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Green Compost Combined with Mycorrhizae and Rhizobia: A Strategy for Improving Alfalfa Growth and Yield Under Field Conditions

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Abstract

Alfalfa (*Medicago sativa* L.) is one of the most important forages and legume crops in the Mediterranean region, where extensive soil exploitation and low soil fertility are among the factor limiting its production. In this study, a field experiment was carried out in the semi-arid area of Morocco, in order to assess the influence of the individual and combined application of autochthonous arbuscular mycorrhizal fungi (AMF), rhizobium strain and green compost supply on alfalfa. Vegetative yield, physiological and biochemical traits were evaluated together with the associated changes in the soil's physico-chemical parameters. The results show that AMF and rhizobium inoculation combined with compost application induced the greatest effect. This treatment increased the dry matter yield, leaves and nodules number, and AMF infection rate. Moreover, the combined use of these biofertilizers further enhanced stomatal conductance, photosystem II efficiency, photosynthetic pigments (chlorophyll and carotenoids), protein and sugar content along with the nutrients uptake (Phosphorus (P), Nitrogen (N), Potassium (K) and Calcium (Ca)). When compared with the initial soil status, the compost (10 t/ha) combined with AMF and rhizobium significantly improved the organic matter, N and P content, decreased soil pH and increased electrical conductivity. This treatment is also efficient in increasing soil glomalin content. This study demonstrated that the interaction between green waste compost, AMF and rhizobium inoculation could have important implications in alfalfa sustainable agriculture.

Keywords Compost · Arbuscular mycorrhizal fungi · Rhizobia · Field conditions · *Medicago sativa*

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Grünkompost in Kombination mit Mykorrhiza und Rhizobien: Eine Strategie zur Verbesserung von Wachstum und Ertrag der Luzerne unter Feldbedingungen

Zusammenfassung
Die Luzerne (*Medicago sativa* L.) ist eine der wichtigsten Futter- und Hülsenfruchtkulturen im Mittelmeerraum, wo die extensive Bodennutzung und die geringe Bodenfruchtbarkeit zu den Faktoren gehören, die ihre Produktion einschränken. In dieser Studie wurde ein Feldversuch in einem semi-ariden Gebiet Marokkos durchgeführt, um den Einfluss von autochthonen arbuskulären Mykorrhizapilzen (AMF), eines Rhizobienstamms und der Grünkompostversorgung – einzeln oder in Kombination – auf die Luzerne zu untersuchen. Vegetativer Ertrag, physiologische und biochemische Parameter wurden zusammen mit den damit verbundenen Veränderungen der physikalisch-chemischen Parameter des Bodens bewertet. Die Ergebnisse zeigen, dass die Inokulation mit AMF und Rhizobium in Kombination mit der Kompostanwendung die größte Wirkung erzielte. Diese Behandlung erhöhte den Trockenmasseertrag, die Anzahl der Blätter, die Anzahl der Knötchen und die AMF-Infektionsrate. Darüber hinaus wurden durch den kombinierten Einsatz dieser Biodünger die stomatäre Leitfähigkeit, die Photosystem-II-Effizienz, die photosynthetischen Pigmente (Chlorophyll und Carotinoide), der Protein- und Zuckergehalt sowie die Nährstoffaufnahme (Phosphor (P), Stickstoff (N), Kalium (K) und Kalzium (Ca)) weiter verbessert. Im Vergleich zum anfänglichen Bodenzustand verbesserte der Kompost (10 t/ha) in Kombination mit AMF und Rhizobium signifikant die organische Masse, den N- und P-Gehalt, senkte den pH-Wert des Bodens und erhöhte die elektrische Leitfähigkeit. Diese Behandlung ist auch effizient bezüglich der Erhöhung des Glomalin-Gehalts im Boden. Die Studie zeigte, dass die Kombination von Grünabfallkompost, AMF- und Rhizobium-Inokulation wichtige Auswirkungen auf eine nachhaltige Landwirtschaft der Luzerne haben könnte.

Schlüsselwörter Kompost · Arbuskuläre Mykorrhizapilze · Rhizobien · Feldbedingungen · *Medicago sativa*

Introduction

In Morocco, as in many parts of the world, land degradation has emerged as one of the most serious problems. The soil fertility has considerably declined due to intensive agriculture, increasing the use of chemical fertilizers and pesticides, over-grazing, water pollution, salinization, deforestation, and accumulation of non-biodegradable waste (Kouba et al. 2018; Ouachoua and Al Karkouri 2020). In addition, climate change effects are further aggravating the situation (Ait El Mokhtar et al. 2019a). The decrease in soil fertility is characterized by the loss of soil's stable organic matter and the increase of plant sensitivity to nutritional imbalances and diseases (Mainville et al. 2006). Thus, integrated farming based on biofertilizers becomes a potential efficient alternative.

Agriculture based on the exploitation of beneficial soil microorganisms and the application of organic amendments can not only ensure food security by providing sufficient nutrients to plants but can also improve biodiversity and soil restoration (Schütz et al. 2018). Therefore, the use of such biofertilizers represents both an economic and an ecological alternative. The application of compost as an organic amendment is highly recommended because of its role in the bioconversion of organic waste, bio-control of plant, improving nutrient availability, and the soil's physicochemical and biological properties (Chaichi et al. 2018; Boutasknit et al. 2020a).

Furthermore, many studies have reported that the use of symbiotic microorganisms can improve crop yields, soil fertility and restore degraded soils by reducing the use of synthetic fertilizers and pesticides (Cavagnaro et al. 2015; Pii et al. 2015; Raklami et al. 2019). Indeed, arbuscular mycorrhizal fungi (AMF) are known for their importance in improving plant growth, through the increase of photosynthetic rate, nutrient and water uptake, as well as soil physicochemical and biological properties (Ait-El-Mokhtar et al. 2019b, 2020a; Boutasknit et al. 2020b). On the other hand, rhizobium could improve yield through the atmospheric nitrogen (N) fixation, ranging between 15 and 20 kg N/ha (Sultana et al. 2014). They can solubilize insoluble phosphate and potassium (Ana et al. 2009; Abdi et al. 2017) and produce active molecules in the soil such as exopolysaccharides (Babu et al. 2015; Primo et al. 2019) and auxin (Defez et al. 2019). Auxin production can promote root growth and architecture (Aloni et al. 2006) while exopolysaccharides play an important role in influencing soil structure and maintaining water film necessary for photosynthetic activity and plant growth (Naseem et al. 2018). The combined use of biofertilizers appears to be very useful in sustainable agriculture by restoring and enhancing degraded lands and increasing plant growth (Ansari and Mahmood 2017; Ben-Laouane et al. 2019; Anli et al. 2020).

Alfalfa (*Medicago sativa* L.) is one of the most important and widespread forages and legume crops in the world, due to its high nutritional value for human food and animal feed (Rashimi et al. 1997; Venkateshwaran and Ané

2011). It is widely cultivated in arid and semi-arid zones, including Morocco, where soil degradation limits the growth of this crop (Zhang et al. 2007). Therefore, special land management strategies may be needed to maintain alfalfa production under these conditions.

Many investigations showed the positive effects of plant-associated microbes (AMF and/or rhizobia) and/or compost on alfalfa growth and development (Campanelli et al. 2013; Bertrand et al. 2015; Zhu et al. 2016; Mbarki et al. 2020). However, the combined effect of AMF, rhizobia, and compost on plants' performances was rarely studied, and according to our knowledge, no data are available for the tripartite combination of these biofertilizers on alfalfa growth. The objective of this work is to investigate the interactions between autochthonous biofertilizers and their underlying physiological and biochemical mechanisms in improving alfalfa performances in the open field.

Materials and Methods

Study Site

The experiment was carried out in an open field in Tamesloht, located at 15 km in southwest of Marrakesh, Morocco (31°54′18″N, 8°02′08″W, 511 m above sea level), where the climate is mediterranean with an annual average temperature of 20.5 °C and annual precipitation of 281 mm. The experimental farm is spread over a total area of 3 ha where the only organic farming system was adopted free from herbicides and chemical fertilizers. The weeds were controlled with hand hoeing as needed.

Biofertilizers Materials

The organic compost used in this experiment was prepared as described by Meddich et al. (2016). It was produced from Quack grass, (*Elymus repens*), which was collected from gardens and green spaces in Marrakesh (Morocco). The composting experiment was conducted for three months on a composting platform at the municipal nursery, Marrakesh, Morocco. The grass waste was placed on plastic layer sheets to prevent run-off and leaching during moistening. The windows were covered with other similarly perforated plastic to reduce evaporation as well as heat loss. Humidity was then adjusted to 60% (optimal value for composting) throughout the composting process as recommended by several authors (Liang et al. 2003; Thomas et al. 2020). During the composting process, the temperature was above 55 °C for 7 days. In addition, the nature of grass waste used in this study was free of pollutants and was applied at 10 t/ha. The compost physico-chemical properties are shown in Table 1.

The arbuscular mycorrhizal fungi (AMF) used was Aoufous mycorrhizal consortium, isolated from the Tafilalet palm grove located at 500 km east of Marrakesh. It contains a mixture of indigenous species: (i) *Glomus* sp. (15 spores/g of soil), (ii) *Sclerocystis* sp. (9 spores/g soil), and (iii) *Acaulospora* sp. (1 spore/g of soil) (Meddich et al. 2015). AMF consortium was multiplied and trapped using corn (*Zea mays* L.) plants for three months under controlled greenhouse conditions, as described by Meddich et al. (2015). According to the most probable number (MPN) test, the trap culture inoculum contained 1056 infective propagules/100 g (Ait-El-Moktar et al. 2020b). Inoculation with AMF was performed by supplying each plot with 0.7 kg of AMF consortium (containing mycorrhizal roots, substrate and AMF spores). Uninoculated plots received the same amount of autoclaved mycorrhizal fungi inoculum.

The rhizobium strain (RhOL1) was delivered by the Microbial Biotechnologies, Agrosciences and Environment Laboratory, Faculty of Science Semlalia, Cadi Ayyad University, Marrakesh, Morocco. It was isolated from nodules of *Medicago sativa* and identified as *Ensifer meliloti* (El-Khalloufi 2012). RhOL1 was tested for different PGPR activities and biochemical characteristics. It can solubilize tricalcium phosphate and potassium. Furthermore, this strain can produce exopolysaccharides and indole acetic acid (IAA). The bacterial suspension was prepared on YEM liquid medium (Vincent 1970), at 28 °C for 48 h to obtain a concentration of 10^9 colony-forming units/mL with an optical density (OD) of 1 at 600 nm, using a UV visible spectrophotometer (UV-3100PC spectrophotometer, VWR). The rhizobium inoculation was performed by incubating alfalfa seeds in a solution of RhOL1 strain (DO = 1) for 30 min in darkness.

Plant Culture and Experimental Treatments

Plots of 1.5 m × 0.8 m were randomly established and spaced by 0.4 m with 1 m interval between the rows. Each row was equipped with two lines of drip irrigation system. The distance between drip lines for the same plots was 0.4 m with a 15 cm distance between internal drippers. The drip hose used is equipped with suitable internal drippers (sheath) which release 2 L/h. The plants were watered every three days for 2 h (50 L/m²).

Table 1 Physico-chemical parameters of compost obtained from grass waste

pH	Total carbon (%)	Total nitrogen (%)	C/N	Ashes (%)	NH_4^+ (mg/g)	NO_3 (mg/g)	P (mg/g)	NH_4^+/NO_3
7.8	30.6	2.1	14.0	49.0	3 10^{-5}	7 10^{-5}	0.2	0.4

Eight treatments were applied: control (Ct) compost alone (C), AMF alone (AM), rhizobium alone (RhOL1) and AM + RhOL1, C + AM, C + RhOL1, C + AM + RhOL1 combinations.

The compost and AMF were applied before sowing the seeds in the 20 cm topsoil layer, at 10 t/ha (1.2 kg each plot) and 6 t/ha (0.7 kg each plot) respectively. For compost and AMF combined treatments, the application of mycorrhizal fungi was made after covering the compost with a layer of soil.

M. sativa seeds (cv. Siriver) obtained from the National Institute for Agricultural Research (Marrakesh, Morocco) were inoculated with rhizobial strain prior to their sowing in February 2017 at 15 kg/ha. An uninoculated control was conducted under the same conditions. To maintain rhizobium viability and nodules development, we conducted a re-inoculation after the 15th day of sowing (720 mL for each plot).

Growth and Yield Parameters

Plants were harvested at 25% of the flowering process, after three months. The following parameters were determined: total dry matter yield (TDM), and leaf numbers. For dry matter measurement, shoots and roots were separated and dried at 80 ºC for 48 h then were weighed.

Microbial Symbiosis Parameters

The infectivity of rhizobium (RhOL1) was assessed by counting the number of nodules. AMF colonization of roots was determined using the modified method of Phillips and Hayman (1970). The method consists of treating the roots of the lateral root system with 10% KOH for 10 min at 90 °C, and were then rinsed with 1% HCl for 10 min. The roots were stained with trypan blue (0.05%) at 90 °C for 10 min. Fine roots of 1 cm length were examined under the Zeiss Axioskop 40 microscope. 30 segments repeated three times for each sample were observed. The frequency (Fa) and intensity (Ma) of AMF infection were estimated according to the following formulae:

$$\text{AMF infection frequency (Fa) (\%)} = \left(\frac{\text{Infected root segments}}{\text{Total root segments}}\right) \times 100$$

$$\text{AMF infection intensity (Ma) (\%)} = \left(\frac{95n5 + 70n4 + 30n3 + 5n2 + n1}{\text{Total root segments}}\right)$$

Where *n* is the number of fragments assigned with the index 0, 1, 2, 3, 4, or 5, with the following infection rates: 100 > n5 > 90; 90 > n4 > 50; 50 > n3 > 10; 10 > n2 > 1; 1 > n1 > 0.

Nutrient Uptake

After harvest, nitrogen (N), phosphorus (P), potassium (K) and calcium (Ca) were measured using finely powdered plant samples dried in an oven at 80 °C for 48 h. The total N was determined according to the Kjeldahl method, while the other minerals analysis was carried out after dry-ashing at 550 °C and digestion of the ash in 6 N HCl. P content was determined according to the Olsen and Sommers (1982) method and K and Ca were measured by flame photometry (AFP 100 flame photometer).

Stomatal Conductance

Stomatal conductance was measured between 10:00 and 12:00 h on a sunny day using a portable steady-state diffusion porometer (Model Sc-1, Decagon Devices, Pullman, USA). Measurements were made on the second healthy and youngest leaf of 4 different plants per treatment.

Chlorophyll Fluorescence

Chlorophyll fluorescence was measured on healthy leaves using a portable fluorometer (Opti-sciences OSI 30p) after 30 min of darkness. The initial (F0), maximal (Fm), and variable (Fv = Fm-F0) relative fluorescence values were measured. The PSII efficiency was expressed as the Fv/Fm ratio (Lutts et al. 1996).

Photosynthetic Pigments

The photosynthetic pigments were extracted in 4 mL of 90% acetone from 50 mg of the fresh leaf. The extracts were centrifuged at 10,000 xg for 10 min. Then, the OD of the supernatant was measured using a UV/visible spectrophotometer at 663, 645 and 440.5 nm (Smith and Benitez 1955). Total chlorophyll, chlorophyll a, chlorophyll b, and carotenoids were estimated according to the formulas provided by (Arnon 1949).

Soluble Sugar Content

The soluble sugar concentration in the alfalfa plant was estimated according to Benzaria et al. (2006). 0.25 mL of the preserved alcoholic extract was added to 0.25 mL of phenol (5%) and 1.25 mL of sulphuric acid. After cooling, the OD was measured at 485 nm using a spectrophotometer. The soluble sugar content was determined using a glucose standard curve and expressed as mg/g FW of leaves.

Protein Content

Fresh leaves (0.1 g) were homogenized in a cold mortar with 5 mL of 0.1 M phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidone (PVPP) and 0.1 mmol of ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 15,000 xg for 15 min at 4 °C. The supernatant was used for soluble proteins measurement according to the method of Bradford (1976).

Soil Physico-chemical Analyses and Glomalin Content

Soil samples of each treatment were collected at 0–20 cm before and after the experiment. The pH and electrical conductivity (EC) were measured in a 1:2 (w:v) aqueous solution. Soil texture was determined using Robinson's method (Baize 1988). Total nitrogen (N) was determined by the Kjeldahl method and the total organic carbon and organic matter were measured by the titrimetric method as described by Anne (1945). Available P was determined by colorimetry according to the method of Olsen and Sommers (1982).

Easily extractable glomalin-related soil protein (EE-GRSP) and total glomalin-related soil protein (T-GRSP) were assessed according to the method described by Wrigth and Upadhyaya (1998). EE-GRSP was extracted from 2 g soil samples by adding 8 mL of 20 mM sodium citrate (pH 7.0), followed by autoclaving the solution at 121 °C for 30 min, while T-GRSP was extracted by autoclaving 2 g soil in 8 mL of 50 mM sodium citrate at pH 8.0 for 60 min. For both EE-GRSP and T-GRSP, the supernatant was collected after centrifugation at 5000xg for 12 min and stored at 4 °C, then the two parameters were quantified by the Bradford method.

Statistical Analysis

Results are means of fifteen replicates per treatment ± standard error (S.E.) for growth and yield parameters (total dry matter yield and leaf numbers as well as nodules) and four replicates for mineral, physiological and biochemical parameters. The statistical analyses were assessed using COSTAT software (version 6.3). Data were subjected to three-way ANOVA analysis. Differences between means were analyzed by Tukey's honestly test ($P \leq 0.05$). Principal component analysis (PCA) was carried out with MS office XLSTAT software (v. 2016).

Results

Growth Parameters

As shown in Fig. 1, the application of AMF, RhOL1, and compost separately or in combination significantly improved total dry mater and leaf number compared with the untreated plants. The maximum improvement was recorded in plants treated with compost-AMF-RhOL1, which increased the total dry matter by 64% and the leaves number by 108% in comparison to untreated plants. Interactions among RhOL1 strain (R), AMF (M), and compost (C) treatments were significant ($P < 0.001$) for the total dry matter.

Symbiosis Colonization

Nodulation and mycorrhization were recorded in roots of treated and untreated alfalfa plants. However, the plants inoculated with AMF and/or RhOL1 had a high mycorrhization rate and a maximum number of nodules compared to uninoculated plants (Fig. 2). Indeed, the values of AMF infection frequency and intensity and nodules number ranged from 73–99%, 21–41% and 211–300 respectively for the inoculated plants, while they varied from 40–59%, 2–21% and 100–203 respectively for the uninoculated plants. It is important to notice that the maximum mycorrhization rate (frequency and intensity), and the highest nodules number were recorded in the dual inoculated plants in the presence of the compost, with an increase of 147%, 241% and 200%

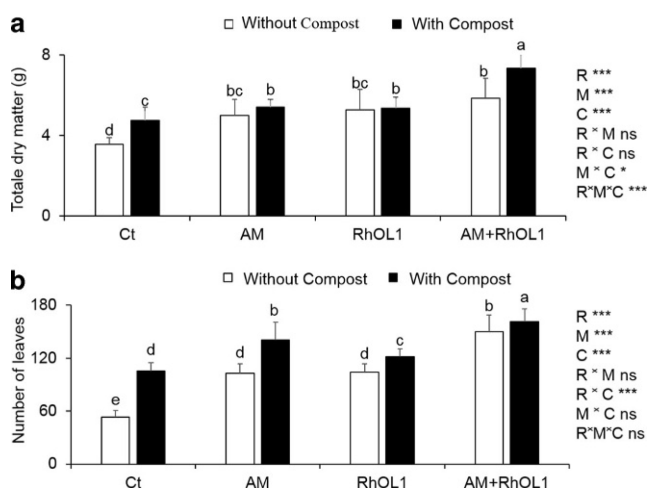

Fig. 1 Total dry matter (**a**) and number of leaves (**b**) of *Medicago sativa*, submitted to different treatments: control (Ct); arbuscular mycorrhizal fungi (AM); rhizobium strain (RhOL1) alone or in combination with AMF (AM + RhOL1), in the presence or absence of compost. Values with different letters are significantly different as determined by Tukey's HSD test, after performing three-way ANOVA ($P < 0.05$) (*ns* not significant, *significant at $p < 0.05$, **significant at $p < 0.01$, ***significant at $p < 0.001$)

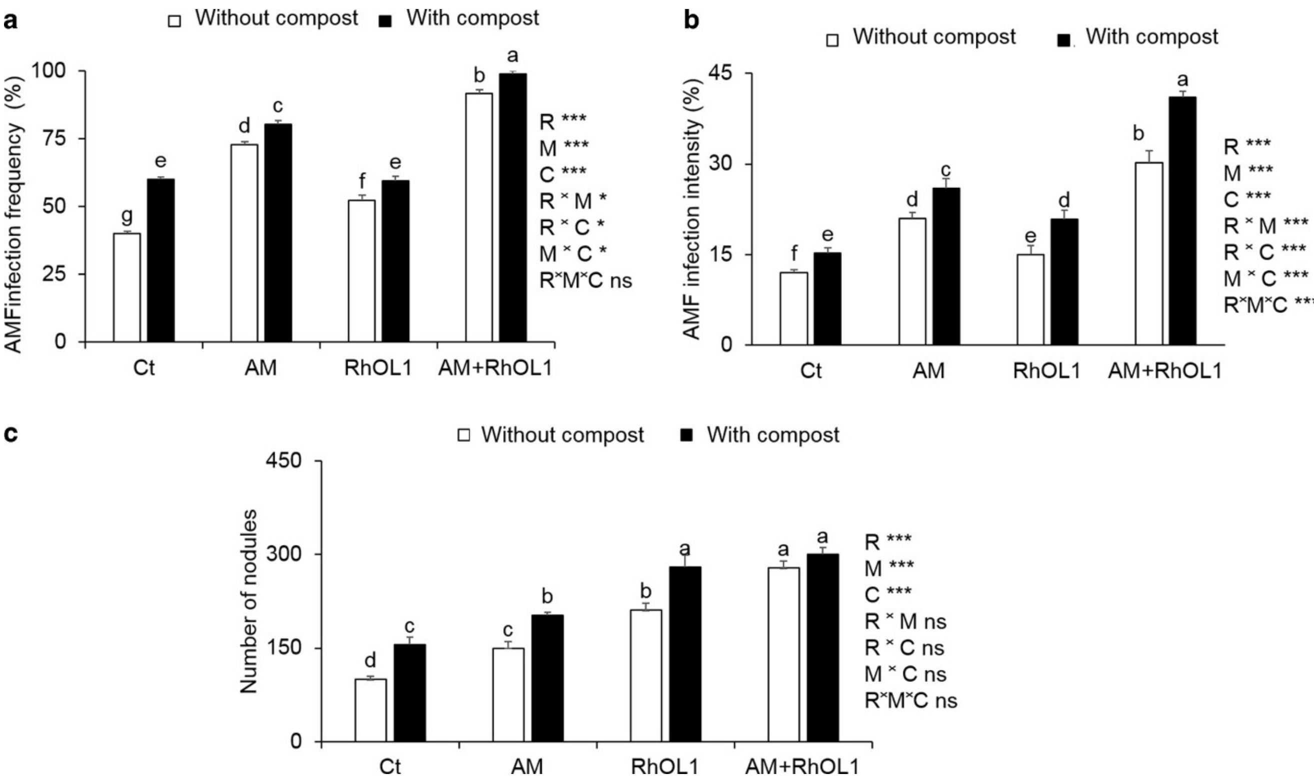

Fig. 2 AMF infection (frequency (**a**) and intensity (**b**)) and number of nodules (**c**) of *Medicago sativa*, submitted to different treatments: control (Ct); arbuscular mycorrhizal fungi (AM); rhizobium strain (RhOL1) alone or in combination with AMF (AM + RhOL1), in the presence or absence of compost. Values with different letters are significantly different as determined by Tukey's HSD test, after performing three-way ANOVA ($P < 0.05$) (*ns* not significant, *significant at $p < 0.05$, **significant at $p < 0.01$, ***significant at $p < 0.001$)

respectively compared to the untreated control. Interactions among R × M × C treatments were significant ($P < 0.001$) for AMF infection intensity.

Nutrient Content

Results from Table 1 indicated that the compost application and/or microbial inoculation significantly influenced the mineral uptake in alfalfa plants compared to the control especially for N, P and K contents. Those elements were more accumulated under C + AM + RhOL1 treatment. The improvements were 20% for N, 80% for P and 55% for K compared to the untreated control. The interaction between RhOL1, AMF, and compost application had a significant ($P < 0.001$) effect on N, P and K contents (Table 2).

Stomatal Conductance and Chlorophyll Fluorescence (Fv/Fm)

Based on the obtained results (Fig. 3), all the applied treatments significantly increased the stomatal conductance and photosynthetic efficiency in comparison with the control. However, the maximum values were recorded in the dual inoculated plants cultivated in compost amended soil. It was about 108% for gs and 15% for Fv/Fm compared to the control plants.

Photosynthetic Pigments

The results of the total chlorophyll, chlorophyll a, chlorophyll b and carotenoid contents, in alfalfa leaves, are shown in Fig. 3. Application of the compost, AMF and RhOL1 separately or in combination improved the photosynthetic pigments compared to control plants. However, plants inoculated with AMF and RhOL1 in the presence of compost showed the highest values (82% for total chlorophyll, 107% for chlorophyll a and b and 74% for carotenoids). Interactions among R × M × C treatments were significant ($P < 0.001$) for total chlorophyll, chlorophyll a and chlorophyll b.

Soluble Sugar and Protein Content

The result of Fig. 4 showed that all the applied treatments significantly enhanced the accumulation of sugars and proteins in alfalfa leaves compared to the control. The greatest enhancement was recorded with the compost + AMF + RhOL1 application, 163% for soluble sugar content and 57% for protein content. However,

Table 2 Mineral concentrations (N: nitrogen; P: phosphorous; K: potassium and Ca: calcium) in the shoots of *Medicago sativa*

	Traitements								Significance level						
	Ct	AM	RhOL1	AM + RhOL1	C	C + AM	C + RhOL1	C + AM + RhOL1	R	M	C	R × M	R × C	M × C	R × M × C
N ($mg \cdot g^{-1}$ DW)	10.0 ± 0.0^{f}	10.0 ± 0.0^{f}	11.2 ± 0.0^{c}	11.9 ± 0.0^{a}	10.5 ± 0.0^{e}	10.9 ± 0.0^{d}	11.5 ± 0.0^{b}	11.9 ± 0.0^{a}	***	***	***	***	***	***	***
P ($mg \cdot g^{-1}$ DW)	0.5 ± 0.0^{e}	0.8 ± 0.0^{b}	0.6 ± 0.0^{c}	0.7 ± 0.0^{b}	0.5 ± 0.0^{e}	0.7 ± 0.0^{b}	0.6 ± 0.0^{d}	0.9 ± 0.0^{a}	***	***	***	***	***	***	***
K ($mg \cdot g^{-1}$ DW)	33.3 ± 4.8^{d}	41.6 ± 0.0^{c}	45.9 ± 0.1^{bc}	51.4 ± 0.0^{ab}	40.7 ± 1.9^{c}	43.8 ± 0.1^{c}	46.7 ± 0.0^{abc}	52.0 ± 0.1^{a}	***	**	ns	ns	ns	ns	ns
Ca ($mg \cdot g^{-1}$ DW)	50.5 ± 0.3^{a}	47.8 ± 1.3^{ab}	44.4 ± 0.1^{abc}	46.1 ± 0.0^{abc}	41.1 ± 5.3^{c}	42.9 ± 0.1^{bc}	45.5 ± 0.0^{abc}	50.4 ± 0.1^{a}	***	***	***	***	***	ns	***

Means (± SE) with different letters are significantly different according to Tukey's HSD test, after performing three-way ANOVA ($P < 0.05$)

ns not significant, *Significant at $p < 0.05$, **Significant at $p < 0.01$, ***Significant at $p < 0.001$

Ct uninoculated and unamended alfalfa control plants, *AM* Alfalfa treated with arbuscular mycorrhizal fungi, *RhOL1* Alfalfa treated with rhizobium strain, *AM + RhOL1* Alfalfa treated with AM and RhOL1, *C* Alfalfa treated with compost, *C + AM* Alfalfa treated with C and AM, *C + RhOL1* Alfalfa treated with C and RhOL1, *C + AM + RhOL1* Alfalfa treated with C, AM and RhOL1

no significant difference was recorded between compost + AMF + RhOL1 and AMF + RhOL1 treatments for protein content. The interaction between RhOL1, AMF, and compost application had a significant effect on these parameters ($P < 0.001$).

Soil Glomalin Content

The results presented in Fig. 5 show that the application of the different biofertilizers improved glomalin content (T-GRSP and EE-GRSP) in alfalfa rhizospheric soil compared to the untreated control. The application of biofertilizers separately did not show significant differences compared to the untreated control soil, especially for EE-GRSP. However, the combination of inoculation and compost (compost + AMF + RhOL1) significantly increased the levels of these two parameters, with an improvement of 42% for T-GRSP and 193% for EE-GRSP. R × M × C interaction had a significant effect on EE-GRSP ($P < 0.05$).

Soil Properties

Results of soil analyses before and after the cultivation of alfalfa and application of different treatments are shown in Table 3. Compared to the initial state, the main soil's physico-chemical properties were significantly improved after 3 months of alfalfa cultivation and biofertilizers application. Indeed, pH values ranged from 8.24–7.94 and a significant decrease in EC was recorded, from 0.3–0.15 mS/cm. The application of different biofertilizers improved also total nitrogen, total carbon, organic matter and available phosphorus content. The interaction between RhOL1, AMF, and compost application had a significant effect on EC ($P < 0.01$) and on carbon, organic matter and phosphorus content ($P < 0.001$).

Principal Component Analysis (PCA)

Results from PCA indicate that most of the parameters measured were influenced by the different biofertilizers treatments. For growth, physiological and biochemical parameters (Fig. 6a), the PC1 explained 76.87% and the PC2 explained 8.58% of the total variance. PC1 separated all the parameters as compared to PC2. AM + RhOL1 treatment in the presence or the absence of compost, as well as Compost + AM, were distinctly separated from control. They were located on the right side of the PC1 axis and closely related to NN, TDM, NL, Fa, Ma and also to the parameters: gs, Fv/Fm, T Chl, Chl a, Chl b, carotenoids, protein, sugar and N, P, K content. Considering the soil's physico-chemical parameters (Fig. 6b), the PC1 explained 64.83%, and PC2 explained 21.06% of the total variance. The most of soil parameters (OM, N, P, C and EC) were

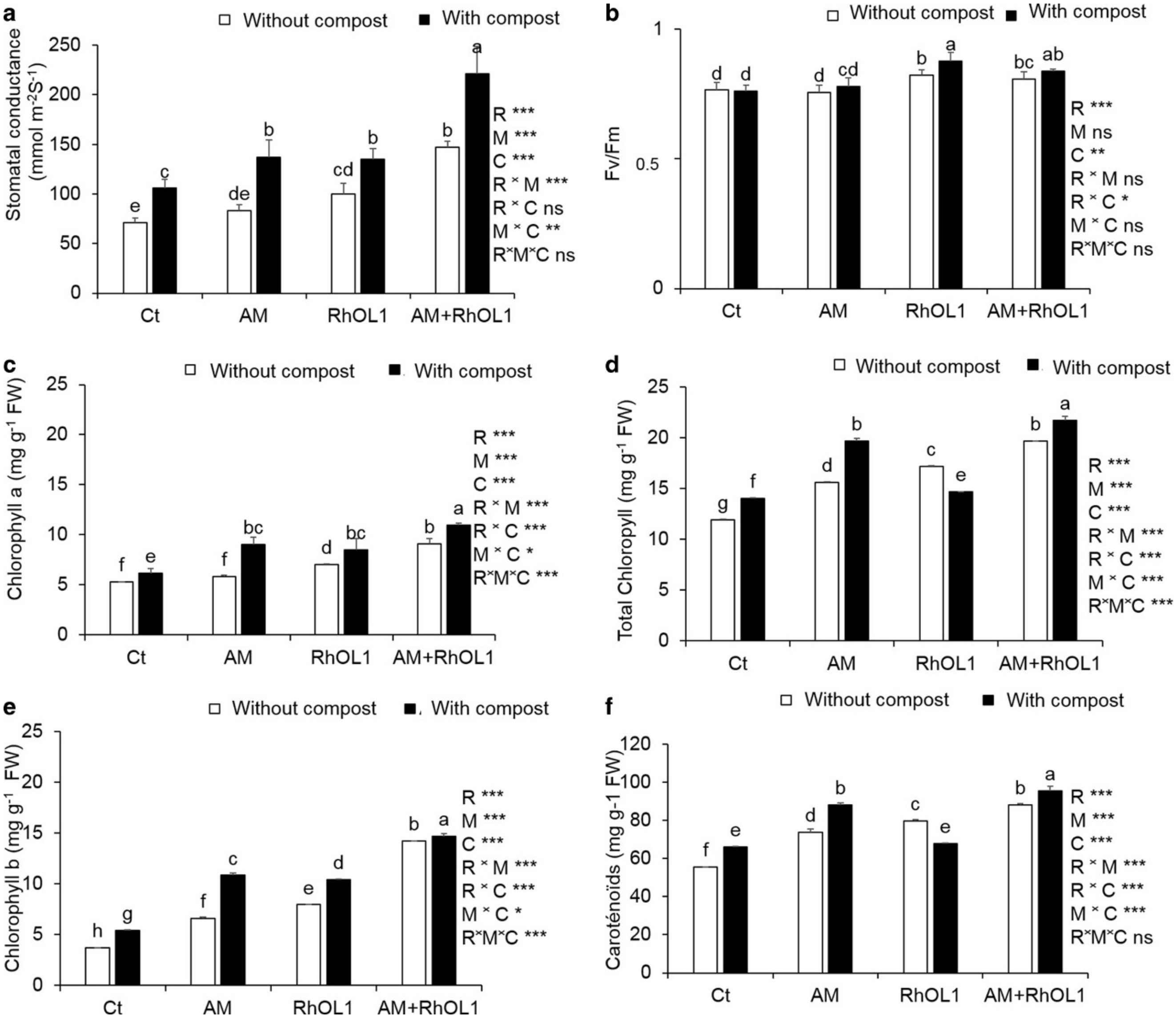

Fig. 3 Stomatal conductance (**a**), chlorophyll fluorescence (**b**), chlorophyll a (**c**), total chlorophyll (**d**), chlorophyll b (**e**), carotenoids (**f**) of *Medicago sativa*, submitted to different treatments: control (Ct); arbuscular mycorrhizal fungi (AM); rhizobium strain (RhOL1) alone or in combination with AMF (AM + RhOL1), in the presence or absence of compost. Values with different letters are significantly different as determined by Tukey's HSD test, after performing three-way ANOVA ($P < 0.05$) (*ns* not significant, *significant at $p < 0.05$, **significant at $p < 0.01$, ***significant at $p < 0.001$)

located along PC1 and closely related to C + AM + RhOL1, AM + RhOL1 and C + AM whereas, pH was associated with RhOL1 and AM and was separated by PC2.

Alfalfa growth parameters as well as soil's physicochemical properties were found to be improved, all by combining inoculation and compost application. Thus, a positive correlation coefficient ($P < 0.001$) was established between plant growth, in terms of total dry biomass, number of leaves, and soil properties (r = 0.71–0.94) (Table 4).

Discussion

Important attention has been recently given to the biofertilizers as an alternative to intensive farming and chemical fertilizers. The biofertilizers, such as AMF, rhizobia and compost, can be potentially used in the agriculture sector to maintain soil fertility and ensure sustainable production. This represents the main objective of the present study in which we evaluate the effect of autochthonous rhizobia, AMF and compost on alfalfa field plantation.

Several studies highlighted the effects of compost application on soil parameters and plant growth (Morales-Corts et al. 2014; Massa et al. 2018; Boutasknit et al. 2020a).

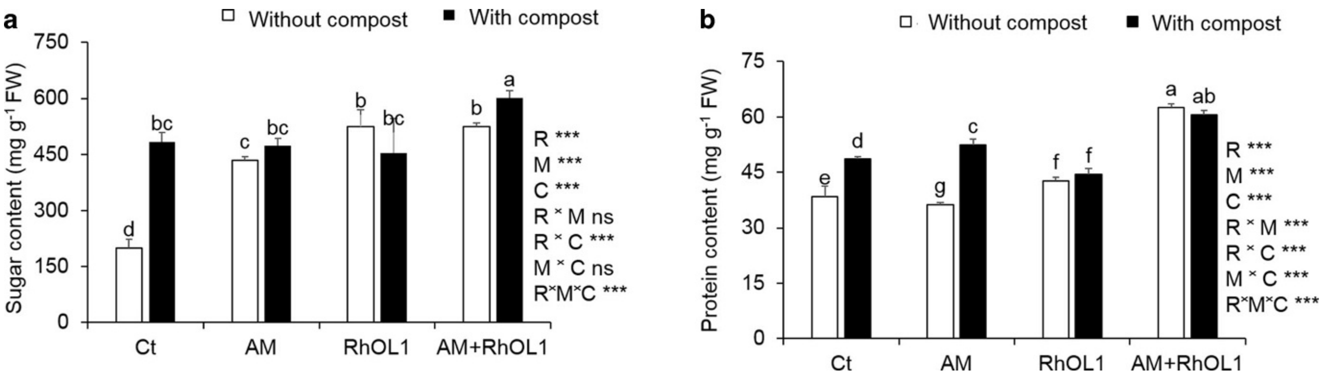

Fig. 4 Sugar (**a**) and protein (**a**) content of *Medicago sativa*, submitted to different treatments: control (Ct); arbuscular mycorrhizal fungi (AM); rhizobium strain (RhOL1) alone or in combination with AMF (AM + RhOL1), in the presence or absence of compost. Values with different letters are significantly different as determined by Tukey's HSD test, after performing three-way ANOVA ($P < 0.05$) (*ns* not significant, *significant at $p < 0.05$, **significant at $p < 0.01$, ***significant at $p < 0.001$)

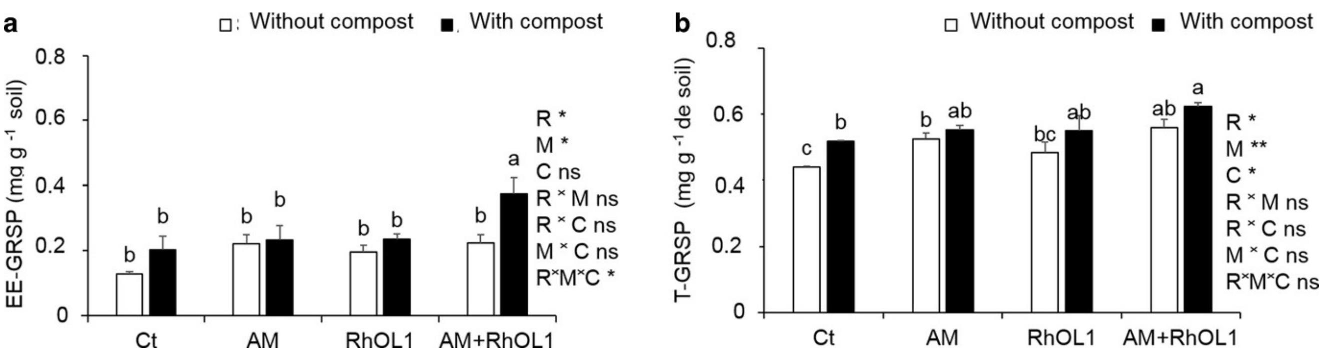

Fig. 5 Average values of easily extractable glomalin-related soil protein (EE-GRSP) (**a**) and total glomalin-related soil protein (T-GRSP) (**a**) of *Medicago sativa*, submitted to different treatments: control (Ct); arbuscular mycorrhizal fungi (AM); rhizobium strain (RhOL1) alone or in combination with AMF (AM + RhOL1), in the presence or absence of compost. Values with different letters are significantly different as determined by Tukey's HSD test, after performing three-way ANOVA ($P < 0.05$) (*ns* not significant, *significant at $p < 0.05$, **significant at $p < 0.01$, ***significant at $p < 0.001$)

The interaction between compost application and PGPR inoculation (Ansari and Mahmood 2017) or compost-AMF (Copetta et al. 2011; Anli et al. 2020) has also been reported. However, the effects of the combined use of composts with the dual inoculation of plants by microorganisms such as AMF and rhizobia were rarely investigated.

Rhizobium strain, applied in this study, acts as plant growth-promoting bacteria, it showed several traits, which make it a good candidate as bio-inoculant to enhance alfalfa yield. It has the ability to improve plant growth by producing exopolysaccharides and auxin, increasing the nutrient status in the rhizosphere, and by providing essential elements such as nitrogen, phosphorus, and potassium (Raklami et al. 2019).

The obtained results have revealed a positive impact of different biofertilizers applied on all measured parameters. However, compost (10 t/ha), AMF and RhOL1 strain combination led to even better improvements regarding growth attributes, nodulation and infection rates as well as nutrient uptake, photosynthetic traits and soil's physico-chemical properties.

Regarding the plant's mineral status, the results showed higher levels of P, N, K and Ca in plants that were dual inoculated and composted. This synergistic effect between AMF, RhOL1 and compost application at 10 t/ha, might be linked, on the one hand, to the fact that the compost provides alfalfa with sufficient nutrients for its cellular needs and an optimal environment for the growth of associated microorganisms (AMF + RhOL1), including nutrients, soil structure and pH (Jat and Ahlawat 2006). On the other hand, AMF and RhOL1 also contribute to nutrient bioavailability either directly or indirectly. Indeed, N good assimilation could be the result of an increased *N*-fixation by rhizobium which can fix 15–20 kg of N/ha increasing crop yield up to 20% (Sultana et al. 2014). Besides its ability to fix N_2 and to solubilize P and K, RhOL1 is able to secrete auxin, which activates root growth. The initiation of lateral and adventitious roots and the stimulation of their elongation and cell division could stimulate nutrient uptake, but also plant mycorrhization by increasing root fungal contact. AMF are known to be effective in improving mineral nutrition, par-

Table 3 Physico-chemical analysis of soil before and after the experiment

Soil properties										Significance level						
Before the experiment		After the experiment														
	–	Ct	AM	RhOL1	AM + RhOL1	C	C + AM	C + RhOL1	C + AM + RhOL1	R	M	C	R × M	R × C	M × C	R × M × C
Texture	Sandy-silty-clayey															
pH	8.2 ± 0.2^{a}	8.0 ± 0.0^{b}	8.0 ± 0.0^{b}	$8.0 \pm 0.0b^{c}$	7.9 ± 0.0^{c}	7.9 ± 0.0^{bc}	7.9 ± 0.0^{bc}	$7.9 \pm 0.0c$	$7.8 \pm 0.0d$	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Electrical Conductivity ($mS\,cm^{-1}$)	0.3 ± 0.0^{a}	$0.1 \pm 0.0b^{c}$	0.1 ± 0.0^{bc}	0.15 ± 0.01^{bc}	0.1 ± 0.0^{b}	0.17 ± 0.0^{b}	0.16 ± 0.0^{b}	0.1 ± 0.01^{bc}	0.2 ± 0.0^{b}	***	***	*	***	Ns	Ns	**
Nitrogen content (mg/kg)	90 ± 1.6^{b}	200 ± 18.6^{a}	252 ± 1.6^{a}	242 ± 18.6^{a}	261 ± 18.6^{a}	224 ± 32.3^{a}	261 ± 49.3^{a}	242 ± 18.6^{a}	280 ± 32.3^{a}	Ns	Ns	Ns	Ns	Ns	Ns	Ns
Carbon content (%)	0.4 ± 0.0^{e}	0.7 ± 0.0^{d}	0.9 ± 0.0^{c}	0.9 ± 0.0^{c}	1.1 ± 0.0^{b}	1.1 ± 0.0^{b}	1.18 ± 0.0^{a}	1.1 ± 0.0^{a}	1.2 ± 0.0^{a}	***	*	***	***	**	Ns	***
Organic matter (%)	0.7 ± 0.0^{e}	1.2 ± 0.0^{d}	1.5 ± 0.0^{c}	1.6 ± 0.0^{c}	1.9 ± 0.0^{b}	1.9 ± 0.0^{b}	2.03 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	***	***	***	***	***	***	***
Phosphorus content (ppm)	20.6 ± 0.0^{d}	23.9 ± 0.3^{c}	27.0 ± 0.9^{b}	27.0 ± 1.0^{b}	27.0 ± 0.9^{b}	26.0 ± 2.0^{b}	27.0 ± 0.9^{b}	26.1 ± 1.5^{b}	38.0 ± 1.7^{a}	***	***	***	***	***	***	***

Means (± SE) with different letters are significantly different according to Tukey's HSD test, after performing three-way ANOVA ($P < 0.05$)

ns not significant, *Significant at $p < 0.05$, **Significant at $p < 0.01$, ***Significant at $p < 0.001$

Ct uninoculated and un-amended alfalfa control plants, *AM* Alfalfa treated with arbuscular mycorrhizal fungi, *RhOL1* Alfalfa treated with rhizobium strain, *AM + RhOL1* Alfalfa treated with AM and RhOL1, *C* Alfalfa treated with compost, *C + AM* Alfalfa treated with C and AM, *C + RhOL1* Alfalfa treated with C and RhOL1, *C + AM + RhOL1* Alfalfa treated with C, AM and RhOL1

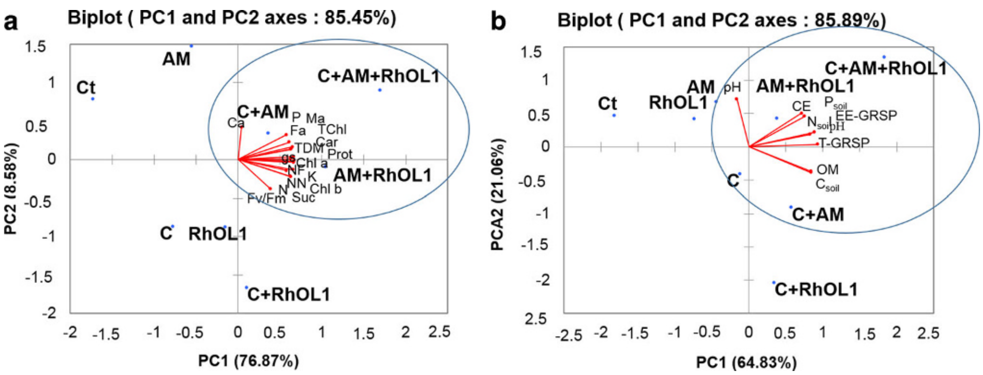

Fig. 6 Principal component analysis based on matrixes of growth, physiological and biochemical parameters (**a**) as well as soil's physico-chemical properties (**b**), under the different biofertilizers treatments (*NN* nodule number, *TDM* Total dry matter, *NL* number of leaves, *gs* stomatal conductance, *Fv/Fm* Chlorophyll fluorescence, *T Chl* total chlorophyll, *Chl a* Chlorophyll a, *Chl b* Chlorophyll b, *Car* Carotenoids content, *Fa* AMF frequency, *Ma* AMF intensity, *Suc* Sugar content, *Prot* Protein content, *N* Nitrogen content in plant, *P* Phosphorus content in plant, *K* Potassium content in plant, N_{soil} Nitrogen content in soil, P_{soil} Phosphorus content in soil, *EC* Electrical conductivity, *pH* Soil acidity, *OM* Organic matter, C_{soil} Carbon content in soil, *EE-GRSP* Easily extractable glomalin-related soil protein, *T-GRSP* Total glomalin-related soil protein, *Ct* uninoculated and un-amended alfalfa control plants, *AM* Alfalfa treated with arbuscular mycorrhizal fungi, *RhOL1* Alfalfa treated with rhizobium strain, *AM + RhOL1* Alfalfa treated with AM and RhOL1, *C* Alfalfa treated with compost, *C + AM* Alfalfa treated with C and AM, *C + RhOL1* Alfalfa treated with C and RhOL1, *C + AM + RhOL1* Alfalfa treated with C, AM and RhOL1)

ticularly P uptake (Evelin et al. 2019; Ait-El-Mokhtar et al. 2020a).

RhOL1 strain increased the AMF root colonization regardless of the presence or absence of compost. These beneficial effects suggest a possible activity of the bacteria RhOL1 as a Mycorrhization Helper Bacteria (MHB), which can stimulate mycorrhization of plants by different mechanisms such as increasing AMF-root contact (Bonfante and Anca 2009). On the other hand, AMF inoculation and compost application has a positive effect on the nodulation of alfalfa plants. The increase in the number of nodules could be explained by the beneficial effect of AMF and compost on plant nutrient status. Improving phosphorus nutrition of the host plant could provide the P needed by rhizobia bacteria for nodule formation and N fixation (Püschel et al. 2017).

Increasing mineral nutrient levels in plants would lead to increased photosynthesis. In this study, soil amendment combined with dual inoculation (AMF + RhOL1) improved alfalfa photosynthetic parameters, such as chlorophyll fluorescence (Fv/Fm), stomatal conductance, and chlorophyll and carotenoids levels. Their improvement indicates a good functioning of the photosynthetic system and an efficiency in CO_2 assimilation. Improved photosynthesis increases protein synthesis and sugar levels. This would positively influence the growth and yield of alfalfa plants. Nitrogen, phosphorus, potassium and calcium are essential nutrients in improving photosynthetic efficiency and plant growth. Thus, improved potassium nutrition plays an important role in stomata opening and closure. It is well known as well that the regulation of stomatal conductance is directly related to CO_2 assimilation for photosynthesis (Auge 2000). Under K deficiency a decrease in chlorophyll level has been reported for many plants (Amtmann et al. 2006). Furthermore, K enhancement could play a major role in the activation of essential enzymes in the metabolism of carbohydrates and the production of amino acids and proteins (Auge 2000). Phosphorus, in synergy with N and other elements, plays a major physiological role in the process of photosynthesis. It is a key element in the maintenance of cellular redox homeostasis since it is a part of several metabolic compounds (Evelin et al. 2019).

Soil analysis results show favorable changes in soil physico-chemical properties after compost application and/or inoculation with RhOL1 and AMF. Indeed, after 3 months of alfalfa cultivation, an improvement of N, C, OM and P soil content was recorded. However, the most significant P, C, OM values were obtained when the application of compost was combined with AMF and RhOL1. These results could be explained on the one hand by the relative richness of the compost provided in organic matter (52.8%) and total carbon (30.65%), and on the other hand by the capacity of the used strains to metabolize different compounds produced by plant roots (amino acids, carbohydrates and organic acids) (Zhou et al. 2016). Organic matter, by releasing the mineral elements which it contains through mineralization, ensures a good supply of major elements N, P, K, but also trace elements (Fe, Mn, Cu, Zn, Bo, Mb) (Scotti et al. 2015). Thus, in this study, the increase of P and N content in soil could be due to the mineralization of organic matter or to the direct supply of these elements by the compost. Furthermore, AMF and rhizobium could improve the chemical and nutritional quality of the soil through different mechanisms such as symbiotic nitrogen fixation, production of siderophores and

Table 4 Pearson correlation showing the relationship between alfalfa growth and different soil physico-chemical characteristics

Variables	pH	EC	N_{soil}	P_{soil}	C_{soil}	OM	EE-GRSP	T-GRSP	TDW	NL
pH	1	–	–	–	–	–	–	–	–	–
EC	0.28 ns	1	–	–	–	–	–	–	–	–
N_{soil}	0.01 ns	0.66***	1	–	–	–	–	–	–	–
P_{soil}	0.11 ns	0.81***	0.74***	1	–	–	–	–	–	–
C_{soil}	–0.40 ns	0.51**	0.65***	0.48*	1	–	–	–	–	–
OM	–0.38 ns	0.49*	0.69***	0.48*	0.99***	1	–	–	–	–
EE-GRSP	–0.09 ns	0.77***	0.85***	0.94***	0.69***	0.70***	1	–	–	–
T-GRSP	–0.17 ns	0.77***	0.89***	0.78***	0.84***	0.85***	0.93***	1	–	–
TDW	–0.05 ns	0.79***	0.92***	0.88***	0.71***	0.73***	0.95***	0.92***	1	–
NL	–0.09 ns	0.72***	0.92***	0.67***	0.85***	0.87***	0.82***	0.93***	0.91***	1

ns not significant, *Significant at $p < 0.05$, **Significant at $p < 0.01$, ***Significant at $p < 0.001$

pH Soil acidity, *EC* Electrical conductivity, N_{soil} Nitrogen content in soil, P_{soil} Phosphorus content in soil, C_{soil} Carbon content in soil, *OM* Organic matter, *EE-GRSP* Easily extractable glomalin-related soil protein, *T-GRSP* Total glomalin-related soil protein, *TDM* Total dry matter, *NL* number of leaves

exopolysaccharides, and solubilization of phosphate and potassium (Naseem et al. 2018; Primo et al. 2019).

Besides, the direct contribution of these biofertilizers to supply nutrients, they could positively influence the construction of soil microbiota, which could further motivate the degradation of organic and inorganic matter and thus be an additional nutrient source for microbes and their host plants (Xue et al. 2013). The compost applied in this study presents interesting microbial biomass of the order of 10^8 of bacterial and 10^5 of fungal biomass. Therefore, it is interesting to assess later the capacity of this waste green compost to improve fertility and increase the microbial biomass of the soil.

The activity of soil microorganisms is higher under near-neutral conditions. Indeed, soil amendment decreases or increases the soil pH depending on their initial pH and also on the type of organic amendment used (Butterly et al. 2013). Soil organic matter provides many negatively charged sites for fixing H^+ in acidic soil, or from which H^+ can be released in basic soil, in order to push the soil solution toward a neutral pH (Mccauley et al. 2017). Alfalfa grows best in soils with a pH above 6.2 conditions in which associated *N*-fixing bacteria and AMF grow well (Mccauley et al. 2017). This result is confirmed in the present study where a decrease in pH from 8.23 (before the experiment) to 7.89 (after application of the organic soil amendment) was recorded.

Changes in the soil chemical properties by microbial inoculation and compost application are often associated with changes in physical properties. For example, increases in organic matter, phosphorus, and nitrogen content are also accompanied by the formation of larger and more stable aggregates (Agegnehu et al. 2015; Naseem et al. 2018; Ji et al. 2019). The stability of aggregates by compost application improves related soil structure, including porosity, aeration and water retention capacity. Besides, aggregate stability promotes water infiltration. This has positive consequences on the soil's resistance to erosion (Agegnehu et al. 2017; Gaiotti et al. 2017). Besides compost, AMF and RhOL1 could also contribute to the stability of aggregates and the improvement of soil structure and fertility. This positive effect is associated with the production of extracellular secretions, including glomalin and exopolysaccharides. The latter, released by bacteria such as rhizobia, act as a true biological glue that plays a decisive role in the stability of aggregates and the maintenance of soil structure (Naseem et al. 2018; Primo et al. 2019). Furthermore, AMF, through their hyphae, promote the formation of aggregates in the soil, through the secretion of glomalin. Several studies have shown a positive correlation between the glomalin produced by AMF hyphae and the stability of soil aggregates (Singh 2012; Ji et al. 2019). This glycoprotein compound acts as a glue that binds soil particles together to form aggregates.

Regarding our results, the application of compost combined with inoculation (AMF + RhOL1) could therefore contribute physically and chemically to the maintaining of soil structure and fertility. This is very useful for nutrient availability and plant development (Gaiotti et al. 2017). A positive correlation coefficient ($P < 0.001$) was established in this study between plant growth, yield and soil properties ($r \geq 0.841$). Therefore, the growth improvement of alfalfa, during application of the compost combined with AMF and RhOL1, as microbial amendments could be the result of improvements in the physico-chemical properties and thus may led to nutrient bioavailability.

Conclusion

In conclusion, the present work shows that AMF and RhOL1 strain inoculation combined with compost amendment at 10 t/ha was the most effective practice to improve alfalfa growth and yield under field conditions. Based on our findings, the synergy between these biofertilizers resulted in the enhancement of soil quality, mineral nutrition, photosynthetic apparatus and therefore the photosynthates accumulation. The positive impacts noticed could explain the complementarity and synergistic effect between these native microorganisms, among themselves and even with the native microflora already existing in the soil. Moreover, this synergy was further significant in the presence of compost at 10 t/ha. Thus, the inoculation of AMF and RhOL1 combined with the compost amendment could be a powerful management strategy for alfalfa production under a bio-sustainable agro-ecosystem. However, this cropping practice requires further investigations to be recommended for the promotion of alfalfa crops in different geographical regions.

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Conflict of interest R. Ben-Laouane, M. Ait-El-Mokhtar, M. Anli, A. Boutasknit, Y. Ait Rahou, A. Raklami, K. Oufdou, S. Wahbi and A. Meddich declare that they have no competing interests.

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