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Effect of arbuscular mycorrhizal fungi (AMF) and water stress on growth, phenolic compounds, glandular hairs, and yield of essential oil in basil (*Ocimum gratissimum* L)

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Abstract

Background: Water stress is one of the most adverse conditions that may affect growth, and synthesis of essential oils in aromatic and medicinal plants. To overcome these climatic conditions, mycorrhiza is an adaptation strategy developed by plants to help them cope with these adverse conditions. For this purpose, we studied the influence of mycorrhizal fungi (*Glomus intraradices*) and water stress on the growth of basil plants (*Ocimum gratissimum* L), the yield of essential oils, and the abundance of glandular hairs.

Results: The analyses show that AMF increases the yield of oils with a maximum recorded in stressed mycorrhizal plants (0.33%) and the lowest in non-stressed non-mycorrhizal plants (0.22%). The contents of total phenolic compounds increase in non-mycorrhizal plants under stress (104% in leaves and 97% in the roots) unlike the mycorrhiza which did not stimulate the synthesis of these compounds. Moreover, the contents of chlorophyll pigments decrease with the application of stress in non-mycorrhizal plants (53%) and increase in mycorrhizal plants. The proline contents increased significantly with the application of water stress; this increase is more pronounced in non-mycorrhizal plants than mycorrhizal plants.

Conclusions: Water stress limits the growth and leads to a decrease in morphological parameters, this reduction is accompanied by a synthesis of several molecules in particular proline and phenolic compounds. However, the AMF stimulates growth, and drives the water status in plants at an optimal level, thus confirming the role of mycorrhizal symbiosis in plant defense against biotic and abiotic stress.

Keywords: Chlorophyll; Essential oils; Glandular hairs; *Glomus intraradices*; Phenolic compounds; Proline; *O. gratissimum*; Water stress

Background

Permanent or temporary water deficit plays an important role in the distribution of natural vegetation and cultivated in performance more than any other environmental factors plants [1]. It limits the growth and leads to changes in the metabolism of plants. Water stress causes the accumulation of phenols in *Bermuda grass* (grass) and in *Gomphocarpus fruticosus* (cotton) [2].

Wahid and Ghazanfar [3], Wahid and Close [4] confirmed these data in other plant species, with increased synthesis of phenolic compounds, flavonoids, and phenylpropanoids. Stress-induced increase in the activity of phenylalanine ammonia-lyase (PAL) may be regarded as the beginning of the acclimation of cells facing water stress.

Other studies have shown that severe water stress has a negative effect on the synthesis of these compounds in tobacco; it will stop the synthesis of anthocyanins in flowers and polyphenols in the leaves [5]. A similar phenomenon is observed for the flowers and leaves of *Begonia gracilis* [6].

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Studies on osmoregulation indicate that proline plays an important role in the fight against water deficit. Ain-Lhout et al. [7,8] reported that the levels of these molecules increased in *Pistacia lentiscus* L and *Halimium halimifolium* L when they are exposed to water stress. These results are confirmed by the work of AJ Delauney and DPS Delauney and Verma [9], Handa et al. [10], and Heuer [11].

Furthermore, Cornic and Fresneau [12] and Kim et al. [13] suggested that the water stress had a negative effect on the chlorophyll contents, which indicates a decrease in photosynthesis due to a disturbance in the metabolism of carbon and of certain enzymes involved in the regulation of these photosynthetic reactions.

To address these climate hazards and struggle against drought, plants develop several defense strategies; mycorrhizal association with soil fungi is an example [14]. These associations improve plant nutrition, mainly nitrogen and phosphate by increasing water intake in plants by increasing the exchange surface root-soil [15]. Many experiments on the effects of mycorrhizae on plant growth showed that the rate of photosynthesis is higher in mycorrhizal plants compared to non-mycorrhizal plants Auge [16], Kucey and Paul [17], and Levy and Krikun [18]. Most studies suggest that AM fungi contribute to increased rate of photosynthesis in improving (P) nutrition in plants, [5,19,20].

For the establishment of this association, it is necessary that both partners agree. This step is initiated via a cross-talk molecule and a change in gene expression [21]. Furthermore, the AMF infection induces a change in the metabolism of host plants resulting in the induction of chemical defense [22,23]. This change affects the synthesis of several families of molecules, terpenoids [24,25], EOs [26-28], and glucosinolates [29], phytoalexins [30,31], and phenolic compounds [32,33]. Several studies have been carried on the synthesis of these molecules on medicinal and aromatic plants: basil (*Ocimum basilicum*) [34,35,26,27], oregano (*Origanum onites*) [28], mint (*Mentha requienii* [36,37] and *Mentha arvensis* [36,38]), dill (*Anethum graveolens*) [39], fennel (*Foeniculum vulgare*) [40], coriander (*Coriandrum sativum*) [40,41], lavender (*Lavandula angustifolia*) [42], pelargonium (*Pelargonium peltatum*) [43], and sage (*Salvia officinalis*) [44].

Ocimum gratissimum L is one of the most used plants in traditional medicine in Morocco. Many studies have been done on this plant [45-47]. This work has focused on improving the yield of EOs. However, the influence of water stress and mycorrhiza on growth and chemical characteristics of this plant is very little studied.

The aim of this work was to evaluate the influence of water stress and inoculation with *Glomus intraradices* on some physiological and biochemical parameters of *O. gratissimum* L. and synthesis of EOs as well as the abundance of secreting glands of these oils.

Material and methods

Pregermination of seeds

The seeds of basil (*O. gratissimum* L) were surface disinfected by a passage in ethanol 95° (1 min 30 s) and immersed in a mercury hypochlorite solution (1%) for 3 to 4 min. Seeds were then rinsed several times in sterile distilled water before being placed on agar medium or on filter paper moistened with sterile distilled water in Petri dishes. Then, placed in an oven at 26°C, in the dark, to allow germination of the seeds.

Culture basil seedlings

After germination, seedlings were transplanted into plastic pots (3 kg capacity) containing the growth substrate in an amount of 50 to 60 plants per pot and were grown in a greenhouse at temperature comprising between 25 and 34°C.

The basil plants (M or NM) are watered daily. After the 50th day, the plants are subjected to different water regimes:

- a) Water regime unstressed (NS): the plants are not deprived of water throughout their growth.
- b) Application of water stress (S): plants are deprived of water for 2 weeks.

Estimation of mycorrhizal root infection

The identification of mycorrhizal root infection is in optical microscopy through a technique not vital staining with trypan blue (TB), described by Hayman [48], revealing the set of fungal biomass.

Root samples, taken at random, are thoroughly rinsed to remove adhering substrate. Then, the root fragments are digested in a solution of potassium hydroxide (KOH) at 10% for 45 min at 90°C in an oven, in order to empty the cell of their cytoplasmic contents which facilitates their coloring. Then, the roots are thoroughly rinsed with distilled water and placed in a solution lactophenol trypan blue (0.5%) at 90°C in the oven for 15 min.

Estimation of mycorrhization

We used the technique described by Trouvelot et al. [49]. This method allows to judge the state of mycorrhization and reflects the potential of the symbiotic system. Colored roots were cut into fragments of approximately 1 cm in length. Thirty random fragments are assembled and crushed between slide and cover slip in lactoglycerol, with 15 fragments per slide. Estimating the endomycorrhizal infection is by observing under light microscope. Several parameters are evaluated as follows:

$$F\% = (\text{Number of mycorrhizal fragments}/N) \times 100$$

- (F%): The frequency of mycorrhization reflects the importance and the percentage of fragments of infected roots.

with: N = Total number of root fragments observed

- ($M\%$): The colonization of the cortex intensity expresses the portion of the cortex colonized with respect to the entire root system.

with: n_5, n_4, \dots, n_1 = number of fragments respectively denoted as 5, 4, ..., 1.

$$\%m = M \times (\text{total number}) / (\text{number of mycorrhizal fragments}) \\ = M \times 100 / F.$$

Mycorrhizal intensity:

$$\%A = a \times (M/100).$$

- ($A\%$): Frequency of arbuscular in the root system.

$$\%a = (100 m_{A3} + 50 m_{A2} + 10 m_{A1}) / 100$$

Arbuscular intensity of the mycorrhizal part:

where m_{A3} , m_{A2} , and m_{A1} are respectively assigned as A_3 notes, A_2 , and A_1 .

with: $m_{A3} = ((95n_5A_3 + 70n_4A_3 + 30n_3A_3 + 10n_2A_3 + n_1A_3) / \text{mycorrhizal number}) \times 100/m$.

Similarly for A_2 and A_1 .

$$\%M = (95 n_5 + 70 n_4 + 30 n_3 + 10 n_2 + n_1) / N.$$

Relative water contents

The relative water content (RWC) is measured on the seventh or eighth leaf fully developed using the following formula according to Bandurska [50]:

$$\text{RWC}\% = 100 \times [(FW - DW) / (WT - DW)]$$

With

FW

the weight of fresh leaf material.

WT

the weight of fresh material from the turgid leaf was submerged in distilled water for 4 h.

DW

dry weight of the sheet material placed in an oven at 70°C for 24 h.

Extraction and determination of total phenols

Oxidation of phenols reduces this reactant in a mixture of the blue oxides of tungsten and molybdenum. The color intensity is proportional to the rate of oxidized phenolic compounds.

- Extraction of TPC.

Fragments of leaves and roots (0.5 g) were ground in a mortar containing a specific volume usually 5 ml, ethanol 50% (water-alcohol solution). Then, we collect the extracts in tubes with lids and well numbered, then leave the tubes in the refrigerator overnight to allow time for

ethanol to extract the maximum amount of phenol present in the extract.

In the tubes containing the leaf extracts, there was a risk of the existence of chlorophylls; we tried to eliminate it by adding in 3 ml of extract 0.5 ml of chloroforms the tubes are vortexed and centrifuged 5 min at $5 \times 1,000$ mtp; two phases were separated, a phase supernatant and pellet.

-Determination of TPC.

The assay of total phenols using the method based on the Folin-Ciocalteu reagent, described by Ribereau-Gayon and Stonestreet [51].

• Prepare in test tubes the following mixture: 0.5 ml of extract, 3 ml of water, 0.5 ml of Na_2CO_3 (20%); mix, wait 3 min, and then add 0.5 ml of Folin-Ciocalteu reagent. Mix and place the tubes for 30 min at 40°C , reading absorbance at 760 nm.

The amount of phenolic compounds was calculated using gallic acid for the standard curve and expressed in milligrams per gram of fresh leaf matter.

Dosage of proline

Proline content was determined according to Bates et al. [52] by measuring the quantity of the colored reaction product of proline with ninhydric acid. The absorbance was read at 520 nm. The amount of proline was calculated using L-proline (Panreac) for the standard curve and expressed in micrograms per gram of fresh leaf matter.

Determination of chlorophylls

Fragments of leaves (1 g) were ground in a mortar previously placed in ice with a pinch of magnesium carbonate and 5 g of anhydrous sodium sulfate. Then, 10 ml of acetone 80% are poured into the ground material, which is filtered on a Buchner; the residue is recovered in tubes essai; and further extractions are carried out with acetone to obtain a filtrate colorless (devoid of all traces of chlorophyll pigments) which the final volume is specified.

OD measurements were made with a spectrophotometer at wavelengths of around 663 to 645 nm for chlorophyll a and chlorophyll b.

McKinney [53] has established systems of equations that calculate the concentrations (g/l) of chlorophyll from absorbance at 663 and 645 nm of an extract of acetone:

$$\text{Chlorophyll a} = (0.0127 D_{663}) - (0.00269 OD_{645})$$

$$\text{Chlorophyll b} = (0.0229 OD_{645}) - (663 \cdot 0.00468 OD)$$

$$\text{Total chlorophyll} = (0.0202 OD_{645}) + (0.00802 OD_{663})$$

Extraction of EOs

One hundred grams of dried aerial parts of *O. gratissimum* were submitted to hydrodistillation with a Clevenger-type apparatus [54] and extracted with 2 l of water for 180 min (until no more EO was obtained). The EO was collected, dried under anhydrous sodium sulfate, and stored at 4°C until analyzed. The EO yield is given by the following formula:

$$\text{YEO (ml/100 g Dm)} = (V/\text{Dm} \times 100) \pm (\Delta V/\text{Dm} \times 100)$$

YEO

essential oil yield of dry matter.

V

the volume of essential oils collected (ml).

ΔV

reading error.

Dm

dry plant mass (g).

Description of environmental scanning electron microscopy

The observations were performed using a Scanning Electron Microscope Environmental Quanta 200 (FEI Company, Hillsboro, OR, USA) category. The microscope is equipped with electron gun tungsten. The analyses are carried out under a partial pressure of water vapor.

Statistical analysis

One-way analysis of variance was carried out for each parameter studied. Tukey's post hoc multiple mean comparison test was used to test for significant differences between treatments (at 5% level). Univariate analysis was used to test significant differences in treatments, accessions, and their interaction for an individual parameter. All statistical analyses were performed with IBM.SPSS statistics, Version 19. The results of each experiment (biochemical essays) were repeated three times (20 times for morphological essays).

Results

Mycorrhizal colonization

Table 1 shows mycorrhizal colonization of basil plants subjected to continuous irrigation (NS) and water stress (S) after disclosure by the trypan blue. This mycorrhizal

Table 1 Mycorrhizal colonization of basil plants (*O.gratissimum*) roots subjected to continuous irrigation (NS) and water stress (S)

Mycorrhizal colonization	NS	S
Mycorrhizal frequency (F%)	78.5	77.3
Mycorrhizal intensity (M%)	35	32
Arbuscular richness (A%)	20	17

colonization, estimated by the mycorrhizal frequency (F%), mycorrhizal intensity (M%), and arbuscular richness (A%), showed no differences between the stressed plant and non-stressed plant differences.

As against, control plants which were grown in a sterilized soil showed no mycorrhizal colonization.

Effect of mycorrhizae and water stress on plant growth and water contents

Mycorrhization and stress influenced significantly on the growth of aerial and root part; this elongation of basil plants are largely driven by mycorrhiza with maximum growth recorded in MNS plants. By cons, we find that water stress inhibits the growth of these two parts mainly in NM plants (Figure 1). In stressed mycorrhizal plants, water supply deficit is offset by the AMF which generates growth at the root and aerial part.

The same observations can be made on the water contents in leaves of basil plant; we recorded a significant variation between the different treatments (Figure 2). In MNS plants, mycorrhiza increases the levels of water to a value which can reach 93%. Non-mycorrhizal plants showed the lowest levels with a dramatic decrease recorded in NMS plants (81%). In MS plants, there have been relatively high water contents (91%) reflecting the role of mycorrhizal fungi to withstand water deficit in stressed plants.

Effect of mycorrhizae and water stress on the contents of chlorophyll pigments

The estimated levels of chlorophyll pigments show that in the absence of water stress, the content of total chlorophyll (Cha + Chb) is slightly higher (10%) in mycorrhizal plants (2.59 mg/g MF) than in NM plants (2.36 mg/g MF). This content is reduced by water stress significantly, especially in NM plants. Furthermore, when comparing the levels of these pigments between NM.S and MS plants, we see that mycorrhizal plants accumulate 72.72% more of chlorophyll (Figure 3).

The same observations can be made about the Chla/Chlb ratio. In the absence of water stress, this ratio is higher in mycorrhizal plants than in NM plants and decreases in times of stress (Figure 3).

Effect of mycorrhizae and water stress on proline contents

During growth, the proline contents in the aerial part significantly change although in case of irrigation or not, in one hand, and mycorrhization or not in the other (Figure 4).

However, in case of water stress, proline contents increased and this increase is more pronounced in NM plants (55 µg/g FM) than in M plants (41 µg/g FM).

At root parts, proline shows the same trend of accumulation for the aerial parts. Thus, proline contents were comparable in unstressed plants inoculated whether or not, while the lack of water leads to an increase in the

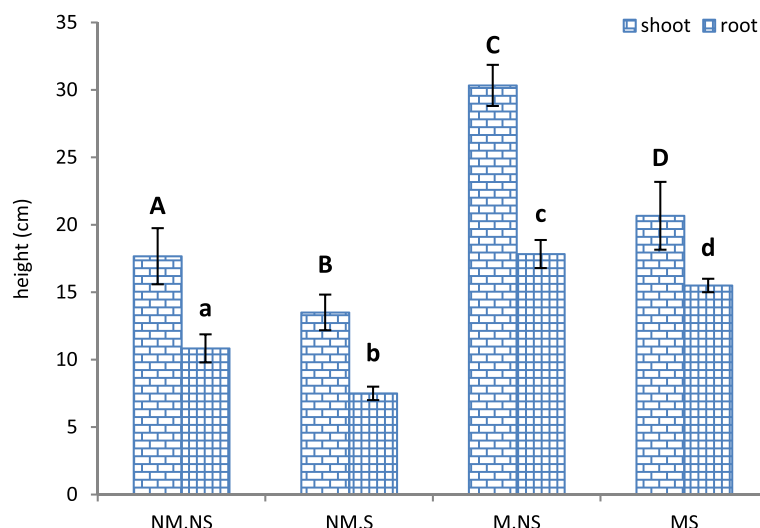


Figure 1 Growth of basil plant. Growth of basil plant (*O. gratissimum* L) inoculated with (*Glomus intraradices*) (M) or not (NM) and subjected to water stress (S) or not (NS). The values followed by different letters are significantly different ($P = 0.05$).

synthesis of proline mainly in non-mycorrhizal plants (31 $\mu\text{g/g}$ FM) (Figure 4).

Effect of mycorrhizae and water stress on phenolic compounds contents

The contents of TPC in aerial parts do not change significantly when the plants are mycorrhized and in the presence or absence of water stress; however, in non-mycorrhizal plants, these levels increase significantly in stressed plants (4.3 mg/g FM) (Figure 5).

In roots, the levels of TPC increase slightly in the NM.S plants. However, in the other treatment, a difference in the level of this compound was not observed.

EO contents

The yield of EOs is very influenced by the mycorrhization since we noted a higher synthesis in both cases stress and irrigation. This increase reaches values up to 37% more than those found in non-mycorrhizal plants; however, water stress did not show a significant

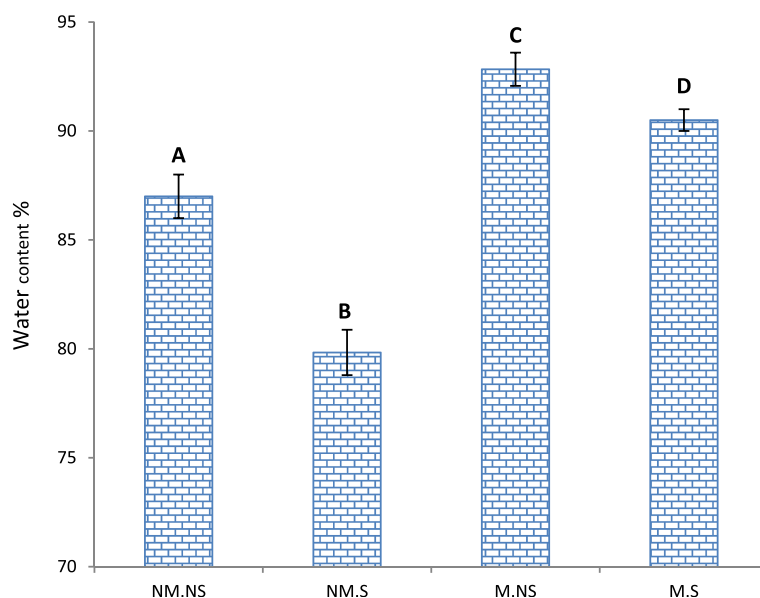
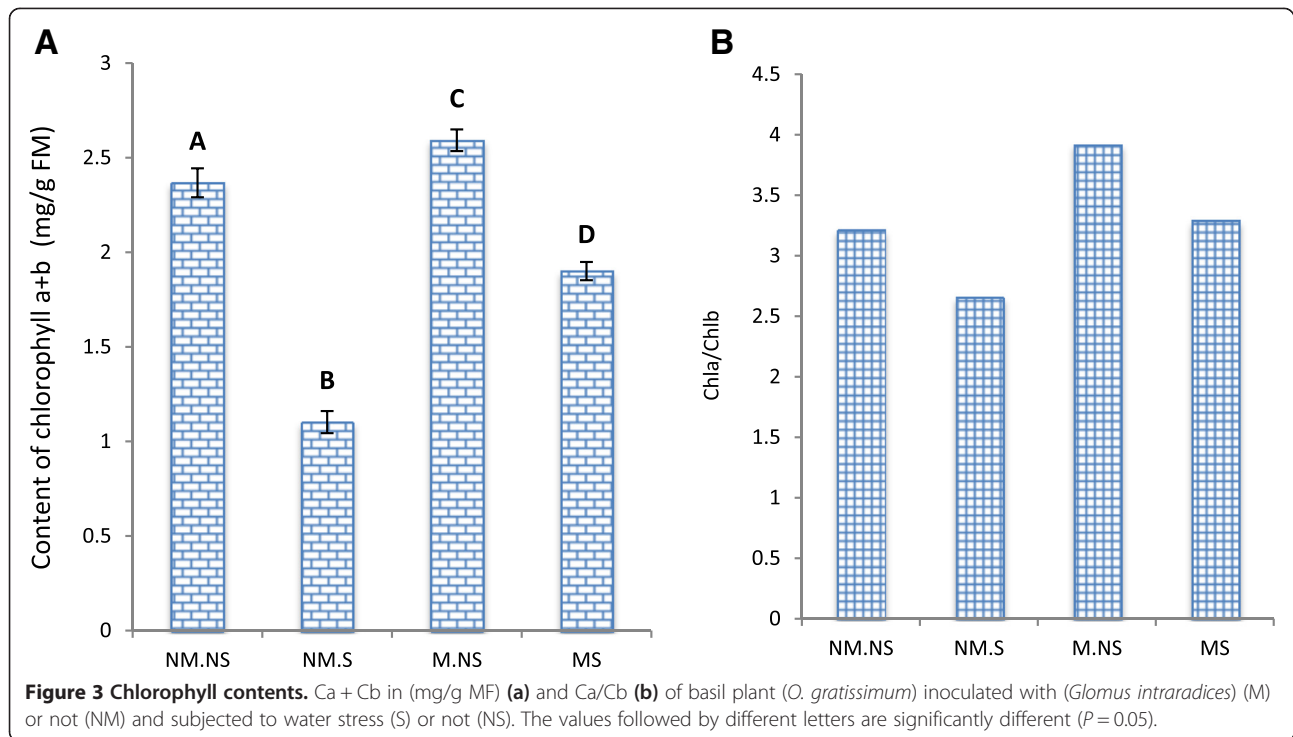
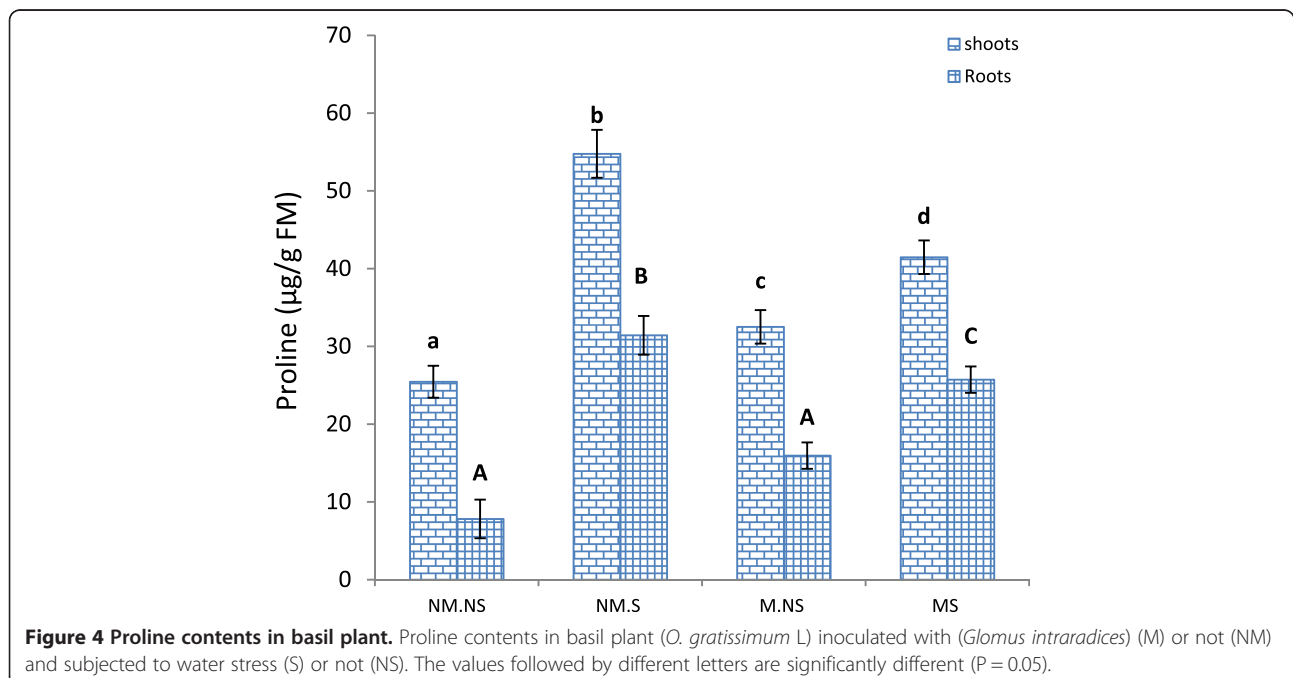


Figure 2 Water contents in basil plant. Water contents in basil plant (*O. gratissimum* L) inoculated with (*Glomus intraradices*) (M) or not (NM) and subjected to water stress (S) or not (NS). The values followed by different letters are significantly different ($P = 0.05$).



influence on the synthesis of EO because we noticed a slight increase (9%) between plants NM and (10%) between M plants. Moreover, the combination of mycorrhization and stress (MS) increases this value to 50%, greater than those found in NM.NS plants (Table 2).

These results can be confirmed by the increase in the number of glandular hairs in basil leaves (Figure 6). The observations in environmental scanning electron microscopy show an increase in the number of glandular hairs in the basal part of mycorrhizal plants compared to basal



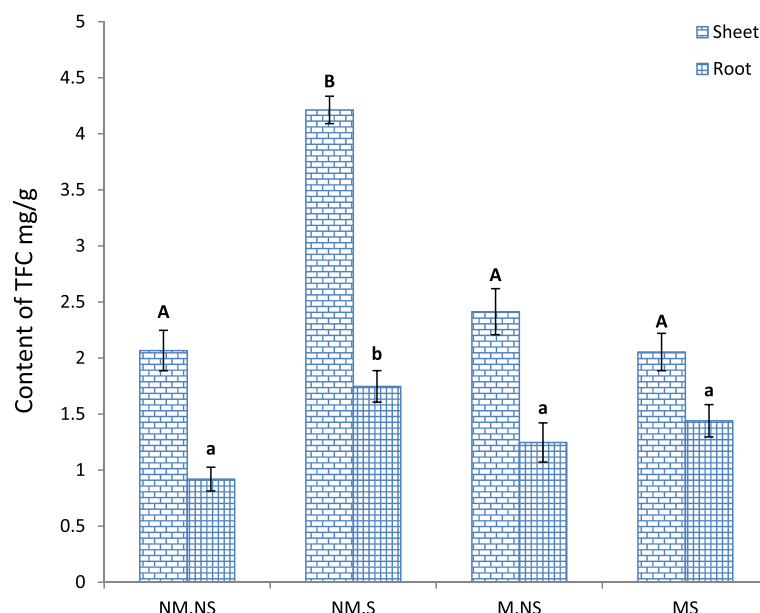


Figure 5 Total phenolic compound contents. Total phenolic compound (TPC) contents in basil plant (*O. gratissimum* L) inoculated with (*Glomus intraradices*) (M) or not (NM) and subjected to water stress (S) or not (NS). The values followed by different letters are significantly different ($P = 0.05$).

areas of non-mycorhizal plant. The mycorrhization can double the number of the glandular hairs.

Discussion

The effect of mycorrhizae on basil plant tolerance to water stress is studied using the fungus *G. intraradices* as mycorrhizal inoculum. Plants inoculated with *G. intraradices* show better growth than non-inoculated plants, both in terms of irrigation, in conditions of water stress. In irrigated conditions, inoculation with *G. intraradices* causes an increase in the length of the aerial part and root of the irrigated plants, respectively, 71% and 75%, while in conditions of water stress, the growth of NM.S plants is limited because stress alters the growth of root parts which influence the water and nutritional status of plants. Under these conditions, mycorrhiza increases the growth of aerial parts of 51% and root parts of nearly 130%. Similar results are observed by many authors, although the magnitude of adverse effects caused by the water stress and the positive effects of mycorrhizal is very

variable: Ferahani et al. [41] showed that mycorrhizal stressed coriander present a normal growth. An-Dong et al. [20], Baslam et al. [2]), Rasouli-Sadaghianil et al. [55], Toussaint et al. [35], and Zolfaghari et al. [56] showed that mycorrhizal fungi *Glomus fasciculatum*, *Glomus etuonicatum*, and *G. intraradices* stimulate the growth and nutritional status of several plants *Lactuca sativa* L., *Lonicera confusa*, *O. basilicum* L, and *Vicia faba* L. Water stress induced a reduction of water contents (WC) of non-mycorrhizal plants. In mycorrhizal plants, there has also been a reduction, but this reduction is very small. It maintains water contents at higher levels under conditions of water deficit through the mycorrhizal association is due to a better exploitation of available water in the soil by fungal hyphae [57].

In the case of irrigation, the plants do not seem affected by mycorrhiza as non-mycorrhizal plants have chlorophyll levels comparable to those of mycorrhizal plants. However, in plants subjected to water stress, mycorrhizal colonization by *G. intraradices* reduced the magnitude of the reduction in chlorophyll and maintains its contents at much higher than those of non-mycorrhizal plants since the improvement rate can reach 72%. These results confirm those found by Kucey and Paul [17], Levy and Krikun [18], Snellgrove et al. [58], and Allen et al. [59]; they have shown that mycorrhizal combination stimulates photosynthesis of many aromatic and medicinal plants. These authors reported stimulation of photosynthesis in improving

Table 2 variation of essential oils contents under conditions of stress and mycorrhizal in *O. gnatissimum* plants

	NM.NS	NM.S	M.NS	MS
Oils content (%)	0.22 a	0.24 a	0.3 b	0.33 c

Three replications were taken for the extraction of essential oils, the values followed by different letters are significantly different ($P = 0.05$).

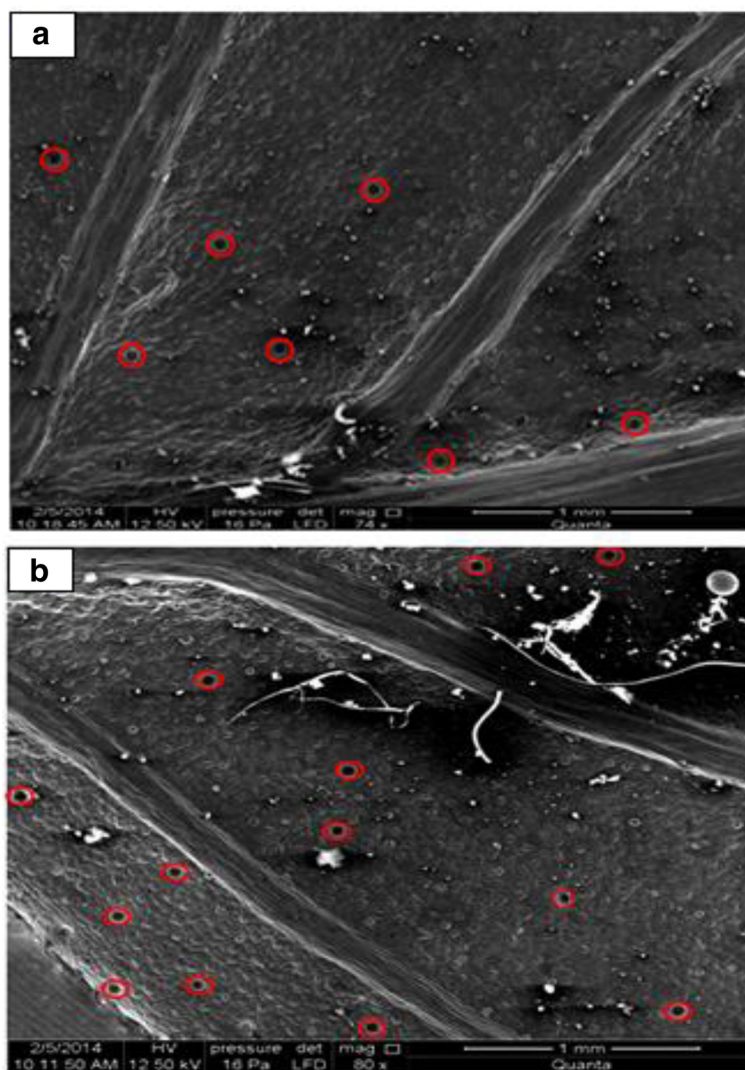


Figure 6 Observation by environmental scanning electron microscopy. Observation by environmental scanning electron microscopy shows the abundance of glandular hairs at the basal part of an unstressed plant and subjected to M (a) or not NM (b).

P nutrition necessary for reactions of CO₂ assimilation in plants. This stimulation can also be linked to an increase in leaf area [60] or associated with increased hydration leaves [61].

Our results show also, with water stress, proline contents as well as in the aerial parts in root parts increased compared to non-stressed plants. This increase is higher in non-mycorrhizal than in mycorrhizal plants. This latter accumulates proline 39% less than non-inoculated plants. Mycorrhiza thus mitigates the increase in proline contents in water stress conditions. Similar results are shown by many authors [62,63] who showed that water stress induced proline accumulation in many plants leaves. Proline is a compound that is considered an osmotic regulator [7,8] to promote tolerance to water stress in the stressed plants by maintaining turgor through osmotic adjustment.

We noted also that in case of stress, the proline contents increased slightly in the aerial and root parts when plants are mycorrhizal. This slight increase was due to the defense mechanism set up by the plant response to mycorrhizal infection. According to Giovannetti and Avio [64], the mycorrhizal colonization would initially be perceived by the plant as a stress or an attack at the very location of colonization by endomycorrhizal, where the synthesis of secondary compounds in the early stages of colonization.

We also showed that the levels of TPC increased mainly in the leaves of NMS plants, in response to water stress. These results are in agreement with those of Iker et al. [65] who showed that the levels of flavanols, which are the monomers of condensed tannins (flavanols epicatechin (EC), epicatechin gallate (ECG), and epigallocatechin gallate

(EGCG), increase in the leaves of *Cistus clusii* in response to hydric deficit. Most phenols plants are also considered stress metabolites [66,67] and their accumulation in plants is affected by several factors.

We also found in this work that the yield of EO increases significantly when the plants are mycorrhizal. This increase may be related to the number of glandular hairs in the leaves. ESM observations of leaves show an increase in glandular hairs in mycorrhizal plants. These results are confirmed by the work of Andrea Copetta et al. [26,27] who showed that Mycorrhizal colonization of *O. basilicum* L plants by *Glomus mosseae*, *Gigaspora margarita*, and *Gigaspora rosea* increases the number of glandular hairs in the leaves compared to non-mycorrhizal plants. Zolfaghari et al. [56], Gupta et al. [38], Freitas et al. [36], and Mucciarelli [68] reported an increase in yield of EOs in *Mentha arvensis* and *Mentha piperita* L inoculated with mycorrhizal fungi, and this increase related to a change in plant secondary metabolism and the number of glands per leaf; this increase in the levels of EO may also be related to fungal colonization. According to Copetta et al. [26,27], the production of terpenoids and constituents of EOs possess fungicidal properties which are considered a defensive response to colonization by the fungus and causing increased yield and increased production of these metabolites in mycorrhizal plants.

Conclusion

This study helps us to understand the reaction of *O. gratissimum* vis-a-vis mycorrhizae and water stress. In the case of non-mycorrhizae, water stress limits the growth and photosynthetic capacity and leads to a reduction of water contents in the plant. This decrease in morphological parameters is accompanied by a synthesis of several molecules in particular proline and phenolic compounds, known for their role in plant tolerance to adverse conditions. However, the AMF stimulates growth and photosynthesis and drives the water status in plant at an optimal level. The results showed a decrease in levels of proline and phenolic compounds, thus confirming the role of mycorrhizal symbiosis in plant defense against biotic and abiotic stress. Note also that this symbiosis leads to an increase in the yield of EOs, regardless of the water status of the plant (stressed or not), and this increase is correlated with the increase of the glandular hairs abundance.

Abbreviation

AMF: arbuscular mycorrhizal fungi; Cha: chlorophyll (a); Chb: chlorophyll (b); ChT: chlorophyll (total); EO: essential oil; M.NS: mycorrhizal unstressed; MS: mycorrhizal stressed; NM.NS: non-mycorrhizal unstressed; NM.S: non-mycorrhizal stressed; TPC: total phenolic compounds.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YM, EhE, and KAJ participated in the data processing and in designing the study and performed the statistical analysis and drafting of the manuscript. All authors read and approved the final manuscript.

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