Nutritional status of orange tree 'Pêra Rio' variety after Huanglongbing disease infection, leaf spray fertilization and application of resistance-inducing bioinductors

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Abstract

Citrus industry is widespread in the world. The Huanglongbing is an important disease of citrus species and has been spread around the world. This research analyzed the nutritional status of tree 'Pêra Rio' variety infected with *Candidatus* Liberibacter spp., under leaf nutrients spray and bioinductors of resistance. The experiment was carried out from September 2013 to June 2014, in a citrus commercial orchard located in the city of Ibitinga (SP). A completely randomized design was used in a factorial $3 \times 4 \times 6$, consisting of three plants status (healthy, infected with asymptomatic HLB, and symptomatic HLB), four rates of spray solutions (0, 2, 4 and 6 L ha^{-1}), six application stages (A_1, A_2, A_3, A_4, A_5, and A_6 with intervals of 45 days between each stage) and four replications. Macro and micronutrients leaf levels were quantified. Data were submitted to exploratory factor analysis with multivariate statistics. Three factors were extracted from the variables, namely, Factor 1 formed by variables S, Cu, Fe, and Zn; Factor 2 by N, K and B, and Factor 3 by Ca, Mg, and Mn. 'Pêra Rio' plants nutritional status was destabilized with the infection of *Candidatus* Liberibacter spp. Progressive treatment with leaf fertilization and resistance bioinductors temporarily re-established nutritional standards of plants affected by Huanglongbing but did not promote stability of the plants nutritional status.

Introduction

Growing fruit plants of the *Citrus* spp. genus is quite widespread (Benfatto et al., 2015). Throughout the world, orange *Citrus sinensis* (L.) Osbeck. lemon. *C. latifolia* Tanaka ex Q. Jiménez, and mandarin *C. reticulata* Blanco are the main growing citrus species (Pereira et al., 2015). The researchers have emphasized that 90% of citrus fruits produced in Brazil are represented by oranges, while lemons and mandarins are represented by 5% and 5%, respectively. Benfatto et al. (2015) reported that the diffusion and importance of citrus have facilitated the sudden spread of pests and diseases. Huanglongbing (HLB), caused by bacteria *Candidatus* Liberibacter spp. (*Ca* L.), also known as greening, is the citrus's most important and destructive disease in the world (Duan et al., 2009). The prefix *Candidatus* is given by the fact that the bacterium cannot be cultivated in culture medium, one of the necessary requirements for the definitive taxonomic classification of a microorganism. Study of genetic variability of *Ca*. L. spp., has facilitated classification of this bacteria into genetically distinct groups or variants. These organisms are classified as α-Proteobacteria, of the order Rhizobiales and family Rhizobiaceae (Coletta Filho et al., 2004; Machado et al., 2010).

HLB is transmitted by psyllid (*Diaphorina citri* and *Trioza erytreae*), insect vectors of the three Gram-negative bacteria: '*Candidatus* Liberibacter americanus', '*Ca*. L. asiaticus', '*Ca*. L. africanus' which colonize phloem (Sauer et al., 2015). The symptoms of the disease depend on the species of the host plant, its age, the culture ecosystem, the time of year, bacterial concentration, nutritional status, involvement by

other pathogens and severity of the disease. After infection by the bacteria, the incubation can vary between six to 12 months without any symptoms. After incubation of the bacterium, the leaves turn yellow; the fruits size and asymmetrical shape is reduced. The irregular external coloration, displaced columella and with aborted seeds can also be expected. The collapse of a large part of the sieve tube and companion cell collapses also occurs, and the pores of the phloem vessels that have not suffered this damage are blocked by callosum and p-protein, reducing the translocation of photoassimilates and nutrients in the phloem (Bové, 2006; Folimonova and Achor, 2010; Koh et al., 2012; Sauer et al., 2015; Wulff et al., 2015).

Analysis of the nutritional status of citrus plants through leaf nutrients contents are important to subsidize plants management and to support scientific community understanding the complex phenomena related to mineral deficiency and phytotoxicity symptoms (Hippler et al., 2015a, b; Rozane et al., 2015).

Nowadays, efforts have been made in order to select management strategies to HBL on citrus production (Hall et al., 2015; Orduño-Cruz et al., 2015a, b), so that using broad spectrum insecticide has been the main citrus growers' strategy to reduce the population of these vectors (Boina and Bloomquist, 2015).

Hall et al. (2015) reported that using resistant plants is a promising strategy to deal with the vector and the HLB causing bacteria. Llorens et al. (2015) reported that natural defense mechanisms of plants may be strengthened by elicitors use. This work aimed analyzing the nutritional status of orange tree 'Pêra Rio' infected with *Ca.* Liberibacter spp. under leaf fertilization and resistance bioinductors to understand plant behavior and expand the management strategies of affected citrus orchards by HLB.

Results

Analysis of factors

Four factors of formations were observed. However, the phosphorus content was removed from the analysis for not fulfilling the basic premises recommended to compose a consistent factor, especially for having a factor from a single variable (Hair et al., 2009). Factor 1 was formed by variables: sulphur (S), copper (Cu), iron (Fe), and zinc (Zn); Factor 2 was formed by variables: nitrogen (N), potassium (K), and boron (B), and Factor 3 by variables: calcium (Ca), magnesium (Mg), and manganese (Mn) (Table 1).

For each factor a significant difference ($p < 0,001$) was observed as a function of plants status, applied solution rates, and the number of applications. Also interactions between plants × rates, plants status × number of applications, solution rates × number of applications have been found, in addition to the triple interaction among these factors (Table 2).

Nutritional process related to redox reactions and carbon compounds

Factor 1 is characterized by nutritional process, where increases in Cu, Fe, and Zn reflected in reductions in S levels. This process was reported by Malavolta et al. (2005) who studied the nutritional composition of leaves of citrus varieties related to HLB. Mattos Jr. et al. (2010) explained that changes in trends on leaf nutrients levels are evident by increasing disease severity. Taiz and Zeiger (2013) classified Cu, Zn, and Fe, according to their biochemical function and level in plant tissues, as nutrients involved in redox reactions

and S as part of carbon compounds. Thus, Factor 1 could be understood as a low nutritional requirement in redox reactions and carbon compounds.

By analyzing the triple interaction, it was observed that on first application (A_1), health plants (P_1), and plants infected by HLB without symptoms (P_2) differ from plants infected by HLB with symptoms (P_3) in the absence of treatment D_1. Under 2 L ha^{-1} treatment (D_2), the reduction in the nutritional process on P_1 were observed matching them to P_3, while 4 L ha^{-1} (D_3) and 6L ha^{-1} (D_4) reduced the process in P_2. With regard to the second application (A_2), P_1 differed from P_2 and P_3 in the absence of treatments. Treatment D_2 decreased the process in P_1, while D_3 was increased in P_2 and P_3. In A_3 and A_4 applications, treatment D_2 decreases the process in all plants. Although this reduction is more pronounced in P_2, there was an increase in P_2 and P_3 under application of D_3. The P_3 has differed from the others in this treatment.

In the fifth (A_5) and sixth (A_6) applications, there was higher healthy plants in relation to others in the absence of treatment. In A_5, rate 2 L ha^{-1} reduced the process in P_1 and increased it in P_2, while treatment D_4 caused a decrease in all plants in relation to D_3.

In the A_6, there is an increase in process P_2 and P_3 under application of D_2. However, there is a significant reduction when the maximum rate of 6 L ha^{-1} was applied. On average, there was a significant increase in process A_2 compared to A_1, with gradual reduction in A_3, A_4 and A_5 with a slight increasing trend in A_6 (Fig 1).

Nutritional process related to carbon, enzymatic activity and osmotic regulation

The N, K, and B content variation characterizes a nutritional profile of the Factor 2. Citric plants affected by HLB expressed reductions in N and K levels, while B was unchanged due to disease (Malavolta et al., 2005). According to the biochemical function classification and the elements in the tissue (Taiz and Zeiger, 2013), Factor 2 can be interpreted as a high nutritional requirement process playing an important role in the formation of carbon compounds and enzyme cofactor as well as the osmotic potential regulator.

From triple interaction, it was found that in A_1 (with 4 L ha^{-1}) an increase in P_3 takes place, while the rate of 6 h L^{-1} reduces the process significantly in P_1 and it differed from P_2. In A_2, the rate of 2 L ha^{-1} reduced the process in P_3, while D_3 significantly reduced the process in P_1 and separated it from the others ones. On the third application, significant reduction in P_1 and an increase in P_3 under 4 L ha^{-1} applications were occurred. This pattern of difference between P_1 and P_3 is maintained with application of 6 L ha^{-1}.

In A_4, under 2 L ha^{-1} rate, significant reduction of the process occured on P_1 and P_3 and this reducing pattern was maintained in P_1 and P_2 at rates of 4 and 6 L ha^{-1}, respectively, while in P_3 there was an increase of the process with 4 L ha^{-1} rate. In fifth application, there was a progressive increase of the process in P_2 and P_3 under 2 and 4 L ha^{-1} application, respectively. In addition, a reduction in the process with 6 L ha^{-1} was observed, while in P_1 there was an increase with 2 L ha^{-1} application followed by stabilization of the system with rates higher than 6 L ha^{-1}. In the sixth application, the process was significantly reduced in P_1 with the increased treatment rates and producing more healthy plants from the other ones. It was also observed a reduction in P_2 with the application of 4 L ha^{-1} rate followed by an increase after application of 6 L ha^{-1}. It is observed that, over time, there is a reduction of process from A_1 to A_2, followed

Table 1. Variables charges values from factors obtained by main component analysis and rotated by Varimax method.

Factors	Variables										
	N	K	Ca	Mg	S	B	Cu	Fe	Mn	Zn	$\sigma^2\%$
Factor 1	0.18	-0.32	-0.09	-0.50	-0.68	0.05	0.84	0.93	0.10	0.60	28.1
Factor 2	-0.58	-0.70	-0.31	-0.27	0.08	-0.83	0.01	0.02	0.25	0.31	18.4
Factor 3	0.22	-0.09	0.80	0.62	0.20	0.13	-0.04	0.03	0.78	-0.21	17.9

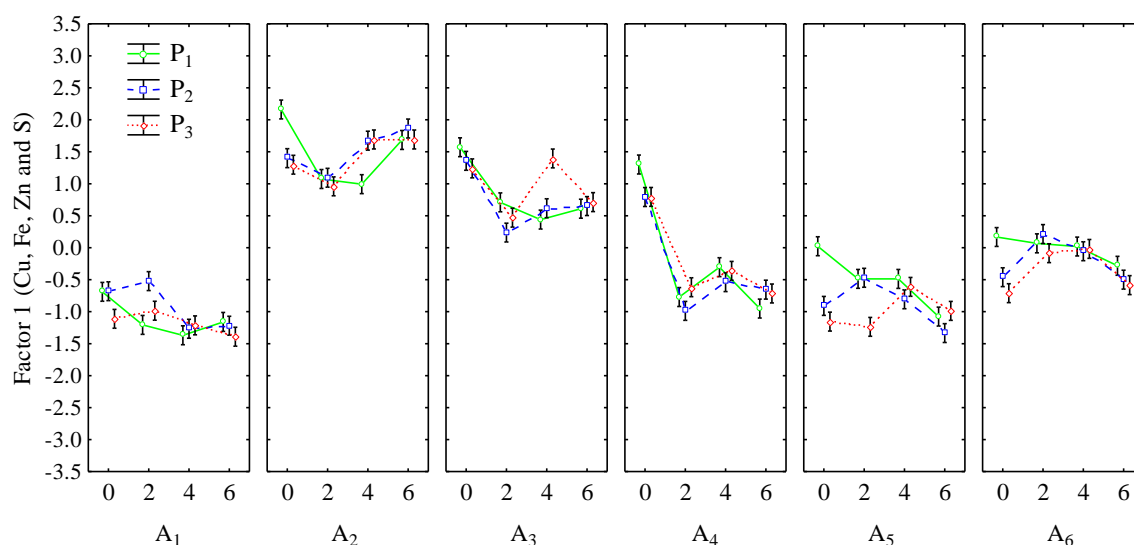$\sigma^2\%$: Proportion of the total variation from explained variance.

Fig 1. Two-dimensional projection from triple interaction among plants status (P), applied solution rates (0 to 6 L ha^{-1}), and the number of applications (A) in the Factor 1.

Table 2. Summary of analyses of variance for three factors from 10 original variables and phosphorus content.

Variation sources	DG	Averages square			
		Factor 1	Factor 2	Factor 3	Phosphorus
Plants status (P)	2	0.56***	26.06***	1.99***	0.86***
Rates of the solutions (R)	3	4.49***	3.35***	16.52***	0.96***
Number of application (A)	5	45.26***	15.46***	13.88***	3.60***
Interaction P x R	6	1.08***	7.04***	10.32***	0.24***
Interaction P x A	10	0.32***	0.51***	2.12***	0.03ns
Interaction R x A	15	1.76***	2.99***	1.14***	0.23***
Interaction P x R x A	30	0.17***	0.47***	1.00***	0.07ns
Residue	216	0.02	0.19	0.15	0.05

***: Significant to 0.1% ($p<0.001$); ns: not significant ($p>0.05$); DG, degrees of freedom.
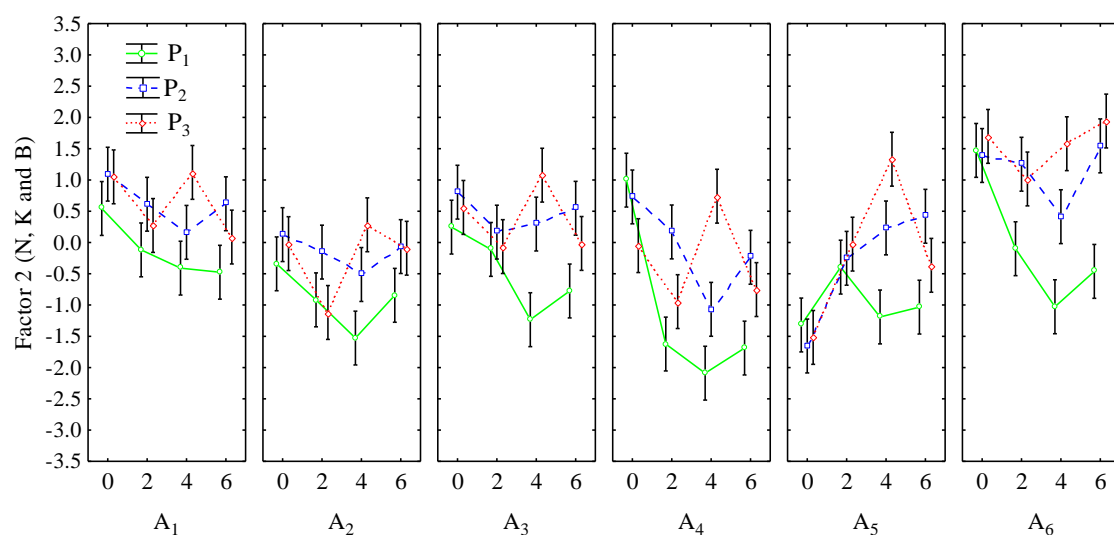

Fig 2. Two-dimensional projection from triple interaction among plants status (P), applied solution rates (0 to 6 L ha^{-1}), and the number of applications (A) in the Factor 2.

Table 3. Soil chemical properties before application of treatments and after treatments completion.

Sample	pH CaCl$_2$	OM g dm^{-3}	P	S	Ca	Mg	K	H+Al
			-----mg dm^{-3}-----		--------------mmol$_c$ dm^{-3}-------------			
Soil$_i$	6.30	19.00	17.00	4.00	44.00	18.00	2.90	12.00
Soil$_f$	5.80	21.00	38.00	5.00	64.00	25.00	3.60	16.00
	BS	CEC	V	B	Cu	Fe	Mn	Zn
	---mmol$_c$ dm^{-3}---		%	--------------------mg dm^{-3}--------------------				
Soil$_i$	64.80	76.80	84.00	0.47	3.20	14.00	16.00	3.90
Soil$_f$	91.90	108.40	85.00	0.57	5.40	42.00	15.20	4.90

Soil$_i$: before treatments application; Soil$_f$: after treatments completion; OM: Organic matter; BS: Base sum; CEC: Cation exchange capacity; V: Base saturation.
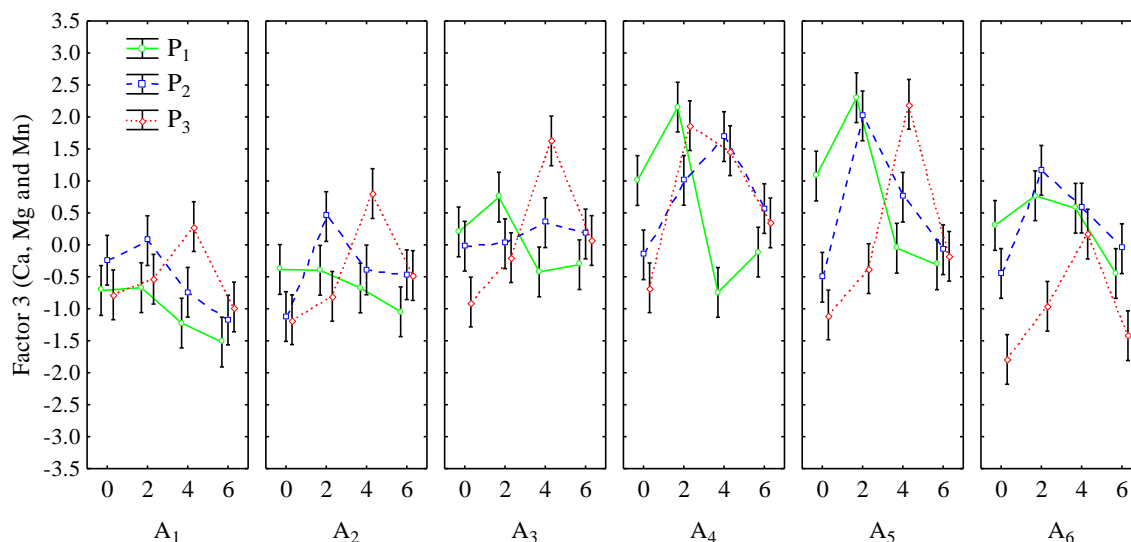

Fig 3. Two-dimensional projection from triple interaction among plants status (P), applied solution rates (0 to 6 L ha^{-1}), and the number of applications (A) in the Factor 3.

Table 4. Chemical properties of the spraying solutions.

SP	N	Ca	Mg	S	B	Fe	Mn	Mo	Zn	HY	HA
	---%---										
S$_1$	0.00	3.00	2.00	0.00	1.00	0.00	0.00	0.00	0.00	63E-3	0.05
S$_2$	0.00	0.00	2.50	4.00	0.02	2.00	1.14	3E-3	1.00	63E-3	0.57
S$_3$	4.00	0.00	0.00	0.00	0.00	0.50	4.00	0.00	4.00	63E-3	0.11

SP: sprayed solutions, obtained from commercial sources: KeyPlex® Blossom DP (S$_1$). KeyPlex® 1.200 DP (S$_2$) e KeyPlex® 1.400 DP (S$_3$); HY: hydrolyzed yeast. HA: humic acid.
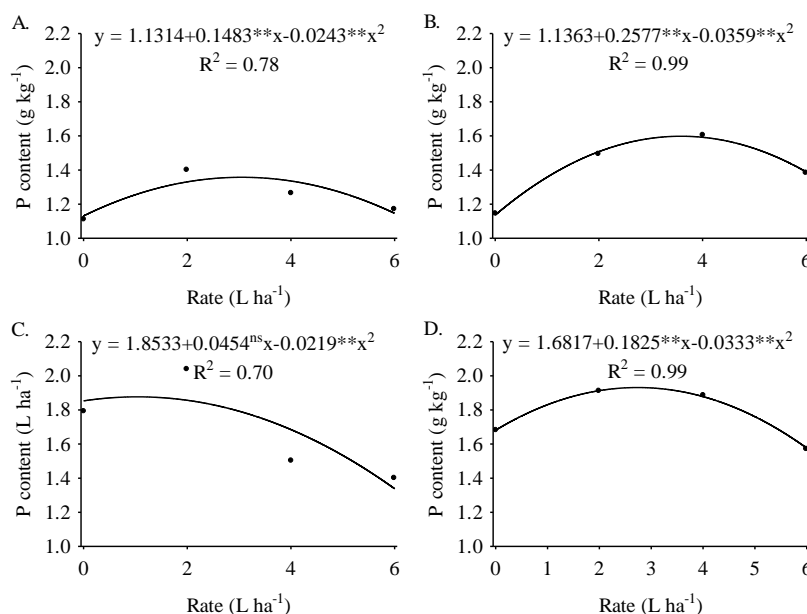

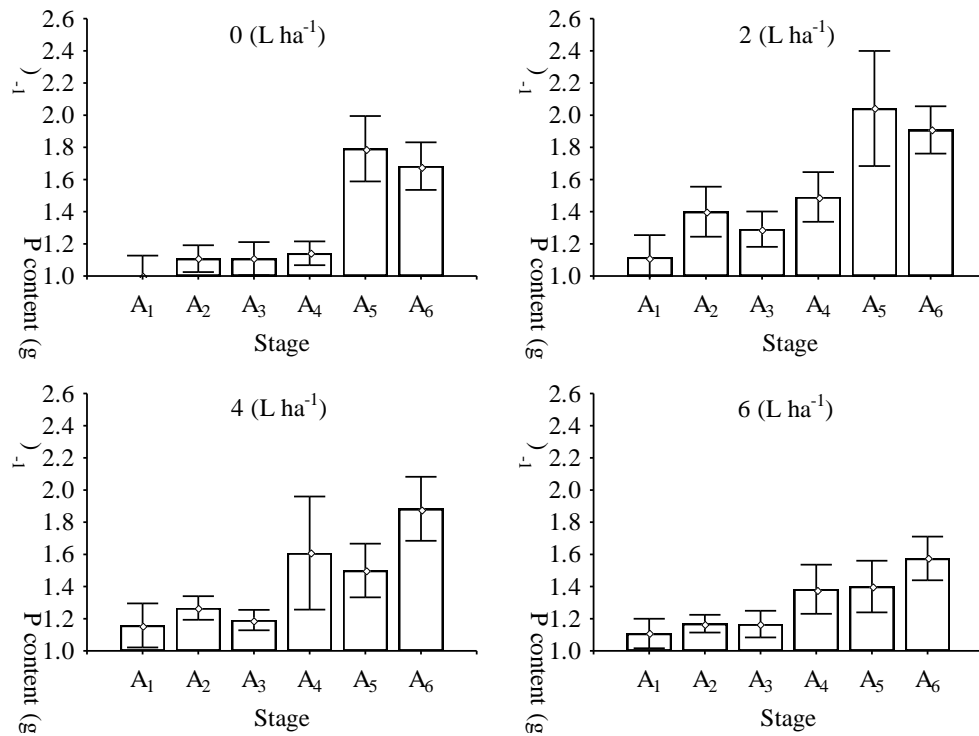**Fig 4**. Unfolding of solutions rates applied within A$_2$ (A), A$_4$ (B), A$_5$ (C) e A$_6$ (D) assessments.

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Fig 5. Unfolding of assessments within each rates of applied solutions.

A.

B.

P_1 y = 1.3252+0.2751**x-0.0461**x^2

R^2 = 0.94

P_2 y = 1.2825+0.0756*x-0.012*x^2

R^2 = 0.89

P_3 y = 1.3563^{ns}
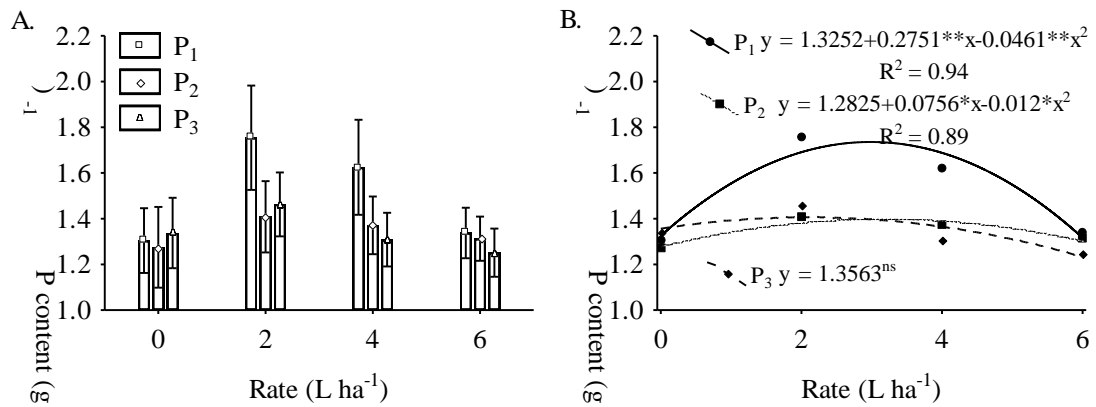
Fig 6. Unfolding of types of plants within the volume (A) and of rates within the kinds of plants (B).
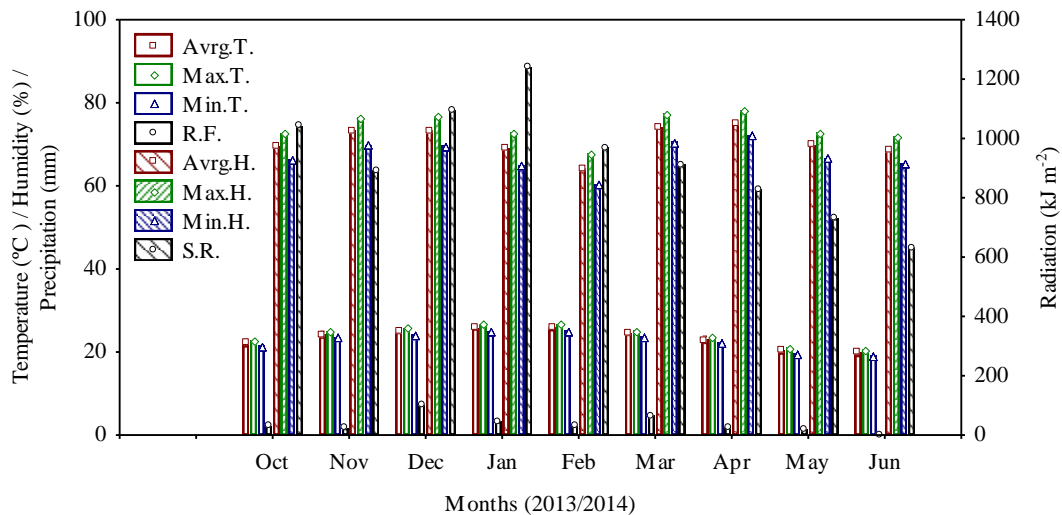

Months (2013/2014)

Fig 7. Climate diagrams variables recorded during the experimental period, average (Avrg.T.), maximum (Max.T.), and minimum temperatures (Min.T.), rainfall (R.F.), average (Avrg.H.). maximum (Max.U.), and minimum (Min.H.) relative humidity and solar radiation (S.R.).

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by the stability among A_2, A_3, and A_4, and recorded increase in A_5 and A_6 (Fig 2).

Nutritional process with nutrients remaining in the ionic form

Factor 3 is formed by variables Ca, Mg, and Mn and it is directly evolved in the process. Malavolta et al. (2005) reported a correlation among these nutrients in plants affected by HLB. As well, Malavolta et al. (2006) studied the distribution of nutrients within Orange trees. These authors suggested that leaf applications of Ca, Mg, and Ni, can improve fruit production under deficiency conditions, mainly through its effect on flowering. According to the classification of biochemical function of the elements proposed by Taiz and Zeiger (2013), it is possible to interpret the factor 3 as a nutritional process with nutrients that remain in ionic form.

In A_1, A_2, and A_3 applications, there is stability from processes P_1 and P_2 responses even in the presence of increasing rates with a strong interaction between P_3 and the others plants status with 4 L ha^{-1} application. With A_4 and A_5 applications, healthy plants showed higher values than others in the absence of treatment. The increase in the process in the three plants status was observed after using 2 L ha^{-1} followed by a significant reduction under higher applied rates (6 L ha^{-1}). This reduction was more pronounced in P_1 after A_4 application, while A_5 in symptomatic plants expressed the peak of the process with application of 6 L ha^{-1}.

In sixth application, healthy and asymptomatic plants showed superior in the process in relation to symptomatic plants in the absence of treatments and 2 L ha^{-1}. This was increase in response profile of the P_2 and P_3 plants with increasing rates of 2 and 4 L ha^{-1} and a reduced profile with the application of the maximum rate of 6 L ha^{-1}, whereas theses variations were more expressive in P_3. On average, it can be observed that a response profile from plant status shows a progressive increase of the process up to the forth application followed by a stability between the forth and the fifth applications and a trend of reduction in the sixth application (Fig 3).

Phosphorus content

Unfolding the effect of rates of solutions within each application, we found that rates did not affect (p> 0.05) leaf phosphorus content in the first and third applications. In the second, fourth, fifth, and sixth applications, averages had quadratic adjustment in response to increasing rates. For these applications, the following P levels were estimated 1.4 g kg^{-1}, 1.6 g kg^{-1}, 1.9 g kg^{-1}, and 1.9 g kg^{-1}, obtained with rates 3.0 L ha^{-1} (Fig 4A), 3.6 L ha^{-1} (Fig 4B), 1.0 L ha^{-1} (Fig 4C), and 2.7 L ha^{-1} (Fig 4D). These rates provided increments of 17%, 29%, 3%, and 13% in leave P content, respectively. From P maximum values, it was noticed that when rates are increased up to of 6 L ha^{-1} they triggered a reduction of 16%, 13%, 29% and 18%, respectively.

Regardless of application rates, no change was observed in P content of leaves, which increased throughout the experimental time. Without treatment (0 L ha^{-1}) a 41% increase in nutrients contents was observed, when compared to the values of the sixth evaluation (1.7 g kg^{-1}) with that on first one (1.0 g kg^{-1}). This behavior was also observed when applications are compared within 2, 4, and 6 L ha^{-1}, with the most significant values achieved on sixth applications (1.9 g kg^{-1}, 1.9 g kg^{-1}, e 1.6 g kg^{-1}). So, for these values gains of

42%, 37%, and 31% for P content were calculated, compared to first application (Fig 5).

It can be observed that healthy plants have had higher P accumulation by comparison with other plants, when treated with 2 and 4 L ha^{-1}. The highest recorded values were 1.7 g kg^{-1} and 1.6 g kg^{-1} for 2 and 4 L ha^{-1}, respectively. In the absence of treatment and at the highest rate applied plant status did not differ (Fig 6A). Unfolding the effects of rates within each plant status, we found that the average values from healthy and asymptomatic plants showed quadratic adjustment of 1.7 and 1.3 g kg^{-1}. The maximum accumulation was achieved in plants with 3.0 and 3.1 L ha^{-1} estimated rates, respectively. Comparing the most significant values of 1.32 and 1.28 g kg^{-1} obtained under control treatment (0 L ha^{-1}), 22% and 1.5% gains were calculated along with 22% decrease in occurrence of disease in the application of maximum rate of 6 L ha^{-1} (Fig 6B).

Discussion

In the case of citrus nutrition, researches have been carrying out optimizing ways to evaluate nutritional status, definition of correct rates, reducing the overuse of fertilizers and their environmental impacts. This is mainly purposed to achieve a nutritional program for citrus cultivation, which can reflect on high fruit productivity and quality and sustainability of citrus industry (Hippler et al., 2015a; Rozane et al., 2015). In Brazilian literature, there are several reports about significant gains in citrus tissue micronutrients content related to leaf fertilization (Boaretto et al., 2003; Malavolta et al., 2006). Increases in leaf nutrient contents are directly related to their amount applied (Boaretto et al., 2003). Increase in leaf nutrient contents are directly related to the amount applied (Boaretto et al., 2003). The same authors explained that there is a nutrient solution runoff when applied to the leaves, which can be accumulated into the soil in field conditions. In this experiment, it is believed that the answers are due to nutritional re-establishment of deficient tissue in response to leaf absorption because the limits of nutrients in the soil are medium, high, and very high according to the recommendations of Raij et al. (1996). Plants with HLB symptomatic and asymptomatic show changes in biochemical constituents compared to healthy plants (Cardinali et al., 2012). These variations are noticeable from nutritional disorders caused by *Ca*. Liberibacter spp., especially in the form of deficiencies and prevention of translocation in phloem (Duan et al., 2009). Giles (2011) reports on successful leaf application of nutrients to promote longevity and better citrus production, when it is infected with HLB (no replicated data available). It is necessary that significant portion of the citrus industry to be joined to nutritional improvement programs (Spann et al., 2009, 2011). In China, Xia et al. (2011) reported that no evidence is available to support the option of leaf fertilization in citrus. Gottwald et al. (2012) studied the effectiveness of nutrition programs via leaf sprays on *Ca*. Liberibacter spp. population dynamics, on fruit production and quality, and disease progression. These authors concluded that this strategy is ineffective in the fight against HLB as well they warned the possibility of accumulation of inoculum and contamination of healthy citrus orchards. Based on this information, it should be noted that the findings of this research contribute to the understanding and management strategies on nutritional status of 'Pêra Rio', mainly due to lack of literature about this cultivar. Thus, the expressive responses on nutritional process, observed in infected plants asymptomatic and symptomatic, are attributed to the increase in volumes of N,

Ca, Mg, S, B, Fe, Mn, Mo and Zn applied through leaf treatment, mainly by plants chlorotic due to the obstruction of sieve pores and impaired translocation of nutrients and photosynthates due to the severity of disease (Cardinali et al., 2012.). According to reports by Duan et al. (2009), *Ca. Liberibacter* spp. use sap nutrients without damaging the host's cellular structure, so that nutritional supplementation by leaf can restore macro and micronutrients responsible for nutritional status highlighted in all three cases (factors) identified on current research. Response patterns of these factors are explained by Spann et al. (2009) who attribute the nutrient reductions to the dilution effect from the starch accumulation in tissue as well dry weight gain in symptomatic leaves. It can be inferred that simultaneous variations among nutrient levels are also linked to this effect, remarkably by the high correlation observed between these original variables and the latent factors obtained.

Phosphorus is an important component to the synthesis of nucleic acids, phosphoproteins, phospholipids and ATP with a great importance to photosynthetic process (Hammond and White, 2008). Increases in P, recorded in this study may be due to its high content in the soil. The re-establishment of the other nutrients supplied through leaves improves the ability of plants in the absorption and distribution of nutrient as long as new applications are carried out (Boaretto et al., 2003). These results are consistent with those mentioned by Zhao et al. (2013) who found differences between healthy and diseased plants to the re-establish of the P content in plants with severe symptoms of HLB under leaf applications of phosphorus. The scenario of HLB control strategies is worrying. Hall et al. (2015) suggested that cultivation of resistant plants is a promising strategy for managing this bacterium. However, the concern lies in the fact that there are no varieties or citrus cultivars resistant to this disease (Gottwald, 2010). Therefore, in addition to efforts for leaf nutrition, alternative treatments with elicitors have drawn scientific attention, justified by the fact that these substances induce signaling and expression of plant's resistance to HLB (Mattos Jr. et al., 2010).

In fact, natural citrus defense mechanisms can be activated by using these natural or synthetic elicitors (Llorens et al., 2013, 2015). Vicedo et al. (2009) reported that the presence of carboxylic acids can induce the production of bioactive molecule jasmonate-isoleucine, which is associated with abscisic and hexanoic acids. This activates mechanisms of resistance and promotes calluses accumulation into the tissues. Graham and Myers (2013) have shown that these acids can be used together with Cu to induce resistance in citrus. There are reports of success in use of yeast as elicitors in plant resistance induction (Zanardo et al., 2009). In the literature, there is lack of reports on citrus resistance induction to HLB, so this justifies the purpose of this study to seek preliminary data that provide the basis for future works to mitigate or eliminate the damage caused by this disease.

Materials and Methods

Experimental area characterization

This research was carried out between September 2013 and June 2014 in Ibitinga, São Paulo state, Brazil, in a commercial orange 'Pêra Rio' (*Citrus sinensis* (L.) Osbeck) orchard grafted on lemon tree 'Cravo' (*Citrus limonia* (L.) Osbeck). Experimental area consisted of 5.6 ha with 21°43'15.3"S of latitude and 48°53'27.1"W of longitude, altitude of 491m high, with a 'Aw' climate according to Köppen's classification (Fig 7).

Soil samples were collected prior to the set up the experiment, packed, and sent to the soil fertility laboratory to be analyzed according to Raij et al. (2001). Soil fertility was again analyzed at the end of experiment. On Table 3 initial and final analyzes are presented.

Experimental design

The experiment was conducted in a completely randomized design in a 3 x 4 x 6 factorial scheme with three types of plant/treatmentss (P_1 = healthy plants; P_2 = plants infected with HLB without symptoms, and P_3 = plants infected by HLB with symptoms). Four rates of the solutions were sprayed (D_1= 0; D_2= 2; D_3= 4; and D_4= 6 L ha^{-1}), and six applications stages (A_1; A_2; A_3; A_4; A_5; and A_6 with intervals of 45 days between each stage), with for replications. Always after plant sampling, each application solution was applied in 45 days interval. It were used three sprayed solutions (S_1; S_2; and S_3), according to phenological stage of the plants. Those solutions were composed of macro and micronutrients and bioinductors (Table 4).

Plant materials

In the beginning of the trial, 48 'Pêra Rio' plants were chosen out of 2700 plant population. All chosen plants were 8 years old and they had been grafted on lemon 'Cravo' rootstock. Sixteen plants were chosen, among 48 'Pêra Rio' variety plants, according to each HLB presence (P_1; P_2; and P_3).

Diagnosis of bacterial infection

For identification of each plants status, they were collected at fully expanded leaves from branches with HLB asymptomatic and HLB symptomatic. All collected leaves were packed in paper bags and sent to Centro de Citricultura Sylvio Moreira laboratory to substantiate the absence of the bacteria '*Ca. Liberibacter* spp.' through the reaction test polymerase chain (PCR) according to Wang et al. (2006).

Treatments application

Initially, treatments were applied according to the following sequence: solution S_1 was used for application A_1; S_3 for applications A_2, A_3, and A_4; solution S_2 for applications A_5 and A_6. All solutions were diluted into 1,000 L of water reservoir at an Arbus 2000® atomizer, with 2,000 L of total volume and water flow of 150 L minute $^{-1}$ at 450 rpm. Atomizer was pulled by a tractor in a constant speed. The orchard was irrigated by a dripping system. The volume of water applied to the plants was calculated through plants water necessity and soil tensiometry. The fertilizers ammonium nitrate (350 kg), calcium nitrate (190 kg), phosphate monoammonium (100 kg), and potassium chlorite (135 kg) were applied through 6 fertigations in the experimental area.

Assessment of nutritional status

In each experimental area and for P_1, P_2, and P_3, thirty fully expanded leaves were harvested from 3rd and 4th position in the branches and close to a growing fruit as reported by Raij et al. (1996). All harvested leaves were packed into paper bags and sent to Department of soil and fertilizers laboratory belonged to Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista to be assessed macro (N, P,

K, Ca, Mg, and S) and micronutrients (B, Cu, Fe, Mn, and Zn) contents.

Statistical analysis

Data were standardized and the scale of the variables was changed in order to obtain average equal to zero and standard deviation equal to one. Afterwards, data were subjected to exploratory factor analysis with multivariate statistics. The temporary values of charge factors were calculated. After that, rotation of the factors by Varimax method was preceded. The eigenvalues (λ) greater than or equal to unity was adopted to determine the number of factors to be used according to the selection criterium by Kaiser (1958). Then, with the factors 'score' the analysis of variance (ANOVA) was calculated. Tukey's test was applied on a 5% probability for multiple comparisons of means of plant status, solutions rates and number of applications, as well as, their interactions within each latent variable (Hair et al., 2009). Due to phosphorus content, an inconsistent factor was formed. This variable was removed from the multivariate analysis and its original data were submitted to analysis of variance, in which a polynomial regression analysis was used to the quantitative factor and Tukey's test for qualitative factors (Barbosa and Maldonado Junior, 2015).

Conclusion

Three cases related to 'Pêra Rio' variety mineral nutrition were assessed. The first one was constituted by variables: S, Cu, Fe, and Zn; the second one by N, K, and B and the third one by: Ca, Mg, and Mn. Nutritional status of that cultivar was destabilized with the infection *Ca.* Liberibacter spp. Progressive treatment with leaf fertilization and resistance bionductors temporarily re-established nutritional standards of 'Pêra Rio' affected by huanglongbing, but it did not promote stability of the nutritional status of these plants at the later stages.

Acknowledgment

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