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Original article

Essential Oil of *Myrtus communis* L. effects *In Vitro* and *In Vivo* Evaluation against Chickpea Blight (*Ascochyta rabiei*) Disease

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Abstract

In this study, (*Myrtus communis* L.) was explored in vitro and in vivo for controlling blight disease using two chickpea genotypes ILC 3279, INRA 199 and a local cultivar Bouazza. Blight caused by *Ascochyta rabiei* Pass. Lab. is major disease affecting chickpea production. To control this disease, chemical harmful fungicides are used hence, developing natural plant protection products as an alternative has become important regarding the environmental impact. The effect of essential oil (EO) extracted from myrtle leaves by hydrodistillation was evaluated on the growth of *Ascochyta rabiei* Pass. Lab. mycelium, by dilution method using chickpea culture medium added with 0, 200, 300, 500 and 1000 ppm. The radial growth of A. *rabiei* Pass.Lab. was inhibited at 40.9, 68.18 and 96.69% with 200, 300 and 500 ppm respectively. Myrtle essential oil used at 1000 ppm inhibited the fungus growth completely (100%). *In vitro* EO efficacy was investigated using detached leaflets method inoculated with 5 µl pathogen spore solution of 106 spores / ml concentration. Efficacy was investigated using 750 ppm EO solution. Disease (DS) Severity and disease incidence (DI) on detached leaflets were determined. Highest disease incidence was observed on Bouazza (100%) followed by ILC 3279 (84.28%) and INRA 199 (17.5%). Myrtle essential oil showed an efficacy in all cases and decreased significantly disease incidence for Bouazza, ILC 3279 and INRA 199 to 63.33, 56.66 and 16% respectively. Disease severity showed the same profile, Bouazza noted the highest rate followed by ILC 3279 and INRA 199 with 60, 40 and 21% respectively and EO showed a significant efficacy and reduced these disease severity rates to 52 32 and 18 %. The bioassay conducted *in vivo* under greenhouse conditions with plants including a negative control with any treatment and two other batches inoculated by hand held sprayer, a spore solution with a concentration of 106 spores/ ml and treated or not with myrtle EO. Disease incidence percentage (DIP) decreased significantly under common myrtle EO compared to control in INRA 199 and ILC 3279 for severity percent index (SPI) no significant difference was observed for all genotypes under *Myrtus communis* L. essential oil treatment effect. This study showed *Myrtus communis* L. essential oil efficacy on chickpea blight disease *in vitro* on fungal radial growth and *in vivo* either on deatached leaflets or whole plants under greenhouse condition. This natural product reduced significantly blight disease incidence and severity. And to control disease, myrtle essential oil extraction as fungicide is a promising alternative against this disease.

Keywords: *Ascochyta rabiei* Pass Lab., efficacy, fungal disease, biocontrol.

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INTRODUCTION

Chickpeas are cultivated by ancient civilizations due to their high protein contents in their seeds (Van der Maesen 1987). It is a very important plant grown in 50 countries covering an area of more than 13 million ha (FAO 2016). During growing season, it is affected by abiotic and biotic factors such as Fusarium wilt, cyst nematodes and blight caused by *Ascochyta rabiei* (Reddy & Singh 1990) a devastating disease reported around the world where chickpea is growing (Shahid *et al*. 2008). Prophylactic measures and chemical fungicides application with negative impact on human health and environment are the main methods adopted against this disease (Davidson & Kimber 2007).

Biological control and biopesticides are an alternative for natural resources and human health preservation is now one of the major agriculture field researcher's areas (Alabouvette *et al*. 2006). Essential oils are chemical compounds mixture that have an antimicrobial effect and can therefore be used as biopesticids in biological control (Roy 1995), hence this work objective is to test the *Myrtus communis* L. essential oils effect on *Ascochyta rabiei* Pass. Lab. chickpea blight disease.

MATERIAL and METHODS

Plant material, EO extraction and amount calculation

Myrtle (*Myrtus communis* L.) leaves were harvested during February 2016 in Bissa forest (45 km northeast of Chlef (Algeria) in the sub-humid Mediterranean bioclimatic zone at 1152 m altitude , 36 ° 27 '06 "N latitude and 1 ° 29' 01" E longitude). They dried in the shade at ambient laboratory conditions (approximate temperature 25–28 °C) for 12 days and then grounded using a commercial blender for the essential oil extraction by hydrodistillation (HD). Essential oil amount expressed as a percentage, is the ratio between extracted oil weight (W1) and used plant material weight (W2), multiplied by 100 (Carée 1953) according to the following formula:

$$R (\%) = W1/W2 \times 100$$

The obtained oil was kept at -20 ° C until use.

The pathogen *Ascochyta rabiei* Pass. Lab. chickpea blight disease agent

The pathogen *Ascochyta rabiei* is cultured on a Chickpea (CP) medium containing 50 g / l of chickpea flour, 20 g of glucose, 15 g / agar, the pH adjusted to 5.6. The cultures are incubated at 25 ° C under 16 hours of light / 8 hours of darkness photoperiod.

Myrtus communis L. essential oil antifungal activity evaluation on *Ascochyta rabiei* Pass. Lab. by dilution method

The antifungal activity was evaluated by dilution method on solid medium to establish inhibition rates of the disease. The oil was previously homogenized with ethyl alcohol to facilitate its diffusion

into the culture medium (Fandohan *et al*. 2004) and then mixed with CP medium placed in Petri dishes. Essential oil inhibitory effect was tested on the fungus mycelial growth. Five mm diameter disks taken from a 14-day *Ascochyta rabiei* Pass. Lab. colony were placed on CP medium mixed with essential oils at different concentrations 200, 300, 500 and 1000 ppm and sterile distilled water for control. Mycelium growth diameter is measured daily and the experiment was repeated five times for each treatment. The inhibition rate is calculated according to the following formula (Bekker *et al*., 2006):

Inhibition rate (%) =100 x (Cd-Ed)/Cd

Cd = Control colony diameter

Ed = Essential oil colony diameter.

The minimum inhibition concentration MIC was determined, corresponding to the first concentration from which no fungal growth was observed

EO effect on *Ascochyta rabiei* Pass. Lab. growth *In vivo* and *in vitro* test

In vitro test on detached leaflets

Myrtle essential oil effect on chickpea genotypes was tested *in vitro* on leaflets, detached from three-week-old plants grow under greenhouse conditions and prepared according to the method described by Harijati & Keane (2012).

Under a laminar flow hood, the leaflets are disinfected with 70 ° alcohol for 30 seconds, then with 2% sodium hypochlorite solution for 1 minute and rinsed three times with sterile distilled water. Ten leaflets are deposited in Petri dishes on sterile filter paper previously moistened with sterile distilled water. The pathogen spore solution with a concentration of 10^6 spores / ml is prepared under a laminar flow hood, from a two weeks aged *Ascochyta rabiei* Pass. Lab. colony.

For each genotype, three batches of ten leaflets repeated 5 times were prepared. The first, untreated, used as control free of inoculum and not treated, the second was inoculated with 5µl of spore solution and also not treated with EO and the third one inoculated as previously described and then treated with 750 ppm EO solution added to 100 ml of a 3% solution (v: v) (containing ethanol and distilled water) and leaflets were sprayed for 3 consecutive days at the same time of the day.

Disease incidence was evaluated according to the Harijati & Keane (2012) scale as soon as the symptoms of chickpea anthracnose appeared.

Disease (DS) severity its incidence (DI) determination on detached leaflets

The observation is carried out after 7 days of the application of the EO solution; the evaluation is done on the treated and untreated samples by using the two following methods:

Disease (DS) Severity on detached leaflet was determined on a scale of 0 to 10,where 0 indicated no lesions,1, 1 necrotic spot, 2 - 3 some necrotic spots,4-5 about 10% of the leaflet surface was infected and 6, 7, 8, 9, and 10 respectively indicated 25%, 30% - 40%, 50%, 60% - 70% and 80% - 100% of the leaflet surface infected by the fungus with the appearance of pycnidia in concentric zones characteristic of chickpea Ascochyta blight (Harijati & Keane, 2012).

$$\mathbf{DS} = \frac{\sum \text{leaflets number by category x category value}}{\text{leaflets total number x 10}} x100$$

Disease incidence (DI) (% of leaflets infected) was determined by the total number of leaflets with lesions (ignoring lesion size) and necrotic lesions (with a diameter, at least ~ 0.5 mm), according to the following formula (Harijati & Keane, 2012):

$$\mathbf{DI} = \frac{\text{leaflets with lesions number} + \text{leaflets with necrotic lesion number}}{\text{leaflets total number}} x100$$

In vivo test on Cicer arietinum L. plants

A bioassay was also conducted *in vivo* under greenhouse conditiond for Bouazza, INRA 199 and ILC 3279 chickpea genotypes, three batches of five pots each (12 cm in diameter and 12 cm deep) filled with an autoclaved mixture of soil and peat (3: 1, v: v) were prepared. Five seeds were sown and then watered twice a week. After three weeks, the first batch served as a negative control and had not received any treatment, the other two batches were inoculated by spraying a spore solution with a concentration of 10^6 spores / ml over the entire aerial part of the plants until runoff. The plants were covered with plastic bags to ensure approximatively75% humidity level maintained constant for the first four days by spraying sterile distilled water under bags and daily watering pots. Half of inoculated pots used as a positive control and the others treated by plants aerial parts vaporization with the biopeticid prepared as described for the *in vitro* test carried out on detached leaflets.

Disease incidence percentage (DIP) was then evaluated in the three inoculated genotypes treated with the biopesticid or not compared to the control according to the formula given by Wheeler (1969).

$$\mathbf{DIP} = \frac{\text{diseased plants number}}{\text{Total plants number}} x100$$

Blight severity is assessed on a nine-level scale (Reddy & Singh 1984).

1- No lesions on all plants (highly resistant).

3- Visible lesions on less than 10% of plants and no broken plant stem (resistant)

5- Lesions visible on 25% of plants with less than 10% broken stems (tolerant).

7- Lesions on the majority of plants and less than 50% are destroyed (sensitive).

9- All plants have broken stems (very sensitive).

Disease severity degree was converted to severity percent index (SPI) according to Wheeler's (1969) formula.

$$SPI = \frac{\text{Individual severity ratings by scale}}{\text{Total number of plants examined x maximum scorex score maximum}} x100$$

Statistical analysis

Data were analysed using One Way variance analysis ANOVA and the difference between means with DUNCAN Multiple Range Test at $p<0.05$ significant level.

RESULTS

Essential oil amount

The average amount of *Myrtus communis* L. leaves essential oil harvested during the month of February 2016 was 0.6%.

***Myrtus communis* L. essential oil effect on *Ascochyta rabiei* Pass. Lab. growth**

Myrtle essential oil effect on *Ascochyta rabiei* Pass. Lab. growth and the minimum inhibitory concentration (MIC) were obtained by using of *Myrtus communis* L. essential oil at different concentrations (200, 300, 500 and 1000 ppm). The mycelial macroscopic results growth follow-up is shown in Figure 1.

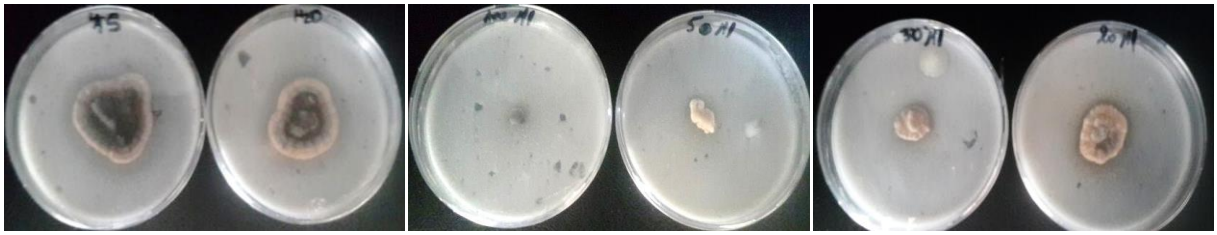

Figure 1. Myrtus communis L. essential oil on Ascochyta rabiei growth after 7 days.

The different essential oil concentrations had a significant $p<0.001$ effect on the *Ascochyta rabiei* Pass. Lab. growth which presented varying diameters after 7 days of incubation.

This growth was statistically similar in both positive and negative controls. All concentrations of myrtle essential oils added to the medium had an effect on mycelium growth. Colony diameters varied from 0.2 cm for 500 ppm concentration, 0.5 cm for 300 ppm, 1.1 cm for 200 ppm and a total *Ascochyta rabiei* Pass Lab. growth inhibition observed with 1000 ppm and the action was significantly more pronounced from the fifth day (Figure 2).

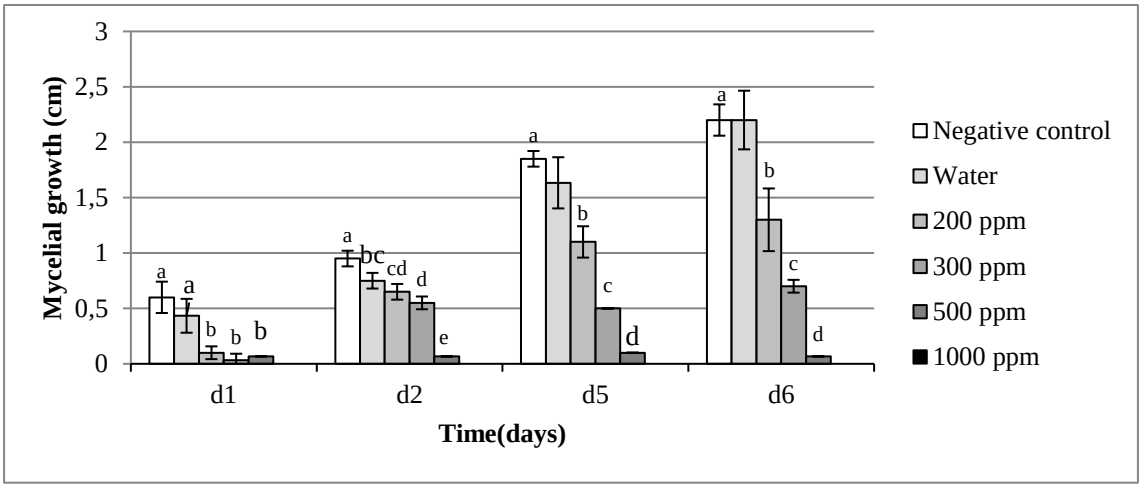

Figure 2. *Ascochyta rabiei* mycelial growth evolution under *Myrtus communis* L essential oil effect at different concentrations

Data are means of 5 replicates. For each day, means with different letters are significantly different by Duncan test ($p < 0.05$)

Inhibition rate determination

Mycelial growth inhibition rate increased with applied EO dose increasing. Inhibition rate estimation revealed that all tested concentrations had an inhibitory effect on mycelium growth (Tab.1). This rate reached 96.96% and 100% with respectively 500 ppm and 1000 ppm and this concentration represented the minimum inhibitory concentration MIC.

Table 1. *Myrtus communis* L. essential oil (Eo) inhibition percentage (%) on *A. rabiei* mycelial growth

EO concentration (ppm	Inhibition rate (%)
200	40.90
300	68.18
500	96.96
1000	100

Effect on detached leaflets

Results obtained with and without myrtle essential oil application revealed that blight expression varied significantly $p < 0.001$ in tested chickpea genotypes.

Analysis of variance (ANOVA) showed that disease incidence and severity varied significantly $p < 0.001$ for different genotypes and local cultivar, highest disease incidence was observed on Bouazza (100%) followed by ILC 3279 (84.28%) and INRA 199 (17.5%). Myrtle essential oil showed an efficacy in all cases and decreased significantly disease incidence for Bouazza, ILC 3279 and INRA 199 to 63.33, 56.66 and 16% respectively (Fig.3).

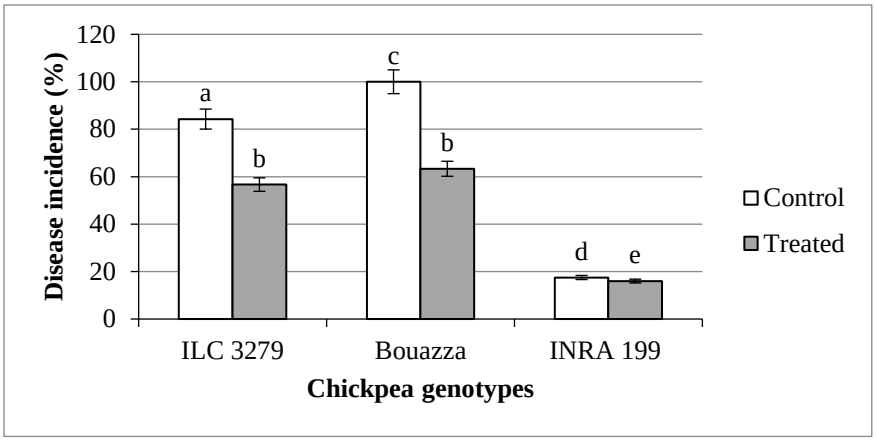

Figure 3. Blight disease incidence on chickpea (*Cicer arietinum*) detached leaflets.

Data are means of 5 replicates. Means with different letters are significantly different by Duncan test ($p < 0.05$)

Disease severity (Fig. 4) showed the same profile, Bouazza local cultivar noted the highest rate followed by ILC 3279 and INRA 199 with 60, 40 and 21% respectively and EO showed a significant efficacy and reduced these disease severity rates to 52, 32 and 18 % (Fig. 4).

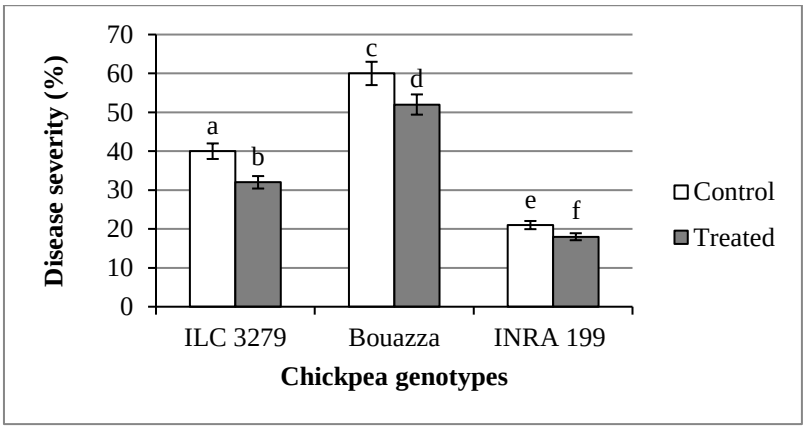

Figure 4. Blight severity on the detached leaflets in chickpea (*Cicer arietinum*)

Data are means of 5 replicates. Means with different letters are significantly different by Duncan test ($p < 0.05$)

Effect of *Myrtus communis* L. EO on inoculated Plants

Chickpea genotypes plants aerial parts were inoculated with a spore suspension concentrated at 10^6 spores / ml using a garden hand pressure sprayer. As soon as the symptoms of anthracnose appeared, the essential oil of the myrtle is sprayed three days in a row, after a week of the first application. The results of the development of the disease are illustrated in Figures 5 and 6.

Figure 5. Blight symptoms on different chickpea (*Cicer arietinum*) genotypes after inoculation with *Ascochyta rabiei* spore suspension

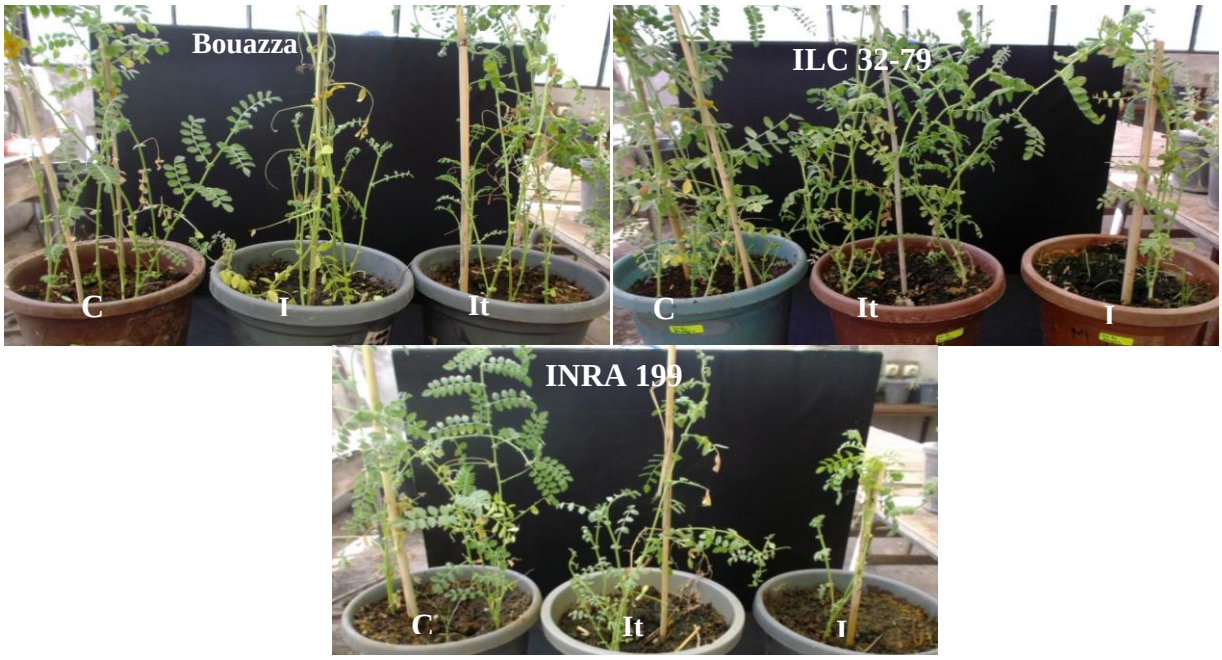

Figure 6. *Myrtus communis* L. essential oil effect on the three of chickpea *Cicer arietinum* L. genotypes one week after application.

C : control ; I : inoculated ; It :inoculated/treated

Analysis of variance showed that the disease incidence percentage (DIP) decreased significantly under the effect of common myrtle EO compared to control in INRA 199 and ILC 3279 (Fig. 7).

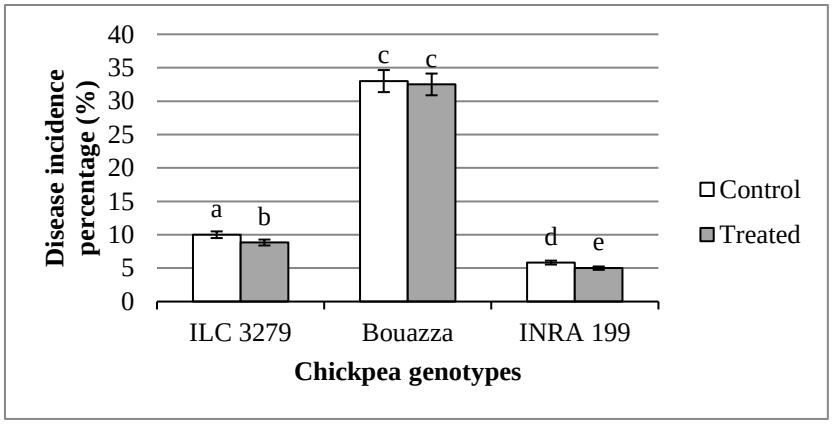

Figure 7. L'incidence de l'anthracnose sur les différents génotypes de pois chiche (*Cicer arietinum).*

Data are means of 5 replicates. Means with different letters are significantly different by Duncan test ($p < 0.05$)

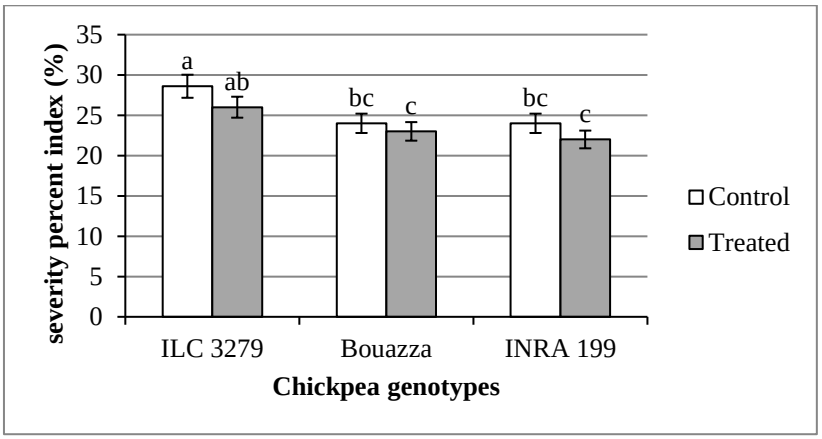

Figure 8. Blight **s**everity percent index in chickpea (*Cicer arietinum*) plants

Data are means of 5 replicates. Means with different letters are significantly different by Duncan test ($p < 0.05$)

For severity percent index (SPI) (Fig.8) no significant difference was observed for all genotypes under *Myrtus communis* L. essential oil treatment effect.

DISCUSSION

Myrtle (*Myrtus communis* L.) essential oil efficacy was evaluated on *Ascochyta rabiei* Pass.Lab. growth, chickpea (*Cicer arietinum* L.) blight agent. Biological control using plant extracts emerged as a promising alternative to chemical compounds against fungi (Zerroug *et al*. 2011).

Essential oil amount after extraction by hydrodistillation from myrtle leaves harvested in February 2016 in the Bissa (Chlef) forest was 0.6%. Taleb-Toudert (2015) obtained respectively 0.5% EO from myrtle leaves. The amount obtained can be influenced by the harvest region, by the month of harvest or plant physiological stage and harvested parts of the plant Wannes *et al*. (2010), report 0.011%yield for fruits.

Myrtus communis L. EO use for *Ascochyta rabiei* Pass. Lab. control was tested *in vitro* by dilution method. EO different concentrations revealed an inhibitory effect on *Ascochyta rabiei* Pass. Lab. from 500 ppm and a total inhibition at 1000 ppm. Previous research had shown that *Myrtus communis* L. essential oil antifungal efficacy against *Penicillium digitatum* (Ladu *et al*. 2015). Kordali *et al*. (2016) reported that the essential oil extracted from four myrtle tested at 1% concentration on 19 types of phytopathogenic fungi could inhibit *Rhizoctonia solani*, *Alternaria alternata* and *Botrytis cinerea*.

Myrtus communis L. EO antifungal activity was evaluated *in vitro* on chickpea (*Cicer arietinum* L.) detached leaflets inoculated with a suspension of *Ascochyta rabiei* Pass. Lab. spores. Compared to untreated but inoculated control, mycelial growth was inhibited inducing a decrease in incidence and severity of blight. This was similar to the results obtained by Tsopmbeng & Fomingia (2015) who tested the effect of methanolic extracts of *Callistemon viminalis* and *Eucalyptus saligna* on Taro leaves inoculated with a spore suspension of *Phytophthora colocasiae*, the causal agent of taro mildew. Antifungal properties appeared five days after application of the methanolic extract from a dose of 5 mg / ml. However, the work of Goufo (2008), based on the inoculation of detached tomato leaflets with a suspension of *Phytophtotra infestans* spores and their treatment with methanolic extracts of 9 different plants originating from Cameroon, was not significant and showed no effect inhibitor on mycelial growth.

Myrtle EO antifungal activity was assessed *in vivo* on both genotypes and local cultivar of chickpea plants inoculated with a suspension of *Ascochyta rabiei* Pass. Lab. spores. Compared with the untreated inoculated control, the development of the disease was not inhibited Nashwa & Abo-elyousr (2012) showed the effectiveness of treatment with extracts from 6 plants collected from Assiut area (Egypt) against the phytopathogenic fungus of tomato *Alternaria solani* at concentrations of 1 and 5 µl applied to the aerial part of infected plants. Similarly for Pereira *et al*. (2012), which showed inhibitory effects from 8 different plants collected in Minas Gerais area (Brazil) on *Hemileia vastatrix*, a fungi causing coffee rust development.

The responses to treatments via the application of plant EO may be different (*in vivo*, under greenhouse and field conditions). According to Gaston *et al*. (2014), *Syzygium aromaticum* extract, effective *in vitro*, was less active under in vivo testing conditions and, contrariwise, *Callistemon viminalis* with lower efficacy *in vitro* was most effective *in vivo*. He suggested that *in vitro*, on the culture medium, the effect of active molecules against *Phytosphtora infestans* extract of *Callistemon viminalis* would be masked by other molecules present in this extract. In addition, the plant extracts active components can act on the pathogens individually or in synergy and inhibit their development. Conversely, there may be antagonistic action between them.

In addition, these bioactive compounds are unstable by heat and therefore rapidly degradable under the sun. Active ingredients under *in vivo* test conditions would be degraded by environmental conditions (Gaston *et al*. 2014).

The difference in efficacy observed *in vivo* and *in vitro* can be related to the *Myrtus communis* L. EO applied amount of but also to the difference in intrinsic behavior of the host when detached leaflets or whole plants as well as experimental conditions.

All of the preliminary results indicated *Myrtus communis* L. EO inhibitory effect on the causative agent of anthracnose (*Ascochyta rabiei* Pass. Lab.) in chickpea (*Cicer arietinum* L.).

The *in vivo* and *in vitro* tests on the incidence and severity of the disease in the different genotypes show a divergence of response, hence the need to study this approach by using a varied range of concentrations (quantity and quality) and study their bioactive effect. The development of incidence and severity of crop diseases at this physiological stage is a criterion used to verify the effect of fungicides on phytopathogenic agents. In addition, it would be desirable to test the action of these essential oils in parallel with synthetic chemical molecules marketed and used by farmers and compare their effectiveness against chickpea blight.

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