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SHORT NOTE

Development and morphological changes in leaves and branches of acid lime (*Citrus aurantifolia*) affected by witches' broom

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Summary. Witches' broom (WB), associated with the presence of '*Candidatus* Phytoplasma aurantifolia', is one of the most serious diseases of acid lime. This study determined incidence, distribution, and development of the disease, and morphological changes in leaves and branches of affected host plants. Survey in different parts of Oman showed that WB occurs in most regions in the country, where 108 out of 158 (68%) surveyed farms were found to have diseased trees. A survey of 6,926 acid lime trees showed that severity of WB was positively related ($r = 0.948$; $P<0.01$) to tree age. The mean percentage of symptomatic branches was 1% in 3-year-old trees compared to 63% in 12-year-old trees. To further characterize morphological changes in WB-affected limes, apical stems (40 cm long) were collected from three infected trees during the autumn of 2009 and spring of 2010. Increases in the numbers of leaves (1,208%), numbers of branches (309%) and total length of branches (712%) were recorded for symptomatic branches relative to non-symptomatic branches. In the spring of 2009 these respective increases were 159%, 243% and 121%.Overall area of leaves in the symptomatic branches was 81% less than for non-symptomatic branches in the autumn of 2009 and 34% less in the spring of 2010. This study is the first to characterize morphological changes in leaves and branches of acid lime affected by WB.

Key words: phytoplasma, '*Candidatus* Phytoplasma aurantifolia'.

Introduction

Acid lime (*Citrus aurantifolia* L.) is a small-fruited citrus type, distinguishable from the seedless, large fruited Tahiti lime (*Citrus latifolia*) and lemons (*Citrus limon*). It is a traditional crop that is used fresh for its juice, or dried, and it is a major fruit crop in Oman. Lime is among the major fruit crops in terms of production, and constitutes 4% of all fruit crops grown in the country. Acid lime is also known as Omani, Indian, Mexican or Key lime (Hodgson, 1967). In addition to the Middle East, lime is also produced in India, Pakistan, Florida, Mexico and other countries. Sun-dried limes were previously a major export commodity from Oman, perhaps second only to dates. In many countries dried limes are still known as "Omani-limes", indicating their exportsource.

Acid lime is affected by several diseases, which include witches' broom (WB), *Citrus tristeza virus* and a number of viroids, fungal diseases and nematodes (Moghal *et al*., 1993; Al-Sadi *et al*., 2012a; 2012b; 2013a; 2013b). Witches' broom, which is associated with '*Candidatus* Phytoplasma aurantifolia', is the most serious disease of lime in the Middle East. This disease was reported in the 1970s and early 1980s in Oman and the United Arab Emirates (Bové *et al*., 1988; Garnier *et al*., 1991; Chung *et al*., 2006). The disease has killed over half a million lime trees in Oman

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since 1990, and has resulted in the loss of more than 50% of the cultivated lime area and 75% of production (FAO, 2013). The disease has been reported in India (Ghosh *et al*., 1999), Iran (Bové *et al*., 2000) and Saudi Arabia (Alhudaib *et al*., 2009). To date, there are no reports of occurrence of host resistance to WB (Al-Sadi *et al*., 2012b). Because the last comprehensive survey of WB in Oman was in the 1980s (Bové *et al*., 1988), little is known about the current distribution of the disease in this country.

Descriptions of WB have shown that infected lime trees develop shoots with small and clustered leaves and branches, which are produced in large numbers (Garnier *et al*., 1991; Chung *et al*., 2006). These symptoms may appear on any part of the canopies of affected trees, with varying degrees of severity during the year. More shoots develop symptoms with time until most trees collapse within 3-8 years of first symptom appearance. However, knowledge of development of WB symptoms in relation to age of lime trees is still lacking. In addition, little is known about differences in the size and numbers of leaves and branches between symptomatic and asymptomatic shoots on individual same trees affected by the disease.

Studies have shown that WB phytoplasma can also spread to other cultivated and wild plants in Oman. This graft-transmissible and presumably vector-borne pathogen can also be transmitted via the parasitizing dodder plant (*Cuscuta campestris*). In the field, severe symptoms appear on citranges and limes, but not on lemon (*C. limon*), sweet lime (*C. limettioides*), sweet orange (*C. sinensis*), pummelo (*C. grandis*), sour orange (*C. aurantium*), grapefruit (*C. paradisi*) or rough lemon (*C. jambhiri*). Experimentally, sweet orange and sour orange were found tolerant or resistant to the WB phytoplasmas, but not Troyer citrange (Garnier *et al*., 1991; Chung *et al*., 2006).

The present study was conducted to investigate: incidence and distribution of WB in different regions in Oman, development of WB in relation to time; and effects of WB on the number and size of leaves and branches of lime trees. The study aimed to provide a basis for future investigations of the anatomical and physiological changes in acid limes affected by WB.

Materials and methods

Incidence and distribution of WB

A survey was carried out from 2009 to 2013 in 158 lime growing farms located in eight geographical regions in Oman (Figure 1). Data were collected on farms with trees showing typical symptoms of WB. In addition, comprehensive data were collected from 73 of these farms, on the number of lime trees grown, age of trees and disease severity. Disease severity on each tree was expressed as percentage of lime branches developing typical WB symptoms out of the total branches.

Figure 1. Map of Oman showing the eight regions which were covered during a survey of occurrence of witches' broom in acid lime farms.

Influence of WB on morphology of branches and leaves

Samples of branches were collected from phytoplasma-infected acid lime trees showing WB symptoms in order to quantify the effects of the disease on leaf and branch morphology. The trees were 11 years old and were growing at the Agricultural Experimental Station of Sultan Qaboos University, Al-Khod, Oman. These trees had shown WB symptoms for more than 6 years. Collection of samples from three trees was carried out in the autumn of 2009 and the spring of 2010. Apical stems were collected from infected

trees with WB leaf symptoms (symptomatic) and no symptoms (asymptomatic). A total of sevensymptomatic branches and seven asymptomatic branches were collected from the trees in autumn 2009, and four symptomatic and three asymptomatic branches were collected from two of the trees in spring 2010.

To characterize morphological changes in leaves and branches of acid lime infected by WB, apical stems (40 cm long) were divided into two batches, symptomatic and asymptomatic. The total number of leaves and main branches were determined in each batch. In addition, the leaf and branch samples were then digitized using a standard optical scanning device. The scanned leaves were then imported to computer software (Assess, APS Press), to determine total leaf area and total length of branches. Comparison with asymptomatic branches from uninfected healthy trees was carried out, to avoid introducing other sources of variation such as tree genotype and cultivation practices.

Data analyses

Data were analyzed using SAS software (SAS Institute). Differences between symptomatic and asymptomatic branches for leaf number, leaf size, number of branches and length of branches were determined using Duncan's Multiple Range Test.

Phytoplasma detection

Young leaf samples were collected from symptomatic and asymptomatic acid lime trees during the survey and also from trees included in the assessment of morphology of leaves and branches. Leaf samples were collected from at least ten trees from each geographical region. Each sample consisted of approx. 20 lime leaves, and the samples were sealed in plastic bags, labeled, and placed in an ice box after field collection. The samples were then stored at -80ºC until assessed. Presence of phytoplasma in the symptomatic and asymptomatic acid lime leaves was confirmed as described previously (Al-Sadi *et al.*, 2012b). In brief, about 5 g of leaf tissue from symptomatic or asymptomatic leaves obtained from the three trees examined in this study was ground into fine powder in liquid nitrogen. DNA was then extracted from 0.1 g samples using GenElute Plant Genomic DNA Extraction Kit (G2N70, Sigma-Aldrich) according to manufacturer's protocol.

The PCR reaction mixture consisted of 1 μL of DNA preparation (approx. 25 ng), 0.4 μM of primers P1 and P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995), PuReTaq™ Ready-To-Go™ PCR beads (HVD Life Sciences) and Milli-Q purified water up to a final reaction mixture volume of 25 μL. The product of the direct PCR was diluted (1:40) prior to re-amplification by nested PCR, using primer pair R16R2 and R16F2n (Gundersen and Lee 1996). The PCR mixture and conditions were as described by Al-Sadi *et al*. (2012b). Negative controls were used in the direct and nested PCRs. After amplification, a 5 μL aliquot from each sample from the direct and nested PCR assays was electrophoresed on 1.5% agarose gel stained with ethidium bromide, and visualized under a UV transilluminator.

Results

Incidence and distribution of WB

Survey in different parts of Oman showed that the symptoms of WB were present in all regions, with the exception of Dhofar (Figure 1; Table 1). Out of 158 farms surveyed, WB symptoms were detected on trees on 108 farms (68%; Table 1). With the exception of Dhofar, incidence of the disease in other regions ranged from 62% to 100% (mean 81%).

Table 1. Incidence of witches' broom (WB) (% affected acid lime farms) in different regions of Oman.

Region	Sample size (No. of farms)	Number of of WB infected farms	% of WB affected farms
Batinah	36	33	92
Buraimi	6	4	67
Dakiliya	21	13	62
Dhahira	14	12	86
Dhofar	25	0	0
Musandam	20	17	85
Muscat	9	9	100
Sharqiya	27	20	74
Overall	158	108	68

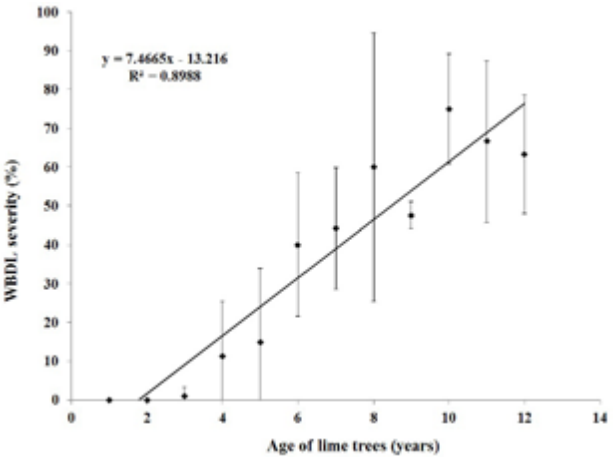


Figure 2. Development of witches' broom symptoms in relation to age of acid lime trees. Disease severity represents the percentage of symptomatic branches in trees. The data are based on a survey of 73 farms and 6,926 acid lime trees, of age 1 to 12 years. Error bars indicate standard deviations.

Detailed investigation of 6,926 acid lime trees from 73 farms showed that typical symptoms of WB begin to appear in trees which are at least 2 years old. Severity of WB was found to have a significant and positive relationship ($r = 0.948$; $P<0.01$) with tree age. The percentage of symptomatic branches on trees was found to increase from a mean of 1% in 3 year old trees to 63% in 12 year old trees (Figure 2). Variation was observed in disease severity, however, for the same tree age within individual farms, and among different farms.

WB influence on morphology of branches and leaves

Affected trees developed WB symptoms which included small, pale green yellow or chlorotic leaves which appeared in clusters. The trees showed a few witches' brooms, with the other parts of the each plant remaining totally symptomless.

The number of leaves in the symptomatic branches was 1,200% more than in the asymptomatic branches, as recorded in the autumn of 2009, and up to 159% more as observed in the spring of 2010 (Figures 3 and 4). In addition, there were increases in the numbers of branches and the lengths of branches by, respectively, 309% and 712% in the autumn of 2009, and by 243% and 121% in the spring of 2010. The increase in the number of leaves and branches was accompanied by an 81% reduction in leaf area in the autumn of 2009, and by 34% in the spring of 2010 (Figure 4).

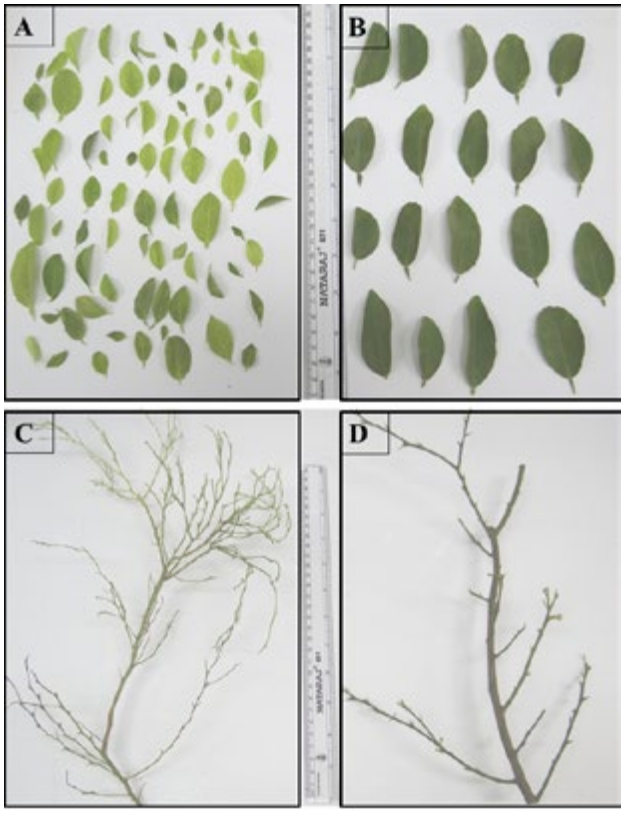
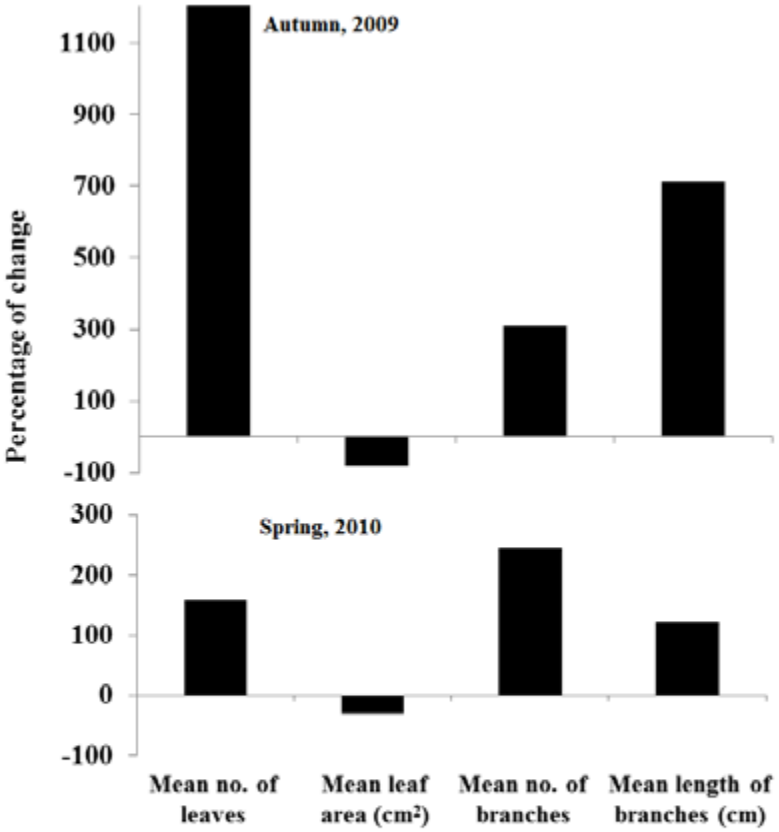


Figure 3. Leaves and branches of acid lime from witches' broom symptomatic (A, C) and asymptomatic (B, D) branches.

Phytoplasma detection

Polymerase chain reaction amplification using primer pairs P1/P7 and R16F2n/R2 yielded, respectively, fragments of 1.8 kb and 1.2 kb. Phytoplasma was detected in all the symptomatic leaves which were collected from different parts of the country. In addition, phytoplasmas were detected in asymptomatic leaves collected from nine samples from Dhofar in which the typical symptoms of WB were not observed. Phytoplasmas were detected also in both symptomatic and asymptomatic leaves collected from trees which were used in the experiment on effect of WB on morphology of leaves and branches.



		Mean no. of leaves	Mean leaf area (cm^2)	Mean no. of branches	Mean length of branches (cm)
Fall	Asymptomatic	63.1 ±14.9 b	9.36 ±0.99 a	5.6 ±1.6 b	109.8 ±25.2 b
	Symptomatic	825.1 ±153.7 a	1.74 ±0.29 b	22.9 ±4.0 a	892.0 ±206.8 a
Spring	Asymptomatic	27.7 ±3.5 a	11.02 ±0.47 a	2.33 ±0.7 b	45.3 ±8.6 b
	Symptomatic	71.8 ±42.2 a	7.27 ±1.76 b	8.0 ±3.6 a	100 ±29.2 a

Figure 4. Percentage changes in mean numbers of leaves, leaf areas, numbers of branches and length of branches in symptomatic acid lime branches affected by witches' broom. Values below the figure indicate mean values and standard errors. Values accompanied by the same letter in the each column and season are not significantly different ($P < 0.05$; (Duncan's Multiple Range Test).

Discussion

This study has shown that typical symptoms of WB are present in all the surveyed regions in Oman, except for Dhofar. Although disease symptoms were observed in about 68% of the visited farms, the proportion of farms which are affected by WB is greater than this. Many lime growers commonly manage the disease by selectively removing symptomatic branches from infected trees. This practice may have led to the removal of WB symptoms in affected farms, leading to under-estimation of proportions of infected trees. This is in addition to farms with lime trees younger than 2-years-old that normally do not show WB symptoms. Detecting phytoplasmas in asymptomatic trees may also indicate that the percentage of farms affected by WB in Oman is greater than the proportion of farms where typical WB symptoms were observed.

Although typical symptoms of WB were not observed on 25 farms in Dhofar in the southern part of Oman, PCR-based detection confirmed the presence of the WB phytoplasma in nine lime trees. This raises a question relating to factors suppressing disease symptoms in the southern part of the country, where

climatic conditions are different from other parts of Oman. The temperature in most areas in Oman usually ranges from 15–22ºC in winter to 40-48ºC in summer. However, the southern part of the country is characterized by a monsoon season between June and September. The temperature usually falls to 20-30ºC during this season, which is associated with rainfall. It is therefore not clear whether the climatic conditions have effects on WB suppression or on the distribution of any potential vectors, and these questions need to be addressed in future studies. Occurrence of WB in some farms in the southern part of Oman raises the possibility of spread of WB to an area which has been considered disease-free. Future studies may also be required to assess the WB situation on farms in the southern part of Oman in order to limit the further spread of the phytoplasma to disease-free districts in the south.

Symptoms of WB usually appear on affected trees as clusters of branches with small leaves. Although many branches remain symptomless at the beginning of phytoplasma infection, more young branches start to develop symptoms with time, which is usually followed by drying and death of the small leaves (Chung *et al.*, 2006). The present study has provided evidence that severity of WB increases with tree age. Although many trees collapse and die within 3 to 5 years from symptom appearance (Chung *et al.*, 2006), findings from our survey showed that some trees can survive more than 8 years after first appearance of symptoms. These observations indicate that unknown factors play roles in the period for affected trees to collapse. It is also possible that there are differences in reaction to the disease among different host genotypes. These factors require elucidation through further investigations.

Previous studies have shown that other phytoplasmas can cause excessive production of host leaves and branches following infection (Al-Zadjali *et al.*, 2012). The present study has provided further evidence that the numbers of leaves, and the numbers and lengths of branches, increased due to phytoplasma infection. The increase in the number of leaves and branches was accompanied by reductions in leaf area in the symptomatic leaves, which may be related to the direct effects of phytoplasma on leaves, or to competition for nutrients among the excessive number of leaves which are produced in the symptomatic branches. Although some studies have addressed interactions of phytoplasma with acid lime (Taheri *et al.*, 2011; Zamharir *et al.*, 2011), the factors affecting the excessive production of leaves and branches and reduction in leaf size deserve further investigation.

This study provides evidence that WB has spread to most regions in Oman. Unless adequate actions are undertaken, all lime growing regions of the country will be affected by the disease. Future studies are required to document spread of the disease in the southern part of the country, investigate factors suppressing symptoms in some locations, and also investigate factors causing reduced size of leaves and increased number of leaves in phytoplasma infected trees.

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Literature cited

Al-Sadi A.M., S.A. Al-Hilali, R.A. Al-Yahyai, F.A. Al-Said, M.L. Deadman, I.H. Al-Mahmooli, G. Nolasco, 2012a. Molecular characterization and potential sources of Citrus Tristeza Virus in Oman. *Plant Pathology* 61, 632–640.

Al-Sadi A.M., H. Al-Moqbali, R. Al-Yahyai, F. Al-Said, I. Al-Mahmooli, 2012b. AFLP data suggest a potential role for the low genetic diversity of acid lime (*Citrus aurantifolia*) in Oman in the outbreak of witches' broom disease of lime. *Euphytica* 188, 285–297.

Al-Sadi A.M., A.G. Al-Ghaithi, N. Al-Fahdi, 2013a. Characterization and pathogenicity of fungal pathogens associated with root diseases of Citrus in Oman. *International Journal of Agriculture and Biology* 16, 371–376.

Al-Sadi A.M., A.N. AL-Wehaibi, R.M. Al-Shariqi, M.S. Al-Hammadi, I.A. Al-Hosni, I.H. Al-Mahmooli, A.G. Al-Ghaithi, 2013b. Population genetic analysis reveals diversity in *Lasiodiplodia* species infecting date palm, Citrus, and mango in Oman and the UAE. *Plant Disease* 97, 1363–1369.

Al-Zadjali A.D., A.M. Al-Sadi, M.L. Deadman, S. Okuda, T. Natsuki, T.A. Al-Zadjali, 2012. Detection, identification and molecular characterization of a phytoplasma associated with beach naupaka (*Scaevola taccada*) witches' broom. *Journal of Plant Pathology* 94, 379–385.

Alhudaib K., Y. Arocha, M. Wilson, P. Jones, 2009. Molecular identification, potential vectors and alternative hosts of the phytoplasma associated with a lime decline disease in Saudi Arabia. *Crop Protection* 28, 13–18.

Bové J.M., M. Garnier, A.M. Mjeni, A. Khayrallah, 1988. Witches' broom disease of small-fruited acid lime trees in Oman: First MLO disease of Citrus. In: *Proceedings of the 10th Conference of the International Organization of Citrus Virologists (IOCV)*, Riverside, 307–309.

Bové J.M., J.L. Danet, K. Bananej, N. Hassanzadeh, M. Taghizadeh, M. Salehi, M. Garnier, 2000. Witches' broom disease of lime (WBDL) in Iran. In: *Proceedings of the fourteenth Conference of IOCV*, Riverside, 207–212.

Chung K.R., I.A. Khan, R.H. Brlansky, 2006. Citrus diseases exotic to Florida: Witches' broom disease of lime (WBDL). *EDIS Publications, University of Florida, Gainesville* PP228, 1–3.

Deng S.J., C. Hiruki, 1991. Genetic relatedness between two non-culturable mycoplasmalike organisms revealed by nucleic acid hybridization and polymerase chain reaction. *Phytopathology* 81, 1475–1479.

FAO, 2013. FAOSTAT-Agriculture. http://faostat.fao.org/site/567/default.aspx#ancor.

Garnier M., L. Zreik, J.M. Bové, 1991. Witches' broom, a lethal mycoplasmal disease of lime trees in the Sultanate of Oman and the United Arab Emirates. *Plant Disease* 75, 546–551.

Ghosh D.K., A.K. Das, S. Singh, S.J. Singh, Y.A. Ahlawat, 1999. Occurrence of witches' broom, a new phytoplasma disease of acid lime (*Citrus aurantifolia*) in India. *Plant Disease* 83, 302.

Hodgson R.W., 1967. Horticultural varieties of citrus. In: *The citrus Industry* (W. Reuther, L.D. Batchelor and H.D. Webber, ed.), University of California Press, Berkeley, CA, USA, 431–591.

Moghal S.M., P. Shivanathan, A. Mani, A.D. Al-Zidjali, T.S. Al-Zidjali, Y.M. Al-Raeesy, 1993. *Status of Pests and Diseases in Oman: Series 1: Plant Diseases in the Batinah*. Ministry of Agriculture and Fisheries, Muscat, Oman.

Schneider B., E. Seemuller, C.D. Smart, B.C. Kirkpatrick, 1995. Phylogenetic classification of plant pathogenic mycoplasmalike organisms or phytoplasmas. In: *Molecular and Diagnostic Procedures in Mycoplasmology* (S. Raszin and J.G. Tully, ed.), Academic Press, New York, NY, USA, 369–380.

Taheri F., G. Nematzadeh, M.G. Zamharir, M.K. Nekouei, M. Naghavi, M. Mardi, G.H. Salekdeh, 2011. Proteomic analysis of the Mexican lime tree response to '*Candidatus* Phytoplasma aurantifolia' infection. *Molecular Biosystems* 7, 3028–3035.

Zamharir M.G., M. Mardi, S.M. Alavi, N. Hasanzadef, M.K. Nekouei, H.R. Zamanizadeh, A. Alizadeh, G.H. Salekdeh, 2011. Identification of genes differentially expressed during interaction of Mexican lime tree infected with '*Candidatus* Phytoplasma aurantifolia'. *BMC Microbiology* 11, 1–9.

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