International Journal of Ecology and Environmental Sciences 44 (1): 77-83, 2018
ISSN: 2320-5199 (Online)

Phytotoxic Effect of the Essential Oils of *Rosmarinus officinalis* L. on the Development of *Lactuca sativa* L. Under Greenhouse

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ABSTRACT

Lettuce, Lactuca sativa L., is a vegetable consumed fresh or after industrial processing. It's cultivation faces several constraints, mainly plant pathogenic fungi. To fight these atacks, chemical method remain the most used despite its bad effects on humans and environment. The present work seeks to find a new method for antifungal treatment. We use the essential oil (EO) of *Rosmarinus officinalis* L. and two widely used chemical fungicides essentially based on mancozeb and azoxytrobine to assess their impact on growth and physiology of the plant. The cultivation of lettuce was conducted under greenhouse. 25 days after the sowing date, weakly treatment with products at different doses has been applied until the harvest. Meanwhile control plants were grown under the same conditions and without fungicide treatment, nor EO. Throughout this study, we monitored the physiological and biochemical parameters relating to the dry biomass of the plant's different parts, the level of chlorophyll and the leaf surface.The results show that the treated plants have a positive response for most biological parameters studied and had significantly increased total biomass compared to its control counterparts. The azoxytrobine has no effect on the same parameters; *Rosmarinus officinalis* has double avantage by improving the performance of lettuce crops without harming the environment.

Key Words : *Lactuca sativa* L.; *Rosmarinus officinalis* L.; Phytotoxicity; Antifungal Treatment; Fungicides; Environment.

INTRODUCTION

The cultivation of lettuce is subject to many constraints related to diseases and attacks by pests which require fairly frequent phytosanitary treatments. The use of these products harms not only the environment but also the plant itself in a way that it may develop a certain resistance toward the chemical molecules.

These molecules, wich are ubiquitous in the environment, are suspected to increase the incidence of certain diseases like cancer. At present, consumer exposure to pesticide mixtures through food is attracting growing interest. However, knowledge in matters of combined effects of low doses of pesticides is still very sketchy. Actually, pollution from these products is relatively well studied in the Moroccan mediterranean coasts in contrast withAtlantic coasts where these studies are very rare (Benbakhta et al. 2007).

Today, searching for alternatives has become a priority. Several products of microbiological or vegetable origin (Rasikari et al. 2005, Wang et al. 2007), easily biodegradable and not persistent, replace conventional pesticides. Other methods are also possible, such as culture control or inert organic pesticides. Phytotoxicity is defined by the effects on physiological processes following an interaction between plants.

Furthermore, plant's competition for space, soil, and light leds some species to develop strategies based on the synthesis of secondary metabolites with allelopathic and phytotoxic effects. These plants can serve as a reservoir of molecules with bioherbicidal activity in a context of sustainable agriculture.

Some previous investigations have shown that vegetable oils from certain herbs can have a bioherbicide effect, particularly essential oils of *Artemisia californica, Salvia apiana* and *Salvia leucophylla* (Muller et al. 1964), of *Tagetes minuta* and *Schinus ariera* (Scrivanti et al. 2003), of *Eucalyptus citriodora* (Batish et al. 2004, Batish et al. 2006, Batish et al. 2008), of *Artemisia vulgaris* (Barney et al. 2005), of *Artemisia scoporia* (Singh et al. 2009), and of *Nepeta meyeri* (Mutlu et al. 2010, Mutlu et al. 2011). Recently, (Rolli et al. 2014) have reported phyto inhibitory activity after testing many EOs taken from different sources on germination and post-germination growth of *Solanum lycopersicum's* seeds.

Rosmarinus officinalis L. is a perennial plant of the *Lamiaceae* family. Its stems are covered by a grayish bark, branches are opposite. Leaves are narrow, with shiny green upper face. This aromatic and medicinal plant produces essential oils used in cosmetics perfumery and natural therapies (El Rjoob et al. 2008). These oils may also have many effects as an acaricide (Miresmailli et al. 2006), bactericide (Burt S. 2004), fungicide (Versaltalab et al. 2012), insecticide (Koschier and Sedy 2003) and nematicide effect (Mattei et al. 2014). Our goal is to evaluate the phytotoxic effect of the essential oils of *Rosmarinus officinalis* on the development of *Lactuca sativa* in comparaison with the effect of two chemical fungicides commonly used in horticulture.

MATERIAL AND METHODS

Plant Material

The cultivated lettuce *Lactuca sativa* L. belongs to the Asteraceae family, genus *Lactuca* which includes about 100 species (Thompson et al. 1941). The variety used for our experiments is named "lettuce Grasse Madrilène". It's a winter lettuce, with soft and refreshing taste. It grows easily, has a fast lifting, and is quite resistant to dryness.

Experimental Protocol

Before any sort of handling, the lettuce seeds are disinfected with a10% bleach for 15 minutes. Then, they are soaked in 70% ethanol for 5 minutes before being rinsed several times with distilled water. Thus treaed seeds are put to germinate for 72 hours at 22°C in petri dishes of 3 wattman paper moistened with distilled water. Produced plantlets are transferred to small pots (30/15 cm): 3 plantlets per pot. Soils in the pots were procured from an agricultural parcel for the production of vegetable crops. After 15 days, we keep one plant per pot, which are cultivated during two months in a greenhouse near Ain Beida locality (Fez).

The soil used in the experiment was analysed by standard methods for texture, pH, carbon, nitrogen and organic matter. Fertilizers were added at the rate of 100kg N/ha by providing $(NH_4)_2SO_4$, 50 P_2O_5/ha and 250 kg K_2O/ha (Herradi et al. 2005). It had a pH of 7.95, carbon 0.64 %, organic matter 1.10 %, nitrogen 0.25 % and a C/N ratio 25.

According to texture analysis, the soil had 12% clay; 39% silt fine; 10% wholesale Limon; 20% fine sand and 19% coarse sand). Accordngly, it was a sandy loam (Jamagne 1968) that is fertile and conducive to the proper development of plants.

In each pot, 2.5 kg of dried and screened substrate was deposited in two layers: the gravel, set in the bottom, covered by a wide layer of fine soil for drainage. Tap water was used for irrigation, with a volume to maintain the pots 90% capacity.

Antifungal Treatments

The tests consist on six treatments distributed in 24 pots (4 replicates per treatment) arranged in a complete random block design.

1. Control: Temoin untreated, No phytosanitary procedure performed.
2. Dithane M45: Based on mancozeb (80%). This is a preventive fungicide of contact multi-site characterized by good efficiency and high selectivity. The recommended dose is 1g L^{-1} of water or 4 kg ha^{-1}. Its commercial formulation is a wettable powder.
3. Ortiva 25Sc: It is a broad spectrum fungicide basic azoxytrobine (25g L^{-1}). It is used to neutralize mildew, *Oidium, Alternaria*, anthracnose and the rusts of vegetable crops. The recommended dose is one L ha^{-1}. Its formulation is a concentrated suspension (SC).

4-6. Essential oil of *Rosmarinus officinalis* used at different doses: 2, 4 and 6 drops L^{-1}.

Various treatments were applied at stages of 7 and 13 leaves.

Monitoring the Development of *Lactuca*

During all stages of the cycle, we observed the growth of salads while comparing leaf area and stem length. At the end of the cycle, lettuce harvested were cut into 4 parts (leaves, stem, roots and pivot), each party weighed in a fresh state and at a dry state after baking at 80°C for a night. The leaf area was calculated after having weighed and scanned the fresh leaves and printed the images on paper 80g m^{-2}. The total leaf surface was calculated by a ponderal relationship equation.

The dosage of chlorophyll was carried out according to the method of Hiscox and Israelstam (1979): 30 to 40 mg of the fifth leaf of each fresh plant is put into a tube with 4ml of DMSO. Then, we perform a read on the spectrometer to measure the optical density (OD) at a wave length of 663nm to 645nm and after passing through the oven at a temperature of 65°C for 30 minutes.The calculating concentrations of total chlorophyll are done with the formula of Arnon (1949):

$$\text{Chl Tot (g L}^{-1}) = 0.0202\text{x}A_{645} + 0.00802\text{x}A_{663} \quad (1)$$

Statistical Analysis

Evaluation of the effect of products used in the cultivation of lettuce was performed by single factor analysis of variance (ANOVA) on each variable studied: fresh and dry biomass, chlorophyll rate, and leaf area. For results that present themselves as a percentage we used a transformation of data to meet the conditions for normality of distribution and equality of variances. This angular transformation was performed according to the following formula (Gulumser et al. 2006):

$$Y = 2\text{ArcSin}\sqrt{x}/100 \quad (2)$$

Statistical analysis was performed by the SYSTAT Version 12 program.

RESULTS

Dry Biomass of the Whole Plant

The whole quantity (the entire plant) of dry biomass is depending on the treatment applied (Figure 1).

By comparing the whole dry biomass produce in the control case and in all the others cases, we note that, except for fungicide P2 (Azoxystrobin based), all others products (fungicide P1) based on mancozeb 80%, and EOs at different concentrations) show an increase of the average quantity of dry biomass.

Compared to the control, plants treated by fungicide P1, and essential oil (OC2) show an increase of 27.85% and 34.26% respectively. Those treated by OC1 and OC3 record the highest values, 44% and 50% respectively. Statistically, the effect of all the treatments (by fungicide P1, fungicide P2, and the three concentrations of *Rosmarinus officinalis* essential oil) on dry matter of the whole plant is significant ($P<0.05$); according to the Tukey test, the difference is significant between the control and OC1 and OC3 treatments.

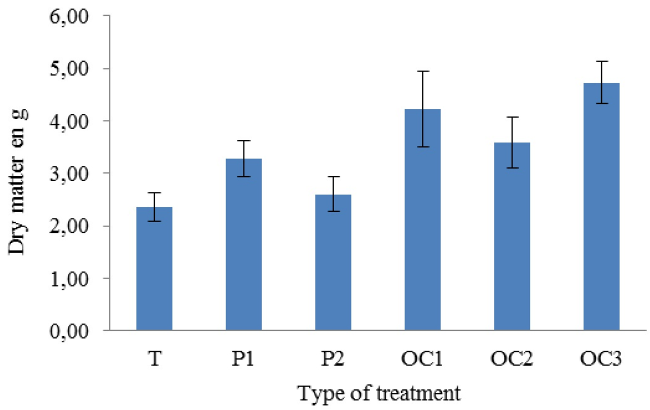

Figure 1. Effect of various treatments on dry biomass production. T: Control, P1: Dithane, P2: Ortiva, OC1: Rosemary 2 drops L^{-1}, OC2: Rosemary 4 drops L^{-1}, OC3: Rosemary 6 drops L^{-1},

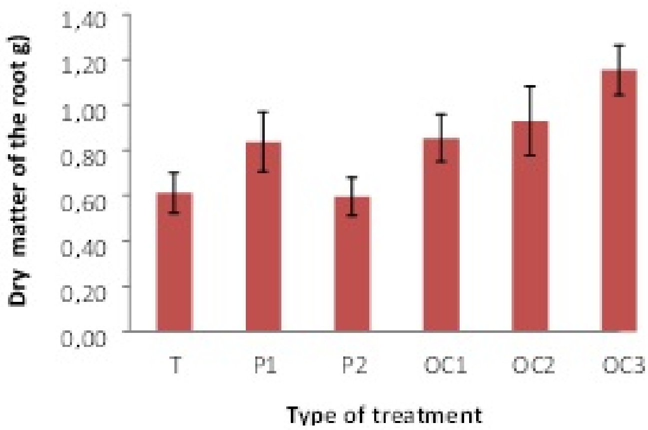

Figure 2. Effect of various treatments on root dry biomass. Treatments as in Figure 1. The values are means of n=4 ±SE

Dry Biomass of the Root

Figure 2 shows that the treatments P1, OC1, OC2 and OC3 caused a significant increase of root dry biomass over that in control. It is worth to underline that the fungicide P1 has caused a 36% increase; the different solutions of the rosemary EO have also developed root biomass in a proportional manner like the applied concentrations : 39.54% increase for OC1, 51.79% for OC2 and 80.56% for OC3 (Figure 2 compared to the control of course). The analysis of variance relative to the dry matter of the entire root has revealed a significant effect ($P<0.05$) for the 5 treatments applied on plants.

Dry Biomass of Taproot

The graphical representation shows that the treatments P1, P2 and the threeconcentrations of rosemary. EO have differents impacts on the growth of the lettuce taproot. Comparing to the control, 41.11% increase of the dry biomass of the root hub is observed with P1 fungicide treatment, while P2 has no significant effect. Meanwhile, treatment with the vegetable products (OC1, OC2 and OC3) has recorded a beneficial effect. The Tukey tests show that the difference is significant only between the control and P1, for the others treatments, there is no significance ($P>0.05$).

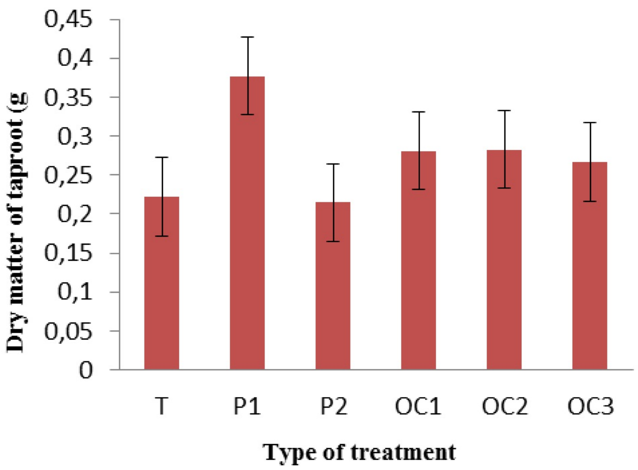

Figure 3. Effect of various treatments on tap root dry biomass. Treatments as in Figure 1. The values are means of n=4 ±SE

Dry Biomass of the Aerial Part

Comparing to the control, fungicide P2 has no impact on the leaves dry biomass, while for all the others products (P1, OC1, OC2 and OC3) the effect is clairly positive. Quantitativly, the aerial biomass has recorded an increase of 32.44%, 38.91% and 33.42% respectively for plants treated by P1, OC1 and OC2; and reached 51.22% for the OC3 treatment (6 drops of rosemary oil per liter of water). Tukey test, show that all these differences are significant ($P<0.05$), more particularly between the control, OC1 and OC2 treatments.

Dry Stem Biomass

Comparing to the control, chemical fungicides (P1 and P2) have no effect on the dry stem biomass, while for the vegetable products (OC1, OC2 and OC3) we have noted an interesting growth in the central part of lettuce proportionaly to the applied doses: The biomass produced increased by 32.84%, 38.8% and 50% respectively for treatments by OC1, OC2 and OC3. The variance analysis relative to the dry matter of the stem has revealed a significant effect for the five treatments applied on the plant ($P<0.05$), this effect is revealed by the Tukey test between the control and treatment OC3.

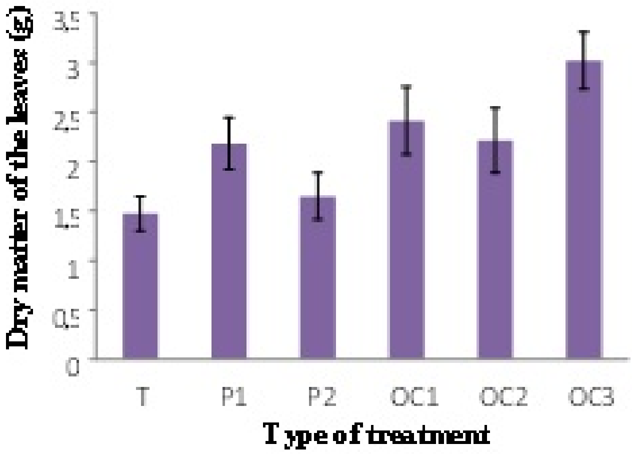

Figure 4. Effect of various treatments on leaf dry biomass. Treatments as in Figure 1. The values are means of n=4 ±SE

The Leaf Area

Comparing to the control, the fungicide treatment P2 does not present a significant effect on the leaf surface; however, treatment with fungicide and EOs at different doses caused an increase of the surface up to 47.64%, 49.6% and 53.4% respectively in plants treated by OC1, OC2 and OC3. These results are logically in accordance with those obtained on a dry leaf biomass (Figure 6).

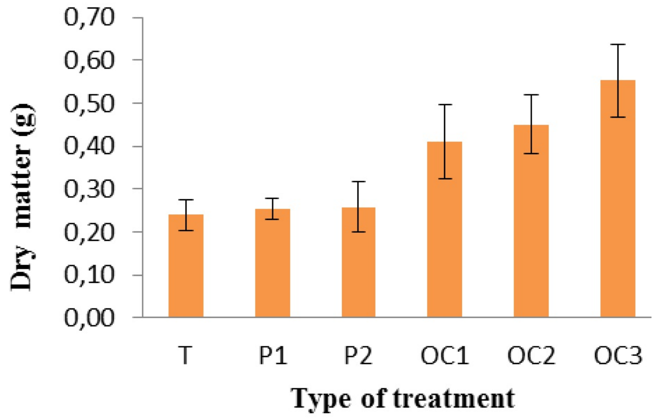

Figure 5. Effect of various treatments on stem dry biomass. Treatments as in Figure 1. The values are means of n=4 ±SE

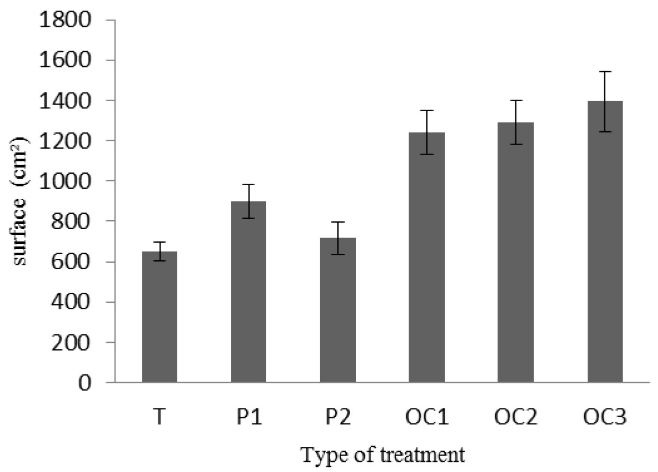

Figure 6. Effect of various treatments on lead area. Treatments as in Figure 1. The values are means of n=4 ±SE

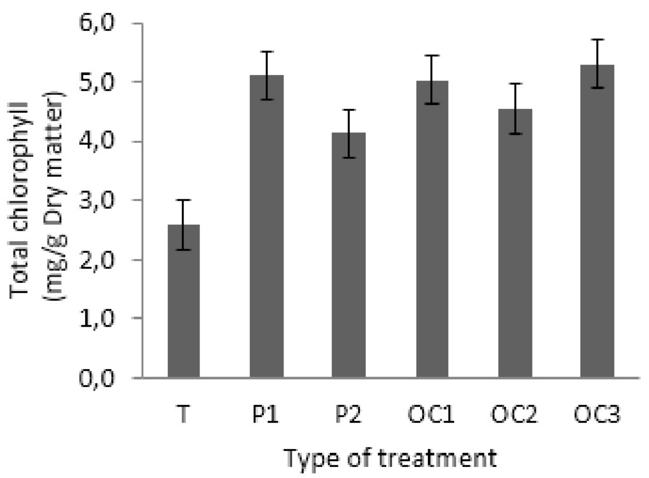

Figure 7. Effect of various treatments on total chlorophyll of leaves. Treatments as in Figure 1. The values are means of n=4 ±SE

Chlorophyll Content

Compared to the control, all the treatments applied to the plants show a remarkable difference in terms of total chlorophyll (Figure 7). The highest increase recorder is 45.65% with P1 treatment. The P2 fungicide has caused a slight increase of 25%, while the three concentrations of essential oils had almost the same effect on the treated plants with a total chlorophyll 43.8% superior to the control. The highest proportion of chlorophyll is represented by chlorophyll A about 80% in the untreated control (equation 1).

DISCUSSION

According to the results observed the products used for all the tests do not have the same effect on different parts of the plant. For instance, all biomasses obtained for the lettuce treated by P2 fungicide based on azoxytrobine were not very different from those recorded in the untreated control. This could be explained by the systemic nature and translaminar action of the active ingredient of this kind of fungicide named Ortiva 25 sc. In fact when the azoxytrobine is brought to crops, this molecule penetrates into the plant under the cuticle but does not spread in all directions; it is characterized by local penetration and low mobility. Therefore, the emerging leaves are not affected by the fungicide and act as control.

The product P1 (Dithane M45) based on mancozeb has positively influenced the physiology of the plant studied. This fungicide has shown a relatively similar effect to that low dose oil *rosmarinus officinalis* sample which is 2 drops / liter. This is similar to an experience conducted by Lambion J. (2005) to fight off B*remia lactucae* with a fungal product based on copper and that had a significant effect on the biomass of treated salads.

An important point to emphasis is that vegetable oils used in our tests have been effective in enhancing the physiological and biochemical performance of *lactuca sativa*. The effect of different concentrations of *Rosmarinus officinalis* EO has been positive for the developpment of the roots (Picture 2) and the aerial part (Picture 4). Treatments by OC1, OC2 and OC3 have caused a significant increase of dry biomass compared with the control, as well as enlarging leaves surfaces and chlorophyll rates.

Plants treated by different concentrations of rosemary EO show a significant increase in their total dry weight, which is consistent with the results obtained from testing each part separatly. The evolutions recorded for leaf area and chlorophyll levels in plants treated by rosemary EO are directly correlated to the concentrations of oil solutions. Thus the results recorded for chlorophyll agree perfectly with those of the leaf surface; the larger is the exchange surface with the air, the more intense the photosynthetic reaction. Based on these results, we can hightlight the remarkable effect that essential oils of *Rosmarinus* have on the productivity and biomass of salad. Our results compare well to those of Lambion and De Scey (2010)who have shown a relative weight evolution in the salads treated by herbal tea of wormwood and Horsetail in order to fight off the fungus *Bremia lactucae*.

CONCLUSION

The antimicrobial activity of essential oils is empirically known since antiquity. The results of our study are added to several others who stress the usefulness of plant species such as *Rosmarinus officinalis* in organic farming. This has a double benefit in the lettuce case: rosemary EO has a good antifungal activity, and in the same times a positive effect on the biomass of the plant. Plants in greenhouses are able to keep the same weather conditions throughout the growing season: neighboring optimum temperature of 26° C and a humidity that not exceed 30%.

The use of vegetable oils for antifungal property and improving plant's biomass is a good mean to preserve the environment against chemical pollution. Nevertheless, the feasibility and profitability of this method remain to be confirmed in the field where ecological conditions are random and uncontrolled.

Lactuca sativa being an edible plant, risks of exposure to chemical pollutants appear more and more inescapable. For cultivation of this species, as for many others, the use of organic products, instead of chemical ones, could be a good alternative; it has been demonstrated that they have positive effect on the physiology and biochemistry of the plants treated, while protecting them from unwanted pathogens.

ACKNOWLEDGEMENTS

This work is a part of Amal Fennane thesis, wich is going on in the laboratory of Functionnal Ecology and Environment, Mohammed Ben Abdallah University in Fès, Morocco. Funding was provided by this laboratory. We are grateful to Professor El Ghadraoui, the Head of the laboratory, who supervised all the steps of this work. Chaïmae Raïs helped with the experimental work and Khadija Boukili helped with the translation from French to English.

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Received 9 October 2017
Accepted 13 February 2018