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Histological characterization of resistance and some alternative control for leaf spot disease in olive tree.

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Abstract - Field trail was conducted on the reaction of four olive cultivars (Chetoui, Picholine, Arbequina and Meski) toward the leaf spot disease caused by *Fusicladium oleagineum*. The results showed that higher disease incidence tends to be associated with thinner cuticle (r=-0.69) and higher trichome density (r=-0.58) and diameter (r=-0.9) of the infected plant. Glasshouse experiments were, then, carried out to study the effects of nitrogen and potassium fertilizing post-inoculation treatment and two antitranspirants (Linseed oil and Vapor Guard) pre-inoculation spray on leaf spot disease incidence on susceptible olive variety, cv. Meski. Results showed that potassium treatment and the Linseed oil 7% or Vapor Gard 3% spray reduced significantly the disease incidence by means of 67% and 80%, respectively.

Keywords: Olive, antitranspirant, nitrogen, potassium, Linseed oil, Vapor Guard, incidence disease.

1. Introduction

Tunisia is the most important olive (*Olea europaea* L.) growing country of the southern Mediterranean region where over 30% of its cultivated land is dedicated to olive growing (1.68 million ha). The annual production is estimated to 376,000 t of olives in 2014 (FAO 2014).

The disease of the peacock eye (also called olive leaf spot or OLS) is considered among one of the most important diseases of the olive tree. It is caused by the fungus *Fusicladium oleagineum* (syn *Spilocaea oleaginea*), a specific biotrophic pathogen with a subcuticular development manifested by lesions on the leaves of olive trees (Gonzales-Lamonthe et al. 2002). The disease causes severe premature defoliation of the olive tree, and sometimes the death of the whole plant (Miller 1949; Azeri 1993). Infections affecting the fruit can cause significant damage on the table olives (cv. Meski). In olive oil cultivars, these infections can cause delayed maturation and reduced yield (Verona and Gambogi, 1964). The disease is widespread in the world and in specialized areas of cultivation of olive trees with yield losses estimated at more than 20% (Wilson and Miller 1949).

So, improvement of cuticle structure, by means of the use of antitranspirants and fertilizers, might be an alternative in fighting of olive to leaf spot disease.

The treatment with a chemical fungicide (dodine) applied before disease onset (spring and fall) is the main method used for the control of this disease (Prota 1995). The continuing use of chemical fungicides in the plant disease control has an undesirable environmental impact, and is leading to the establishment of the phenomenon of resistance of phytopathogenic fungi. This problem has spawned the use of products respecting of the Environment leading to reduce the effects of widespread use of fungicides in plant protection (Coats et al. 2003).

As alternative to fungicides, antitranspirants are spread in a thin continuous layer on the surface of the sheet to prevent any penetration of fungi and other pathogens (Marcelis et al. 2005). They have no toxicity to the environment or to the pathogen. The antitranspirant may therefore exclude fungi by physical separation through preventing contact between the pathogen and the tissue of the host plant that stimulates the pathogen penetration structures. Protection using antitranspirants is shown to be more effective than treatment with fungicides against powdery mildew, Septoria spots, mildew and rust on different crops (Nasraoui et al. 1996).

The use of fertilizers in the protection of plants against pathogens is to avoid stress phenomena and promote plants to benefit from the contributions despite pathogen (Palti 1981). But a mineral element can decrease the severity of certain diseases and other increases it in different environments. It is therefore necessary to establish a nutritional balance for optimal response of the plant (Huber and Haneklaus 2007).

The aim of this study is to evaluate the effects of fertilizers and antitranspirants on the reaction of the plant to the OLS disease on one susceptible variety and to investigate the relationship between some structural characteristics of the plant (cuticle and trichomes) and the infection severity by this disease on some olive varieties.

2. Materials and methods

2.1 Plant material

Field studies were carried out on the olive trees of four cultivars (Chetoui, Picholine, Arbequina and Meski) at the Experimental Station of the Higher School of Agriculture of Kef in the north west of Tunisia (36°.12'E; 8°.7' N, 516 m above sea level) during 2010, 2011 and 2012. To study the incidence of leaf spot disease under natural infection, 100 fresh leaves from each cultivar (5 trees and 4 branches) were collected during spring time, the main period of olive attack by OLS in orchards.

For a glasshouse trial (16-23°C, 30-60% RH), experiments were conducted on 2-year-old Meski cultivar, which is highly susceptible to OLS (Graniti 1993). Plants were grown individually in 9 liter plastic pots containing a mixture of composted bark and pumice (4:1, v/v) with slow release fertilizer (N:P:K ¼ 15:4:7.5). The youngest ten fully expanded leaves were marked. The moisture was supplied by a manual spraying with distilled water for 10 min/day. Cultural practices were similar for all plants and followed the standard procedures described in the North of Tunisia manual for commercial olive growers (AMT 2007).

2.2 Fungal material

To inoculate plants of the galss-house trial, suspensions of conidia of *F. oleagineum* were prepared from fresh diseased leaves of olive tree (cv Meski, susceptible), showing typical symptoms of OLS caused by F. *oleagineum*. In order to obtain the suspension, the infected leaves were dried at room temperature for 2 weeks and stored at 5°C in darkness (Trapero et al. 1994). Then, these leaves were agitated in sterile distilled water for 2 h at 80 rpm. The obtained conidial suspension was filtered through a double layer of cheesecloth to eliminate vegetable matter (Alonso et al. 2006). The concentration of the conidial suspension was adjusted at 5 10^4 conidia/ml by using a Malassez cell.

2.3 Plant inoculation

Meski cultivar planted in the glasshouse was inoculated by F. *oleagineum* conidia. The previously prepared suspension was sprayed onto plants using an atomizer to just before run-off. For the next days, the plants were enclosed within a clear polythene tent (plastic bag) constructed around each plant to increase relative humidity and provide sufficient moisture for infection. Control was treated with distilled water.

2.4 Antitranspirant spray

To evaluate the protective activity of the antitranspirants in the glasshouse trial, Linseed oil and Vapor Gard antitranspirants were pre-inoculation used both at two concentrations: 3 and 7% (v/v). Linseed oil consists in pure linseed oil and contained the α-linolenic acid, linoleic acid, oleic acid and saturated fatty acids; its diode index is about 170. Vapor Gard (VG; di-1-*p*-menthene) is a water-emulsifiable organic compound for use on plants to reduce water transpiration. Antitranspirants were applied with a hand-held spray bottle until runoff, using a volume of 10-15 ml/plant (Ansary and Al-Humaid 2005).

2.5 Fertilizer treatment

Two fertilizers were post-inoculation used in the glasshouse trial. The first, based on nitrogen, was the ammonitrate (NH_4NO_3); the second was the monopotassium phosphate (KH_2PO_4). A total of 18 plants were divided on two groups. The first group of trees received two doses of nitrogen (55 and 100 g/pot); the second group was treated also with two doses of potassium (40 and 130 g/pot). The first

treatment by nitrogen and potassium were applied in March, one week post-inoculation. Fertilizers were applied as a solution for individual pots. After treatments, plants were returned to their tents.

2.6 Infection evaluation

The determination of the infection incidence was performed using the method of Alonso et al. (2006). It consists in dipping leaves in a 5% NaOH solution for 20 min and then symptoms were observed. For the natural infection field trial, a total of 100 leaves were collected from three trees of each variety and NaOH treated. Regarding the inoculation glasshouse experiment, the same treatment has concerned 10 leaves collected from each plant, 12 weeks after inoculation. In both cases of trials, disease incidence assessment was performed by measuring the percentage of the infected leaves.

2.7 Measurements of trichome diameter and density

Trichomes are cells that lie on the upper leaves of olive cultivars. Because these cells are difficult to detach, we proceeded to use their scares left after bonding with ribbon of scotch on the upper side of the leaf. Once the ribbon removed carefully, the scare of trichomes is glued on the scotch. After cleaning the graded blades with alcohol, slides were observed by microscope for each collected leaf to measure the trichome density and diameter of leaves of the field trial (Larkin et al. 1996).

2.8 Cuticle thickness measurement

The preparation of sections of leaves from each variety of the field trial was carried out by cutting transversally by a sharp blade without sawing to keep intact the sheet structures. The cuts must be fine and perpendicular to the axis of the leaf. The sections were washed with water, and then 2 to 3 drops of iodine were added for duration of 10 s. Finally, the cut pieces were placed on a slide on which some drops of carmine were filed and then covered with a cover slip. The prepared slides were observed by means of microscopic (x400) and measurements of the thickness of the cuticle for each collected leaf were performed. Photos are taken with a Canon digital camera (Rhouma et al. 2013).

2.9 Data analysis

For the field trial, infection assessment was based on a total of 100 leaves taken randomly from each cultivar with 20 leaves/branch on five branches selected in various directions. The sampling was repeated three times and data were transformed to meet the requirement for normal distribution.
In the glasshouse, experiments were carried out according to the completely randomized block design with three replications. So there are three plants for each block with three pots for each. The pots were placed separately for each concentration in polyethylene bags.
The results were analyzed using analysis of variance (ANOVA) and Duncan's multiple range tests (SAS 1985).

3. Results and Discussion

3.1 Leaf anatomy

Leaf anatomical characteristics were varied by cultivar (Figure 1). Indeed, we observed differences of the anatomical parameters assessed. The cuticule of 'Meski' were thinner than those in 'Picholine', 'Chetoui' and Arbequina leaves. Similarly,'Meski' and 'Arbequina' had a lower density of trichomes compared to 'Chetoui' and 'Picholine'.

3.2 Infection incidence

The results showed an important difference in the susceptibility of the four cultivars to the disease. The analysis of variance and the means comparison revealed significant differences between these cultivars ($p<0.001$). The cultivar Picholine was the most resistant to the disease since almost no infection was observed. The cultivars Chetoui and Arbequina were less resistant with an incidence infection between 15 and 20%. However, cv. Meski was the most susceptible to the disease, with the percentage of infected leaves exceeding 60 %.

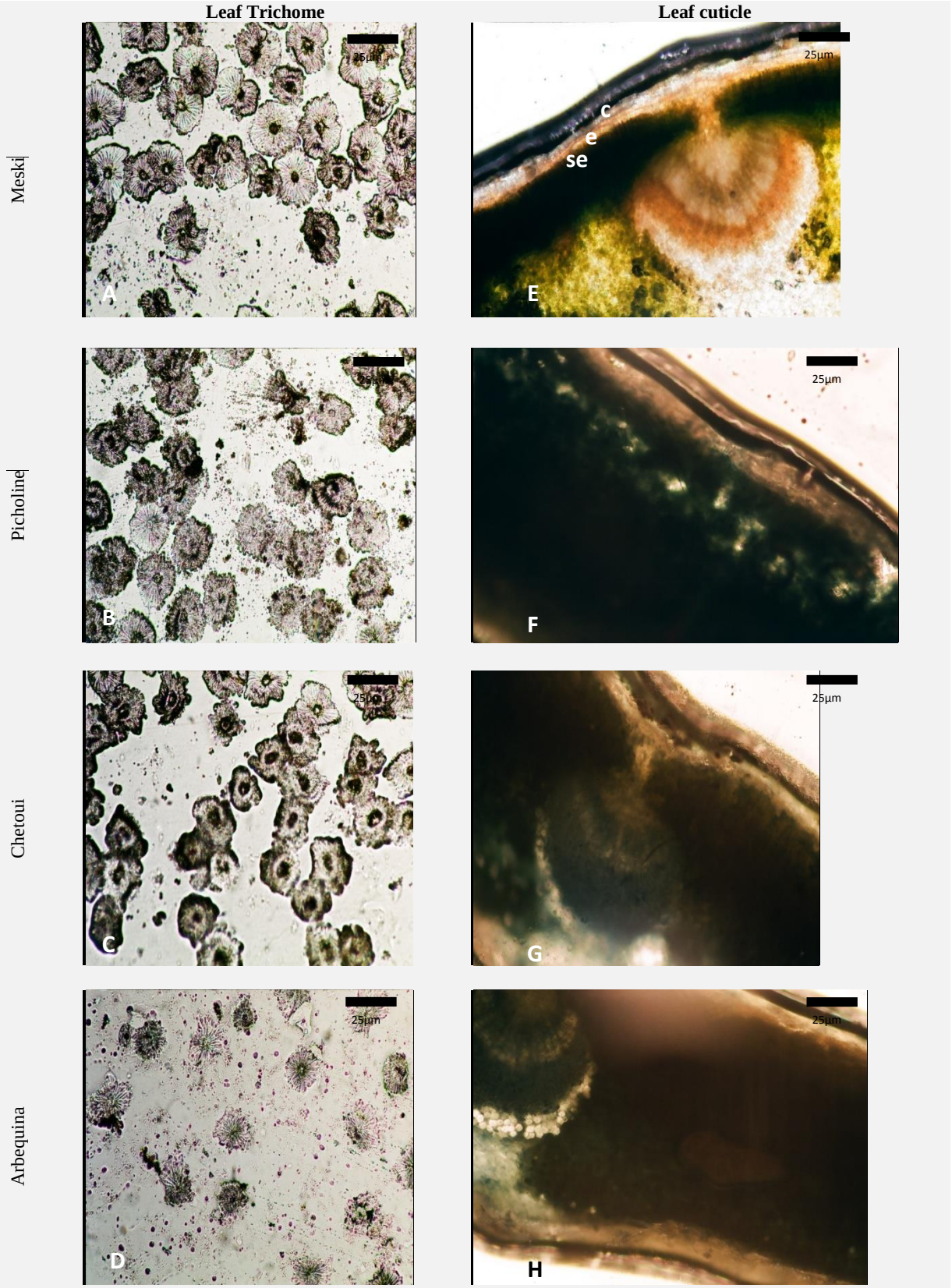

Figure 1 : Trichome distribution in the abaxial epidermis of the leaf and Transverse cuts of the leaf tissus of four olive cultivars (D : Arbequina Cultivar and A : Meski cultivar showing a less trichomes leaf distribution than the others B et C . E: Transverse sections of Meski. Leaf showing normal epidermal and sub-epidermal cells and properly arranged parenchyma cells with more chloroplasts. F: Transverse sections of Piccholine leaf showing larger epidermal and bigger sub-epidermal cells, loosely arranged parenchyma cells with few chloroplasts, G and H are similar. c: Cuticle, e:epidermis, se: sub-epidermis.)

3.3 Cuticle thickness

The results on Figure 2 show that the cultivars Picholine and Arbequina have significantly the same thickest cuticle (between 8 and 9 µm). In contrast, the cultivars Chetoui and Meski have the thinnest cuticle, around 5 µm. Compared on infection incidence, there are a significant difference between Meski cv. and the others cvs. (Figure 2). Analysis of results showed that the cuticle thickness is raised more than the percentage of infestation is low, inversely proportional (r=-0.69).

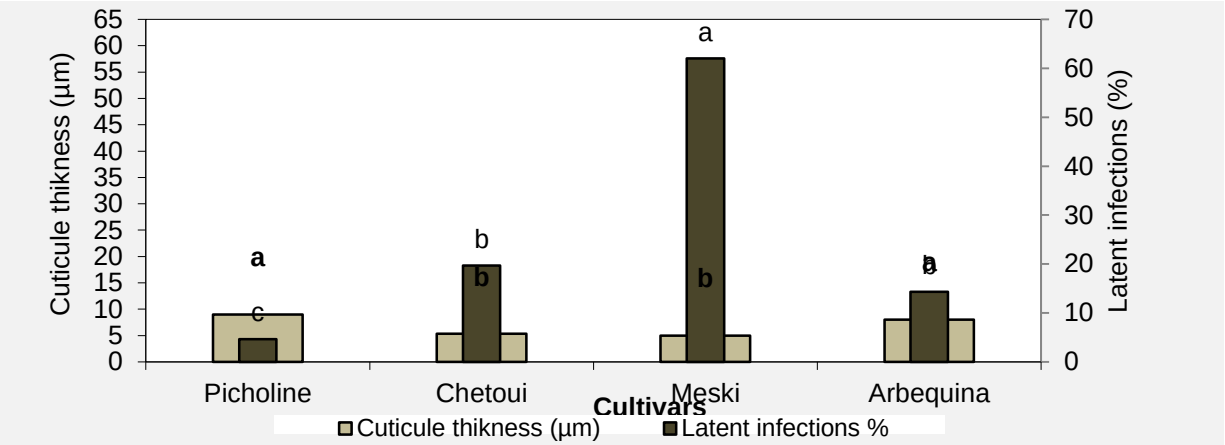

Figure 2. Cuticle thickness (■) of four olive cultivars studied regarding the leaf spot latent infections (■) caused by Fusicladium oleagineum.

3.4 Trichome size and density

Regarding the trichome density, Chetoui and Arbequina have the highest density (more than 25 trichomes/mm²) whereas cv. Meski had the lowest density of trichome (less than 15 trichomes/mm²). Cv. Picholine has an intermediary density with around 20 trichomes/mm² (Figure 3)

Measurements of the size of trichomes showed a difference in their diameters according to the cultivars. Cv. Meski has the smallest diameter of trichome (less than 90 µm) as compared to the others cultivars where the diameters were between 115 and 145 µm (Figure 4). Results showed that percentage of infestation decreased significantly when the dimension and the density of trichomes increased (r=-0.9 and r=-0.58 respectively).

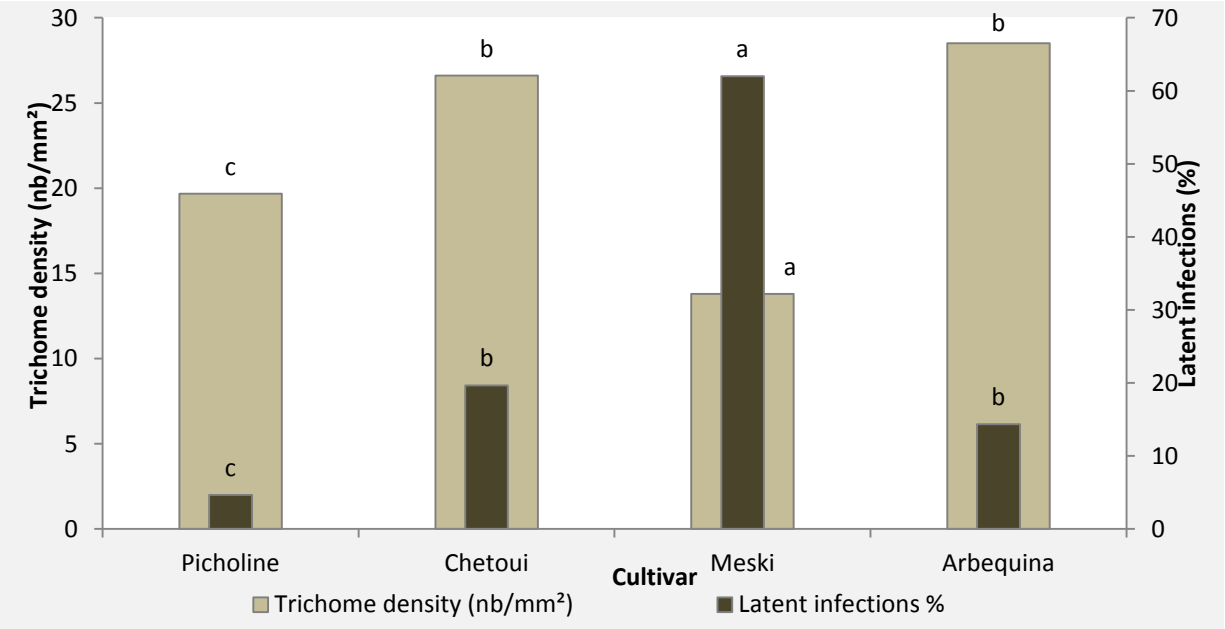

Figure 3. Trichome density (■) of four olive cultivars studied regarding the leaf spot latent infections (■) caused by *Fusicladium oleagineum.*

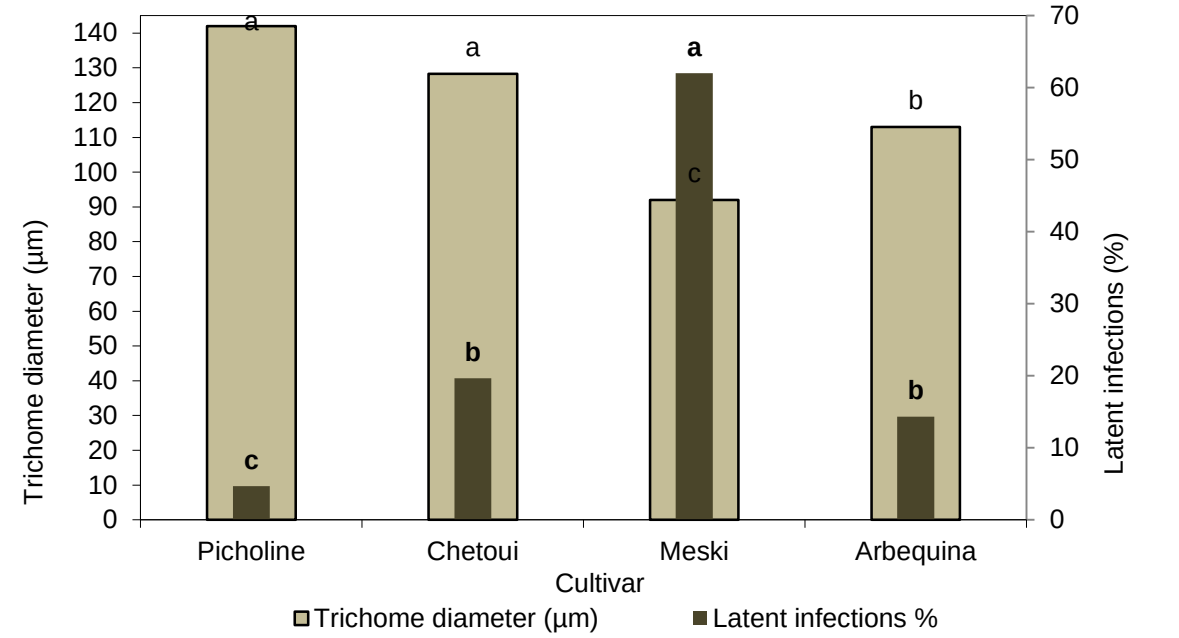

Figure 4. Trichome diameter (■) of four olive cultivars studied regarding the leaf spot latent infections (■) caused by *Fusicladium oleagineum.*

3.5 Antitranspirant spray effect

The effect of antitranspirant treatment was significant ($P < 0.05$). At 12 weeks after inoculation, plants that have received Linseed oil at 7% or Vapor Gard at 3% showed fewer leaf lesions (less than 20%), but without significant difference compared to the control (Figure 5). The control and the treatment with Linseed oil at 3% were associated to intermediary infection intensities (between 30 and 40%). The highest incidence infection was observed with Vapor Gard 7% treatment (near to 60%), without significant difference with the control.

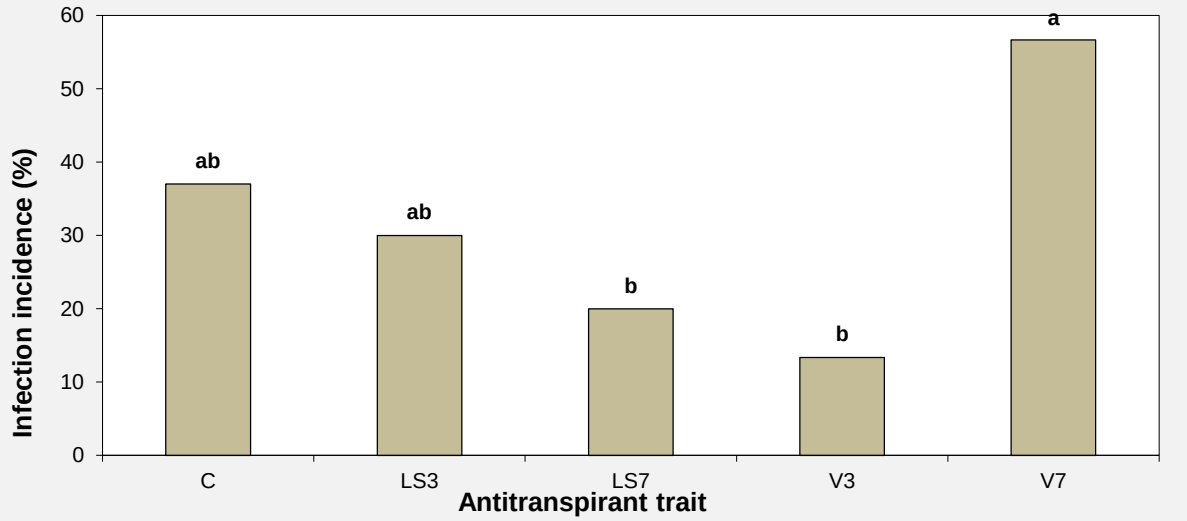

Figure 5. Effect of antitranspirant spray on the leaf spot disease caused by *Fusicladium oleagineum* on olive (cv. Meski), expressed by means of the infection incidence. C: control, LS3: Linseed oil at 3%, LS7: Linseed oil at 7%, V3: Vapor Gard at 3%, V7: Vapor Gard at 7%.

3.6 Fertilizer treatment effect

For all potassium treatments, there was an important decrease in disease incidence than in untreated plots throughout the glasshouse experiment (from 30% to less than 10%). In contrast, increase in disease incidence was recorded with the nitrogen treatments (figure 6).

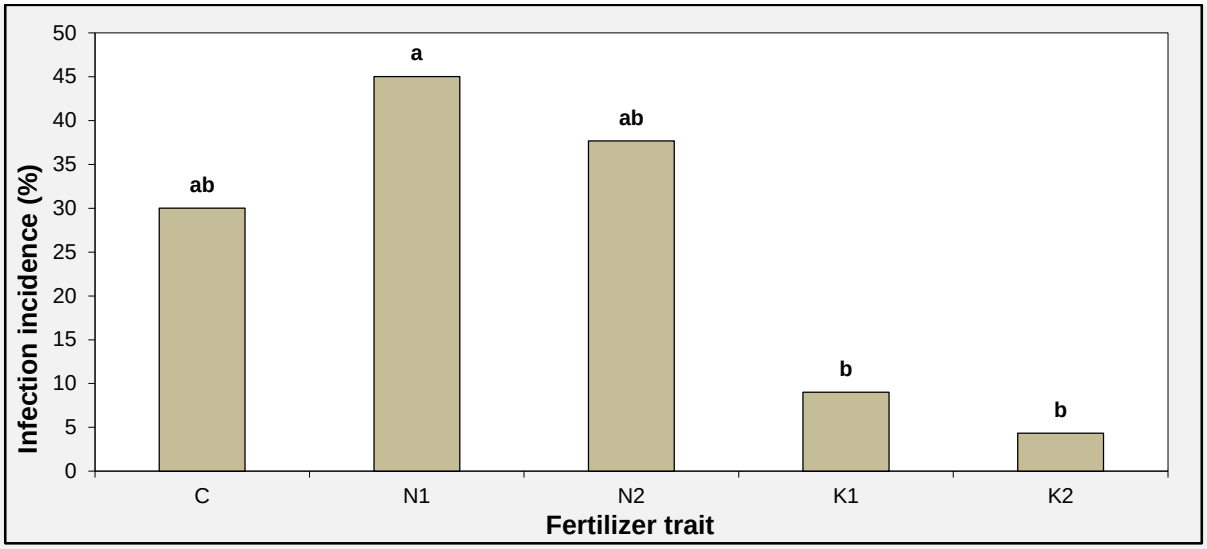

Figure 6. Effect of fertilizer treatment on the leaf spot disease caused by *Fusicladium oleagineum* on olive (cv. Meski), expressed by means of the infection incidence. C: control, N1: nitrogen 55 g, N2: nitrogen 100 g, K1: potassium 40 g, K2: potassium 130 g.

Field studies were carried out on four olive cultivars in the north west of Tunisia in order to investigate their resistance to OLS disease. Many studies showed that the cuticle presents a significant physical barrier against the penetration of the pathogen and then the thicker cuticle can prevent this penetration (Nawrath 2002). The analysis of results showed a difference between varieties in their cuticle thickness, and that thicker cuticle more difficult to overcome may act directly in reducing the OLS diseases infection. The same observation might be recorded regarding the trichome density and diameter in a way that more and bigger trichomes are unfavorable to the settlement and germination of conidia. Cv. Meski which is the most susceptible to OLS disease has the thinnest cuticle and the lowest values of trichome density and diameter. In contrast, cvs. Picholine and in a less extend Chetoui and Arbequina have their low susceptibility associated with thicker cuticle and/or higher density and diameter of trichomes. These results confirm those of Triki et al. (2003) who has recorded that Meski cultivar is the most susceptible and Chetoui cultivar is less susceptible whereas Picholine cultivar is more resistant to OLS disease.

In the glasshouse trial, antitranspirants provided preventive activity against OLS after inoculation. The efficacy of the antitranspirants tested in this study was greater for Linseed oil 7% and Vapor Gard 3%. This result confirms the worth of the use of antitranspirants instead fungicides because they can reduce the development of conidia such as those of *Venturia inaequalis* on apples and *Pyrinophora oryzae* on rice. These Antitranspirants have acted as a barrier that prevents the penetration of the pathogen through the cuticle (Shaffer and White 1985). The natural oils based formulations may be a good alternative to chemical fungicides (Wicks et al. 1999). The antitranspirant efficacy is a function of the thickness of the layer of the film, separating the film of the free water and disorientation germ tubes of the pathogen by the structural change in the leaf area (Sutherland et al. 2001).

The development of an appropriate plan of a mineral nutrition that contributes to the reduction of disease is an additional tool for minimizing the use of pesticides which means beneficial effects for the environment. In our glasshouse trial, the use of potassium seems to reduce the OLS disease intensity whereas nitrogen tends to increase it. This converges to other work results where nitrogen improves the development of several epidermal diseases caused by biotrophic fungi in such as wheat powdery mildew (*Blumeria graminis*) (Bainbridge 1974; Olesen et al. 2003), yellow rust (*Puccinia striiformis* f. sp. *tritici*) (Mascagni et al., 1997) and by semi biotrophic fungi such as Septoria leaf blotch disease (*Septoria tritici*) (Haward et al. 1994) and other fungi such as *Fusarium* wilt of cotton (Huber and Thompson 2007) and *Rhizoctonia* on wheat (Huber, 1980). In contrast, excessive potassium intake reduces the severity of diseases and this effect is related to the equilibrium K/N (James, 1996). This element can have a direct effect on the penetration of the pathogen, lesion size and density of the inoculums (Marschner 1999). A potassium deficiency predisposes plants to disease (Prabhu et al. 2007).

4. Conclusion

Our study demonstrated that genotype choice, antitranspirant spray and fertilizer treatment may be sufficient for effective control of OLS disease on olive tree. Four applications in spring of potassium fertilizer and autumn spray of Linseed oil 7% or Vapor Gard 3% have best results in control of the disease but further research is required to determine how many applications of which antitranspirants and fertilizers are likely to provide optimum control.

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