reed, 1996a). The economic cost of RO water limits its application to nursery/greenhouse production systems, and is frequently only used in tissue culture and subsequent acclimatization of plant material (F.T. Davies, Jr., personal communication, May 2004).

Under field conditions, dealing with high alkalinity is both difficult and expensive (Wallace and Wallace, 1986). Bicarbonate induced Fe chlorosis may be treated with varying success by injecting Fe solutions into the trunk of trees and/or using Fe chelates and/or acid reaction fertilizers (Mortvedt, 1991, Reed et al., 1988; Tagliavini et al., 2000; Wallace and Wallace, 1986). Iron sulfate application has been reported to be ineffective in field production systems unless application exceeds 200-560 $kg \cdot ha^{-1}$ (Mortvedt, 1991). Other potential means of alleviating HCO_3^- induced Fe chlorosis, include application of acidic solutions to the foliage (Tagliavini et al., 2000) and/or application of vivianite (a synthetic $Fe_3(PO_4)_2 \cdot 8H_2O$) to calcareous soils (Rombolà et al., 2003).

The development and use of Fe-efficient/HCO_3^- resistant genotypes and rootstocks are currently the only economically feasible solution to the problems associated with high HCO_3^- in irrigation water and substrates (Jolley et al., 1996; Mortvedt, 1991). However, priority has not been given to conduct this research and/or development (Mortvedt, 1991). Therefore agricultural/horticultural production will have to increasingly rely on other economically and environmentally sound solutions to the problems associated with high HCO_3^- in irrigation water and substrates.

Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi [AMF; Division Zygomycota/Glomeromycota, Order Glomales/Glomerales (Morton and Benny, 1990; Schüßler et al., 2001)] exist in symbiotic (mutually beneficial) associations with the fine young roots of higher plants (Smith and Read, 1997). The plant supplies the fungus with carbon (from photosynthesis) (Douds et al., 2000), while AMF enhances the plant nutrient and water

uptake, and helps alleviate cultural and environmental stresses (Smith and Read, 1997). These AMF associations evolved concurrently with the first colonization of land by plants, some 400-500 million years ago (Paleozoic Era, Ordovician period) (Cairney, 2000; Heckman et al., 2001; Taylor et al., 1995), and persist in 80-90% of all extant (not extinct) terrestrial plants (angiosperms, gymnosperms, pteridophytes, gametophytes of some mosses, lycopods, and Psilotales) (Cairney, 2000; Clapp et al., 2002).

NUTRIENT UPTAKE

Studies have demonstrated that AMF associations play an important role in plant nutrition, specifically the enhanced acquisition of P, N, Zn, Cu, and other ions (Clark and Zeto, 2000; Marschner and Dell, 1994). Arbuscular mycorrhizal fungi associations are thought to utilize nutrient cycles through greater effective root area/penetration provided by AMF roots and/or hyphae and through the activation and excretion of various enzymes by the hyphae to enhance nutrient acquisition (Aerts, 2002; Kodie, 1991; Miller and Jastrow, 2000; Smith et al., 2001; Saito, 2000; George, 2000). Hyphal nutrient uptake may be just as efficient as the uptake of roots and hyphae together (Pearson and Jakobsen, 1993). However, the presence of hyphae does not necessarily imply effective nutrient uptake (Jakobsen et al., 1992a, 1992b; Pearson and Jakobsen, 1993).

While, inorganic nutrients in the substrate solution constitute the primary nutrient source for the AMF, the fungi also appear to have some influence on availability of nutrients from organic sources. Extracellular phosphatase activity has been detected in excised mycelium of *Glomus intraradices* Schenck & Smith (Danish isolate 28A, BEG 87) and *Glomus claroideum* Schenck & Smith (Danish isolate SC09, BEG 14) extracted from root free sand of two compartmental pot cultures of *Trifolium subterraneum* L. cv. Mount Baker (subterranean clover) (Joner and Johansen, 2000). *Glomus intraradices* has been shown to take up P from adenosine monophosphate (^{32}P-labelled AMP) in compartmented monoxenic cultures (system void of organisms other than the two symbionts; AMF and plant) of root inducing transferred (RiT)-DNA transformed

Daucus carota L. (carrot) root (Joner et al., 2000a). Hawkins et al. (2000) demonstrated that *G. intraradices* transported N from glycine and glumate in compartmented monoxenic cultures RiT-DNA transformed *D. carota* roots. However, this uptake did not contribute significantly to N uptake by the roots, but may be important for fungal nutrition. Irrespective of N form, little is known about N transfer mechanisms (transporter) at the biotrophic interface between plants and AMF and/or whether new transport mechanisms are switched on as a result of this association (Smith et al., 1994). The N form translocated within the extraradical hyphae is also unclear, although Smith et al. (1994) suggest that N-rich amides (Asn or Gln) are the major form in which N is transported.

ADDITIONAL BENEFITS

Arbuscular mycorrhizal associations alter the biosynthesis of phytohormones (Ludwig-Müller, 2000), increase rates of photosynthesis and stomatal conductance (Estrada-Luna et al., 2000), and improve resistance to drought (Augé, 2001; Davies et al., 2002a; Estrada-Luna and Davies, 2003; Estrada-Luna et al., 2000). Arbuscular mycorrhizal associations contribute to the sustainable maintenance of plant health and soil fertility (Jeffries et al., 2003) and play an important role in soil aggregation and soil structure (Rillig et al., 2002). Arbuscular mycorrhizal associations may tolerate adverse external pH conditions by modifying the pH of the mycorrhizosphere (Pacovsky, 1986). Arbuscular mycorrhizal associations may enhance plant tolerance under alkaline conditions (calcareous soils) (Bavaresco et al., 2000; Bavaresco and Fogher, 1992). Arbuscular mycorrhizal associations improve resistance to some root pathogens (Azcón-Aguilar and Barea, 1997; Kasiamdari et al., 2002), and reduce stunting on fumigated soils (Azćon-Aguilar and Barea, 1997; Munyanziza et al., 1997; Ryan and Graham, 2002). Arbuscular mycorrhizal associations improve transplant establishment and growth (Estrada-Luna et al., 2000), biomass production (Mathur and Vyas, 1999), and may be essential for the survival of many plant species in natural competitive situations (Hart and Klironomos, 2002).

In addition, AMF associations have been reported to play an important role in the establishment and growth of plant material under saline conditions (Al-Karaki and Hammad, 2001; Copeman et al., 1996; Mohammad et al., 2003) and in industrial waste substrates/pollutants (Malcová et al., 2001), including hydrocarbon-polluted soil (Cabello, 2001) stimulated acid-rain and aluminum (Vosátka et al., 1999), arsenate (Gonzalez-Chavez et al., 2002), chromium (Davies et al., 2002b), and uranium (Rufyikiri et al., 2002; Rufyikiri et al., 2003) toxicity.

AGRICULTURAL/HORTICULTURAL PRODUCTION

The potential beneficial application of AMF to numerous plants of economic importance in agricultural/horticultural production systems has been demonstrated (Azćon-Aguilar and Barea, 1997; Davies, 2000; Davies et al., 2000; Munyanziza et al., 1997; Ryan and Graham, 2002). However, current commercial production practices, high economic cost, and variable results limit their application (Azćon-Aguilar and Barea, 1997; Davies, 2000; Munyanziza et al., 1997; Ryan and Graham, 2002).

ARBUSCULAR MYCORRHIZAL-HOST PLANT RESPONSES

Plant species and cultivars within species can differ markedly in their response to AMF species (Parke and Kaeppler, 2000; Sanders, 2002). Efficiencies among AMF species may vary (Jakobsen et al., 2002; Parke and Kaeppler, 2000) and each AMF isolate originating from a specific environment may represent an ecotype adapted to that particular environment (Entry et al., 2002). Therefore, the beneficial application of AMF may depend on plant species and/or cultivars within species used, plant interaction with the AMF species, and the difference between the environment from which the AMF isolate was obtained and the experimental system under study.

Rosa

MORPHOLOGICAL DESCRIPTION

The genus *Rosa* L. (rose) belongs to the family Rosaceae Juss. and includes

approximately 100 species, which can be divided into four subgenera [*Platyrhodon*, *Hesperhodos*, *Hulthemia*, and *Eurosa* (Lynch, 2002)], of prickly shrubs, which sometimes trail and/or climb, and have a natural distribution throughout the temperate parts of the Northern Hemisphere (Bailey, 1960; Bailey and Bailey, 1976). The modern rose is within the subgenera *Eurosa* (the remaining subgenera have contributed little to the history of the cultivated rose) (Lynch, 2002).

Rosa spp. have alternate leaves which are mostly odd-pinnately compound (5-7 oval leaflets with rounded or pointed tips, which are sometimes toothed), deciduous or persistent, with stipule fused to the petiole (Bailey, 1960; Bailey and Bailey, 1976). Leaves are often aromatic-glandular (Bailey, 1960). Stems usually bear thorns and/or prickles (Brickell and Cole, 2002). Their flowers range in color (rarely blue) and are commonly solitary or corymbose or paniculate (Heywood, 1993). Flowers have petals in multiples of 5, petals are broad and mostly rounded at the end (Bailey, 1960). Whorled stamens and pistils are indefinite and numerous (Bailey, 1960; Bailey and Bailey, 1976). Petals and stamens are inserted on a disk at the edge of the hypanthium, and pistils are borne in the inside of the hypanthium (Bailey, 1960). Fruits are fleshy hips (ripened hypanthium), containing hairy achenes as if they were seeds (Bailey, 1960; Bailey and Bailey, 1976). Their typical chromosome number is 2n=14 (Darlington and Wylie, 1956).

AGRONOMIC/HORTICULTURAL VALUE

Rosa spp. have high landscape and ornamental value, and are an important field/greenhouse cut flower crop (Bailey and Bailey, 1976; Heywood, 1993). *Rosa* spp. are also an important source of essential oils used in perfumes and aromatherapy (Cutler, 2003). *Rosa* petals and/or hips contain malic acid which is used as a pH adjuster in cosmetics (Culter, 2003).

Rosa extracts also have a wide variety of medicinal uses, including the prevention and/or cure of depression, insomnia, fatigue, hangovers, headaches, dizziness, stomach-ache, indigestion, diarrhea, and constipation (Cutler, 2003). Extracts

have also been used as purgatives, astringents, anti-inflammitories, and for the treatment of wounds and scars (Cutler, 2003). *Rosa* petals and/or hips contain quercitrin which may be beneficial for individuals suffering from hepatitis, cirrhosis, and hepatic cancers (Cutler, 2003). Quercitrin is also thought to prevent build up of arterial plaque, and may also regulate cell growth (Cutler, 2003). *Rosa* hips also contain leucoanthocyanins which is an antioxidant which may increase tone and elasticity of capillary walls, improving resistance to haemorrhages and infections (Cutler, 2003).

Rosa hips can be used as a nutritional supplement, as they are a good source of vitamin C, B, carotenoids, folic acid, pectins, and also contain small amounts of vitamins A, B_3, D, E, and Fe, K, and Zn, which may be lost in processing (Cutler, 2003). *Rosa* petals and hips are also used as flavorings and coloring in confectionery (sweets, cakes, jams, and jellies), and beverages (wine, tea, and juices) (Culter, 2003).

Thus, *Rosa* spp. are an important horticultural/agricultural crop, and it is estimated that there are approximately 5000 named *Rosa* cultivars in cultivation (Heywood, 1993), with a global retail market value of approximately $720 million (Lynch, 2002). *Rosa multiflora* Thunb. ex J. Murr. are an important commercial rootstock and have been demonstrated to be susceptible to physiological stress on alkaline soil, although some resistance was reported for some cultivars (Reed et al., 1992). *Rosa multiflora* have also been reported to form AMF associations (Davies, 1987; Davies et al., 1987).

CHAPTER III

ARBUSCULAR MYCORRHIZAL FUNGI ENHANCE TOLERANCE TO BICARBONATE IN *Rosa multiflora* cv. Burr

Introduction

Water is a finite yet renewable resource (Ripl, 2003), which is essential for socioeconomic development and a critical requirement for the maintenance of viable and productive (sustainable) ecosystems (Wallace et al., 2003). Population growth, infrastructure inadequacies, resource mismanagement (massive governmental subsidies and distorting incentives), fragmentation of legislation and regulation, and the inability to adequately quantify the effects of extraction, irrigation, and drainage projects, threaten the long-term sustainability of global water resources (Schultz and De Wrachien, 2002). New sources of water are increasingly expensive to exploit and decreasing in availability and quality (Wolff and Hübener, 1996). Environmental concerns and intensifying competition for municipal and industrial water will reduce the availability of good quality water for agricultural/horticultural production (Hamdy et al., 2003; Rosegrant and Ringler, 1998).

Agricultural/horticultural production will increasingly have to preserve and augment good quality water resources through enhanced water efficiency and utilization of non-conventional water resources. This may include utilization of water of marginal quality, high in total dissolved solids (non-volatile solutes) (Oster, 1994; Shalhevert, 1994). The potential bicarbonate (HCO_3^-) content and associated high pH of this irrigation water may be detrimental/limiting to plant growth, due to its adverse effects on availability and solubility of nutrients (P, N, Mg, Fe, Mn, Zn, Cu, and other ions) (Bailey, 1996; Marschner, 1995). Prolonged nutrient deficiency results in significant reductions in growth, yield, and marketability of plant material. Thus, sustainable agricultural/horticultural production will increasingly have to rely on economically

feasible and environmentally sound solutions to the problems associated with high levels of HCO_3^- in irrigation water.

Arbuscular mycorrhizal fungi [(AMF); Division Zygomycota/Glomeromycota, Order Glomales/Glomerales (Morton and Benny, 1990; Schüßler et al., 2001)] exist in symbiotic (mutually beneficial) associations with the fine young roots of higher plants (Smith and Read, 1997). Studies have demonstrated that AMF enhance plant nutrient acquisition (P, N, Zn, Cu, and other ions) (Clark and Zeto, 2000), water relations (Augé, 2001), and alleviate cultural and environmental stresses (Jeffries et al., 2003) through greater effective root area and penetration of substrate(s) (direct access to nutrients outside the zone of nutrient depletion that develop close to roots and to nutrients in inaccessible microsites), and activation and excretion of various enzymes by AMF roots and/or hyphae (Marschner, 1995; Smith and Read, 1997).

Therefore, we proposed a model study to determine if inoculation of plant material with AMF would enhance plant growth and nutrient acquisition under high levels of HCO_3^- in irrigation water. An important arbuscular mycorrhiza genus is *Glomus* Tulasne & Tulasne, which colonize a variety of host species (Marschner, 1995; Smith and Read, 1997), including *Rosa multiflora* Thunb. ex J. Murr. (Davies, 1987; Davies et al., 1987). Rootstocks of *Rosa multiflora* are susceptible to growth stress on alkaline soils (Reed et al., 1992). Our hypothesis was that inoculation of *R. multiflora* cv. Burr stem cuttings with an AMF mixed *Glomus* species isolate (ZAC-19) would enhance plant growth and nutrient acquisition under high levels of HCO_3^- in irrigation water. Objectives of this research were to determine if ZAC-19 can enhance *R. multiflora* cv. Burr tolerance to HCO_3^- stress as determined by plant growth and nutrient acquisition.

Materials and Methods

CULTURAL CONDITIONS

This study was conducted under glasshouse (Rough Brothers, Inc., Cincinnati,

Ohio) conditions at Texas A&M University, College Station, Texas, lat. 30°36'02'N and long. 96°18'44'W, from 5 Mar. 2003 to 17 May 2003. Temperature and relative humidity were measured and recorded hourly for the duration of this study (Model 150-Temp/RH, WatchDog™ Data Logger, Spectrum Technologies, Inc., Plainfield, Ill.). Average day/night temperature and relative humidity were 28.1±0.2°C/23.4±0.1°C, and 66.1±0.8%/83.1±0.5%, respectively. Photosynthetic photon flux density (PPFD) was measured daily at approximately solar noon for the duration of the study (Model LI-189 and LI-190SA, LI-COR® Quantum/Radiometer/Photometer and LI-COR® Quantum Sensor, LI-COR Biosciences, Lincoln, Nebr.). Average PPFD was 403.6±28.0 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Substrate temperature was measured daily at approximately solar noon for the duration of the study (Model 90900-00, TempTestr™, Cole Parmer Instrument Co., Vernon Hills, Ill.). Average substrate temperature was 27.4±0.1°C.

Rosa multiflora cv. Burr stem cuttings were obtained from stock plants at Texas A&M University Agricultural Research and Extension Center, Overton, Texas, lat. 32°16' 32'N and long. 94°58'20'W, in late Feb. 2003. Original virus indexed stock came from the University of California, Davis, Calif. lat. 30°33'18'N and long. 121°44'09'W (H.B. Pemberton, personal communication, June 2003). Cuttings were stored in the dark in a cold room (Bally Case and Cooler, Inc., Bally, Pa.) at 2°C until used. Cuttings with axillary buds intact were trimmed (Felco® 7 Sécateurs, Felco SA, Geneveys-sur-Coffrane, Switzerland) to approximately 15 cm in length and treated with 0.3% indole-3-butyric acid (Hormodin® 2, E.C. Geiger Inc., Harleysville, Pa.) and planted in 0.09 L black plastic cells (Landmark Plastic Corporation, Akron, Ohio). The container substrate (Redi-Earth®, The Scotts Co., Marysville, Ohio) had high organic matter (15.6%), pH 4.8, electrical conductivity (EC) 1.14 $dS \cdot m^{-1}$, and nutrient levels with the following $\mu g \cdot g^{-1}$: 82 N, 8 P, 255 K, 717 Ca, 326 Mg, 0.38 Zn, 17.84 Fe, 6.64 Mn, 0.34 Cu, 337 Na, 158 S, and 0.32 B (Soil, Water, and Forage Testing Laboratory, Texas A&M University, College Station, Texas). Substrate was previously autoclaved (Model SSR-2A, Consolidated Stills & Sterilizers, Boston, Mass.) on 2 consecutive days for 20 $min \cdot d^{-1}$ at 121°C (21psi).

Cuttings were fogged [Fogg-It Nozzle (3.785 $L \cdot min^{-1}$), Fogg-It Nozzel Co., San Francisco, Calif.] manually as required with reverse osmosis (RO) water. The RO water had pH 7.2, EC 0.06 $dS \cdot m^{-1}$, and nutrient levels with the following $\mu g \cdot ml^{-1}$: 0.06 NO_3-N, 0.06 P, 0 K, <1 Ca, 12 SO_4, <1 Mg, , <0.01 Fe, <0.01 Zn, <0.01 Mn, <0.01 Cu, 0.28 B, 9 Cl^- , 11 Na, 0 CO_3, 38 HCO_3, 37 TDS, and <0.010 As, 0.011 Ba, 0.028 Ni, 0.010 Cd, <0.010 Pb, and 0.020 Cr, (Soil, Water, and Forage Testing Laboratory, Texas A&M University, College Station, Texas). Cuttings were irrigated 25 d after planting with a formulation of Long Ashton nutrient solution (LANS) (Hewitt, 1966) modified to supply P ($NaH_2PO_4 \cdot H_2O$) (EM Science, Darmstadt, Germany) at 31 $mg \cdot L^{-1}$ P.

Uniform rooted cuttings were selected 30 d after planting, their roots rinsed free of commercial propagation substrate with RO water, and transplanted into 1.4 L green plastic containers (Dillen Products, Middlefield, Ohio). The container substrate (Landscapers Pride® Play Sand, Louisiana Pacific, New Waverly, Texas) had textural analysis of 92% sand, 4% silt, and 4% clay, low organic matter (0.08%), pH 6.9, EC 0.12 $dS \cdot m^{-1}$, and nutrient levels with the following $\mu g \cdot g^{-1}$: 2 N, 1 P, 13 K, 267 Ca, 27 Mg, 0.13 Zn, 2.79 Fe, 0.46 Mn, 0.05 Cu, 368 Na, 23 S, and 0.14 B (Soil, Water, and Forage Testing Laboratory, Texas A&M University, College Station, Texas). The container substrate was previously steam pasteurized with aerated steam (Model SA150 and TC-424, LINDIG Manufacturing Corporation, St. Paul, Minn.) on 2 consecutive days for 3 $h \cdot d^{-1}$ at 80°C.

ARBUSCULAR MYCORRHIZAL INOCULATION

Half the rooted cuttings were non-colonized (non-AMF). Remaining rooted cuttings were inoculated at transplanting with approximately 700 spores of a mixed *Glomus* species isolate (ZAC-19): *Glomus albidum* Walker & Rhodes, *Glomus claroideum* Schenck & Smith, and *Glomus diaphanum* Morton & Walker (Chamizo et al., 1998). The ZAC-19 inoculum was applied directly to the dibble hole at transplanting (approximately 10 g per pot) and included hyphae and colonized root segments of *Carica papaya* L. used for isolate multiplication. The textural analysis of the inoculum

was 85% sand, 5% silt, and 10% clay, low organic matter (0.2%), pH 8.1, EC 0.45 $dS \cdot m^{-1}$, and nutrient levels of the following $\mu g \cdot g^{-1}$: 4 N, 24 P, 125 K, 512 Ca, 38 Mg, 14.65 Zn, 2.44 Fe, 3.7 Mn, 0.11 Cu, 393 Na, 26 S, and 0.22 B (Soil, Water, and Forage Testing Laboratory, Texas A&M University, College Station, Texas).

The ZAC-19 isolate was originally collected from non-irrigated (annual precipitation of approximately 450 mm), low nutrient (16 $\mu g \cdot g^{-1}$ P), and low organic matter (1.1%) sandy-loam soil (pH 5.4) used for commercial *Phaseolus vulgaris* L. production in Zacatecas, Mexico, lat. 22°45'53"N and long. 102°33'25"W (A. Alarcón, personal communication, November 2003). The ZAC-19 isolate was propagated under glasshouse conditions in *C. papaya* pot culture (Brundrett et al., 1996) in 1999 at Texas A&M University, College Station, Texas, and root balls were harvested and stored in the dark in a cold room at 2°C until used.

Transplanted *R. multiflora* rooted cuttings were irrigated with approximately 150 mL of a formulation of LANS, modified to supply P ($NaH_2PO_4 \cdot H_2O$) at 31 $mg \cdot L^{-1}$ P and Fe [Fe-DTPA (ferric diethylenetriaminepentaacetic acid)] (Becker Underwood Incorporated, Ames, Iowa) at 5 $mg \cdot L^{-1}$ Fe, every 3 d for 12 d. Plants were irrigated to achieve approximately 20% leachate fraction by volume.

BICARBONATE APPLICATION

Twelve days after transplanting, plants were irrigated with approximately 300 mL of a formulation of LANS, modified to supply P ($NaH_2PO_4 \cdot H_2O$) at 31 $mg \cdot L^{-1}$ P, Fe as (Fe-DTPA) at 5 $mg \cdot L^{-1}$ Fe, and HCO_3^- [$KHCO_3$ (Sigma, St. Louis Mo.), $NaHCO_3$ (Fisher Scientific, Pittsburgh, Pa.), and NH_4HCO_3 (Mallinckrodt Lab. Chemicals, Phillipsburgh, N.J.)] at 0, 2.5 (250 $mg \cdot L^{-1}$ $KHCO_3$), 5 (500 $mg \cdot L^{-1}$ $KHCO_3$), and 10 (600 $mg \cdot L^{-1}$ $KHCO_3$, 220 $mg \cdot L^{-1}$ $NaHCO_3$, and 120 $mg \cdot L^{-1}$ NH_4HCO_3) mM HCO_3^- every 4 d for 28 d (Table A1 and A2). Average solution pH were 6.03±0.13, 7.10±0.08, 7.34±0.07, and 7.59±0.12, and EC ($dS \cdot m^{-1}$) were 1.95±0.14, 2.08±0.10, 1.86±0.13, and 2.38±0.16, respectively (Table A3). Plants were treated to achieve approximately 20% leachate fraction by volume. Leachate was collected at each irrigation (n=5) using the

pour through method apparatus (Wright, 1987), and pH and EC were analyzed (Model B-213, Compact pH Meter and Model B-173, Compact Conductivity Meter, HORBIA Ltd., Kyoto, Japan).

ASSESSMENT OF PLANT GROWTH

Final growth measurements were recorded at harvest (n=9) 32 d after transplanting, and included leaf area (Model LI-3000, LI-COR® Portable Area Meter, LI-COR Biosciences, Lincoln, Nebr.), leaf, stem, root, and total fresh and dry mass (DM) (Model 1601A MP8-1, Sartorius Balances & Scales, Sartorius Corporation, Goettingen, Germany). Tissue samples were dried (Model 214330, Tru-Temp Oven, Hotpack Corporation, Philadelphia, Pa.) for 7 d at 70°C and leaf, stem, root, and total DM were recorded. Root/shoot ratio ($g \cdot g^{-1}$), leaf area ratio {(LAR) [leaf area (cm^2)]/[plant DM (g^{-1})]}, and specific leaf area {(SLA) [leaf area (cm^2)]/[leaf DM (g^{-1})]} were calculated. The mycorrhizal inoculation effect (MIE) was calculated by the formula MIE (%) = (total DM of AMF plant – total DM of Non-AMF plant)/(total DM of Non-AMF plant)$^{-1}$ x 100 (Plenchette et al., 1983; Sylvia, 1994).

LEAF NUTRIENT ANALYSIS

Physiologically mature leaves from nine randomly selected plants per treatment were collected at harvest, leaves were pooled (plants # 1, # 2, and # 3, plants # 4, # 5, and # 6, and plants # 7, # 8, and # 9) into three replicate samples (n=3) and ground (Wiley Mill, Arthur H. Thomas Co. Scientific Apparatus, Philadelphia, Pa.) to pass a 40-mesh screen. Complete tissue analysis (N, P, K, Mg, Ca, S, Na, Fe, Mn, Zn, Cu, Al, B, and Mo) was conducted on an inductively coupled plasma atomic emission spectrophotometer (Model Optima 4300V ICP-OES, PerkinElmer Life and Analytical Sciences, Inc., Boston, Mass.) (MDS Harris Laboratory Services, Lincoln, Nebr.).

LEAF CHLOROPHYLL CONTENT

Leaf chlorophyll content was determined at harvest (n=3), by extraction of

chlorophyll with acetone (Harborne, 1998). Procedure was modified as follows, representative semi-mature leaflets were collected and surface area was determined. Leaflets were placed in 5 mL of 80% acetone (Mallinckrodt Lab. Chemicals, Phillipsburgh, N.J.) and stored in the dark for 7 d at 4°C. Supernatant was quantified with a spectrophotometer (Beckman Coulter™ Du® Series 640 UV/Vis Spectrophotometer, Beckman Coulter, Inc. Fullertan, Calif.) at 645 and 663 nm, and compared to an 80% acetone blank standard. Total chlorophyll content was expressed as $mg \cdot cm^2$ of leaflet area.

ROOT IRON REDUCTASE ACTIVITY

Root iron reductase enzymatic activity was determined at harvest (n=3), based on the formation of Fe(II)-BPDS (Bathophenanthroline-Disulfonic Acid) complex (Brüggemann and Moog, 1989; Rosenfield et al., 1991). Procedure was modified as follows, roots were rinsed in nanopure water (Model D3700, Barnstead NANOpure II, Sybron Corporation/Barnstead Co., Boston, Mass.) and tips excised (approximately 0.5 cm), 1.2 g of root tissue per sample were immersed in 40 mL of Fe^{3+} reductase assay solution containing 5 mM Mes (2-[N-Morpholino]ethanesulfonic acid) (Sigma St. Louis, Mo.) (pH 5.5), 0.5 mM $CaSO_4$ ($CaSO_4 \cdot 2H_2O$) (Sigma, St. Louis, Mo.) , 0.1 mM Fe^{3+}EDTA, and 0.3 mM BPDS (4,7-Diphenyl-1,10-phenanthroline-disulfonic acid) (Sigma, St. Louis, Mo.). Samples were aerated [Model 200 (160 $L \cdot h^{-1}$), Aquarian®/Rena Air Pump, Mars Inc., McLean, Va.] and incubated in the dark at 21°C for 4 h. The appearance of Fe^{3+} BPDS was quantified with a spectrophotometer (Bausch & Lomb Spectronic® 21 UVD, Bausch & Lomb Instrument & System Division, Rochester, N.Y.) at 535 nm, and compared to a standard concentration series (0, 3.58, 7.15, 21.45, 35.75, and 57.2 μM) of authentic Fe(II)-BPDS.

ROOT PHOSPHATASE ACTIVITY

Root acid phosphatase (ACP) and alkaline phosphatase (ALP) enzymatic activity (soluble and extractable) were determined at harvest (n=3), based on the hydrolysis of *p*-

nitrophenyl phosphate (*p*-NPP) substrate to yield *p*-nitrophenol (*p*-NP) and inorganic phosphatase (Eivazi and Tabatabai, 1977; Tabatabai and Bremner, 1969). Procedure was modified as follows, roots were rinsed in nanopure water and tips were excised (approximately 0.5 cm), 100 mg of root tissue per sample were transferred into a 600 µL of extraction modified universal buffer (MUB) [100 mM THAM (Tris(hydroxymethyl)aminomethane) (Bio-Rad Lab., Hercules, Calif.), 100 mM maleic acid (Toxilic Acid; *cis*-Butenedioic Acid) (Fisher Scientific, Pittsburgh, Pa.), 5 mM citric acid ($C_6H_8O_7$) (Sigma, St. Louis, Mo.), and 100 mM boric acid (H_3BO_3) (EM Science, Darmstadt, Germany)] pH 5.5 for ACP and pH 9.0 for ALP. Extractable ACP and ALP root samples were macerated (Kontes Pellet Pestle®, Vineland, N.J.). All root samples were centrifuged (Model 235B, Fisher® Micro-Centrifuge, Fisher Scientific Intl. Inc., Hampton, N.H.) at 13,000 g_n for 15 min at 4°C. The reaction mixture consisted of 400 µL of supernatant, and 150 µL of 0.003 M *p*-NPP ($C_6H_4NO_6PNa_2{\cdot}6H_2O$) (Sigma, St. Louis, Mo.). The mixture was then incubated (Precision® 180 Series Water Bath, Precision Scientific Inc., Chicago, Ill.) at 37°C for 45 min for ACP and ALP. The reaction was stopped by the addition of 100 µL 500 mM $CaCl_2$ ($CaCl_2{\cdot}2H_2O$) (Sigma, St. Louis, Mo.) and 400 µL 500 mM NaOH (Sigma, St. Louis, Mo.). Precipitate was recovered by centrifugation at 13,000 g_n for 15 min. The *p*-NP content of the supernatant was quantified with a spectrophotometer (Beckman Coulter™ Du® Series 640 UV/Vis Spectrophotometer, Beckman Coulter, Inc. Fullertan, Calif.) at 420 nm, and compared to a standard concentration series (0, 1.4, 2.8, 4.2, 5.6, and 7.0 mM) of authentic *p*-NP ($C_6H_5NO_3$) (Sigma, St. Louis, Mo.).

ASSESSMENT OF ARBUSCULAR MYCORRRHIZAL DEVELOPMENT

For AMF analysis of roots, 1-cm root segments from three randomly selected plants per treatment were sampled at harvest and pooled to assess colonization percentage through clearing with KOH and staining of root samples with trypan blue (Philips and Hayman, 1970). Twenty five 1-cm stained root pieces were placed on each slide and three observations (the top, the middle, and the bottom) per 1-cm root piece

were made with a microscope (Nikon Alphaphot YS, Nikon Inc., Instrument Division, Garden City, N.Y.) at 40X. The presence of arbuscules, vesicles, and hyphae was determined (Biermann and Linderman, 1981). There were nine slides per treatment (n=675 observations per treatment from 225 1-cm root pieces).

STATISTICAL DESIGN

The experiment was a 2 x 4 factorial in a completely randomized design with two AMF levels (AMF and Non-AMF) and four levels of HCO_3^-: 0, 2.5, 5, and 10 mM HCO_3^-. There was one rooted *R. multiflora* cv. Burr cutting per container, with each container as a single replicate. Data were analyzed using Analysis of Variance (ANOVA) and LSD multiple comparison test (SAS Institute Inc., 2000) and regression models were also determined (SPSS, Inc., 2003). The number of replications were: leachate analysis (n=5), growth data (n=9), nutrient analysis (n=3), chlorophyll content (n=3), iron reductase activity (n=3), phosphatase activity (n=3), and AMF observations (n=675).

Results

LEACHATE ANALYSIS

Bicarbonate caused a significant ($P \leq 0.001$) increase in leachate pH (Fig. 1). In general HCO_3^- caused a significant increase in leachate EC (Fig. 2). In general, pH and EC increased over the duration of the study regardless of AMF treatment.

PLANT GROWTH

Bicarbonate caused a significant ($P \leq 0.01$) reduction in all plant growth parameters (Tables 1 and 2). In general, most growth parameters were significantly ($P \leq 0.05$) affected by AMF, except for root DM, root/shoot ratio, and leaf number. The HCO_3^- x AMF interaction was not significant for any growth parameters, except SLA.

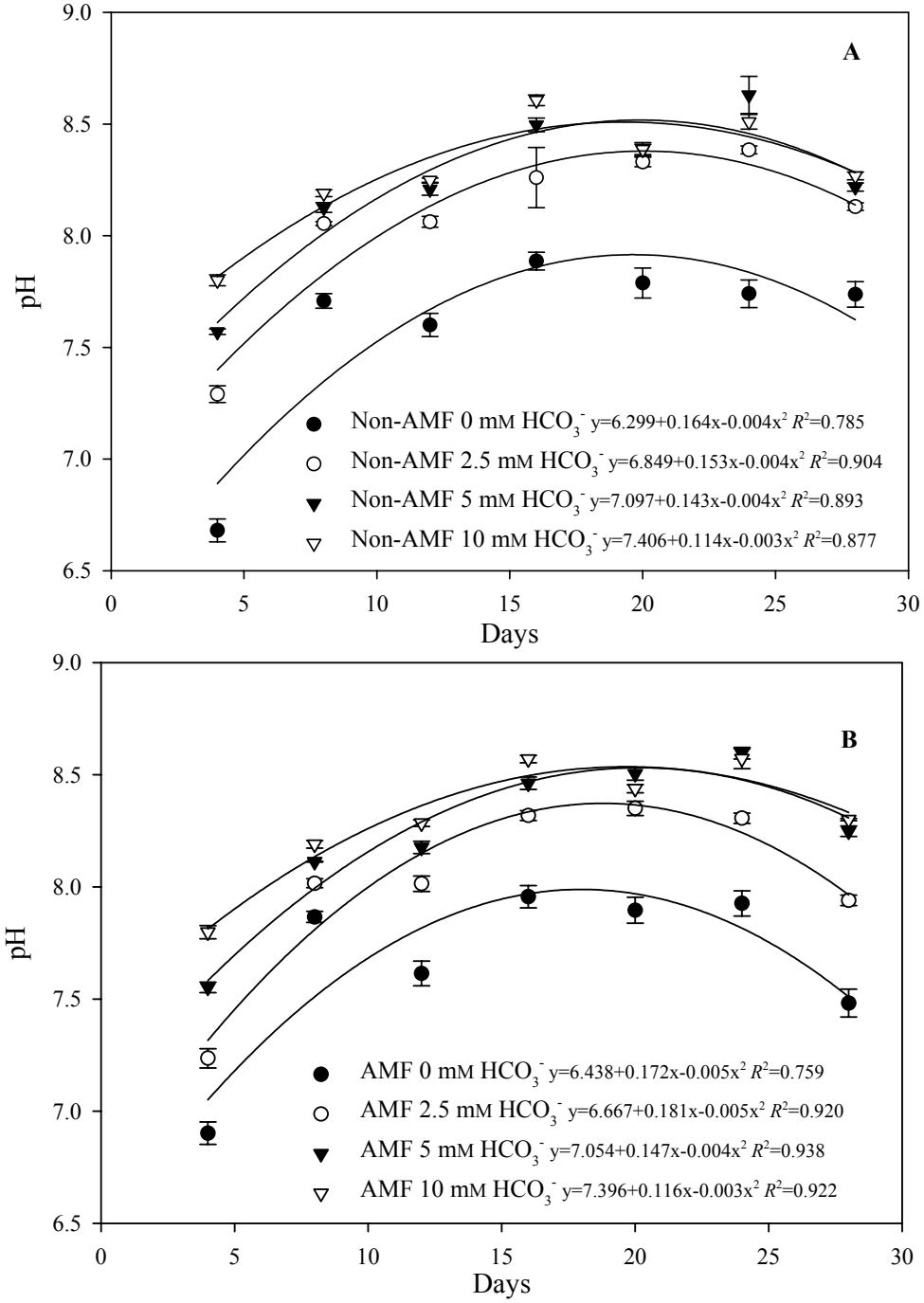

Fig. 1. Effect of bicarbonate (HCO_3^-) and arbuscular mycorrhizal fungi (AMF) on pH of *Rosa multiflora* cv. Burr substrate for duration of study. (A) Non-AMF plants. (B) AMF plants. Treatment effect of HCO_3^- was significant ($P \leq 0.001$) at every irrigation. Treatment effect of AMF and AMF x HCO_3^- interaction was nonsignificant at every irrigation. Means±standard error (n=5).

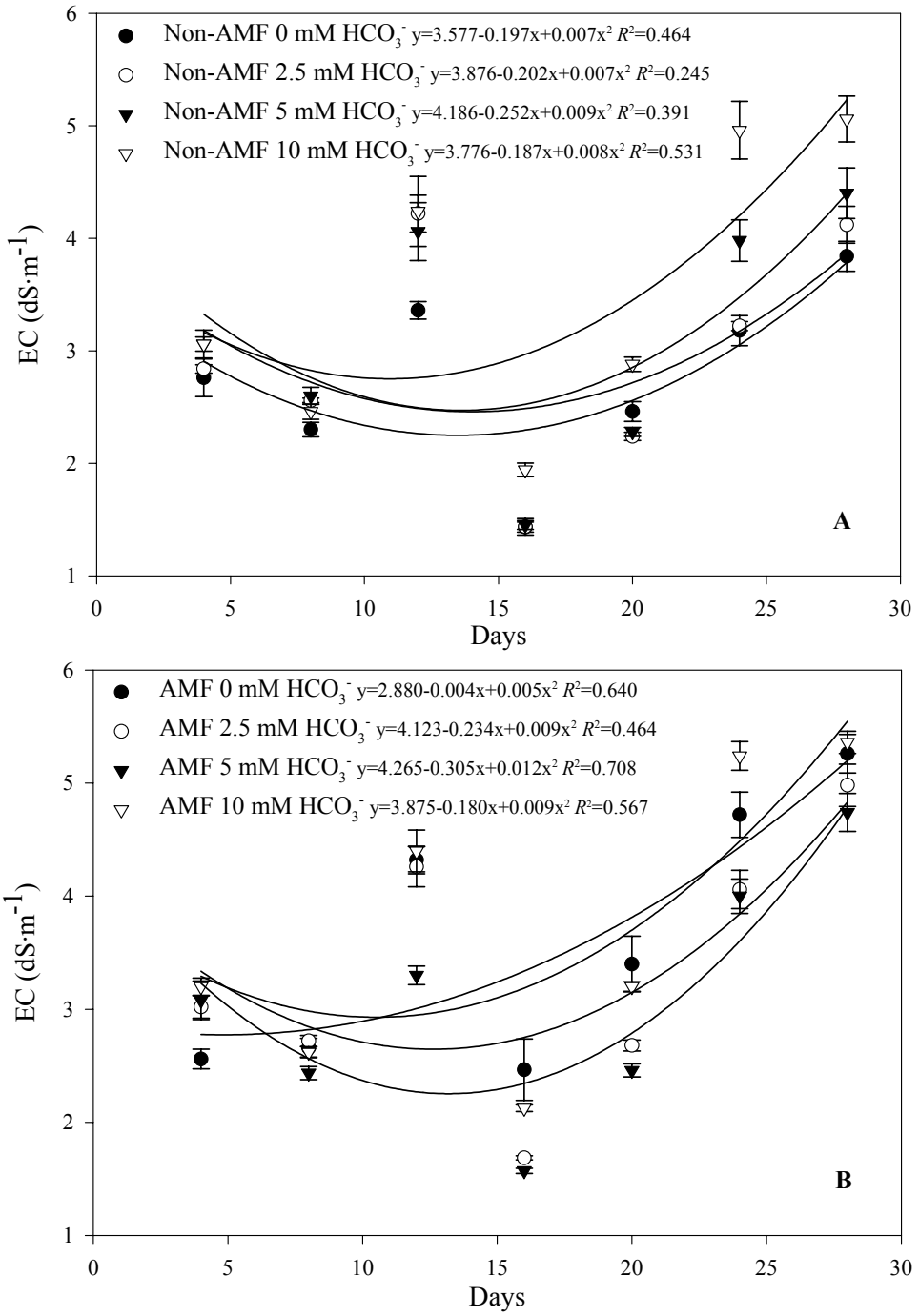

Fig. 2. Effect of bicarbonate (HCO_3^-) and arbuscular mycorrhizal fungi (AMF) on electrical conductivity (EC) of *Rosa multiflora* cv. Burr substrate for duration of study. (A) Non-AMF plants. (B) AMF plants. Treatment effect of AMF was significant ($P \leq 0.05$, 0.01, 0.01, and 0.01) at day 16, 20, 24, and 28, respectively. Treatment effect of HCO_3^- was significant ($P \leq 0.05$, 0.01, and 0.001) at day 16, 20, and 24, respectively. Treatment effect of AMF x HCO_3^- interaction was nonsignificant. Means±standard error (n=5).

Table 1. Effect of bicarbonate (HCO_3^-) and arbuscular mycorrhizal fungi (AMF) on growth of *Rosa multiflora* cv. Burr plants.

HCO_3^- (mM)	AMF Inoculation	Leaf DM (g)	Stem DM (g)	Root DM (g)	Total Plant DM (g)	Root/Shoot Ratio ($g \cdot g^{-1}$)	MIE[z] (%)
0	No	4.4±0.5[y]	4.6±0.4	0.9±0.1	9.9±0.9	0.09±0.01	
	Yes	5.0±0.3	4.6±0.2	1.0±0.1	10.6±0.5	0.10±0.00	7
2.5	No	2.8±0.3	3.5±0.3	0.6±0.1	6.9±0.6	0.09±0.01	
	Yes	3.9±0.3	4.3±0.4	0.8±0.1	8.9±0.7	0.08±0.00	29
5	No	2.4±0.2	3.2±0.2	0.5±0.1	6.1±0.4	0.09±0.01	
	Yes	3.1±0.2	3.7±0.2	0.6±0.1	7.4±0.4	0.09±0.00	21
10	No	2.1±0.1	2.9±0.2	0.7±0.1	5.7±0.3	0.12±0.00	
	Yes	2.6±0.2	3.4±0.3	0.7±0.1	6.7±0.5	0.10±0.01	18
Significance[x]							
AMF		**	*	NS	**	NS	-
HCO_3^-		***	***	***	***	**	-
Interaction		NS	NS	NS	NS	NS	-

[z] Mycorrhizal inoculation effect [MIE (%) = (total DM of AMF plant – total DM of non-AMF plant)/(total DM of non-AMF plant)$^{-1}$ x 100].
[y] Means±standard error (n=9).
[x] Significance according to ANOVA, NS, *, **, ***, nonsignificant and significant $P \leq 0.05$, 0.01, 0.001, respectively.

Table 2: Effect of bicarbonate (HCO_3^-) and arbuscular mycorrhizal fungi (AMF) on leaf growth of *Rosa multiflora* cv. Burr plants.

HCO_3^- (mM)	AMF Inoculation	Leaf Number	Leaf Area (cm^2)	SLA[z] ($cm^2 \cdot g^{-1}$)	LAR[y] ($cm^2 \cdot g^{-1}$)
0	No	58.2±5.0[x]	1088.0±118.4	247.4±14.3	108.6±10.1
	Yes	54.1±6.5	1162.3±35.5	236.6±21.3	110.3±10.8
2.5	No	49.4±3.8	560.3±56.1	203.7±11.8	80.6±11.0
	Yes	44.7±3.9	878.2±45.5	227.6±21.9	99.7±13.4
5	No	44.6±3.8	442.8±58.9	183.6±20.3	72.4±11.9
	Yes	41.7±3.6	669.1±22.9	218.1±11.9	90.6±9.1
10	No	37.8±4.6	412.3±61.3	195.9±20.3	73.1±7.8
	Yes	38.2±4.4	549.6±54.5	206.5±16.8	81.0±11.5
Significance[w]					
AMF		NS	***	***	***
HCO_3^-		**	***	***	***
Interaction		NS	NS	**	NS

[z] Specific leaf area.
[y] Leaf area ratio.
[x] Means±standard error (n=9).
[w] Significance according to ANOVA, NS, *, **, ***, nonsignificant and significant $P \leq 0.05, 0.01, 0.001$, respectively.

Total Plant Dry Mass

Increasing concentrations of HCO_3^- significantly ($P \leq 0.001$) reduced total plant DM (Table 1). On average, plants inoculated with AMF exhibited a significantly ($P \leq 0.01$) greater total plant DM (+17%) compared to non-AMF plants. The HCO_3^- x AMF interaction was not significant, indicating that increasing HCO_3^- stressed plants, regardless of AMF. However, AMF alleviated HCO_3^- stress as indicated by greater total plant DM at all treatment levels.

In non-AMF plants treated with 2.5 mM HCO_3^-, there was a reduction (-30%) in total plant DM compared to control (0 mM HCO_3^-) plants. Non-AMF plants treated with 5 and 10 mM HCO_3^- exhibited decreased total plant DM (-38% and -42%, respectively).

Plants inoculated with AMF had a greater tolerance to HCO_3^- stress. In AMF plants treated with 2.5 mM HCO_3^-, there was a reduction (-16%) in total plant DM compared to AMF control (0 mM HCO_3^-) plants. Inoculated plants treated with 5 and 10 mM HCO_3^- exhibited decreased total plant DM (-30% and -37%, respectively).

Arbuscular mycorrhizal plants partially alleviated plant HCO_3^- stress at 2.5, 5, and 10 mM with increased (+29%, +21%, and +18%, respectively) plant DM compared to non-AMF plants.

Stem Dry Mass

Increasing concentration of HCO_3^- induced a significant ($P \leq 0.001$) decrease in stem DM (Table 1). On average, plants inoculated with AMF exhibited a significant ($P \leq 0.05$) increase (+13%) in stem DM compared to non-AMF plants. The HCO_3^- x AMF interaction was not significant.

In non-AMF plants treated with 2.5 mM HCO_3^- there was a reduction (-24%) in stem DM compared to the control (0 mM HCO_3^-) plants. Non-AMF plants treated with 5 and 10 mM HCO_3^- exhibited a decrease in stem DM (-30% and -37%, respectively).

In AMF plants treated with 2.5 mM HCO_3^- there was a reduction (-7%) in stem DM compared to AMF control (0 mM·HCO_3^-) plants. Inoculated plants treated with 5 and 10 mM HCO_3^- exhibited decreased stem DM (-20% and -26% respectively).

Arbuscular mycorrhizal plants partially alleviated plant HCO_3^- stress at 2.5, 5, and 10 mM with increased (+23%, +16%, and +17%, respectively) stem DM compared to non-AMF plants.

Root Dry Mass

In general, increasing concentrations of HCO_3^- significantly ($P \leq 0.001$) reduced root DM (Table 1). On average, plants inoculated with AMF did not exhibit a significant difference compared to non-AMF plants. The HCO_3^- x AMF interaction was not significant, indicating that HCO_3^- stress occurred to the same degree regardless of AMF.

In non-AMF plants treated with 2.5 mM HCO_3^- there was a reduction (-33%) in root DM compared to control (0 mM HCO_3^-) plants. Non-AMF plants treated with 5 and 10 mM HCO_3^- exhibited a decrease in root DM (-44% and -22%, respectively).

In AMF plants treated with 2.5 mM HCO_3^- there was a reduction (-20%) in root DM compared to AMF control (0 mM HCO_3^-) plants. Inoculated plants treated with 5 and 10 mM HCO_3^- exhibited decreased stem DM (-40% and -30% respectively).

Regardless of AMF treatment there was a decrease (-26%, -42%, and -26%) in root DM at 2.5, 5, and 10 mM HCO_3^-, respectively.

Root/Shoot Ratio

The root/shoot ratio was significantly ($P \leq 0.01$) affected by the HCO_3^- concentration (Table 1). Plants inoculated with AMF and the HCO_3^- x AMF interaction was not significant. The results were inconsistent and there was no clear tendency for treatments.

Mycorrhizal Inoculation Effect

The MIE was very low (7%) in plants subjected to 0 mM HCO_3^- (Table 1). At 2.5 mM HCO_3^- MIE greatly increased (29%). Plants treated with 5 and 10 mM HCO_3^- decreased MIE values (21% and 18%, respectively).

Leaf Growth

Leaf number was significantly ($P \leq 0.01$) reduced by increasing HCO_3^- concentration (Table 2). Plants inoculated with AMF and the HCO_3^- x AMF interaction were not significant. However, increasing concentration of HCO_3^- significantly ($P \leq 0.001$) reduced both leaf area and leaf DM (Tables 1 and 2). On average, plants inoculated with AMF exhibited a significantly ($P \leq 0.01$) greater leaf area and leaf DM (+30% and +25%, respectively) compared to non-AMF plants. However, in AMF plants the negative effect of HCO_3^- was mitigated to a certain degree.

In non-AMF plants treated with 2.5 mM HCO_3^- there was a reduction in leaf area and leaf DM (-49% and -36%, respectively) compared to control (0 mM HCO_3^-) plants. Non-AMF plants treated with 5 and 10 mM HCO_3^- exhibited decreased leaf area (-59% and -62%, respectively) and leaf DM (-46% and -52%, respectively).

Plants inoculated with AMF had a greater tolerance to HCO_3^- stress. In AMF plants treated with 2.5 mM HCO_3^- there was a reduction in leaf area and leaf DM (-24% and -22% respectively) compared to AMF control (0 mM HCO_3^-) plants. Inoculated plants treated with 5 and 10 mM HCO_3^- exhibited a decrease in leaf area (-42% and -53%, respectively) and leaf DM (-38% and -48%, respectively).

Arbuscular mycorrhizal plants partially alleviated plant HCO_3^- stress at 2.5, 5, and 10 mM with increased (+39%, +29%, and +24%, respectively) leaf DM compared to non-AMF plants. Arbuscular mycorrhizal fungi had a greater effect in alleviating HCO_3^- stress in leaf area ($P \leq 0.001$) than leaf DM ($P \leq 0.01$). Furthermore, AMF partially alleviated plant HCO_3^- stress at 2.5, 5, and 10 mM with increased (+57%, +51%, and +33%, respectively) leaf area compared to non-AMF plants.

The SLA and LAR were significantly ($P \leq 0.001$) reduced by increasing HCO_3^- concentration (Table 2). On average, plants inoculated with AMF exhibited a significantly ($P \leq 0.01$) greater SLA and LAR (+7% and +14%, respectively) compared to non-AMF plants. The SLA HCO_3^- x AMF interaction was significant ($P \leq 0.01$). However, the LAR HCO_3^- x AMF interaction was not significant.

In non-AMF plants treated with 2.5 mM HCO_3^- there was a reduction in SLA and LAR (-18% and -26%, respectively) compared to control (0 mM HCO_3^-) plants. Non-AMF plants treated with 5 and 10 mM HCO_3^- exhibited a decrease in SLA (-26% and -21%, respectively) and LAR (-33% and -33%, respectively).

In AMF plants treated with 2.5 mM HCO_3^- there was a reduction in SLA and LAR (-4% and -10%, respectively) compared to AMF control (0 mM HCO_3^-) plants. Inoculated plants treated with 5 and 10 mM HCO_3^- exhibited a decrease in SLA (-8% and -13%, respectively) and LAR (-18% and -27%, respectively).

LEAF NUTRIENT ANALYSIS

Leaf nutrient content of N, P, Ca, Fe, Cu, and B was significantly ($P \leq 0.05$) reduced by increasing HCO_3^- concentration, whereas K, Na, Mn, Mg, S, Zn, and Al were unaffected or had no consistent response (Tables 3 and 4). Conversely, levels of Mo increased with higher HCO_3^- concentrations. At selected concentrations of HCO_3^-, plants inoculated with AMF had significantly ($P \leq 0.05$) increased leaf nutrient content of N, P, K, Ca, Mg, S, Na, Fe, Zn, Cu, Al, B, and Mo, compared to non-AMF plants. Neither AMF nor HCO_3^- affected Mn levels (Table 4). The HCO_3^- x AMF interaction was significant ($P \leq 0.05$) for Na, Fe, Zn, Cu, Al, and Mo.

Non-Arbuscular Mycorrhizal Plants

In non-AMF plants, HCO_3^- concentration induced decreased leaf nutrient content, however there was no clear pattern as the HCO_3^- concentration increased. The most affected nutrients were P, S, Fe, Zn, Cu, and Al (with a maximum decrease of -58%, -50%, -50%, -63%, -54%, and -54%, respectively). The lowest leaf N, P, and B content was obtained at 10 mM HCO_3^-. The lowest leaf Ca, S, Fe, and Cu content was obtained at 5 mM HCO_3^-. The lowest leaf K, Mg, Na, Zn, and Al content was obtained at 2.5 mM HCO_3^-. Leaf Mo increased with increasing HCO_3^- concentration (maximum increase of 362% at 5 mM HCO_3^-).

Table 3: Effect of bicarbonate (HCO_3^-) and arbuscular mycorrhizal fungi (AMF) on leaf macronutrient and Na content of *Rosa multiflora* cv. Burr plants.

HCO_3^- (mM)	AMF Inoculation	N ($g \cdot kg^{-1}$)	P ($g \cdot kg^{-1}$)	K ($g \cdot kg^{-1}$)	Ca ($g \cdot kg^{-1}$)	Mg ($g \cdot kg^{-1}$)	S ($g \cdot kg^{-1}$)	Na ($g \cdot kg^{-1}$)
0	No	32.4±3.1[z]	2.4±0.3	24.0±2.2	6.9±0.7	2.0±0.2	2.0±0.2	0.3±0.0
	Yes	36.3±0.3	2.6±0.1	27.1±0.8	7.3±0.2	2.2±0.0	2.3±0.0	0.3±0.0
2.5	No	22.1±0.9	1.5±0.1	15.5±1.6	4.6±0.5	1.4±0.1	1.2±0.1	0.2±0.0
	Yes	30.0±3.0	2.1±0.2	26.9±2.5	7.4±0.9	2.4±0.2	1.9±0.2	0.3±0.0
5	No	19.6±1.4	1.1±0.1	16.7±1.9	4.4±0.4	1.4±0.1	1.0±0.1	0.2±0.0
	Yes	24.3±1.9	1.4±0.1	23.8±1.7	6.1±0.5	1.8±0.1	1.3±0.1	0.4±0.0
10	No	18.1±2.7	1.0±0.1	17.5±2.0	4.8±0.8	1.4±0.2	1.0±0.2	0.3±0.0
	Yes	21.1±1.9	1.2±0.1	23.4±2.8	5.7±0.7	1.7±0.2	1.3±0.1	0.4±0.1
Significance[y]								
AMF		**	**	**	**	**	**	**
HCO_3^-		***	***	NS	*	*	***	NS
Interaction		NS	NS	NS	NS	NS	NS	*

[z] Means±standard error (n=3).

[y] Significance according to ANOVA, NS, *, **, ***, nonsignificant and significant $P \leq 0.05$, 0.01, 0.001, respectively.

Table 4. Effect of bicarbonate (HCO_3^-) and arbuscular mycorrhizal fungi (AMF) on leaf miconutrient content of *Rosa multiflora* cv. Burr plants.

HCO_3^- (mM)	AMF Inoculation	Fe ($\mu g \cdot g^{-1}$)	Mn ($\mu g \cdot g^{-1}$)	Zn ($\mu g \cdot g^{-1}$)	Cu ($\mu g \cdot g^{-1}$)	Al ($\mu g \cdot g^{-1}$)	B ($\mu g \cdot g^{-1}$)	Mo ($\mu g \cdot g^{-1}$)
0	No	66.9±3.2[z]	35.3±3.7	10.7±0.6	3.5±0.5	16.9±0.5	90.3±9.4	2.6±1.0
	Yes	70.1±2.7	34.7±1.3	12.7±0.4	3.9±0.0	15.3±0.7	99.6±2.6	2.6±0.7
2.5	No	38.0±2.4	26.6±2.1	4.0±0.6	2.0±0.3	7.7±0.9	56.5±5.8	2.1±0.7
	Yes	69.0±6.5	32.8±4.2	14.6±1.2	4.2±0.7	16.0±1.3	98.8±9.7	3.0±1.1
5	No	33.6±2.5	30.6±3.4	5.0±0.7	1.6±0.1	8.4±0.9	57.3±5.7	12.0±0.8
	Yes	52.1±4.2	32.5±4.5	11.7±1.5	2.7±0.2	20.2±2.6	80.6±3.9	18.1±0.5
10	No	34.5±5.7	25.5±5.8	7.4±1.7	2.2±0.3	9.9±1.3	53.4±7.4	10.9±2.0
	Yes	44.6±6.2	27.7±2.4	11.3±0.9	2.4±0.1	16.9±2.4	64.2±5.8	11.0±1.3
Significance[y]								
AMF		**	NS	***	**	***	**	*
HCO_3^-		***	NS	*	*	NS	**	***
Interaction		*	NS	**	*	**	NS	*

[z] Means±standard error (n=3).

[y] Significance according to ANOVA, NS, *, **, ***, nonsignificant and significant $P \leq 0.05$, 0.01, 0.001, respectively.

Arbuscular Mycorrhizal Plants

In AMF plants, HCO_3^- induced decreased leaf nutrient content, however there was no clear pattern as the HCO_3^- concentration increased. The most affected nutrients were N, P, and S (-42%, -54%, and -43%, respectively) compared to AMF control (0 mM HCO_3^-) plants. The lowest leaf N and P content were obtained at 10 mM HCO_3^-, and the lowest leaf S content was obtained at 5 mM.

Inoculated plants had a greater leaf N, P, and S content compared to non-AMF plants. At 2.5 mM HCO_3^- concentration, AMF plants exhibited greatest leaf N, P and S content (+36%, +40%, and +58%) compared to non-AMF plants.

Leaf K, Ca, Mg, Fe, and B content remained constant in inoculated plants treated with 2.5 mM HCO_3^- compared to control AMF (0 mM HCO_3^-) plants. At 2.5 mM HCO_3^- concentration AMF plants exhibited greatest leaf K, Ca, Mg, Fe, and B content (+74%, +61%, +71%, +82%, and +75%, respectively) compared to non-AMF plants. Bicarbonate concentrations >2.5 mM decreased leaf K, Ca, Mg, Fe, and B content. The lowest leaf K, Ca, Mg, Fe, and B content (-14%, -22%, -23%, -36%, and -36%, respectively) was obtained at 10 mM HCO_3^-.

At 2.5 mM HCO_3^- concentration AMF plants exhibited greater leaf Zn and Cu content (+15% and +8%, respectively) compared to control (0 mM HCO_3^-) AMF and (+36% and +20%, respectively) non-AMF plants. In AMF plants leaf Zn content remained constant with increasing HCO_3^- concentration compared to control (0 mM HCO_3^-) non-AMF plants. However, in AMF plants leaf Cu content decreased with increasing HCO_3^- concentration compared to control (0 mM HCO_3^-) non-AMF plants.

At 0 mM HCO_3^- concentration AMF plants did not have increased leaf Mo, Na, and Al content compared to non-AMF plants. In AMF plants increasing HCO_3^- concentration increased leaf Mo, Na, and Al content compared to non-AMF plants. At 5 mM HCO_3^- concentration AMF plants exhibited greatest (+51%, +100%, and +140%) leaf Mo, Na, and Al content compared non-AMF plants.

TOTAL CHLOROPHYLL CONTENT

Total leaf chlorophyll content of AMF and non-AMF was significantly ($P \leq 0.001$) reduced by increasing HCO_3^- concentration (Fig. 3). Arbuscular mycorrhizal plants had greater leaf chlorophyll content than non-AMF plants at all HCO_3^- concentrations. At 0 mM, 2.5 mM, 5 mM, and 10 mM HCO_3^- concentrations, AMF plants exhibited significantly ($P \leq 0.001$) increased (+310%, +494%, +63%, and +26%, respectively) total chlorophyll content compared to non-AMF plants. The HCO_3^- x AMF interaction was significant (<0.01).

In non-AMF plants treated with 2.5 mM HCO_3^- there was a reduction (-79%) in total chlorophyll content compared to control (0 mM HCO_3^-) plants. Non-AMF plants treated with 5 and 10 mM HCO_3^- exhibited a decrease in total chlorophyll content (-33% and -35%, respectively).

In AMF plants treated with 2.5 mM HCO_3^- there was a reduction (-70%) in total chlorophyll content compared to AMF control (0 mM HCO_3^-) plants. Inoculated plants treated with 5 and 10 mM HCO_3^- exhibited a decrease in total chlorophyll content (-73% and -80%, respectively).

ROOT IRON REDUCTASE

The Fe reductase activity was not significantly reduced by increasing HCO_3^- concentration (Fig. 4). On average, AMF plants had significantly ($P \leq 0.01$) decreased (-18%) Fe reductase activity compared to non-AMF plants. The HCO_3^- x AMF interaction was not significant.

ROOT PHOSPHATASE ACTIVITY

Soluble Acid Phosphatase

The soluble ACP activity was significantly ($P \leq 0.001$) decreased by increasing HCO_3^- concentration (Fig. 5A). Plants inoculated with AMF and the HCO_3^- x AMF interaction were not significant. In AMF plants treated with 0 mM HCO_3^- there was greater (+21%) enzymatic activity compared to non-AMF control (0 mM HCO_3^-) plants.

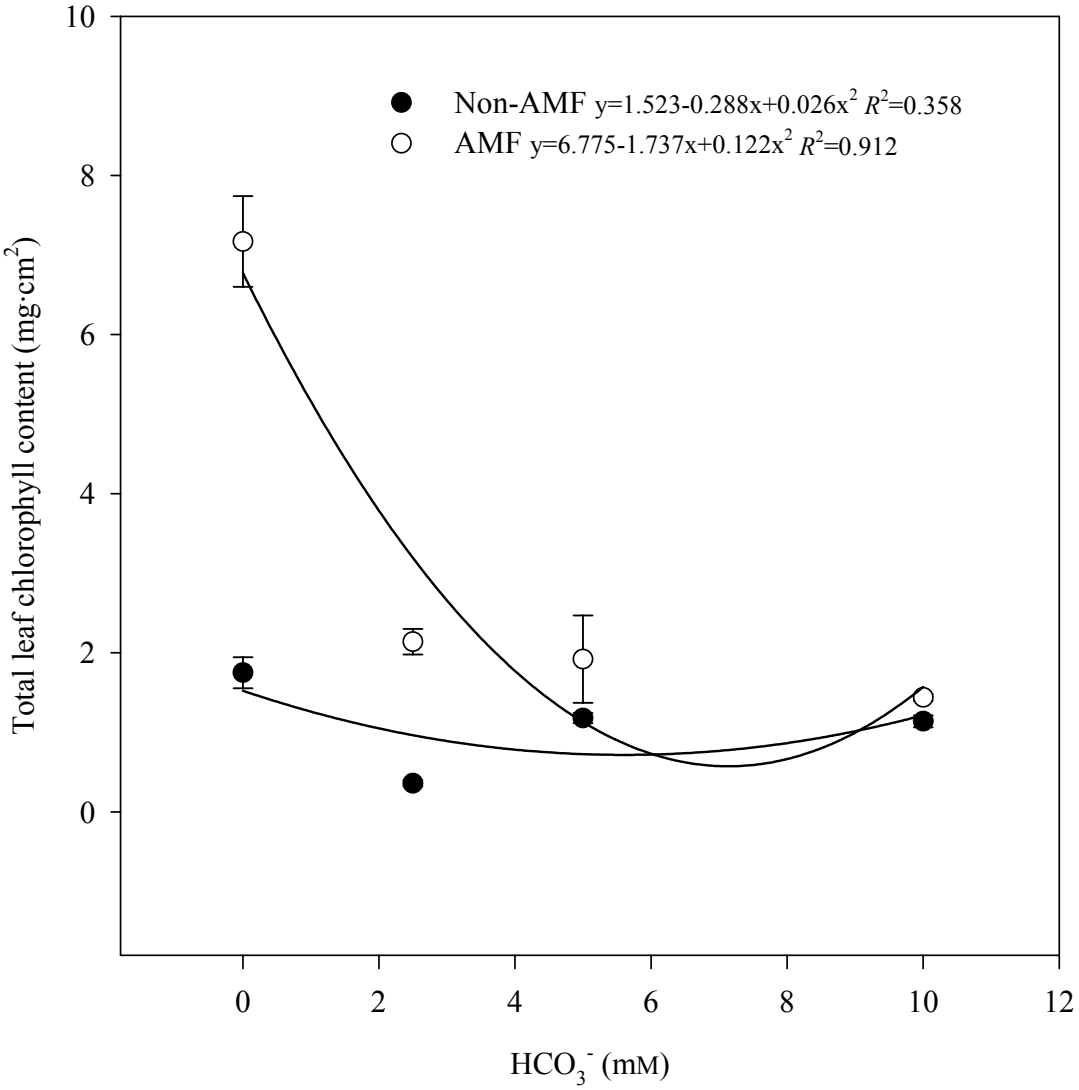

Fig.3. Effect of bicarbonate (HCO_3^-) and arbuscular mycorrhizal fungi (AMF) on total leaf chlorophyll content of *Rosa multiflora* cv. Burr plants. Treatment effect of AMF, HCO_3^-, and AMF x HCO_3^- interaction were significant ($P \leq 0.001$, 0.001, and 0.01, respectively). Means±standard error (n=3).

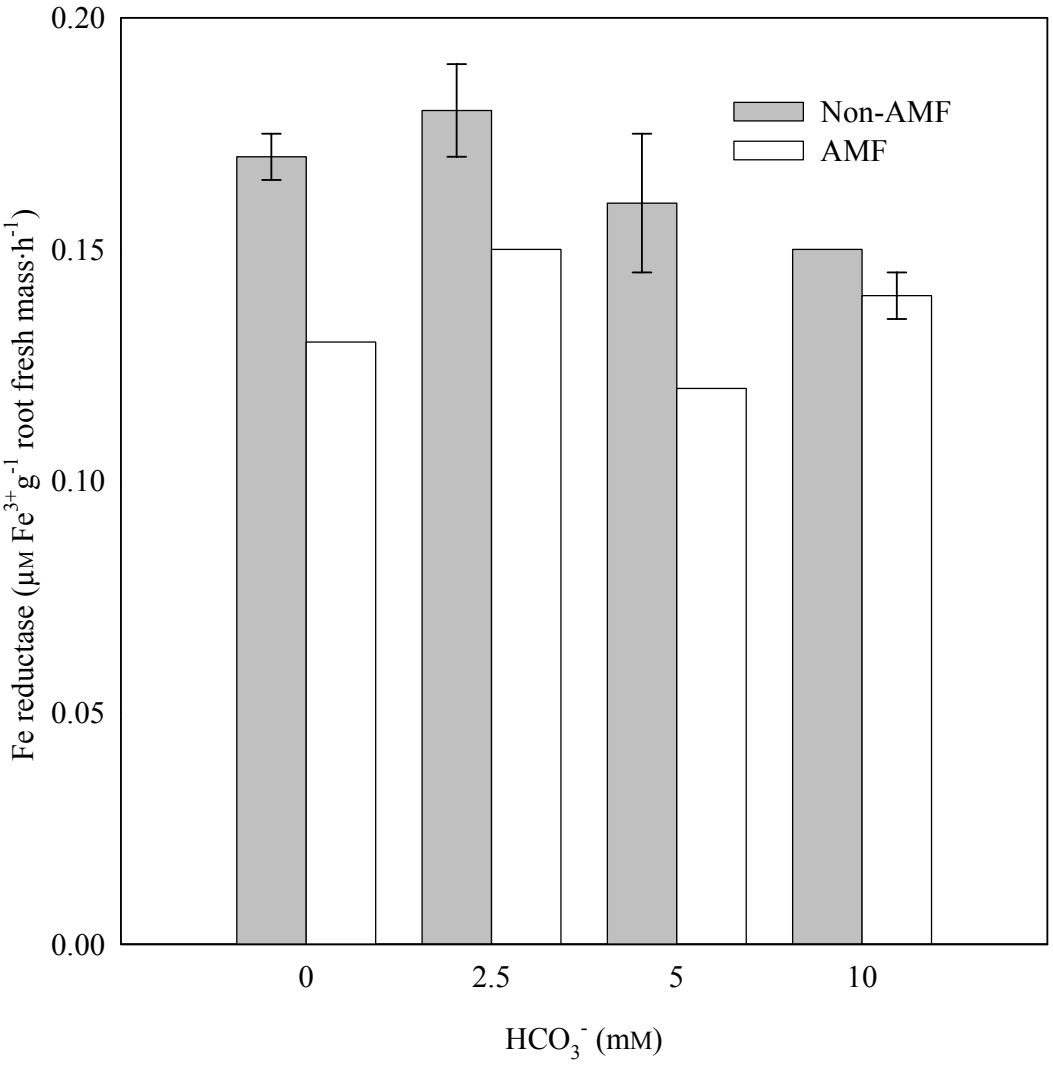

Fig.4. Effect of bicarbonate (HCO_3^-) and arbuscular mycorrhizal fungi (AMF) on root Fe reductase activity in *Rosa multiflora* cv. Burr plants. Treatment effect of AMF was significant ($P \leq 0.01$). Bicarbonate and AMF x HCO_3^- interaction were nonsignificant. Means±standard error (n=3).

Inoculated plants treated with 2.5 and 5 mM HCO_3^- exhibited comparable enzymatic activity between AMF and non-AMF plants, while plants treated with 10 mM HCO_3^- exhibited greater (+16%) enzymatic activity compared to non-AMF plants.

Wall-Bound Acid Phosphatase

The wall-bound ACP activity was also significantly ($P \leq 0.01$) decreased by increasing HCO_3^- concentration (Fig. 5B). Plants inoculated with AMF treated with 0 mM HCO_3^- exhibited a significant ($P \leq 0.01$) decrease (-28%) in enzymatic activity compared to non-AMF control (0 mM HCO_3^-) plants. The HCO_3^- x AMF interaction was significant ($P \leq 0.05$). In non-AMF plants treated with 0 and 2.5 mM HCO_3^- there was greater (+39% and +168%, respectively) enzymatic activity compared to AMF plants. Inoculated and non-AMF plants treated with 5 and 10 mM HCO_3^- exhibited comparable enzymatic activity.

Soluble Alkaline Phosphatase

Soluble ALP activity was not significantly affected by increasing HCO_3^- concentration or AMF inoculation (Fig. 6A). The HCO_3^- x AMF interaction was significant ($P \leq 0.05$). In non-AMF plants treated from 0 to 5 mM HCO_3^- there was increased (+59%) enzymatic activity. At higher HCO_3^- concentration (>5 mM HCO_3^-) there was a decline in the enzymatic activity, however enzymatic activity of AMF plants increased with increasing HCO_3^- concentrations. At 10 mM HCO_3^- concentration AMF plants exhibited increased enzymatic activity (+36%) compared to AMF control (0 mM HCO_3^-) plants.

Wall-Bound Alkaline Phosphatase

Wall-Bound ALP activity was significantly ($P \leq 0.01$) increased by increasing HCO_3^- concentrations (Fig. 6B). Inoculated plants and the HCO_3^- x AMF interaction were not significant. In general, non-AMF plants exhibited greater enzymatic activity than AMF plants. In AMF and non-AMF plants treated from 0 to 5 mM HCO_3^- there

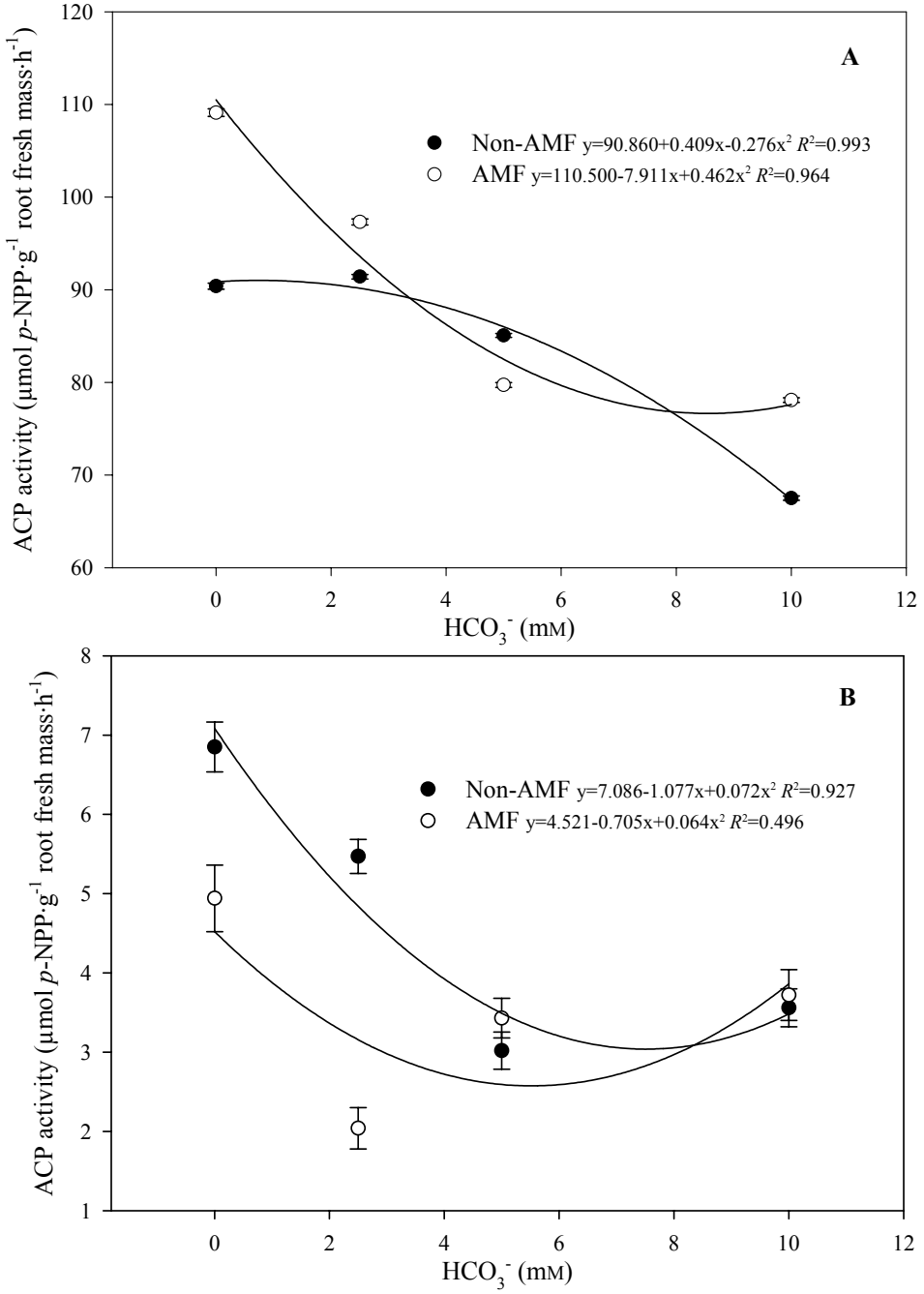

Fig.5. Effect of bicarbonate (HCO_3^-) and arbuscular mycorrhizal fungi (AMF) on root acid phosphatase (ACP) activity in *Rosa multiflora* cv. Burr plants. (A) Soluble ACP activity. Treatment effect of HCO_3^- was significant ($P \leq 0.001$). Arbuscular mycorrhizal fungi and AMF x HCO_3^- interaction were nonsignificant. Means±standard error (n=3). (B) Wall-bound ACP activity. Treatment effect of AMF, HCO_3^-, and AMF x HCO_3^- interaction were significant ($P \leq 0.01$, 0.01, and 0.05, respectively). Means±standard error (n=3).

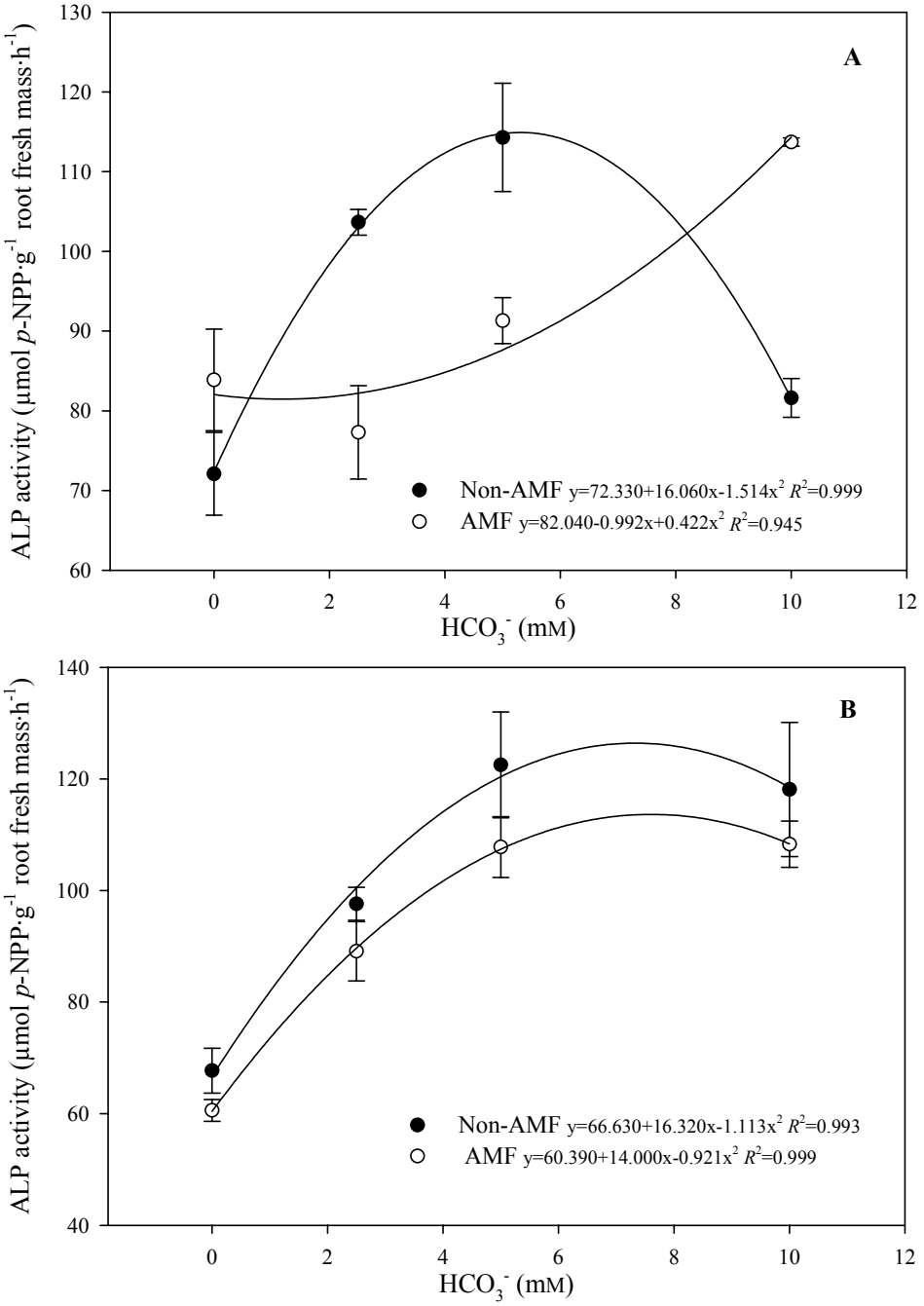

Fig.6. Effect of bicarbonate (HCO_3^-) and arbuscular mycorrhizal fungi (AMF) on root alkaline phosphatase (ALP) activity in *Rosa multiflora* cv. Burr plants. (A) Soluble ALP activity. Treatment effect of AMF x HCO_3^- interaction was significant ($P \leq 0.05$). Treatment effects of AMF and HCO_3^- were nonsignificant. Means±standard error (n=3). (B) Wall-bound ALP activity. Treatment effect of HCO_3^- was significant ($P \leq 0.01$). Arbuscular mycorrhizal fungi and AMF x HCO_3^- interaction were nonsignificant. Means±standard error (n=3).

was increased (+78% and +81% respectively) enzymatic activity compared to control (0 mM HCO_3^-). At higher HCO_3^- concentration (>5 mM HCO_3^-) enzymatic activity plateaued.

ARBUSCULAR MYCORRHIZAL FUNGI COLONIZATION

No AMF colonization occurred with non-inoculated plants. Greatest depression of AMF occurred with increasing HCO_3^- concentration, however colonization was still achieved (Table 5). Total colonization and hyphae levels were ≤40%. Inoculated plants treated with 2.5 mM HCO_3^- exhibited significantly ($P \leq 0.001$) decreased (-35%) hyphae and total colonization compared to control (0 mM HCO_3^-) AMF plants. Inoculated plants treated with 5 and 10 mM HCO_3^- exhibited significantly ($P \leq 0.001$) decreased (-81% and -62% respectively) hyphae and total colonization compared to control (0 mM HCO_3^-) AMF plants. No vesicles were observed in any treatments.

Discussion

To our knowledge, this is one of the first reports that AMF enhance tolerance of *R. multiflora* cv. Burr to HCO_3^- stress.

LEACHATE ANALYSIS

Increasing HCO_3^- concentration caused a significant increase in substrate pH, which was attributed to the neutralization of H^+ by HCO_3^- (Lindsay, 1979). Bicarbonate accumulation in the substrate was a result of frequent (every 4 d for 32 d) irrigation with water high in HCO_3^-. Thus, accumulation of HCO_3^- caused a slight increase in substrate pH overtime (Fig. A1). In general, increasing HCO_3^- concentration caused a significant increase in substrate EC.

We suggest that the high pH and EC reported in this study may not be a true reflection of actual substrate pH and EC. Substrate was not brought to container capacity prior to leachate collection as recommended (Wright, 1987). Thus, data

Table 5. Effect of bicarbonate (HCO_3^-) on percentage arbuscules, hyphae, and vesicles in root cortical cells of arbuscular mycorrhizal *Rosa multiflora* cv. Burr plants.

Bicarbonate (mM)	AMF[z] Inoculation	Arbuscules (%)	Vesicles (%)	Hyphae (%)	Total Colonization (%)
0	Yes	5.0±0.6[y]	0.0±0.0	40.0±3.3	40.0±3.3
2.5	Yes	1.8±0.3	0.0±0.0	26.1±2.0	26.1±2.0
5	Yes	1.5±0.1	0.0±0.0	15.4±1.5	15.4±1.5
10	Yes	0.4±0.4	0.0±0.0	7.6±0.7	7.6±0.7
Significance[x]					
HCO_3^-		***	NS	***	***

[z] Arbuscular mycorrhizal fungi.
[y] Means±standard error (n=675).
[x] Significance according to ANOVA, NS, *, **, ***, nonsignificant and significant $P \leq 0.05$, 0.01, 0.001, respectively.

reported in this study maybe an overestimation of actual substrate pH and EC. By bringing the substrate to container capacity, salts present in the substrate solution diffuse into applied irrigation water, diluting concentration of salts and potentially decreasing pH and EC. This may explain the high EC towards the end of the study, with no visible signs of salt toxicity in the plant material.

In addition, modified LANS Na content (Table A1 and A2) was at levels reported to cause little or no toxic effect to plant growth (Petersen, 1996). Leaf Na content irrigated with high levels of HCO_3^- were not significantly different from control AMF and non-AMF plants.

PLANT GROWTH

Plants treated with increasing HCO_3^- concentrations (≥2.5 mM) exhibited significantly inhibited growth. Reduced plant growth may be caused by the inhibitory effect of HCO_3^- on metabolic processes and/or impairment of root activity/growth (Alhendawi et al., 1997; Bialczyk and Lechowski, 1992; Bialczyk et al., 1994; Kosegarten et al., 1999) and/or nutrient solubility (Alcántara et al., 1988; Bialczyk et al., 1994; Pearce et al., 1999). Root physiology and nutrient solubility are affected by the buffering capacity of HCO_3^-, which is related to an increase in substrate pH (Marschner, 1995). Bicarbonates react with H^+ resulting in higher pH, increasing the concentration of OH^- (Lindsay, 1979). However, plant species and cultivars may differ in their tolerance to HCO_3^- stress (Alcántara et al., 1988; Alhendawi et al., 1997; Gharsalli and Hajji, 2002).

In general, AMF helped to partially alleviate HCO_3^- stress, as indicated by greater plant growth (leaf, stem, and total plant DM, leaf area, SLA, and LAR) compared to non-AMF plants. The MIE was low in plants treated with 0 mM HCO_3^-, suggesting that under control (non-stress) conditions *R. multiflora* cv. Burr plants were only moderately AMF dependant (Bagyaraj et al., 1988; Plenchette et al., 1983). However, at ≥2.5 mM HCO_3^-, MIE values increased indicating that plants became more AMF dependent, as shown by the percent increase in total plant DM of AMF plants compared

to non-AMF plants (Bagyaraj et al., 1988; Plenchette et al., 1983). Treatment with >2.5 mM HCO_3^- decreased MIE indicating that while AMF partially alleviated HCO_3^- stress, increasing HCO_3^- concentration also had a negative effect on AMF.

There was a nonsignificant trend of lower leaf number in AMF plants compared to non-AMF plants. Specific leaf area and LAR were higher in AMF plants treated with HCO_3^- compared to non-AMF plants. Arbuscular mycorrhizal plant leaves were thinner (generally higher SLA) and had a larger photosynthetic area per plant (greater LAR) in relative terms, as a result of carbon cost necessary to maintain AMF associations (Wright et al., 1998a, 1998b).

LEAF NUTRIENT CONTENT

Increasing HCO_3^- concentration (≥2.5 mM) decreased leaf N, P, Ca, Fe, Cu, and B content. Leaf K, Na, Mn, Mg, S, Zn, and Al content were unaffected or had no consistent response with increasing HCO_3^- concentration. Decreased nutrient content may be caused by the inhibitory effect of HCO_3^- on metabolic processes and/or impairment of root activity/growth (Alhendawi et al., 1997; Bialczyk and Lechowski, 1992; Bialczyk et al., 1994; Kosegarten et al., 1999). Reduced solubility of nutrients in the substrate at high pH as a result of high HCO_3^- concentration may also be a factor (Alcántara et al., 1988; Marschner, 1995). In addition, plant nutrient uptake may vary between species and cultivars in response to HCO_3^- stress (Alcántara et al., 1988; Alhendawi et al., 1997; Gharsalli and Hajji, 2002).

In general, AMF plants had significantly increased leaf nutrient content compared to non-AMF plants. Arbuscular mycorrhizal fungi enhance nutrient acquisition through greater effective root area and penetration of substrate(s) (direct access to nutrients outside the zone of nutrient depletion that develop close to roots and to nutrients in inaccessible microsites), and activation and excretion of various enzymes by AMF roots and/or hyphae (Clark and Zeto, 2000; George, 2000; Marschner, 1998). Arbuscular mycorrhizal fungi may tolerate adverse external pH conditions by modifying the pH of the mycorrhizosphere during nutrient uptake (Pacovsky, 1986). Increased P

nutrition as a result of AMF associations may also indirectly increase uptake of other ions, including N, Cu, Fe, and Zn (Marschner and Dell, 1994). Zinc efficiency may be related to enhanced tolerance to HCO_3^- stress (Yang et al., 1993).

TOTAL CHLOROPHYLL CONTENT

Total chlorophyll content was significantly reduced by increasing HCO_3^- concentrations. However, AMF plants had higher chlorophyll content compared to non-AMF plants. Higher chlorophyll content may reflect the higher photosynthetic rate necessary to support the carbon cost of AMF associations (Wright et al., 1998a, 1998b; Trimble and Knowles, 1995). The majority of carbon to support the metabolism of AMF originates directly from host plant photosynthesis (Douds et al., 2000).

Increased photosynthesis of AMF plants maybe mediated by enhanced Fe uptake, as Fe is essential for various plant metabolic reactions, including chlorophyll synthesis and photosynthesis (Marschner, 1995). Increased photosynthesis of AMF plants may also be mediated by increased P nutrition. Cytoplasmic inorganic phosphate (Pi) levels in leaves regulate carbon export, and thus photosynthesis via the triose-P/Pi translocator in the chloroplast membrane (Huber, 1982; Marschner, 1995). Low levels of Pi lead to a build up of starch in the chloroplast, which can decrease photosynthesis (Chatterton et al., 1972; Thorne and Koller, 1974). Pulse/chase experiments with $^{14}CO_2$ show a greater percentage of labeled photosynthates are transported out of leaves of AMF plants during the chase period compared to non-AMF plants (Douds et al., 1988).

ROOT IRON REDUCTASE ACTIVITY

Leaf Fe content decreased with increasing HCO_3^- concentration (Fig. A2). In strategy I plants Fe deficiency enhances the activation of plasma membrane-bound inducible reductase, enhanced net excretion of protons, and enhanced release of reductants/chelators as a means of alleviating Fe stress (Marschner and Römheld, 1994; Moog and Brüggemann, 1994). In our study, root Fe reductase activity was not

significantly enhanced by increasing HCO_3^- concentrations, suggesting that plant material was not Fe efficient under study conditions.

However, in general AMF plants had significantly lower root Fe reductase activity and higher leaf Fe content compared to non-AMF plants (Fig. A2), suggesting that AMF enhanced plant Fe uptake under HCO_3^- stress. Arbuscular mycorrhizal fungi may enhance Fe uptake through greater effective root area and penetration of substrate(s) (direct access to nutrients outside the zone of nutrient depletion that develop close to roots and to nutrients in inaccessible microsites), and activation and excretion of various compounds by AMF roots and/or hyphae to mobilize/convert substrate Fe into plant available forms (Caris et al., 1998; Clark and Zeto, 1996). Arbuscular mycorrhizal fungi may produce Fe chelating compounds, for example siderophores (Cress et al., 1986), as do other fungi, including ectomycorrhizal fungi, to enhance Fe uptake (Leyval and Reid, 1991). However, in other studies the effect of AMF on Fe uptake is variable and inconsistent, and acquisition of Fe has been both enhanced (Al-Karaki and Clark, 1998; Al-Karaki et al., 1998; Clark and Zeto, 1996; Treeby, 1992) and reduced (Clark et al., 1999; Kothari et al., 1990a, 1990b) by AMF depending on experimental conditions. Bacterial levels were not tested in this study, however AMF may indirectly stimulate bacterial populations (Bianciotto and Bonfante, 2002; Vázquez et al., 2000), which may enhance Fe availability and uptake (Carrillo-Castañeda et al., 2003; Cowart, 2002).

ROOT PHOSPHATASE ACTIVITY

Biochemical and biophysical processes involved in P metabolism, and phosphatase synthesis, activity, and efficiency are inconsistent and not well understood (Ezawa et al., 2002; Joner et al., 2000a, 2000b; Tarafdar et al., 2001; van Aarle et al., 2002b). In this study, leaf P content decreased with increasing HCO_3^- concentration (Fig. A3), and increasing HCO_3^- concentration decreased soluble and wall-bound ACP activity in AMF and non-AMF plants, suggesting that high HCO_3^- concentration and associated high pH impaired ACP synthesis, release, and/or stability (Tabatabai, 1994). Conversely increasing HCO_3^- concentration and associated high pH resulted in increased

soluble and wall-bound ALP activity in AMF and non-AMF plants, suggesting that high HCO_3^- concentration and associated high pH had limited effect on ALP synthesis, release, and/or stability (Tabatabai, 1994). Bicarbonate concentrations >5 mM resulted in a sharp decrease in soluble ALP activity in non-AMF plants, suggesting that high HCO_3^- concentrations and associated high pH are inhibitory to plant ALP synthesis, release, and/or stability. This may attributed to: 1) lower Fe uptake potentially reducing chlorophyll synthesis and photosynthetic rates resulting in lower carbon accumulation and transport to the roots affecting ALP synthesis; 2) an internal alkalization of root cell symplast impairing cell metabolism and affecting ALP synthesis, release, and/or stability; and 3) phosphorus precipitation with Ca limiting induction of ALP.

Arbuscular mycorrhizal plants had significantly lower soluble ALP activity at <10 mM HCO_3^- and in general higher leaf P compared to non-AMF plants (Fig. A3), suggesting AMF plants had enhanced P uptake and transport under HCO_3^- stress. Arbuscular mycorrhizal fungi may also enhance P uptake through greater effective root area and penetration of substrate(s) (direct access to nutrients outside the zone of nutrient depletion that develop close to roots and to nutrients in inaccessible microsites), and activation and excretion of compounds by AMF roots and/or hyphae to mobilize/convert substrate P into plant available forms (Bolan, 1991; Joner et al., 2000a, 2000b; Miyasaka and Habte, 2001). However, HCO_3^- concentrations >5 mM induced a sharp increase in soluble ALP activity in AMF plants, suggesting that at high HCO_3^- concentrations and associated high pH were inhibitory to AMF P availability/uptake. Bacterial levels were not tested in this study, however AMF may indirectly stimulate bacterial populations (Bianciotto and Bonfante, 2002; Vázquez et al., 2000), which may enhance phosphatase activity, P availability, and uptake (Gryndler et al., 2002; Rodríguez and Fraga, 1999; Villegas and Fortin, 2002).

ARBUSCULAR MYCORRHIZAL COLONIZATION

Arbuscular mycorrhizal colonization (arbuscules, vesicles, and hyphae) was adversely affected by increasing HCO_3^- concentrations, which may be due to the high

pH associated with increasing HCO_3^- concentrations (Hayman and Tavares, 1985; Medeiros et al., 1994; van Aarle et al., 2002a). However, AMF colonization still occurred at the highest HCO_3^- concentrations. Because of technical problems with the clearing and staining of root pieces, we suggest that results obtained in this study may be an underestimation of actual AMF colonization.

Conclusion

This is one of the first reports to demonstrate that AMF enhance plant tolerance to HCO_3^- stress, as indicated by the enhanced growth, nutrient uptake, leaf chlorophyll content, MIE, low iron reductase activity, and low soluble wall bound ALP activity. At 2.5 mM HCO_3^-, AMF plant growth was comparable to non-AMF and AMF plants at 0 mM HCO_3^-, indicating the potential beneficial application of AMF for alleviation of plant HCO_3^- stress.

We suggest that if this study had been conducted using a commercial substrate with a higher buffering capacity (compared to the low buffering capacity of the sand substrate used in this study), the beneficial application of AMF for enhanced plant tolerance to HCO_3^- stress may be greater at higher HCO_3^- concentrations.

However, plant species and cultivars within species can differ markedly in their response to HCO_3^- stress (Alcántara et al., 1988; Alhendawi et al., 1997; Gharsalli and Hajji, 2002) and AMF species (Parke and Kaeppler, 2000; Sanders, 2002). Efficiencies among AMF species vary (Jakobsen et al., 2002; Parke and Kaeppler, 2000), and each AMF isolate originating from a specific environment may represent an ecotype adapted to that particular environment (Entry et al., 2002). Therefore, the beneficial application of AMF to enhance plant tolerance to HCO_3^- stress may depend on plant species and/or cultivars within species used, plant interaction with the AMF species, and the difference between the environment from which the AMF isolate was obtained and the experimental system under study.

CHAPTER IV
CONCLUSION

Agricultural/horticultural production will increasingly have to preserve and augment good quality water resources through more enhanced water efficiency and utilization of non-conventional water resources; this may include water of marginal quality, high in total dissolved solids (non-volatile solutes) (Oster, 1994; Shalhevert, 1994). The potential bicarbonate (HCO_3^-) content and associated high pH of this irrigation water may be detrimental/limiting to plant growth, due to its adverse effects on availability and solubility of nutrients (P, N, Mg, Fe, Mn, Zn, Cu, and other ions) (Bailey, 1996; Marschner, 1995).

Rooted stem cuttings of *Rosa multiflora* Thunb. ex J. Murr. cv. Burr (rose) treated with increasing HCO_3^- concentrations (≥2.5 mM) exhibited significantly inhibited growth and nutrient uptake. Inoculation with a mixed *Glomus* Tulasne & Tulasne species inoculum of arbuscular mycorrhizal fungi (AMF), *Glomus* ZAC-19 (containing *Glomus albidum* Walker & Rhodes, *Glomus claroideum* Schenck & Smith, and *Glomus diaphanum* Morton & Walker), significantly enhanced plant tolerance to HCO_3^- stress as indicated by generally greater growth, nutrient uptake, leaf chlorophyll content, higher mycorrhizal inoculation effect (MIE), lower Fe reducatase activity, and generally lower soluble and wall-bound alkaline phosphatase activity. At 2.5 mM HCO_3^-, AMF plant growth was comparable to non-AMF and AMF plants at 0 mM HCO_3^- , indicating the potential beneficial application of AMF for alleviation of plant HCO_3^- stress.

However, plant species and cultivars within species can differ markedly in their response to HCO_3^- stress (Alcántara et al., 1988; Alhendawi et al., 1997; Gharsalli and Hajji, 2002) and AMF species (Parke and Kaeppler, 2000; Sanders, 2002). Efficiencies among AMF species vary (Jakobsen et al., 2002; Parke and Kaeppler, 2000), and each AMF isolate originating from a specific environment may represent an ecotype adapted to that particular environment (Entry et al., 2002). Therefore, the beneficial application of AMF to enhance plant tolerance to HCO_3^- stress may depend on plant species and/or

cultivars within species used, plant interaction with the AMF species, and the difference between the environment from which the AMF isolate was obtained and the experimental system under study.

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APPENDIX

Table A1. Formulations and composition of modified Long Ashton nutrient solution to supply bicarbonate (HCO_3^-).

Salts	HCO_3^- (mM)			
	0	2.5	5	10
		$g \cdot L^{-1}$		
$Ca(NO_3)2$	0.94	0.94	0.94	1.24
NaH_2PO_4	0.14	0.14	0.14	0.14
$MgSO_4$	0.37	0.31	-	-
$Mg(NO_3)_2$	-	0.06	0.38	0.38
KNO_3	0.40	0.35	0.10	-
K_2SO_4	0.18	-	-	-
NH_4NO_3	0.12	0.12	0.12	-
$KHCO_3$	-	0.25	0.50	0.60
$NaHCO_3$	-	-	-	0.22
NH_4HCO_3	-	-	-	0.12
Fe-DTPA	5.0	5.0	5.0	5.0
		$mg \cdot L^{-1}$		
$MnSO_4 \cdot H_2O$	2	2	2	2
$ZnSO_4 \cdot 7H_2O$	0.22	0.22	0.22	0.22
$CuSO_4 \cdot 5H_2O$	0.08	0.08	0.08	0.08
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.2	0.2	0.2	0.2
H_3BO_3	2.8	2.8	2.8	2.8

Table A2. Elemental formulation and composition of modified Long Ashton nutrient solution to supply bicarbonate (HCO_3^-).

Element	HCO_3^- (mM)			
	0	2.5	5	10
	(mM)			
NO_3^-	15.45	15.46	15.69	15.7
NH_4^+	1.5	1.5	1.5	1.5
P	1	1	1	1
K^+	6.03	5.99	5.99	6.00
Ca^{2+}	5.7	5.7	5.7	5.7
Mg^{2+}	1.5	1.49	1.46	1.46
SO_4^{2-}	2.53	1.26	1.26	1.26
Na^+	1	1	1	3.63
Fe	0.09	0.09	0.09	0.09
Zn	0.0008	0.0008	0.0008	0.0008
Cu	0.0003	0.0003	0.0003	0.0003
Mn	0.011	0.011	0.011	0.011
Mo	0.0011	0.0011	0.0011	0.0011
B	0.005	0.005	0.005	0.005

Table A3. Average pH and electrical conductivity (EC) of modified Long Ashton nutrient solution to supply bicarbonate (HCO_3^-).

HCO_3^- (mM)	pH	EC ($ds \cdot m^{-1}$)
0	6.03±0.13	1.95±0.14
2.5	7.10±0.08	2.08±0.10
5	7.34±0.07	1.86±0.13
10	7.59±0.12	2.38±0.16

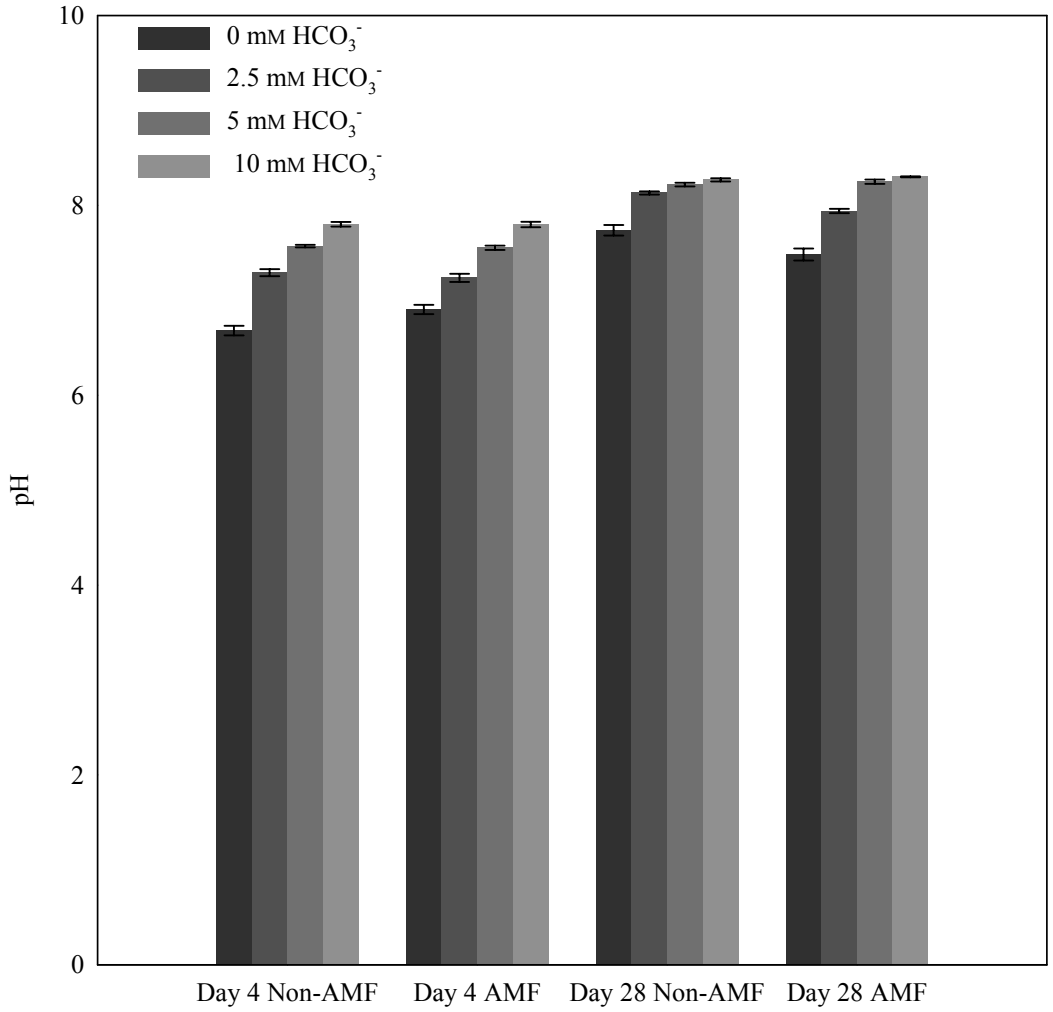

Fig. A1. Effect of bicarbonate (HCO_3^-) and arbuscular mycorrhizal fungi (AMF) on pH of *Rosa multiflora* cv. Burr substrate at days 4 and 28. Treatment effect of HCO_3^- was significant ($P \leq 0.001$) at days 4 and 28. Treatment effect of AMF and AMF x HCO_3^- interaction was nonsignificant at days 4 and 28. Means±standard error (n=5).

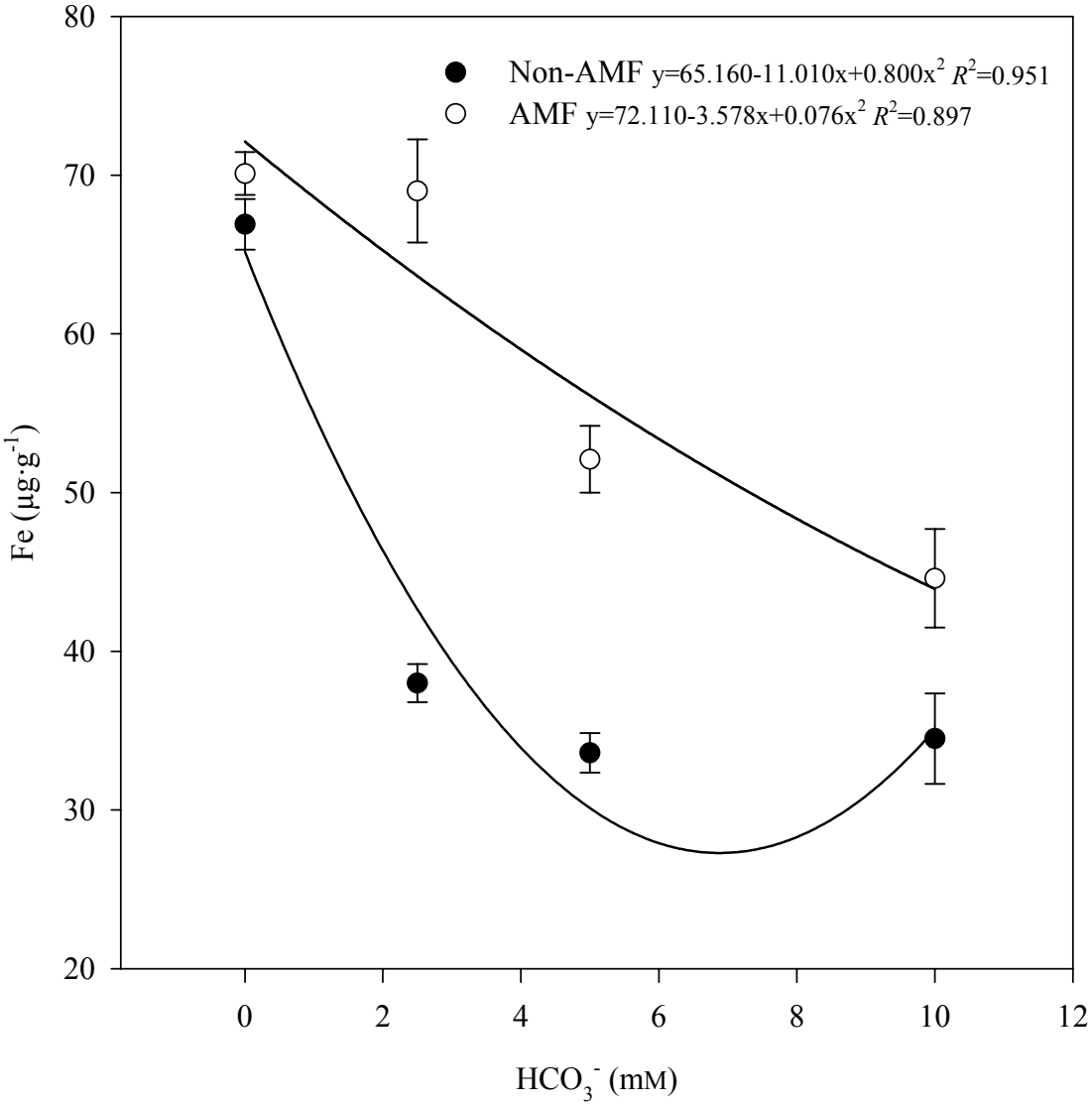

Fig. A2. Effect of bicarbonate (HCO_3^-) and arbuscular mycorrhizal fungi (AMF on total leaf Fe content of *Rosa multiflora* cv. Burr plants. Treatment effect of AMF, HCO_3^-, and AMF x HCO_3^- interaction were significant ($P \leq 0.01$, 0.001, and 0.05, respectively. Means±standard error (n=3).

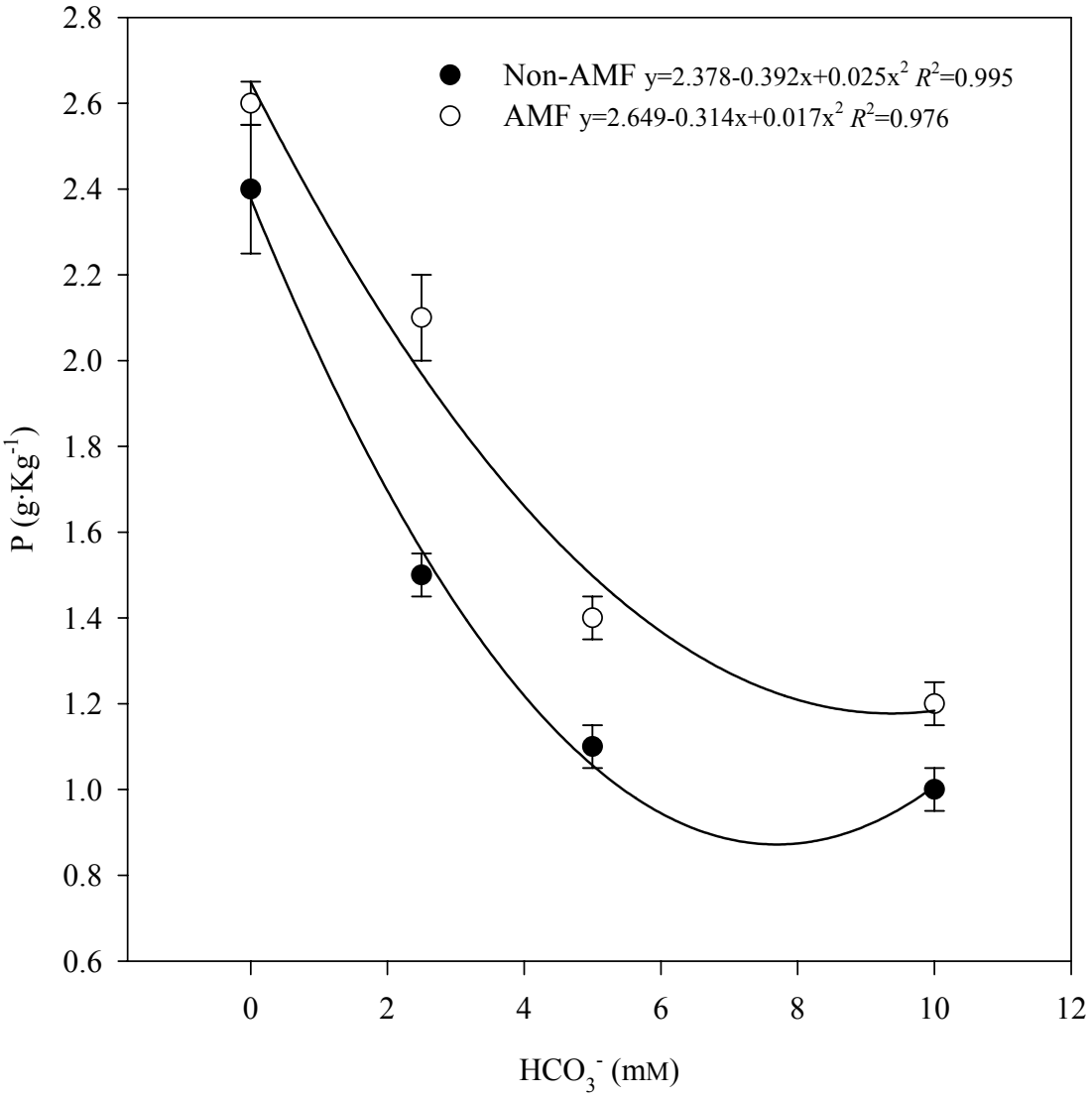

Fig. A3. Effect of bicarbonate (HCO_3^-) and arbuscular mycorrhizal fungi (AMF) on total leaf P content of *Rosa multiflora* cv. Burr plants. Treatment effect of AMF and HCO_3^- were significant ($P \leq 0.01$ and 0.001, respectively). Treatment effect of AMF x HCO_3^- interaction were nonsignificant. Means±standard error (n=3).

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