

Symbiotic and physiological performances of *Bradyrhizobium*-nodulated cowpea plants subjected to varying concentrations of manganese and iron

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Abstract

The objective of this study was to analyse the response of *Bradyrhizobium*-inoculated cowpea plants to increasing Mn concentrations from 0.1 to 80 μM . For each Mn concentration, Fe was provided at a ratio of 0.5, 1 and 5, as Fe-EDTA. Concentrations of Mn from 0.1 to 20 μM did not enhance the Mn concentration in leaves. Only the lowest Fe:Mn ratio had a positive effect. Except for plants grown at 5 and 10 μM Mn, the leaf Fe concentration increased with increasing Fe:Mn ratio until 60 μM Mn. Concomitantly, plant growth, nodulation and the catabolism of ureides in leaves were hindered at 0.1 up to 2 μM Mn and above 10 μM Mn, at Fe:Mn ratios equal or higher than 1. A decrease in the total reducing sugar concentration in leaves and in the first internode of the main stem occurred between 0.1 and 2 μM Mn. Leaf chlorophyll contents showed a decrease at Mn concentrations higher than 2 μM , regardless of the Fe:Mn ratio. These results indicate the very narrow range for Mn and Fe fertilization in cowpea for optimal plant growth, nodulation and catabolism of ureides in leaves.

Keywords: Cowpea, iron, manganese, *Vigna unguiculata*, ureides

1. Introduction

Cowpea (*Vigna unguiculata* L.) is a legume crop of immense benefit within small-scale cropping systems in the tropics. Among the most relevant advantages of this crop are its tolerance to heat and dry conditions and ability to establish effective association with indigenous bradyrhizobia (Sicardi de Mallorca and Izaguirre-Mayoral, 1994; Mayz et al., 2003). Cowpea plants, however, are characterized by a poor response to *Bradyrhizobium* inoculation, in spite of the extensive investigations to which plants have been subjected during the last two decades (Fening et al., 2001; Deepak and Sangwan, 2005).

In Venezuela, the low nutrient content of savanna soils (Izaguirre-Mayoral et al., 1992a) seems to be the main constraint for crop yield of cowpea plants depending solely on the symbiotic nitrogen fixation for their nitrogen

economy. *Bradyrhizobium*-nodulated cowpeas always display smaller total dry mass when compared to that of plants fertilized with nitrate, in spite of the soil amendment with optimal phosphate concentrations (Izaguirre-Mayoral et al., 2002). This observation pointed out that deficiency of other nutrients, besides P, must be involved in the low symbiotic performance of local cowpea varieties.

Among soil cations, manganese (Mn) can be considered as a possible candidate underlying poor yields of cowpea crops under tropic conditions, since Mn-deficiency often occurs in leached tropical or lime-amended soils (Marschner, 1995; Voundi-Nkana et al., 2001; Hue et al., 2001). At the plant level, Mn participates in a large number of cellular activities (Lidon et al., 2004 and citations in that paper), and in the symbiotic N_2 fixation process acting as a cofactor in the catabolism of the ureides in the leaves (Todd and Polacco, 2004) of ureide-producing species like cowpeas (Izaguirre-Mayoral et al., 1992b). Interpretation of plant responses to differing Mn concentrations is difficult, however, because of the close relationship between Mn and

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iron (Fe) at the cellular level (Moroghan, 1992; Korshunova et al., 1999; Lovyagina et al., 2005) due to the existing antagonism between both cations (Izaguirre-Mayoral and Sinclair, 2005a).

In legumes, Fe actively participates in metabolic pathways involving Mn (Hell and Stephan, 2003) and it is a key factor in the symbiotic activity in ureide-producing legume species (Reddy et al., 1995; Abdelmajid and Chedly, 2003). As for Mn, iron deficiency also occurs in savanna ultisols (Méndez-Natera and Mayz-Figueroa, 2000) and calcareous soils (Marschner, 1995) where cowpeas are usually planted.

To make the situation even more complex, deficient or toxic levels of Mn lead to an accumulation or depletion of zinc (Zn) in roots (Thomine et al., 2003), and an impairment in the copper (Cu) uptake rates (Shingles et al., 2004). Both microelements play an important role in the symbiotic N₂-fixing process of legumes (Athar and Ahmad, 2002).

There are, however, no published investigations on the performance of *Bradyrhizobium*-nodulated cowpeas over a wide range of Mn concentrations, on the Fe:Mn interactions and on the nutrient content that give full documentation of plant response. As far as we could verify, published reports deal only with the effect of toxic levels of Mn upon growth of cowpea plants (Iwasaki et al., 2002; Fecht-Christoffers et al., 2003a).

The present investigation was, therefore, undertaken to analyze the response of *Bradyrhizobium*-inoculated cowpea plants to increasing Mn concentrations in solution culture, starting at deficient levels of 0.1 μM up to concentrations of 80 μM considered as toxic for many plant species (Marschner, 1995). The impact of each Mn concentration was analyzed in the presence of varying concentration of Fe to yield Fe:Mn ratios of 0.5, 1 and 5. It could be assumed that by keeping the Fe:Mn ratio constant the deficiency or toxicity of Mn or Fe induced on the plants by the excess or deficiency of any of the two micronutrients could be avoided.

Plants were analyzed in term of growth, symbiotic N₂-fixing performance, nutrient content, chlorophyll and total reducing sugar concentrations in different plant compartments. Determination of the optimal Fe:Mn ratio for the growth of plants has important consequences for future soil fertilization programs to improve cowpea yields in soils characterized by a low nutrient content, and for understanding the interactions among micronutrients at the plant level.

2. Materials and Methods

Plant material and growth conditions

Seeds of cowpea (*Vigna unguiculata* (L.) Walp. var. Tuy) were surfaced-sterilized with 95% ethanol and rinsed six

times with sterile distilled water. One seed per jar was sown at a depth of 1 cm in sterilized Leonard jars containing 0.9 kg of sand and 0.8 L of a nutrient solution. The basic solution contained CaCl₂ (3.3 mM), MgSO₄ (2 mM), K₂SO₄ (1.3 mM), dibasic potassium phosphate buffer (2 mM, solution pH maintained at 6.1), H₃BO₃ (4 μM), ZnSO₄ (1.6 μM), CuSO₄ (1.6 μM) and NaMoO₄ (0.1 μM).

To analyze the response of cowpea to manganese (Mn) and iron (Fe) nutrition, nutrient solutions were supplied with MnSO₄ at one of nine concentrations (0.1, 0.5, 2, 5, 10, 20, 40, 60 and 80 μM). For each Mn concentration, Fe was provided at a ratio to Mn of 0.5, 1 and 5, as Fe-EDTA. The appropriated nutrient solution was added daily to each jar to replace transpirational losses, and every 4 days the solution was completely replaced. Flasks and solution containers were covered with black plastic bags to avoid photodegradation of Fe-EDTA (Albano and Miller, 2001).

Before sowing, 1 g of a commercial mixture of peat-based bradyrhizobial strains (Nitragin, Milwaukee, WI, USA) was spread as an uniform layer on the sand bed for the seeds in each jar. The jars were placed in a growth chamber with a distance of 20 cm between jars. Growth conditions were of 30/24°C mean day/night temperatures and 12 h photoperiod. Photosynthetic photon flux density was of 460 $\mu\text{mol m}^{-2} \text{s}^{-1}$ emitted by a combination of fluorescent and incandescent lamps.

Plant analyses

Six plants from each of the treatment combinations were harvested 30 d after germination. Chlorophyll content was determined in 1 cm² discs removed from the central area of each of the three leaflets of the upper most mature, fully expanded leaf (Izaguirre-Mayoral et al., 1992a). The plants were then separated into shoot, root, and nodule mass and the components were individually oven-dried at 80°C until constant weight. Plant growth under each treatment was analyzed taking into account the dry weight of individual compartments.

Subsamples of the leaves, first internode of the main stem and nodules were used to measure colorimetrically ureide and total reducing sugar contents (Izaguirre-Mayoral et al., 1992a). The concentrations of potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu) were determined in leaf subsamples with an atomic absorption spectrometer (SpectrAA 55B, Varian Techtron, Victoria, Australia) in nitric-perchloric acid digestions (Miller, 1998).

Statistical analyses

The data were analyzed statistically using a one-way ANOVA (significant level of $p < 0.05$) to evaluate differences between treatments. Significant differences among treatments are expressed by different letters in the figures.

3. Results

Aerial, root and nodule growth

Maximum aerial mass was detected in plants subjected to solution Mn concentrations of 5 and 10 μM , with virtually no change in the mass among those two solution Mn concentrations (Fig. 1A). In all cases, aerial mass decreased with increasing Fe:Mn ratio in the solution. The greater accumulated root mass also occurred in plants grown at solution Mn concentrations of 5 and 10 μM and Fe:Mn ratios of 0.5 (Fig. 1B). At higher solution Mn concentrations, there was very little variation in root mass with differing Mn concentrations, and the Fe:Mn ratio had no influence on the root mass. Nodule mass in response to the varying solution Mn and Fe concentrations were much the same as with aerial mass (Fig. 1C). The nodule mass was insensitive to increasing solution Mn concentrations between 0.1 up to 2 μM . For all Mn concentrations, nodule mass decreased with increasing Fe:Mn ratio.

Chlorophyll, total reducing sugars and ureide concentrations

Chlorophyll contents per unit area of $28 \pm 3 \mu\text{g cm}^{-2}$ were detected in mature leaves of plants growing at Mn concentrations of 0.1, 0.5 and 2 μM . At higher solution Mn concentrations there was a marked decrease in chlorophyll content with an average of $20 \pm 2 \mu\text{g cm}^{-2}$ for all Mn concentrations. None of the plants displayed deficiency or toxic symptoms such as interveinal chlorosis at the lowest or highest solution Mn concentrations, respectively. Differences in solution Fe concentration did not result in differences in chlorophyll contents among plants, and did not elicit Fe-deficiency or Fe-toxicity symptoms such as bronze speckles in leaves.

The lowest total reducing sugar concentrations were detected in leaves and first internode of plants growing at 0.1 up to 2 μM solution Mn concentrations (Fig. 2A and B). At higher solution Mn concentrations there were 6–10 fold increases in the total reducing sugar concentration in leaves but much smaller increases (about 2–3 fold) in the first internode. Concentration of total reducing sugars in leaf or first internode was not affected by the Fe:Mn ratios at which plants were grown.

At all Fe:Mn ratios, the greater leaf ureide concentrations were detected in plants growing at solution Mn concentrations of 0.1 up to 2 μM (Fig. 2C). Whereas, a sharp decline in the leaf ureide concentrations took place in plant growing at 5 and 10 μM Mn, followed by a progressive increases mainly in plants subjected to a Fe:Mn ratio of 5. Leaf ureide concentrations also displayed an inverse relationship with the Fe:Mn ratio at solution Mn concentrations between 0.1 and 40 μM . No effect of the Fe:Mn ratio was detected in plants grown at 60 and 80 μM solution Mn concentrations. In contrast, differences in the ureide concentration in the first internode were stable within

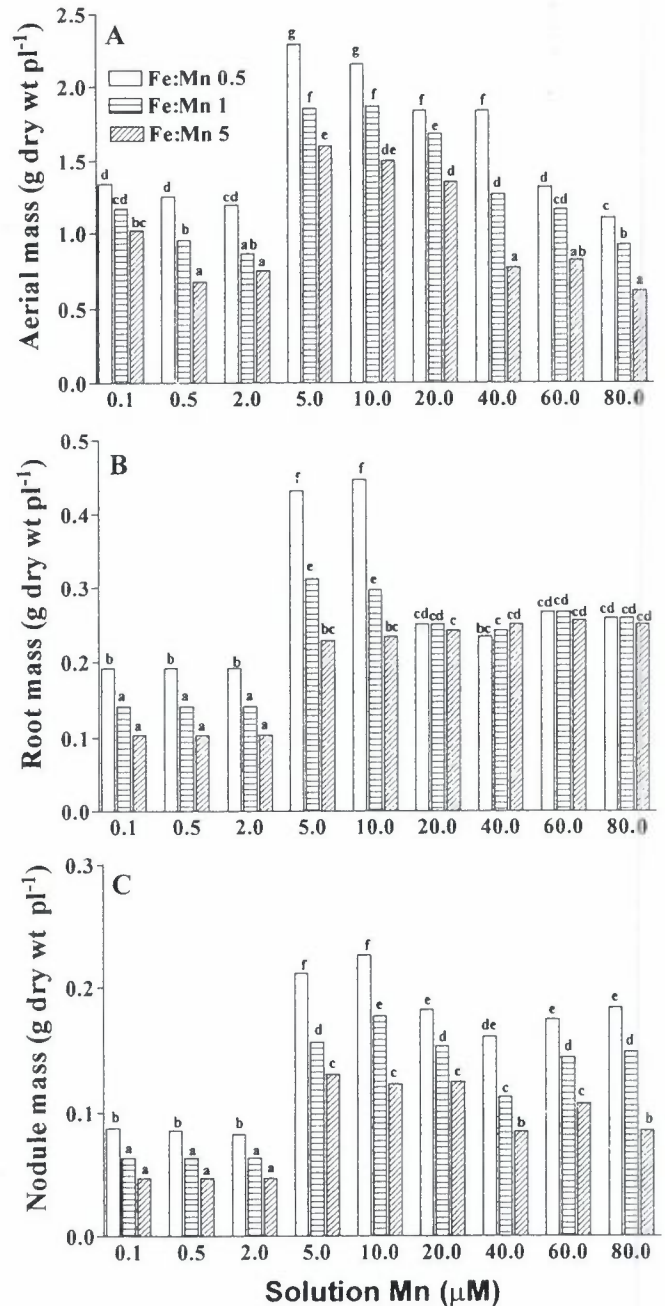


Figure 1. Aerial, root and nodule mass in cowpea plants grown at increasing Mn concentrations and Fe:Mn ratios. Value in each column represents the statistical mean ($n=6$). Different letter(s) represent significant differences between treatments ($p < 0.05$).

two clearly differentiated group of plants delineated by plants growing at 0.1–2 and 5–80 μM Mn (Fig. 2D). Within each group, the Fe:Mn ratio made no difference in ureide concentration (Fig. 2B). In nodules, neither Mn contents nor the Fe:Mn ratio altered the ureide concentration (data not shown).

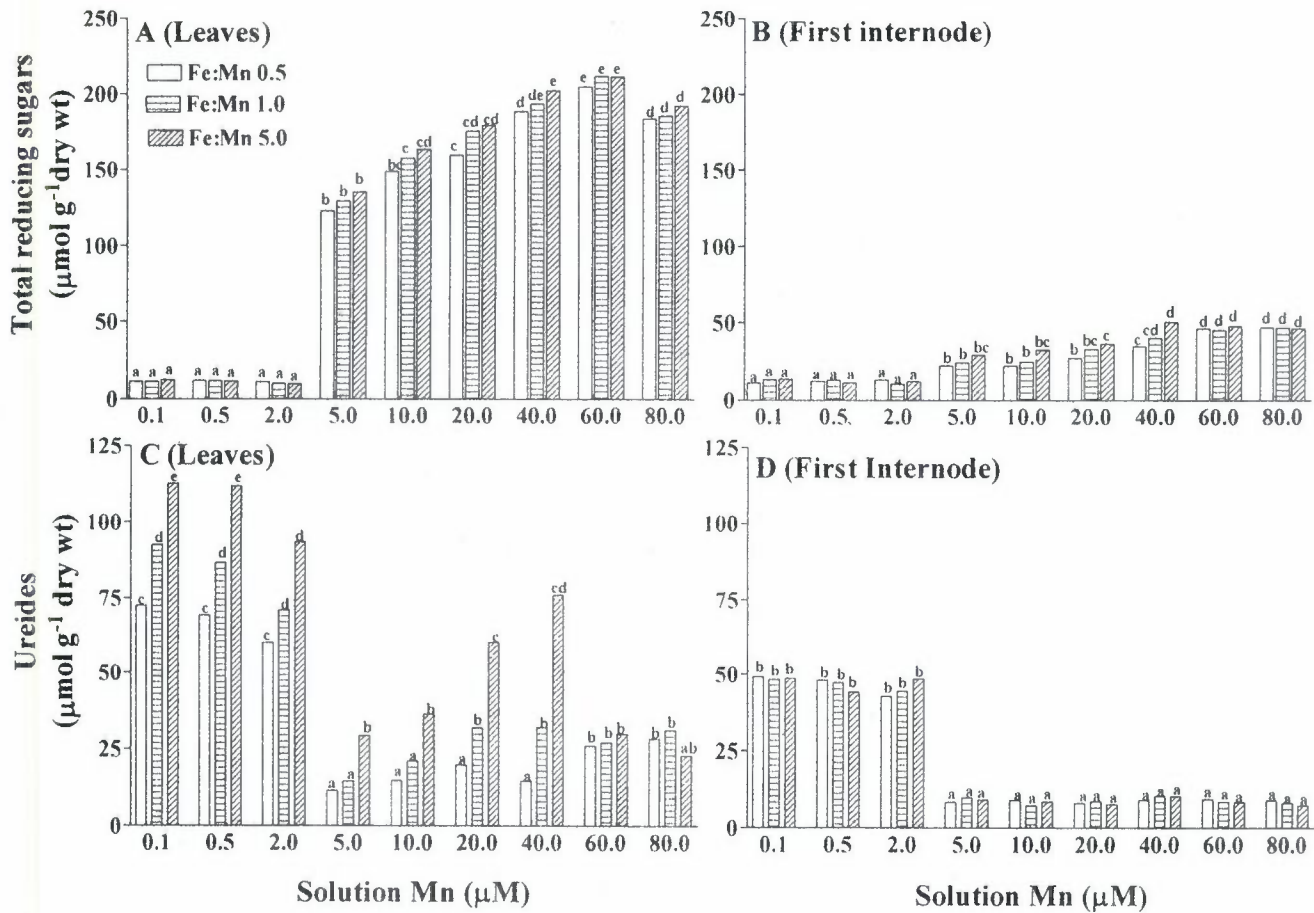


Figure 2. Total reducing sugar and ureide concentrations in leaves and first internode of cowpea plants grown at increasing Mn concentrations and Fe:Mn ratios. Value in each column represents the statistical mean ($n=6$). Different letter(s) represent significant differences between treatments ($p<0.05$).

Nutrient concentrations in leaves

Leaf Mn concentration did not increase with increasing solution Mn concentration up to 10 μM , but a steady increase in leaf Mn was observed at solution Mn concentrations equal and higher than 20 μM (Fig. 3A). At Mn concentrations equal or lower than 10 μM there was a tendency for the lowest Fe:Mn ratio to enhance the accumulation of Mn in leaves. A marked effect of Fe:Mn ratios was detected at higher solution Mn concentrations, with the highest Mn concentrations in leaves of plants subjected to a Fe:Mn ratio of 0.5.

Leaf Fe concentration response to nutrient solution treatments was more complicated than leaf Mn concentrations (Fig. 3B). At low solution Mn concentrations of 0.1 to 2 μM , there was a significant accumulation of leaf Fe, followed by an abrupt decline when solution Mn was increased to 5 and 10 μM , followed by a steady increase mainly at the highest Fe:Mn ratio. Except for plants grown at 5 and 10 μM solution Mn

concentrations, the leaf Fe concentration tended to increase with increasing Fe:Mn ratio until 80 μM solution Mn when the Fe:Mn ratio did not alter the leaf Fe concentrations.

Potassium concentrations of 100–110 mmol kg^{-1} dry wt were detected in leaves of plants grown in solution Mn concentrations of 0.1 up to 2 μM , regardless of the Fe:Mn ratio (Fig 4A). A drastic decrease was observed when solution Mn was increased to 5 and 10 μM followed by a progressive increase with increasing solution Mn concentrations. An effect of the Fe:Mn ratio upon leaf K concentrations occurred at solution Mn concentrations equal or above 20 and up to 60 μM , with the highest values observed at the higher Fe:Mn ratios. Concomitantly, the higher leaf concentrations of Cu and Zn were detected in plants subjected to extreme solution Mn concentrations (Fig. 4B and 4C). For both Cu and Zn, the observed tendency of lower concentrations at the highest Fe:Mn ratio in leaves of plants grown at lower solution Mn concentrations was reverted once the Mn concentration in

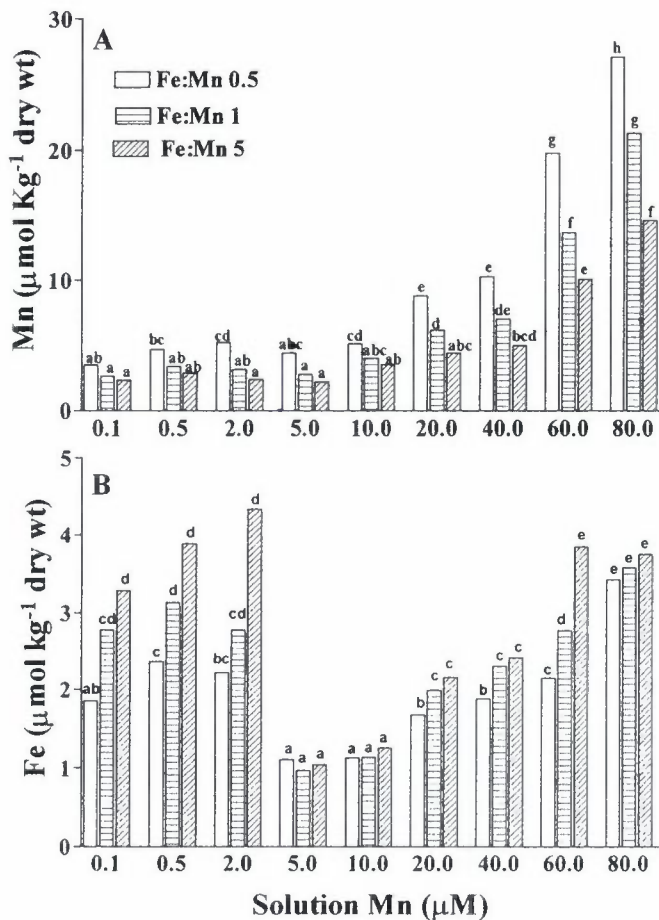


Figure 3. Manganese and Fe concentrations in leaves of cowpea plants grown at increasing Mn concentrations and Fe:Mn ratios. Value in each column represents the statistical mean ($n=6$). Different letter(s) represent significant differences between treatments ($p<0.05$).

the solution was equal or exceeded 40 μM . Neither increasing solution Mn concentrations nor the Fe:Mn ratio at which plant were grown affected the Ca and Mg content in leaves (data not shown). Plants contained an average of 250 ± 30 and 110 ± 10 mmol kg^{-1} leaf dry weight of Ca and Mg, respectively.

4. Discussion

The previously reported complex relationship between Mn and Fe for the nutrition of soybeans (Izaguirre-Mayoral and Sinclair, 2005a,b) is now evident in cowpea. Leaf Mn concentrations were apparently not related to solution Mn within a wide range of treatment concentrations until a threshold value of 20 μM that triggered the Mn accumulation in leaves. On the other hand, the recognized antagonism between Mn and Fe was exclusively observed at solution Mn concentrations of 0.1 up to 2 and above 20 μM . Increasing solution Mn concentrations also altered the

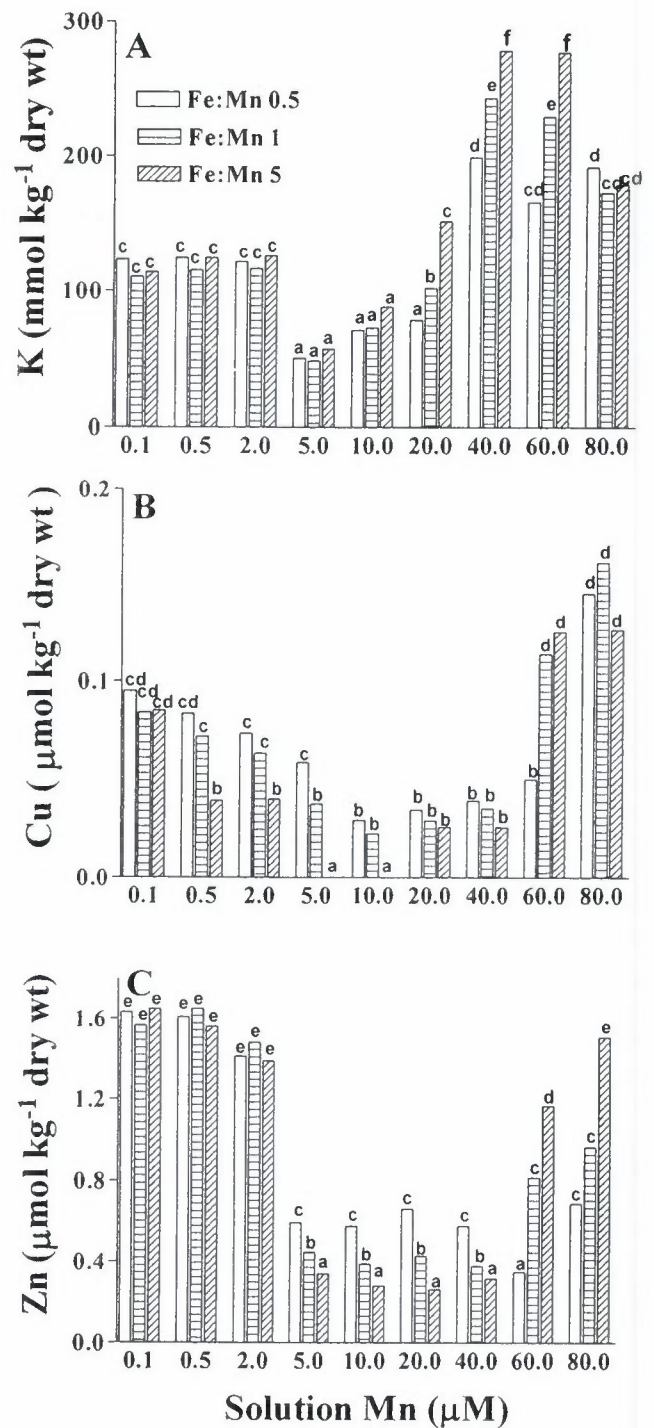


Figure 4. Potassium, Cu and Zn concentrations in leaves of cowpea plants grown at increasing Mn concentrations and Fe:Mn ratios. Value in each column represents the statistical mean ($n=6$). Different letter(s) represent significant differences between treatments ($p<0.05$).

uptake of K and Zn in the same pattern as that of Fe in leaves versus solution Mn concentrations, while there was

a parallelism between leaf Cu, Zn and Mn concentrations. To complicate the situation even further, leaf concentrations of Mg and Ca were independent of the solution Mn concentrations and Fe:Mn ratio at which plants were grown.

In general, solution Mn concentrations equal to or lower than 2 μM proved to be deficient for supporting plant growth. Similarly, solution Mn concentrations of 60 and 80 μM as well as Fe:Mn a ratio of 5 hindered the growth and nodulation of plants, regardless of the Mn concentration at which plant were grown. This direct influence of Mn upon growth and nodulation seems to be the result of an impaired hydrolysis of ureides in leaves, likely as a result of the requirement for Mn as a cofactor for ureide catabolism (Izaguirre-Mayoral and Sinclair, 2005b). The accumulation of ureides in the leaves could be, in turn, the cause for the observed ureide accumulation in the first internode at deficient solution Mn concentrations, via feedback inhibition of translocation.

Present results also suggest the direct participation of Fe in the catabolism of ureides in leaves, since the greater accumulation of ureides was detected in plants subjected to a Fe:Mn ratio of 5. On the other hand, the low Fe concentration in leaves of plants grown at low Fe questions the relevance of the iron III chelate reductase in the cellular Fe homeostasis in cowpea, as reported for strategy I plants (Zaharieva and Romheld, 2000). There is a possibility that accumulation of ureides at deficient Mn could also be attributed to the lack of reductant power due to the extremely low total sugar concentration in leaves resulting from an impairment of the photosynthetic apparatus. This assumption was, however, ruled out since all plants displayed high chlorophyll contents and did not have visible symptoms of Mn and Fe deficiency or toxicity such as brown speckles, intervenial chlorosis and necrosis of mature leaves (Marschner, 1995; Fecht-Christoffers et al., 2003b; Ksouri et al., 2005).

The most interesting aspect of present results is the clear relationship between optimal plant growth and the lower ureide content in leaves brought about by solution Mn concentrations of 5 and 10 μM . This range of solution Mn concentration corresponded with the average 8 μM Mn soil concentration measured in the principal areas where cowpeas are planted in Venezuela (Méndez-Natera and Mayz-Figueroa, 2000), considered as adequate for promoting cowpea growth (Fageria et al., 1997). This observation also indicates that the capacity of leaves to metabolize the ureide exported from nodules constitutes the main constrain for the nitrogen economy of cowpea plants relying on the symbiotic process for growth. Nevertheless, the amendment of plant with an optimal solution Mn concentration for maximum growth promotion and ureide catabolism did not enhance plant dry wt mass above that previously reported for plants grown at optimal 2 mM P and 10 mM KNO_3 solution concentrations (Izaguirre-Mayoral et al., 2002).

It is also of interest to note that the minimum concentrations of Fe, Mn and Cu in leaves occurred at solution Mn concentrations that elicited maximal plant growth and nodulation. The possibility exists that such low nutrient concentrations resulted from a dilution due to the relative greater partitioning of biomass to plant supporting structures as the plant grow larger. However, the determination of nutrients only in leaf blades with petioles removed does not support this suggestion. Concomitantly, leaf concentration values of Mn, Ca, Mg and Zn are within the range considered as sufficient to support the growth of cowpeas (Fageria et al., 1997). Rather, the deficiency of Fe and K concentrations as well as the low K/Ca ratio in plants displaying maximal growth might be the factors limiting growth when solution Mn concentration reaches optimal values (García-Hernández et al., 2005).

In spite of the apparent failure of increasing Mn and Fe:Mn ratio amendments to enhance plant growth, the present data provides new insights into the understanding of the relationships between growth, symbiotic nitrogen fixation and plant nutrition. As shown, the inverse relationship between Mn and Fe concentration in cowpea leaves seems to be more complicated than a simple antagonism between these two cations since increasing solution Mn concentration had only a small influence, if any, on Mn concentration in leaves when solution Fe:Mn ratio was low.

On the other hand, the demonstrated interactions between leaf Fe and Mn with Zn, Cu and K but not with Mg and Ca indicate the complexity of the nutrition in cowpeas. Even more, the determination that solution Mn concentrations lower than 5 or higher than 10 μM as well as a Fe:Mn ratio equal or larger than 1 negatively affected the growth of the plants indicates that the cowpeas has a very narrow range for optimal Mn and Fe fertilization. The reasons for the sharp transition of concentrations of ureides and total reducing sugars between Mn solution concentrations of 2 and 5 μM needs further investigation.

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