

The presence of *Lens culinaris* at different nodulation stages alters the expression of the genes TdNAR2.2, TdNRT1.1, TdAMT1.1, and TdAMT1.2 in an intercropping system with *Triticum durum*

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Research Article

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Abstract

Background and Aims

Nitrogen (N) is an essential macronutrient for plant growth and key physiological processes. Consequently, the application of nitrogen fertilizers in agriculture is a widespread practice aimed at meeting crop nutritional demands. Excessive use of these fertilizers lead to environmental degradation. An alternative approach involves biological nitrogen fixation (BNF), which is carried out by legumes through symbiosis with rhizobia. Legume/cereal intercropping systems provide an opportunity to exploit this beneficial trait of legumes.

Methods

In the present study, we evaluated the effect of *Lens culinaris* at different stages of nodulation: non-nodulated (2 days), early nodulation (15 days), and late nodulation (30 days) on the growth and gene expression of *Triticum durum* in an intercropping system. Specifically, the expression of nitrate (NO_3^-) transporter genes and ammonium (NH_4^+) transporter genes. The intercropping system simulated alternating rows of both species, with or without KNO_3 application to mimic nitrogen fertilization.

Results

The presence of lentil at the late nodulation stage improved wheat growth when intercropping was combined with KNO_3 , while lentil growth was not markedly affected. Furthermore, wheat presence and nitrogen deficiency synergistically stimulated nodule formation in lentil at both early and late stages of interaction. Finally, the presence of non-nodulated lentil induced the expression of *TdNAR2.2*, *TdNRT1.1*, *TdAMT1.1*, and *TdAMT1.2* in intercropped wheat under fertilized conditions.

Conclusion

Our results suggest that *T. durum* growth benefits from intercropping with *L. culinaris*, due to enhanced nodulation in the legume and the upregulation of NO_3^- and NH_4^+ uptake genes.

Introduction

The presence of healthy soil is one of the primary requirements for achieving favorable agricultural yields. However, soil quality is affected by various elements, including abiotic factors (Kumar et al., 2020). In general, factors such as droughts, floods, rising temperatures, and variations in rainfall (driven by climate change) reduce crop yields, promote the proliferation of weeds and pest, and lead to crop failures in the short term and decreased production in the long term (Irrgang et al., 2022; Malhi et al., 2021; Sivaraj et al., 2017). Additionally, human activities have a significant impact on soil quality,

particularly through the use of synthetic agricultural inputs applied to fertilize crops (Díaz-Rodríguez et al., 2021). Nitrogen-based fertilizers are among the most widely used agricultural inputs, as nitrogen (N) plays a critical role in several essential plant processes, including growth, leaf area expansion, yield, chlorophyll synthesis, and phytohormone production. However, the excessive application of these fertilizers is a common practice among farmers, resulting in economic losses and serious environmental impacts such as water eutrophication, increased greenhouse gas emissions, soil acidification, and degradation (Anas et al., 2020).

An alternative to the use of nitrogen fertilizers is biological nitrogen fixation (BNF), a well-established process in leguminous plants in which atmospheric nitrogen (N_2) is fixed by specialized root structures called nodules. These nodules result from the legume–rhizobia symbiosis, where the rhizobia may belong to five different genera: *Rhizobium*, *Azorhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Bradyrhizobium* (Andrews and Andrews, 2017). BNF can also occur without nodule formation, in a process known as associative nitrogen fixation, wherein microorganisms reside on the surfaces or in the intercellular spaces of the host plant. These microorganisms utilize the plant's photosynthates to fix N_2 , supplying only the excess nitrogen to the plant. Both associative and symbiotic nitrogen fixation contribute to nitrogen input in agricultural systems, accounting for approximately 50–70 teragrams (Tg) and 21.5 Tg annually on a global scale, respectively (Guo et al., 2023). In BNF, atmospheric N_2 is converted into a plant-available form, primarily ammonium (NH_4^+) (Igarashi et al., 2003). NH_4^+ , along with nitrate (NO_3^-), can also be found in soils as inorganic forms of nitrogen, which are absorbed by plants and transported across the root plasma membrane. Consequently, depending on the nitrogen source, plants activate distinct transport pathways (Masclaux-Daubresse et al., 2010).

NO_3^- is taken up by plants and transported by various proteins belonging to the NRT1 and NRT2 families. The NRT1 family constitutes a diverse group of nitrate peptide transporters (NPF). NRT1 genes are organized into subfamilies, with NRT1.1 and NRT1.2 primarily responsible for NO_3^- uptake in roots. In contrast, the NRT2 family comprises a smaller and more specific group of transporters that function under low NO_3^- availability. Among them, *NRT2.1* and *NRT2.2* are the most important genes for nitrate uptake within the NRT2 family (Zayed et al., 2023). NRT2 proteins require the formation of complexes with an associated partner protein from the NAR2/NRT3 family (Nitrate Assimilation Related Protein/Nitrate Transporter 3). These are small membrane-associated proteins that play a crucial role in the NO_3^- uptake system and are essential for the stabilization and localization of NRT2 proteins in the plasma membrane (Kotur et al., 2012; Wirth et al., 2007). Additionally, two other families of NO_3^- transporters exist: CLC (Chloride Channel Family) and SLAC/SLAH (Slow Anion Channel/Slow Anion Channel Homolog). However, only transporters from the NPF and NRT2 families are significantly involved in nitrate uptake by plant roots (Noguero et al., 2016; Nacry et al., 2013). Regarding NH_4^+ uptake, plants utilize AMT transporters, which belong to a multigene family. Based on their sequence and structure, AMTs are classified into two subfamilies, AMT1 and AMT2, both of which are mainly expressed in plant roots (Akhtar et al., 2024).

In the context of Biological Nitrogen Fixation (BNF) by legumes, intercropping is a common agricultural practice that involves the mixed cultivation of two or more agriculturally important plant species in the same area. The most frequent combination is a legume/cereal intercrop. The incorporation of legumes significantly enhances soil organic carbon, available phosphorus (P) and total soil nitrogen. Generally, legume crops are a source of nitrogen in the soil through atmospheric nitrogen fixation, due to their symbiotic interaction with rhizobia (*Rhizobium* spp.). Additionally, the sole cultivation of legumes provides several soil-related benefits such as BNF, the recovery of acidic soils, more efficient phosphorus use, improved soil porosity, enhanced water storage and infiltration capacity, better soil particle aggregation, increased microbial activity, and higher organic matter content, among others (Chamkhi et al., 2022).

The integration of legumes in intercropping systems is particularly relevant when combined with maize (*Zea mays*) or wheat (*Triticum aestivum*), given their importance in human diets (Tsubo et al., 2005). For instance, durum wheat (*Triticum durum*) is intercropped with the economically valuable legume *Lens culinaris* (lentil), which helps reduce lodging of the legume stems, as wheat stems provide mechanical support (Loïc et al., 2018). Wheat-lentil intercropping also enhances wheat grain yield (Koskey et al., 2022). Furthermore, the presence of *L. culinaris* in intercropping with *T. durum* increases mycorrhizal formation (Lorenzetti et al., 2024).

In summary, in a legume/cereal intercropping system, the presence of the legume increases N availability in the soil, thereby enhancing the yield of the cereal component (Montejano-Ramírez & Valencia-Cantero, 2024). Additionally, cereals release root exudates that promote nodulation and, consequently, BNF (Li et al., 2016). However, to the best of our knowledge, the effect of legume presence on nitrogen uptake pathways in cereals within intercropping systems has not yet been studied. Therefore, the present study evaluated the interaction of lentils at different nodulation stages with *Rhizobium leguminosarum* UMER8 in an intercropping system with *T. durum*, and its effect on the expression of *TdNAR2.2* and *TdNRT1.1* genes involved in the NO₃⁻ uptake pathway, as well as the *TdAMT1.1* and *TdAMT1.2* genes associated with NH₄⁺ uptake.

Materials and Methods

Culture Media

PY medium was used for the cultivation of the nodulating bacterium *R. leguminosarum* UMER8. The PY medium was prepared as follows: 3 g of yeast extract per liter, 5 g of casein peptone per liter, 15 g of bacteriological agar per liter, and 7 mL of CaCl₂ per liter.

Growth of *R. leguminosarum*

R. leguminosarum UMER8 was streaked onto PY medium and incubated for 24 hours at 34°C prior to use.

Seed Germination

Seeds of *L. culinaris* and *T. durum* were surface-sterilized using commercial bleach for 4 and 3 minutes, respectively, and then rinsed six times with sterile deionized water. After sterilization, *L. culinaris* seeds were incubated for 24 hours in an inoculum of *R. leguminosarum* UMER8 (optical density at 595 nm adjusted to 0.1 absorbance units). Subsequently, seeds were sown in rows and kept separate by species (lentil seeds in one container and wheat seeds in another) in sterilized peat moss. The substrate was moistened with deionized water, and the containers were transferred to a Percival® growth chamber set to a photoperiod of 16 h light / 8 h dark at 24°C until germination.

T. durum - L. culinaris Intercropping Assay

Three days after germination, seedlings were transplanted into pots containing sterile peat moss substrate. Six plants were placed per pot in two rows of three, arranged either as 6 wheat and 6 lentil plants, or as 3 wheat and 3 lentil plants per row, to simulate planting furrows and intercropping. Each treatment consisted of 8 replicate pots. The treatments included: wheat-lentil under nitrogen-deficient conditions (TLN⁻, LTN⁻), wheat-lentil under nitrogen-sufficient conditions (TLN⁺, LTN⁺), lentil alone under N⁻ (LN⁻) and N⁺ (LN⁺) conditions, and wheat alone under N⁻ (TN⁻) and N⁺ (TN⁺) conditions. To induce nitrogen deficiency, plants were irrigated according to substrate moisture with deionized water and fertilized weekly (in the case of 15 and 30 day interaction treatments) or once (in the case of 2 day interaction treatments) using the Broughton and Dilworth (1970) nutrient solution, supplemented or not with KNO₃ (Final concentration of 49 mM) depending on the treatment. Following transplantation, lentil plants were inoculated with 1 mL of *R. leguminosarum* UMER8 (optical density at 595 nm adjusted to 1.0 absorbance units). The plants were then placed in a Percival® growth chamber set to 16 h light / 8 h dark at 24°C. Growth and gene expression parameters were measured at three time points corresponding to nodulation stages in lentils: 2 days (non-nodulated), 15 days (early nodulation), and 30 days (late nodulation).

Nodule Counting

At 15 and 30 days after the onset of the wheat-lentil intercropping interaction, total nodules and pink nodules were counted in *L. culinaris* plants.

RNA Extraction and cDNA Synthesis

To evaluate the expression of the genes *TdNRT1.1*, *TdNAR2.2*, *TdAMT1.1*, and *TdAMT1.2*, total RNA was extracted from the roots of *T. durum* plants grown in intercropping with lentils at different nodulation stages. RNA extraction was performed using the TRI Reagent (Cat. No. T9424, Sigma-Aldrich). Complementary DNA (cDNA) was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad). Prior to reverse transcription, RNA samples were treated with DNase I to eliminate genomic DNA contamination.

RT-qPCR reactions

RT-qPCR reactions and the oligonucleotide sequences used for amplification of the genes *TdNRT1.1*, *TdNAR2.2*, *TdAMT1.1*, *TdAMT1.2*, and the housekeeping gene, *Td18s* were based on the protocol described by Fileccia et al. (2017).

Statistical Analysis

Data were analyzed using two-way analysis of variance (ANOVA), followed by Duncan's multiple range test at a significance level of $p \leq 0.05$.

Results

The Presence of *L. culinaris* Alters the Growth of *T. durum* in an Intercropping System

Since it has been widely reported that legumes enhance nitrogen fixation in the soil through nodule formation via symbiosis with rhizobia (Ghosh et al., 2007), we analyzed the effect of *L. culinaris* presence at different nodulation stages, specifically non-nodulated (2 days), early nodulation (15 days), and late nodulation (30 days) on the growth of *T. durum* in an intercropping system under both nitrogen-sufficient (N+) and nitrogen-deficient (N-) conditions. This analysis aimed to elucidate the relationship between nodule presence, including both nodule number and developmental stage and the nitrogen contribution to the intercropped cereal (wheat). In wheat shoots (Fig. 1), the parameters evaluated included shoot length, biomass, and number of leaves.

After two days of interaction between wheat and lentil, the TN- treatment showed a decrease in shoot biomass compared to the control. No statistically significant differences were observed in shoot biomass or leaf number across treatments at this stage. At 15 days of interaction, a consistent pattern was observed in shoot length, biomass, and leaf number: both TLN + and TLN- treatments exhibited values comparable to the control, unlike TN-, indicating that nitrogen deficiency began to impair wheat growth at this time point. However, the presence of lentil in the early nodulation stage appeared to mitigate this effect. At 30 days, shoot length in the TLN + treatment remained comparable to the control, while TLN- showed values similar to TN-. This suggests that the presence of lentil at a late nodulation stage was not sufficient to counteract the negative effects of nitrogen deficiency on wheat growth. Furthermore, shoot biomass and leaf number increased in the intercropping treatment under nitrogen-sufficient conditions. In contrast, under N- conditions, these parameters decreased regardless of lentil presence. At this final interaction stage, nitrogen fertilization with KNO_3 was essential to observe a growth-promoting effect associated with lentil presence.

In the case of roots, the key parameters evaluated were root length, root biomass, and the number of lateral roots (Fig. 2).

After two days of interaction, no statistically significant differences were observed in any of the root parameters. After 15 days, the TLN- treatment showed a 36% increase in root biomass and a 25% increase in the number of lateral roots compared to the control. Finally, at 30 days, increases were observed in the intercropping treatments supplemented with KNO_3 ; both root biomass and the number of lateral roots were higher than in the control. However, no statistically significant differences were found in root length. Overall, the behavior of root parameters followed the same pattern observed in the shoot.

Based on these results for shoot and root growth parameters in wheat, it is suggested that the interaction between wheat and lentil promotes the growth of the cereal, particularly when the lentil is at an early nodulation stage (15 days). This suggests that the interaction facilitates greater nitrogen availability, sufficient to meet the demands of both species during the initial phase of nitrogen deficiency stress. However, under prolonged nitrogen deficiency (30 days), fertilization becomes necessary; when combined with intercropping with lentil at a late nodulation stage, a growth-promoting effect on wheat can be observed.

L. culinaris Growth in an Intercropping System with T. durum is Affected by Nitrogen Availability

In general, legume/cereal intercropping systems are mutually beneficial for both species (Chamkhi et al., 2022). To assess whether the presence of wheat influences lentil growth, shoot (Fig. 3) and root (Fig. 4) development parameters were measured at 2, 15, and 30 days of interaction under both nitrogen-sufficient and nitrogen-deficient conditions.

During the first two days of interaction, an increase in shoot biomass was observed exclusively in the fertilized intercropping treatment (LTN+), accompanied by a reduction in the number of nodes. At the early nodulation stage (15 days), nitrogen deficiency affected only shoot biomass in lentil; however, this reduction was counteracted by the presence of wheat. At the late nodulation stage (30 days), lentil shoot biomass decreased in the intercropping system with wheat, even under nitrogen-fertilized conditions (LTN+). This suggests a potential competition between the two species for nitrogen under prolonged stress, as this effect was not observed under nitrogen-deficient conditions.

Root length (Fig. 4b) was not significantly affected under any of the interaction conditions evaluated, regardless of nitrogen availability.

Root biomass was higher in the LN- treatment, both in non-nodulated lentils and those at the early nodulation stage. Only specific parameters, such as root biomass at 30 days and the number of lateral roots at 15 days, were affected by the presence of wheat, even when plants were fertilized (LTN+). Overall, the data on lentil shoot and root growth parameters indicate that lentil development is generally not negatively affected by the presence of wheat. Minor competitive interactions for KNO_3 may occur under certain conditions, but these do not significantly impair legume growth.

The Interaction with *T. durum* Enhances Nodulation in *L. culinaris* Under Both Nitrogen-Sufficient and Nitrogen-Deficient Conditions

As previously reported, the number of nodules tends to increase in legumes grown in intercropping systems with cereals (Liu et al., 2017). Based on this, we aimed to determine whether the presence of wheat influences nodule formation in lentil. This question is especially relevant given that, in the wheat–lentil interaction, particularly during early and late nodulation stages under both nitrogen-sufficient and nitrogen-deficient conditions, we observed increases in several wheat growth parameters without any detrimental effects on lentil development, which remained comparable to the control. To assess this, we quantified total and pink nodules in lentil plants inoculated with *R. leguminosarum* UMER8 and intercropped with wheat for 15 days or early nodulation (Fig. 5) and 30 days or late nodulation (Fig. 6).

During early nodulation, nitrogen deficiency combined with wheat presence (LTN-) significantly increased nodule formation, with total nodule number rising by 96%. This trend was also observed for pink nodules. Notably, even under nitrogen-fertilized conditions (LTN+), the number of total and pink nodules remained elevated in the intercropped treatments. In contrast, lentil monocultures under nitrogen-deficient conditions exhibited an increase in pink nodules relative to the control, but this was not accompanied by a corresponding increase in total nodules. Given that the highest nodulation levels occurred in the intercropped treatments, we suggest that the presence of wheat increases nitrogen demand, triggering an adaptive response in lentil by enhancing nodulation to meet the nutritional needs of the intercropping system.

On the other hand, at 30 days during the late nodulation stage (Fig. 6), it was observed that both nitrogen deficiency (LN-) and the presence of the intercrop under this condition (LTN-) induced a significant increase in total nodule formation, with increases of 132% and 200%, respectively.

Similarly, a significant increase in the number of pink nodules was observed, with increments of 161% and 555%, respectively. Additionally, plants grown under control conditions or in intercropping with sufficient nitrogen did not show a significant increase in nodulation at this stage, despite such an increase being observed at 15 days.

These results indicate that the increase in nodulation observed at 15 days, due to the presence of wheat in the lentil intercrop, is sustained over time, particularly in the TLN – treatment, where a considerable increase in both total and pink nodules was observed. This suggests that under prolonged nitrogen-deficient conditions, the lentil response remains similar to that observed at 15 days; that is, an enhanced formation of nodules as a strategy to meet the nitrogen nutritional demands of the intercrop.

The Presence of *L. culinaris* Affects NO_3^- and NH_4^+ Uptake Pathways in Intercropped *T. durum*

Given that the results from wheat growth indicate that the presence of lentil in the intercrop, particularly during the late nodulation stage, enhances the development of the cereal in both shoot and root related parameters, and considering that the formation of total and active (pink) nodules in lentil increases in the

presence of wheat, the expression of key genes involved in nitrogen transport in the cereal was evaluated. The objective of this analysis was to establish a potential relationship between the observed phenotypes and the uptake of different nitrogen sources through the regulation of genes associated with NO_3^- and NH_4^+ transport.

To analyze the NO_3^- uptake pathway, the relative expression of the *TdNAR2.2* and *TdNRT1.1* genes was assessed (Fig. 7).

The expression of *TdNAR2.2* was induced (7.78-fold increase in TLN + and 6.81-fold increase in TLN-) by interaction with lentils during early nodulation, regardless of nitrogen deficiency or sufficiency. This induction persisted at 15 days (4.01-fold increase in TN-); however, only nitrogen deficiency affected gene expression, as the presence of lentil during early nodulation did not exert a synergistic effect. During late nodulation in lentil (30 days), gene expression increased under nitrogen-deficient conditions (6.65-fold increase) as well as in the intercrop fertilized with KNO_3 (9.74-fold increase).

On the other hand, the expression of *TdNRT1.1* was induced only by fertilizer application and in combination with the presence of lentil at non-nodulated (2.58-fold increase) or early nodulation stages (2.32-fold increase). In the absence of fertilizer, gene expression was either repressed (at 2 days) or unchanged (at 15 days). Interestingly, at 30 days of interaction (late nodulation), gene expression was inhibited in the TLN + treatment (1.11-fold reduction), likely due to sufficient NO_3^- uptake or functional compensation by *TdNAR2.2*.

To analyze the behavior of the NH_4^+ uptake pathway, two key reference genes were evaluated: *TdAMT1.1* and *TdAMT1.2* (Fig. 8).

The expression of both genes increased within the first two days of interaction, with the presence of lentil playing a notable role. The highest expression levels were observed in the intercrop fertilized with KNO_3 (42.29-fold increase for *TdAMT1.1* and 16.34-fold increase for *TdAMT1.2*), and expression remained elevated under nitrogen-deficient conditions (4.34-fold increase) only for the *TdAMT1.1* gene. At 15 days, in the presence of lentil at the early nodulation stage, gene induction was maintained in the TLN + treatment for both genes (3.83-fold increase for *TdAMT1.1* and 2.20-fold increase for *TdAMT1.2*). Furthermore, nitrogen deficiency alone induced gene expression in the wheat monoculture when compared to the control (3.01-fold increase for *TdAMT1.1* and 1.46-fold increase for *TdAMT1.2*).

The peak of maximum expression reached in the genes *TdAMT1.1* and *TdAMT1.2*, at two days, with induction maintained at 15 days in the TLN + treatment, decreased at 30 days (lentil with late nodulation) in the *TdAMT1.1* gene (and remained at levels comparable to the control in the *TdAMT1.2* gene. However, in the latter gene, an induction in expression was observed in the TLN- (1.65-fold increase) treatment, which highlights a differential function between the two genes and is associated with the observed phenotype of increased nodulation in the legume under this treatment.

Discussion

Nitrogen is an essential element for plant growth, as it plays a role in all stages of development, including plant height, leaf number, stem diameter, and leaf area (Mohan, 2024). It is also vital for structural functions and for participating in the synthesis of key molecules for growth, such as proteins, nucleic acids, phospholipids, chlorophyll, hormones, vitamins, and alkaloids (Wang et al., 2024). The mechanism by which plants acquire nitrogen is through their roots (Muratore, 2021).

Nitrogen makes up 79% of the atmosphere in its diatomic form (N_2); however, its high availability does not prevent many plants from facing deficiencies of this nutrient due to the poor content of assimilable nitrogen forms in some agricultural soils (Fathi, 2022). To mitigate this deficiency, the use of nitrogen fertilizers is a common practice, with approximately 120 million tons applied annually worldwide (Yadav et al., 2017), increasing production costs and causing environmental issues, such as nitrogen leaching into groundwater and soil degradation due to salinization (Fathi, 2022).

An alternative to address nutrient deficiency in plants of agricultural interest is intercropping, where two or more crops are planted in the same soil and during the same growing season. The objective of this practice is to increase production in a specific area of land by maximizing its resources, which would not be possible in a monoculture system (Willey, 1979). Generally, legume/cereal intercropping is one of the most widely used systems due to its advantages, such as increasing harvest yields, improving soil properties, controlling weeds, and, most importantly, enhancing the availability of bioavailable nitrogen for plants through the increased presence of nitrogen-fixing bacteria (Yin et al., 2018; Nasar et al., 2020; Carton et al., 2020; Solanki et al., 2019). In addition to increasing the presence of nitrogen-fixing bacteria, legumes fix nitrogen symbiotically through root nodules. This fixed N is particularly advantageous for the legume when growing in nitrogen-limited conditions (Jensen, 1996), and it can potentially benefit crops present in an intercropping system (Isaac et al., 2012).

Based on this concept, our research evaluates the effect of intercropping wheat with lentil at different stages of nodulation and the nitrogen contribution from the legume to the grass, through changes in the expression of genes involved in NO_3^- and NH_4^+ uptake pathways.

Growth of *T. durum* and *L. culinaris* in an Intercropping System

In the growth results obtained, we observed that the presence of *L. culinaris* during early nodulation was able to meet the nitrogen nutritional requirements of *T. durum* plants in the intercrop. Shoot length, weight, and leaf number were similar to those in fertilized intercrop treatments, while root weight and the number of lateral roots were higher than in the fertilized controls. Furthermore, after 30 days of interaction, the addition of KNO_3 was necessary to observe a growth-promoting effect on shoot weight and leaf number of *T. durum*, induced by the presence of *L. culinaris* in a late nodulation stage. This growth pattern was also reflected in root weight and the number of lateral roots (Figs. 1 and 2). Generally, the objective of a legume/cereal intercrop is to enhance cereal growth due to the multiple benefits provided by the legume (Meena et al., 2018). Growth promotion of grasses intercropped with legumes has been widely reported, particularly in cereal species of agricultural, industrial, and nutritional importance, such as *Zea mays*, showing increased plant height, chlorophyll index, and yield (Ciarlo et al.,

2013; Latati et al., 2014). In the specific case of *T. durum* intercropped with *L. culinaris*, increases in biomass (Lorenzetti et al., 2023), flowering (Koskey et al., 2023), and yield (Koskey et al., 2022) have been reported. The presence of a legume in the intercrop promotes grass growth primarily by increasing soil nutrient availability, particularly nitrogen (Ghaley et al., 2005), through enhanced biological BNF (Garland et al., 2017), which in turn increases nitrogen rhizodeposition by the legume. This process is determined by the total nitrogen assimilation by the legume, overall root production, and plant age; meaning nitrogen rhizodeposition increases during senescence as roots release nitrogen into the soil (Wichern et al., 2008). Moreover, under nitrogen deprivation, intercrop legumes exude large amounts of ammonium, functioning as a unidirectional nitrogen transfer process from the legume to the grass (Paynel and Cliquet, 2003), which aligns with our results showing enhanced growth of *T. durum* in intercrop with *L. culinaris* even under nitrogen-deficient conditions. Additionally, nitrogen is deposited in the soil due to the continuous turnover of roots and nodules (Walker et al., 2003), supporting our findings that nodule presence is crucial in the intercrop. Fertilization does not affect nitrogen rhizodeposition by legumes (Xi Quan et al., 2021), which is consistent with our observed increased growth promotion in *T. durum* plants intercropped with *L. culinaris* and fertilized with KNO_3 . Although legume nodules typically have a lifespan of 10 to 12 weeks, their nitrogen-fixing capacity begins to decline 3 to 5 weeks after inoculation (Puppo et al., 2005), marking the onset of senescence. Considering that nitrogen rhizodeposition increases with plant age (Wichern et al., 2008), this may explain why *T. durum* plants showed greater growth after 30 days of interaction with *L. culinaris* in the late nodulation stage.

Although some studies have reported a reduction in legume growth when intercropped with cereals, a limitation that has been addressed by reducing the density of the cereal to minimize competition (Lorenzetti et al., 2024; Latati et al., 2014), our results did not show a reduction in most of the evaluated parameters. However, a slight competitive interaction between *T. durum* and *L. culinaris* could be inferred, as a decrease in shoot biomass was observed in the LTN + treatment at 30 days of interaction. Additionally, a reduction in root biomass at 15 and 30 days, as well as a lower number of lateral roots at 15 days, was noted under the same conditions (LTN+). In contrast, other studies have reported an increase in the yield of *L. culinaris* when intercropped with *T. durum* (Leoni et al., 2023), attributing this benefit to the structural support provided by the cereal stems, which helps prevent lodging of the legume (Loïc et al., 2018).

Effect of *T. durum* Presence on *L. culinaris* Nodulation

Considering that the amount of N assimilated by the legume influences the amount of N exuded into the soil through rhizodeposition (Wichern et al., 2008), and that nodules play a central role in the biological nitrogen fixation (BNF) process (Andrews and Andrews, 2017), the present study also evaluated nodulation in *L. culinaris*. During the early nodulation stage, the presence of *T. durum* increased both the total number of nodules and the number of pink nodules in *L. culinaris*, even under fertilized conditions (Fig. 5). However, in the late nodulation stage, this effect was only observed in intercropping systems under nitrogen-deficient conditions, with increases of 161% in total nodulation and 555% in pink nodules compared to the control (Fig. 6). In legume/cereal intercropping systems, increased nodulation in the

legume component has been previously reported. For example, in *Vicia faba*, the presence of *Triticum aestivum* has been shown to enhance total nodulation even under water-deficit stress, with a greater effect observed in the deeper soil layer (15–30 cm). Bargaz et al. (2016) suggest that this phenotype results from interactions between the root systems of both species, along with the low nutrient availability, as the plants were unfertilized for 49 days. Consequently, nodule formation was likely a response to promote N acquisition, a pattern consistent with the observations in our LN- and LTN- treatments. Furthermore, the presence of a grass species alone has been shown to stimulate nodulation in legumes through the release of root exudates. In the case of *Z. mays*, these exudates enhance flavonoid exudation in *V. faba*, which in turn mediates the chemoattraction of rhizobia. This increase in flavonoid production is driven by the induction of the *chalcone-flavanone isomerase* gene, as well as other nodulation-related genes such as *nodulin-like 4 (NODL4)*, *early nodulin-like 2 (ENODL2)*, and *early nodulin 93 (ENOD93)*, the latter remaining upregulated at 35 days (Li et al., 2016). These findings help explain the increased nodulation observed in *L. culinaris* in the TLN+ treatment, and the stronger effect observed when intercropping is combined with N deficiency (TLN-). Additionally, the nodule density in legumes intercropped with grasses has been positively correlated with ammonium (NH_4^+) concentrations in the rhizosphere. Moreover, both NO_3^- and NH_4^+ levels have been reported to be higher in intercropping rhizospheres (Qiao et al., 2016). Taken together, these findings, along with the increased nodulation observed, suggest a potential correlation with the enhanced growth observed in *T. durum* plants intercropped with *L. culinaris*.

Changes in the Expression of *T. durum* Genes Involved in NO_3^- and NH_4^+ Uptake Due to Intercropping with *L. culinaris*

To establish a relationship between the increased growth of *T. durum* in the presence of *L. culinaris* and nitrogen (N) availability, as well as the enhanced nodulation of *L. culinaris* when intercropped with *T. durum*, our study also evaluated the expression of genes involved in NO_3^- and NH_4^+ uptake pathways, in order to assess the N nutritional status of the cereal in the intercropping system. In the NO_3^- uptake pathway, we observed that at 2 days, the expression of the *TdNAR2.2* gene increased in both the TLN+ and TLN- treatments. Although expression levels declined by day 15, induction relative to the control was maintained in the TN- and TLN- treatments. Notably, the highest expression observed throughout the interaction period occurred at 30 days in the TLN+ treatment; however, induction was also detected in the TN- treatment (Fig. 7). It is well established that NAR2 proteins play a role in the activation of high-affinity transport systems (HATS) (Orsel et al., 2007), such as NRT2, which functions under low NO_3^- concentrations (Wang et al., 2012). In *Arabidopsis thaliana*, the existence of an oligomer, proposed to be a tetramer composed of two AtNRT2.1 and two AtNAR2.1 subunits, has been demonstrated (Yong et al., 2010). Furthermore, the expression of *AtNAR2.1*, *AtNRT2.1*, and *AtNRT2.2* was shown to be tightly coordinated with the regulation of the HATS nitrate uptake system, being induced by low NO_3^- concentrations and nitrogen deprivation, and suppressed by high NO_3^- supply (Feng et al., 2011). These findings explain the *TdNAR2.2* gene induction observed in the TN- and TLN- treatments at 15 days, and in TN- at 30 days. However, at 2 days, it is possible that *T. durum* detects the presence of *L. culinaris*, thereby activating the NO_3^- uptake machinery despite the application of KNO_3 (TLN+). This hypothesis is

supported by the induction observed in TLN⁻, where the constant variable is the presence of *L. culinaris*. Moreover, the increased *TdNAR2.2* induction observed at 30 days in the TLN⁺ treatment may be attributed to NH₄⁺ rhizodeposition (Paynel & Cliquet, 2003) associated with the late nodulation stage of *L. culinaris*, which could lead to the presence of an NH₄⁺/NO₃⁻ mixture. This could influence the expression patterns of NO₃⁻ transporters, as such behavior has been reported at 25%/75% NH₄⁺/NO₃⁻ ratios (Zhang et al., 2022). Additionally, NH₄⁺ itself inhibits the expression of NO₃⁻ transporters (Aslam et al., 1996), which may explain why, in the TLN⁻ treatment, where NH₄⁺ levels are presumably higher due to nodulation, the *TdNAR2.2* expression levels are similar to the control and are not activated by N deprivation. The other gene evaluated in the nitrate uptake pathway was *TdNRT1.1*, whose expression was induced in the TLN⁺ treatment at both 2 and 15 days of interaction with *L. culinaris*. However, this induction declined by day 30, showing even repression at this time point. In contrast, nitrogen N deficiency led to a downregulation of *TdNRT1.1* expression in the TN⁻ treatments at both 2 and 15 days. Interestingly, under N-deficient conditions, the presence of *L. culinaris* restored gene expression levels to values similar to those observed in the control treatment. The NRT1.1 transporter is responsible for the majority of nitrate (NO₃⁻) uptake in plants via the roots (Léran et al.), thereby regulating the distribution of NO₃⁻ to different plant tissues and contributing primarily to plant growth (Fang et al., 2021). Moreover, NRT1.1 acts as a key nitrate sensor and regulates various physiological and developmental processes in response to NO₃⁻, in addition to activating the expression of genes involved in the nitrate uptake pathway (Bouguyon et al., 2015). These functions explain the expression pattern of *TdNRT1.1* observed in the fertilized treatments (TLN⁺), where NO₃⁻ is available, as well as the repression in the TN⁻ treatments, where nitrogen is absent and thus unable to activate the nitrate transport machinery. The expression levels of *TdNRT1.1* observed in the TLN⁻ treatments, which were similar to the control, could be attributed to ammonium (NH₄⁺) exudation by *L. culinaris*. In *Arabidopsis thaliana*, it has been demonstrated that concentrations of 5 mM NH₄⁺ can induce the expression of the *AtNRT1.1* gene (Li et al., 2025).

In relation to the NH₄⁺ uptake pathway, we found that the expression of both *TdAMT1.1* and *TdAMT1.2* (Fig. 8) followed a similar pattern, with the highest induction observed at 2 days (no-nodulation stage) under the TLN⁺ treatment. Although expression levels decreased by 15 days (early nodulation stage), the presence of *L. culinaris* in the fertilized intercropping condition (TLN⁺) was key to sustaining gene induction. Additionally, the absence of nitrogen per se (TN⁻) also led to an increase in expression compared to the control. The most distinctive result between the expression patterns of *TdAMT1.1* and *TdAMT1.2* was observed at 30 days (late nodulation stage), where *TdAMT1.2* exhibited an increase in expression under TLN⁻ treatment. AMT1 proteins are high-affinity NH₄⁺ transporters. In *Arabidopsis thaliana*, *AtAMT1.1* and *AtAMT1.3* account for approximately 30–35% of NH₄⁺ uptake in nitrogen-deficient roots (Loqué et al., 2006), whereas *AtAMT1.2* contributes about 18–26% (Yuan et al., 2007). Furthermore, *AMT1.1* and *AMT1.2* exhibit distinct expression patterns: while *AMT1.1* is expressed in both leaves and roots, *AMT1.2* is mainly root-specific (Engineer & Kranz, 2007; Yuan et al., 2007; Wu et al., 2015). Generally, the expression of *AMT1.1* and *AMT1.2* is downregulated in plants fertilized with 25 mM KNO₃ (Huang et al., 2015), while nitrogen deficiency upregulates *AMT1* transporters (Yuan et al.,

2007). These data highlight that, in the TLN + treatments, the presence of *L. culinaris* is responsible for inducing the expression of *TdAMT1.1* and *TdAMT1.2*, rather than KNO_3 fertilization. This also explains why nitrogen deficiency at 15 days of interaction induces these genes. During intercropping, root-to-root communication occurs between legumes and non-legumes through exudates (Homulle et al., 2022), which may explain the early expression (at 2 days) of *TdAMT1.1* and *TdAMT1.2* in response to *L. culinaris* presence. Moreover, the increased expression of *TdAMT1.2* observed at 30 days under nitrogen deficiency may be explained by NH_4^+ rhizodeposition from *L. culinaris* (Paynel & Cliquet, 2003), which is likely perceived by *T. durum*. Alternatively, root contact and nitrogen transfer from the legume (Heijden & Horton, 2009) could also be responsible, especially considering that *AMT1.2* is predominantly expressed in roots (Yuan et al., 2007).

Conclusion

our research demonstrates that the presence of *L. culinaris* in an intercropping system with *T. durum* promotes the growth of the grass, particularly during the late nodulation stage, at 30 days. This growth promotion is associated with an increase in nodulation of the legume, as well as an induction in the expression of *T. durum* genes involved in NO_3^- and NH_4^+ uptake pathways; *TdNAR2.2*, *TdNRT1.1*, *TdAMT1.1*, and *TdAMT1.2*. Notably, this gene induction occurs as early as the initial stages of legume/cereal interaction (2 days).

Abbreviations

TN+ *T. durum* fertilized with KNO_3

TN- *T. durum* unfertilized with KNO_3

TLN+ *T. durum* intercropped with *L. culinaris* and fertilized with KNO_3

TLN- *T. durum* intercropped with *L. culinaris* unfertilized

LN+ *L. culinaris* fertilized with KNO_3

LN- *L. culinaris* unfertilized

TLN+ *L. culinaris* intercropped with *T. durum* and fertilized with KNO_3

TLN- *L. culinaris* intercropped with *T. durum* unfertilized

Declarations

Competing Interest:

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

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Conceptualization, F.J.C.-M, V.M.-R. and E.V.-C.; methodology, F.J.C.-M and V.M.-R.; validation, V.M.-R. and E.V.-C.; formal analysis, V.M.-R and F.J.C.-M investigation, F.J.C.-M and V.M.-R resources, E.V.-C.; writing—original draft preparation, F.J.C.-M.; writing—review and editing, V.M.-R and E.V.-C.; funding acquisition, E.V.-C.

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Figures

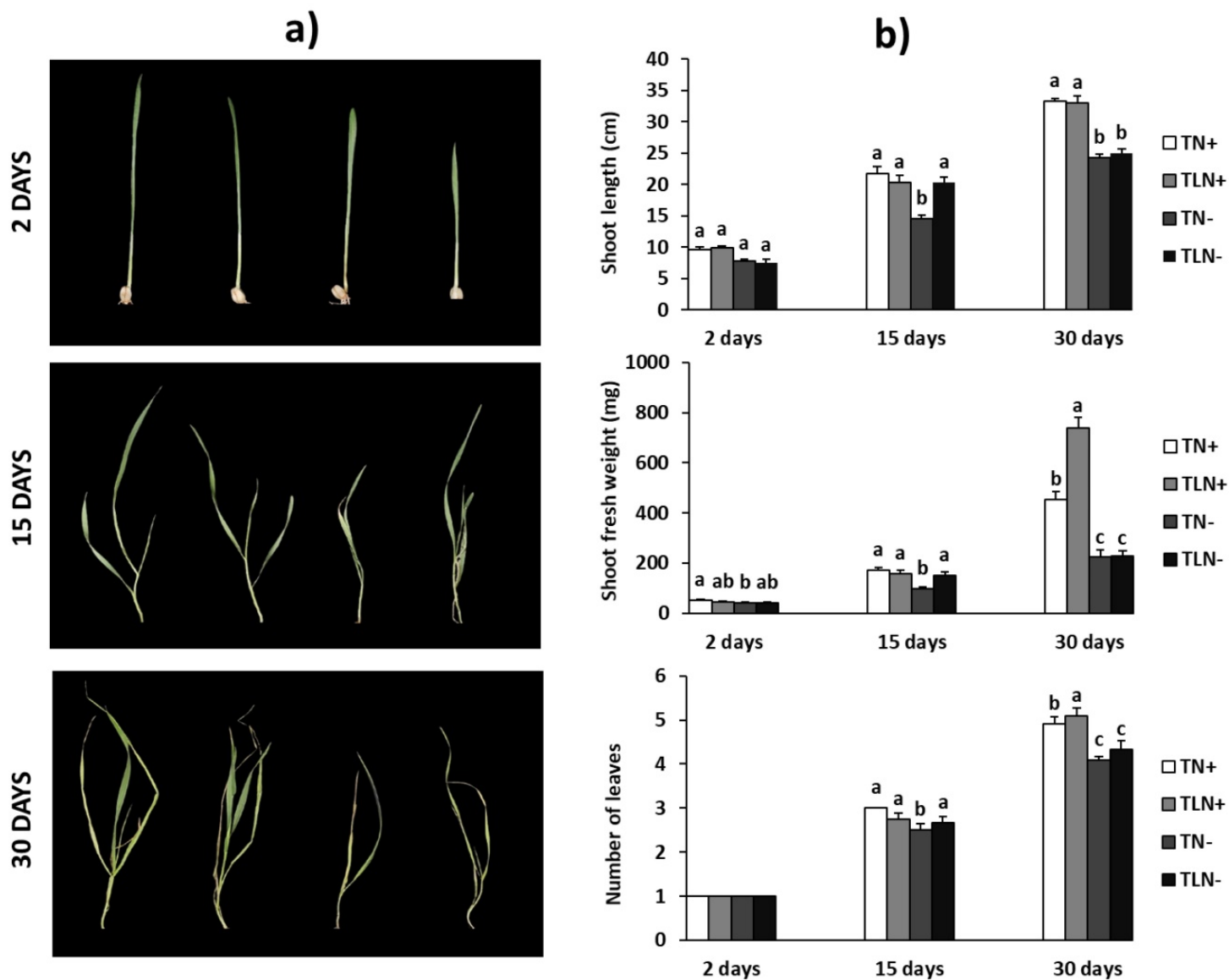


Figure 1

Analysis of growth parameters in wheat shoots intercropped with non-nodulated lentils, and lentils exhibiting early and late nodulation. a) Shoots at 2, 15 and 30 of growth. b) Growth parameters evaluated in wheat shoots. The white control bars correspond to the TN+ treatment, representing wheat plants fertilized with KNO_3 . The second bar represents wheat and lentil plants both fertilized with KNO_3 . The third bar corresponds to the TN- treatment, consisting of wheat plants grown under nitrogen-deficient conditions. The fourth bar represents the TLN- treatment, comprising wheat and lentil plants grown under nitrogen deficiency. The labels on the x-axis indicate the number of days of intercrop interaction. Different lowercase letters above the standard error bars indicate statistically significant differences among treatments, as determined by two-way ANOVA followed by Duncan's multiple range test ($P < 0.05$; $n = 12$).

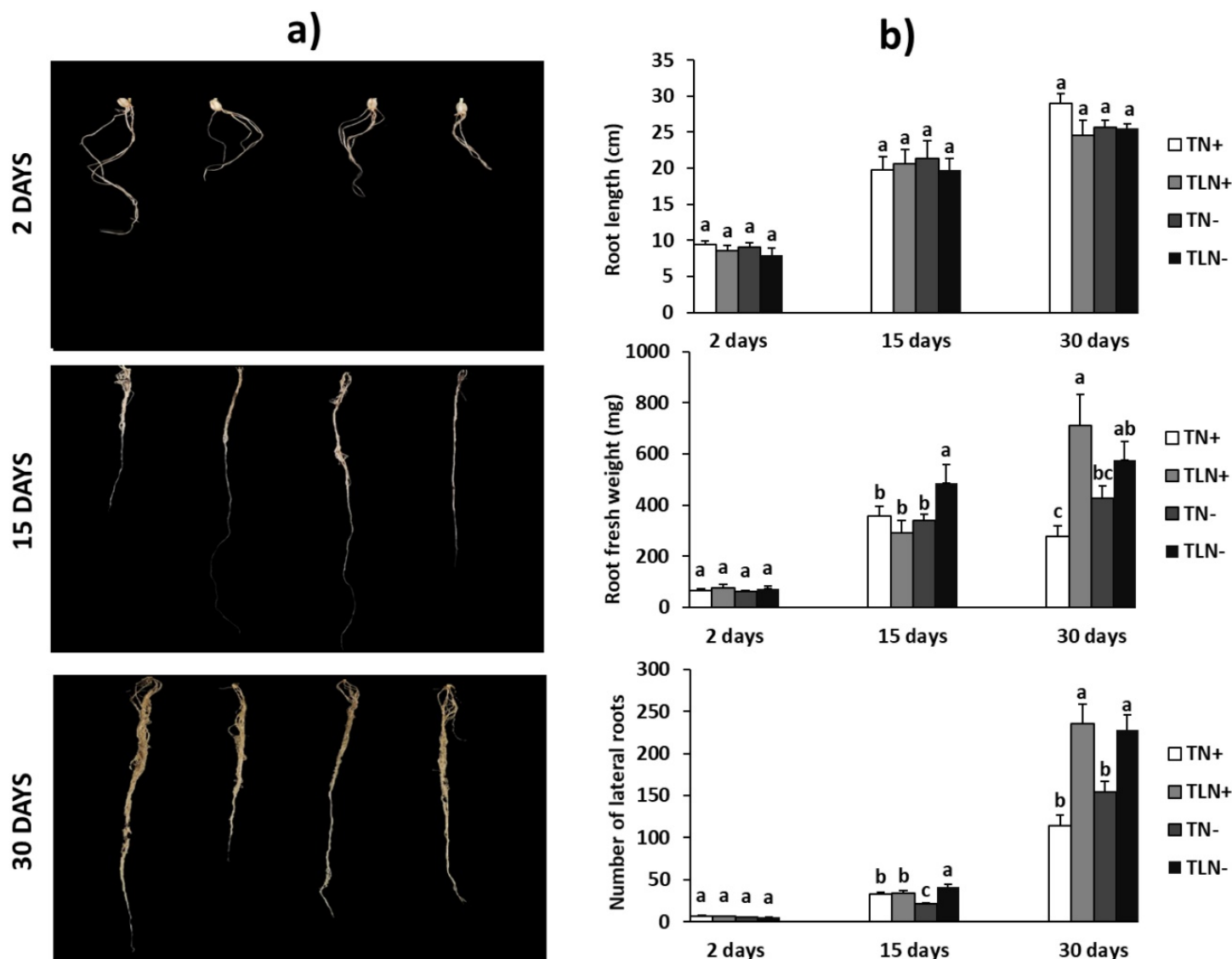


Figure 2

Analysis of root growth parameters in wheat intercropped with non-nodulated lentils, and lentils undergoing early and late nodulation. a) Roots at 2, 15 and 30 days of growth. b) Growth parameters evaluated in wheat roots. The white control bars correspond to the TN+ treatment, representing wheat plants fertilized with KNO_3 . The second bar corresponds to wheat and lentil plants both fertilized with KNO_3 . The third bar represents the TN- treatment, consisting of wheat plants grown under nitrogen-deficient conditions. The fourth bar corresponds to the TLN- treatment, consisting of wheat and lentil plants grown under nitrogen-deficient conditions. Labels on the x-axis indicate the number of days of intercropping interaction. Different lowercase letters above the standard error bars denote statistically significant differences between treatments, as determined by two-way ANOVA followed by Duncan's multiple range test ($P < 0.05$; $n = 12$).

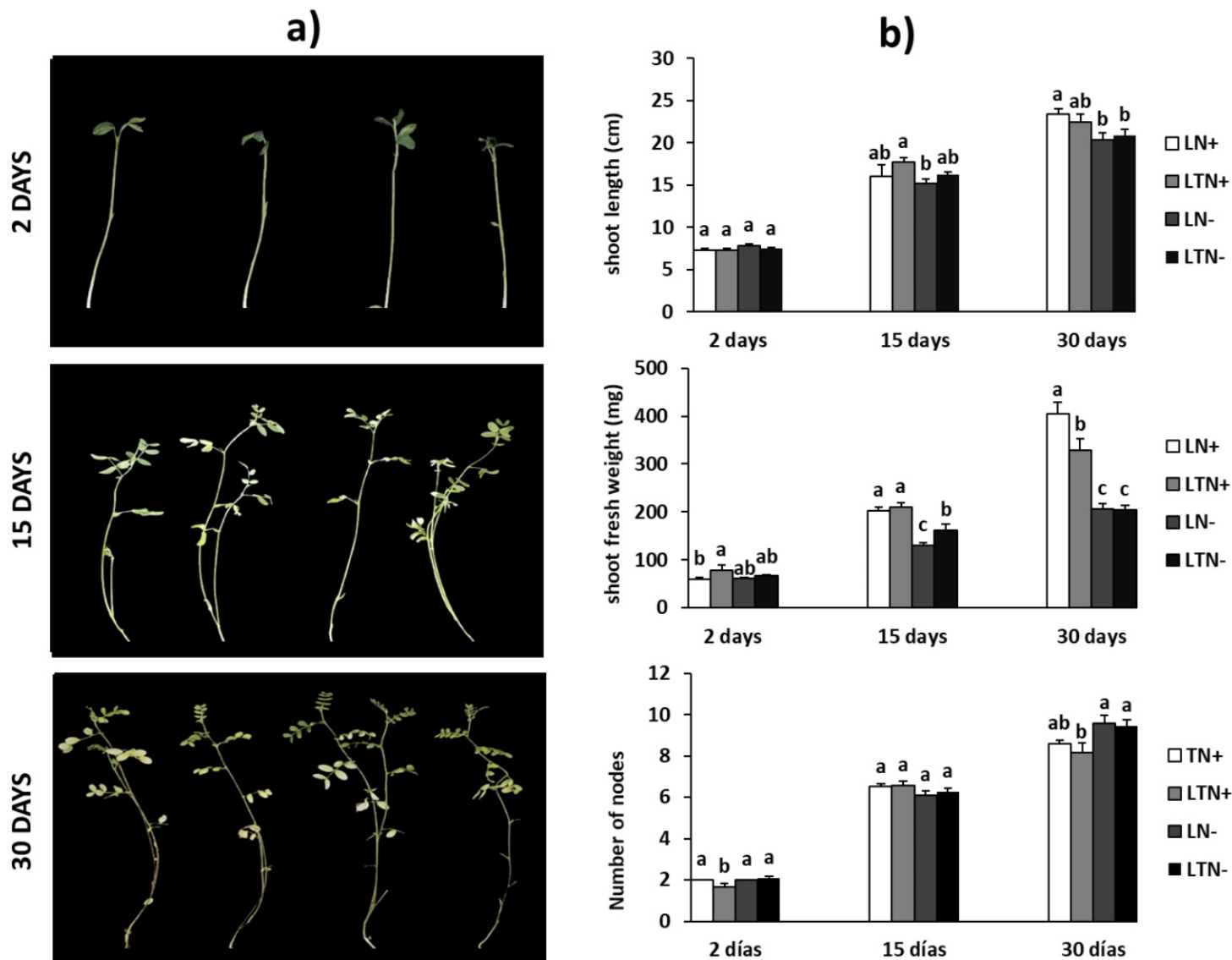


Figure 3

Analysis of shoot growth parameters in lentils non-nodulated, early nodulated, and late nodulated intercropped with wheat. . a) Shoots at 2, 15 and 30 of growth. b) Growth parameters evaluated in lentil shoots. The white control bars correspond to the TN+ treatment, representing wheat plants fertilized with KNO_3 . The second bar represents wheat and lentil plants both fertilized with KNO_3 . The third bar corresponds to the TN- treatment, consisting of wheat plants grown under nitrogen-deficient conditions. The fourth bar represents the TLN- treatment, in which wheat and lentil plants were grown under nitrogen-deficient conditions. The x-axis labels indicate the number of days of intercropping interaction. Different lowercase letters above the standard error bars indicate statistically significant differences among treatments, determined by two-way ANOVA followed by Duncan's multiple range test ($P < 0.05$; $n = 12$).

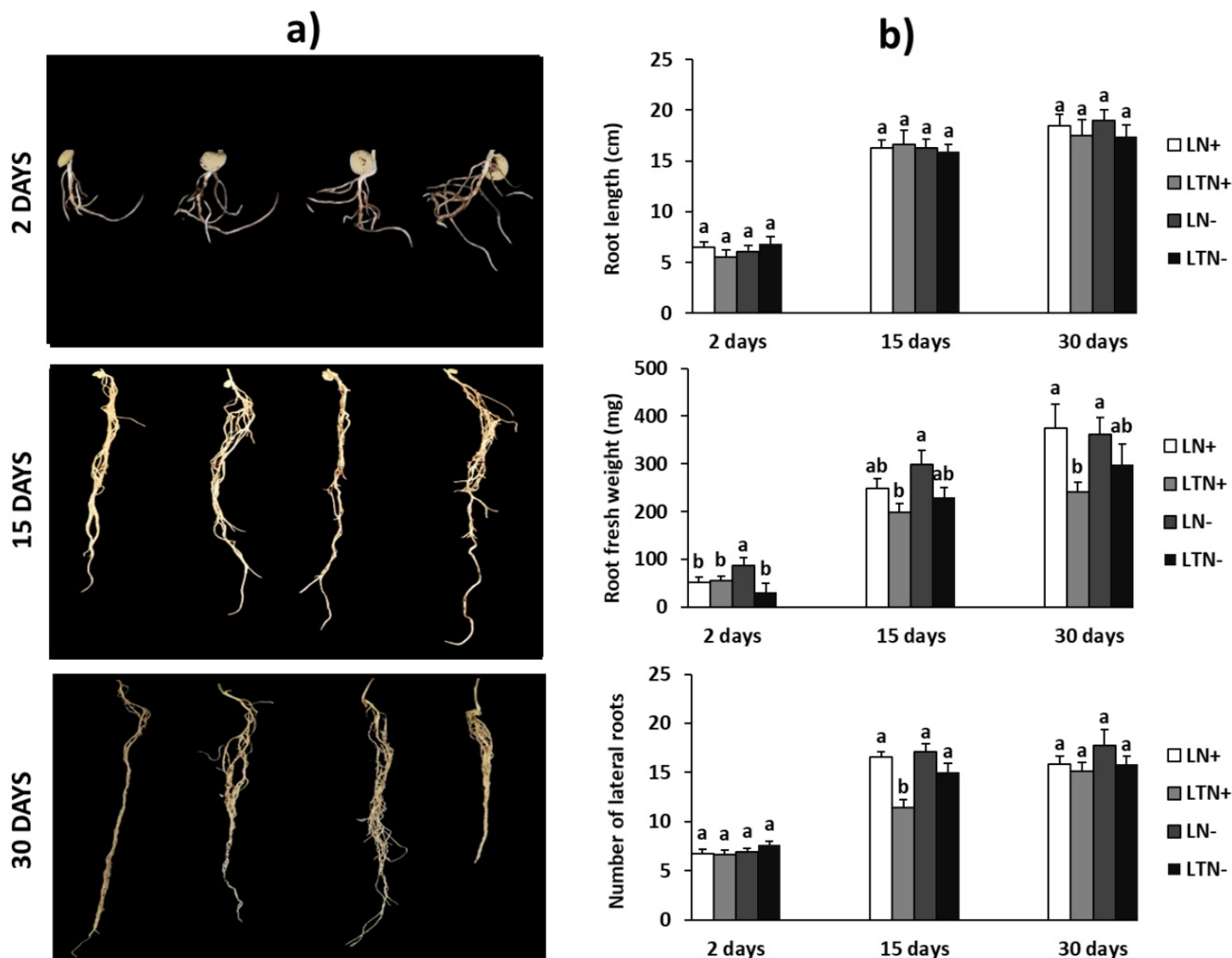


Figure 4

Analysis of root growth parameters in lentils non-nodulated, early nodulated, and late nodulated

intercropped with wheat. A a) Roots at 2, 15 and 30 days of growth. b) Growth parameters evaluated in lentil roots. The white control bars correspond to the TN+ treatment, representing wheat plants fertilized with KNO_3 . The second bar represents wheat and lentil plants both fertilized with KNO_3 . The third bar corresponds to the TN- treatment, consisting of wheat plants grown under nitrogen-deficient conditions. The fourth bar represents the TLN- treatment, in which wheat and lentil plants were grown under nitrogen-deficient conditions. Labels on the x-axis indicate the number of days of intercropping interaction. Different lowercase letters above the standard error bars indicate statistically significant differences among treatments, as determined by one-way ANOVA followed by Duncan's multiple range test ($P < 0.05$; $n = 12$).

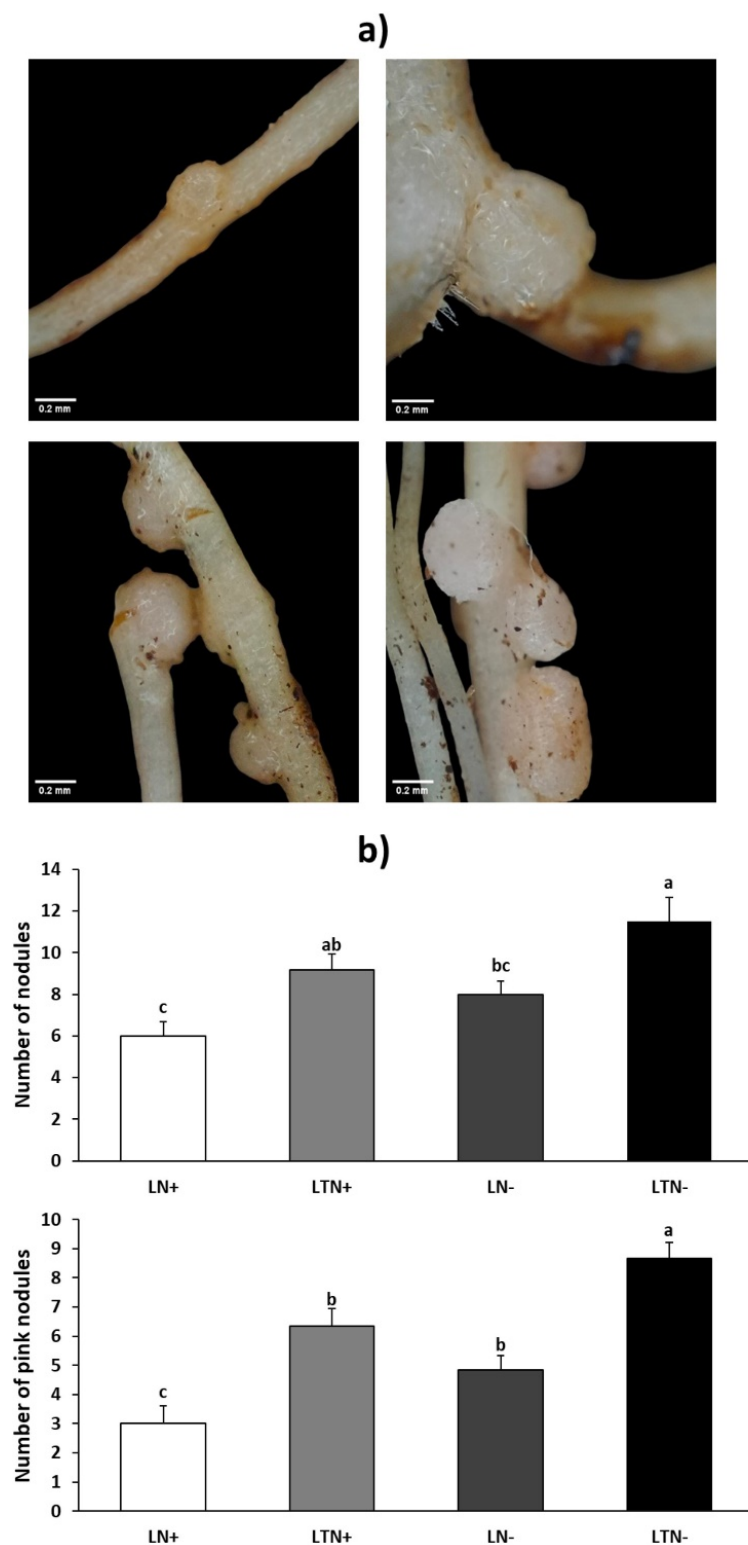


Figure 5

Effect of nitrogen deficiency and the presence of wheat on lentil nodulation after 15

days of interaction. a) Root nodules in lentils under different treatment conditions. b) Parameters evaluated in nodules. The white control bars correspond to the TN+ treatment, representing wheat plants fertilized with KNO_3 . The second bar represents wheat and lentil plants both fertilized with KNO_3 . The

third bar corresponds to the TN- treatment, consisting of wheat plants grown under nitrogen-deficient conditions. The fourth bar represents the TLN- treatment, in which wheat and lentil plants were grown under nitrogen-deficient conditions. Labels on the x-axis indicate the number of days of intercropping interaction. Different lowercase letters above the standard error bars indicate statistically significant differences among treatments, as determined by two-way ANOVA followed by Duncan's multiple range test ($P < 0.05$; $n = 12$).

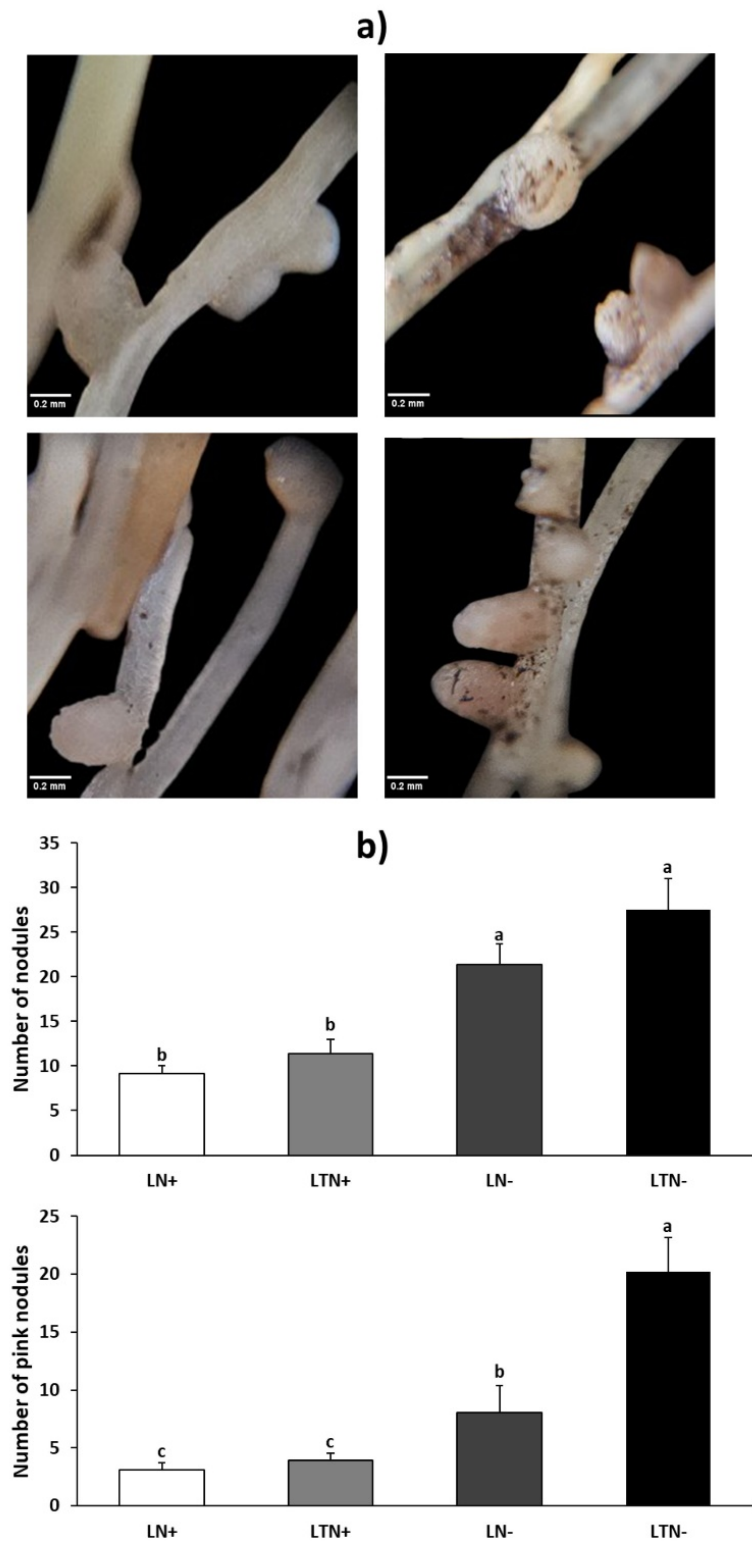
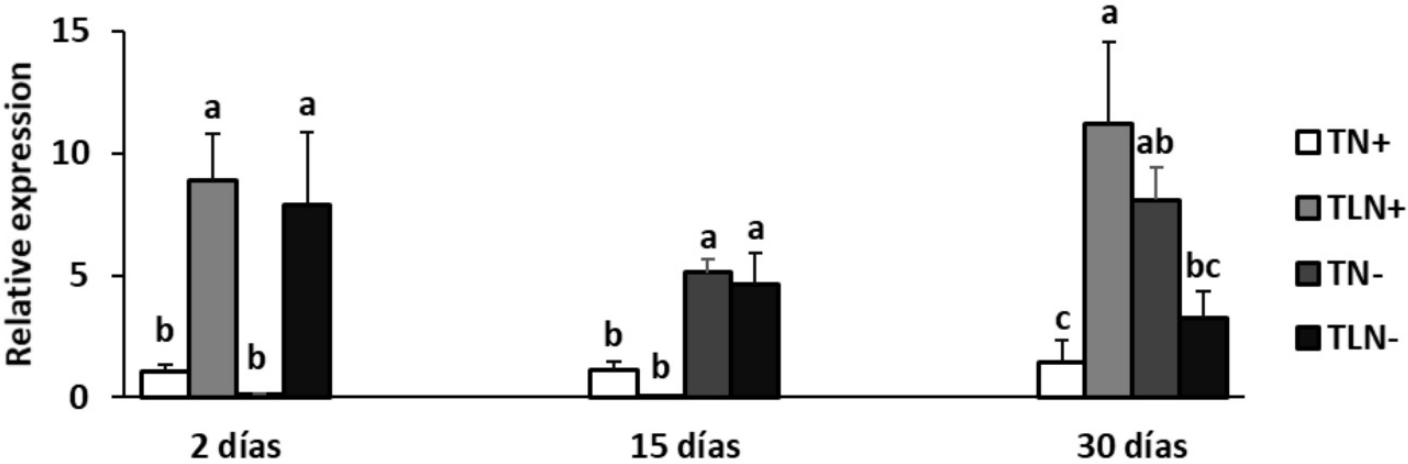


Figure 6

Effect of nitrogen deficiency and the presence of wheat on lentil nodulation after 30 days of interaction.a) Root nodules in lentils under different treatment conditions. b) Parameters evaluated in nodules. The white control bars correspond to the TN+ treatment, representing wheat plants fertilized with KNO₃. The second bar represents wheat and lentil plants both fertilized with KNO₃. The third bar corresponds to the TN- treatment, consisting of wheat plants grown under nitrogen-deficient conditions. The fourth bar represents the TLN- treatment, in which wheat and lentil plants were grown under nitrogen-deficient conditions. Labels on the x-axis indicate the number of days of intercropping interaction. Different lowercase letters above the standard error bars indicate statistically significant differences among treatments, as determined by two-way ANOVA followed by Duncan's multiple range test (P < 0.05; n = 12).

a)

TdNAR2.2



b)

TdNTR1.1

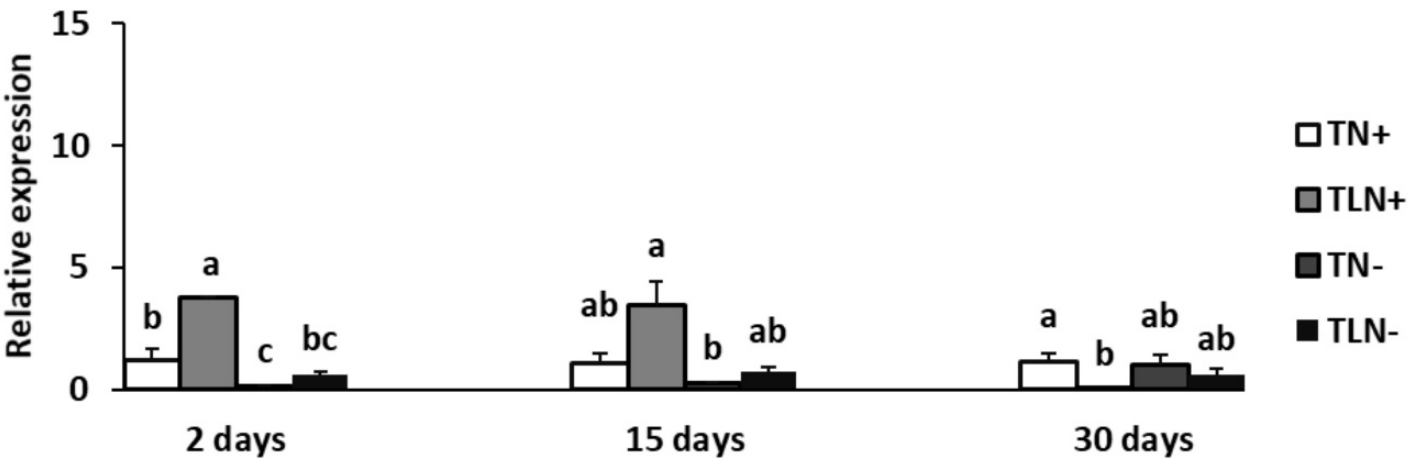


Figure 7

Relative expression of *tdNAR2.2* and *tdNRT1.1* genes involved in nitrogen uptake and transport in wheat at different time intervals. A) Relative expression of *tdNAR2.2*. B) Relative expression of *tdNRT1.1*. Labels on the x-axis indicate the number of days of intercropping interaction. Different lowercase letters above the standard error bars indicate statistically significant differences among treatments, as determined by two-way ANOVA followed by Duncan's multiple range test ($P < 0.05$; $n = 3$).

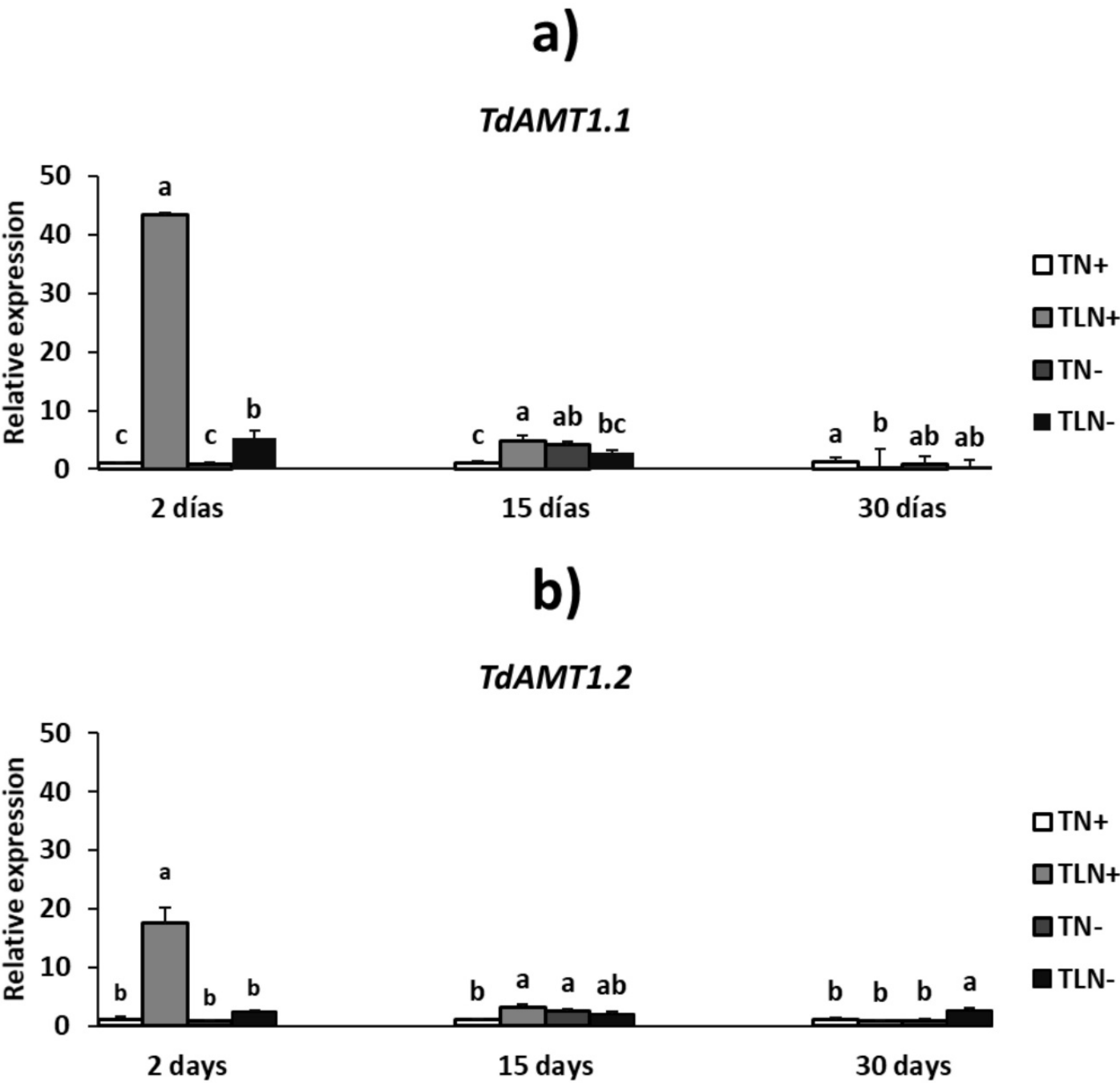


Figure 8

Relative expression of *tdAMT1.1* and *tdAMT1.2* genes involved in nitrogen uptake and transport in wheat at different time intervals. A) Relative expression of *tdAMT1.1*. B) Relative expression of *tdAMT1.2*. Labels on the x-axis represent the number of days of intercropping interaction. Different lowercase letters above the standard error bars indicate statistically significant differences among treatments, as determined by two-way ANOVA followed by Duncan's multiple range test ($P < 0.05$; $n = 3$).