Implications of ectomycorrhizal inoculation for drought stress tolerance of Atlas cedar (Cedrus atlantica (Endl.) Carrière) seedlings

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Abstract

Background: Ectomycorrhizal inoculation is a promising strategy to minimise the initial transplant shock and increase plant survival and growth during the first years of out-planting in the field. The aim of this research was to investigate the effect of sporal inoculum of three ectomycorrhizal fungi: Cortinarius cedretorum, Amanita vaginata and Inocybe geophylla on tolerance levels of Atlas cedar (Cedrus atlantica (Endl.) Carrière) seedlings subjected to applied drought stress in nursery conditions.

Methods: Carpophores, seeds and organic forest soil were collected under pure stands of Atlas cedar. After fifteen months of growth, seedlings were subjected to drought stress by withholding water for thirty days; we assessed morphological and physiological variables of all seedling batches (inoculated and uninoculated, controlled and stressed seedlings).

Results: All roots of inoculated stressed seedlings were mycorrhizal. The mycorrhization rates were 67%, 64.6% and 53.6% for stressed seedlings inoculated with Cortinarius cedretorum, Amanita vaginata, Inocybe geophylla, respectively. This root mycorrhization was accompanied by a significant improvement in seedling growth, especially height and length of the main root (10.2 cm, 52 cm) reached in stressed seedlings inoculated with Cortinarius cedretorum. There was a significant increase in relative water content, total chlorophyll, carotenoids, soluble sugars and starch, superoxide dismutase and ascorbate peroxidase enzyme activities in inoculated stressed seedlings compared with uninoculated seedlings.

Conclusions: Inoculation of Atlas cedar seedlings with spores of ectomycorrhizal fungi remains a very effective alternative for improving growth and the morphological and physiological status of seedlings under drought conditions. Cortinarius cedretorum appears to be consistently advantageous followed by Amanita vaginata and Inocybe geophylla.

Keywords: Cedrus atlantica, drought stress, sporal inocula, forest soil.
low soil fertility, etc.) during the establishment phase (Lamhamedi et al. 2000). The use of biological methods as a practical way to alleviate soil stresses, including drought, on plant growth has received increased attention (Miransari et al. 2009). Ectomycorrhizal inoculation is a promising strategy to minimise the initial transplant shock and increase plant survival and growth during the first years of out-planting in the field. (Bouckim et al. 2002). Ectomycorrhizal fungi can improve growth and physiological status of seedlings by enhancing their photosynthetic capacity and by increasing the uptake of water and mineral nutrients (Read et al. 2004), promote soil aggregation, and improve plant health by increasing protection against biotic (pathogen attacks) and abiotic (drought, salinity and heavy metals) stresses (Rillig & Mumme 2006). Indeed, ectomycorrhizal fungi enhance water uptake under dry conditions by exploring a larger volume of soil than non-mycorrhizal roots, and hyphae can enter small soil pores that are not accessible to the short roots of a plant (Lehto & Zwiazek 2011) thus improving plant water relations under low water conditions (Dunabetia et al. 2004).

In natural conditions, Atlas cedar trees develop symbiotic associations with many different ectomycorrhizal species (Hocine et al. 1994). However, it is difficult to form mycorrhizae even after artificial inoculations. Therefore, the application of the inoculum to Atlas cedar has been limited by the difficulty in obtaining a widespread and reproducible colonisation of root systems after their inoculation by mycelia of different fungal species (Nezzar-Hocine 1998).

Abourouh (2000) and Lamhamedi et al. (2009) reported that no comprehensive studies have yet been conducted to assess the mycorrhization of Atlas cedar seedlings with spores collected from the same site and their possible behaviour in nursery. There have been few studies associated with the benefit of ectomycorrhizal symbiosis to Cedrus atlantica under water stress and it is not yet known how the mycorrhizae play an important role in the growth and water relations of forest trees.

Inoculation of Atlas cedar plants using ectomycorrhizal spores collected from the same seed collection site and soil is a more efficient technique for reaching a satisfactory mycorrhization rate in the nursery. In this way, the spore inoculation may ultimately provide an inexpensive and viable strategy for nursery growers that they can integrate easily into nursery operations for forest trees. In addition, unlike solid and liquid inoculum where only one strain (i.e. a genotype) is used, the spore inoculum allows the introduction of a variety of fungal genotypes and increase of the probability of obtaining compatible genotypes (fungus-cedar) that can promote good colonisation of the roots.

The inoculation of Atlas cedar seedlings with fungal spore inoculum of Cortinarius cedretorum (R. Maire), Amanita vaginata (Fries) Vittidini and Inocybe geophylla (Pers.) had the best mycorrhization rate and improved Atlas cedar seedling growth (Gaba-Chahboub et al. 2016). Although different studies have shown that ectomycorrhizal fungi confer drought tolerance for several forest species (Lamhamedi et al. 1992; Lehto & Zwiazek 2011), studies concerning the effects of inoculation by spore-based Atlas cedar seedlings on growth parameters, as well as physiological and biochemical parameters in response to drought stress are limited, and little is known about the effect of ectomycorrhizal inoculation on reactive oxygen species metabolism. Therefore, the aim of this research was to determine whether mycorrhizal symbiosis minimises the damage caused by oxidative stress via the regulation of antioxidant enzymes.

We assume that inoculation of Atlas cedar seedlings with ectomycorrhizal spores enhances their growth when subjected to drought stress. The purpose of this study is to test the controlled mycorrhization of Atlas cedar seedlings by ectomycorrhizal spore inoculation under water limited conditions and to demonstrate their effectiveness on morphological, physiological and biochemical variables in Cedrus atlantica seedlings as compared to uninoculated stressed seedlings.

Materials and methods

Study site

The experiment was carried out in January 2013. Both soil and Atlas cedar seeds were collected from the same stand located in the Chrea National Park (36 ° 25’ N, 2° 52’ E, elevation: 1600 m, rainfall: 1400 mm/year) located in the north-western Tell Atlas Mountains of Algeria where prevailing winds are from the northwest and tend to be wet. The rainy period is about ten months (September to June) and the dry period typically occurs over two months, July and August. The mean maximum temperatures of hottest month oscillate between 26.3 °C and 33.6 °C, and the mean minimum temperatures of the coldest month can vary between 0.4 °C and 7.3 °C (Halimi 1980).

Organisms and soil used in the study

The forest soil was collected from the A1 horizon (located at depths varying between 25 to 30 cm in the main zone prospected by the roots) of lower Cretaceous schists that were classified as more or less clayey (Zaidi 2002). Granulometric analysis of the collected soil showed the following composition: 13.9% clay, 20.3% fine silt, 25.2% coarse silt, 2.3% fine sand and 38.2% coarse sand (Aubert 1978). The soil is slightly acid, rich in organic matter and little limestone (1.2%) with a total carbon of 5.2%, total nitrogen of 0.08% (John 1970), assimilable phosphorus 0.9 mg/kg soil (Olsen et al. 1954), organic matter of 9.1% (Anne 1945); a pH of 5.9 and an electrical conductivity of 2.9 ds/m.

The Atlas cedar seeds were collected from ten trees located within a distance of 20 m from each other. The seeds were surface sterilised with 30% hydrogen peroxide (H₂O₂) for 5 min, rinsed three times with distilled water and then stratified at 4 °C for two weeks in the dark, under semi-xenic conditions. Seeds were transferred to Petri dishes lined with wet filter paper before they were germinated in a growth chamber at 25 °C day / night and humidity of 65%.
Three ectomycorrhizal fungi were collected from the same seed collection site. These are *Cortinarius cedrorum* (CC), *Amanita vaginata* (AV) and *Inocybe geophylla* (IG). The fresh carpophores were gently cleaned by a small brush to remove root fragments and soil particles still adhering to them. They were dried firstly in the open air and then in an oven at 30 °C for 24 hours to remove all traces of moisture. Subsequently, they were ground using a grinder for a very short time (2 min). The spore inoculums were screened using a sieve with a mesh size of 200 μm. These spore suspension inoculums were kept in the refrigerator (4 °C) until use. The forest soil was sterilised by autoclaving at 120 °C for 20 min twice over 48 hours period.

**Experimental design**

The experiment was conducted in a greenhouse at the National Forest Research Institute (Bainem arboretum) located at 15 km west of Algiers, 36° 45’ N, 3° 25’E). The randomised experimental design included four treatments side by side: three treatments that consisted of Atlas cedar seedlings each inoculated with CC, AV and IG, and a control treatment that consisted of uninoculated control seedlings. Twenty seedlings were randomly allocated to each treatment. After one month of growth in Riedacker containers (height: 17 cm, diameter: 10 cm) with a "W" or "M" shaped section (Thermoflan, Molières-Cavaillac, Le Vigan, France), each seedling was inoculated with 0.1 g of ground spore inoculums mixed with 250 mL of sterile distilled water. Using a pipette, the spore inoculum was placed at a depth of 4 cm in direct contact with the roots of each seedling (Abourouh 1994) for the three treated groups and seedlings of the control group were left uninoculated. All the seedlings were watered three times per week with sterile distilled water in order to maintain soil moisture levels near saturation to promote plant and fungus growth. The experiment was conducted in the greenhouse with a 16-hour day length. Day and night-time temperatures were 25 and 18 °C, respectively and relative humidity was 65%.

After fifteen months of growth, while all seedlings were still actively growing and kept in the same position, a drought stress treatment was applied by withholding water for thirty days. Each batch of inoculated and uninoculated seedlings was further subdivided into two batches: stressed and control seedlings. Overall, there were eight batches of seedlings: uninoculated control seedlings (UCS); uninoculated stressed seedlings (USS); inoculated control seedlings (ICS, CC, AV; and IG); and inoculated stressed seedlings (ISS, CC, AV; and IG).

**Mycorrhizal colonisation and growth measurements**

The extent of mycorrhizal colonisation was assessed based on the number of mycorrhizal apices, number of fine roots, and the rate of mycorrhization of the roots. These assessments were made on root systems that had been thoroughly and carefully washed to ensure that they were free of soil. Growth data (stem height, length of main root and whole plant dry mass) were collected on ten samples from each batch that had been oven dried at 80 °C for 48 hours.

**Measurement of physiological and biochemical variables**

Relative water content (RWC) was determined according to (Clark & McCaig 1982). Needles were sampled randomly from the last rosette of each seedling (five replicate from each batch). Fresh weight (FW) was determined from the needles sampled in the upper part of seedlings; Turgid weight (TW) was obtained by floating the needles in tubes with deionised water for at least 48 h in the dark before being weighed again. Dry weight (DW) was determined after drying needles at 80°C for 48 h. RWC was calculated from the following equation: RWC = [(FW – DW) / (TW – DW)] × 100.

Biochemical analysis was done on samples of fresh needles that were collected from the upper part of five seedlings from each batch (five replicates from five seedlings for each biochemical variable). Chlorophyll and carotenoid contents were determined in 80% acetone extract. The absorbance readings were obtained at 663 nm (Chlorophyll a), 647 (Chlorophyll b) and 470 nm (carotenoids) by an Optizen Pop spectrophotometer and contents were calculated according to Lichtenthaler (1987). The soluble sugars and starch contents were determined using anthrone reagent (McCready & McCready 1950). Proline content was estimated by the method described in Rasio et al. (1987); absorbance was read at 528 nm. Proline content was expressed as µmole proline/g of dry matter (DM). Lipid peroxidation was determined by measuring the amount of Malondialdehyde (MDA) produced by the thiobarbituric acid reaction (Ali et al. 1995).

**Antioxidant enzyme activity assay**

Catalase (CAT, EC 1.11.1.6) activity was assayed by measuring the rate of disappearance of H₂O₂ according to the method described in Aebi et al. (1983). 150 mg of fresh vegetal material was ground with 1 mL of 0.1M Tris-HCl buffer (pH = 8.1) and the mixture was centrifuged at 12,000 rpm. For the assay of the enzymatic activity, 100 μL of the supernatant was added to 2 mL of potassium phosphate buffer (0.1 M, pH = 7) and the optical density was read by adding 20 μL H₂O₂ at 6% every 30 seconds over the course of 3 to 4 minutes. The enzyme activity was expressed in U mg⁻¹ protein (U=1 μM of H₂O₂ reduction min⁻¹ mg⁻¹ protein).

Total ascorbate peroxidase (APX, EC 1.11.1.1) activity was assayed according to Nakano & Asada (1981). The reaction was started with the addition of H₂O₂, and ascorbate oxidation measured at 290 nm for one minute; a decline in optical density is proportional to enzyme activity. Total superoxide dismutase (SOD, EC1.15.1.1) assay was performed according to Marklund (1974) with some modifications. The evaluation of the autooxidation of pyrogallol was carried out by differential measurement between a control and a test at 420 nm every thirty seconds for four minutes.

**Statistical analysis**

The data from these measurements and assays were subjected to a descriptive statistical analysis and analysis of variance (ANOVA). The two-way ANOVA included two
fixed factors (inoculum and water stress), and means were compared according to the method of Newman and Keuls (Dagnelie 1999). All analyses were undertaken using the Statistica software (TIBCO Software Inc, Palo Alto, USA).

Results

Effect of mycorrhization on Atlas cedar seedlings growing under drought stress conditions

For uninoculated seedlings, significant reductions in seedling height (57%), length of the main root (20.8%), dry weight of whole seedling (42%) and number of fine roots (53.8%) were observed in stressed seedlings (US) compared with control seedlings (UC). Relative to uninoculated stressed seedlings, stressed seedlings that had been inoculated all had significantly higher values of the size and morphological characteristics that were measured. For example, values of seedling height in the CC, AV, and IG were 191%, 171% and 125% greater, respectively compared with US. The mean length of the main root in inoculated stressed seedlings was also significantly higher compared to uninoculated stressed seedlings. In the CC, AV, and IG treatments, mean root lengths were 108%, 68% and 52.8% greater, respectively compared with US. We found highly significant increases in the average number of fine roots between US and ISS. These enhancement rates were: 130%, 100% and 52.3%, respectively in CC, AV, and IG compared to US. There was no reduction in mycorrhization rate or the number of mycorrhizal apices number in ISS compared to ISS (Table 1).

Effect of drought stress on relative water content (RWC)

In UCS, the relative water content was 83% while in US it was reduced significantly to 60% reflecting the installation of physiological drought. In ICS, RWC was higher than in UCS; they were 88%, 87.7% and 86.8%, respectively in CC, AV, and IG. Moreover, ISS maintained their water status and their RWC were near to that of ISS compared with control seedlings (UCS). Relative to UCS, the relative water content was 88%, 87.7% and 86.8%, respectively in CC, AV, and IG compared to UCS. Relative to UCS, the RWC was higher than in UCS; they were 88%, 87.7% and 86.8%, respectively, respectively compared with UCS. In ISS, RWC was 278 ± 0.002 for CC, AV, and IG, respectively in CC, AV, and IG, respectively compared with US. The mean length of the main root in inoculated stressed seedlings was also significantly higher compared to uninoculated stressed seedlings. In the CC, AV, and IG treatments, mean root lengths were 108%, 68% and 52.8% greater, respectively compared with US. We found highly significant increases in the average number of fine roots between US and ISS. These enhancement rates were: 130%, 100% and 52.3%, respectively in CC, AV, and IG compared to US. There was no reduction in mycorrhization rate or the number of mycorrhizal apices number in ISS compared to ICS (Table 1).

Total chlorophyll content (chl a+b)

In the needles of UCS, the total chlorophyll content was 225 ± 0.007 µg/g DW. This content increased significantly and reached 278 ± 0.002 µg/g DW, 263 ± 0.004 µg/g DW and 253 ± 0.004 µg/g DW, respectively, in CC, AV, and IG. Under drought stress, the total chlorophyll content was reduced significantly in US (161 ± 0.006 µg/g DW) was 28.4% lower compared to UCS. In CC, AV, the total chlorophyll content was 255 ± 0.003 µg/g DW and 243 ± 0.006 µg/g DW, respectively, and were significantly higher than values recorded in US (Fig. 1).

Carotenoid content

Needle carotenoid content in CC, AV, and IG were 50.4 ± 0.09 µg/g DW, 49.6 ± 0.09 µg/g DW and 49 ± 0.10 µg/g DW, respectively, and were significantly higher than in UCS 7.2%, 4.8%, 3.6% increase, respectively in CC, AV, and IG. In US, carotenoid content reduced significantly to 40.6 ± 0.05 µg/DW. Otherwise, carotenoid contents were significantly higher in ISS; we recorded mean values of 48.6 ± 0.03 µg/g DW, 48 ± 0.08 µg/g DW and 47.6 ± 0.05 µg/g DW respectively in CC, AV, and IG (Fig. 1).

Malondialdehyde content

The average MDA content in needles of UCS was 4.1 ± 0.027 nmole/mg DW. This content decreased in ICS, where it was 1.9 ± 0.003 nmole/mg DW, 2 ± 0.03 nmole/mg DW and 2 ± 0.04 nmole/mg DW, respectively, in CC, AV, and IG. Under drought stress, we found that the average MDA content increased to 5 ± 0.088 nmole/mg DW (20.6% increase) in US compared to US indicating that drought stress aroused lipid peroxidation. In ISS it was significantly lower than in US with mean values of 3.5 ± 0.025 nmole/mg DW, 3.6 ± 0.075 nmole/mg DW, 3.6 ± 0.02 nmole/mg DW, respectively, in CC, AV, and IG, reductions of 28.8%, 27% and 26.4%, respectively (Fig. 1).

Soluble sugars

The soluble sugars content in the needles of UCS was 1.9 ± 0.007 mg/g DW. This content increased significantly in ICS where mean values of 2.2 ± 0.012 mg/g DW, 2.1 ± 0.006 mg/g DW and 2.1 ± 0.006 mg/g DW, respectively, were obtained for CC, AV, and IG. These corresponded to increases of 15.9%, 13% and 11%, respectively. For the ISS, the soluble sugars content was significantly lower than in ISS. Mean values of 2.1 ± 0.14 mg/g DW, 2 ± 0.057 mg/g DW and 1.9 ± 0.006 mg/g DW, respectively, were obtained for CC, AV, and IG. Compared with US, these contents corresponded to decreases of 41.8%, 39.5% and 38%, respectively.

Starch content

There was no significant difference in starch content between ICS and ISS. The following mean values were recorded for CC, AV, and IG: 1.8 ± 0.006 mg/g DW, 1.7 ± 0.005 mg/g DW, and 1.7 ± 0.006 mg/g DW, respectively. Small differences in starch content seemed were observed between UCS and US (1.5 ± 0.01 mg/g DW and 1.3 ± 0.008 mg/g DW, respectively) (Fig. 1).

Free proline content

The drought stress induced a significant increase in proline content in US, where a mean value of 8.9 ± 0.039 µg/g DW was recorded. This content was lower in ISS where values of 8 ± 0.117 µg/g DW, 8.1 ± 0.085 µg/g DW and 8.4 ± 0.045 µg/g DW, respectively, were recorded in CC, AV, and IG, corresponding to decreases of 11%, 9.3%, 5.1%, respectively, but still higher than values observed in ICS (Fig. 1).

CAT activity

There were no significant differences between the CAT activity of needles in inoculated and uninoculated seedlings control and stressed seedlings. In ISS, the CAT activity was 95 ± 0.02 µmole H₂O₂ min⁻¹ mg⁻¹ protein, 95 ± 0.01 µmole H₂O₂ min⁻¹ mg⁻¹ protein and 96 ± 0.01 µmole H₂O₂ min⁻¹ mg⁻¹ protein, respectively, in CC, AV, and IG (Fig. 1).
TABLE 1: Impact of mycorrhization on growth traits of 15-month-old *Cedrus atlantica* seedlings subjected to drought stress. Each variable was the average of 10 independent measurements. For each variable, the different letters showed significant differences between uninoculated stressed seedlings (USS) and inoculated stressed seedlings (ISS) based on the Student-Newman-Keuls test (P < 0.05).

| Growth and mycorrhization variables | Mycorrhizal inoculation and stress situation of seedlings |
|-------------------------------------|----------------------------------------------------------|
|                                     | Uninoculated control seedlings (UCS)                     |
|                                     | Uninoculated stressed seedlings (USS)                    |
|                                     | Inoculated control seedlings (CCc)                       |
|                                     | Inoculated stressed seedlings (CCs)                      |
|                                     | Inoculated control seedlings (AVc)                       |
|                                     | Inoculated stressed seedlings (AVs)                      |
|                                     | Inoculated control seedlings (IGc)                       |
|                                     | Inoculated stressed seedlings (IGs)                      |
| Seedling height (cm)                | 5.5 ± 0.49                                               |
|                                     | 3.5 ± 0.56<sup>a</sup>                                   |
|                                     | 10.5 ± 0.54                                              |
|                                     | 10.2 ± 0.32<sup>b</sup>                                  |
|                                     | 10.0 ± 0.35                                              |
|                                     | 9.5 ± 0.50<sup>b</sup>                                   |
|                                     | 8.7 ± 0.47                                               |
|                                     | 7.9 ± 0.61<sup>c</sup>                                   |
| Length of the main root (cm)        | 30.2 ± 0.7                                               |
|                                     | 25.0 ± 0.57<sup>a</sup>                                  |
|                                     | 56.0 ± 1.28                                              |
|                                     | 52.0 ± 2.34<sup>b</sup>                                  |
|                                     | 47.0 ± 0.82                                              |
|                                     | 42.0 ± 0.79<sup>b</sup>                                  |
|                                     | 43.0 ± 2.65                                              |
|                                     | 38.2 ± 3.96<sup>c</sup>                                  |
| Whole-seedling dry weight (g)       | 0.3 ± 0.01                                               |
|                                     | 0.21 ± 0.02<sup>a</sup>                                  |
|                                     | 3.5 ± 0.83                                               |
|                                     | 2.5 ± 0.57<sup>b</sup>                                   |
|                                     | 2.1 ± 0.30                                               |
|                                     | 2.3 ± 0.11<sup>b</sup>                                   |
|                                     | 2.0 ± 0.37                                               |
|                                     | 1.7 ± 0.25<sup>c</sup>                                   |
| Mycorrhization rate (%)             | -                                                        |
|                                     | -                                                        |
|                                     | 70.0 ± 1.64                                              |
|                                     | 67.0 ± 1.95                                              |
|                                     | 68.0 ± 1.57                                              |
|                                     | 64.6 ± 1.96<sup>b</sup>                                  |
|                                     | 57.0 ± 1.61                                              |
|                                     | 53.6 ± 1.51<sup>c</sup>                                  |
| Number of mycorrhizal species       | -                                                        |
|                                     | -                                                        |
|                                     | 130.0 ± 5.72                                             |
|                                     | 124.0 ± 3.53                                             |
|                                     | 124.0 ± 8.36                                             |
|                                     | 116.4 ± 9.20                                             |
|                                     | 90.0 ± 8.25                                              |
|                                     | 82.2 ± 8.79                                              |
| Number of fine roots                | 10.0 ± 1.15                                              |
|                                     | 6.5 ± 1.20<sup>a</sup>                                   |
|                                     | 22.0 ± 2.43                                              |
|                                     | 15.0 ± 3.91<sup>b</sup>                                  |
|                                     | 21.0 ± 3.20                                              |
|                                     | 13.0 ± 2.86<sup>b</sup>                                  |
|                                     | 17.0 ± 0.38                                              |
|                                     | 10.0 ± 0.54<sup>c</sup>                                  |
FIGURE 1: Impact of mycorrhization on physiological and biochemical variables of Cedrus atlantica seedlings under drought stress (NI: uninoculated seedlings, CC: seedlings inoculated with Cortinarius cedretorum, AV: seedlings inoculated with Amanita vaginata, IG: seedlings inoculated with Inocybe geophylla). (five replicates for each parameter) Letters indicate the significant differences between batches of seedlings, homogeneous groups have approximatively the same mean values and the same letter following the Newman-Keuls test (P < 0.05).
Total APX activity
Results showed that the highest needle APX activity was observed in IG₂ and IG₃. However, no significant differences were observed between these two treatment groups. The increase of APX activity was approximately 13.5% in IG₂ compared to USS (Fig. 1).

Total SOD activity
Among the uninoculated and inoculated seedlings, SOD activity of needles in ISS was significantly higher than in ICS. Mean values of SOD activity of 35.4 U mg⁻¹ protein min⁻¹, 34.5 U mg⁻¹ protein min⁻¹ and 32.3 U mg⁻¹ protein min⁻¹, respectively, were observed in CC₃, AV₂ and IG₃. No significant differences in SOD activity were observed between UCS and ICS (Fig. 1).

Discussion
Our results showed that inoculated stressed seedlings had better growth than the uninoculated stressed seedling. This enhancement occurred for all growth variables recorded in this study: seedling height, length of the main root, total dry matter and number of fine roots. An increase of the ratio of the length of the main root to the height of seedlings was also observed.

The adaptation of Atlas cedar to drought is due to the extensive root growth, its ability to explore the soil thoroughly and the alternating waves of aerial growth with waves of growth root (Ducrey 1994). Thus, it has been shown in some forest species as Cedrus atlantica (Aussenac 1985), Pinus ponderosa (McMillin 1995) and Fagus sylvatica (Van Hees 1997) that water constraint induces preferentially high biomass allocation to the roots when the stress intensity is important. The enhancement of the root part at the expense of the aerial part is considered as a criterion for drought resistance (Pallardy 1993; Liu et al. 2014). In another study, plant tolerance to drought could be primarily due to a large volume of soil explored by roots and the extra-radical hypheae of the fungi (Songsi et al. 2008, Kambiranda et al. 2011, Zhang et al. 2016). Cedar trees can be considered as an anisohydric species because they are adapted to short episodic drought (McDowell et al. 2008). Atlas cedar is characterised by a very high dynamic potential of water absorption by roots, which colonise mainly the upper layers of soil. In addition, cedar plants respond quickly to rainfall via the efficiency of its ectomycorrhizal root system at 20 to 50 cm of depth. Under water stress, Atlas cedar maintains its physiological activity up to very pronounced drought levels (Bouahmed et al. 2019).

Relative water content is a key indicator of cell and tissue hydration, and it is important for optimal physiological functioning and the growth processes (Altinkut et al. 2001). In our study, the RWC of ISS is higher than that of USS. This confirms the effectiveness of the three-spore inoculum on the upkeep of a high RWC in the needles of inoculated seedlings. Our results also showed that the total chlorophyll content (a + b) and carotenoids content decreased significantly in USS and that total chlorophyll and carotenoids content in the needles of ISS are significantly higher than those of USS. This suggests that there is a beneficial effect of mycorrhizal symbiosis on the induction chlorophyll and carotenoids accumulation (Baslam et al. 2011). Similar results were obtained in six-month-old Pseudotsuga menziesii plants inoculated with the ectomycorrhizal fungus Rhizopogon vinicolor and subjected to water stress conditions, which had higher chlorophyll levels than the stressed uninoculated plants (Dixon & Hiol-Hiol, 1992).

The ISS also reacted by significantly increasing soluble sugars and starch content compared to USS. Indeed, the relative increase in soluble sugars content could contribute to an osmotic adjustment that would allow the plant to balance its water content and to maintain a high cellular integrity in plant tissues during water deficit periods (Rai 2002; Chaves et al. 2003). Proline accumulation under stress conditions is due to the induction of the gene encoding the enzyme P5CS (pyrroline-5-carboxylatesynthetase) and inhibition of the proline dehydrogenase (ProDH) gene (Szabados & Savouré 2010). The accumulation of proline is concomitant with soluble sugars in some plant species (Clifford et al. 1998) and the ability of some genotypes to accumulate the osmotica (proline and sugars) is used as a selection criterion of drought tolerant genotypes (Nouri 2002). Mycorrhizal symbiosis protects plants against a variety of abiotic stresses using various processes such as improved photosynthetic rate, uptake and accumulation of mineral nutrients, accumulation of osmo-protectants and changes in the rhizosphere ecosystem (Yin et al. 2016). It is known that drought stress induces oxidative stress with the generation of reactive oxygen species (ROS). They attack the membrane polyunsaturated fatty acids (PUFA) resulting in their breakdown into small hydrocarbon fragments like ketones or Malondialdehyde (MDA) or other related products (Zeriri et al. 2012). In our study, MDA content of leaves in USS was significantly higher than that of ISS. In previous studies, it was reported that mycorrhizal plants had lower MDA content than non-mycorrhizal plants, suggesting that mycorrhization decreased lipids oxidative damages during stress conditions (Wu et al. 2006). Our experiments on antioxidant enzymatic activities CAT, APX and SOD indicated that applied drought stress to Atlas cedar seedlings did not significantly modify the CAT enzyme activity and that the highest needle APX activity was observed in IG₂ and IG₃, while the highest needle SOD activities were observed in inoculated stressed seedlings relative to USS. It is known that when plants are subjected to stress, the first reactive oxygen species (ROS) scavenging enzyme active in the enzymatic mechanism is SOD which plays a key role in cellular defences against ROS (Scandalios 1993). The increase of SOD activity in leaves is closely related to a higher ability to scavenge active oxygen radicals under water stress (Abedi & Pakniyat 2010). Moreover, there is a correlation between the SOD and CAT activities because the SOD is responsible for the formation of hydrogen peroxide H₂O₂ and the increase of this last is correlated to CAT activity (Khalifa et al. 2011).
The importance of root antioxidant enzymes in the regulation of cellular metabolism under water stress conditions has been demonstrated by Shvaleva et al. (2006). The balance between APX and CAT activities is critical for suppressing toxic levels of ROS in cells (Apel & Hirt 2004). In most of the previous studies, mycorrhizal application enhanced the antioxidant system (Lambais et al. 2003). Increased SOD activity in mycorrhizal plants is not related to their nutritional status but is the direct effect of mycorrhizal association in response to drought treatment of the host plant (Ruiz-Lozano & Azcon 1995). Ectomycorrhizal plants show high resistance to drought stress by preventing oxidative stress and effectively removing ROS (Bartels 2001). Coordinated activation of SOD and CAT can prevent cellular damage to the host plant during ectomycorrhizal formation between 
*Castanea sativa* Mill and *Pisolithus tinctorius* (Baptista et al. 2007). The increase in antioxidant enzymatic activities induced by mycorrhizae is associated with an increase in photosynthetic activity, biomass and plant nutrition. However, the stimulation of antioxidant molecules and antioxidant enzymatic activities depends on the host plant and mycorrhizal fungi (Roldán et al. 2008). It seems that mycorrhizal inoculation notably influences the activity of antioxidant enzymes in *citrus* leaves under water stress and an increase in the activity of antioxidant enzymes alleviates water stress (Roldán et al. 2008).

**Conclusions**

This study showed that inoculating Atlas cedar seedlings with ectomycorrhizal spores were effective in improving host morphological and physiological status of Atlas cedar seedlings under drought conditions. Among the three mycorrhizal fungi inoculants, *Cortinarius cedretorum* appears to be consistently advantageous for *Cedrus atlantica* seedlings. It exhibited the greatest impact on the cedar seedlings growth under water drought, followed by AV and IG; this last had the least effect on cedar seedlings, probably reflecting its poor symbiotic efficiency under these drought conditions.

This study provides interesting perspectives and a very valuable framework for further studies focusing on integrated analysis of the ectomycorrhizal symbiosis effects under drought stress. It also showed the services delivered by ectomycorrhizal symbiosis, which has important implications to forest ecosystems of Atlas cedar in water-limited environments and could potentially lead to development of an efficient conservation strategy in order to reach sustainable objectives in forest ecosystem productivity of this endemic species.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

CH undertook the conception and design, acquisition of data, drafting of the manuscript, analysis and interpretation of data, and final approval of the version to be published. LMS critically revised the manuscript for important intellectual content. ABO interpreted data, drafted the manuscript and undertook analysis and interpretation of data.

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