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Interaction between potassium (K) and calcium (Ca) on the severity of Yellow Sigatoka in banana plants

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The main control measure of Yellow Sigatoka (*Pseudocercospora musae*) in banana plants (*Musa* spp.) has been the planting of resistant varieties, and fungicide application. However, the use of adequately nourished plants is also emphasized as a complementary control method. This study evaluated the influence of interaction between potassium (K) and calcium (Ca) in nutrient solution on the severity of Yellow Sigatoka in banana. Evaluation included severity of disease, chlorophyll *a* and *b* contents, nutrient contents, and total dry weight (TDW). There was no interaction between concentrations of K and Ca for area under the disease severity progress curve (AUDSPC), although the AUDSPC increased in leaves 1 and 2 with increasing concentrations of K from 1 to 6 mmol L^{-1}. Increasing K led to a reduction in chlorophyll *a* and *b* contents, and in nutrients N, P, Mg, B, Cu, Zn, and Mn. TDW increased with increasing K. Therefore, high concentration of K causes nutritional imbalance in banana plants, and favors the severity of Yellow Sigatoka.

Key words: Hydroponics, *Musa* spp., nutritional imbalance, *Pseudocercospora musae*.

INTRODUCTION

Banana (*Musa* spp.) is grown worldwide in tropical and subtropical countries. Yellow Sigatoka leaf spot disease, caused by the fungus *Pseudocercospora musae* Zimm (teleomorph *Mycosphaerella musicola* Leach), is a major factor affecting global production, particularly when susceptible varieties are grown in favorable microclimates (Aman and Rai, 2015; Rocha et al., 2012).

The disease reduces photosynthetic area and plant growth, thus affecting fruit quality with obvious consequences for productivity (Castelan et al., 2013). Disease management basically involves genetic and chemical control (Cordeiro and Matos, 2005; Ferreira et al., 2003; Patel, 2009).

In many cases, genetic control for resistance displeases the consumer with a supply of unpalatable varieties without commercial appeal. Also, misuse of chemical control agents results in risks to the applicator, environment, and selection of fungicide resistant

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populations. It also weighs the cost of production, thus reducing producer profits.

However, an alternative method using properly nourished plants can reduce severity of disease (Pozza and Pozza, 2012) without altering fruit taste. Among mineral nutrients, potassium (K) and calcium (Ca) participate in various plant defense responses to plant pathogens (Belan et al., 2014; Garcia Júnior et al., 2003; Pinheiro et al., 2011; Sugimoto et al., 2005).

In general, K-deficient plants are susceptible to infection (Balardin et al., 2006; Sharma et al., 2005; Marschner, 2012; Uchôa et al., 2011). This behavior is not a rule, however, as some studies report increased disease with increasing concentration of K, such as in the cases of *Cercospora coffeicola* in coffee and *Colletotrichum gloeosporioides* in strawberry, due to an imbalance of K and Ca (Garcia Júnior et al., 2003; Pozza et al., 2001; Nam et al., 2006).

Potassium participates in regulating various physiological pathways, such as enzyme activation, protein synthesis, photosynthesis, osmoregulation, transport, and stress resistance (Marschner, 2012). Potassium contributes to improving plant resistance to alterations in protein or amino acid availability, decreased cell permeability, and decreased susceptibility of tissue to maceration and penetration (Prabhu et al., 2007).

With respect to calcium, studies have shown reduction in intensity of diseases with increasing concentration of Ca up to balance point with other ions (Pozza and Pozza, 2012). Calcium plays a key role in recognizing invading pathogens in the plasma membrane, acting as a second messenger (Yang et al., 1997) to maintain biomembrane stability, thus avoiding the outflow of low molecular weight compounds from the cytoplasm to the apoplast (Marschner, 2012). Calcium also acts to build and strengthen the cell wall, thus hindering pathogen infection (Bateman and Lumsden, 1965).

In addition to the isolated effect, it is important to know how the interaction of these nutrients in plants influences the intensity of disease. According to Marschner (2012), cations K^+ and Ca^{2+} compete with each other, and with other nutrients for the same absorption sites, which results in unbalanced plant nutrition.

In other cultures, imbalance of K^+/Ca^{2+} ratio promoted changes in nutritional status, and favored pathogen infection (Carvalho et al., 2013; Garcia Júnior et al., 2003; Lima et al., 2010; Pinheiro et al., 2011; Pozza et al., 2001). In the case of banana plants, the intensity of Yellow Sigatoka was higher in plants grown in K-deficient nutrient solution (Freitas et al., 2015b), and lower in plants grown in soil with higher contents of Ca (Freitas et al., 2015a; Gerald et al., 2003).

Although there is no report emphasizing the effects of interaction of these nutrients in plants prone to *P. musae*, the culture was found to be sensitive to nutritional imbalance (Silva et al., 2008). Thus, knowledge of nutrient effects on the severity of Yellow Sigatoka can help develop management strategies, consequently reducing crop protection applications and increasing environmental and financial sustainability of banana crops. This study evaluated K and Ca interaction in nutrient solution in the severity of Yellow Sigatoka in banana plants.

MATERIALS AND METHODS

Plant material and growth conditions

The present study was conducted under greenhouse at the Plant Pathology Department, Federal University of Lavras (UFLA), Lavras. Lavras is situated in the Southeast region of Brazil at 21°14'S (latitude) and 45°00'W (longitude) at an altitude of 918 m above the mean sea level. The relative humidity and average temperature of greenhouse during the conduction of the experiment was 80% e 25°C, respectively.

Seedlings of micropropagated banana plants (*Musa acuminata* 'Grande Naine AAA Cavendish') with 49 days age obtained from tissue culture were adapted in trays containing 16 L nutrient solution (Hoagland and Arnon 1950) at 50% ionic strength, with continuous ventilation for 15 days.

Once adapted, plants were transferred to 6-liter pots containing nutrient solution with continuous aeration. Treatments consisted of five concentrations of K (1, 2, 4, 6, and 8 mmol L^{-1}) combined with five concentrations of Ca (1, 3, 5, 7, and 9 mmol L^{-1}) in a total of 25 treatments in factorial analysis of variance (5 x 5). The experiment was conducted in randomized block design with three replicated. The experimental unit consisted of one plant per pot, and the whole experiment was repetition once.

Concentrations of N (15 mmol L^{-1}), P (1 mmol L^{-1}) Mg (2 mmol L^{-1}), S (2 mmol L^{-1}) and micronutrients (1 mL of L^{-1} micronutrient stock solution) were the same in all treatments. Micronutrient stock solution was composed of 2.86 g L^{-1} boric acid, 1.81 g L^{-1} manganese chloride, 0.10 g L^{-1} zinc chloride, 0.04 g L^{-1} copper chloride, and 0.02 g L^{-1} molybdic acid. Iron was provided by adding 1 mL of iron stock solution (33.3 g Na_2 EDTA, 100.4 mL NaOH 1N, 24.9 g $FeSO_4.7H_2O$ and 4 mL HCl 1N) L^{-1} in the nutrient solution (Lima et al., 2010).

The pH of nutrient solution was monitored weekly, and kept at 5.5-6 with addition of HCl 0.1 mol L^{-1} or NaOH 0.1 mol L^{-1}. When necessary, volume of pots was supplemented with deionized water. Depletion of ions from the nutrient solution was checked weekly with a Compaction Meter device for K^+ (Horiba-CARDY®). Nutrient solution was changed in all treatments when depletion reached 30% of the initial value of K^+ (Braccini et al., 1999).

Inoculum preparation and inoculation

The isolate of *P. musae* came from diseased banana leaves (Cordeiro et al., 2011), and conidia was obtained using the method described by Freitas et al. (2015b) with modifications. Ten mycelial fragments (5 mm diameter) taken from the colonies after 26 days of growth in Petri dishes containing malt medium (20 g malt extract, 20 g agar and 1,000 mL distilled water) were macerated in mortar and pestle. Then, the mash was diluted in 15 mL tomato juice (200 mL tomato juice, 1g $CaCO_3$, and 900 mL distilled water) and transferred to a 9-cm Petri dish with solid tomato juice (200 mL tomato juice, 20g agar, 1g $CaCO_3$, and 900 mL distilled water).

To facilitate drying, dishes were left open in BOD incubator at 25°C with a 24 h photoperiod, and four 20-watt fluorescent bulbs. Once the culture medium was dry, about two days after incubation, 10 mL sterilized distilled water was added to each Petri dish, and

conidia were released from the dry mycelium with a toothbrush. This suspension was filtered on a double layer of cheesecloth, and concentration was adjusted to 4 x 10^4 conidia mL^{-1} using a hemocytometer, establishing an average of 4 readings.

Inoculation was carried out two months after transferring plants to the nutrient solution containing the treatments. Leaves 1, 2, 3 and 4 were inoculated on the abaxial surface by spraying 0.7 mL conidial suspension in a 36 cm^2 area delineated in the median region. After inoculation, plants were individually covered with transparent plastic bags for 60 h, remaining at 23°C, and relative humidity 92%.

Assessment of disease severity

Severity of disease was evaluated on the abaxial surface of each inoculated area after the first symptoms appeared. Five evaluations were performed at five-day intervals, based on the diagrammatic scale described by Stover (1971) and modified by Gauhl (1994). Scores ranged from 0 to 5, being: 0 - inoculated area without symptoms, 1- inoculated area with up to 10 stains, 2- inoculated area with 1 to 5% stains, 3 - inoculated area with 6 to 15% stains, 4 - inoculated area with 16 to 33% stains, and 5 - inoculated area with 34 to 50% stains. All five evaluations were integrated in the area under the disease severity progress curve (AUDSPC) according to the equation proposed by Shaner and Finney (1977):

$$\text{AUDSPC} = \sum_{i=1}^{n-1} \left(\frac{(y_i + y_{i+1})}{2} \right) (t_{i+1} - t_i)$$

In which:

yi proportion of disease in i-th observation
ti time, in days, in i-th observation
n total number of observations.

Chlorophyll assessment

Chlorophyll content was measured in samples of fresh leaf tissue of banana variety 'Grande Naine' with different shades of green, using a portable SPAD-502® for calibration. After reading, 0.2 g plant tissue was weighed, and macerated in liquid nitrogen, then transferred into tubes containing 10 mL of 80% acetone (v/v), remaining for 24 h in cold chamber protected from light.

After 24 h, extracts were filtered, and reading was performed with spectrophotometer at wavelengths 663 and 645 nm for chlorophyll *a* and *b*, respectively. Determination of chlorophyll (mg gmf^{-1}) was based on the following equations, according to Whitham et al. (1971):

$$\text{Chlorophyll } a = \frac{\left(12{,}7 \text{ x } A_{663} - 2{,}69 \text{ x } A_{645}\right) V}{1000 \text{ MMF}}$$

$$\text{Chlorophyll } b = \frac{\left(22{,}9 \text{ x } A_{645} - 4{,}68 \text{ x } A_{663}\right) V}{1000 \text{ MMF}}$$

In which:

A = absorbance at the indicated wavelength
V = final volume of chlorophyll-acetone extract
MMF= fresh weight in grams of plant material (mg $(g\ MF)^{-1}$).

Once calibration curve was built, readings were taken with SPAD-502® near the inoculated areas, 24 h before inoculation. With the data obtained, contents of chlorophyll a and b were determined using the following equations:

Ya = -0.0035 + 0.0007356X
Yb= -0.0001734 + 0.0002983X

In which:

X= SPAD reading
Ya = chlorophyll *a*
Yb = chlorophyll *b*.

Determination of leaf nutrient contents

Nutrient contents were determined at the end of the experiments, after evaluations were completed. Leaves 3, except the central ribs, were washed in distilled water, placed in paper bags and dried in oven at 60°C until constant weight, as established by Martinez et al. (1999). Then, samples were ground and analyzed according to the method proposed by Malavolta et al. (1997) to determine the contents of nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, boron, copper, zinc and manganese.

Determination of plant dry weight

Total dry weight of plants was determined after evaluations were completed at the end of the experiments. Leaves, stems, and roots were washed, packed in paper bags, and placed on a greenhouse bench for preliminary drying, then dried in an oven at 60°C until constant weight.

Data analysis

The Shapiro-Wilk test (Shapiro and Wilk, 1965) was applied to the data of each repetition to evaluate normal distribution. As data were normally distributed, the variables required no transformation. Thus, data from both experiments were subjected to joint analysis over time to determine differences between them. Variables underwent analysis of variance (ANOVA) in a factorial 5 x 5, that is, 5 concentrations of potassium and 5 of calcium, in a total of 25 treatments. Linear regression models were fit to significant variables in F test ($p \leq 0.05$). Response surfaces were fit in case of significant interaction. Normal distribution of data was analyzed using R software, while the remaining analyses were performed using PROC GLM procedure in SAS software (v.9.2, SAS Institute Inc.).

RESULTS

Joint analysis of data

Joint analysis of variables over time showed no significant difference ($p \leq 0.05$) between experiments. Thus, results are the average of two repetitions.

Severity of Yellow Sigatoka

Early symptoms of Yellow Sigatoka in areas inoculated with *P. musae* were observed 26 days after inoculation.

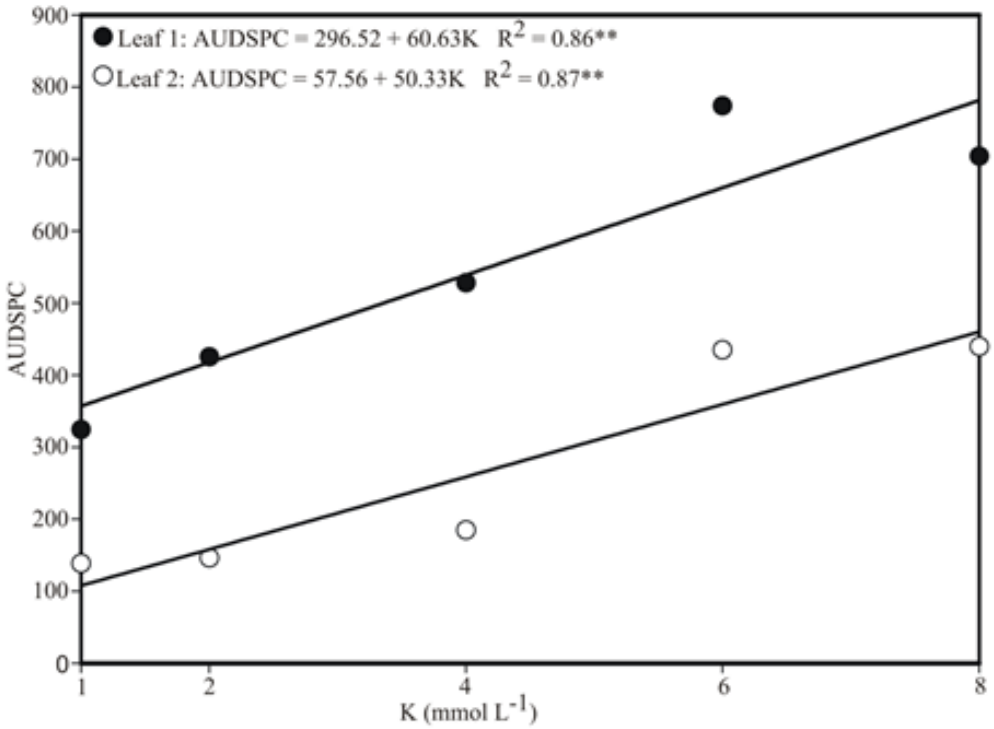

Figure 1. Area under the disease severity progress curve (AUDSPC) in leaves 1 and 2 of banana plants, depending on increase in potassium (K) concentrations in nutrient solution (**Significant (p≤0.01)).

There was no interaction between K and Ca concentrations for the area under the disease severity progress curve (AUDSPC). There was no effect of isolated concentrations of Ca on AUDSPC with overall average 551.17 and 268.96 in leaves 1 and 2, respectively.

However, increase in K concentrations from 1 to 6 mmol L^{-1} influenced (p≤0.01) increase in AUDSPC from 357.15 to 660.3 and 107.89 to 359.57 in leaves 1 and 2, respectively. AUDSPC in leaf 1 was on average 51.37% higher than in leaf 2 (Figure 1). AUDSPC in leaves 3 and 4 was not affected either by interaction of K and Ca concentrations or by isolated effect of these nutrients (Figure 1).

Content of chlorophyll *a* and *b*

There was no interaction between K and Ca concentrations for leaf contents of chlorophyll *a* and *b*. However, increase of these nutrients reduced the content of chlorophyll *a* and *b* in leaves in an independent way. With increasing concentrations of K from 2 to 8 mmol L^{-1}, leaf content of chlorophyll *a* and *b* decreased from 0.037 to 0.035 mg gmf^{-1} and 0.016 to 0.015 mg gmf^{-1}, respectively (Figure 2 a and b). Increase of Ca from 1 to 9 mmol L^{-1} decreased leaf contents of chlorophyll *a* and *b* from 0.038 to 0.035 mg gmf^{-1} mg, and 0.016 to 0.015 mg gmf^{-1}, respectively (Figure 2c and d).

Nutritional aspects of banana plants

There was an interaction between K and Ca concentrations for leaf contents of sulfur (S) and calcium (Ca). The highest Ca leaf content (25.77 g kg^{-1}) was found in concentrations 9 and 2 mmol L^{-1} of Ca and K, respectively (Figure 3 k).

Regarding S, the highest content (6.5 g kg^{-1}) was observed in the combination of concentrations 1 and 8 mmol L^{-1} of Ca and K, respectively (Figure 3 l). Separately, increasing K and Ca concentrations influenced nutrition of banana plants.

Regarding K, increase in concentrations from 1 to 8 mmol L^{-1} raised K leaf content from 28.80 to 40.14 g kg^{-1} respectively (Figure 3 a). However, increasing K (Figures 3 b to h) reduced leaf contents of N (46.34 to 37.45 g kg^{-1}), P (3.95 to 2.35 g kg^{-1}), Mg (5.31 to 2.41 g kg^{-1}), micronutrients B (46.44 to 32.62 mg kg^{-1}), Cu (7.73 to 3.4 mg kg^{-1}), Zn (15.16 to 12.1 mg kg^{-1}) and Mn (289.29 to 122.62 mg kg^{-1}).

Increased concentration of calcium from 1 to 9 mmol L^{-1} influenced reduction in N leaf content from 44.80 to 39.47g kg^{-1} (Figure 3 i). In addition, Mg leaf content decreased from 4.27 to 3.39 g kg^{-1} up to 4.70 mmol L^{-1},

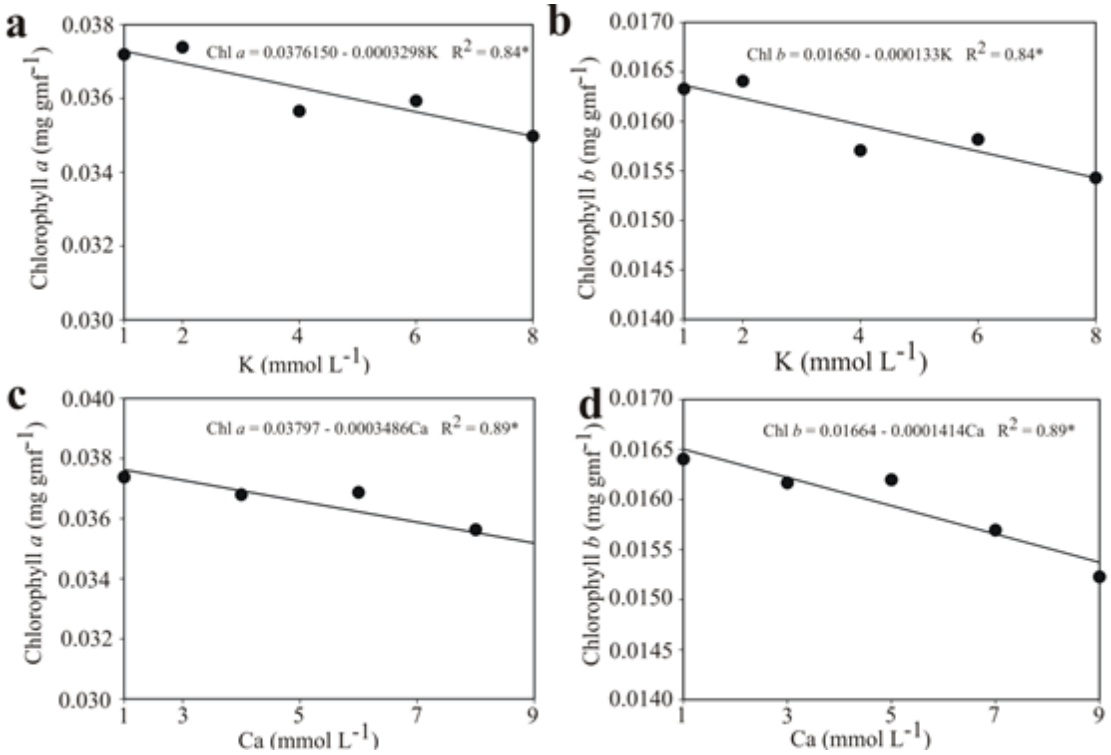

Figure 2. Leaf content of chlorophyll a (a and c) and b (b and d) in banana leaves, depending on increase in potassium (K) and calcium (Ca) concentrations in nutrient solution. gmf: gramm of fresh weight (*Significant (p≤0.05)).

and increased from this concentration up to 4.57g kg^{-1} at 9 mmol L^{-1} (Figure 3 j).

Plant dry weight

Total dry weight (TDW) of plants was not influenced either by interaction of K and Ca concentrations or by isolated effect of Ca concentration. However, increased K concentrations significantly influenced TDW increase, ranging from 59.43 to 170.52 g $plant^{-1}$ at K concentrations 1 and 8 mmol L^{-1} respectively, that is, TDW increased 15.87 g per 1 mmol L^{-1} of K supplemented in nutrient solution (Figure 4).

DISCUSSION

Addition of K to nutrient solution influenced increase of area under the disease severity progress curve (AUDSPC) for *P. musae* in banana plants. However, Uchôa et al. (2011) found reduction in severity of Black Sigatoka (*M. fijiensis*) from 260 to 117 intensity with increasing potassium concentration in soil of planting areas from 28 to 57.5 mg dm^{-3}, respectively.

Conversely, Freitas et al. (2015b) found that absence of K in nutrient solution resulted in higher severity of *P. musae* in banana plants. The findings of this study are different from those reported by Uchôa et al. (2011), obviously due to experiment conditions. Several soil factors can influence the nutritional status of plants. In nutrient solution, by contrast, it is possible to isolate the effect of each nutrient, enabling the study of relationship between nutrient effects and intensity of disease (Lima et al., 2010).

Experiments also conducted in nutrient solution for other pathosystems showed similar results to this study. The area under the progress curve of cercospora leaf spot (*C. coffeicola*) of coffee increased from 14.6 to 17.39 with increase of K from 4 to 7 mmol L^{-1}, respectively (Garcia Júnior et al., 2003).

Also in coffee, phoma leaf spot (*Phoma tarda*) increased the area under incidence progress curve (AUIPC), and area under severity progress curve (AUSPC) with K contents above 6.59 and 6.57 mmol L^{-1}, respectively (Lima et al., 2010). Excess of potassium, 30 mmol L^{-1}, also influenced increase of anthracnose (*Colletotrichum gloeosporioides*) in strawberries (Nam et al., 2006). Potassium applied appropriately in soil, 8% K_2O, reduced anthracnose (*Discula destructiva*) in *Cornus florida* L.

However, opposite results were found with double amount of K, 16% K_2O (Holzmueller et al., 2007). That is, as the effect of K on intensity of disease depends on

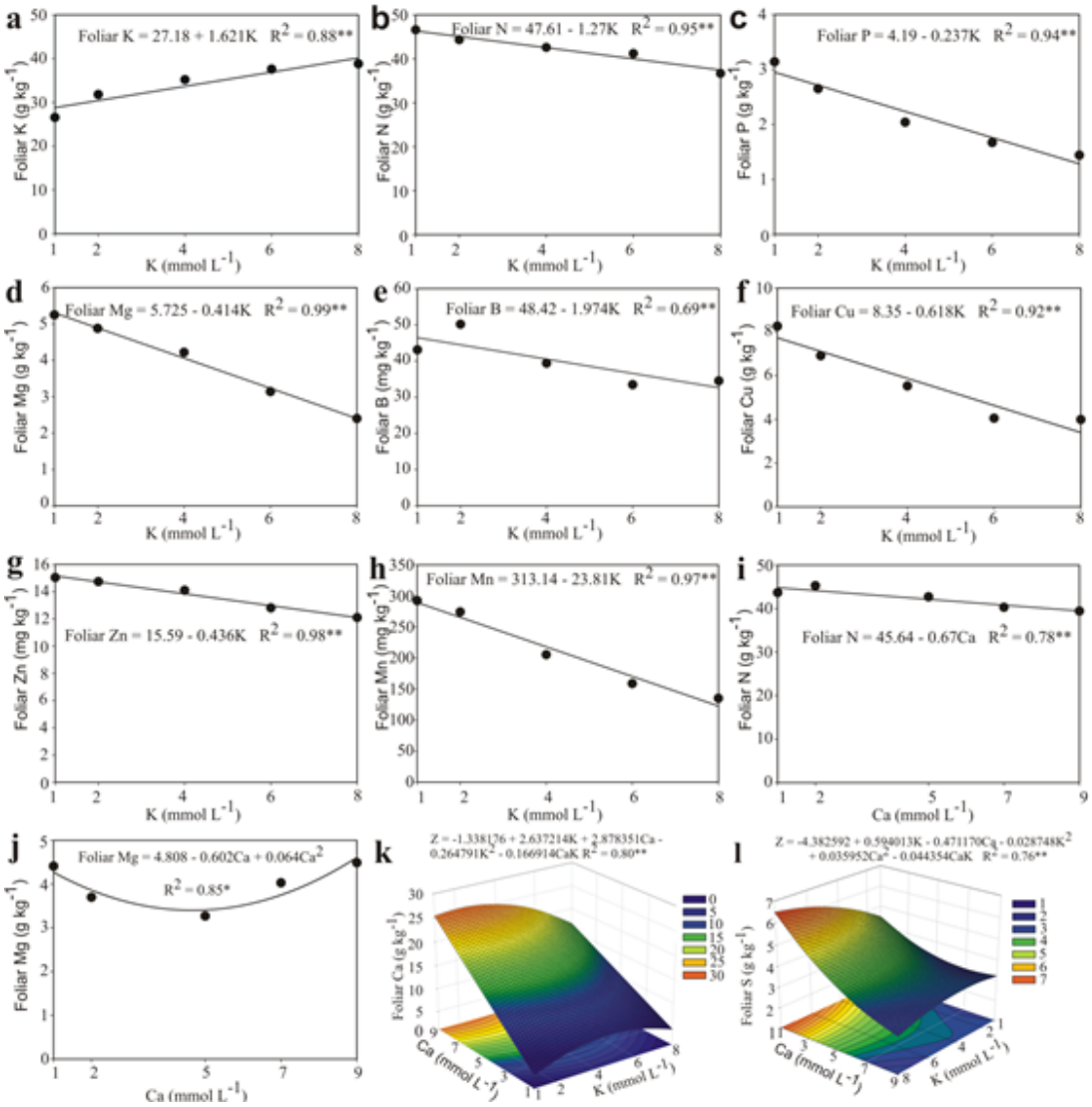

Figure 3. Leaf contents of potassium (a), nitrogen (b and i), phosphorus (c), magnesium (d and j), boron (e), copper (f), zinc (g), manganese (h), calcium (k) and sulfur (l) in banana leaves, depending on increase in potassium (K) and calcium (Ca) concentrations in nutrient solution. **Significant (p≤0.01) (*Significant (p≤0.05)).

the pathosystem, it is not possible to generalize the findings. Soil factors such as interaction between nutrients, organic matter and pH, and buffering capacity, texture, and structure should also be considered. Hosts may also influence this interaction depending on their nutritional requirements at each stage of life cycle. As it is known in the literature, banana plants require large amounts of K (Borges and Souza, 2004). Overall, leaf nutrient contents were outside both the range considered appropriate (Borges and Souza, 2004), and the reference values (Martinez et al., 1999) established for banana growing.

Thus, it can be concluded that increased severity of Yellow Sigatoka was due to plant nutrient imbalance. Freitas et al. (2015b) also found increasing severity of Yellow Sigatoka caused by nutrient imbalance in banana plants. Similarly, imbalance due to high contents of S and low contents of P, Ca, and Mg in the soil resulted in increased severity of Black Sigatoka from 117 to 340 intensity (Uchôa et al., 2011).

In coffee, increase of K contents up to 7 mmol L^{-1} reduced absorption of N and Ca, making plants more susceptible to cercospora leaf spot (Garcia Júnior et al., 2003; Pozza et al., 2001). In the same culture, imbalance in ratio N/K also promoted changes in the nutritional status of plants and favored infection by *P. tarda* (Lima et al., 2010).

According to Huber and Haneklaus (2007), nutrient

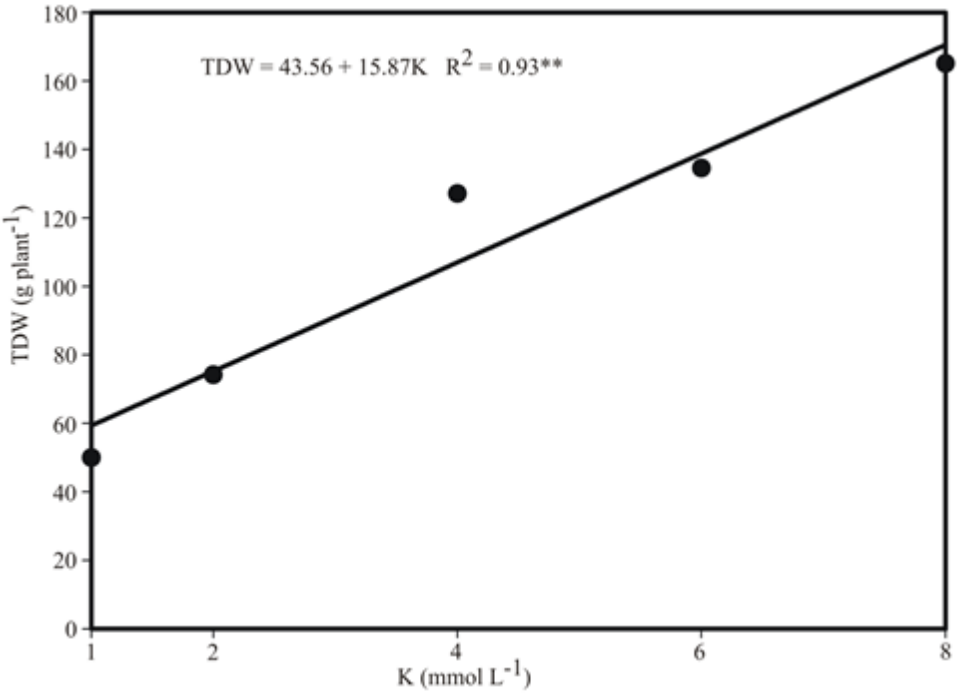

Figure 4. Total dry weight (TDW) of banana plants depending on increase in potassium (K) concentrations in nutrient solution (**Significant (p≤0.01)).

imbalance can be as harmful to plant resistance to disease as nutrient deficiency. According to Marschner (2012), high concentrations of K can cause nutrient imbalance by reducing the uptake of other nutrients. This result was confirmed by Pinheiro et al. (2011), who found a reduction of 26.4 % in Ca contents in soybean leaves with increasing K in nutrient solution.

Increasing K also reduced leaf contents of chlorophyll *a* and *b*. Chlorophylls are green pigments specialized in absorbing light and transferring energy and electrons during photosynthesis, and are thus related to this process efficiency (Taiz and Zeiger, 2013).

However, leaf chlorophyll content can be reduced under physiological stress (Larcher, 2004). Thus, reduction in contents of chlorophyll *a* and *b* can be attributed to nutritional imbalance, as, besides being part of plant molecular structure, mineral nutrients also participate in important reactions during chlorophyll synthesis (Neves et al., 2005).

As an example, Mg, N and Mn show contents outside the suitable range for banana. As magnesium is part of the structure of chlorophyll molecule (Marschner, 2012), Mg-deficient plants are more susceptible to disease (Huber and Jones, 2013).

In coffee, Alves et al. (2009) found higher intensity of rust (*Hemileia vastatrix*) in leaves and cercospora leaf spot in leaves and fruits in crops with Mg, S, N, and Cu deficiency. In rice, sheath blight (*Rhizoctonia solani*) was reduced with increasing Mg concentration from 0.062 to 0.5 mmol L^{-1} (Schurt et al., 2014).

Regarding N and Mn, the former is important as a chlorophyll constituent, and the latter for its role in photosynthetic reaction, in which oxygen is produced from water (Taiz and Zeiger, 2013). The positive effect of these nutrients in reduction of plant diseases has been demonstrated in other studies. Furtado et al. (2009) found higher incidence of Panama disease (*Fusarium oxysporum* f. sp. *cubense*) in banana plants with lower contents of nitrogen. Incidence of cercospora leaf spot of coffee was reduced by 20.7% with increase of N from 3 to 15 mmol L^{-1} (Pozza et al., 2001). In wheat, severity of spot blotch (*Bipolaris sorokiniana*) was reduced with increasing Mn in leaves (Zanão Júnior et al., 2009).

In addition to nutrient imbalance, plant growth stage may explain the difference in susceptibility to *P*. *musae*, as AUDSPC was higher in young leaves. Similar results were found in banana plants infected with *M. fijiensis* (Kablan et al., 2012; Romero, 1995). Kablan et al. (2012) attributed these results to the formation of physical or physiological barriers in old leaves, acting either during or after fungal invasion. In this study, as there was no significant difference for AUDSPC in leaves 3 and 4, the formation of physical barriers such as lignification and cuticle thickening helped reduce the intensity of disease.

Although addition of K had increased AUDSPC, and reduced leaf contents of N, P, Mg, B, Cu, Zn, Mn, and chlorophyll *a* and *b*, there was increase in total dry weight of plants. Likewise, Silva et al. (2008) found higher dry

weight of banana plants grown with higher doses of K in soil, up to 1.600 mg dm^{-3}. Increased dry weight in contrast to reduction in contents of most nutrients can be explained, since contents were still close to the suitable range for banana even after reduction (Borges and Souza, 2004; Martinez et al., 1999).

In addition, availability of water and nutrients to plants is constant during experiments in nutrient solution, as water level is kept constant and nutrients are changed when depletion reaches 30% of the initial value (Braccini et al., 1999). Under these conditions, plant growth is possible even with low contents of some nutrients.

According to Marschner (2012), although high concentrations of K decrease the contents of other nutrients, plant growth may increase. In banana culture, K and N are the most important nutrients for plant growth (Borges and Souza, 2004).

Thus, in this study, the favorable conditions of hydroponics and increase of K were responsible for increasing plant weight. Reduction in contents of chlorophyll *a* and *b* was insufficient to affect plant growth. Also, as disease manifested only in the inoculated area and not in the full leaf, there was no influence on total dry weight of plants.

Conclusions

High concentrations of K in nutrient solution promoted nutritional changes in banana plants, mainly by reducing leaf contents of N, P, Mg, B, Cu, Zn, and Mn. As a result of this imbalance, there was an increase in severity of Yellow Sigatoka. Therefore, proper and balanced fertilization can minimize nutritional changes in banana plants, and reduce the number of fungicide sprays for controlling *P. musae*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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