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ORIGINAL PAPER

Exploring the Role of *Glomus iranicum* in Managing Mild Drought Events in *Triticum aestivum* cv Peralba

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Abstract
The use of arbuscular mycorrhizal fungi (AMFs) represents a promising tool for improving the agronomical performance of crops. Our aim was to evaluate the effects of a commercial formulation of *Glomus iranicum*, also in combination with the biocontrol agent *Trichoderma asperellum*, on *Triticum aestivum* in response to mild drought stress. *G. iranicum* was applied singularly or in association with *T. asperellum*, as seed coating on wheat, and the effects on seed germination and vigor were evaluated as compared to untreated seeds. The efficacy of microbial treatment was evaluated in a greenhouse experiment on singularly, co-inoculated, and untreated plants in control and mild drought stress condition, on six plants for each experimental condition. Physiological, developmental, metabolic, and molecular parameters were measured. *G. iranicum*, either alone or in combination with *T. asperellum*, promoted faster seed germination, while *T. asperellum*, despite its role as a biofungicide, did not adversely affect the mycorrhization index or seedling development. *G. iranicum* and *T. asperellum*, singularly or in combination, determined a significant increase of leaf and root biomass at soil relative water content (SRWC 80%), while in mild stress conditions (SRWC 40%) the sole *G. iranicum* was effective. Abscisic acid (ABA) content increased in plants treated with *G. iranicum*, either under water-limiting or control conditions, but the combination with *T. asperellum* did not attenuate this response. This study demonstrated that *G. iranicum* and *T. asperellum* were effective in increasing the resilience of wheat in mild drought stress conditions, potentially increasing the production yield in unfavorable climatic conditions and supporting the development of combined sustainable agronomic practices.

Keywords AMF · Biocontrol agents · *Glomus* · Mild drought stress · *Triticum aestivum* · Wheat

Abbreviations

ABA	abscisic acid
AMF	arbuscular mycorrhizal fungi
BCA	biocontrol agents
Chl	chlorophylls
Car	carotenoids
FW	fresh weight
DPPH	(2,2-diphenyl-1-picrylhydrazyl radical)
DW	dry weight
GAE	Gallic acid equivalents
G.I.	Germination Index
qRT-PCR	Quantitative reverse transcriptase PCR
RWC	relative water content
SRWC	soil relative water content
S.V.I.	Seed vigor index

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1 Introduction

Cereals represent one of the most important crops, as they play a key role in human nutrition. Bread wheat (*Triticum aestivum*) provides 30% of the World's cereal production, and with an ever-increasing population, estimated to reach

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9.7 billion by 2050, the need for nearly one billion additional tons of annual cereal production is foreseen to meet the growing food demand (FAO, 2025). This scenario is further aggravated by climate change, that compromises crop productivity and quality: in fact, the increasing frequency and severity of adverse events such as drought, heat waves, and water deficit combined with crop pathogens occurrences represent a real challenge for agriculture (World Economic Forum, 2023). Water availability is considered the main factor determining wheat yield (Langridge et al. 2022), and the effects of water scarcity usually associated with heat stress are increasing progressively under climate change. Therefore, it will be necessary to develop strategies to increase crops resilience to climate stresses based on the promotion of sustainable agricultural practices (Verma et al. 2024).

Severe drought events are known to cause, in wheat, a significant reduction of plant growth and yield, negatively affecting seed germination as well as several morphological, physiological and biochemical aspects of the plant development (Dhakal 2021). However, the fluctuating environmental parameters that crops must continuously face, not necessarily threaten plant survival; the adverse effects of moderate (mild) drought stress, for example, strongly depend on the developmental stage impacted to cause yield losses (Harb et al. 2010; Des Marais et al. 2012). A plethora of strategies aimed at maintaining cell turgor through osmotic adjustment and cellular plasticity are attained by plants to cope with different extents of water deficiency (Morgan 1984; Takahashi et al. 2020); among them, the biosynthesis and compartmentalization of osmoprotective compounds and the production of antioxidant molecules/enzymes that reduce or prevent the accumulation of reactive oxygen species (ROS) were found to strongly support the steady growth and development during the exposition to mild drought, by triggering the activation of both primary and specialized metabolisms (del Río 2015; Men et al. 2018; Laxa et al. 2019). For example, osmocompatible aminoacids as well as terpenoids and phenolics were elicited during controlled drought stress applied to some medicinally relevant crops (Nowak et al. 2010; Wu et al. 2016; Araniti et al. 2024).

Biostimulants are considered eco-friendly agricultural tools that have the potential to mitigate climate-induced biotic and abiotic stresses in plants without compromising soil health, plant growth and the environment (Bibi and Rahman 2023; Mandal et al. 2023; Jiang et al. 2024). They are usually applied in small amounts and represent a sustainable alternative to chemical fertilizers, as several studies have demonstrated their efficacy in maintaining ecological balance in agroecosystems by reducing the use of chemicals, pesticides, and heavy metals in agricultural practices (Sun and Shahrajabian 2023). In fact, the use of pesticides has promoted over decades the development of fungicide-resistant pathogens through the increase of genetic adaptation phenomena in target populations, or, in the case of broad-spectrum active compounds, they brought to undesirable effects on non-target organisms (Beckerman et al. 2023; Islam et al. 2024). Microbial-based biostimulants, that include arbuscular mycorrhizal fungi (AMF) and biocontrol agents (BCA), have recently attracted the attention because of their numerous positive effects in promoting various eco-physiological processes associated with the solubilization and uptake of macro- and micronutrients, primary and secondary metabolism promotion and augmented resilience to both biotic and abiotic stress (Abdi et al. 2021, 2024; Gowtham et al. 2022; Islam et al. 2024). As well, the plant–microbe interaction is considered very useful for leading crops to overcome heat, salinity and drought (Sbrana et al. 2014; Sun and Shahrajabian 2023). AMF are found in nearly all soil ecosystems and types, and are known to establish a symbiotic relationship with a wide number of plant species by colonizing the roots with intra-radical hyphae, arbuscules, and vesicles (Kivlin et al. 2011); several investigations pointed out that Glomus spp. are typical of semi-arid Mediterranean ecosystems, being able not only to grow under water deficit (Stahl and Christensen 1991; Mohammad et al. 2003; ÖPik et al. 2006), but also to ameliorate stress detrimental effects in plants thanks to the regulation of biochemical pathways, promoting osmolytes accumulation and triggering ABA signaling (Gong et al. 2012; Ruiz-Lozano et al. 1995; Fernández-Lizarazo et al. 2016; Bahadur et al. 2019). Interestingly, recent findings showed that ABA production in mycorrhizal roots locally suppresses the salicylic acid-dependent defense mechanisms, with the result of increasing the susceptibility of plants to AM infection and suggesting that the modulation of ABA biosynthesis is not only favorable for water deficit management, but it is also necessary for the arbuscule formation (Herrera-Medina et al. 2007).

As integrated farming systems with reduced inputs of fertilizers and pesticides have been developed, the contextual use of biostimulants, AMF, and BCAs is significantly increasing. The most applied biopesticides are fungal species belonging to the genus *Trichoderma*, that has been reported to suppress a great number of plant diseases by its ability to colonize the rhizosphere. Given their interspecific competitiveness for the ecological niche and mycoparasitic behavior, several *Trichoderma* strains can effectively control soil-borne phytopathogens, enhancing plant root growth and nutrients solubilization/uptake (such as P and Fe) as well (Harman et al. 2004; Zin and Badaluddin 2020; Poveda et al. 2021). However, when it comes to the interaction between *Trichoderma* and AMF, available results widely differ, for example, *T. harzianum* has been found to exert

antagonistic, neutral, and stimulating effects (Rousseau et al. 1996; Godeas et al. 1999; Green et al. 1999; Vázquez et al. 2000; Martínez-Medina et al. 2009) in different plant species.

In wheat, the exploitation of *Trichoderma* and/or AMF-based biostimulants has been variously described for both the enhancement of growth and development performance, and the resilience against biotic and abiotic stressors (Colla et al. 2015; Oljira et al. 2020; Illescas et al. 2022; Abdelaal et al. 2024; Younes et al. 2025; Sun et al. 2025). However, few information is available regarding the plant physiological and metabolic response to mild drought stress, and studies that have investigated these aspects with respect to the contextual application of drought mitigation strategies –such as soil biostimulants – are also limited (Pons and Muller 2022; Shakya et al. 2023) and are related to the use of experimental strains; in turn, to our knowledge, no data are available about the validation of *Trichoderma*/AMF-based formulations as wheat biostimulants during mild-drought events. We explored the hypothesis that, under mild drought stress conditions, inoculation with *G. iranicum* could improve wheat physiological performance by enhancing plant water relations, root growth, and stress-related hormonal signaling (e.g. ABA), and that co-inoculation with *Trichoderma asperellum* may exert additive or complementary effects without impairing AMF colonization or function. Therefore, the aims of this study were: to investigate the effect of a commercial formulations of *G. iranicum var. tenuihypharum* applied alone or in combination with *T. asperellum* on seed germination and vigor; to test its compatibility with *T. asperellum*; to compare the efficacy, alone or in combination, on wheat development, also during a mild drought event, by the evaluation of physiological, metabolic and molecular parameters typically used as drought stress markers in plants.

2 Materials and Methods

2.1 Plant Material and Seed Coatings

Seeds of bread wheat (*Triticum aestivum cv*. Peralba) were kindly provided by Consorzio Nazionale Sementi (Co.na.se Soc. Coop. Agr., Conselice, RA, Italy). For the AMF coating treatment, the procedure suggested by the manufacturer was followed: seeds were wetted with H_2O (1% referred to seed weight) and dusted with *Glomus iranicum* var. *tenuihypharum* (Symborg, Murcia, Spain; WO2015/000612) at a dose of 100 g/q using a rotating pan. The inoculum is guaranteed to have a concentration of 30 propagule/g. *Trichoderma asperellum* strain TV1 was applied directly to the soil in powder format at a dose of 0.5 Kg/m^3 (1×10^7 CFU/g). Biofungicide and biostimulant products were kindly supplied by Serbios s.r.l. (Badia Polesine, RO, Italy).

2.2 Plant Growth Conditions and Physiological Parameters Evaluation

2.2.1 Phytotron Trials

For germination rate and vigor assessment, 50 seeds for each treatment were sown in polystyrene seedbeds filled with sterilized horticultural soil (CompoSana®, Compo GmbH, Münster, Germany), placed in a phytotron and incubated in controlled conditions (24 ± 1 °C; 16/8 h light/dark photoperiod; 300 µmol m^{-2} s^{-1} PAR, 75% RU). Three different experimental conditions were set up: control (CNT; no treatment), seeds with *G*, *iranicum* coating (Glo) and seeds with *G. iranicum* coating and *T. asperellum* addition (Glo-T). The number of germinated seeds was recorded daily for ten days, then germination rate (%G) and Germination Index (G.I.= $\sum_{i=1}^{k} \frac{Ni}{Ti}$ where T_i is the time from the start of the experiment to the *i*th interval, N_i is the number of seeds germinated in the *i*th time interval and *k* is the total number of time intervals) were calculated according to ISTA (International Seed Testing Association) protocol. Seedlings were grown for 13 days, then plantlets were sampled, roots and shoot length were both measured, and Seed Vigor Index (S.V.I.) was then calculated by multiplying % G and seedling length (Abdul-Baki and Anderson 1973).

2.2.2 Greenhouse Trials

Wheat plants were grown in a greenhouse (23 ± 1 °C; 16/8 h light/dark photoperiod) in pots, size 21 × 21 cm, filled with a mixture of four parts potting soil (CompoSana®, Compo GmbH, Münster, Germany) and three parts of vermiculite. The experimental design was a randomized complete block design with six experimental conditions: control plants (CNT; not treated); plants not treated and exposed to mild drought stress (CNT-D); plants treated with *G. iranicum* (Glo); plants treated with *G. iranicum* and subjected to mild drought stress (Glo-D); plants treated with *G. iranicum* and *T. asperellum* (Glo-T); plants treated with *G. iranicum* and *T. asperellum* and subjected to mild drought stress (Glo-T-D). For each condition, six pots were prepared, with two seeds in each pot.

The plants were maintained at 80% SRWC (see Sect. 2.4) and grown for 47 days until reaching the tillering stage. Afterward, the control theses (CNT, Glo and Glo-T) were maintained at 80% SRWC, while the mild drought stressed theses (CNT-D, Glo-D, and Glo-T-D) gradually brought to 40% SRWC. These conditions were maintained for 15 days,

then the entire plant was removed, cleaned from soil, leaves and roots were separated, immediately frozen in liquid nitrogen and stored at -80 °C until further analyses.

Chlorophyll contents were measured with the portable device Chlorophyll Meter SPAD-502plus (Konica Minolta, Europe GmbH), to obtain the SPAD (Soil Plant Analysis Development) index, which provides an indication of the health status and photosynthetic apparatus of the plant.

Plant growth and development were evaluated by measuring the leaves and root length using a ruler with a sensitivity of 1 mm. Leaves and roots were separated, fresh weight (FW) was recorded, and dry weight (DW) was measured after desiccation for 48 h at 60 °C.

2.3 Determination of the Relative Water Content (RWC) in Soil and Tissues

The evaluation of SRWC was obtained weighing the soil before sowing to obtain the dry weight (DW) and the turgid weight (FW) for each pot. During the experiment pots were weighed daily at 10:00 am, and the lost water was replenished based on the weight differences. The following formula was used to calculate the amount of water (g) necessary to obtain 80% and 40% SRWC respectively:

$$g\ (H_2O) = 0.8 \times (FW - DW) + DW$$
$$g\ (H_2O) = 0.4 \times (FW - DW) + DW$$

The leaves RWC was determined measuring leaf FW, DW, and turgid weight (TW) according to De Pascali et al. (2022), followed by the application of the formula:

$$RWC\,(\%) = [(\,FW - DW)/(TW - DW\,)] \times\ 100$$

2.4 Mycorrhization Efficacy Assessment

Three plants were randomly selected from each condition; their roots were reduced to 1 cm length fragments and preserved in 50% ethanol. For each plant, three slides were prepared, each containing ten root fragments, and a total of 30 fragments were analysed for each condition. The staining protocol was conducted according to the method of Phillips et al. (1970) with some modifications: briefly, roots were immersed in 5 mL of 10% KOH and heated at 60 °C for 2 h. After 1 h, KOH was replaced with fresh solution, then an equal amount of 10 vol H_2O_2 was added. After 30 min of incubation, 7 mL of 1% HCl were added to samples that were incubated overnight. Finally, 5 mL of staining solution dye (500 mL glycerol, 450 mL water, 50 mL 1% HCl, 0.05% w/v TrypanBlue®) were added: samples were then incubated at 70 °C for 3 h, to two rinses with distilled water and finally stored in staining solution without TrypanBlue®.

The quantification of mycorrhizal structures (hyphae, vesicles and arbuscules) was achieved following the 'Magnified intersection method' (McGonigle et al. 1990). Observations were performed under a light microscope (CHK-G, Olympus optical co., LTD. Tokyo, Japan) at 100X and 400X magnification.

2.5 Quantification of Total Chlorophylls and Carotenoids Content

Chlorophylls and carotenoids were spectrophotometrically determined according to Lichtenthaler et al. (1987). A total of 20 mg lyophilized leaf tissue was extracted in 1 mL of 96% ethanol, vortexed 10 s then heated at 55 °C for 20 min in a thermomixer. After 5 min centrifugation at 8,000 rpm, 30 µL of supernatant were diluted in a final volume of 1 mL of 96% ethanol, then absorbance was measured with UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at A 470, 648.6 and 664.2 nm. The concentration of chlorophylls (Chl) and carotenoids (Car) was calculated as follows:

$$Chl\ a(\mu g/mL) = (13.36) \times (A664.2) - (5.19 \times A648.6)$$

$$Chl\ b\ (\mu g/mL) = (27.43 \times A648.6) - (8.12\ \times A664.2)$$

$$Chl\ a + b\ (\mu g/mL) = (5.24 \times A664.2) + (22.24 \times A648.6)$$

$$Car(\mu g/mL) = [(1000 \times A\ 470) - (2.13 \times Chl\ a) - (97.64 \times chl\ b)]\,/209$$

2.6 Abscisic Acid Quantification in Leaves

ABA was extracted from 200 mg of lyophilized leaf tissue and quantified using a competitive ELISA kit (Enzyme-Linked Immunosorbent Assay; AS20 4392, Agrisera AB Ltd., Vännäs, Sweden) following the manufacturer's instruction. ABA amount was expressed as µg mL^{-1}.

2.7 Quantification of Free Proline Content in leaves

Free proline was extracted and quantified following the protocol described by Potestio et al. (2024). Briefly, 20 mg of lyophilized leaf tissue were extracted with 1 mL of 96% ethanol, vortexed for 10 s then heated at 55 °C for 20 min in a thermomixer and centrifuged at 8,000 rpm for 1 min. A volume of 250 µL of the supernatant was recovered and added to 1 mL of reaction buffer (1% w/v ninhydrin, 60% v/v acetic acid, 20% v/v ethanol). Samples were heated at 90 °C for 20 min in a thermomixer, rapidly ice cooled for few seconds then centrifuged at 7,000 rpm for 1 min. Absorbance

was measured at 520 nm using a UV-Vis spectrophotometer (Agilent Technologies). Free proline concentration was calculated according to a standard curve built with serial dilutions of a 1 M L-proline stock solution from 0.0125 to 1 mM. Data were expressed as mg mg^{-1} of leaf DW.

2.8 Quantification of Scavenging Antioxidant Activity, Total Polyphenol Content, and Total Flavonoids Content

A total of 20 mg lyophilized leaf tissue was extracted with 2 mL 70% methanol, incubated 1 h at RT, then centrifugated at 14,000 rpm for 30 min. The supernatant was carefully collected and used for the following analyses. The radical-scavenging activity was evaluated with the DPPH (2,2-diphenyl-1-picrylhydrazyl radical) assay (Brand-Williams et al. 1995). The antioxidant activity was calculated as .

$$(Abs0 - Abs1)/Abs0) \times 100$$

where Abs0 is the absorbance of the control and Abs1 is the absorbance of the sample.

DPPH solution was prepared at 1 mM concentration; the calibration curve was built from a 10 mM Ascorbic acid stock solution serially diluted from 10 to 300 µM. Fifty µL of extracts were added to 700 µL of 70% methanol and 250 µL of DPPH solution, vortexed, and incubated in the dark for 30 min. Absorbance was measured at 517 nm. DPPH radical-scavenging activity was expressed as Ascorbic acid equivalents per gram of dry weight (AA E/g DW).

Total phenolic content in samples was estimated according to Folin-Ciocalteu's method (Singleton and Rossi 1965). Fifty µL of extracts were added to 75 µL and 125 µL of Folin-Ciocalteu reagent; after 10 min of incubation in the dark at RT, then 1.25 mL of $NaHCO_3$ 7% (w/v) were added to the mixture. Samples were incubated in the dark at RT for 30 min, and the absorbance was acquired at 765 nm. The standard curve was built by serial dilutions of Gallic acid prepared in methanol 70%, ranging from 0.12 to 0.6 mg/mL. Data were expressed as Gallic acid equivalents per gram of dry weight (GAE/g DW).

Total flavonoids content was determined according to the aluminum chloride methods (Zhishen et al. 1999) with slight modifications: 100 µL of extract were brought to a final volume of 250 µL with dH_2O and added to 75 µL of 5% $NaNO_2$ (w/v). The mixture was vortexed thoroughly and incubated at RT for 5 min. Then, 75 µL of $AlCl_3$ solution (w/v) were added, and samples were vortexed, incubated 5 min at RT, added to 500 µL of 1 mM NaOH, and incubated in the dark for 15 min. The standard curve was built by serial dilutions of a 1 mg/mL (+)-Catechin stock solution in 70% methanol, ranging from 2.5 to 40 µg/mL. Absorbance was measured at 510 nm using a UV-Vis spectrophotometer. Data were expressed as Catechin equivalents per gram dry weight (CAE/g DW).

2.9 Gene Expression Analysis

2.9.1 Selection of Target Genes and Primers Design

Target genes of *T. aestivum* were identified in the Ensembl database (plants.ensembl.org), and primers were designed using Primer Blast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) or were found in the literature and were synthesized by Eurofins (Ebersberg, Germany) (Table S1). Primer specificity was checked in silico with the use of Ensembl Plants database.

2.9.2 RNA Extraction, cDNA Synthesis and Quantitative RT-PCR

Total RNA was isolated from 80 mg of lyophilized tissue using the kit EasyPure Plant RNA (TransGen Biotech Co., Ltd, China) according to supplier's instructions. Extracted RNA samples were quantified by Nanodrop spectrophotometer (Thermo Scientific™ NanoDrop™ 2000/2000c Spectrophotometers) and stored at −20 °C. The cDNA was obtained starting from 1 ug of total RNA by using the HiScript III RT SuperMix for qPCR (+gDNA wiper) R323 kit (Nanjing Vazyme Biotech Co., China), following supplier's instructions, and cDNA was quantified by Nanodrop spectrophotometer and stored at −20 °C.

Quantitative reverse transcriptase PCR (qRT-PCR) was performed in a CFX96 apparatus (Biorad, Hercules, CA, USA). For each reaction, 25 ng of cDNA were amplified in 10 µL of mix containing 1× ACEQ Universal SYBR qPCR Master Mix (Nanjing Vazyme Biotech Co., China) and 0.25 µM of each forward (FW) and reverse (REV) primers. Oligos information are reported in Supplementary Table 1. The cycling program consisted of a 3 min preincubation at 95 °C, followed by 40 cycles at 95 °C for 10 s and at 62 °C for 30 s. The temperature range for amplicon dissociation was between 65 and 95 °C, with an increment of 0.1 °C s^{-1}. Each qRT-PCR assay consisted of three independent biological replicates and three technical replicates. The 18 S gene was selected as a reference, and the quantification of expression was performed according to the ΔΔCt method (Livak and Schmittgen 2001). Relative gene expression levels were obtained by calculating the mean fold change from the $2^{-\Delta\Delta Ct}$ value.

2.10 Data Analysis

The data collected by physiological and metabolic analyses were analysed by two-way analysis of variance (ANOVA) followed by Tukey's HSD test. Data were considered significantly different at $p \leq 0.05$. The correlations between physiological and metabolic traits were studied using Principal Components Analysis (PCA). All the analyses were performed using the software Past 4.03 (© Copyright Hammer 1999–2020).

The transcriptomic data were analyzed with CFX Maestro™ Software vs. 2.2 (Biorad, Hercules, CA, USA). The heatmap was produced using the software Heatmapper (Babicki et al. 2016) available online (http://www.heatmapper.ca/).

3 Results

3.1 Effect of *G. iranicum* either Alone or in Combination with *T. asperellum* on Seed Germination and Vigor

An initial evaluation of the biostimulants efficacy was conducted at the germination stage: seed germination percentage, G.I., and S.V.I. were assessed. As reported in Table 1, no significant differences were observed in germination percentage comparing the different treatments, but Glo and Glo-T coated seeds showed a germination rate higher than the CNT. Similarly, G.I. and S.V.I. were positively affected by the treatments. Roots length was significantly increased under biostimulants application ($p < 0.05$) whereas epicotyl length was not affected by the treatment. As expected, the mycorrhization level, estimated with microscopic analysis, confirmed the presence of hyphae, arbuscules, and vesicles in Glo and Glo-T seedling roots but not in the CNT roots (Table 1; Fig. 1b, c).

The seed germination was followed for 13 days (Fig. 1a). Untreated seeds had slower germination and reached the maximum of 83.3% at 10 days after sowing (das), while Glo and Glo-T treated seeds germinated faster than and at 10 das reached the maximum of 95.8%. The Glo-T treatment did not inhibit the stimulating effect of *G. iranicum*. Therefore, we can conclude that Glo treatment stimulated the early stages of development in wheat, and the action of *G. iranicum* was not inhibited by *T. asperellum.*

3.2 Effect of *G. iranicum* on Mild Drought Stress Response

3.2.1 Mycorrhization Efficiency

To evaluate the biostimulant potential upon application of mild drought stress, plants were grown in greenhouse for 47 days and maintained at two different water regimes: 80% SRWC (control) and 40% SRWC (mild drought stressed). The level of mycorrhization was evaluated in all samples as described in Material and methods section. Results of the two-way ANOVA analysis indicated that, under both water regime conditions, plants treated with Glo and Glo-T had a significant increase in mycorrhization index with respect to CNT (Table 2). The Glo-D treatment showed the highest mycorrhization index (69.1%). However, in Glo-T samples a partial reduction in the mycorrhization index (31.7%) was observed, suggesting that *T. asperellum* may have exerted a competitive -or inhibitory- effect on root colonization by *G. iranicum*.

3.2.2 Physiological and Developmental Traits

In all samples we evaluated foliar RWC, the values measured in stressed plants were lower than those of watered plants, placing them in a state of moderate water stress, as shown in Table 2. No effect of the biostimulants was observed for this trait. The analysis of growth parameters revealed substantial disparities resulting from the combined influences of stress and the application of the two microorganisms. Root length was increased in Glo-D and Glo-T-D plants, although the difference was not significant compared to CNT; the leaf length was not modified in any treatment. A significant increase of leaf FW was observed in the Glo-T treatment (mean value of 13.2 ± 2.5), in comparison to CNT

Table 1 Seed germination percentage, shoot and root length, germination index (G.I.), seed vigor index (S.V.I.) and mycorrhization were evaluated on wheat seedlings obtained from seeds untreated (CNT), treated with *Glomus* (Glo), and treated with *Glomus* plus *Trichoderma* (Glo-T) in phytotron trials. Each treatment was represented by 50 seeds

Treatment	Germination (%)	Length (cm)		G.I.	S.V.I.	Mycorrhization (%)		
		Shoots	Roots			Hyphae	Arbuscules	Vescicles
CNT	83.3	22.8 ± 4.4	16.4 ± 4.3^{b}	26.9	3268.3	0.0	0.0	0.0
Glo	95.8	25.3 ± 3.7	22.6 ± 5.6^{a}	32.0	4584.7	1.8	0.05	0.0
Glo-T	95.8	26.6 ± 3.4	20.9 ± 5.4^{ab}	31.2	4470.6	1.6	0.06	0.1

Values are reported as mean ± SD. Different letters in each column represent significant differences ($p < 0.05$)

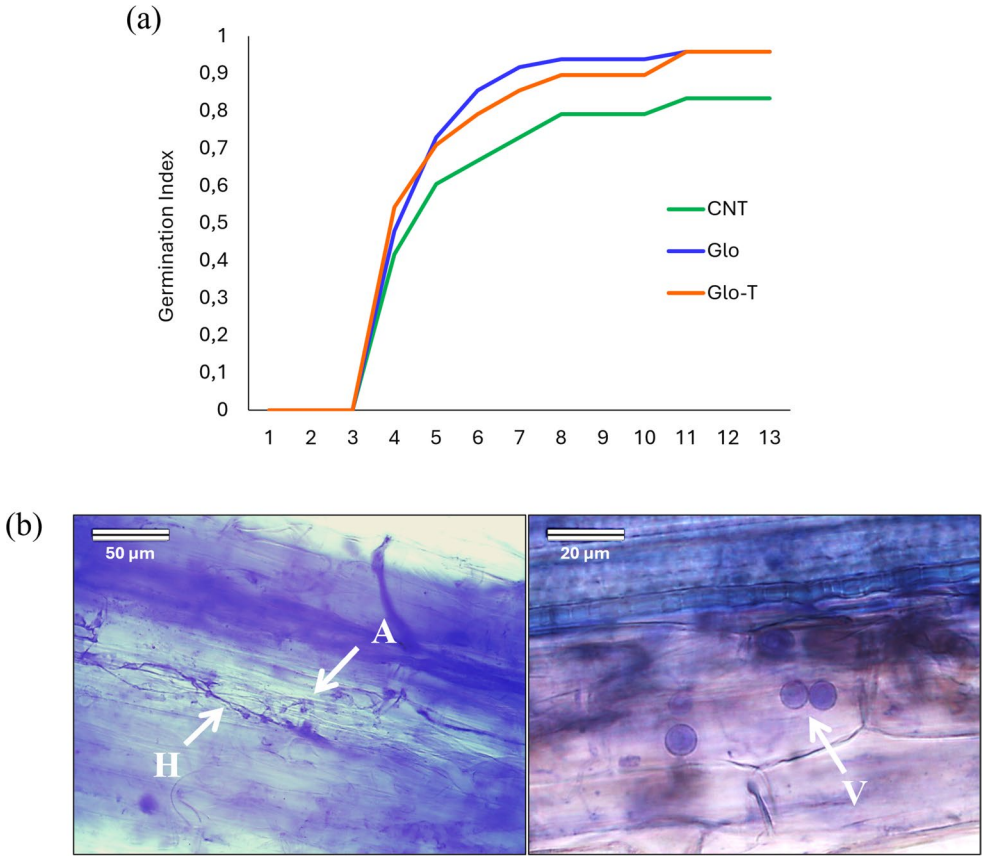


Fig. 1 (**a**) Germination index progression over 13 days under three treatments: CNT (control, green line), Glo (plants inoculated with *G. iranicum*, blue line), and Glo-T (plants inoculated with *G. iranicum* and *T. asperellum*, orange line). (**b**) Microscopic observation of root mycorrhization in plants inoculated with *G. iranicum*. Roots were stained with trypan blue. Images show fungal structures, including hyphae (H), arbuscules (A), and vesicles (V). Images were acquired with an Olympus CHK-G optical microscope (Olympus Corporation, Tokyo, Japan) in brightfield at 400x magnification. Left bar 50 mm, right bar 20 mm

Table 2 Physiological parameters and mycorrhization level measured in roots and leaves of wheat plants grown in greenhouse conditions and treated with *Glomus* (Glo) and *Trichoderma* (T) in control conditions (CNT) or under mild drought stress (D). Bolded numbers represent the highest values for each trait. The results of two-way ANOVA are reported

Trait		Treatment						ANOVA	
		CNT	CNT-D	Glo	Glo-D	Glo-T	Glo-T-D	Stress	Seed coating
Length (cm)	Roots	34.36±2.6	37.26±1.4	41.56±4.3	50.93±2.4	46.3±4.9	**59.10±13.4**	n.s	n.s
	Leaves	52.01±1.5	49.5±1.1	**52.93±1.3**	51.3±1.8	52.71±1.7	52.02±1.9	n.s	n.s
FW (g)	Roots	2.23±0.6^{ab}	1.62±0.4^{ab}	2.02±0.5^{ab}	2.24±0.6^{ab}	**3.45±1**^{a}	1.50±0.4^{b}	***p*=0.027**	n.s
	Leaves	7.48±1.6 ^{b}	6.4±1.4 ^{b}	8.96±0.9 ^{b}	9.2±2.1 ^{ab}	**13.2±2.4**^{a}	8.87±0.9 ^{b}	***p*=0.035**	***p*=0.001**
DW (g)	Roots	0.29±0.1	0.25±0.04	0.24±0.05	0.40±0.1	**0.41±0.1**	0.28±0.03	n.s	n.s
	Leaves	1.03±0.3 ^{b}	1.05±0.3 ^{ab}	1.25±0.1 ^{b}	1.58±0.3 ^{ab}	**1.92±0.3**^{a}	1.44±0.2 ^{ab}	n.s	*p*=0.001
RWC (%)	Leave	0.96±0.03^{a}	0.93±0.01^{b}	0.96±0.01^{a}	0.93±0.02^{b}	0.95±0.02^{a}	0.93±0.01^{b}	***p*=0.01**	n.s
Mycorrhization (%)	Roots	n.d	n.d	44.8±3.2 ^{b}	**69.1±9.1**^{a}	31.7±3.7 ^{c}	37.6±1.9 ^{c}	n.s	***p*=1.15E-5**

FW: fresh weight; DW: dry weight; RWC: relative water content. Values are reported as mean±SD. Different letters in each row represent significant differences ($p<0.05$ two-way ANOVA and Tukey's HSD test)

(7.48±1.6) and to the Glo treatment (8.96±0.88). Under water-limiting conditions, leaf FW in CNT-D was significantly lower compared to Glo-T and Glo-T-D. Root FW exhibited a notable response to stress application, with a significant difference observed for Glo and Glo-D (2.02±0.5 and 2.24±0.63 respectively) as compared to Glo-T-D (1.5±0.38). The Glo-T treatment led to an increase in leaf dry weight (1.95±0.29) compared to control (1.03±0.28) and Glo (1.25±0.09) treatment. However, neither the stress application nor treatments significantly impacted root DW and length.

3.3 Metabolic Response

3.3.1 Photosynthesis and Antioxidants Profile

At metabolic level, the interaction between the mild drought stress and biostimulants application determined a significant increase of the SPAD index (Fig. 2a) in Glo-T treatment (40.96±0.9) and Glo (38.96±0.9) conditions as compared to control (37.07±1.5), while in stress conditions all samples had similar SPAD values. The total chlorophylls (a+b) content was reduced in Glo plants (4.5±0.5) with respect

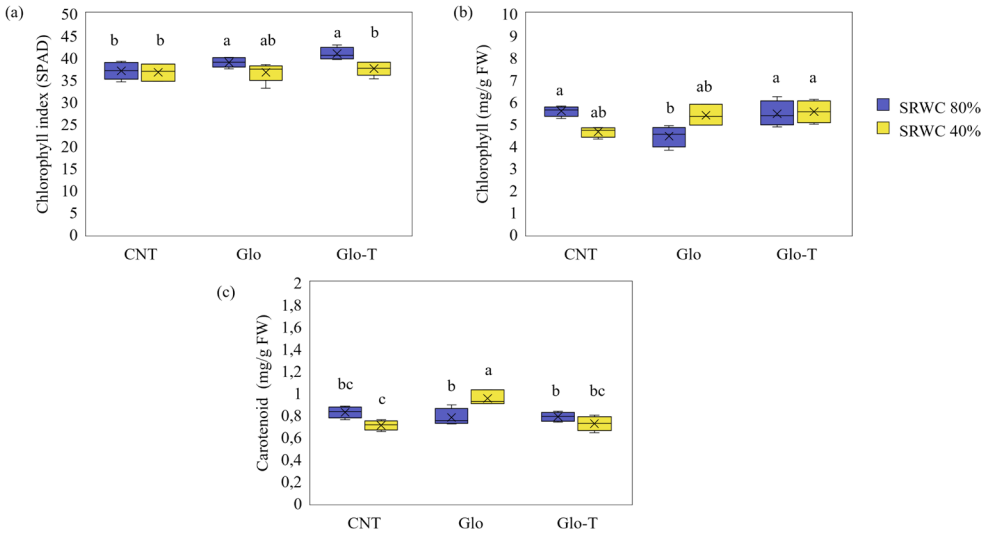


Fig. 2 Effect of mild drought stress on photosynthetic pigments in wheat leaves: (**a**) chlorophyll content measured as SPAD index; (**b**) chlorophyll content in mg/g of fresh weight (FW); (**c**) carotenoid content in mg/g FW. CNT, control condition; Glo, treatment with *G. iranicum*; Glo-T treatment with *G. iranicum* and *T. asperellum*. Plants were grown at 80% soil relative water content (SRWC) (blue bars) or at 40% SRWC (orange bars) considered as mild drought stress. Different letters indicate significant differences between the groups, determined by ANOVA followed by Tukey's test ($p<0.05$)

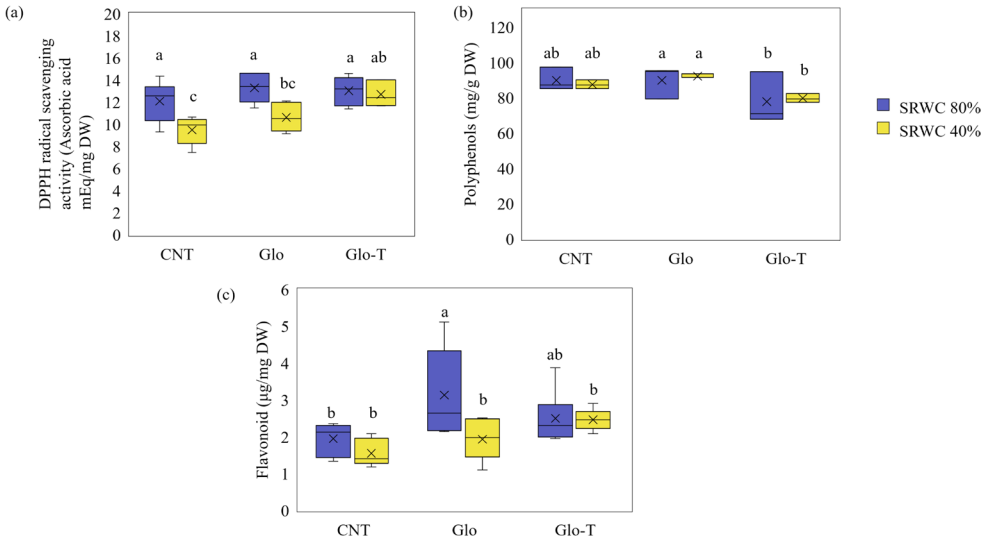


Fig. 3 DPPH radical scavenging activity (Ascorbic acid mEq/mg DW) (**a**), polyphenols content (**b**), and flavonoid content (**c**) were measured in leaves of wheat plants under different experimental conditions. CNT, control condition; Glo, treatment with *G. iranicum*; Glo-T treatment with *G. iranicum* and *Trichoderma*. Plants were grown at 80% soil relative water content (SRWC) (blue bar) or at 40% SRWC (orange bar). Different letters indicate statistically significant differences between the groups. These differences were determined by ANOVA followed by Tukey's test ($p<0.05$)

to CNT (5.6±0.2) and Glo-T (5.5±0.6) (Fig. 2b). In Glo-D and Glo-T-D, total chlorophylls showed a value similar to CNT. The carotenoid content increased significantly in Glo-D as compared to all other conditions (Fig. 2c).

The scavenging activity of the extracts of the leaf samples on the DPPH radical is shown in Fig. 3a: at 80% SRWC, no differences were observed in response to the presence of the two biostimulants, while at 40% SRWC the scavenging activity of the extracts in both CNT (9.48±1.24) and Glo (10.62±1.22) conditions were significantly lower than in Glo-T.

The total polyphenols content was similar in CNT and Glo samples, also in response to drought, while in Glo-T we observed significantly lower values compared to CNT and Glo treatments in both water regime conditions as shown in Fig. 3b. Flavonoids content was higher in the Glo samples (3.13±1.22) as compared to CNT (1.95±0.44); at 40% SRWC, however, the amount of flavonoids was reduced in Glo-D samples (1.94±0.56). In addition, the combined Glo-T treatment had a lower flavonoid content than Glo, although not statistically significant (Fig. 3c).

3.3.2 Abscisic Acid and Proline Content

ABA level was increased under stress conditions in all samples, as expected (Table 3). In Glo samples, the ABA levels were higher than in CNT and Glo-T, but the difference was not statistically significant.

The amount of proline was increased in Glo-T-D samples; however, no significant differences were found either between treatments or under water-limiting conditions.

Therefore, it is assumed that the presence of *Glomus* and its combination with *Trichoderma* may modulate proline levels, which could influence the stress response.

Table 3 Abscisic acid (ABA) and proline content measured in leaves of wheat plants treated with *Glomus* (Glo) and *Trichoderma* (T) in control conditions (CNT) or under drought stress (D). The results of two-way ANOVA are reported

	Treatment						ANOVA	
	CNT	CNT-D	Glo	Glo-D	Glo-T	Glo-T-D	Stress	Seed coating
ABA (µg/mL)	0.09 ± 0.01	0.13 ± 0.1	0.17 ± 0.03	0.18 ± 0.1	0.11 ± 0.07	0.14 ± 0.02	*n.s*	*n.s*
Proline (µg/mL)	11.9 ± 0.8	11.8 ± 1.1	12.4 ± 2.7	11.3 ± 1.9	13.1 ± 1.2	14.9 ± 1.1	*n.s*	*n.s*

Values are reported as mean ± SD

3.3.3 Multivariate Analysis of Traits Evaluated in Greenhouse Experiment

Figure 4 reports the biplot of the PCA model calculated on the investigated parameters on wheat plants grown in the presence of biostimulants and in different water regimes. The first two components of the PCA model represented more than half of variation in the dataset (61.6%). PC1 clearly divided samples grown in control conditions from samples treated with the two biostimulants. Samples treated with the combination of *Glomus* and *Trichoderma* were separated also along PC2 for parameters linked to metabolic properties such as TPC and antioxidant activities of leaves extracts. Overall, Glo-T treatments determined positive effects on plant growth in control conditions, while in response to 40% SRWC improved the metabolic traits.

3.4 Gene Expression Profiling

Gene expression modulation in response to both treatments and water-limiting conditions was evaluated for eight target genes (Tab. S1), selected for their role in different pathways related to drought stress response.

For the ABA biosynthetic pathway, zeaxanthin epoxidase (ZEP) and 9-cis-epoxycarotenoid dioxygenase (NCED), a limiting step in ABA synthesis, were selected (Zareen et al. 2024). The transcription factor MYC2 plays a significant role in the regulation of secondary metabolites and,

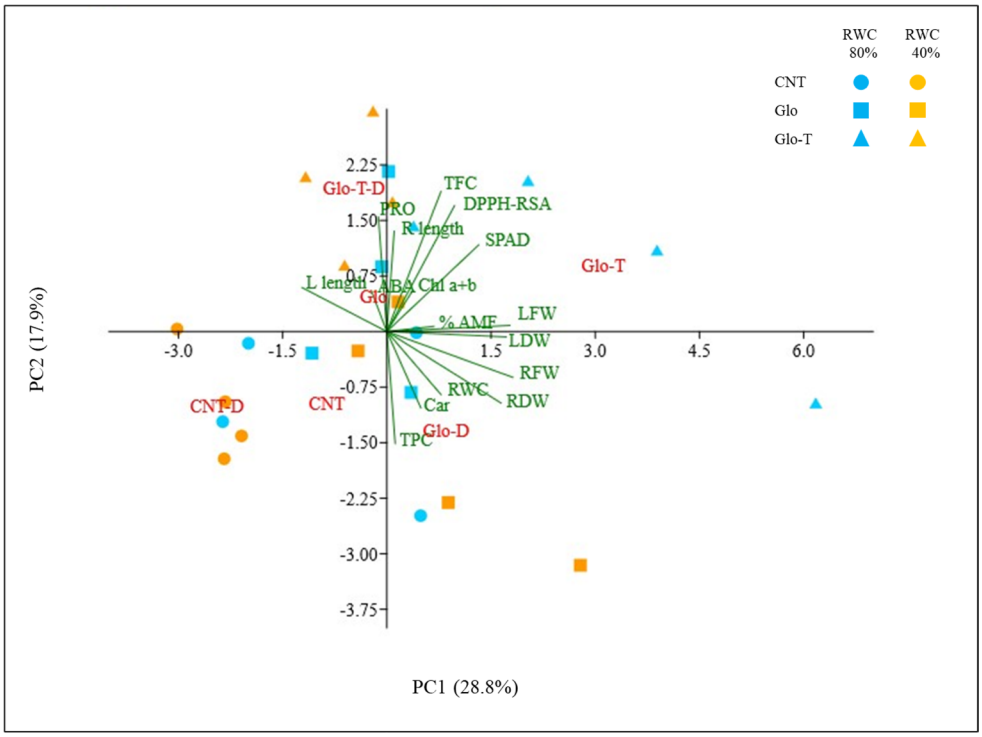


Fig. 4 Principal Component Analysis (PCA) of growth parameters and metabolic traits evaluated in wheat plants grown in different conditions. Growth parameters were: fresh and dry weight of leaf (LFW, LDW) and root (RFW, RDW); leaf and root length (L length, R length); root mycorrhization (% AMF). The metabolic traits are: total phenolic (TPC); carotenoid (Car); chlorophyll (Chl a + b); chlorophyll as Spad index (SPAD); DPPH radical scavenging activity (DPPH-RSA): proline (PRO): abscisic acid (ABA). Plants grown at 80% soil relative water content (SRWC): Control condition (CNT, blue circles); treatment with *G. iranicum* (Glo, blue squares); treatment with *G. iranicum* and *Trichoderma* (Glo-T, blue triangles); plants grown at 40% SRWC: control conditions (CNT-D, orange circles); treatment with *G. iranicum* (Glo-D, orange squares); treatment with *G. iranicum* and *Trichoderma* (Glo-T-D, orange triangles)

above all, has a key role in regulating plant responses to abiotic stress, as it controls the expression of downstream genes involved in improving the response of plants to these stresses (Zeng et al. 2025). BCAT genes encode aminotransferases involved in the metabolism of branched-chain amino acids. During drought stress, the expression of these genes increased in flag leaves in durum wheat, as previously reported (Buffagni et al. 2020). For the proline biosynthetic pathway, pyrroline-5-carboxylate reductase (P5CR) gene was targeted, since it is known that its expression is up-regulated in drought conditions (Dudziak et al. 2019; Jamshidi Goharrizi et al., 2022). The β-cyanoalanine synthase gene (CYSC1) takes active part in the cyanide detoxification pathway, which plants activate in response to stress-induced ethylene and cyanide production. Drought often elevates ethylene synthesis, which can in turn increase cyanide generation; then, CYSC1 detoxifies cyanide by converting it to β-cyanoalanine and contributes to stress tolerance. CS120 is a cold-shock protein, member of the large dehydrins family, which are responsive to several abiotic stresses, including drought stress (Kosová et al. 2014). Aquaporins are a multigene family encoding transmembrane proteins that allow water absorption and transport between plant tissues. Among them, the PIP2 gene was selected since a modulation by mycorrhizal inoculation was reported (Asadollahi et al. 2023; Ye et al. 2023).

As shown in Fig. 5, all the target genes were up-regulated by drought stress in the absence of biostimulants. In plants treated with *Glomus*, the expression of ZEP, BCAT2, and MYC2 was downregulated under stress condition compared with the CNT, with ZEP showing the strongest reduction Similarly, Glo-T treated plants showed a reduced expression of the same genes under both SRWC conditions. CS120 gene followed a similar pattern as well: in fact, a reduced expression in Glo and Glo-D treatment was observed. The combination with *Trichoderma* determined an opposite trend, with an increased level of expression; however, expression decreased under mild water-stress (Glo-T-D) when compared with the control, especially for CS120. P5CR (pyrroline-5-carboxylate reductase) and NCED (9-cis-epoxycarotenoid dioxygenase) genes were downregulated by Glo and Glo-T treatments. PIP2 (aquaporin PIP2-5) expression was induced in Glo and Glo-T-D treatments, while it was downregulated in Glo-D and especially in the Glo-T treatment.

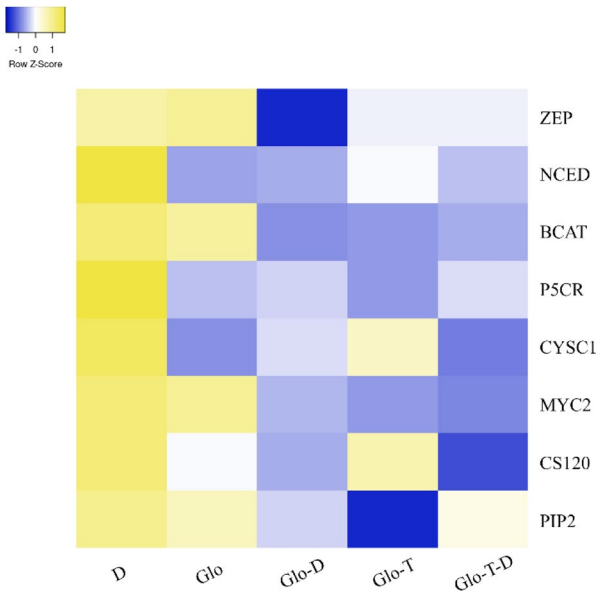


Fig. 5 Heat-map showing the relative expression of *ZEP*, *NCED*, *BCAT*, *P5CR*, *CYSC1*, *MYC2*, *CS120*, and *PIP2* measured in leaves of wheat plants grown in different conditions. CNT, control condition; Glo, treatment with *G. iranicum*; Glo-T treatment with *G. iranicum* and *Trichoderma*. Plants were grown at 80% soil relative water content (SRWC) (CNT, Glo, Glo-T) or at 40% SRWC (CNT-D, Glo-D, Glo-T-D). Heatmap represents the log2 fold change of gene expression values normalized with respect to CNT. Each row represents a gene, and each column represents a treatment. Color code from yellow to blue correspond to gene expression levels from high to low

4 Discussion

Drought stress is one of the main abiotic stress conditions that exerts negative effects on plants growth and productivity, and the effects of water deprivation have been studied in wheat for many years. As ecological disturbance in agroecosystems, drought effects can be categorized as either a press or pulse disturbance, depending on their duration, frequency, and ecological impact (Jentsch and White 2019), and mild drought stress in particular represents a highly relevant, realistic condition in agricultural systems, where crops are more often exposed to moderate and transient water deficits rather than severe stress. However, it is less commonly perceived as a threat for agriculture with respect to severe water-limiting events, despite it triggers early physiological and biochemical adjustments that determine whether a plant will later tolerate or succumb to harsher conditions; in fact, mild stress affects growth, stomatal behavior, root development, and hormone signaling in subtle ways that often go unnoticed but can reduce yield over time (Chen et al. 2021; Haider et al. 2024). Under these conditions, plants maintain active metabolism and can engage adaptive responses (Clauw et al. 2016), providing a suitable context to reveal functional plant–microbe interactions. These moderate events can also act as selective pressures that influence plant adaptation strategies and microbial community composition, ultimately affecting ecosystem resilience and productivity: for example, if intense drought destabilizes the microbiome network, reducing its role in plant growth (De

Vries et al. 2018), low drought stress has been demonstrated to reduce the bacterial and fungal diversity while increasing the network complexity of soil microbiomes (Li et al. 2023). Therefore, focusing on mild drought stress allows for a more nuanced understanding of biostimulants action under realistic agronomic conditions, complementing the existing literature largely centered on severe stress scenarios.

This study was designed to endorse the efficacy of current sustainable strategies, aimed at mitigating the impact of drought stress in crops, that exploit the application of beneficial rhizosphere microorganisms during mild-drought events: we evaluated the effectiveness of commercial formulations containing the AMF *G. iranicum*, used alone and in combination with the biocontrol agent *T. asperellum*, in supporting seed vigor and the plants' ability to cope with mild drought stress conditions, as, at our knowledge, the combination of them has not been reported in wheat so far.

The first step was to investigate whether the co-application of *Glomus* and *Trichoderma* would impact seed germination or vigor, since AMFs are known to play an essential role in plant growth and stress resistance through a "chemical dialogue" between the root apparatus and fungal mycelium, and *Trichoderma*, a biofungicide, can modify the rhizosphere and promote plant growth by triggering defense mechanisms (Manzar et al. 2022) and, more interestingly, it can stimulate root growth, enhance nutrient mobilization, and induce systemic stress tolerance.

Our experiments, conducted in phytotron, showed that the presence of *G. iranicum* positively influenced roots length during the germination phase, suggesting that the use of *Glomus* as seed-coating can facilitate the accumulation of essential resources for the plant growth through the root colonization in the early stages of plant development. We also observed that the higher germination index, the seed vigor index, and the mycorrhization level determined by *G. iranicum* were not reduced by the addition of *Trichoderma.* Similar findings were reported by Fernandez-Zarate et al. (2022), who observed that the commercial mixture of fungi belonging to the *Glomus* genus determined a higher level of mycorrhization and a better performance in germination parameters of *Cinchona officinalis* treated plants; however, in contrast with our results, this improvement was not statistically significant. In wheat, seed vigor is related to the following plant performance in the field, and several techniques for seed hardening have been tested to ameliorate this trait (Basra et al. 2006). Hence, the use of biostimulants such as AMF and *Trichoderma* constitutes a strategy with potential long term efficacy during plant whole life cycle. Effectively, *G. iranicum var. tenuihypharum* tested on different crops under field conditions determined an increase in plant productivity in all the species analyzed (Martin et al. 2017). Early microbial seed treatments with *Trichoderma* in association with AMF were found to increase germination rates, seedling vigor, and biomass in several crops (Cardarelli et al. 2022), while the hormonal profile induced may promote seedling establishment under various conditions (Martinez-Medina et al. 2011). Besides, once germination begins, *Trichoderma* can stimulate root hair formation, enhance root morphology, and mobilize nutrients, supporting and promoting the completion of the germination process itself (Ferreira et al. 2024; Yang et al. 2024).

The combined use of mycorrhizal fungi with *Trichoderma* already proved to enhance plant tolerance to drought stress because both organisms improve root function and soil interactions in complementary ways, for example by promoting hormonal balance and strengthening plant defenses, that in turn results in better hydration and resilience under limited water availability. Their synergistic actions was attributed to an establishment of a more robust root–microbe ecosystem that sustained plant growth during severe drought conditions (Eftekhari et al. 2025; Zhang et al. 2025).

We decided to investigate if, under a mild drought stress, the combined presence of *G. iranicum* and *T. asperellum* on wheat would to provide the plant with key features that can help in mitigating further drought events at different levels, and if, due to a positive effect exerted by *Trichoderma* on the plant root apparatus, the *Glomus* mycorrhization level would be partially reduced. The increase of mycorrhization by *G. iranicum* obtained under mild stress treatment in our experiments was consistent with the effect of a short-term drought exposition in soybean (Oliveira et al. 2022). However, the root colonization was partially reduced in plants subjected to the co-application of *Glomus* and *Trichoderma* both at 80% and 40% SRWC and this is not completely surprising, as the presence of non-AMF in the rhizosphere has been already reported to partially inhibit the intraradical proliferation of AMF hyphae - with a reduction of AM fungal phosphorus transport to the plant (Gryndler et al. 1996) - consistently with the mycoparasitic/antagonistic action of *Trichoderma* (De Jaeger et al. 2011). It can be speculated that in drought experiments reporting that water limitation tends to reduce AMF colonization in terms of arbuscule abundance (Zhang et al. 2024), results usually depend on drought intensity, plant species, and AMF strains. Additionally, very severe drought often damages fungal hyphae or precludes colonization, while mild or moderate stress may allow AMF to persist. This suggests that expecting a "stress-driven boost" of mycorrhization may be optimistic; rather, success likely depends on the resilience of the specificity of AMF–plant interaction, drought intensity, and soil conditions. There is experimental evidence that co-application of AMF and *Trichoderma* alters physiological and metabolic traits associated with stress resilience: in

broccoli, for example, the co-inoculation was reported to relieve the effects of water deficiency through a positive effect on SPAD index, antioxidant enzymes and phenolics accumulation (Metwally et al. 2025), but various are the features involved in plant response to drought stress that result enhanced by the synergistic activity of these microorganisms (such as increased biomass, higher photosynthetic performance, improved ROS management and changes in primary/secondary metabolites), and that we observed in this study.

Among the downstream effects of AMF inoculation some are aimed at protecting photosynthetic pigments, maintaining proline levels, and detoxification of ROS. The combination of these mechanisms determines a synergic effect on photosynthetic characteristics and osmotic adjustments, particularly through the accumulation of compounds such as proline, chlorophyll, and carotenoids (Ouhaddou et al. 2025). Not only: in fact, mycorrhizal establishment directly activates the phenylpropanoid pathway by upregulating phenylalanine ammonia lyase (PAL) and related enzymes, which increase synthesis of phenolic compounds and anthocyanins (both acting as antioxidants and osmoprotectants) during oxidative stress caused by limited water availability. In maize, for example, the mycorrhization improved photosynthetic activity under mild water stress conditions (Khan et al. 2024), due to an increase in the amount of photosynthetic pigments and the ability to maintain ROS scavenging activities, but a significant increase of flavonoids and total antioxidant phenolics was also found in other crops inoculated with AMFs and posed under water-limiting conditions (Cecatto et al. 2016; Liu et al. 2022; Nahuelcura et al. 2023). In wheat cv Peralba we observed a similar effect, even if less remarkable; moreover, consistently with our results, the biopriming of wheat seeds with *Trichoderma* has been reported to modulate phenolics content (Shukla et al. 2015), even if responses are more variable and often species-, genotype- or stress-intensity dependent (Nahuelcura et al. 2023).

ABA is a key hormone in the plant's response to water stress that accumulates in leaves in response to drought conditions, causing the stomata closure to reduce water loss and activating other defense mechanisms (Waadt et al. 2022). So far, 255 AMF species have been identified, and it is now well established that AMF mediated regulatory mechanisms significantly influence plant responses to abiotic stress, also by strongly modulating ABA biosynthesis, depending on the host plant, the type of fungus, and the context in which the interaction occurs (Khan et al. 2025). In the present study, the increase in ABA content observed in *Glomus* treated plants, together with the enhancement of biomass under both well-watered and mild drought stress conditions, suggests that AMF inoculation promotes a primed physiological state that optimizes water use and stress acclimation rather than simply mitigating damage. However, the combination with *Trichoderma* significantly increases biomass but not ABA level, indicating that it may help plants cope more efficiently with stress without requiring high levels of ABA suggesting that microbial interactions are strongly context-dependent and may not necessarily result in synergistic effects under moderate water limitation. Under strong water deficiency, AMFs were demonstrated to induce ABA biosynthesis, helping the plant to minimize water loss by transpiration and reducing the detrimental effects of drought (Chitarra et al. 2016). It has been found that mycorrhizal plants perform better under water stress compared to non-mycorrhizal plants. Therefore, the enhanced ABA biosynthesis in mycorrhized stressed plants not only improves drought tolerance but also strengthens and establishes the AM symbiosis (Bahadur et al. 2019). Some studies, however, showed that colonization with AMF can reduce ABA concentrations in plant tissues under drought conditions, while others have demonstrated that it can increase them (Li et al. 2016; Xie et al. 2018). In *S. lycopersicum*, AMF enhanced plant drought tolerance by regulating the 14-3-3 genes (*TFT1-TFT12*) in the ABA signaling pathway and improved plant water relations (Xu et al. 2018). In *Lycium barbarum* L. (Goji), after inoculation with AMF, the ABA content in the leaf increases, contributing to the maintenance of osmotic balance and enhancing resistance to salt stress (Liu et al. 2016). However, the role of ABA in regulating drought resistance in mycorrhizal plants still needs to be studied and better understood.

Proline is a key metabolite in plant responses to drought and salinity, regulating osmotic potential, protecting proteins and cellular membranes, and acting as a ROS scavenger (Renzetti et al. 2024). Some studies have shown that plants inoculated with AMF accumulate more proline than non-mycorrhized plants under drought or salt stress, while others report the opposite. This variation may depend on the plant species, the specific AMF strain, or the type of environmental stress (Chun et al. 2018). In maize, Kandowangko et al. (2009) investigated the effect of *Azospirillum* sp. and AMF on the physiological response under drought stress, and it was observed that proline accumulation in the leaves was higher in the combined treatment with *Azospirillum* and AMF as compared to non-inoculated plants. Furthermore, the combination of *Azospirillum* sp. and AMF resulted in greater proline accumulation than single inoculations, suggesting a synergistic effect between the two microorganisms. These results are consistent with our study, in which the combined Glo-T treatment led to an increased proline content.

At the molecular level, the modulation of genes involved in abscisic acid (ABA) biosynthesis and secondary metabolites accumulation during drought stress provides valuable

insight into the functional efficacy of root mycorrhization, since these pathways are central to plants perception of stress and adaptation. ABA regulates key drought responses (stomatal closure, root architecture adjustment, stress-responsive gene expression), while osmoprotective metabolites contribute to antioxidant protection and osmotic balance, and AMF, by influencing both hormonal signaling and metabolic pathways, can actively improve plant water status, nutrient acquisition, and root–shoot communication, stress modulation, enhancing plant resilience under water-limiting conditions (Degola and Palmano 2024). Hence, the modulation of gene expression might provide mechanistic evidence of how mycorrhization enhances plant resilience under drought progression.

In our water-limiting condition, the target genes ZEP and NCED involved in ABA biosynthesis (Zareen et al. 2024) and already reported to increase in wheat leaves under severe drought, enhancing the plant's tolerance (Ma et al. 2016), were both up regulated. NCED1 in particular is strongly induced by drought stress in leaves of a variety of crop species (Iuchi et al. 2001), but here the application of 40% RWC to plants treated with Glo and Glo-T resulted in a down-regulation of both genes, suggesting a mitigating effect of the two biostimulants even at an early stage of stress establishment. A similar pattern has been observed for MYC2, a transcriptional activator induced in plants subjected to drought conditions (Wang et al. 2021), that plays a significant role in the regulation of secondary metabolites and controlling the expression of downstream genes involved in plant responses to abiotic stress (Luo et al. 2023; Zeng et al. 2025). And for other stress-related target genes commonly up-regulated by drought, such as BCAT (Buffagni et al. 2020), P5CR (Dudziak et al. 2019; Goharrizi et al. 2023). However, for the latter, the down regulation determined in Glo, Glo-D and Glo-D-T treatments does not agree with what reported by Wang et al. (2025), that described how in *Populus cathayana* inoculated with *Rhizophagus irregularis* in both watered and stress conditions the gene remained induced. This was not completely unexpected, as AMF symbioses are highly variable: in fact, different combinations of host plants and AMF are known to produce different outcomes on both nutrition efficiency and gene expression (Feddermann et al. 2010).

Like NCED and P5CR, the β-cyanoalanine synthase gene (CYSC1) resulted down-regulated by the presence of the AMF alone also (Glo). CYSC1 takes active part in the cyanide detoxification pathway, which plants activate in response to stress-induced ethylene and cyanide production: drought often elevates ethylene synthesis, which can in turn increase cyanide generation; then, CYSC1 detoxifies cyanide by converting it to β-cyanoalanine. Though, an increased activity under drought suggests a protective adjustment by supporting oxidative stress tolerance under abiotic stress (Machingura et al. 2013). As β-cyanoalanine synthase activity responds to water deficit and contributes to stress tolerance by participating in cyanide detoxification and associated oxidative stress regulation, we speculated that, since ethylene biosynthesis produces cyanide as a by-product and water stress events as well as both AMF and *Trichoderma* interaction can modulate ethylene signaling, it is plausible that CYSC1 expression can be influenced by *Glomus*/*Trichoderma*-altered ethylene dynamics. To our knowledge, this is the first evidence about the CYSC1 gene response during mycorrhizal interaction under water-limiting conditions, that fills a knowledge gap and represents an opportunity for future targeted research.

Aquaporins are a multigene family encoding transmembrane proteins that allow water absorption and transport between plant tissues. The expression of PIP family genes has been observed to vary depending on the wheat genotype: for example, it was found that the expression of the PIP2-24 gene was clearly up-regulated in tolerant genotypes, whereas in the susceptible genotypes the expression levels were down-regulated (Madrid-Espinoza et al. 2018). In a study conducted on rice varieties treated with *T. harzianum*, the expression of aquaporin was reduced in the genotype subjected to drought; however, this effect was attenuated with a higher dose of *T. harzianum*. This suggests that this interaction improves drought tolerance through molecular interactions between the two organisms (Pandey et al. 2016). The regulation of PIP2 genes is highly dynamic and sensitive to mycorrhizal inoculation; inoculation has a more significant impact on aquaporin expression than drought alone, in which *pip2* family genes show differential responses depending on stress or symbiosis conditions, suggesting complex regulation (Asadollahi et al. 2023; Ye et al. 2023). This complex regulatory pattern is also time dependent, as observed in common bean where mycorrhizal symbiosis induces a modulation of aquaporin gene expression, leading either to an up- or down-regulation of specific isoforms. PIP2-5 gene showed lower expression levels in mycorrhizal plants exposed to water deficit compared to non-mycorrhizal ones in the early stages of stress, followed by a subsequent up-regulation (Recchia et al. 2018).

Overall, the expression profiles observed in Glo-D and Glo-T-D conditions were the opposite as compared to the CNT-D condition suggesting an alteration in drought sensing.

An additional aspect of our study relates to the use of commercial products based on AMF, which often results ineffective in establishing a profitable mycorrhization, as reported in a recent review (Boussageon et al. 2025) in which 85% of the commercial AMF inoculants tested could not colonize the plant roots. Amongst the factors determining

this low reliability of market formulas, it can be argued that the major challenge for using AMF in agriculture is the host-specificity/fungus–crop mismatch (Tran et al. 2019): "one-size-fits-all" AMF inoculation may not succeed, and effectiveness strongly depends on compatibility between fungal strain and specific crop species - or even cultivar. Species belonging to genus *Glomus* are known to establish symbiotic relationships with a wide variety of plants (Ouhaddou et al. 2025), being diffused worldwide and widespread in diverse ecosystems. *G. iranicum* was identified in *T. aestivum* cultivated in southwestern Iran (Błaszkowski et al. 2010)d *iranicum* var. *tenuihypharum* is claimed to possess unique and exclusive characteristics that differentiate it from other mycorrhiza-forming fungi and achieve the highest level of mycorrhizal symbiosis (production of very small spores, occurring of sporulation outside the root hairs, neither blocking nor breaking them as other mycorrhizal species, a higher abundance of hyphae that explore the soil, up to four times than other AMF, and the ability to develop in saline soils with values of up to 6 dS/m and pH 4–9) (https://www.global-agriculture.com/global-agriculture/what-is-glomus-iranicum-var-tenuihypharum-and-why-is-it-better-than-other-mycorrhiza-forming-fungus/ accessed 12/9/2025). However, to our knowledge, no experimental studies are available that test *G. iranicum* var. *tenuihypharum* inoculation on wheat under field or greenhouse conditions, as most studies on this AMF target maize and non-cereal crops (Martin et al. 2017; Luciani et al. 2019; Contreras-Pino et al. 2025). Our results not only represent one of the few reports on the use of this commercial product on wheat but also confirmed its specificity for *T. aestivum*.

5 Conclusions

Our results demonstrate that the combined application of *Glomus iranicum* var. *tenuihypharum* and *Trichoderma* can elicit measurable, though moderate, improvements of *Triticum aestivum* cv Peralba performance under mild drought conditions. While the physiological and metabolic responses observed were less pronounced than those typically reported under more severe water deficits, the perceivable enhancement of stress-related traits indicates that the dual inoculation is biologically active and potentially synergic even when water limitation is not extreme. Under mild stress, plant performance seemed more tightly linked to water relations and hydraulic traits (Arbuscular Mycorrhizal Fungi-dominated mechanisms), while *Trichoderma*-mediated growth promotion may be less effective when water becomes the primary limiting factor. On the other hand, the increase of abscisic acid content observed in AMF-treated plant mirrors a fine-tuned acclimation rather than a stress shutdown, as if these plants may operate in a "primed" state, balancing growth and water conservation more efficiently than control plants. These findings suggest that such assembled beneficial microbial consortia can prime wheat for early or low-intensity drought episodes, potentially increasing resilience before stress becomes critical. Such early-stage modulation of plant responses highlights the value of microbial-based strategies as preventive tools in sustainable crop management.

Future research should extend beyond controlled or single-site experiments, by incorporating field trials across diverse agroecological zones: such trials would allow to evaluate the effectiveness of *Glomus/Trichoderma*-based formulations when different soils, climatic conditions and management practices -that characterize real agricultural systems- are combined with water-limiting conditions, where plant–microbe–environment interactions are less predictable than under severe stress. Moreover, multi-site studies would enable the assessment of consistency, scalability, and economic feasibility of inoculation strategies, while also identifying potential constraints (such as native microbial competition or soil physicochemical limitations) that could interfere with the soil-dependent microbial performance. Being the mild-water stress a "grey zone" where the success of microbial biostimulants is neither guaranteed nor absent, but highly dependent on local conditions, multi-location field trials are useful to disentangle this complexity and to determine whether the observed benefits are robust, reproducible, and agronomically meaningful across realistic production scenarios.

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Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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