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Screening for tolerance of different citrus rootstocks against zoospores of *Phytophthora nicotianae* in infested soil

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Abstract

Citrus gummosis, caused by *Phytophthora nicotianae*, becomes one of the most destructive diseases in citrus production in Tunisia. Thus, the selection of resistant rootstocks is needed to reduce the damage caused by this disease. In the present study, five citrus rootstocks (Citrange carrizo (*Citrus sinensis* x *Poncirus trifoliata*), Citrumelo Swingle-4475 (*Citrus paradisi* x *Citrus trifoliata*), Citrus volkameriana (*Citrus limon* x *Citrus aurantium*), Citrange C-35 (*Citrus sinensis 'ruby blood'* x *Poncirus trifoliata*) and Sour orange (*Citrus aurantium*)) were screened against *P. nicotianae* by inoculating young citrus seedlings with the freshly zoospores suspension. After three months of inoculation, the disease severity was evaluated based on root damaged, plant height, stem and root weights (fresh and dry) and density of *P. nicotianae* population in infested roots. The evaluation of the rootstocks response revealed different levels of susceptibility against *P. nicotianae*. Regarding to all the parameters studied, results showed that the rootstock Citrumelo Swingle-4475 and the rootstock Sour orange are tolerant to *P. nicotianae*. The rootstock Citrange C-35 was considered as a moderately tolerant, while the two rootstocks Citrange carrizo and Citrus volkameriana were very susceptible. The index severity was ranged from 1 for the tolerant rootstocks to 3.16 for the very susceptible one. The highest percent of the stem growth rate was 75.5 % recorded with the tolerant rootstocks, while it ranged between 18.25 % and 19 %, respectively, for the moderately tolerant and for the susceptible rootstocks. In the case of the other parameters like the fresh and the dry weight of stem and root, the tolerant rootstocks showed the minimum percent of reduction (17.8 %). However, the minimum percent of reduction of the moderately tolerant and of the very susceptible rootstocks was ranged between 38.5 and 30.8, respectively. The lowest number of propagules of *P. nicotianae* was found in the tolerant rootstocks (4.25), the highest number was found in the very susceptible rootstocks (24.5). However, the number was 12.25 in the case of the moderately tolerant one. It is believed that the rootstock Citrumelo Swingle-4475 should be involved with the resistance of citrus plants to *P. nicotianae*.

Key words: Citrus, gummosis, *P. nicotianae*, rootstocks, screening.

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Introduction

Citrus is one of the most important tree fruits and is widespread in the word (Cimen & Yesiloglu, 2016). In Tunisia, citrus is now grown on over 22 000 ha, located mainly in the Cap-bon regions of Nabeul government (CTA, 2016). In the last years, symptoms of gummosis have been observed in the major citrus producing areas of Tunisia and became a serious problem for citrus production. The infection by the gummosis occurs near the ground level of the scion and produces lesions which extend down to the bud union on resistant rootstocks, or up the trunk into the major limbs of the tree (Graham & Feichtenberger, 2015). Ten species of *Phytophthora* have been reported infecting citrus around the world (Erwin & Ribeiro, 1996), but *P. nicotianae* is the most common species occurring in subtropical areas of the world (Graham & Feichtenberger, 2015). It's also one of the most important soil-borne pathogen of citrus which causes mortality of trees (Verniere et al., 2004). Gummosis is responsible for 10 to 30 percent of losses in citrus grown around the world (Timmer et al., 2000). It remains a threat and a persistent problem wherever, citrus is grown that can result in substantial tree loss, particularly trees on susceptible rootstock (Whiteside, 1973). The management of *Phytophthora* diseases of citrus tends to be an integrated approach that includes the production and the use of *Phytophthora* free nursery stock, of resistant/ tolerant rootstocks, an appropriate cultural practices to mitigate disease development, of chemicals as prophylactic and/or cure of the diseases and of biological antagonists (Naqvi, 2004). Losses attributable to *Phytophthora* gummosis have been reduced through the use of the systemic fungicides such as fosetyl-Al and metalaxyl and a single application of either fungicide can provide maximum protection from colonization by *P. citrophthora* and *P. nicotianae* for at least 3 months (Matheron & Matejka, 1988). However, *Phytophthora* spp. are known to develop resistance to metalaxyl after its repeated use (Timmer et al., 1998). Also, they are not always desirable due to the high costs of application, potential hazards to the environment and the development of fungicide-resistant strains (Faldoni et al., 2015). The use of tolerant rootstock with desirable horticultural characteristics is the best management strategy of *Phytophthora* diseases in order to reduce the costly applications of fungicides (Naqvi, 2004). Also, rootstock choice is one of the most important aspects in orchard management because scion cultivars respond differently to growth, fruit quality and nutrient accumulation when grown on diverse rootstocks (Dubey & Sharma, 2016). However, the resistance of the rootstock is relative (Benyahia et al., 2004). Thus, selection and breeding for resistance to *Phytophthora* in citrus species becomes necessary to control the disease (Boava et al., 2011). As a solution, the screening and the development of new rootstocks became a vital aspect in citriculture (Graham, 1995). A variety of methods have been used, in the past, to determine the susceptibility of citrus rootstocks to *Phytophthora* species (Matheron et al., 1998). After all, the rootstock growth, the vigor and the ability to proliferate roots in infested soil have been noted as

important plant defense traits that protect against pathogens (Graham, 1995). The most used rootstock in Tunisia and in the Mediterranean region is the rootstock Sour orange (*Citrus aurantium*) due to its wide adaptability to soil types, its better affinity with most commercial varieties and its good resistance to *Phytophthora* gummosis (Benyahia et al., 2004). However, it is observed in many citrus orchards in Tunisia that all the infected trees of citrus by gummosis are grafted onto sour orange rootstock. Thus, the main objectives of this investigation were to: (i) screen the resistance of different rootstocks of citrus for the soil infection of *P. nicotianae,* and (ii) to identify aggregates of rootstocks that induced similar characteristics to *P. nicotianae* inoculation, using a multivariate clustering analysis technique.

Materials and methods

Plant material and growth conditions: The experiment was carried out, between October and December 2015, in a plastic tunnel at temperatures between 25°C and 30°C, at the Higher Institute of Agriculture of Chott Mariem (Tunisia). In this assay, five 3 months old cultivars of citrus rootstocks, provided by the Technical Centre of Citrus of Tunisia (C.T.A), were investigated for their tolerance against two isolates of *P. nicotianae*. The cultivars of rootstocks used were Sour orange (*Citrus aurantium L.*), Citrange carrizo (*Citrus sinensis* x *Poncirus trifoliata*), Citrumelo Swingle-4475 (*Citrus paradise* x *Citrus trifoliata*), Citrange C-35 (*Citrus sinensis 'ruby blood'* x *Poncirus trifoliata*) and Citrus volkameriana (*Citrus limon* x *Citrus aurantium*). We have chosen the rootstock Sour orange, who is the most rootstock used in Tunisia as reference to accurately estimate the tolerance of the other rootstocks. The different rootstocks were placed in plastic pots about 15 cm deep and 8 cm in diameter, containing a filled with a mixture of sterile sand-peat. The seedlings were irrigated daily.

Inoculum used: Two isolates of *P. nicotianae* (P.15 and P.128), mating type A2, obtained from citrus infected trees by gummosis in Tunisia, have been used in this assay. These pathogens were isolated on PARP-BH selective medium (CMA (Corn Meal agar) amended with Pimaricin, Ampicilin, Rifampicin, Benomyl, Pentachloronitrobenzene and Hymexazol) as described by Erwin and Ribeiro (1996). The identity of isolates was confirmed by their morphological traits (Erwin & Ribeiro, 1996) and by polymerase chain reaction (PCR) using species-specific (White et al., 1990) (Table 1). These isolates had been shown in previous studies to be pathogenic to citrus trees. For a long-term storage, a 5-mm-diameter of agar plug from the edge of each isolate was placed onto a 25-ml tube with ~15 ml of sterile soil solution and they were maintained in the collection of the laboratory of Phytopathology, Department of Biological Science and Plant Protection, I.S.A of Chott Mariem, in Tunisia. Fresh cultures were prepared by transferring agar disks with mycelium of each isolates to PDA medium. The plates were then transferred to an incubator at 25±1°C, for 5 days, until the mycelium covered the agar surface.

Preparation of inoculum: The two

isolates of *P. nicotianae* were maintained on V_8 medium (2 g $CaCO_3$, 200 ml V_8 juice and 15 g agar in 800 ml distilled water) and incubated for 5 days in the dark, at 25±1°C. Plugs were cut from the V_8 medium with a cork borer. Then, zoospores suspensions were produced by the method of Henderson et al. (1986). Zoospore densities were estimated with haemocytometer and the suspensions were diluted in deionized water to obtain $4x10^4$ zoospore mL^{-1}. Inoculum suspensions were plated on PARP-BH selective medium (containing 20 g of corn meal agar amended with 10 µg pimaricin, 200 µg ampicillin, 10 µg of rifampicin, 25µg of pentachloronitrobenzene and 10 µg of benomyl) (Jeffers and Martin, 1986), before and after inoculation to determine viable zoospore densities (Tsao, 1969).

Method of inoculation by zoospores of *P. nicotianae*: Rootstocks of citrus were inoculated with 30 ml of the zoospores suspension of *P. nicotianae* by making 5-6 cm deep and 2 cm diameter holes in the pot mixture around the root zone of seedlings, to facilitate the diffusion of zoospore suspension. Pots were watered regularly to maintain the moisture for pathogen development. For the control, the plants have been inoculated with 30 ml of sterile distillated water.

Estimation of disease severity: The response of the seedlings to zoospores treatment was determined by recording the occurrence of symptoms in roots of rootstocks after 3 months of inoculation by *P. nicotianae*. Infected plants were removed from the plastic pots and were carefully washed in running tap water and observed. The symptoms of root rot rating of each rootstock was recorded using a scale (1-5) given by Grimm and Hutchinson (1973) as:

1= No visible symptoms, 2= A few roots with symptoms (1-25 % rotted), 3= Majority of roots with symptoms (26-50 % rotted), 4= All roots infected, cortex sloughed from major roots (51-75% rotted), 5= Majority roots, dead or missing (>76% rotted).

To confirm that these symptoms are caused by *P. nicotianae*, re- isolation was made from the treated root of each rootstock, using PARP-BH medium. The identification of *P. nicotianae* was accomplished according the keys of Erwin and Ribeiro (1996).

Growth measurement: Stem height (SH) was measured for each plant before and after three months of *P. nicotianae* treatments. Then, stem growth rate (SGR) was estimated from these parameters according to the following formula:

$$SGR = (Hf - Hi)/Hi$$

Where, H= Height; i= before inoculation; f= after 3 months of inoculation.

At the end of the experiment, plants were harvested and divided into roots and stems for biomass determination. Fresh weigh of each part was measured, whereas dry weight was determined after drying tissue at 60°C for three days.

Estimation of colonization of roots by *P. nicotianae*: Roots from each rootstock were collected for the evaluation of the colonization of roots by propagules of *P. nicotianae.* Two grams of each fresh

roots sample were added to 20 ml of sterile water and ground for 2 min in a blender containing 20 ml of sterile distilled water. A sample of 1 ml of this solution of homogenate roots was spread in a Petri dish (90 mm) containing the PARP-BH selective medium. Three Petri dishes were prepared for each sampled of rootstock. These plates were incubated for 4 days in darkness and at 25±1°C, and then the colonies of *P. nicotianae* are counted. The analysis results obtained have been expressed as the amount of germs / g fresh tissue.

Experimental design and statistical analysis: The experiment was carried out in a completely randomized design with six replications by rootstock and treatment. All data were subjected to variance analysis (Anova). Significant Anova tests were followed by multiple comparisons of means using Fishers Least Significant Difference procedure ($P<0.05$). Data of all parameters tested of each rootstock were submitted to a multivariate statistical method of cluster analysis, in order to classify rootstocks in groups of closer similarity, according to their response to the inoculation by *P. nicotianae*. Hierarchical cluster analysis was applied by standardized means of the evaluated variables by using the single linkage method, and is shown on a dendrogram. All analyses were performed with STATISTICA version 12.

Table 1: Isolates of *P. nicotianae* employed in the study, host, geographic origin, and year of isolation and GenBank accession number of sequences.

Isolates	Date of collection	Origin	Organ of isolation	GenBank accession number of sequences
P.15	31/05/2012	Takilsa	Soil	KU248805
P.128	19/09/2013	Gobba	Crown	KU248812

Results

Estimation of disease severity: Symptoms of root damages were observed in roots of different rootstocks, planted for 3 months in a *P. nicotianae*-infested soil, while no damaged has been observed in non-inoculated plants. The results of the re-isolation of *P. nicotianae* form all the treated rootstocks were positive. Overall, citrus rootstocks were more susceptible to isolate P.128 than to isolate P.15. Nevertheless, no statistical difference has been observed between the two isolates of *P. nicotianae,* and all rootstocks were grouped within the same susceptibility group. Also, no interaction of *P. nicotianae* and rootstocks was observed (Table 2). However, a higher statistical difference has been observed between the five rootstocks used to indicate a clear difference regarding the tolerance of the rootstocks to *P. nicotianae* (Figure 1). The severity index, resulting from the inoculation of *P. nicotianae,* ranged from 1 to 3.16. The maximum infection indicated by the root damaged rating of 3.16 and 3 was observed, respectably, in rootstocks Citrange carrizo and Citrus volkameriana, indicating the susceptibility of these two rootstocks to *P. nicotianae,* while the minimum roots damaged rating of 1 was noted when the rootstock of reference Sour orange and

the rootstock Citrumelo Swingle-4475 were used. These rootstocks were followed by the rootstock Citrange C-35 with a root damaged by 2.66 (Table 3).

Table 2: Univariate testing custom Significance for severity index based on "*P. nicotianae* *Rootstocks" interaction.

	Type III Sum of Squares	df	Mean Square	F	Sig.
P. nicotianae	0.41667	1	0.41667	1.66667	0.266265
Rootstocks	51	4	12.75	51	0.001095
P. nicotianae * Rootstocks	1	4	0.25	1	0.5

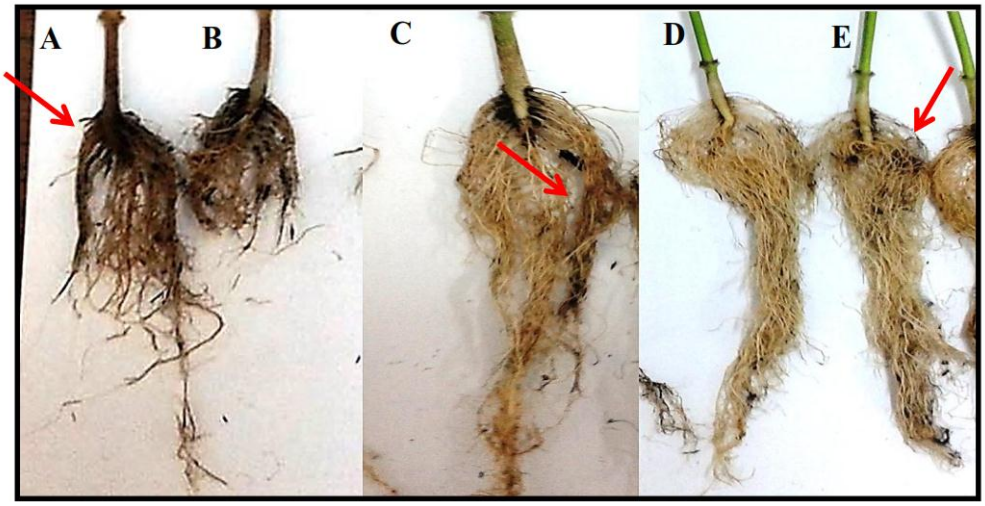

Figure 1: Appearance of roots of five citrus rootstocks (A: Citrange carrizo; B: Citrus volkameriana; C: Citrange C-35; D: Sour orange; E: Citrumelo Swingle 4475), after 3 months of inoculation with zoospores suspension of *P. nicotianae*.

Table 3: Comparison of the severity of root damaged of five citrus rootstocks that were growing into *P. nicotianae*-infested pots for 3 months, according to the scale of Grimm and Hutchinson (1973).

	Isolates of *P. nicotianae*	
Rootstocks used	P.15	P.128
Citrange carrizo	3.16±0.63^{a*}	3.16±0.4^{a}
Citrumelo Swingle 4475	1±0^{c}	1±0^{b}
Citrus volkameriana	3±0.89^{a}	3±0.89^{a}
Sour orange	1±0^{c}	1±0^{b}
Citrange C-35	2.66±0.89^{b}	2.66±0.51^{a}

Effect of inoculation with *P. nicotianae* on stem growth rate (SGR): The studies of the growth attributed to the plant height revealed that the disease has a significant effect on rootstocks height. Indeed, the data showed that inoculation with *P. nicotianae* caused a significant reduction in stem growth of rootstocks used compared to the control. Also, a highly significant difference was found between SGR of the rootstocks in their responses to zoospores inoculation (Figure 2). The highest percent of SGR was recorded for rootstock of reference Sour orange, while the lower one was in rootstock Citrange C-35, following with Citrumelo Swingle-4475. However, a significant difference was found between these two rootstocks and the others rootstocks used (SRG between 25 % and 18.25 %).

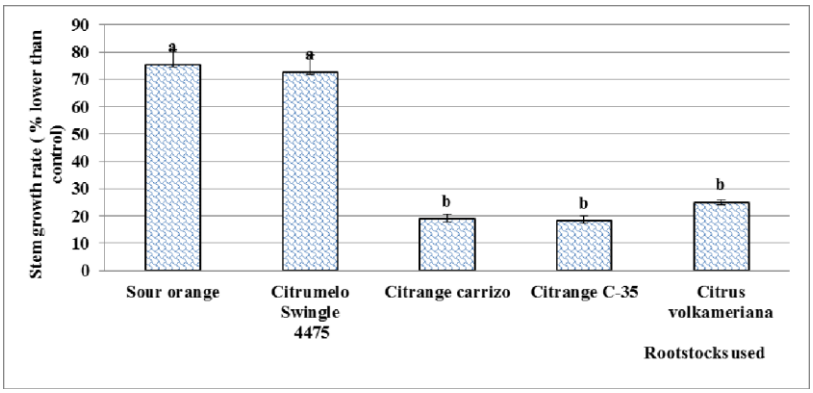

Figure 2: Effect of *P. nicotianae* inoculation on stem growth rate of the five rootstocks studied expressed as % of control plants.

Effect of inoculation with *P. nicotianae* on stem and root weight: Infection by *P. nicotianae* caused a significant reduction in all parameters considered and a high difference was also found between the different rootstocks in their response to this pathogen. Compared to their respective controls, the rootstock of reference Sour orange and the rootstock Citrumelo Swingle-4475 appeared the most tolerance against *P. nicotianae* in all parameters tested. The observation based on the fresh weight stem revealed that the important percent of decrease (38.5 %) was observed for Citrange C-35, while Sour orange (18 %) and Citrumelo Swingle-4475 (17.8 %) showed susceptible response with the lowest percent of decrease (Figure 3). For fresh weight root the decrease of this parameter was observed for Citrumelo Swingle-4475 (37.33 %) and Citrange carrizo (46.7 %) (Figure 4). The dry weight stem was revealed a higher value for Citrange carrizo (49 %) followed, respectively, by Citrange C-35(45.3 %), Sour orange (28.3 %), Citrumelo Swingle-4475 (26.8 %) and Citrus Volkameriana (25.2 %) (Figure 5). Dry weight root values varied between 41.2 for rootstock Citrumelo Swingle-4475 and 59.7 % for rootstock Citrange C-35 (Figure 6).

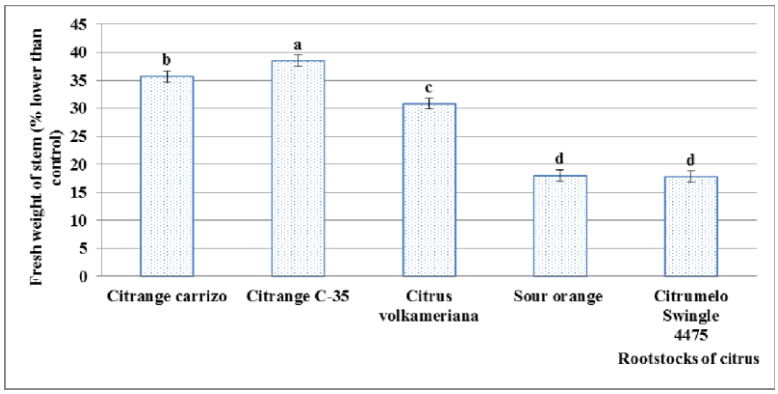

Figure 3: Fresh weight stems percent of five rootstocks of citrus, after 3 months of zoospore inoculation with *P. nicotianae*.

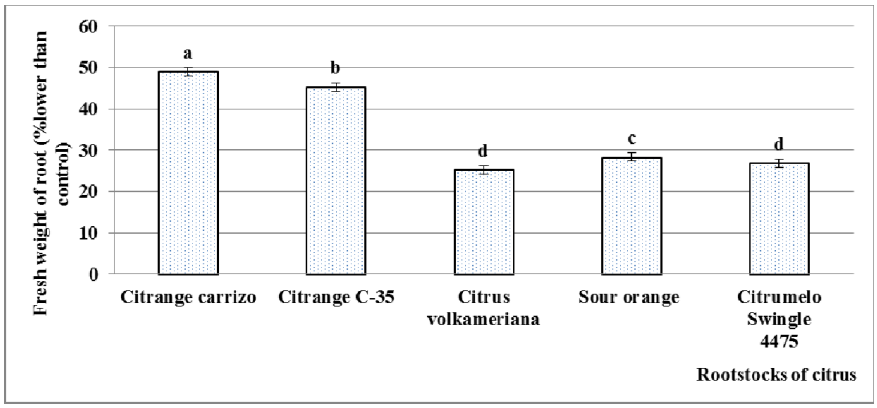

Figure 4: Fresh weight root percent of five rootstocks of citrus, after 3 months of zoospore inoculation with *P. nicotianae*.

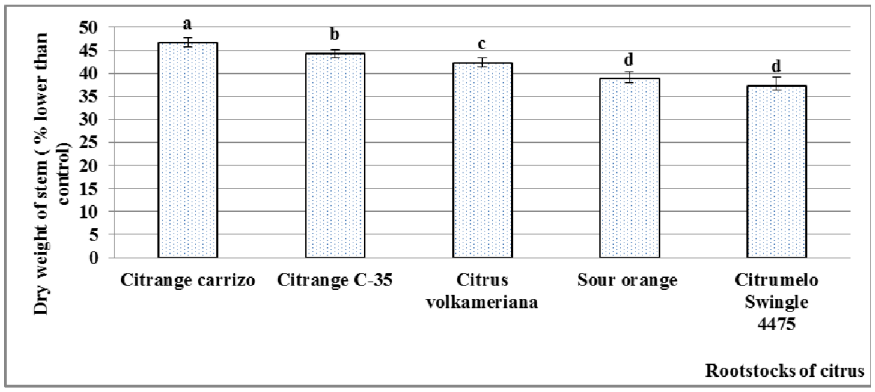

Figure 5: Dry weight stem percent of five rootstocks of citrus, after 3 months of zoospore inoculation with *P. nicotianae*.

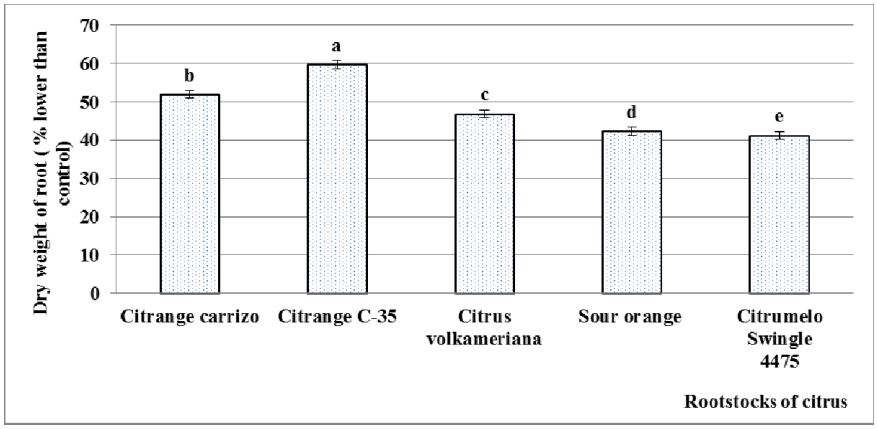

Figure 6: Percent of growth of dry weight root of five rootstocks of citrus, after 3 months of zoospore inoculation with *P. nicotianae*.

Estimation of root colonization by *P. nicotianae*: Mean populations of *P. nicotianae* varied significantly for the five rootstocks tested (Figure 7). Counts ranged from 4.25 propagules per mg of root for the rootstock of reference Sour orange to 24.5 propagules per mg of root for Citrus volkameriana which had significantly higher populations than all other rootstocks. The rootstock Sour

orange was followed by the rootstock Citrumelo Swingle-4475 (5.5 propagules per mg of root). The rootstock Citrange C-35 was following the rootstock Citrus volkameriana with 19.5 propagules mg of the root, then the rootstock Citrange Carrizo (12.25 propagules mg of root). Based on symptoms of damaged observed in roots, the growth parameters measured and the density of population of *P. nicotianae* found in infected roots, a comparison was made between the five rootstocks tested, in order to better understand the response of the different rootstocks to *P. nicotianae*. The hierarchical cluster analysis applied to aggregate rootstocks with similar effects, identified three different clusters of rootstocks (Figure 8). The first cluster of rootstocks with similar effects was formed by the rootstock of reference Sour orange and the rootstock Citrumelo Swingle-4475. These two rootstocks were the most tolerant to *P. nicotianae*. A second cluster included the rootstock Citrange C35 who appeared moderately tolerant rootstock. A third cluster of similarity was formed by the rootstocks Citrus volkameriana and Citrange Carrizo, who exhibited the slightly tolerant reaction against *P. nicotianae*.

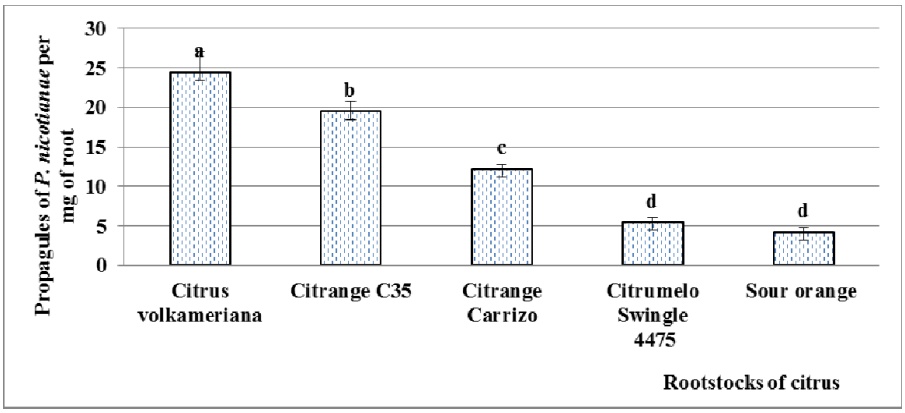

Figure 7: Average number of propagules of *P. nicotianae*, recovered from roots of five rootstocks of citrus, after 3 months of soil inoculation.

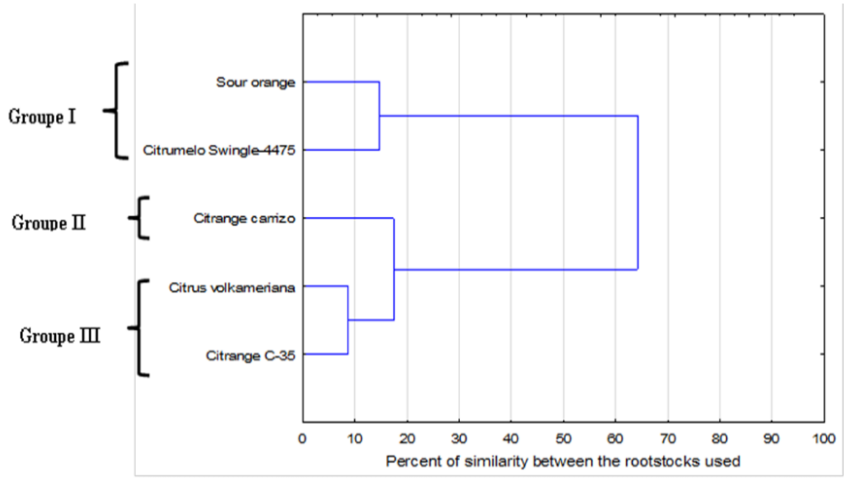

Figure 8: Hierarchical classification of five rootstocks of citrus based on their susceptibility to *P. nicotianae*.

Discussion

The results found in this investigation showed that citrus rootstocks response to *P. nicotianae* different from one to another. None of the tested rootstocks totally fulfilled the criteria tested. However, the rootstocks used could be classed into three susceptibility groups (low, moderate and high susceptibility) against *P. nicotianae* infection. The low susceptibility group included the two rootstocks Sour orange and Citrumelo Swingle-4475. The rootstock Citrange C-35 was moderately susceptible. However, the two rootstocks Citrange carrizo and Citrus volkameriana were consistently the most susceptible rootstocks. The present findings are in agreement with other authors. Broadbent et al. (1971) revealed that rootstock Sour orange exhibited tolerant reaction against *Phytophthora* species. Vanderweyen (1973) reported that the rootstock Sour orange were more resistant, whereas Citrus volkameriana was least susceptible rootstocks against *P. nicotianae*. Graham et al. (2014) reported that the rootstock Citrumelo was resistant to gummosis. Rogers et al. (1996) noted the rootstock Citrange carrizo as susceptible whereas the rootstock Citrus citrumelo as tolerant against *P. nicotianae*. The rootstocks Citrange carrizo and Citrus volkameriana may not be suitable for orchards planted in fields where conditions are favorable for infection by *P. nicotianae*. Moreover, if rootstocks with moderate susceptibility stress by prolonged flooding conditions they may, also, become more susceptible to infection (Wilcox & Mircetich, 1985). When the gummosis of citrus has been reorganized, in the past in Tunisia, the search for resistant rootstocks started and seedlings were gradually replaced by the *Phytophthora* tolerant rootstock Sour orange. This rootstock is one of the most common citrus rootstocks, in the world, especially in areas with high soil pH and calcareous soils and it often supports high densities of *P. nicotianae* (Graham, 1995). The consequence of this orientation induced the suppression of this disease in the country. Recently, the gummosis has been appeared again in this area. Also, the rootstock Sour orange has been noted as susceptible to viruses and other diseases (Graham, 1995). This lets suggest the existence of change or an increase in the population of *P. nicotianae* existing in the soil or a change in the behavior of the rootstock Sour orange against this pathogen. The present study showed a similarity of the response of the rootstock Sour orange with the rootstock Citrumelo Swingle-4475, in their susceptibility to zoospores infection by *P. nicotianae*. This result has important implications for the proper management of gummosis of citrus under orchard conditions, because the rootstock Citrumelo Swingle-4475 could substitute the rootstock Sour orange. The use of the rootstock Citrumelo Swingle-4475 may become an integral part of gummosis of citrus disease management in the future in Tunisia. However, at this point, the use of new varieties is not typically considered by the farmers how still used the rootstock Sour orange. Also, it is prudent to study the effect of scion varieties on the susceptibility of the rootstock and a population of the pathogen (Ippolito et al., 1997). However, from the present study, it was concluded that none of the citrus rootstocks tested were completely resistant to *P. nicotianae*, which suggests

that the use of resistance rootstock alone will not control this disease unless new rootstocks with complete resistance are released. Matheron et al. (1988) noted that the development of *Phytophthora* gummosis is influenced by several variables in addition to the innate resistance of the citrus host. These variables include the age, the nutrient status, the succulence, the vigor, and the scion of the infected rootstock as well as soil characteristics and soil temperature and moisture levels (Broadbent, 1977). However, the kind of pattern used can influence the chemical composition and the antioxidant activity of the fruit (Sanchez-Rodriguez et al., 2012). The use of resistant rootstocks together with chemical control methods and cultural practices such as water management is necessary for managing *Phytophthora*-induced diseases (Graham et al., 2014).

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