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Zinc Sulfate and Bordeaux Mixture Treatment Towards Witches' Broom Disease of Mexican Lime in South of Iran

Shadab Faramarzi[*1], **Olly Sanny Hutabarat**[2]

[1]*Department of Plant Production and Genetics, Faculty of Agricultural Science and Engineering, Razi University, Kermanshah, Iran*
[2]*Hasanuddin University, Department of Agricultural Engineering, Makassar, South Sulawesi, Indonesia*

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ABSTRACT

Mexican lime growing in Hormozgan province is an awesome example of industrial horticultural crops production in Iran. However, over the last two decades, witch's broom disease of Lime (WBDL), caused by *Candidatus* Phytoplasma aurantifolia, has devastated Mexican lime orchards in south of Iran. The disease can result in shortened internodes and small leaves in the infected trees, then gradually leading to dry trees out within five to eight years. Furthermore, infected trees undergo significant changes in phenol compounds and enzymes related, protein pattern, and chlorophyll as well as carotenoid contents. Despite about 30 years of progress this disease, the necessity to combat WBDL is still of interest. In the current study, the role of zinc sulfate and Bordeaux mixture in controlling WBDL was assessed. The study was conducted in completely randomized design in autumn 2017 with three replications, so that each WBDL-infected tree was considered as an experimental replicate. WBDL-infected trees were foliar sprayed with zinc sulfate and Bordeaux mixture. The morphological characteristics, chlorophyll and carotenoid content, total phenol content and protein content were analyzed before and 40 days after treatments. Our obtained data indicated that zinc sulfate treatment can positively change morphological parameters including length leaf (125.2 ± 35.5 %), width leaf (158.3 ± 32.1%) and internode length (231.1 ± 48.8%), while application of Bordeaux mixture showed increasing influence on chlorophyll a, b and total chlorophyll content in WBDL-infected trees. The total phenol content remained almost steady in zinc sulfate treatment (3.7 ± 1.01 %) in comparison to Bordeaux mixture (-64.7 ± 11.06 %). In addition, Bordeaux mixture and zinc sulfate enjoyed significant influence on PI, about 65 % and 50 % respectively. Their effectiveness on Fv/Fm were 7.7 % in Bordeaux mixture and 5.2 % in zinc sulfate.

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1. Introduction

Mexican lime orchards in Hormozgan province are an awesome example of industrial horticultural crops production in Iran. However, over the last two decades, witch's broom disease of lime (WBDL) has devastated many areas of Mexican lime orchards in south of Iran (Mardi *et al*., 2011). The distribution of the disease was first encountered in Oman (Bové, 1986) from where it spread to the UAE, India, Pakistan (Ghosh *et al*., 1999) and later Iran (Bové, 2000). The causal agent of WBDL is *Candidatus Phytoplasma aurantifolia*, which is transmitted by the leafhopper vector *Hishimonus phycitis*. Many fruit species have been reported to host *Candidatus* phytoplasma species, such as papaya (Guthrie *et al*., 1998), foxtail Palm (Naderali *et al*., 2013) date palm (Alhudaib *et al*., 2014) coconut (Gurr *et al*., 2016), and jujube (Wang *et al*., 2018). In acid lime trees infected by phytoplasma, a bunch of shoots with shortened internodes and small leaves will emerge in one part of the tree and gradually spread to other parts. Eventually, the fruit yield of this trees decreases, and will die within three to eight years (Chung *et al*., 2006). In phytoplasma-infected trees significant changes in phenolic compounds and enzymes involved in the

[*] Corresponding author.
E-mail address: sh.faramarzi@razi.ac.ir

phenylpropanoid pathway (Mollayi *et al.*, 2016) auxin level and protein pattern (Zafari *et al.*, 2012) carbohydrate (Zafari *et al.*, 2012) chlorophyll and carotenoid content (Bertamini *et al.*, 2001; Taheri *et al.*, 2011; Faramarzi and Hassanzadeh, 2018) were observed.

The molecular mechanism and function of phytoplasma in host plants is still poorly understood. However, it has been shown that it is distributed through phloem to the meristem areas of the branch and other parts of the plant (Christensen *et al.*, 2004). Phytoplasma distribution in infected plants has been revealed by real-time PCR and bio-imaging (Christensen *et al.*, 2004) and also unevenly distribution has been reported, so that infected leaves contain a higher level of phytoplasma (Oropeza et al., 2011).

In the light of the above, the necessity to control of WBDL motivated us to compare the effects of zinc sulfate and Bordeaux mixture on phytoplasma-infected Mexican lime trees in order to elucidate their role. To address this issue, different parameters such as morphological characteristics, chlorophyll and carotenoid content, total phenol content and total protein were investigated. Considering WBDL as biotic stress, performance index (PI) and quantum yield (ratio of variable fluorescence and maximum fluorescence (Fv/Fm)) were determined.

2. Materials and methods

The study was conducted in a completely randomized design with three replications in a commercial citrus orchard located in Rudan county (Hormozgan province) in autumn 2017. The experiment was performed on 12-year-oldtrees were WBDL infected. Zinc sulfate ($ZnSO_4$) and Bordeaux mixture ($CuSO_4$ + CaO) (purchased from Merck Co.) were applied in the concentrations of 10,000 ppm and 20,000 ppm, respectively. The treatments were applied by means of foliar spraying. Leaves were collected before spraying and 40 days after treatments. Eventually, the following morphological and biochemical characteristics were assessed.

2.1. Morphological characteristics

The leaf length, leaf width and internode length were measured before and after treatment with a millimeter ruler.

2.2. Chlorophyll and carotenoid content

Half gram of fresh leaf was crushed in 10mL of 80% acetone and centrifuged at 6000 rpm for 10 minutes. A determined amount of supernatant was transferred to a cuvette and its absorbance was measured by spectrophotometer at 663nm, 645nm, and 470nm. From these values relative concentrations of chlorophyll and carotenoids (Lichtenthaler and Welburn, 1983) were calculated as follows:

$$\text{Chlorophyll a } (\mu g/mL) = 12.7\, A_{663} - 2.69\, A_{645}; \quad (1)$$

$$\text{Chlorophyll b } (\mu g/mL) = 22.9A_{645} - 4.68A_{663}; \quad (2)$$

$$\text{Total Chlorophyll } (\mu g/mL) = 20.2A_{645} + 8.02A_{663}; \quad (3)$$

$$\text{Total Carotenoid } (\mu g/mL) = (1000A_{470} - 3.27[\text{Chl a}] - 104[\text{Chl b}]) /227. \quad (4)$$

2.3. Total phenol content

From freeze-dried leaves, 0.2 g with 2 mL of ethanol (80%) were crushed and centrifuged at 1000 rpm for 15 minutes. Then, 25µL of extract was added to 175µL distilled-water, 1mL Folin-Ciocalteu (diluted1:10) and 800µL sodium carbonate (7.5%), and subsequently incubated for 2h at room temperature in darkness. After incubation, samples were measured using a spectrophotometer at 765nm (D'Angelo *et al.*, 2007).

2.4. Total protein content

The total protein content of the leaves was determined using a Kjeldahl method (Kjeltec 8100 model, Denmark). From freeze-dried leaf, 0.1g was digested with 0.5 g of a mixture of sulfates (200g potassium sulfate, 40 g copper sulfate and 2g selenium), followed by the addition of 5mL of sulfuric acid (98%), and the temperature increase to 200°C for 1h. Then the temperature was slowly increased to 380°C and kept there for 3h-4h. At the end of the digestion the extract was of green color. It was washed several times and finally the extract was made up to 50 mL (Rowell, 1994; Walling *et al.*, 1989). Then, protein content was obtained according the following formulas:

$$\%N = [1.4007\times(Va-Vb)\times N]/W \quad (5)$$

whereas Va stand for volume of acid used for sample titration, and likewise, Vb: volume of acid used for the blank, N: Normality of acid; W: sample weight in grams; 1.4007: conversion factor milliequivalent weight of nitrogen N percent; and eventually: Percent Crude Protein (CP) %CP = %N × F; F = 6.25. (6)

2.5. Chlorophyll fluorescence

The ratio of variable to maximum chlorophyll fluorescence of photosystem (F_v/F_m) and performance index (PI) was measured using a chlorophyll fluorimeter (Pocket PEA, chlorophyll fluorimeter) in non-phytoplasma-infected and phytoplasma-infected leaves (Strasser *et al*., 1995).

2.6. Data analysis

The percentage changes in the traits were calculated according to the following formula:

$$\Delta X\% = (X_{t2} - X_{t1})/X_{t1} \times 100 \quad (7)$$

whereas X= trait, t_1= before treatment; t_2 = after treatment.

3. Results and discussion

3.1. Morphological traits

Fig. 1 shows the positive changes of leaf size and internode length as a result of both treatments applied used in this study. The highest percentage of changes was related to $ZnSO_4$ treatment, with the following values: length leaf: 125.2 ± 35.5 %, width leaf: 158.3 ± 32.1 and internode length: 231.1 ± 48.8; while the values corresponding to the changes of length leaf, width leaf and internode length were 12.8 ± 3.3 %, 65.9 ± 22.4 % and 251.6 ± 45.6 %, respectively. Previous results revealed that foliar application of GA3 (600 ppm) can positively affect leaf length and internode elongation (Faramarzi and Hasanzadeh, 2018). In addition,the utilization of copper sulfate (20,000 ppm) can cause recovery effect through regenerating new shoots with no phytoplsma (Faramarzi *et al*., 2018)..Due to the important role of zinc in biosynthesis and metabolism of GA3, foliar application of zinc sulfate can significantly improve leaf morphology. Ćurković Perica (2008) reported that the use of auxin in vitro can improve recovery of infected shoots in periwinkle with different '*Candidatus* Phytoplasma' species. It was also showed that BA (cytokinin) can cause methylation of periwinkle genome infected with *Ca*. P. asteris, but the IBA did not have such an effect (Leljak-Levanić *et al*., 2010).

3.2. Chlorophyll and carotenoid content

Fig. 2 shows that Chlorophyll a, chlorophyll b, total chlorophyll content as well as total carotenoid content possess higher changes in Bordeaux mixture treatmentin comparison to zinc sulfate treatment, so that the percent of changes were obtained 16.6 ± 4.1 % I Chlorophyll a, 14.46 ± 2.6 in chlorophyll b and 21.59 ± 4.1 % in total chlorophyll content. On the contrary, changes of total carotenoid content was negative with the utilization of zinc sulfate and Bordeaux mixture (-56.6 ± 10.2 % and – 55.7 ± 12.8 %, respectively). It would be suggested that Bordeaux mixture can boost photosynthesis efficiency in phytoplasma-infected Mexican lime trees. Zafari et al. (2012) reported that the presence of phytoplasma in Mexican lime can reduce chlorophyll content in the leaf. In addition to decreasing the amount of chlorophyll a and b in phytoplasma-infected leaves, the ratio of chlorophyll a to b also decreased, which is an indicator for the level of photosynthesis activity. In grape, phytoplasma-infected leaves showed low levels of chlorophyll and carotenoid content (Bertamini and Nedunchezhian, 2001).

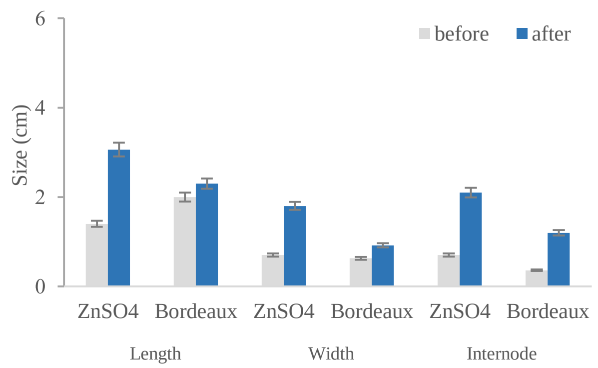

Figure 1. Comparison of leaf size (length and width) and internode length changes before and 40 days after zinc sulfate and Bordeaux mixture treatments in this study.

3.3. Total phenol content and total protein content

The total phenol content (TPC) remained almost steady in zinc sulfate treatment after application (3.9 ± 1.01 %) in comparison to Bordeaux mixture (-64.7 ± 11.06 %), demonstrating a negative effect of Bordeaux mixture on TPC (Table 1). Mollayi et al. (2016) showed that in acid lime trees infected with phytoplasma, the catechin and epicathechin increased by 390% and 250%, respectively after 30 days, while after 150 days a stark decrease had occurred. They also reported that changes in catechin and epicathechin are associated with changes in phenolic pathway's enzymes such as phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) and malondialdehyde (MDA).

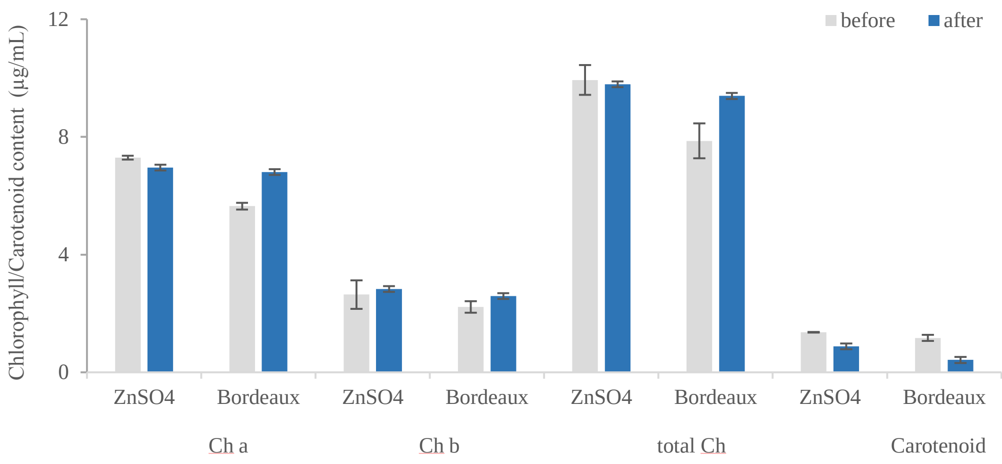

Figure 2. Changes of chlorophyll and carotenoid content before and 40 days after zinc sulfate and Bordeaux mixture treatments.Ch a, Ch b, and total Ch stand for chlorophyll a, chlorophyll b and total chlorophyll,respectively.

In regard to total protein content, both treatments did not prevent decreasing of total protein content. The percent of changes was observed -36.7 ± 3.7 % in zinc sulfate and -6.5 ± 1.34 % in Bordeaux mixture (Table 1) Although Bordeaux mixture was more effective than zinc sulfate concerning total protein content reduction. Zafari et al. (2012) demonstrated that phythoplasma agent of WBDL can decrease total protein content in lemon leaves. The results of SDS-PAGE in order to compare the protein pattern showed a significant difference in protein pattern in healthy lime (control) and phytoplasma- infected lime. They found a 76-kDa protein in healthy plants and two proteins of 15 and 26 kDa in infected- phytoplasmatic plants as well. Phytoplasma -infected leaves in grape also possessed lower levels of total soluble protein compared to healthy leaves (Bertamini and Nedunchezhian, 2001).

Table 1. Percent of changes in total phenol content (mg GAE/ g DW) and total protein content before and 40 days after zinc sulfate and Bordeaux mixture treatments (kjeldah method).

Trait / Treatment	% changes	
	Total phenol content	Total protein content
ZnSO4	3.7 ± 1.01	-36.7 ± 3.7
Bordeaux mixture	-64.7 ± 11.06	-6.5 ± 1.34

Table 2. Percent of changes in PI and Fv/Fm values seen in non-WBDL shoots and treated WBDL-shoots before and 40 days after zinc sulfate and Bordeaux mixture treatments.

Shoot type / Treatment	PI non-WBDL/non-treated	PI WBDL/treated	Fv/Fm non-WBDL/non-treated	Fv/Fm WBDL/treated
$ZnSO_4$	0.317 ± 0.05^{a}	0.640 ± 0.13^{a}	0.720 ± 0.08^{a}	0.760 ± 0.21^{a}
Bordeaux mix	0.220 ± 0.03^{b}	0.643 ± 0.1^{a}	0.683 ± 0.11^{b}	0.740 ± 0.14^{ab}

Numbers with the same letters are not significantly different according to the PLSD test.

3.4. Chlorophyll fluorescence

Considering WBDL as biotic stress, the comparison between uninfected and infected leaves was carried out from PI and Fv/Fm points of view (Table 2). It was observed that both treatments had the significant effects on PI in infected leaves comparing to non-phytoplasma-infected leaves. In fact, Bordeaux mixture enjoyed higher influence on PI, about 65 % and zinc sulfate showed nearly 50 % effectiveness. PI (Performance Index) reflects the function of both photosystems I and II; whereas F_v/F_m (Quantum Yield) is defined as the ratio of variable florescence and maximum florescence of PS II. The effect of both treatments on Fv/Fm were observed 7.7 % and 5.2 % in Bordeaux mixture and zinc sulfate, respectively (Table 2). Photosynthesis in phytoplasma-infected leaves was reduced due to diminish chlorophyll content and as such, these leaves cannot produce enough carbohydrates to meet their needs. However, the amount of starch in infected leaves significantly is increased (Tan *et al*., 2015).

4. Conclusion

We conclude that application of zinc sulfate and Bordeaux mixture can improve phytoplasma –infected Mexican lime trees from morphological and biochemistry points of view. Since, trees infected by WBDL are dried out within 5-8 years, the study of this disease is too problematic. However, combination therapy such as cutting infected branches out along with foliar application of mineral compounds may be an effective approach. Accordingly, it can be suggested to study divers concentrations of ZnSO4 and Bordeaux mixture individually and/or combined as well as different applications times on WBDL- infected Mexican lime. Taking into account the results obtained during our study, we are highly optimistic that this work will provide a route-map for tailoring the anti-phytoplasma mineral compounds that could help in finding promising phytoplasma inhibitors for WBDL.

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