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Potential of silicon fertilization in the resistance of chestnut plants to ink disease (*Phytophthora cinnamomi*)

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***Abstract*—** *The European chestnut (Castanea sativa Mill.) is a specie with great economic importance in Europe that have been present for thousands of years. In Portugal, the chestnut helps to maintain a positive trade balance, by contributing to the gross national product (GDP). One of the biggest threats for the chestnut is the ink disease caused by Phytophthora cinnamomi, this disease is problematic to chestnut crop with a damaging impact. Silicon (Si) is classified as a beneficial nutrient, having the ability to make plants more resistant to attacks by pathogens. Studies on the effect of silicon on chestnut are practically non-existent, so the aim of this study was to evaluate the impact of silicon in the resistance of chestnut plants to P. cinnamomi. The plants were treated by 0 mM, 5 mM, 7.5mM and 10 mM SiK® with the analyzed mad at 0, 15 and 30 days after inoculation by P. cinnamomi. These findings showed that the Si-treated plants had higher survival rate resulted from the presence of phytoliths in root tissues, that acted as a mechanical barrier reducing the development of pathogenic structures and they arealso associated with the improvement on antioxidant activity through the increase of CAT and SOD, higher values of total phenols compounds and less oxidative damage. The presence of Si in PDA medium reduced the growth of P. cinnamomi all over the time, presenting high PI. This work shows that the Si fertilization in chestnut plants contributes to increase the resistance against P. cinnamomi infection.*

***Keywords*—Biotic stress, Castanea sativa Mill, Fungitoxic, Phytophthora cinnamomi, Resistance, Silicon.**

I. INTRODUCTION

The sweet chestnut (*Castanea sativa* Mill.) is present in all countries of the Mediterranean Sea basin, playing an important role in the economy of these countries (Corredoira *et al*., 2012),covering large areas in France, Greece, Italy, Portugal, Spain, Turkey and the United Kingdom (Fernandez-López and Alia, 2003). This chestnut species has an important historical and cultural value, playing a key role in the economy and environmental sustainability of the mountain areas (Marinoni *et al*., 2013). However, the European chestnut has been strongly threatened by ink disease. *Phytophthora cinnamomi*was detected for the first time in Japan. In Spain and Portugal, it emerged during the 19th Century andwas subsequently reported in other countries of Europe (Italy, Greece, Switzerland, Turkey, France and the United Kingdom) (Vannini and Vettraino, 2001; Vettraino *et al*., 2005; Corredoira *et al*., 2012).

In Portugal, this disease has been responsible for the disappearance of more than 50% of the chestnut-producing areasince the 20th Century (Seabra *et al*., 2001; Martins and Abreu, 2007). This soil oomycete, which has asexual reproduction, attacks the root system and produces a black exudate that stains the surrounding soil leading the collapse of xylem and consequently to the death of the tree (Vannini and Vettraino, 2001). The *P. cinnamomi* representing one of the most devastating root rot pathogens of chestnuts (Balci and Halmschlager, 2003), by these reasons is essential to search for alternative strategies that can help the trees to increase their resistance against this pathogen.

In this context, the fertilization with Si in chestnut plants appear as a possible inducer of resistance against *P. cinnamomi* infection, considering the potential of Si as

an important and promising plant protector against several biotic stresses allowing to decrease the intensity of diseases in different crops in the world (powdery mildew and rice blast). Several authors verified that Si fertilization reduces the infection of angular leaf spot and *Colletotrichum lindemuthianum* in cotton (Oliveira *et al*., 2012) and bean plants (Polanco *et al*., 2014), respectively. On the other side, Côrtes *et al*. (2015) added that the Si is classified as an elicitor with potential through enzymes defense suppress the rice blast.

Several diseases were also suppressed by Si application (Rodrigues and Datnoff 2005). In this context, Seebold *et al*. (2001) have tested the effects of Si on several components of resistance to rice diseases using susceptible, partially resistant and completely resistant rice varietys. They found that the number of sporulation per lesion, lesion size, rate of lesion expansion, number of spores per lesion and diseased leaf area were significantly reduced by Si application. Moreover, the presence of brown spot, stem rot, sheath brown rot on rice, *Fusarium* and *Corynespora* leaf spot on cucumber decreased with the increase of Si supplied. Datnoff *et al*. (2001), suggesting that production inputs can be better managed by using Si, allowing the reduction of pesticide elimination, as well as improved plant resistance. Furthermore, Bakhat *et al*. (2018) note that Si can reduce diseases such as blast to the same level as a fungicide, reducing costs and providing positive environmental benefits.

The objetive of the present study was to investigate the effect of Si fertilization in chestnut plants on the resistance toink disease (*P. cinnamomi*).

II. MATERIAL AND METHODS

Plant material and growing conditions

The experiments used 160 chestnut seeds (*Castanea sativa* Mill var. Sousã) from the same tree growing in the Germobank of University of Trás-os-Montes e Alto Douro (UTAD), Vila Real, Portugal (41° 17′ 20″ N, 7° 44′ 0″ W). The seedlings were planted in 2 L filled pots with 3:1 turf and perlite and randomly organized into 4 groups with 40 pots each. The plants with 4 months old were then placed in the growing chamber, with a 12h photoperiod, radiation 1600 µmolfotões.m^{-2}.s^{-1}, 26 ºC, and watered on a daily basis.

Silicon Treatments

Silicon was applied 45 days after the plants were potted, as potassium silicate (SiK®), according to Ma and Takahashi (2002). In this way, four treatments were prepared and evaluated: 0 mM, 5 mM, 7.5 mM and 10mM SiK®. The silicon solutions were adjusted to pH 6.9 using 30 M hydrochloric acid (HCl). Each plant was fertilized with 50 mL of a SiK® solution, which was directly applied to the soil.

Isolation of *P. cinnamomi*

The *P.cinnamomi* isolate (IMI 340340) used in the inoculation was selected due to its virulence in accordance with previous tests (Abreu *et al*., 1999). The high pathogenicity of this isolate in European chestnuts was also confirmed by Dinis *et al*., (2011). The inoculum was prepared for growth in PDA (potato dextrose agar) during 6 days at 25°C in the dark.

Leaf mineral analysis

The samples were analysed using the standard procedures of the University of Trás-os-Montes and Alto Douro Soil Analysis Laboratory. The preparation and analysis of chemical macronutrients (N, P and K) in leaves from Si-treated plants and untreated plants (0 mM SiK®) were done using the methods described by Malavolta *et al*., (1997). The content of Si in chestnut leaves was analyzed by the method described by Korndörfer*et al*., (2004).

Resistance tests to ink disease

Leaf disks inoculation with P. cinnamomi

The inoculation of leaves with *P. cinnamomi* was made according to Gouveia and Abreu (1994), with some changes to verify if there was a correlation between this inoculation form and roots inoculation. Six leaves per treatment were sampled from the non-inoculated plants. In the middle part of each one, 3 disks with 2 cm of diameter, including midrib, were punched. The disks were placed in petri dishes on a damp filter paper to maintain humidity conditions to the development of *P. cinnamomi*. An 8 mm disc of PDA inoculated with *P. cinnamomi* was placed on top of each leaf disc, as described earlier. The time, in hours, between the inoculation and the visible symptoms was evaluated daily over a period of 7 days, recording observations about theappearance of chlorosis in leaf disks was recorded.

Preparation of P. cinnamomi inocolum

The inoculum of *P. cinnamomi* was prepared from a mixture of potatoes, sugar and distilled water until boiling and then drained. The mixture was autoclaved for 20 minutes at 120°C and after the cooling period was inoculated with *P. cinnamomi* mycelium disks of about 8 mm diameter, from colonies with 10 days and posteriorly incubated in the oven at 25°C for 8 days.

Root inoculation with P. cinnamomi

The *P. cinnamomi* inoculum (50 mL) was applied in 20 chestnut plants per treatment (described in the plant material) directly in the soil, 60 days after SiK® fertilization. The plants were then monitored for 4 months, registering the time whenever a plant died.

Histopathology analysis

With a hand microtome, cross sections (1 µm thick) of secondary roots were obtained from untreated (0 mM SiK®) and Si-treated plants (5 mM, 7.5 mM and 10 mM SiK®) at 150 days after inoculation (Monteiro *et al*., 2017). The root samples were collected from three different plants per

treatment, avoiding lignified zones and the root tips. Sections were stained with a solution of 0.1 % toluidine blue-O solution in citrate buffer (pH 0.5) (Ruzin, 1999) to stain vegetative tissues in general and for specific detection of phenolic compounds in the middle lamella of woody species (Ruiz-Gómes *et al.*, 2015) and then sealed with a mounting medium (Entellan; Merck).

Pictures from sections were taken with an Olympus IX 51 inverted microscope (Olympus optical Co., GmbH, Hamburg, Germany) using an Olympus BX50.

The set of images obtained, five per sample, contained different tissue types: outer cortex containing epidermal cells, the central cylinder with medullary parenchyma, the vascular cambium, and the vascular tissue system such as xylem and phloem. The amorphous silicon bodies were analyzed with an optical microscope in the same root samples treated with SiK® mentioned above (Monteiro *et al*., 2017).

The histology analysis was performed to analysis the effect of Si fertilization on the resistance of root tissues against to *P. cinnamomi* infection.

Evaluation of the effect of soluble silicon application in PDA medium on the mycelial growth of *P. cinnamomi*

Filter sterilized solutions with different concentrations of Si (5 mM, 7.5 mM and 10 mM SiK®) were added to autoclaved PDA culture for Si+PDA medium by mixing 200 mL of Si solution with 350 mL of PDA (proportion of 1:2) being this process made for each concentration under study. The Si+PDA solutions were mixed with magnetic stirrers to ensure even distribution of Si and subsequently were decanted into sterilized Petri plates, according to Kaiser (2005). On the other hand, the control (0 mM SiK®) was represented by non-ameliorated PDA medium.

Then a 1 mm square of *P. cinnamomi* from 15 days old culture on PDA were transferred to the center of Petri plates (90 mm in diameter) containing SiK®+PDA and control plates (0 mM SiK®). The present methodology was adapted by Bekker *et al*., (2009) and was performed for each treatment (5 mM, 7.5 mM and 10 mM SiK®) under study.

The Petri plates from all treatments were incubated in a chamber at a temperature of 25ºC in the darkness. The evaluations were carried by measuring the daily diameter of *P. cinnamomi* using a ruler, ending when the control colonies reached the entire surface of the Petri plate. The percentage of inhibition (PI) was used to calculate the colony diameter growth of *P. cinnamomi* according to the formula (Ebrahimi *et al*., 2012):

$$PI = (C-I)/C * 100\%$$

PI – Percentage of mycelial growth inhibition

C – Diameter of mycelial growth in control treatment

I – Diameter mycelial growth of Si treatment

This methodology was made to evaluated the impact directly of Si application on growth of *P. cinnamomi* in PDA medium. Data presented were resulted by twenty replicates for each treatment.

MDA and hydrogen peroxide contents

Lipid peroxidation in the leaves was measured in terms of malondialdehyde (MDA), a product of lipid peroxidation which is formed in a reaction mixture containing thiobarbituric acid. The MDA amount was determined at 532 nm, followed by correction for the non-specific absorbance at 600 nm using an extinction coefficient of 155 $mM^{-1}cm^{-1}$ as described by Farooq *et al*., (2013).

Hydrogen peroxide (H_2O_2) amount was determined according to Schurt *et al*., (2014). The absorbance was measured at 390 nm and the H_2O_2 content was computed by using the extinction coefficient of 0.28 $mmol^{-1}cm^{-1}$.

The MDA and H_2O_2 content quantification was measured to analysis the impact of Si fertilization on oxidative damage. These measurements were held between 0 and 30 days after inoculation and were replicated 6 times per treatment (n=6).

Total phenols compounds determination

The total phenols were determined according to the Folin-Ciocalteu's procedure of Singleton and Rossi (1965) with the remainder alcoholicextractof the photosynthetic pigments. The absorbance of these metabolites was quantified at 795 nm. These measurements were held between 0 and 30 days after inoculation and were replicated 6 times per treatment (n=6).

Antioxidant activity determination

The antioxidant activity determination was made for evaluated the role of Si application in host defense system.The activity of catalase (CAT) was measured by Wu *et al.*, (2014) method. CAT activity was determined as the rate of disappearance of H_2O_2 at 240 nm, for 1 minute. Reaction mixture (3 mL) included 50 mM potassium phosphate buffer (pH 7), and the activity was expressed as µgmol/min/g FW. The activity of superoxide dismutase (SOD) was assayed by Roohizadeh *et al*. (2014) and was expressed as U g^{-1} FW. Reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 1.3 µM riboflavin, 0.1 mM EDTA, 13 mM methionine, 63 µM NBT, 0.05 M sodium carbonate (pH 10.2) and enzyme extract, was used. The photoreduction of NBT was measured at 560 nm.

These measurements were held between 0 and 30 days after inoculation and data from all the enzymes corresponded to four replicates per treatment (n=4).

Statistical analysis

Data were expressed as means. For statistical analysis, the Turkey's test ($P< 0.05$) was applied.

III. RESULTS

Leaf mineral analysis

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Table 1 presents the composition in mineral nutrients and Si amount of Si-free plants and Si-treated plants. The Si-treated plants showed a significant increase on Si content, presenting an increase of 298% between 0 mM SiK® and 10 mM SiK® treatments. In addition, data showed that the Si content increases in Si-fertilized chestnuts with the enhance of the Si concentration applied, 1.81, 2.45 and 3.98 mg $Si.g^{-1}$ recordedin 5 mM, 7.5 mM and 10 mM SiK® treatments, respectively while control plants (0 mM SiK®) showed only 1.00 mg $Si.g^{-1}$ (Tab. 1).

Table.1: Amount of mineral nutrients and silicon content in chestnut leaves of all treatments (0 mM, 5 mM, 7.5 mM and 10 mM SiK®) under study (n=3).

Treatment	Si (mg $Si.g^{-1}$)	N (g kg^{-1})	P (g kg^{-1})	K (g kg^{-1})
0 mM SiK®	1.00 ± 0.001	25.3 ± 0.025	3.0 ± 0.004	30.5 ± 0.037
5 mM SiK®	1.81 ± 0.003	30.7 ±0.033	3.5 ± 0.001	40.3 ± 0.056
7.5 mM SiK®	2.45 ± 0.005	34.5 ± 0.019	3.9 ± 0.002	41.6 ± 0.068
10 mM SiK®	3.98 ± 0.001	40.2 ± 0.048	4.8 ± 0.002	42.1 ± 0.026

Additionally, as shown in Tab. 1, the Si application promoted a significant increase in the content of N, P and K, where the percentage increases with the Si concentration applied in chestnut plants, in 10 mM SiK® treatment was 59% N, 60% P and 38% comparatively to control treatment (0 mM SiK®).

Resistance tests for ink disease and survival analysis

Analyzing the resistance tests, the Figure 1a showed clearly that in Si-fertilized leaf disks, the development of chlorosis was significantly delayed and reduced compared to non Si-fertilized plants (0 mM SiK®). The results indicate that the plants behavior differs in relation to *P. cinnamomi* inoculation, on the Si absent plants (0 mM SiK®), the symptoms of oomycete infection appeared after 78h. In contrast, in the Si-fertilized leaf disks with 7.5 mM and 10 mM SiK® (Fig. 1a) it took 156 and 139 h for chlorosis to appear and/or necrosis in leaf disks. These results are consistent with those shown in Figure 1b, where the number of non-affected disks increase with Si concentration applied. On 10 mM SiK® treatment, 72% of the disks did not present chlorosis, while in the control (0 mM SiK®) only 11% of the disks remained free of chlorosis.

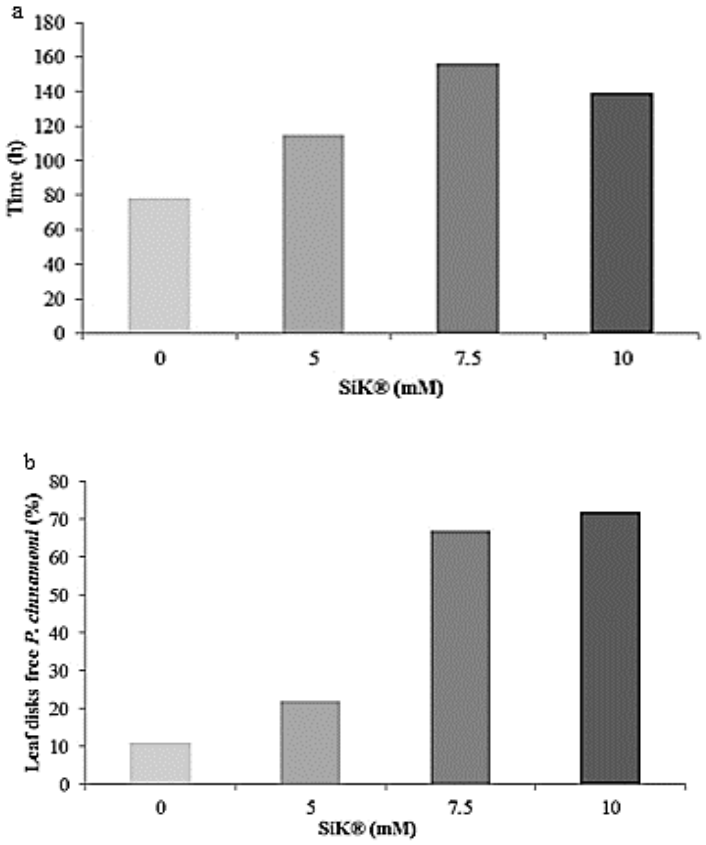

Fig.1: Resistance test to P. cinnamomi. a - Meantime (h) of chlorosis appearance in leaf disks from 0 mM, 5 mM, 7.5 mM and 10 mM SiK®. b - The percentage of leaf disks free from P. cinnamomi (n=6).

The Figure 2 presents the survival rate of chestnut plants from the different treatments under study against to *P. cinnamomi* infection. The higher values of survival rate demonstrated higher resistance to the severity of ink disease.

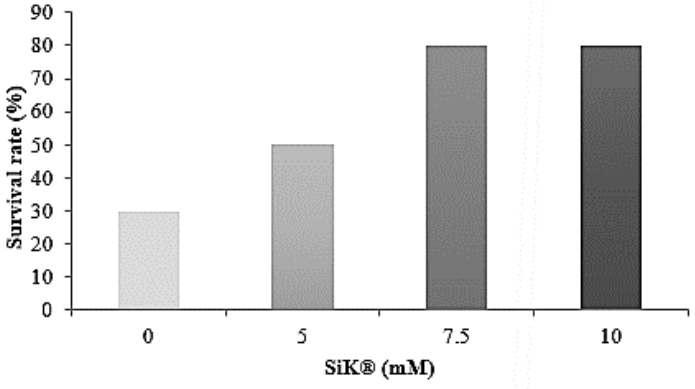

Fig.2: Survival rate of non Si-fertilized (0 mM SiK®) and Si-fertilized (5 mM, 7.5 mM and 10 mM SiK®) plants 150 days after inoculation with P. cinnamomi (n=20).

http://dx.doi.org/10.22161/ijeab/2.5.60 *ISSN: 2456-1878*

Consistently, after 150 days of inoculation, only 40% of control plants (0 mM SiK®) remained alive, unlike Si-fertilized plants, of which 80% survived for the 7.5 mM and 10 mM SiK® (Fig. 2). Therefore, the present findings suggesting that the highest concentrations of Si (7.5 mM and 10 mM SiK®) have more resistance against to *P. cinnamomi* inoculation compared to untreated plants (0 mM SiK®).

Histopathology analysis

Figure 3a illustrates the degree of infection by *P. cinnamomi* in the root cortex and cortical parenchyma from each one of the treatments (0 mM, 5mM, 7.5mM and 10 mM SiK®).

The degree of infection by *P. cinnamomi* was assessed by the amount of the oospores in the cortex and vascular cylinder root cells. Roots from the Si absent plants (0 mM SiK®) showed a high degree of infection in the cortical parenchyma, whichwere fullycolonized by the oospores (Fig. 3b and arrows a), reason why a high number of these pathogenic structures is observed in the parenchyma cells of the vascular cylinder and leading to the disruption and occlusion of xylem vessels (Fig. 3a). In the root cells of cortical parenchyma many oospores were detected, dispersed throughout the cortical tissue. However, as the Si concentration increased in plants a decrease in the infection degree was observed, both in cortical parenchyma and vascular cylinder tissues, (Fig. 3b and arrows a), suggesting that Si fertilization might reduce the incidence of *P. cinnamomi* infection in chestnuts plants.

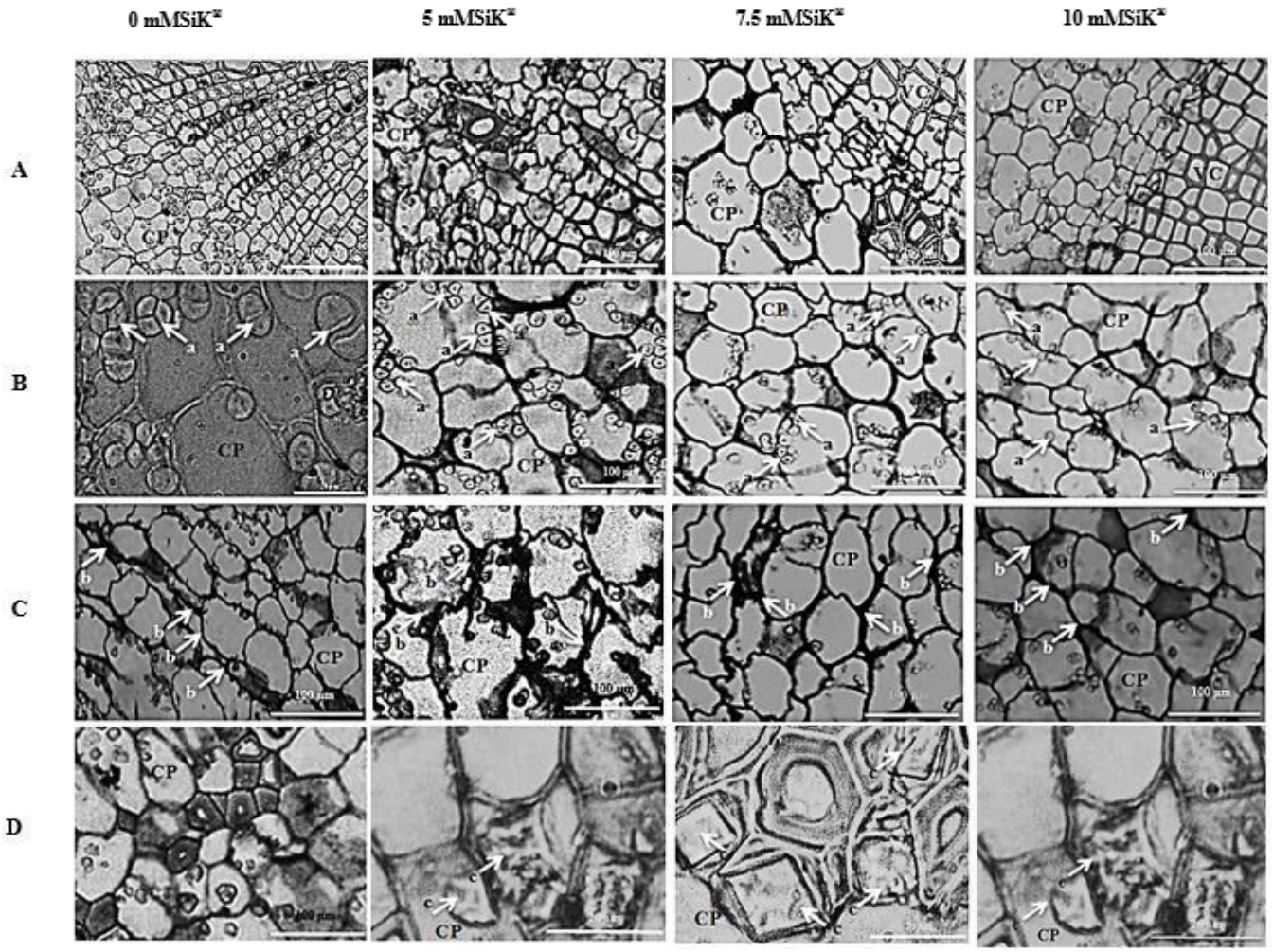

Fig.3: Cross section of 0 mM,5 mM, 7.5 mM and 10 mM SiK® treatment chestnut roots at 150 days after P. cinnamomi infection, analyzing the degree of infection (A), the presence and number of oospores (B), the occurrence of hyphae (C) and the presence of phytoliths (D).CP. Cortical parenchyma, VC. Vascular cylinder, E. Endoderm, arrow a. oospores,arrow b. hyphae,arrow c. phytoliths. Bars = 100 µm in A, B, C and D (0 mM SiK®). Bars = 20 µm *in D of 5 mM, 7.5 mM and 10 mM SiK®* treatments.

Otherwise, multiplication of oospores in the root cortical parenchyma seems to be influenced by SiK® fertilization, since higher rates are visible on untreated plants (0 mM SiK®) and 5 mM SiK® treatment than in 7.5 mM and 10 mM SiK®concentrations (Fig. 3b and arrows a). The presence of these pathogenic structures in the xylem cells was responsible for the reduction of water translocation in the xylem vessels inducing their cavitation. Thus, water stress and, consequently, the death of the plants is the result of the action of this pathogen, as can be observed in the

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results showed in Fig. 2, where the percentage of surviving plants in the control treatment was only 30%.

In addition, a high number of *P. cinnamomi* hyphae (Fig. 3c and arrows b) was detected in the cell walls of root tissues from the Si deprived plants (0 mM SiK®). Their presence was indicated by the strong black color in the cell wall of root tissues (Fig. 3c). In Si-fertilized plants, the number and intensity of these structures (hyphae) in the cortical parenchyma was lower than in the former plants, decreasing with the increase of SiK®concentration (Fig. 3c and arrows b). After 150 days *P. cinnamomi* inoculation, hyphae (Fig. 3c, arrows b) was identified in the cortical parenchyma and the oospores (Fig. 3b and arrows b) in the same tissue and also in the vascular cylinder in non-treated plants (0 mM SiK®), while in SiK® groups they were practically nonexistent inside the vessels. On the other hand, only in Si-treated plants it is possible to observe the presence of the amorphous silicon deposits (phytoliths), crystals with cubic form in cortex tissue next to endoderm, involving all the vascular cylinder (Fig. 3d arrows c).Data suggested a direct correlation between the Si contents (Tab. 1) and the number of phytoliths in chestnuts plants.

The presence of phytoliths in the roots of inoculated plants appears to have a protective effect against pathogen penetration in the vascular system of plants.

Evaluation of the effect of soluble silicon on the radial growth of *P. cinnamomi*

Regarding the effect of different concentrations of Si on the growth of *P. cinnamomi* (Figs. 4, 5 and 6), the results show significantly differences between Petri plates contain Si (5 mM, 7.5 mM and 10 mM SiK®) in PDA medium and control (0 mM SiK®) with only PDA medium. Analyzing the Fig. 4 it can be observed that all Petri plates containing Si+PDAdidn't present any growth of *P. cinnamomi*, demonstrating an evident percentage of inhibition (PI) of 100% at 24h after incubation while the control (0 mM SiK®) showed a faster growth of this pathogen, presenting therefore a PI of 70% (24h, Fig. 4).

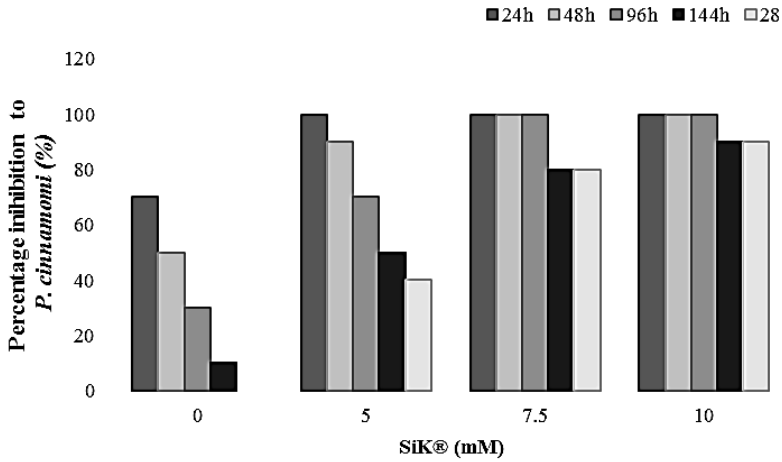

Fig.4: Percentage inhibition (PI) against to P. cinnamomi development in control petri plates (0 mM SiK®) and Si+ PDA petri plates (5 mM, 7.5mM and 10 mM SiK®). The PI were measured at 24, 48, 96, 144 and 288 h after incubation (n=20).

At 48h the 7.5 mM and 10 mM SiK® treatments, still maintain the total inhibitional against *P. cinnamomi*, showing a 100% of PI, followed by 5 mM and 0 mM SiK® treatments with 90 and 50% of PI, respectively (Figs. 4 and 5). As shown in Fig. 5 a great development of *P. cinnamomi* were observed in control and 5 mM SiK® treatments, while the high inhibition capacity were found in the highest concentration of Si applied in PDA medium (7.5 mM and 10 mM SiK®). Between 48h and 144h, the control treatment showed a reduction of 60% on PI comparatively to Si treatments (Figs. 4, 5 and 6).

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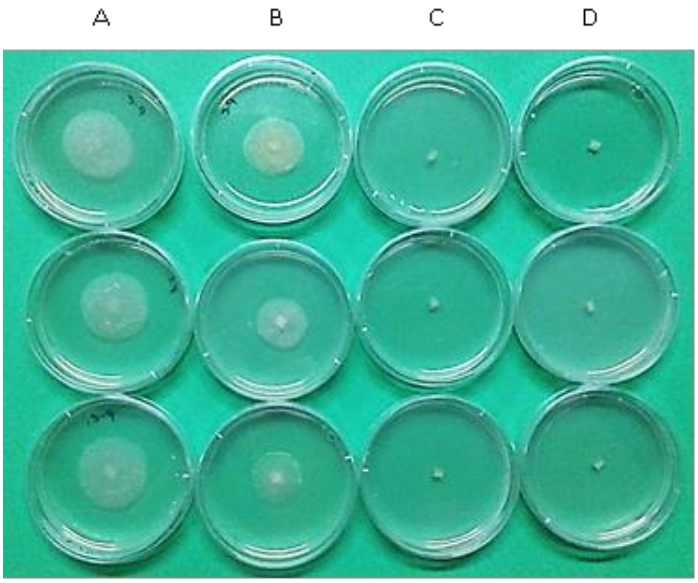

Fig.5: Mycelial grown of P. cinnamomi in response to 0 mM (A), *5 mM* (B), *7.5mM (C) and 10 mM SiK® (D) application on PDA at 48h after incubation.*

In the current study, the soluble Si application was effective in inhibiting the growth of *P. cinnamomi* in vitro which was directly associated to the augment of Si concentrations applied, recording 50%, 80% and 90% of PI in 5, 7.5 and 10 mM SiK® treatments, respectively (Figs. 4 and 6). In these treatments was also noted that from early period of incubation (24h) the inhibition of growth *P. cinnamomi* is quickly manifested by the presence of Si in PDA medium, demonstrating a great ability to reduces the growth and development of this problematic oomycete. Data is consistent with the results obtained previously, indicating that the application of Si to soil can help to reduce their propagation capacity of ink disease in chestnut plants.

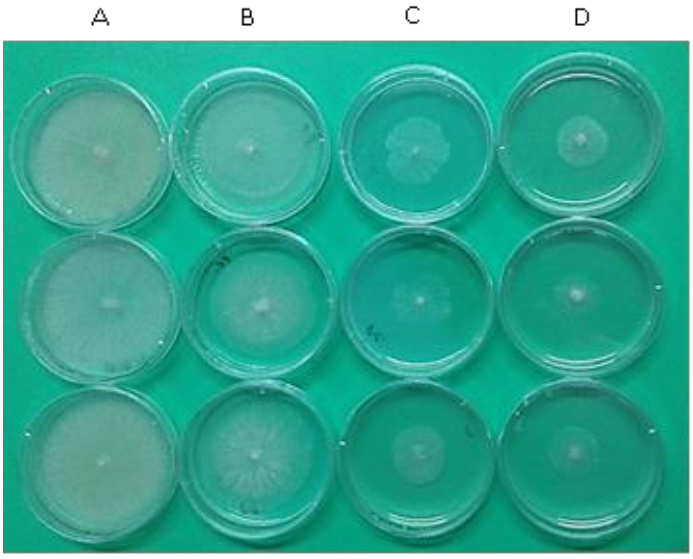

Fig.6: *Mycelial grown of P. cinnamomi in response to 0 mM* (A), *5 mM* (B), *7.5 mM (C) and 10 mM SiK® (D) application on PDA at 288h after incubation.*

MDA and hydrogen peroxide contents

MDA is considered an indicator of lipid peroxidation in the cell wall membrane. In the present study, a significant reduction on MDA amount were observed between 0 mM and 10 mM SiK® treatments, 61% and 68% at 15 and 30 days after inoculation (Fig. 7). Moreover, the 7.5 mM and 10 mM SiK® treatments recorded the lower values of MDA, 0.840 and 0.610 μmol g^{-1} FW (Fig. 7) respectively, compared to control treatment (0 mM SiK®) that achieved the higher value, 1.891 μmol g^{-1} FW at 30 days after

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inoculation (Fig. 7), indicating that Si application can affect the ability of *P. cinnamomi* to infected the plants reducing the damage in cell membrane, while the untreated plants (0 mM SiK®) showed a significative augmented on MDA level in same time.

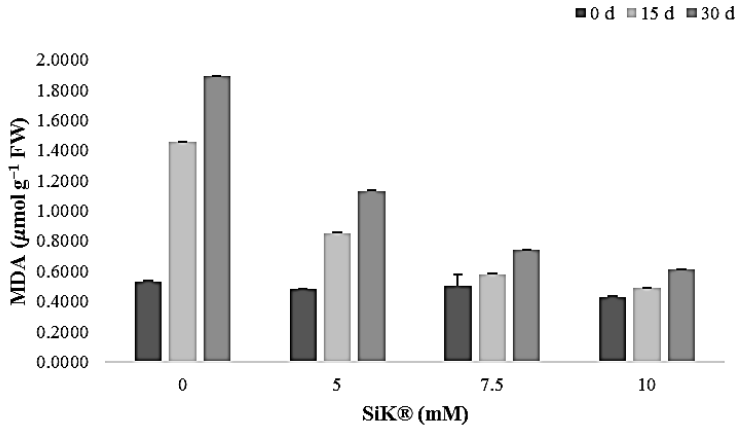

Fig.7: Effect of Si application (0 mM, 5 mM, 7.5mM and 10 mM SiK®) on malondialdehyde (MDA) amount at 0, 15 and 30 days after inoculation by P. cinnamomi (n=6).

A similar trend was observed in H_2O_2 amount, the Si-treated plants (10 mM SiK®) recorded a significant decrease on this parameter, 37% at 15 days and 54% at 30 days after *P. cinnamomi* infection compared to Si-deprived plants (Fig. 8). At 15 days after inoculation the lower values of H_2O_2 amount were 0.410 and 0.390 mmol g^{-1} FW in plants treated with the highest concentrations of Si (Fig. 8). Comparing the results between 0 and 30th day, the H_2O_2 content (Fig. 8) increased 357% in Si-free plants while in Si-supplied plants (10 mM SiK®) recorded an increase of only 14%. These results suggest that Si application contributes to reduce the lipid peroxidation and oxidative stress in chestnut plants inoculated by *P. cinnamomi*.

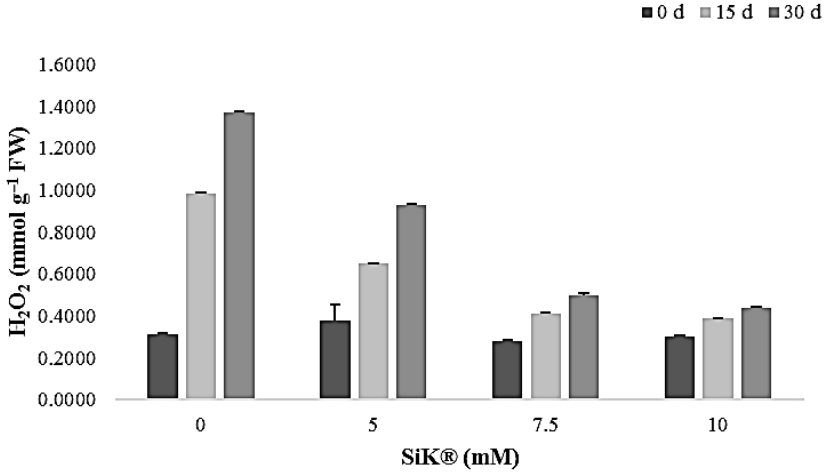

Fig.8: Effect of Si application (0 mM, 5 mM, 7.5mM and 10 mM SiK®) on hydrogen peroxide (H_2O_2) amount at 0, 15 and 30 days after inoculation by P. cinnamomi (n=6).

Total phenols compounds

As shown in Figure 9, the Si fertilization increases the total phenol compounds (TP) content in response to *P. cinnamomi* infection, with an augment of 180% and 194% in 7.5 and 10 mM SiK® treatments, respectively at 15 days after inoculation. At 30 days, the synthesis of TP was more significative, with an increase of 350% and 393% (Fig. 9) recorded by the chestnut plants treated with the highest concentrations of Si (7.5 and 10 mM SiK®). Moreover, it is important highlight that the TP amount increased significative from 0.20 to 0.81 mg g-1 FW^{-1}, between 0 and 10 mM SiK® treatments, representing an increase of about 305% at 30 days after *P. cinnamomi* infection (Fig. 9).

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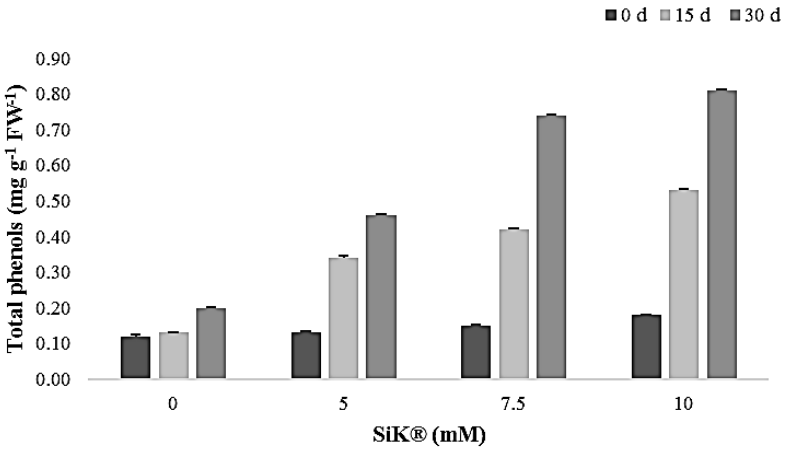

Fig.9: The effect of Si application (0 mM, 5 mM, 7.5mM and 10 mM SiK®)on the total phenol compounds at 0, 15 and 30 days after inoculation by P. cinnamomi (n=6).

Antioxidant activity

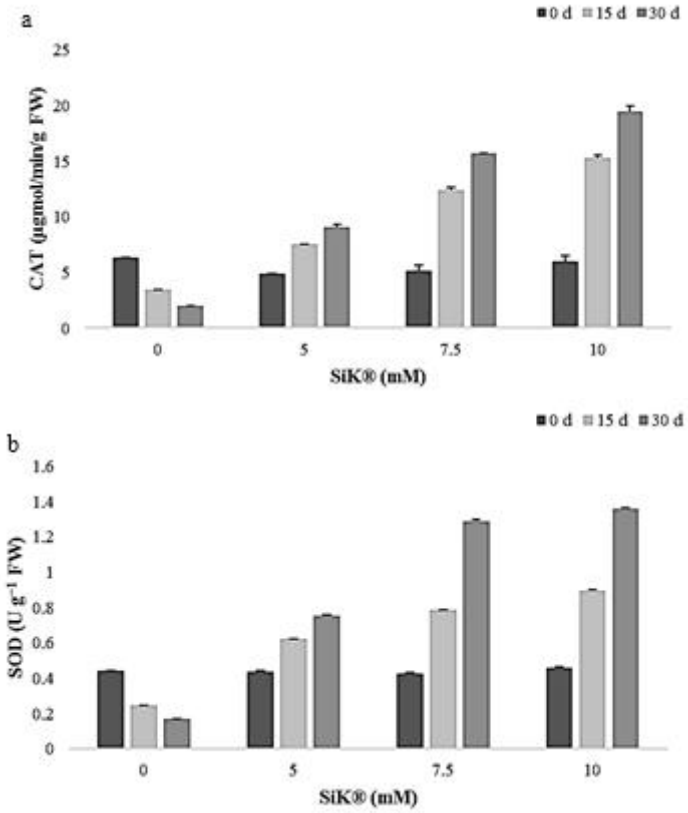

Fig.10: *Activities of catalase (CAT) and superoxide dismutase (SOD) in untreated (0 mM SiK®) and Si-treated plants(5 mM, 7.5 mM and 10 mM SiK®) at 0, 15 and 30 days after P. cinnamomi infection (n=4). a. CAT activity expressed as µgmol/min/g FW. b. SOD activity expressed as U g^{-1} FW.*

The CAT and SOD activity on untreated and Si-treated plants are illustrated in Fig. 10a and 10b, respectively. Data showed that Si application enhanced significantly the CAT activity in response to inoculation, recording an increase of 73% and 102% (10 mM SiK®, Fig. 10a) at 15th and 30th day, while the control (0 mM SiK®) presented a reduction of 46% (15th day, Fig. 10a) and 64% (30th day, Fig. 10a). Similar tendency was observed in SOD activity (Fig. 10b), theSi fertilization influenced significantly the activity of this enzyme, conferring higher protection of the host plants against to this root diseases through improvement their defense system than control plants (0 mM SiK®). The SOD activity increased significantly in 7.5 mM and 10 mM SiK® treatments than control treatment (0 mM SiK®). As shown in Fig. 10b, in Si-treated plants (10 mM SiK®) was recorded a significant increase on SOD activity from 0.456 to

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0.895 U g^{-1} FW between 0 and 15th day after inoculation, however in Si absent plants (0 mM SiK®) is observed an decrease from 0.443 to 0.244 U g^{-1} FW. Furthermore, the values of SOD activity were significantly higher at 30th day than those in 0 day, increasing 198% in 10 mM SiK® treatment, while in untreated plants (0 mM SiK®) suffer a reduction of 62% (Fig. 10b). As shown in Fig. 10b, the SOD activity increased with time in all Si-treated plants by 30%, 50% and 70% for of 5 mM, 7.5 mM and 10 mM SiK® treatments, respectively, reached the highest amount at 30 days (Fig. 10b).

IV. DISCUSSION

The Si have a beneficial role in the protection of agricultural crops against diseases, however the effect of this element in chestnut plants against ink disease, was never approach, reason for why we decided investigated.

The exogenous application of Si enhanced significantly the Si amount and promoted a greater absorption of N, P and K (Tab. 1) in chestnut plants, being this nutrients benefits to the growth and development of crops. Similar results were found by Pati *et al*., (2016) in rice plants. The improvement of mineral nutrition observed in Si-treated plants demonstrates that Si applicationenabled the augment of the solubility and the absorption of the Si, N, P and K, as verified by Epstein, (1999); Bekker *et al*., (2007); Lima Filho and Tsai, (2007).

The increase of Si amount in Si-treated plants can be explainedby the augment of Si availability in the soil and with the enhance of root system, stimulating the chestnuts to absorb more Si from soils. These results are in accordance with Datnoff and Rutherford (2004); Lima Filho and Tsai (2007) who reported a significant increase in the percentage of Si accumulated in bermudagrass, wheat and oat leaves treated with the higher rates of calcium silicate.

The resistance of plants to ink disease is generally determined by the greater or lesser ability of the host plant to limit penetration, development and/or reproduction of this phytopathogen in their tissues after being inoculated by the invading agent (Gouveia and Abreu, 1994).

The beneficial role of Si in plants is associates frequently to the decrease diseases intensity, by their translocation and accumulation in tissues (Pozza *et al*., 2015). Data showed a correlation between the inoculation of leaves (Figs. 1a and b) and roots inoculation method (Fig. 2). In this work, the 7.5 mM and 10 mM SiK®treatments demonstrated a greater resistance against *P. cinnamomi* inoculation by hindering and avoid the appearance of chlorosis in the leaf discs, promoting a larger percentage of free chlorosis disks, as well as a high survival rate (80%) compared to control treatment (30%), suggesting that Si contributes to the augment of resistance in chestnut plants against to ink disease. Consistently, the presence of Si in PDA medium reduces the radial growth of *P. cinnamomi*, presenting a PI of 90% at 288h (Figs. 4 and 6), comparatively to Si-free plants that showed a PI of 0% in the same time. These results can be explained by the phytotoxic effect of Si, which increase with the rise Si concentration applied (Fig. 4 and 6). These findings confirmed previous studies of Ebrahimi *et al*., (2012) and Farahani *et al*., (2012 a, b), who reported that addition of increasing concentrations of Si to PDA medium completely inhibited the mycelial growth of *P. expansum* and *Candida membranifaciens* in apple plants allowing the biocontrol of these pathogens. Additionally, Kaiser *et al*., (2005) and Mahdikhani *et al*., (2008), referred that the application of SiK® in Petri plates has the capacity to reduce the growth of *P. cinnamomi* and *Fusarium oxysporum* in avocado and melon plants, respectively.

The roots histopathology carried out allows to analyze and understand the state of the roots tissues and the degree of colonization of these by *P. cinnamomi* infection in the different treatments under study (Fig. 3). In Si-fertilized plants were observed the accumulation of cubic amorphous Si bodies, denominated phytoliths (Yoshida *et al*., 1962) in the root tissues. The observation of phytoliths with cubic form were also reported by Ma and Yamaji (2006) and Neethirajan *et al*. (2009) in grass plants. Phytoliths act as a mechanical barrier due to the silicified of cells lead to the augment the resistance of the cell wall difficulting the entry of the oomycete, its development and their respective colonization making their walls thicker and more rigid, being one of the explains to the reduced number of hyphae and oospores observed in root cuts from Si-treated plants compared to control, as also verified by Monteiro *et al*. (2017). The presence of oospores in infected root tissues has also been reported by others authors in avocado, soybean and holm oak (Mircetich and Zentmyer; 1967; Ruiz-Goméz *et al*., 2012).These results suggest that the cell wall fortification of chestnut roots induced by Si-addition may be closely associated with the enhance of host resistance to ink disease. Indeed, plants treated with the highest concentration of Si showed a higher total phenols compounds (TP, Fig. 9), a higher minerals content (Tab. 1) and a higher percentage of tolerant plants to ink disease than non-treated plants (Fig. 2). Similar results were also reported by Amaral *et al*., (2008) in coffee plants.

Several authors also observed pathogenic structures (hyphae and oospores) in holm oak, *Quercus suber* (Ruiz-Gómes *et al*., 2015) and *Quercus ilex* (Ebadzad *et al*., 2015) infected with *P. cinnamomi*. These findings are in accordance with the studies of Oh and Hensen (2007) in oregon ceder plants and Monteiro *et al*., (2017) in chestnut plants, who observed the presence of oospores inside phloem and xylem cells in non Si-fertilized plants (0 mM SiK®) and a very lower number in Si-treated plants (10 mM SiK®, Fig. 3). Moreover, the high number of

pathogenic structures (hyphae and oospores, Fig. 3) are justified by oomycete's need for nutrients increased after reaching the parenchymal cells of the central cylinder, allowing for faster growth and, finally, for expansion towards new unexplored root areas through vascular tissues (Ruiz-Gomés *et al*., 2015).

Data also indicate that the augment in concentration of Si applied increase the number of phytoliths in plants tissues and consequently reducethe infection in root tissues by the oomycete responsible for ink disease. Similar results were found by Huang *et al*., (2011), who reported that Si reduce the damages of *Fusarium crown* in root tomato plants, indicating its ability to block the progression of the fungus.

Relatively to the chemical defense proportionated by Si fertilization in chestnut plants, the CAT and SOD are considered important antioxidant enzymes responsible for the defense responses and are frequently associated with the reduction on the reactive oxygen species (ROS) in resistant plants against diseases, according to Sakr, (2016). In the current study, Si applicationenhance significantly CAT and SOD activity (Fig. 10) after infection by *P. cinnamomi*, that suggesting the Si reduce the H_2O_2 amount and restricts the development of this pathogenby inducing synthesis of antioxidant enzymes and phenols compounds in host plants.

CAT is an enzyme that decompose the H_2O_2 into water and oxygen (Song *et al*., 2016). In this context, the results demonstrated high CAT activity in Si-treated plants (Fig. 10a) lead to lower H_2O_2 amount in tissues than control plants (0 mM SiK®, Fig. 10a). The extent of the damage caused by *P. cinnamomi* inoculation can be associated to the oxidative stress, the infection of chestnut plants by ink disease promote an accumulation of H_2O_2(Fig. 8) and consequently, higher MDA amount (Fig. 7), the first product in membrane lipid peroxidation that allow to index the degree of injury to the cells.

The beneficial effect of Si in CAT and SOD activity were also reported by Fortunato *et al*., (2012) and Schurt *et al*., (2014) in banana and rice plants against *Fusarium wilt* and *Rhizoctonia solani*, respectively.

Data showed that the high SOD activity in Si-treated plants (Fig. 10b) reduced MDA level (Fig. 7), while the augment in CAT activity (Fig. 10a) decreased the H_2O_2 accumulation (Fig. 8), demonstrated that Si application as associated with their ability to resist biotic stress. These results are in agreement with Mohaghegh *et al.*, (2011) who reported that the Si addition in cucumber plants inoculation by *P. melonis* increase the CAT and SOD activity, enzymes involved in the plant-pathogen tolerance.

The MDA and H_2O_2 amount (Figs. 7 and 8) in chestnut plants at 30 days after inoculation was significantly higher than 15 days, suggesting that the injury caused by *P. cinnamomi* increased with a prolonged infection period in plants. Our results reinforce that Si addition protects antioxidant enzyme system in chestnut plants helping to increase their resistance caused by ink disease infection. The Si-absent plants recorded a decline in CAT and SOD activity, while Si-treated plants exhibited higher values of this enzymes, indicating that these enzymes reduced the free radical damage and thus improve their resistance, because Si promotes the systemic acquired resistance defense, by plant signaling against pathogens and synthesis of defense compounds, reinforcing the previous studies of Lu *et al*., (2008); Mohaghegh *et al*., (2011) and Fortunato *et al*., (2012) in asparagus, cucumber and banana plants, respectively.

The present results demonstrate that the antioxidant enzyme activities improve with time after *P. cinnamomi* inoculation in 7.5 Mm and 10 Mm SiK® treatments. Additionally, the Si-treated plants recorded the highest levels of TP (Fig. 9), indicating that Si stimulate a quickly and efficient production of this compounds. Phenols in plants are important to their defense against this type of biotic stress by increasing their natural chemical defense. These results are supported by the researches of Chérif et al., (1992, 1994), who suggested that phenols increased in Si-fertilized cucumber plants after infection with *Pythium ultimum*, compared to control plants. Similar results were found by Han *et al*. (2016) in rice plants, who reported that Si interacts with the defense-associated signaling pathways and seems to regulate a range of physiological activities in plant stress defense.

The highest TP content (Fig. 9) recorded by Si-supplied plants are consistently with the highest values of *P. cinnamomi* free leaf discs (67% and 72%) (Fig. 1a) andsurvival rate (80%) (Fig. 2). Furthermore, these metabolites promote defense of the chestnut plants and help to maintain the healthy root tissue resulting in the decrease of pathogenic structures (Fig. 3). The resistance of fertilized plants with SiK® against *P. cinnamomi* may be associated with the physical barrier, composed by phytoliths as mentioned before (Fig. 3c) and with the high phenol amount.

The induction of antioxidant activity by the chestnut fertilization with Si may represent one of the mechanisms of action against the attack of *P. cinnamomi*. Several studies have shown that Si assists plants in defense against phytopathogens by inducing the defense reactions, biosynthesis of phytoalexins, enzymes and PR's proteins (Song *et al*., 2016; Wang *et al*., 2017).

The augment of plants defense by Si application has been associated to the increase of signaling components amount and the defense hormones (such as salicylic acid, jasmonic acid and ethylene), which are important to establish the plant's innate immune system and are associated with resistance. Besides that, after perceiving a pathogen-derived signal, the plant would create a faster and stronger immune

response to the pathogenic agents, the prophylactic effect of silicon is considered to be the result of both passive and active defense (Van Bockhaven *et al*., 2013). In addition, Si also regulates the genes defense as referred by Ye *et al*., (2013), who suggest that silicon application in plants can facilitate the accumulation of inactive cellular proteins involved in signal transduction, such as MAPKs, and lead to the rapid activation of these inert signaling components, thus increasing the host's defensive processes and/or the speed with which they are activated.

The Si-fertilized chestnut plants responded quickly and effectively to *P. cinnamomi* inoculation, rapidly activates the natural defense mechanisms of the host, and provides physical protection and chemical defense that help in increasing the resistance of plants to the attack of pathogen. Data reveal that Si improve the physical and biochemical defense of chestnut plants from 7.5 mM and 10 mM SiK® treatments which demonstrated more resistance against to this oomycete responsible by ink disease.

The present research suggests that SiK® fertilization could be successfully used in control of ink disease and can represent a control method that is efficient, cost-effective and not harmful to the environment. For these reason is necessary the divulgation of the knowledge about Si to farmers in order to helpcontrol chestnut diseases, increase the resistance of their plants and consequently improve the chestnut fruits production and quality.

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