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Effect of seasonal and citrus rootstocks on inoculum density of *Phytophthora* spp. in Citrus orchard in a heavy soil of the Gharb region of Morocco

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ABSTRACT

Phytophthora root rot is considered as the most damaging disease for citrus production in Morocco. *Phytophthora* species are generally present as propagules in citrus fields colonizing the root zone of rootstocks and representing a source of infection for the aerial parts and a risk of dissemination to new fields. This study aimed to monitor the seasonal evolution of the inoculum density of *Phytophthora* spp. in heavy ground at the Gharb region in Morocco in the rhizospher of two rootstocks: *Citrus aurantium* L. and *Citrus macrophylla*. Soil samples were taken at monthly intervals over a period of 14 months between April 2013 and June 2014. The inoculum density of *Phytophthora* species was estimated using the dilution technique in conjunction with the selective BARPHY 72 medium. *Phytophthora* isolates were identified based on colonies' morphological and taxonomic criteria as well as mycelial characteristics. In both plots, *Phytophthora parasitica* was the predominant isolated species, followed by *P. citrophthora*. The inoculum density of *Phytophthora* fluctuated throughout sampling period in spring and summer, depending on environmental conditions, and rootstocks.

Keywords: Root rot, *Phytophthora*, seasonal evolution, *P. parasitica*, *P. citrophthora*.

INTRODUCTION

P*hytophthora* gummosis and root rot are among the most important fungal diseases of citrus (Klotz,. 1978).). In Morocco, three species were reported on citrus: *P. citrophthora*, *P. parasitica* and *P. syringae* (Vanderweyn, 1963; 1966; 1974; 1982). However, only *P. citrophthora* and *P. parasitica* are known to cause damage in citrus crops, the first one being reported as the most common species in the Mediterranean region as suggested by its frequent isolation in this area (Serrhini, 1981 ; 1986 ; Donald *et al*., 1996 ; Benyahia, 1998). Nevertheless, Benyahia (1998); Benyahia (2007) reported that the most frequent species in soil of citrus orchards in Morocco is *P. parasitica*, while *P. citrophthora* was found predominant in

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cases of trunk gummosis of citrus tree. Losses attributable to *Phytophthora* gummosis and root rot have been reduced through the use of the systemic fungicides fosetyl-Al and metalaxyl and a single application of either fungicide can provide maximum protection from colonization by P. *citrophthora* and P. *parasitica* for at least 3 months (Matheron and Matejka,. 1988).

Also, the use of resistant rootstocks such as *Troyer citrange* and sour orange (*Citrus aurantium* L.) was found to be an efficient approach. Indeed, considering its high resistance to *Phytophthora spp*. diseases and its wide adaptability to soil conditions, sour orange has became the most used rootstock in Moroccan citrus orchards. However, the resistance of this rootstock is relative and its performance depends highly on soil and irrigation water quality (Benyahia *and al.*, 2004). For instance, salinity increased the severity of root rot, caused by *Phytophthora parasitica*, when Troyer citrange was used as rootstock (Blacker and MacDonald, 1986; Benyahia *and al.*, 2004). Furthermore, it has been suggested that salinity and soil moisture may predispose citrus rootstocks (particularly sour orange) to trunk gummosis due to *P. citrophthora* (Sulistyowati and Keane, 1992; Benyahia *et al.*, 2004 ; Benyahia, 2007). It is also accepted that the rootstock not only can limit the development of the fungus in attacked tissue, but may also have an influence on the inoculums density in soill (Agostini *and al.*, 1991). It is known that the increase in *Phytophthora* density in soil may constitute a source of infection for the aerial part which inevitably results in yield reduction or dieback.

The population density of *Phytophthora spp*. in an orchard is a potential indicator of the probability of significant disease development. Threshold values for soil populations of *P. citrophthora* or *P. parasitica* have not been established definitively in morocco; however, researchers have suggested that orchards with mean densities of less than 10 propagules of *P. parasitica*/cm3 of soil likel cannot be treated economically with fosetyl- Al or metalaxyl, whereas a population of the same pathogen in the range of 15 to 20 propagules/g of rhizosphere soil can reduce citrus yield by 20% (Menge 1986; Timmer *and al.* 1988).

In Morocco, although some information about *Phytophthora* density in soil of citrus orchard are available (Serhini, 1981 ; Farih *et al.*, 1993., Benyahia, 1998), quantitative data concerning the evolution of the inoculum density over time with respect to the rootstock used are lacking although such information may be useful for the development of an efficient phytosanitary strategy to control this pathogen. In this sens, Matheron, *et al* (1997) reported that to gain maximum biological, economic, and environmental benefits from fosetyl-Al or metalaxyl, a treatment program should be initiated only when significant disease development is likely to occur.

Moreover, due to CTV threat, new rootstocks are introduced nowadays at a national level to replace sour orange in citrus orchards but little is known about the interaction of these roostocks with *Phytophthora spp* in soil.

The aims of our research studies were to determine the seasonal evolution of inoculums density of *Phytophthora spp*. in the rhizosphere of two citrus rootstocks: *Citrus aurantium* L. and *Citrus macrophylla* in a heavy soil at gharb region in Morocco.

MATERIALS AND METHODS

Collection of soil samples:

Monitoring *Phytophthora spp*. inoculum was carried out over a 14 months period from April 2013 to June 2014 in two citrus orchards located in the Gharb region in Morocco. The used rootstock in the first orchard was sour orange, while *Citrus macrophylla* was used in the second one. In each orchard, three trees were randomly selected and four soil subsamples were collected according to the four cardinal directions. The samples were taken using an auger at one meter from the trunk and at a depth of 5 to 20 cm (Timmer *et al*., 1988 ; Timmer *et al*., 1993 and Zouheir Talbi et al**, 2015**).

Estimation of inoculum density:

Soil samples were crushed, passed through a 2 mm sieve, and then stored at an ambient temperature of 21 to 24°C (Tsao, 1983; Timmer *et al*., 1988; Timmer *et al*., 1989). To accurately estimate the density of *Phytophthora spp.* inoculum in soil samples, we used the dilution technique. 10 g of soil from each subsample, representing a specific geographic orientation, were diluted in 90 mL of 0.25% water agar. After stirring for 20 min, 1 mL of the mixture is spread on a Petri dish containing BARPHY72 (Benyahia, 1998; 2007), which is a selective media culture used for isolating *Phytophthora* species. This medium was developed in the laboratory after preliminary tests. The composition of BARPHY medium is as follows : 15 $g.L^{-1}$ malt extract, 20 $g.L^{-1}$ agar, 250 $mg.L^{-1}$ Ampicilline, 10 $mg.L^{-1}$ Rifampicine, 15 $mg.L^{-1}$ Benomyl, 10 $mg.L^{-1}$ Pimaricine and 72 $mg.L^{-1}$ Hymexazol (Benyahia, 1998).

For each suspension, three Petri dishes were prepared. The incubation was performed in the dark at 28°C for 48 hours (Timmer *et al*., 1988; 1989). For counting the number of propagules of *Phytophthora* spp, we proceeded first to wash the dishes by steril distilled water in order to remove soil particles, and then we counted the colonies. These are then transferred separately into test tubes containing Corn Meal Agar medium (CMA). Knowing the amount of soil deposited in each dish, the approximative number of *Phytophthora* propagules per gram of soil was calculated.

Identification of *Phytophthora* species:

The identification of *Phytophthora* species was based on taxonomic (Waterhouse, 1963; Stamps *et al*., 1990) and morphologic criteria of the colonies, mycelium characteristics, Aspect of the species on the Potato Dextrose Agar and Corn Meal Agar mediums, presence or absence of chlamydospores, mophology of hyphae and size of sporangia.

To induce the sporangia formation, a 10 g soil sample was put in an Erlenmeyer where it was mixed with 90 mL of water agar. After 20 minutes of mechanical stirring using a stirrer, the solution was allowed to settle. Twelve mycelia discs were then isolated from the edge of five days-old colonies on PDA medium and placed in Petri dishes containing 8 mL of the soil extract (Benyahia, 2003).

RESULTS

Estimation of inoculum density:

Propagules of *Phytophthora spp.* were identified in soil samples of both orchards in the

Figure 1: Seasonal variation of the density of *Phytophthora spp* inoculum in the rhizosphere of *Citrus macrophylla* (bars correspond to the standard deviation)

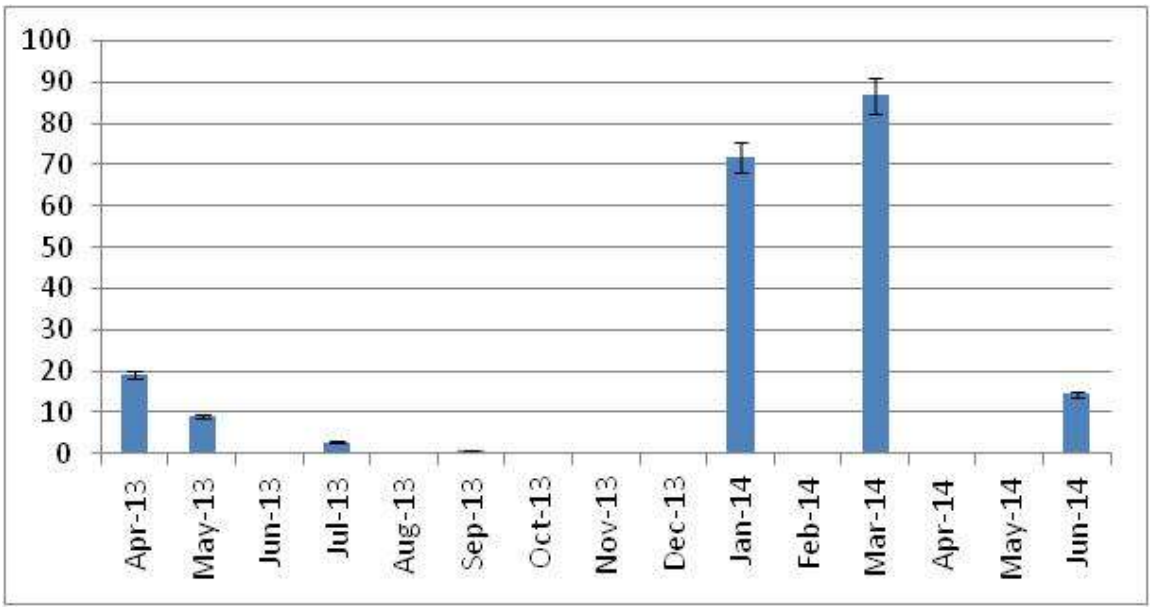
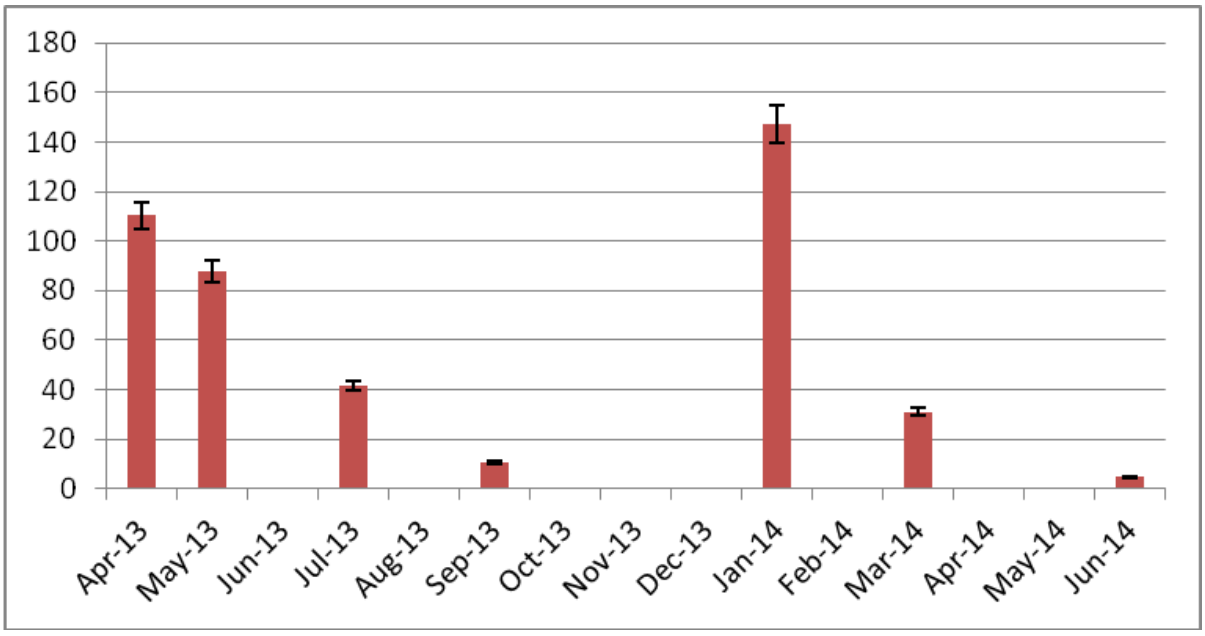
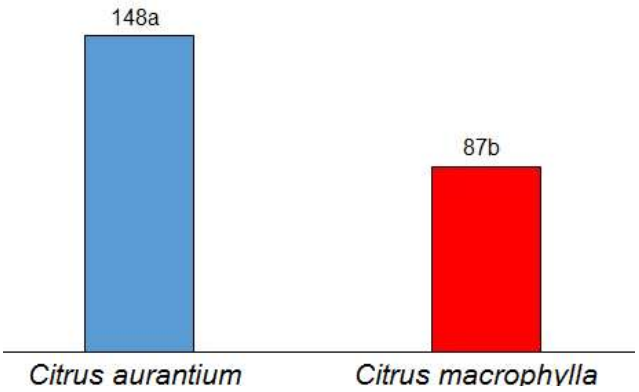


Figure 2: Seasonal variation of the density of *Phytophthora spp* inoculum in the rhizospher of *Citrus aurantium* (bars correspond to the standard deviation)



first plot where *Citrus macrophylla* was used as rootstock, figure 1 show that the density of *Phytophthora spp.* inoculum varied significantly depending on the date of estimation. Indeed, the density values recorded from March to October were lower than 20 propagules, whereas those recorded later showed a high increase to reach a density of 90 propagules in March.

Figure 3: Inoculum density of *Phytophthora spp* in the rhizosphere of tow citrus rootstocks (two affected results two letters differ significantly at the 5% level).

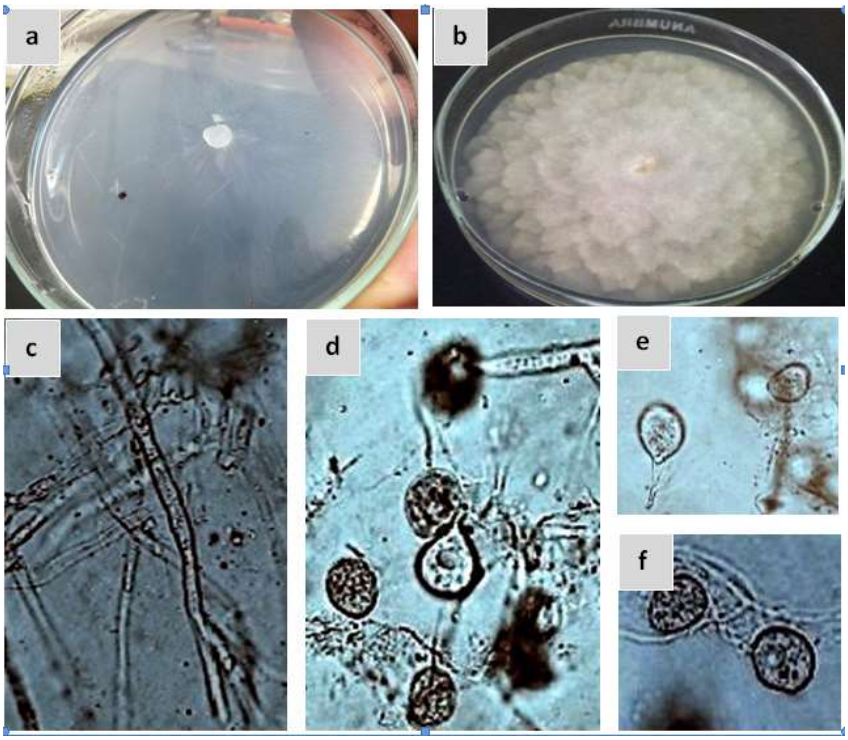


Regarding the other plot, where sour orange was used as rootstock, figure 2 shows clearly that the density of *Phytophthora* spp. inoculum in soil did not remain constant over time but varied significantly over the months. Indeed, the density values recorded during March were higher in this case than the ones recorded in the first plot where *Citrus macrophylla* was used as rootstock. The estimated value for the second plot was 110 propagules per gram of dry soil. From March onwards, there was a decrease of inoculum in soil until reaching a minimum value of 10 propagules per gram of dry soil in August. However, between September and January, the inoculum increased again until reaching a maximum value of 140 propagules per gram of dry soil in January. Beyond January, the inoculum undergoes a progressive drop until reaching a minimum value in May.

Basing on data in figure 1 and 2, the evolution of *Phytophthora* inoculum seems to vary depending on the rootstock. Indeed, in presence of *Citrus macrophylla* as rootstock, the maximum inoculum density was recorded in March, while it was recorded in January in the case of sour orange.

Furthermore, when expressed by average values (Figure 3), the density of *Phytophthora spp.* in the root zone of *Citrus macrophylla* was 87 propagules per gram of soil, whereas it was 148 propagules per gram of soil in the root zone of sour orange.

Figure 4 : *Phytophthora citophthora*. *a : colony growth After 8 days of culture on CMA medium ; b: : colony growth After 5 days of culture on PDA medium ; c : Coenocytic hyphae ; d: Spores with different shapes and sizes. e: Sporangium observed by light microscopy * 40. Bar = 1µm ; f : chlamydospore observed by light microscopy * 40, Bar = 1µm;*



Identification and quantification of *Phytophthora* species:

The observation of microscopic and macroscopic characteristics of the *Phytophthora* isolates and the comparison of descriptions reported by Waterhouse (1963) and Stamps *et al.* (1990) allowed us to identify two species, namely *Phytophthora parasitica* and *Phytophthora citrophthora.*

On PDA medium, The *Phytophthora spp.* colonies grow rapidly covering the entire medium surface after five days of incubation at a temperature of 24 to 26°C. At young stages, the colonies are beige, but become white over the time. A large part of mycelium is immersed in the culture medium.

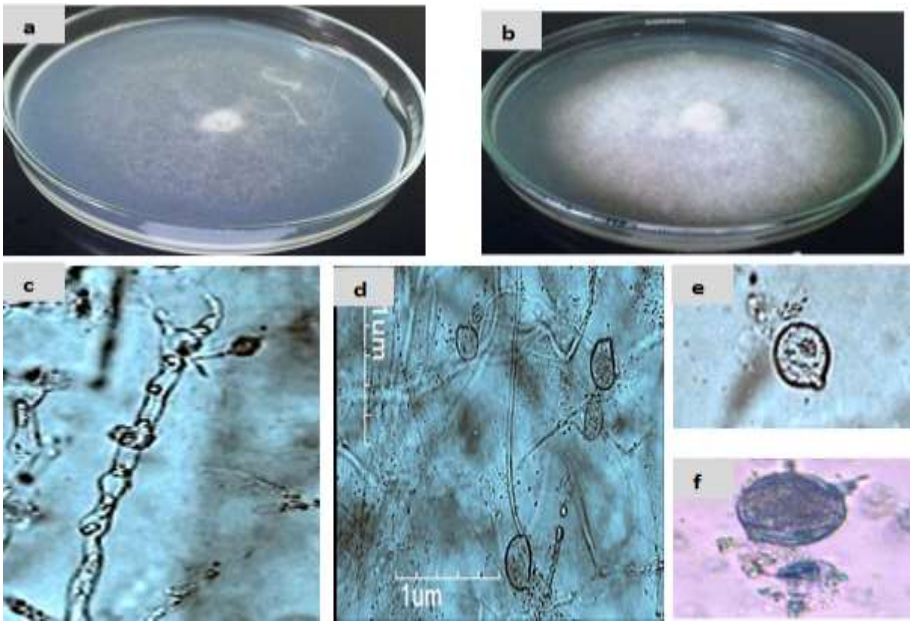
On CMA, the mycelium of *P. parasitica* is much ramified, less regular and has diffuse edges. Sporangia elliptical, oval or peer-shaped with papillae. chlamydospores were also present. On the same medium (CMA), *P. citrophthora* has a petaloid aspect. The mycelium is slightly ramified, regular and thick. Sporangia have a wide range of shapes and sizes and their papillae are well differentiated.

Furthermore, the results of our study suggested that, in both plots, the incidence of *P. parasitica* was high during all the period of the study, whereas the incidence of *P. citrophthora* reached the highest values in January for sour orange plot and Ferbruary for *Citrus macrophylla* plot (Figure 4)

DISCUSSION

In this study, significantly high densities of *Phytophthora* incoculum were found. In most cases, the densities were above 20 propagules per gram of soil. In Morocco, Serrhini (1981) reported much higher inoculum densities in two soils of the Gharb region. For instance, his results have shown that the density of *P. citrophthora* was 128 propagules per gram of a clay soil, and 47 propagules per gram of a sandy soil.

Figure 5 : *Phytophthora parasitica.* *a: colony growth after 8 days of culture on CMA medium ; b: colony growth after 5 days of culture on PDA medium ; c: Coenocytic hyphae ; d: Spores with different shapes and sizes. e: Sporangium observed by optical microscopy * 40. Bar = 1µm f: Chlamydospore observed by optical microscopy * 40. Bar = 1µm ;*



The high number of the obtained *P. citrophthora* propagules could be related to the humidification of soil samples that may occur during storage. Indeed, water may stimulate inoculum development which can distort the estimation of its real density (Farih *et al*. (1995; Benyahia, 2007), by spreading soil particles on Petri dishes containing a selective medium, obtained densities of 32, 75 and 78 propagules per gram of soil of a citrus orchard located in the Gharb region, respectively in three successive years. However, the used method in that experiment was qualitative rather than quantititative, and can be used only when the density of *Phytophthora* inoculum in soil is very low (Tsao, 1983; Donald *et al*., 1996).

On the other hand, by using the technique of spreading soil suspension on a selective medium and avoiding the humidification of soil during storage, also high densities of *Phytophthora* spp. inoculum, which were in most cases above 20 propagules per gram of dry soil (Benyahia, 1998).

The increase in inoculum density of *Phytophthora* in soil is an important factor of infection for the aerial part of the tree (Timmer *et al*., 1988). Moreover it may affect the performance of the root system causing yield decline or even the dieback of trees (Timmer *et al.*, 1988). The use of fungicide treatments is usually expensive, especially when soil contains high densities of *Phytophthora* populations (Timmer *et al*., 1989; Lutz and Menge, 1986; Donald *et al*., 1996). In Florida, the density of *Phytophthora parasitica* populations was estimated by the number of propagules by gram of soil and fungicide treatments are only recommended if the inoculum density exceeds a threshold of 15 to 20 propagules per gram of dry soil (Lutz and Menge, 1986; Sandler *et al.*, 1989).

In the present work, the highest densities of *Phytophthora* inoculum was recorded in the two orchards indicate clearly that the distribution of that inoculum in the root zone of the trees is randomized in both orchards. These observations are consistent with those reported by Timmer *and al.* (1989) and Benyahia (1998). According to these authors, the distribution of *Phytophthora* propagules in citrus orchards varies depending on the severity of root attack. Indeed, it was

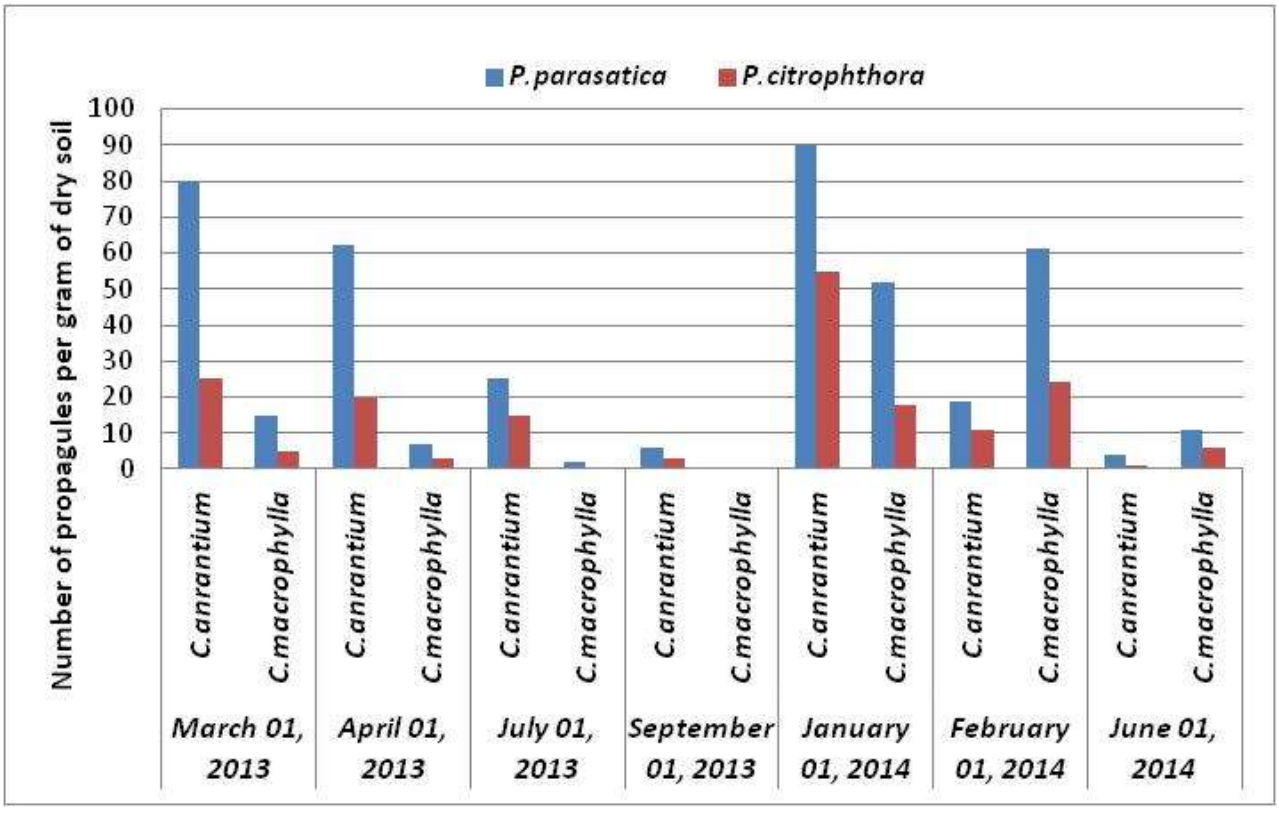
found that the distribution was randomized in orchards where the severity of root rot was high, and grouped into foci in healthy orchards. Thus, the randomization of *Phytophthora* inoculum observed in this study probably reflects a severe attack of the root system of sour orange and *Citrus macrophylla* which were used as rootstocks. According to Graham (1990), sensitive rootstocks such as *Citrus aurantium* may undergo severe root rot when the inoculum density is high enough. Similarly, Lutz and Menge (1986) estimated that an inoculum density of 15 to 20 propagules per gram of soil of *P. nicotianae* may cause root damage in sour orange. In our study, the recorded densities in the root zone of sour orange exceeded 20 propagules per gram of soil.

Although sour orange is known by its tolerance to *Phytophthora* (Cutuli and Salerno, 1998), IR values obtained suggest a high susceptibility of this rootstock to root rot. This observation was explained in the literature by a specific susceptibility of sour orange to *P. nicotianae* (Cutuli and Li Destri Nicosia, 1976), or by a genotype-dependant resistance in sour orange (Salerno and Cutuli, 1981; Benyahia, 1993). We should note also that many factors might influence the susceptibility of sour orange such as the microclimate of rhizosphere, soil physic-chemical properties, and particularly soil salinity. Indeed, Benyahia (1998) found a positive correlation between soil salinity and the density of *Phytophthora* inoculum in the root zone of sour orange in Moroccan orchards.

A review of literature shows many inconsistencies regarding the ranking of citrus rootstocks in terms of their resistance to root rot and trunk gummosis caused by *Phytophthora* spp. Indeed, according to Graham (1990 ; 1995), sour orange was ranked as sensitive to root rot based on the low ability of its root fibers to regenerate after being destructed by *Phytophthora parasitica*. A similar ranking was reported by Widmer *et al*. (1998).

Concerning *Citrus macrophylla*, the lower densities of *Phytophthora* inoculum recorded in the root zone of this rootstock as compared to

Figure 6: Evolution of the inoculum density of *P. parasitica* and *P. citrophthora* in the root zone of the two rootstocks expressed by propagules per gram of dry soil



sour orange suggest the existence of a defense mechanism employed by *C. macrophylla* to limit the multiplication of *Phytophthora* spp. at root level probably by the secretion of some substances that inhibited the growth of this fungus. Similarly, the works of Graham (1995) reported a low density of *Phytophthora parasitica* in the root zone of trifoliate rootstocks. The author suggested that the colonization of soil by *Phytophthora spp.* may be inhibited by a biochemical mechanism operating at root level. In other works, Graham and Timmer (1994) also considered that the resistance of citrus rootstocks to *Phytophthora* spp. is generated by a possible mechanism of phytoalexin production at root tips.

On the other hand, some authors reported that *C. macrophylla* is tolerant to *Phytophthora* gummosis and reacts well to root attacks thanks to its high ability to regenerate injured roots (Anonyme., 2002; Aubert and Vullin, 1997). Similarly, Hutchison and Grimm (1973) described *C. macrophylla* as resistant to *P. parasitica*. Their works showed that not only the studied genotype displayed a high resistance to root rot, but can also transmit this resistance property to its hybrid descendance. These results confirmed those reported earlier by Carpenter and Furr (1962).

In this study, the analyze of soil samples that were collected from the root zone of two rootstocks showed the existence of two *Phytphpthora* species, namely *P. citrophthora* and *P. parasitica*. These two species were isolated from citrus orchard soils in the Gharb region by Serhini, (1981); Farih *et al.*, 1998; and Benyahia (1998). Furthermore, our results showed the dominance of the species *P. parasitica* in these soils consistently to the results of Benyahia (1998). Indeed, this author reported that *P. parasitica* was more frequent than *P. citrophthora* in citrus orchard soils of Gharb, Beni Mellal and Berkane regions. According to Zitko *et al.* (1994), *P. parasitica* was easily detectable by its symptoms at root level, and particularly at root tips, contrastively to *P. citrophthora* which attacks the aerial part of the tree.

The study of seasonal evolution of *Phytophthora spp* in soil showed a fluctuation of *Phytophthora* populations depending on season and the rootstock used. Generally, the populations tended to increase from December onwards. We should note that the extent of this variation is similar to the one observed for *Phytophthora* species in Egyptian citrus nurseries (Salama, 2008; Ahmed and D'Onghia, 2012) and Italian orchards (Ippolito *et al*., 1992). The determination of seasonal variation of the *Phytophthora* spp density populations may have important implications in the choice of the most efficient moment for the application of control measures. Also, the knowledge of seasonal variation of the citrus rootstocks sensitivity may indicate periods when the disease is severe the most or when soil temperature stimulates its development. Such information may also be helpful for the choice of the right moment for the application of these control measures (Matheron and Matejka, 1993).

Despite the presence of *Phytophthora spp.* propagules in citrus orchards, the development of the infection is not systematic and the harmfulness may vary depending on rootstock susceptibility and inoculum density Benyahia (2007). For example, the threshold of inoculum density, reported for sour orange by Lutz and Menge (1986) is 15 to 20 propagules per gram of soil. It is evident that such thresholds may be exceeded even in disadvantageous conditions.
The highest obtained inoculum density in this study could be the result of a severe root attack caused by *Phytophthora*. Indeed, many authors agree that soil inoculum density is correlated positively to the severity of *P. parasitica* root rot in citrus orchards (Timmer *et al*., 1988; Sandler *et al*., 1989; Duncan *et al.*, 1993). Furthermore, it has been established that environmental conditions and the presence of root fibers may promote the development of *P. parasitica* root rot in sour orange, which results in the increase of inoculum density in soil (Agostini *et al*., 1991). However, although the effects of soil temperature (Matheron and Matejka, 1992; Matheron *et al*., 1997; Dirac *et al*., 2003), duration of soil inundation and water potential (Wilcox and Mircetich, 1985 ; Woods and

Duniway, 1986) on the severity of *Phytophthora* diseases have been proven, little is known about the influence of meteorological variables on the severity of infections by this pathogen on citrus.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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