

Article

Trichoderma parareesei Favors the Tolerance of Rapeseed (*Brassica napus* L.) to Salinity and Drought Due to a Chorismate Mutase

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Abstract: Both drought and salinity represent the greatest plant abiotic stresses in crops. Increasing plant tolerance against these environmental conditions must be a key strategy in the development of future agriculture. The genus of *Trichoderma* filament fungi includes several species widely used as biocontrol agents for plant diseases but also some with the ability to increase plant tolerance against abiotic stresses. In this sense, using the species *T. parareesei* and *T. harzianum*, we have verified the differences between the two after their application in rapeseed (*Brassica napus*) root inoculation, with *T. parareesei* being a more efficient alternative to increase rapeseed productivity under drought or salinity conditions. In addition, we have determined the role that *T. parareesei* chorismate mutase plays in its ability to promote tolerance to salinity and drought in plants by increasing the expression of genes related to the hormonal pathways of abscisic acid (ABA) under drought stress, and ethylene (ET) under salt stress.

Keywords: *Trichoderma*; *Brassica napus*; chorismate mutase; salinity

1. Introduction

Although the demand for food continues to increase year after year, agricultural productivity faces several abiotic stresses (i.e., water limitation, salinity, extreme temperatures) that reduce it, which are often associated with global warming [1]. Abiotic stresses have a negative impact on plant growth, reducing the yield of all the major crops and resulting in considerable losses [2]. Climate change is predicted to be very harmful for agriculture production, particularly at low latitudes in developing countries. In this sense, plants have to adapt to the changing conditions in the environment they grow [3]; therefore, improving plant stress resistance is critical for agricultural productivity and also for environmental sustainability [4].

The most important environmental stress that constrains crop yield is drought. In this sense, more than 40% of the world is classified as dry land, an area that increases each year due to the increase in the world temperature as a result of global warming [5]. Thus, water is the single most important abiotic factor limiting crop productivity [6]. Therefore, it is estimated that the damage caused by drought in crops will reduce their productivity by 36% [7], although it can induce a reduction in yield between 13% and 94% in several crops, depending on the intensity and duration of the stress [8].

On the other hand, soil salinity is a global problem in crop productivity due to land clearing and unsustainable irrigation practices. To avoid the osmotic damage caused by salt in their roots, and to be able to absorb the water present in the soil, plants must pay a high energy cost. Thus, a reduced yield leads to significant economic damage—up to losses of 33% of profits for farmers. Moreover, a constant increase in soil salinity may mean that cropping becomes impossible [9].

In this context, improving farming technologies and crop varieties has been the main driver to increase farm productivity during the last century, but, to ensure an environmentally sustainable and

socially responsible food supply, we need the development of new agricultural practices that focus on minimizing soil degradation, environmental pollution, and the adverse effects of climate change, basically, environmentally friendly biological strategies. In this sense, the soil microbiome has great potential to provide solutions due to its capacity as a driver of soil functions and agricultural crop productivity. The role of microorganisms in plants, particularly those inhabiting the rhizosphere, includes performing core functions such as the supply of nitrogen, phosphorus, potassium, sulphur, and micronutrients. Such host-microbiome interactions are crucial for plant health, as microbes can affect plant growth and/or development at multiple stages, including germination, morphogenesis, flowering, and hence, productivity. Microbial symbionts of plants also act as a functional extension in plant defense against biotic and abiotic stresses [10]. As far as abiotic stress is concerned, beneficial microorganisms are able to counteract environmental stress by improving plant performance, enhancing plant growth and productivity, interacting with several processes involved in plant responses to stress, and increasing the accumulation of antioxidant compounds that allow a decrease in plant stress sensitivity, with interesting results in their application today [8]. Examples of this can be found in the microorganisms' capacity of reactive oxygen species (ROS) scavenging, to provide membrane stability and osmoprotection, to promote stomatal regulation and xylem hydraulic conductance, and to regulate root zone water availability and root ethylene (ET) and abscisic acid (ABA) levels [11,12].

Among the so-called beneficial microorganisms for agriculture, *Trichoderma* is a genus of soil-borne filamentous fungi that includes species widely used as biocontrol agents in agriculture, which are able to colonize diverse substrates under different environmental conditions. These species stand out for their capacity to antagonize plant pathogens through different mechanisms such as mycoparasitism, antibiosis, competition, and triggering plant defense; however, some strains are also able to stimulate plant growth and induce tolerance to abiotic stress [13–15]. Regarding this last mechanism, in 2010, Mastouri et al. described how the treatment of tomato seeds with *T. harzianum* accelerates their germination, increases the vigor of the seedlings and reduces the effects caused by thermal, osmotic, saline and water stress, as it would induce physiological changes in the plant against oxidative damage, decreasing the damage caused by the accumulation of ROS that occurs in stressed plants due to the reduced accumulation of lipid peroxides and the production of antioxidant compounds such as glutathione [16]. This was also observed by Ghorbanpour et al. (2018) in tomatoes subjected to low temperatures [17], as well as by Yasmeen and Siddiqui (2018) in corn and rice subjected to salinity [18], and also in tomatoes—with a reduction in stomatal conductance and transpiration, and the expression of increased levels of the genes involved in signaling abscisic acid-, ethylene-, and salicylic acid-dependent pathways [19]. As far as ABA is concerned, it is the crucial plant-hormone in regulating stomatal aperture, which is required to limit water loss from leaves under drought conditions, also, ABA induces the expression of many genes whose products are important for stress responses and tolerance such as enzymes for osmoprotectant synthesis [20]. On the other hand, ET signaling positively regulates salt tolerance, thanks to altering Na^+/K^+ ratio and selectively activating salt-tolerance genes in plants, such as ETR1, ETR2, EIN4, CTR1, ECIP1, SIED1, ERF1, and/or ESE1 [21].

As far as *T. parareesei* is concerned, there are very few studies on this strain, so some of its potential applications in agriculture may be yet to be discovered. *T. parareesei* is considered the ancestor of *T. reesei*, a common strain used in the biotechnological industry for the production of cellulolytic and hemicellulolytic enzymes [22]. Its genome was annotated by Yang et al. in 2015 [23].

In agriculture applications, *T. parareesei* has shown biocontrol potential against fungal (*Rhizoctonia solani* and *Botrytis cinerea*) and oomycete (*Pythium irregularulare*) phytopathogens, the promotion of root growth in tomato seedlings, and the induction of tolerance to salinity and resistance against *B. cinerea* in tomato plants [24]. Continuing this work, Pérez et al., in 2015, determined the key role of the *T. parareesei* *Tparo7* gene, encoding a chorismate mutase (CM), a shikimate pathway branch point leading to the production of aromatic aminoacids, in *T. parareesei* mycoparasitic capacity against *R. solani*, *B. cinerea* and *Fusarium oxysporum* and its ability to trigger systemic plant defenses by root colonization [25].

Considering the need to find new biological strategies to increase agricultural productivity in situations of abiotic stress, in this work, we have inoculated rapeseed or canola (*Brassica napus*) plants with *T. parareesei* and induced stress situations with salt and drought. This was undertaken due to the absence of studies with rapeseed and *Trichoderma* and the search for new plant-beneficial microorganisms not yet studied—such as *T. parareesei*. On the other hand, rapeseed is the second most important oilseed crop of the world. It is an indispensable component of crop rotations in major growing areas to maintain soil fertility and contributes to sustainable production. Moreover, it contributes to farmers' incomes and, therefore, helps to stabilize rural populations, because rapeseed is a raw material for vegetable oil and extraction meal [26]. Moreover, we have tried to elucidate the possible role of CM in the ability of the fungus to improve the tolerance of plants against abiotic stress because its relationship with biotic stresses has already been elucidated [25].

2. Materials and Methods

2.1. Plant Material and Growth

B. napus cv. Jura were the plants used in this study, whose seeds were surface-sterilized by vigorous sequential shaking in 70% ethanol and 2% sodium hypochlorite solutions for 10 min each and then washed thoroughly four times in sterile distilled water, as previously described [19].

Seeds were plated on Murashige and Skoog (MS) (Duchefa, Haarlem, the Netherlands) solid medium (agar 1%) with sucrose (1%) in a growth chamber at 22 °C, 40% relative humidity (RH) and a 16 h light/8 h dark photoperiod at 80–100 $\mu\text{E m}^{-2}\cdot\text{s}^{-1}$, for seven days. Rapeseed seedlings were individually transferred to 5 L pots containing a mixture of peat/vermiculite (3:1) and maintained in a greenhouse at 22 ± 2 °C and a 16 h light/8 h dark photoperiod, as previously described [27], placed in random trays on the tables (Figure S1) and watered with 2 L of water to every four pots (a plate) (bottom pot irrigation) every two days.

2.2. Abiotic Stress and Productivity Quantification

Regarding saline stress, rapeseed plants were watered with a 200 mM NaCl solution every two days, starting from the development of the third true leaf (three-week-old plants) until the end of its development cycle (19 weeks). The drought stress was developed due to a progressive reduction in watering from the development of the third true leaf (three-week-old plants). The pots were irrigated twice a week in weeks 4 and 5, once a week in weeks 6 and 7, and once every two weeks until the end of the life cycle (weeks 8–19).

The siliques from 15 rapeseed plants per condition were collected at the end of the life cycle and counted (19 weeks).

2.3. *Trichoderma* Cultures and Inoculation

T. harzianum CECT 2413 (Spanish Type Culture Collection, Valencia, Spain, referred to as strain T34) and *T. parareesei* (formerly *T. reesei*) IMI 113135 (CABI Bioscience, Egham, UK, referred to here as the T6 strain) were used throughout this study. Moreover, we use from the work of Pérez et al. in 2015, the Tparo7-S4 *T. parareesei* transformant was obtained by the silencing of the *Tparo7* gene, together with the transformation control strain Tp-TC with pJL43b1 [25].

Spores were harvested from seven-day-old potato dextrose-agar (PDA, Sigma-Aldrich, Madrid, Spain) dishes as previously described [24], after being grown at 28 °C.

Each plant was inoculated by *Trichoderma* with 1 mL of a conidial suspension containing 2×10^7 spore mL^{-1} , one week after the seedlings had been transplanted. Twenty plants were used for each condition and inoculant, and each assay was repeated three times, at different times.

2.4. Quantification of *Trichoderma*-Root Colonization and Gene Expression Studies

For the analyses of fungal root colonization and the expression of abiotic tolerance-related genes, for each condition and inoculant, roots from five plants per each treatment were pooled and root pools from three independent assays were considered. Roots were collected during the formation of the floral primordia, in 10-week-old rapeseed plants [28,29]. All root material was washed with water until there was no remaining substrate, immediately frozen with liquid nitrogen, and pulverized with a mortar.

The quantification of *Trichoderma* colonization was performed by the quantification of *Trichoderma* DNA in the roots of rapeseed, by quantitative PCR (qPCR) as previously described [28]. The DNA was extracted from rapeseed roots of T34, T6, Tp-TC and Tparo7-S4 inoculated plants. A mix was prepared in a 10- μ L volume using 5 μ L of Brilliant SYBR Green QPCR Master Mix (Roche, Penzberg, Germany), 10 ng of DNA, the forward and reverse primers at a final concentration of 100 nM, and nuclease-free PCR-grade water to adjust the final volume. The *Actin* genes of *Trichoderma* and rapeseed were used for the quantification, as in other *Trichoderma*-rapeseed interaction studies [28,29], and their corresponding primer pairs are indicated in Table 1. Amplifications were performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), programmed for 40 cycles under the following conditions: denaturation, 95 °C for 15 s; annealing, 60 °C for 1 min; extension, 72 °C for 1 min. Each PCR was performed in triplicate by using the DNA extracted from three root pools of five plants each—one for each treatment and plant type. Cycle threshold values served to calculate the amount of fungal DNA using standard curves. The values of *Trichoderma* DNA were referred to the amount of rapeseed DNA in every corresponding sample.

Table 1. Oligonucleotides used in this work.

Code	Sequence (5'–3')	Use	Reference
Act-T-F Act-T-R	ATGGTATGGGTCAGAAGGA ATGTCAACACGAGCAATGG	Endogenous <i>Trichoderma</i> gene	[29]
Act-Bn-F Act-Bn-R	CCCTGGAATTGCTGACCGTA TGGAAAGTGCTGAGGGATGC	Endogenous rapeseed gene	[29]
ACCO1-Bn-F ACCO1-Bn-R	ATTTTGGGAAGAGATTGGAG GCTGGATAGTTGCTCACCTTA	Synthesis gene of ET in rapeseed	[30]
ERF1-Bn-F ERF1-Bn-R	AGTCACGGCGTTACAAT GTGGTGACAACGGCGAGAA	Response gene to ET in rapeseed	[31]
NCED3-Bn-F NCED3-Bn-R	GTGGAAGTCGGAGTTACAGATAG CCAAGTCACTAGCTCCATAAA	Synthesis gene of ABA in rapeseed	[32]
PYL4-Bn-F PYL4-Bn-R	CGGTCCTAACCAGTGTGCTC GCTGAAACTAATGTCGTGCTCT	Response gene to ABA in rapeseed	[33]

For gene expression studies, the pooled roots were used for RNA extraction with the TRI reagent (Ambion, Austin, TX, USA), following the manufacturer's instructions. cDNA was synthesized from 2 μ g of RNA, which was treated with DNase RQ1 (Promega Biotech Ibérica, Alcobendas, Spain), and then used for reverse transcription with an oligo(dT) primer with the Transcriptor First Strand cDNA Synthesis Kit (Takara Bio, Inc., Tokyo, Japan), following the manufacturer's protocol. Gene expression was analyzed by RT-qPCR, using an ABI PRISM 7000 Sequence Detection System with Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA). All PCR reactions were performed in triplicate in a total volume of 10 μ L for 40 cycles under the following conditions: denaturation, 95 °C, 30 s; annealing, 60 °C, 1 min; extension, 72 °C, 1 min. Threshold cycles (C_T) were determined using the 7000 SDS System Software (Applied Biosystems, Foster City, CA, USA), and C_T values were calculated using the rapeseed *Actin* gene as an endogenous control. The primers used are given in Table 1: genes of the ACC-oxidase 1 (*ACCO1*), ethylene response factor 1 (*ERF1*), lipoxigenase 1 (*LOX1*),

nine-cis-epoxycarotenoid dioxygenase 3 (*NCED3*), and ABA-receptor (*PYL4*). Data are expressed using $2^{-\Delta\Delta CT}$ method [34]. The choice of genes for synthesis and response to ABA and ET is based on the importance of both hormones in the response to abiotic stresses in plants.

2.5. Indirect Quantification of ROS in Roots

The indirect quantification of ROS in roots was carried out by measuring electrolyte leakage similar to the method used by Aguilar et al. in 2015 for leaf discs, which really measures cellular oxidative damage related to the production of ROS [35]. From the root pools formed to carry out colonization and gene expression analyses (Section 2.4), 100 mg of fresh tissue was briefly mixed with water and floated on 5 mL of double-distilled water for 6 h at room temperature. The conductivity of the water was measured using a Crison™ Conductimeter GLP31 (Crison, Barcelona, Spain). This represented the electrolyte leakage from the roots (Reading 1). Then, samples were boiled for 20 min at 90 °C. After the liquid cooled down, the conductivity of the water was measured again. This represented the total ions present in the roots (Reading 2). Electrolyte leakage, an indirect measurement of ROS, was represented as the percentage of total ions released [(Reading 1/Reading 2) × 100].

2.6. Statistical Analysis

The statistical analysis of the data was carried out using Statistix 8.0 software (Miller Landing Rd, Tallahassee, FL, USA). One-way ANOVA using Tukey's multiple range test at $p < 0.05$ was used for pairwise comparisons; the different letters indicate the significant differences. The combined effects of *Trichoderma* strain T34 and abiotic stress were analyzed by two-way ANOVA followed by Sidak's multiple comparison test, indicating significant differences as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ***** $p < 0.00001$. The G×E interaction was performed following the methodology described by Mondo et al. in 2019 [36]. Using the interactions between rapeseed, different stress conditions, and different inoculations (using both parameters as different environments), we determined the absence of G×E interaction in our study.

3. Results

3.1. Rapeseed Productivity

Without stress, a significant increase in rapeseed seed yielding in interaction with T6 strain, the transformation control (Tp-TC), and the *Tparo7* silenced mutant (Tparo7-S4)—compared with not-inoculated plants—was observed. Furthermore, this increased productivity was even higher after root inoculation with the T34 strain (Figure 1A).

Under drought stress, all the root treatments showed a significantly better yield compared with the un-inoculated plants (control). The highest yield was observed in the root treated by T6 and Tp-Tc, and the lowest values were observed in the un-treated plants, followed by Tparo7 (Figure 1B). Similar results were observed under salt stress conditions (Figure 1C), although there are no significant differences between inoculation with T34 and with the rest of *Trichoderma*.

3.2. *Trichoderma*-Roots Colonization

Without stress, the levels of root colonization by T6, Tp-TC, and Tparo7-S4 are significantly lower than in the case of T34 (Figure 2). Under stress due to drought and salinity, the levels of every *Trichoderma* strains colonization increase significantly in comparison with T34 colonization without stress, being significantly higher still in the case of T6, Tp-TC, and Tparo7-S4 (Figure 2).

3.3. Expression Levels of Different Hormone-Related Genes

Rapeseed root colonization by the T34 strain without stress induces higher gene expression in ET-related genes such as *ACCO1*, involved in ethylene biosynthesis, as is the case with Tparo7-S4, and *ERF1*—involved in ethylene signaling, compared to rapeseed without inoculation (Figure 3).

Contrastingly, a significantly lower gene expression in ABA-related genes, such as *NCED3* and *PYL4*, involved in ABA metabolism and action, respectively, was observed (Figure 3). Concerning to ABA-related gene expression, similar results were observed in the rapeseed plants root-inoculated with different *T. parareesei* strains, whereas the higher gene expression of ET-related genes was also observed—although significant differences were only detected in the case of the silenced mutant Tparo7-S4 (Figure 3).

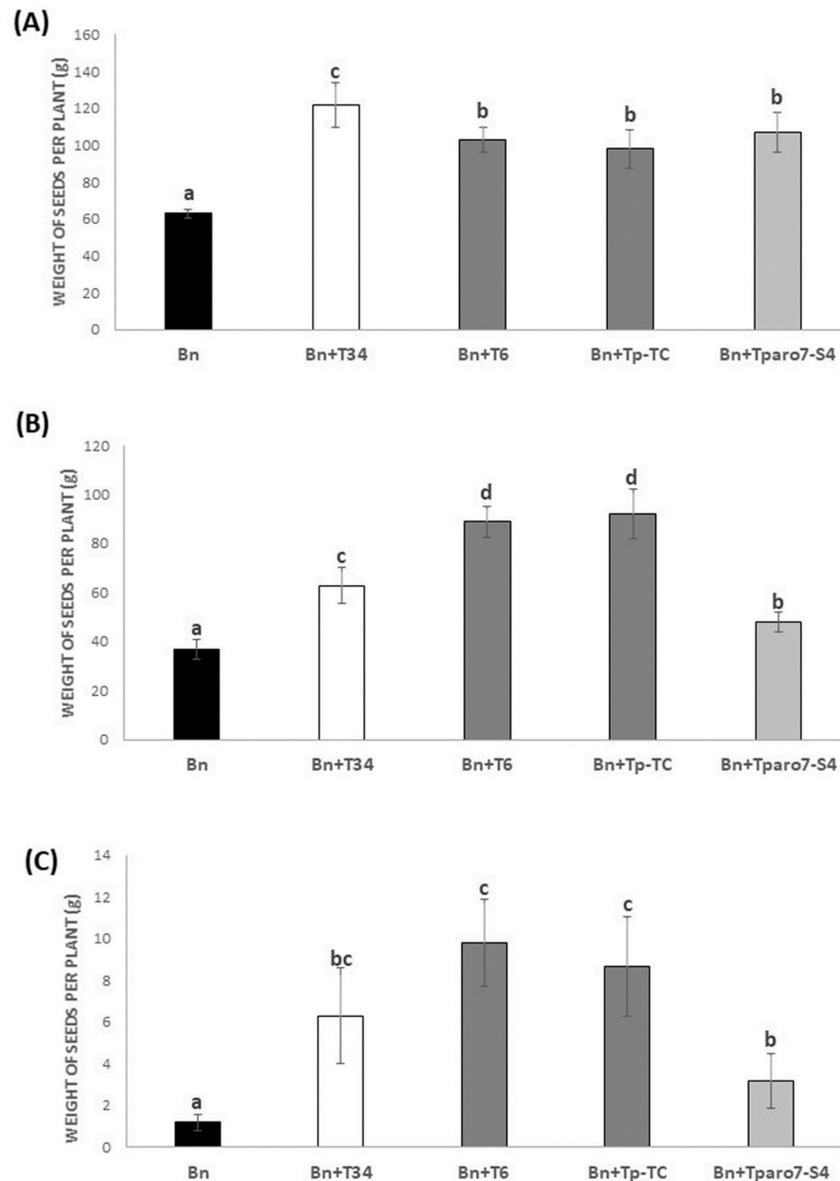


Figure 1. Rapeseed productivity. The weight (g) of the seeds collected from the rapeseed (Bn) and the different *Trichoderma* inoculations: *T. harzianum* (+T34), *T. parareesei* (+T6), *T. parareesei* transformation control (+Tp-TC), and the transformant with the silenced *Tparo7* gene (+Tparo7-S4) per plant without stress (A), under drought stress (B) and under salt stress (NaCl 200mM) (C). Data are the mean of three biological replicates for each condition with the corresponding standard deviation, and 15 plants per replicate were used. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($p < 0.05$).

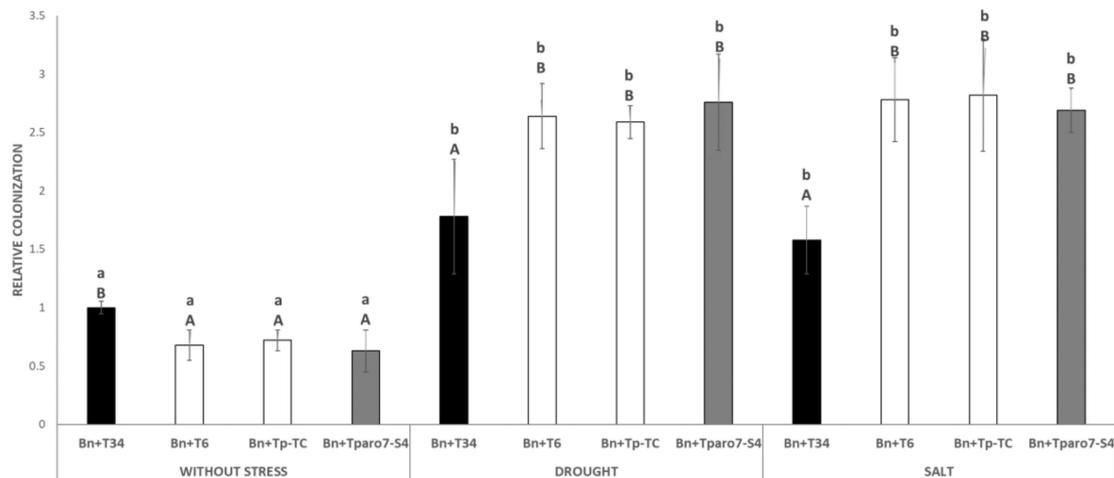


Figure 2. Measurements of rapeseed-root colonization by *T. harzianum* (+T34), *T. parareesei* (+T6), *T. parareesei* transformation control (+Tp-TC), and the transformant with the silenced *Tparo7* gene (+Tparo7-S4) without stress, under drought stress and under salt stress (NaCl 200mM). To quantify rapeseed root colonization, the DNA of the fungus was quantified by qPCR from radicular samples using the *actin* genes from both the plants and the fungus. Fungal DNA/plant DNA ratio was normalized to 1 in the case of T34 and was calculated based on this data for the rest of the situations and inoculants. Data are the mean of three biological replicates for each condition with the corresponding standard deviation, and for each biological replicate and condition, roots from five plants were used. One-way analysis of variance (ANOVA) was performed, followed by the Tukey’s test. Different letters represent significant differences ($p < 0.05$), comparing between environment within each treatment (strain)- and control-(identify by small letters) and treatment- and control- (strain) within each specific environment (identify by capital letters). Also, two-way analysis of variance (ANOVA) was performed, shown in Supplementary Table S1.

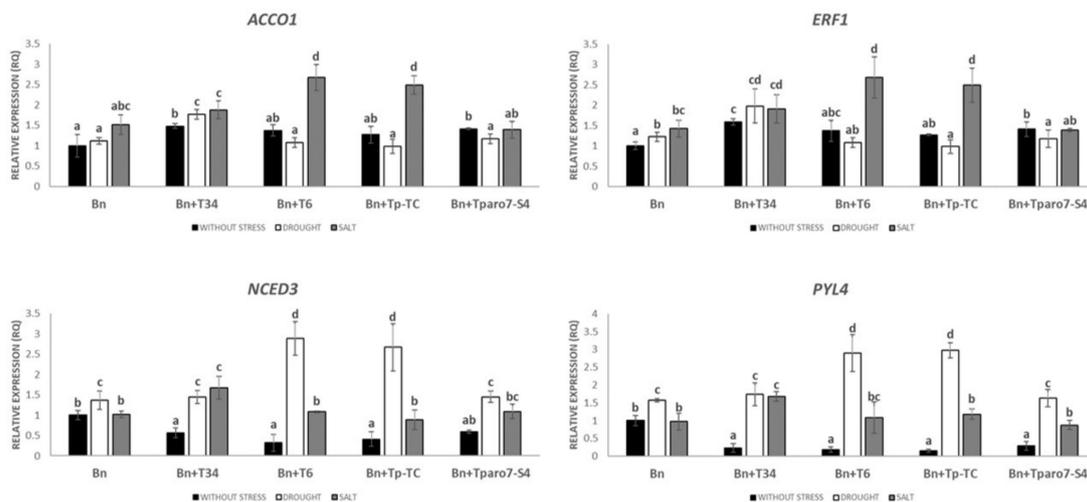


Figure 3. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of the expression of some tolerance-abiotic-related genes in the roots of rapeseed (Bn) inoculated with *T. harzianum* (+T34), *T. parareesei* (+T6), *T. parareesei* transformation control (+Tp-TC), and the transformants with the silenced *Tparo7* gene (+Tparo7-S4). Values correspond to relative measurements against plants without stress neither *Trichoderma*-inoculation ($2^{-\Delta\Delta C_t} = 1$). Data are the mean of three biological replicates for each condition with the corresponding standard deviation, and for each biological replicate and condition, roots from five plants were used. One-way analysis of variance (ANOVA) was performed, followed by the Tukey’s test. Different letters represent significant differences ($p < 0.05$) between plants with and without stress and *Trichoderma*-inoculation.

Under abiotic stress conditions, a significantly higher gene expression in the levels of ET-related genes was only observed in rapeseed plants root-inoculated with the T6 and Tp-TC strains under drought conditions, whereas a significantly higher gene expression in ABA-related genes were detected under salt stress conditions in rapeseed plants interacting with *T. parareesei* strains, except in the case of the silenced mutant T (Figure 3). In addition, rapeseed root inoculation with T34 also induces the expression of ET-related genes, although this increase is only significant in the case of salt stress. Concerning ABA-related genes, a significantly higher gene expression in the corresponding expression levels was only observed under drought stress conditions.

3.4. ROS in Roots

With rapeseed root inoculation with *Trichoderma*, a significant decrease in the relative leakage of the roots is observed—an indirect measure of oxidative stress by ROS (Figure 4). Under salinity, there is a significant increase in the oxidative stress of rapeseed, which decreases significantly with the application of T34, the application of T6 and Tp-CT being even more beneficial; however, it does not occur with the application of Tparo7-S4. Similar results were observed under drought.

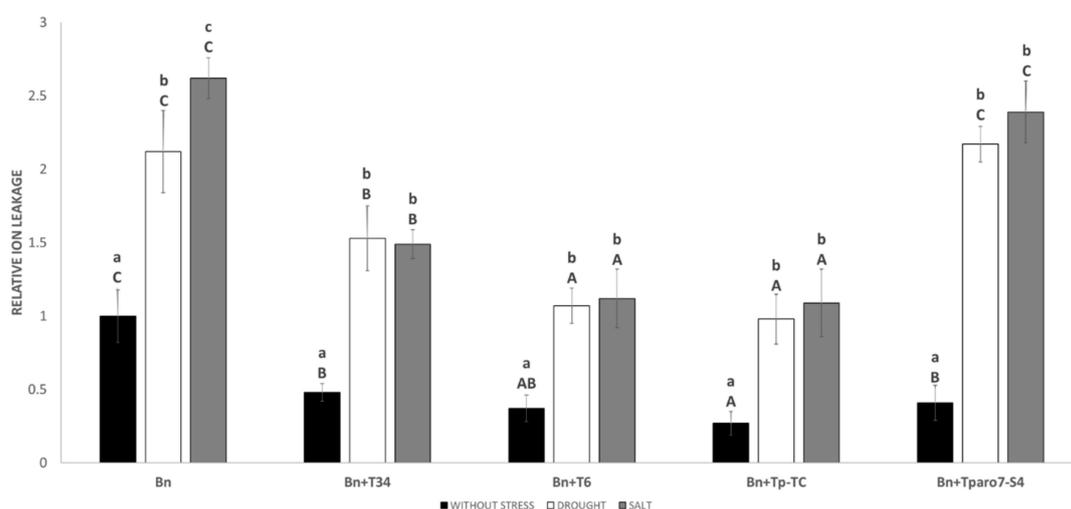


Figure 4. Indirect quantification of ROS in roots by relative ion leakage in the roots of rapeseed (Bn) inoculated with *T. harzianum* (+T34), *T. parareesei* (+T6), *T. parareesei* transformation control (+Tp-TC), and the transformants with the silenced *Tparo7* gene (+Tparo7-S4). Data are the mean of three biological replicates for each condition with the corresponding standard deviation, and for each biological replicate and condition, roots from five plants were used. One-way analysis of variance (ANOVA) was performed, followed by the Tukey's test. Different letters represent significant differences ($p < 0.05$), comparing between environment within each treatment (strain)- and control-(identify by small letters) and treatment- and control-(strain) within each specific environment (identify by capital letters). Also, two-way analysis of variance (ANOVA) was performed, shown in Supplementary Table S2.

4. Discussion

Rapeseed is important for edible oil production in semi-arid areas, where abiotic stresses threaten its production [37], as observed under salt and drought stress (Figure 1). In this sense, little is known about the transcriptomic changes that occur in rapeseed in response to abiotic stress—particularly salinity and drought [38]. In spite of this, it has been observed how salinity stress provokes an inhibition of seed germination and seedling establishment in rapeseed [39], as well as a reduction in biomass, size, and number of leaves, but not in photochemical activity [40]. Moreover, the way in which drought stress significantly inhibited germination and seedling growth led to oxidative stress from excessive H_2O_2 generation and reduced the chlorophyll content [41], resulting in growth inhibition, oil content reduction, and yield loss during the reproductive stage [42].

In our study, we have verified how the inoculation without stress of rapeseed plants with different strains of *Trichoderma* represents a significant increase in the weight of seeds produced by each plant (Figure 1A), something observed previously, although without knowing exactly what are the fungal mechanisms involved in it of all those described for the *Trichoderma*-plant interaction, with the application of *T. harzianum*, also increasing the weight of seeds per plant [29]; *T. asperellum*, with an increase in seed weight, lipid content, and total seed yield [43]; *T. viride*, increasing fresh and dry biomass [44]; and *T. atroviride*, with a significantly larger root and shoot biomass, but without a systemic induction of plant-defenses against the caterpillars *Plutella xylostella* [45]. However, the induction of systemic resistance in rapeseed has been observed with the rapeseed root application of *T. harzianum* against the leaf fungi *Sclerotinia sclerotiorum* [46] and *Erysiphe cruciferarum* [47].

Moreover, the inoculation with T34 increases significantly rapeseed productivity more than T6, Tp-TC and Tparo7-S4 (Figure 1A). It is probably due to the greater capacity of rapeseed root colonization observed in T34 compared to the other fungi (Figure 2). In this sense, the key role of the ThKEL1 protein of T34 has been demonstrated in the colonization of crucifer roots, such as rapeseed and *Arabidopsis thaliana*, but not in other groups of taxonomically more remote plants, such as tomatoes, due to the modification of their myrosinase activity involved in the hydrolysis of glucosinolates—secondary metabolites present in this group of plants [29]. *T. parareesei* does not present any homologous gene to *Thkel1* in its genome; therefore, the productivity increase observed with T34 could be due to the presence of *Thkel1* in its genome, as a consequence of an increase in its ability to root-colonize in crucifers [29].

Under abiotic stresses, little has been studied of the benefits of rapeseed radicular inoculation with beneficial fungi, due to the accumulation of fungicidal allochemicals in its roots [48]. The application of bacteria has resulted in an improvement in the growth, physiology, and antioxidant activity, and reduced the chromium uptake of rapeseed in chromium-contaminated soil by *Burkholderia phytofirmans* [49]; furthermore, it has improved major seed germination, root length, shoot length, amount of lateral roots, and chlorophyll content in rapeseed seedlings under salt stress by *Enterobacter cloacae* [50] and *Pseudomonas stutzeri* [51]. Nonetheless, we have observed how the rapeseed-root-inoculation with *Trichoderma* also increases its tolerance against drought (Figure 1B) and salinity (Figure 1C)—demonstrated in its productivity. Similarly, an increase in the salt-tolerance was observed with the inoculation of tomatoes with *T. harzianum* [16,19] and *T. parareesei* [24], of cucumber with *T. harzianum* [52], and of wheat with *T. longibrachiatum* [53,54]; also, regarding drought-tolerance, with the inoculation of tomato with *T. longibrachiatum* or *T. harzianum* [55,56]—never before analyzed in the case of rapeseed.

Moreover, the inoculation with T6 and Tp-TC implies a significant increase in tolerance to abiotic stresses against inoculation with T34 (Figure 1B,C), probably due to the greater capacity for root colonization of T6 and Tp-TC that we have reported (Figure 2). In this sense, it has been demonstrated how, in situations of abiotic stresses, *Trichoderma* colonizes the plant's roots to a greater extent, in order to improve the plant's ability to tolerate stress. [57]. On the other hand, the rapeseed-inoculation with Tparo7-S4 under salt or drought stress had the same colonization levels as T6 and Tp-TC (Figure 2), but its ability to increase tolerance was significantly reduced to the levels observed for T34 (Figure 1C) or even lower (Figure 1B), never reaching the productive levels of uninoculated plants. This is due to the presence of the *Tparo7* gene in *T. parareesei*, a chorismate mutase protein/group of proteins implicated in tolerance to salt and drought stress in tomato [58].

Plants under abiotic stresses differentially express a range of genes involved in their tolerance to each stress, and the same can be said for rapeseed plants [59]. Under drought-stress, the key phytohormone in plant tolerance is ABA [60], so it increases the expression of its synthesis and response genes, including its PYL receptors [4] and *NCED* synthesis gene in rapeseed [32], as we have been able to observe in our study (Figure 3). Meanwhile, ET acts against salt stress [60], although an increase in the expression of the *ERF* response gene has been observed in both salinity and drought situations in *Brassica rapa* [61], as we have also observed with rapeseed (Figure 3).

The ability of *Trichoderma* to produce ACC-deaminase and prevent the accumulation of ET in the roots [57,62] has been extensively studied. In this sense, the inoculation of rapeseed with T34 under drought stress implies an increase in the expression of ET-related genes, something not observed in other studies, while under salt stress, the ABA-related genes increase, as observed by Contreras-Cornejo et al. (2010) in *A. thaliana* with the application of *T. virens* and *T. atroviride* [63]. In contrast, the inoculation with T6 and Tp-TC causes a significant increase in the expression of ABA-related genes in drought-stress and of ET-related genes in salt-stress, specific effective responses against both stresses and, therefore, the plants have a higher tolerance [60]. As far as ABA is concerned, the increase in the expression of the genes related to this hormone by inoculation with T6 and Tp-TC in rapeseed would have a direct relationship with its greater capacity for tolerance against drought, possibly due to plant physiological mechanisms of response to ABA as the regulation of stomatal aperture, key mechanism to limit water loss from leaves under drought conditions, and to induce the osmoprotectant compounds synthesis [20]. On the other hand, the increase in tolerance in rapeseed after inoculation with T6 and Tp-TC under salt stress would be directly related to an increase in the expression of genes related to ET, a hormonal route implied in tolerance against this type of stress thanks to alter Na^+/K^+ ratio and selectively activating salt-tolerance genes in plants [21].

Moreover, the plant accumulates ROS, which are a strong oxidative stress in the cells [64,65], and have been proven to cope with abiotic stresses such as salinity and drought. We have verified how the application of *Trichoderma* reduces this type of stress on rapeseed roots (Figure 4), as have other authors with tomato [16], *Arabidopsis* and cucumber [53], checking how the presence of CM in T6 plays a key role in this reduction, and verifying its importance in the regulation of the expression of ABA-related genes (Figure 3) in the plant [66,67].

This differential response between inoculation with T34 and T6 could be due to CM encoded by the *Tparo7* gene. We have verified that with *Tparo7* silenced, *Tparo7-S4*, loses its ability to increase tolerance against both abiotic stresses in rapeseed, while also ceasing to induce the expression of ABA- and ET-related genes that we can observe in the Tp-TC transformation control and in T6. This indicates the key role of CM in the induction of these genes and plant abiotic tolerance. Pérez et al. (2015) determined the relationship between CM and T6 biocontrol capacity, as to the prevent the synthesis of secondary metabolites resulting from the shikimate pathway that capacity is reduced [25]. The importance of CM in the plant response to salinity and drought has been proven in tomato—being involved in the synthesis of crucial aromatic amino acids against stress [58], in *Arabidopsis* [68], in *Musa acuminata* [69], and in *Salicornia herbacea* [70]. In addition, Pérez et al. (2015) observed a bigger production of SA in the transformants with silenced CM, and as this increase in SA levels leads to an increase in the expression of the gene related to ET *EIN2* in tomato after root colonization [25]. Under drought and salinity stress, SA increases the activity of antioxidant enzymes that alleviate oxidative stresses; therefore, the role of SA in salt and drought tolerance has been extensively evidenced in many crops [71]. Furthermore, a possible decrease in SA due to the presence of CM could be involved in increasing the tolerance of rapeseed against these abiotic stresses, thanks to the antagonism between SA-ET [72,73] and SA-ABA [74,75]. Therefore, the best response against abiotic stresses observed in rapeseed by inoculation with T6 and Tp-CT, through a specific response mediated by ABA and ET against drought and salinity, respectively, could be directly a consequence of a reduction in SA levels by CM, increasing the expression of the antagonist pathway genes.

In conclusion, *T. parareesei* represents a more efficient alternative than *T. harzianum* in increasing rapeseed productivity in drought or salinity conditions, probably thanks to a reduction in oxidative stress and the regulation of the expression of hormone genes related to abiotic stresses in the plant. Moreover, CM present in *T. parareesei* plays a key role in this phyto-fortifying capacity by increasing the expression of genes related to the plant's response to abiotic stresses through the hormonal pathways of ABA and/or ET.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/1/118/s1>, Figure S1. Block and sub block random distribution in the greenhouse; Table S1. Table of ANOVA two ways for

measurements of rapeseed-root colonization by *T. harzianum* (+T34), *T. parareesei* (+T6), *T. parareesei* transformation control (+Tp-TC), and the transformant with the silenced Tparo7 gene (+Tparo7-S4) without stress, under drought stress and under salt stress (NaCl 200mM); Table S2. Table of ANOVA two ways for indirect quantification of ROS in roots by relative ion leakage in the roots of rapeseed (Bn) inoculated with *T. harzianum* (+T34), *T. parareesei* (+T6), *T. parareesei* transformation control (+Tp-TC), and the transformants with the silenced Tparo7 gene (+Tparo7-S4).

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