Influence of beneficial fungi on NRT2.4 expression in *Arabidopsis thaliana* grown under stress condition

Master's Thesis

Submitted by

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Jena, Germany, 2019

This Master thesis was conducted at Max Planck Institute for Chemical Ecology, in the independent research group “Plant defense Physiology” under supervision of PD Dr. Axel Mithöfer in collaboration with Prof. Dr. Ralf Oelmüller’s laboratory at the Friedrich-Schiller-Universität Jena.

চিত্ত যেথা ভয়শূন্য, উচ্চ যেথা শির,

জ্ঞান যেথা মুক্ত, যেথা গৃহের প্রাচীর

রবীন্দ্রনাথ ঠাকুর

Where the mind is without fear and the head is held high

Where knowledge is free

Rabindranath Tagore

To my family and friends.

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1. Introduction

1.1. Nitrogen in the environment

Nitrogen (N) is one of the most abundant elements found in the environment. It is one of the essential regulators for plant growth processes such as seed dormancy, root growth, leaf development as well as an essential component of proteins, nucleic acids, and chlorophyll (Alboresi et al. 2005). In the environment nitrogen availability influences the plant growth. Nitrogen increase the crop yield; therefore, the use of N fertilizers is important to meet the requirement of increasing food demand. The deficiency of nitrogen in plants is physiologically visible (Figure 1) (Fan et al. 2017; Goel & Singh 2015). Interestingly approximately 78% in the atmospheric environment is nitrogen, which is nevertheless not directly accessible to the plant. Nitrogen-fixing bacteria such as Rhizobium, Azotobacter, Clostridium have a major role in nitrogen fixation and generate ammonium that can be converted into nitrate and nitrogen containing compounds. In the soil, nitrogen is available in the form of inorganic or organic components. Plant roots are able to absorb nitrogen from the soil in the form of ammonium (NH_4^+), nitrate (NO_3^-) and amino acids (Fan et al. 2017). Among those, nitrate is the major inorganic nitrogen source for plants in aerobic soils. To acquire nitrate from soil, plants have to compete with microbes and other plants. Some plants attract soil fungi, which contribute to nitrate absorption as well as to plant growth in general. Plants efficiently acquire nitrogen and distribute it from source to sink organs under various environmental conditions. From nitrate acquisition to nitrate translocation plants have evolved different strategies and systems (Kiba et al. 2012).

https://labmodules.soilweb.ca/nutrients-nitrogen/

Figure 1: Wheat plants grown in no nitrogen (right) and full nitrogen (left) soil-conditions. Nitrogen deficiency reduces the growth of plants (right) and also induces chlorosis.

1.2. Nitrogen acquisition

1.2.1. Transportation of Nitrate

Nitrate is the main water-soluble nitrogen source in soil. Plant roots are able to take up nitrogen in the form of nitrate but not in the form of nitrite. In order to survive in soil environments with different amounts of nitrate present, plants have evolved different transport systems to take up nitrate (Krapp et al. 2014).

Based on the nitrogen acquisition, plants have two classes of transporter systems to ensure the efficiency of nitrate uptake: nitrate transport systems with high affinity, called High-Affinity Transporters (HATs) and with low affinity called Low-Affinity Transporter (LATs) (Kiba et al. 2012). Nitrate transporters have a diverse role in nitrate acquisition from soil. So far, four nitrate transporter families have been identified, those are *NRT1/PTR* (*NPF*, nitrate transporter 1/peptide transporter family), *NRT2* (nitrate transporter), *CLC* (chloride channels) and *SLAC1/SLAH* (slow anion channel-associated 1 homologues) (Figure 2) (Wang et al. 2012).

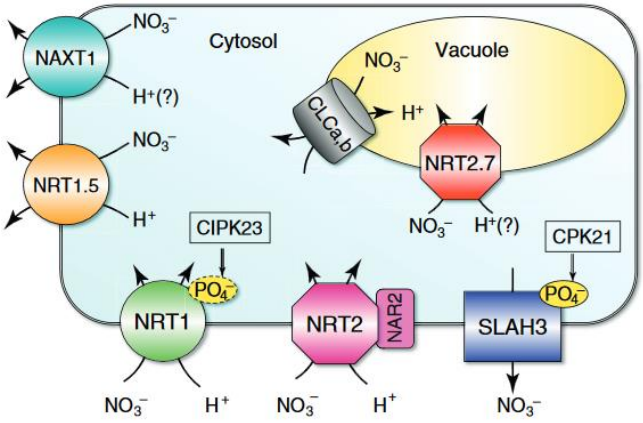


Figure 2: Different types of nitrate transporters and the direction of nitrate transport. NRTs are symporters, CLC are antiporter and SLAH are uniporter (Wang et al. 2012).

In Arabidopsis, the nitrate transporter work in different physiological range. For example, LATs are activated when nitrate concentrations are higher than 0.5 mM, HATs are already activated below 0.5 mM. Nitrate transporters are mainly proton-nitrate symporters that transport nitrate along with protons.

NRT1 transporters, which comprises 53 members, belong to LATs, except for NRT1.1 which acts as dual affinity transporter. Both NRT1.1 and NRT1.2 are involved in nitrate acquisition in the roots. NRT1.1 also participates in auxin transport. NRT1.6 is expressed in seeds and transports nitrate from maternal tissue to embryos (Figure 3). Other NRT1s members also play different roles in nitrate transport (Kiba et al. 2012; Orsel et al. 2004).

CLC transporters which comprise seven members belong to antiporters that transport nitrate into the vacuole. CLCa and CLCb are selectively responsible for nitrate transport and storage into vacuoles. Other CLC transporter-like CLCc, CLCd and CLCg, have been shown to be involved in chloride transport (Figure 2) (Czechowski 2005; Kiba et al. 2012).

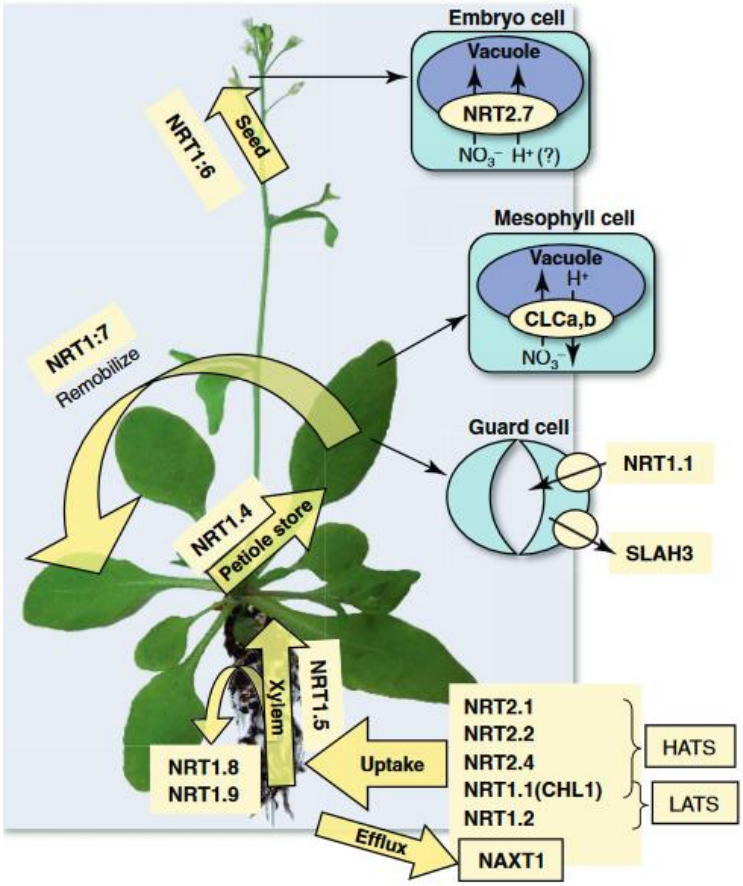


Figure 3: Nitrate transporter gene families involved in nitrate uptake and their localization inside plants. Four types of transporter genes are *NRT1/PTR, NRT2, CLC* and *SLAC1/SLAH* (Wang et al. 2012)**.**

SLAC1/SLAH is another nitrate transporter, located in the guard cells. It is an uniporter and the exact nitrate selectivity of this transporter is still not known (Reddy & Ulaganathan 2015; Wang et al. 2012).

1.2.2 Nitrogen assimilation and translocation

In nature, nitrogen is generally present in the form of urea, ammonia, ammonium and nitrate salts. It can be removed from the soil in different ways, such as by washout or being converted into organic compounds or it may be absorbed by plants.

Once nitrate enters into the plant cell, it is converted into nitrite by nitrate reductase (NR) using NADH or NADPH in the cytosol. NR enzymes are coded by *NIA1* and *NIA2* genes in *A. thaliana*. A small amount of nitrate is also stored in the vacuole. It has to be stated that nitrite can react with amines thereby forming toxic nitrosamines. Therefore, it is important for plants to further reduce toxic nitrite into ammonia. For the shoot, nitrite is transported into the chloroplast whereas for the root it is being transported into the plastids, where it is converted to ammonia by nitrite reductases. The produced and also toxic ammonia is then incorporated into amino acids by following the GS/GOGAT pathway (Dechorgnat et al. 2012; Goel & Singh 2015; Zhang et al. 2018). The whole process is known as nitrogen assimilation (Figure 4 A). Along with nitrate assimilation, few nitrates are stored inside the vacuole. The overall nitrate content in vacuoles influences the total nitrate content in plants. The stored amount of nitrate inside the vacuoles is always maintained at higher concentration than the nitrate concentration in the cytosol (Figure 4 B). CLC and NRT2.7 are the main transporters for vacuole nitrate transport (Fan et al. 2017; Wang & Shen 2011; Zhang et al. 2018).

Another step is the nitrate mobilization, which means to load and / or unload nitrate into the vascular tissue. Different NRT transporters take part in the nitrate mobilization. NRT1.5 or NRT2.3 are responsible for loading nitrate into the xylem, while NRT1.4 and NRT1.8 are responsible for the unloading process (Reddy & Ulaganathan 2015).

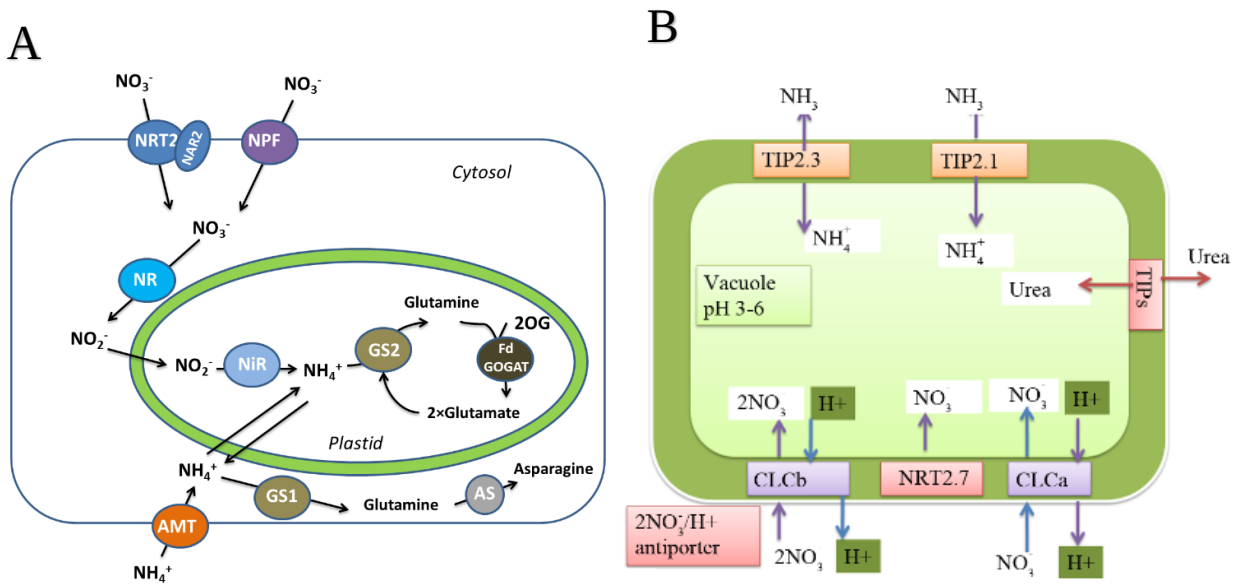


Figure 4: Nitrate acquisition and assimilation in plant cells. A, nitrate and ammonium are transported by nitrate and ammonium transporters respectively. Nitrate is converted into different forms of nitrogen and later incorporated into amino acid synthesis by the GS/GOGAT pathway. **B**, Nitrate storage into the vacuole. CLCs and NRT2.7 are involved in nitrate transport and storage into the vacuole (Reddy & Ulaganathan 2015).

1.3. Nitrate transporter 2 family (NRT2)

The NRT2 family consists of seven members, belonging to the high-affinity transporters (HATs). The NRT transporter family plays a major role in the nitrate uptake from soil in the environment. In Arabidopsis, the expression and localization of the NRT2 family members is tissue specific. This versatile mechanism of nitrate transport helps the plant to cope up with different N conditions in soil environment. NRT2 family has a major contribution of nitrate influx into roots. NRT2.1, NRT2.2, NRT2.4 and NRT2.5 are important for plants to survive with optimal nitrate limitation. The specific expression location of NRT2.7 is in seeds (Chopin et al. 2007). Previous studies showed that NRT2.1 is mainly expressed in the older part of the main root in both nitrate full or limited conditions (Kiba et al. 2012). It has been revealed that the expression pattern of NRT2.2 is similar to the one observed in NRT2.1 (Lezhneva et al. 2014). Interestingly, the preferential expression of NRT2.5 is in the root hair zone, the older part of the lateral roots (side roots) and in the shoot (veins), but not observed in the main root (Lezhneva et al. 2014). NRT2.6 displays a higher expression in roots, rosettes, and is only weakly present in stems and flowers (Dechorgnat et al. 2012).

Expression of NRT2.4 was observed in lateral roots (side roots) and younger parts of the main root under nitrogen starvation. NRT2.4 is not expressed under nitrate starvation, which revealed that NRT2.4 is specific to nitrate starvation. NRT2.4 transcript is also detected in shoots but at very low level. NRT2.4 is located in the epidermis of lateral roots (side roots) and vascular tissue in shoots (Kiba et al. 2012). Intracellular, NRT2.1 and NRT2.2 are located in the plasma membrane. Among the NRT family and compared with NRT2.1, NRT2.2 shows a high amino acids similarity in amino acids (91%) while NRT2.7 shows a low amino acid similarity (57%); NRT2.4 has 88% similarity with NRT2.1 (Orsel 2002).

Among NRTs, NRT2.1 is the main component of HATs under both nitrate starvation and full nitrate conditions. It has been shown that under nitrate limitation, double mutant *nrt2.1-2* in *A. thaliana* showed 20 % reduced fresh weight compared to the wild type (Col-0). Additionally, *nrt2.1-2* × *nrt2.5* triple mutant showed 10% reduction compared to the wild type (Lezhneva et al. 2014). In mutant *nrt2.4,* the nitrate level in phloem exudates decreased by 40% compared to the wild type; however, there was no phenotype observed. Further analyses revealed that the double knockout mutant *nrt2.4* × *nrt2.5* displayed strongly decreased nitrate contents compared to the wild type (around 21-26%) (Kiba et al. 2012). This research demonstrates that the NRT2 family especially NRT2.1 and NRT2.4, play a major role in the maintenance of optimal plant growth under different nitrate conditions.

1.4. *Arabidopsis thaliana* as a model system

Arabidopsis thaliana is a small dicotyledonous flowering plant with a short life cycle. It belongs to the Brassicaceae family and is a non-mycorrhizal host plant. Since 1987, *Arabidopsis thaliana* has been used as a model plant in plant biology. It has a well-sequenced genome, with over 25,000 identified genes. For the nitrate starvation experiment, *Arabidopsis thaliana* is one of the major model plants. Even though it's a non-mycorrhizal plant, it can interact with microorganisms, for example with beneficial and pathogenic fungi. Moreover, for its known genome sequence and short life cycle, it is very convenient to monitor the plant growth and gene expression across a full generation under a controlled environment (Koornneef & Meinke 2010; Meinke et al. 1998).

1.5. Expression of NRTs induced upon biotic and abiotic stress conditions

In the environment, plants are faced with many types of stresses. Two major types of stress are abiotic stress (drought, salinity, heat and cold stress) and biotic stress (fungi or other microorganisms both beneficial and pathogenic). The majority of plants interacts with endophytes by for example forming a symbiosis. These interactions help plants against biotic and abiotic stress as well in a facilitated nutrients and water acquisition from the soil, which leads to growth promotion (Vahabi et al. 2015).

NRTs have a major role in plant stress management. NRT gene expression is regulated by fungal interaction (Vahabi et al. 2015). Among the NRT family, NRT2.1 expression is high with and without nitrate present and is reduced after addition of alternative nitrate sources like ammonium or glutamine, whereas NRT2.4 is highly expressed only under nitrate starvation. Both NRT2.1 and NRT2.4 genes are expressed mainly in the roots and have a very low expression in the shoot (Kiba et al. 2012). NRT gene expression is also regulated by the presence of pathogenic or beneficial fungi. Previous studies showed that NRT2.6 and NRT2.1 gene expression was induced by the bacterium *Erwinia amylovora* and *Pseudomonas syringae* respectively (Dechorgnat et al. 2012). The root colonizing endophytic fungus *Mortierella hyalina* promotes aerial growth of many plant species by supplying nutrients and increasing the plants resistance against both biotic and abiotic stresses (Meents et al. 2019). Another beneficial fungus, *Piriformospora indica* (also known as *Serendipita indica*), induced stress resistance as well as plant growth, too. *P. indica* can protect Arabidopsis from the fungal pathogen *Verticillium dahlia* (Sun et al. 2014).

Abiotic stresses also stimulate phytohormone production and regulate the expression of nitrate transporter genes. Previous findings revealed that ethylene and jasmonic acid (JA) signaling downregulates the gene expression of NRT1.5 while NRT1.8 expression was upregulated (Zhang et al. 2014). Another phytohormone, auxin, plays an important role in plant development, which interacts with other genes to influence them in different consequence. Phytohormone induction or accumulation sometimes is microbe specific, such as Arabidopsis co-cultivation with endophytic fungus *P. indica* often induces SA while *M. hyalina* induces JA (Meents et al. 2019). Along with phytohormones, biotic or abiotic stresses also affect amino acid concentrations (Ali et al. 2019) which has a consequence on the whole crop yield (up to 70 % loss) (Shekari & Javanmardi 2017).

1.6. Usage of nitrogen in agriculture: Importance of nitrate transporters

To achieve the maximum crop demand an excessive amount of synthetic nitrogen fertilizer has been used since the 1850s. A study revealed the use of N fertilizer in the US has risen from 0.22 g N/m^2yr in 1940 to 9.04 g N/m^2yr in 2015 (Peiyu et al. 2018). However, only 30%-50% of applied nitrogen is taken up by plants and more than 50% is lost through leaching, washed-out by rain, ammonification or denitrification via specific bacterial species. The washed-out N fertilizer, especially water-soluble nitrate, can pollute drinking water and causes environmental pollution as well as affect human health. Excess presence of nitrate in fresh-water causes algal blooms thereby resulting in the disruption of the aquatic systems in rivers, lakes and oceans. These, along with nitrous oxide also plays a role in global warming and ozone depletion (Mcallister et al. 2012).

To enhance the nitrate uptake, researchers use genetically modified plants with manipulated nitrate transporter patterns to increase the nitrate uptake from the environment. Overexpression of genes enhances the nitrate utilization rates of plants. As an example, nitrate transporter TaNRT2.1 in wheat plays a role in post-flowering nitrate uptake, which is upregulated by transcription factor coded by the *NAC2* gene. In tomato, overexpression of the nitrate transporter *LeNRT2.3* promoted nitrate acquisition as well as increased the fruit weight and root to shoot transporter (Fan et al. 2017). In rice, *OsNRT2.1* expression is promoted by the co-expression with *OsNAR2.1*. Overexpression of *OsNRT2.3b* also improved rice grain yield. Besides the grain yield, nitrate transporter genes also improve resistance against heavy metals, salt stress and soil acidity by changing the nitrate acquisition and allocation. In Arabidopsis, NRT1.8 and NRT1.5 promote salt

and cadmium tolerance by mediating SINAR (stress-initiated nitrate allocation to roots). SINAR enhances sodium and salt stress through ethylene / jasmonic acid signaling module. In soybean or maize, NRT1.1 improves plant tolerance to soil acidity via maintaining H^+ ions in the rhizosphere (Fang et al. 2016). Thus, in agriculture over-expression of nitrate transporter genes is considered as a major target for promoting grain yield and soften stress impacts.

2. Aims of the study

Even though several studies on nitrate transporters revealed their role under stress conditions (Orsel et al. 2002, Dechorgnat et al. 2012, Lezhneva et al. 2014), the effects of beneficial fungi on NRTs still remain unknown. Previous studies showed that interaction with beneficial fungi can promote plant growth. These finding also indicated that nitrate transporters and their responses to fungi might play a major role to maintain optimal plant growth as well as to cope with a harsh environment. Co-cultivation with beneficial fungi like *Piriformospora indica* and *Mortierella hyalina* promotes the growth of *Arabidopsis thaliana* (Varma et al. 2012, Meents et al. 2019). In this thesis, one main goal was to monitor nitrate transporter NRT2.1 and NRT2.4 expressions upon co-cultivation with beneficial fungi under nitrate starvation. Those two genes were selected because of their unique and versatile expression pattern. Additionally, NRT2.4 expression should be also investigated upon JA and IAA treatment. The concentration of phytohormones is depended on environmental stress. Amino acid concentrations are also affected by stress conditions. Thus, I also wanted to check whether amino acids and phytohormones production were directly related and affected by nitrogen starvation or fungal induction. Furthermore, photosynthetic parameters were investigated along with nitrate content measurements to investigate the relation of the NRTs effects on plant growth.

3. Materials and Methods

3.1. Plant material and growth medium

3.1.1. *Arabidopsis thaliana* lines:

All transgenic seeds of *Arabidopsis thaliana* were kindly provided by Prof. Dr. Anne Krapp (INRA, IJPB, Versailles, France). Transgenic, ProNRT2.4: GFP or *nrt2.4* (Col-0 background) as well as wild-type (Col-0) *Arabidopsis thaliana* seeds were used in this work. ProNRT2.4: GFP constructs were stably introduced into *Arabidopsis* Columbia (Col-0) type. The *nrt2.4* single mutant was constructed in Col-0 via introduction of T-DNA at the 3rd exon position (Kiba et al. 2012) (Figure 5). Col-0 seeds were collected from PD Dr. Axel Mithöfer, MPI-Jena.

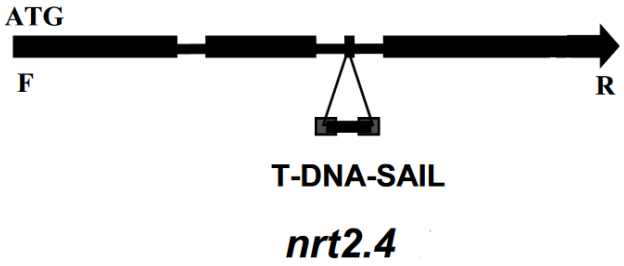


Figure 5: Insertion sites in *nrt2.4* mutant. *nrt2.4* mutant carrying the T-DNA insertion in the 3rd exon (Kiba et al. 2012).

3.1.2. Medium compositions:

MGRL plant culture medium was prepared without or with different NO_3^- concentration to investigate the *NRT2.4* expression under varying conditions. Medium compositions were chosen according to (Naito et al. 1994) and (Orsel et al. 2004) with minor modifications, such as the addition of $Na_2Mo_7O_{24}\cdot 4H_2O$ instead of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$. In MGRL medium without nitrate, KNO_3 and $Ca(NO_3)_2\cdot 4H_2O$ were replaced with KCL and $CaSO_4.2H_2O$ (Table 1). All media were supplemented with 1.2% agar-kobe I (Carl Roth, Germany) and 1% sucrose (Carl Roth, Germany).

Table 1: The MGRL medium composition

Compositions for 1L	10mM NO_3^-	7mM NO_3^-	0.25mM NO_3^-	0.1mM NO_3^-	0 mM NO_3^-
KNO_3	6mM	3mM	0.125mM	0.05mM	NA
$Ca(NO_3)_2 \cdot 4H_2O$	2mM	2mM	0.0625mM	0.025mM	NA
Sodium phosphate buffer pH5.8	1.75mM	1.75mM	1.75mM	1.75mM	1.75mM
$MgSO_4 \cdot 7H_2O$	1.5mM	1.5mM	1.5mM	1.5mM	1.5mM
Na_2-EDTA$\cdot 2H_2O$	67µM	67µM	67µM	67µM	67µM
H_3BO_3	30µM	30µM	30µM	30µM	30µM
$MnSO_4 \cdot 5H_2O$	10.3µM	10.3µM	10.3µM	10.3µM	10.3µM
$FeSO_4 \cdot 7H_2O$	8.6µM	8.6µM	8.6µM	8.6µM	8.6µM
$CuSO_4 \cdot 5H_2O$	1µM	1µM	1µM	1µM	1µM
$CoCl_2 \cdot 6H_2O$	130nM	130nM	130nM	130nM	130nM
$Na_2 \cdot Mo_7O_{24} \cdot 4H_2O$	24nM	24nM	24nM	24nM	24nM
$ZnSO_4 \cdot 7H_2O$	1µM	1µM	1µM	1µM	1µM
1.0% w/v sucrose	10 gram	10 gram	10 gram	10 gram	10 gram
1.2% purified agar-agar kobe I	12 gram	12 gram	12 gram	12 gram	12 gram
KCL	NA	NA	NA	NA	3mM
$CaSO_4.2H_2O$	NA	NA	NA	NA	2mM

3.1.3. Seeds sterilization and plant growth conditions:

Arabidopsis thaliana seeds were surface sterilized by adding 500 µl of distilled water, 500 µl of sodium hypochloride (ACROS Organics™, Germany) and 3µl of Triton X-100 (Sigma-Aldrich, Germany). After shaking for 7 min the seeds were gently spin down for 3 s, and washed with sterile water 7-8 times under sterile conditions to avoid contaminations. *Arabidopsis thaliana* seeds (10-15 seedlings per plate) were grown on square plates (120×120×16mm) (ThermoFisher Scientific, Germany) containing MGRL medium.

Platted seeds were kept in a dark room at 4°C for 48 h for stratification. Afterwards, plates were transferred into the light chamber (climate chamber) and placed the plates vertically to allow the roots downward (Figure 6). Plants grown for 14 days under long-day conditions at 20 ± 2°C (light intensity was 80 µmol/m^2×sec; Day/night, 16 h/8 h). All growth experiments were conducted at MPI-CE, Jena, Germany.

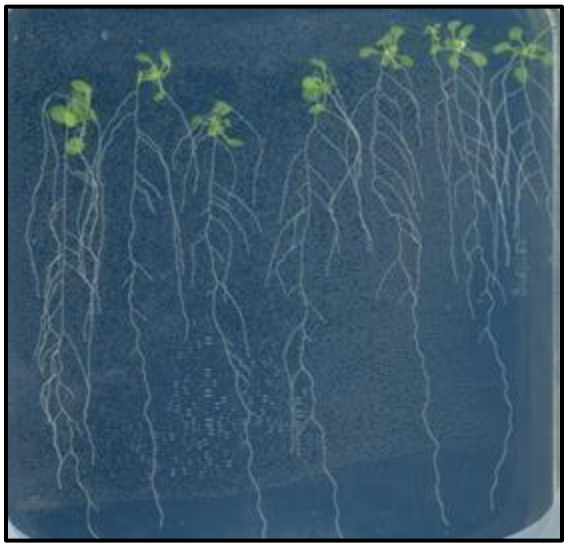


Figure 6: 14 days old *Arabidopsis thaliana* plants grown on MGRL medium containing 7 mM nitrate under long day conditions.

3.2. Fungal growth

Piriformospora indica was provided by Prof. Ajit Verma (School of Life Science, JNU, India). *Mortierella hyalina* was provided by Prof. Ralf Oelmüller (Friedrich-Schiller-Universität Jena). *M. hyalina* and *P. indica* were grown on Potato Dextrose Agar (PDA) medium (Sigma-Aldrich, Germany) (Table 2) at pH 6-6.5 (Michal Johnson et al. 2014) for 1 week (Figure 7 A & B). Therefore, a single fungal plug (5 mm diameter) was placed on the center of a PDA plate. Afterwards the PDA plates were kept at 22°C with 75% relative humidity and 12 / 12 h day-night illumination. *P. indica* and *M. hyalina* grew well in both nitrate and no nitrate (Figure 7 C) MGRL medium.

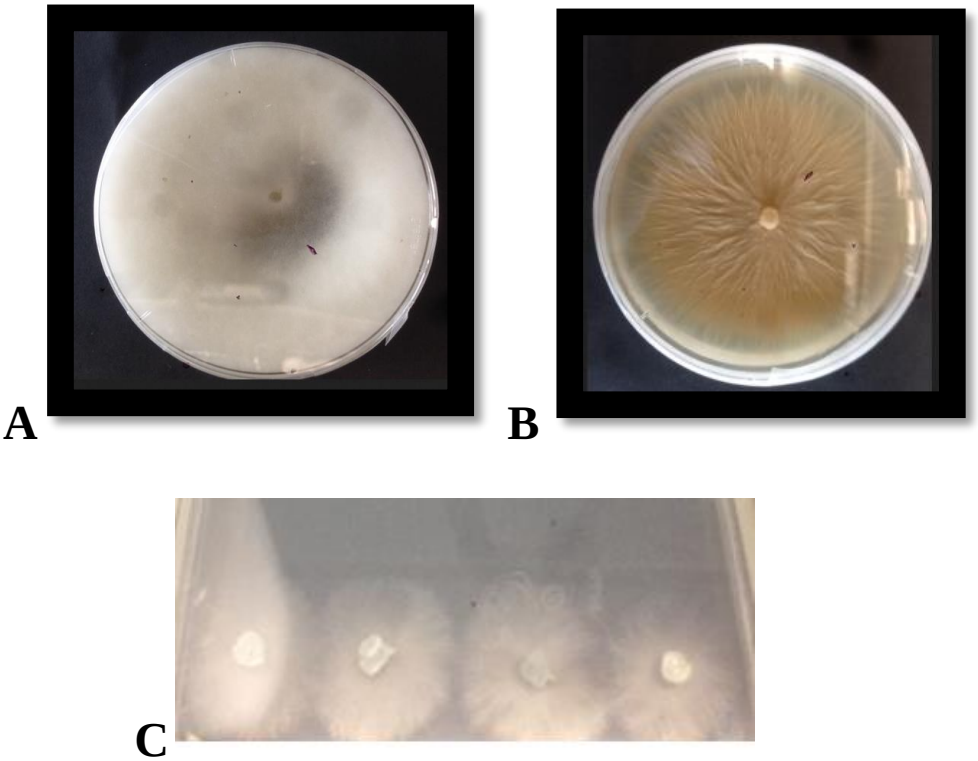


Figure 7: *M. hyalina* (A) and *P. indica* (B) growing on PDA medium. Growth of *M. hyalina* on no nitrate MGRL medium (C). There was no visible growth difference of *P. indica* and *M. hyalina* grown on PDA, no nitrate, or 7 mM nitrate medium.

Table 2: Potato Dextrose Agar (PDA) Medium. For 1L

Compositions	Amount (gram)
Potato Dextrose	20
Agar-Agar kobe I	10
pH	6.5

3.3. Co-cultivation of *A. thaliana* with beneficial fungi

14 days old *A. thaliana* seedlings grown on MGRL / 7 mM nitrate medium (to ensure the proper nutrient) were exposed to beneficial fungus (*M. hyalina* and *P. indica*) on no nitrate medium (square plates). Fungal plug (5 mm) is placed few cm up from the bottom of *A. thaliana* plants placed such a way that its root tip touches the mycelium of *M. hyalina* or *P indica* respectively (Figure 8). Control plants were exposed only with a PDA plug of 5 mm diameter. GFP fluorescence was observed in different time intervals (1 d, 2 d, 4 d, 6 d and 8 d) by using ZEISS Axio Zoom.V16 (Zeiss microscopy GmbH, Germany).

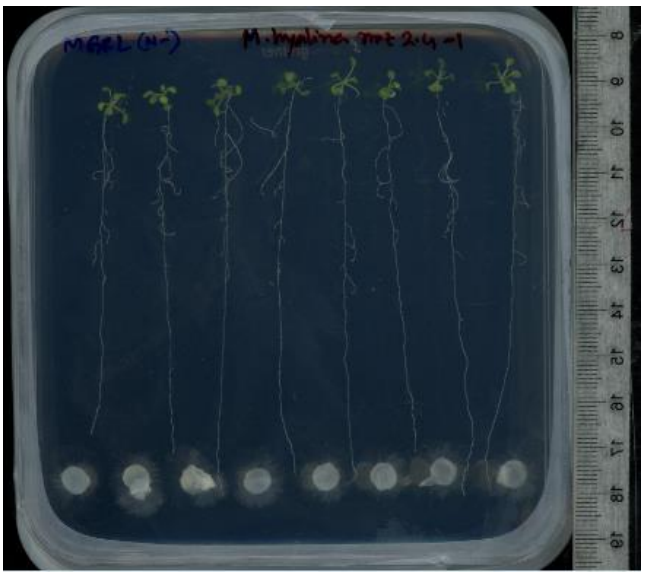


Figure 8: Co-cultivation of *A. thaliana* with beneficial fungi (*M. hyalina* or *P. indica*). Root tip was touched to the mycelium of fungal plug. Each plate contains 4-8 plants for treatment or control.

3.4. Treatment with Jasmonic acid (JA) / Indole-3-acetic acid (IAA)

14 days old *Arabidopsis thaliana* (ProNRT2.4: GFP) grown on 0.25 mM MGRL medium were exposed to JA (Sigma-Aldrich, Germany) or IAA (Merck, Germany). JA or IAA was applied exogenously with different concentration of JA or IAA, such as 1 mM of JA or 1 µl IAA solution to either the root or shoots of Arabidopsis. For root treatments, 10 µl of JA or IAA were applied to the root tips of the plant. For shoot treatment, 20-30 µl of JA or IAA were sprayed to the shoot of the plant. The control plants were sprayed with sterile water with 0.01% Tween 20 solution (Biotium) or added 10 µl onto the root tip. Treatment and control plates were observed at different time intervals, directly before treatment (-0), after the treatment (+0), 1, 2, 3 and 6 h after treatment by using ZEISS Axio Zoom.V16 (Figure 9).

3.5. Fluorescence microscopy

To observe the fluorescence of the fungal-colonized Arabidopsis seedlings expressing reporter constructs ProNRT2.4: GFP and ProNRT2.1: GFP, the following settings were used :- For GFP, camera- Axiocam 506 mono with 1×camera adapter, excitation wavelength 488 nm, emission wavelength 509 nm, exposure time 2s, depth of focus 38.56 µm. Imaged was processed with a combination of several tiles with object PlanNeoFluar Z 1.0x, zoom 3.1, magnification 50X, light intensity 100% and channel name EGFP. For bright filed image was processed using camera Axiocam 506 color with 1× camera Adapter, depth of focus 282.02 µm with exposure time 54ms. Light source intensity was 100% and channel name RL bright field. For ProNRT2.1: GFP observation was done using the same settings with modification in an exposure time of 280 ms.

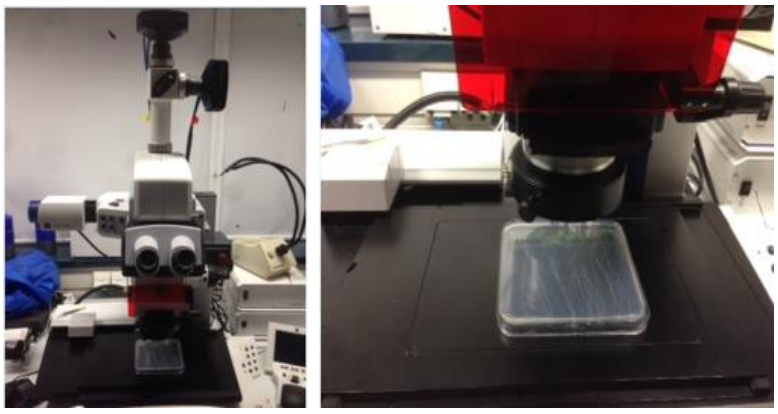
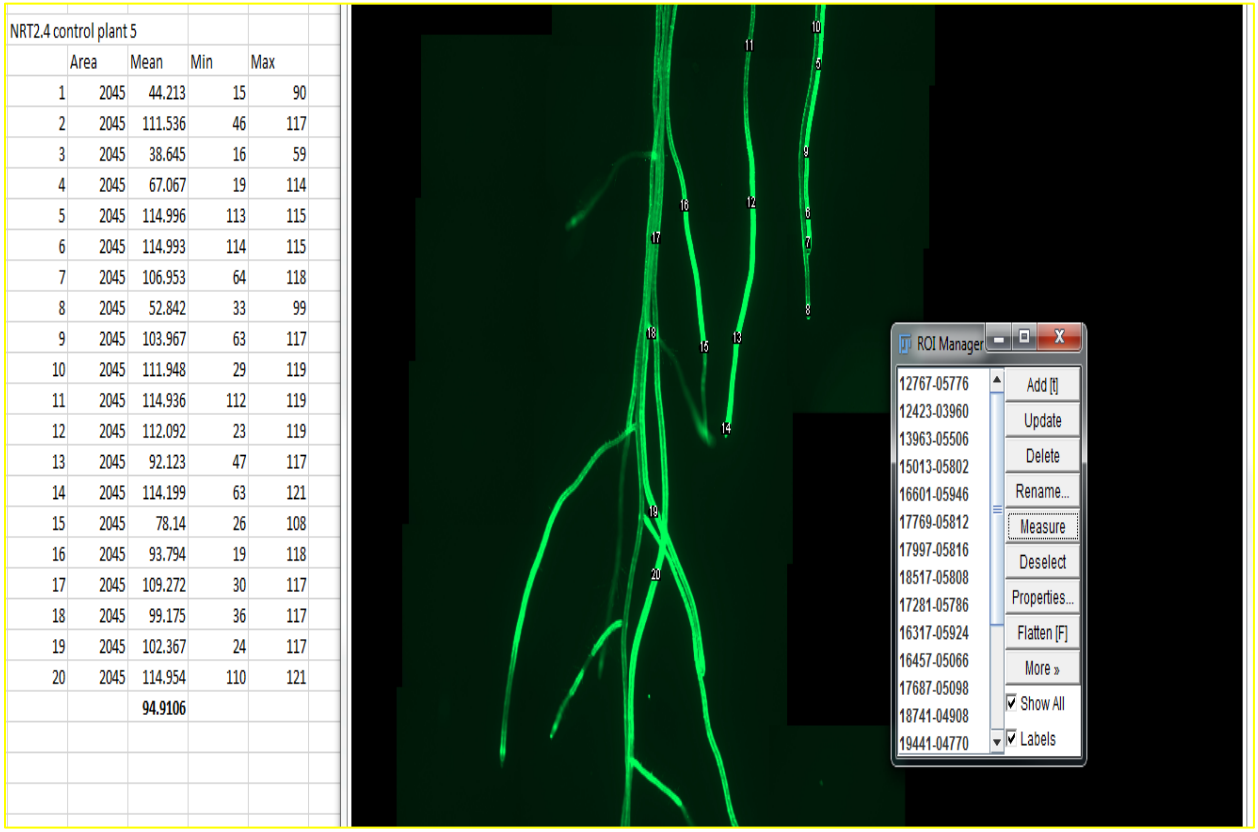


Figure 9: Axio Zoom.V16 to observe the fluorescence expression of transgenic plant NRT2.4: GFP.

3.6. Image analysis

Images were processes with Zen software (Zeiss, Germany). Fluorescence images were analyzed by using Java-based image-processing software 'ImageJ' (Version 1.52s 10). The Image was first compiled and converted into jpg files. Analysis was performed by following settings: Analysis > Tools > ROI manager > Select Points >Shift + T (to get value) > Measure to get data (Figure 10). Main root, Root tip (means few mm along with root tip) and side roots were analyzed separately.



NRT2.4 control plant 5

	Area	Mean	Min	Max
1	2045	44.213	15	90
2	2045	111.536	46	117
3	2045	38.645	16	59
4	2045	67.067	19	114
5	2045	114.996	113	115
6	2045	114.993	114	115
7	2045	106.953	64	118
8	2045	52.842	33	99
9	2045	103.967	63	117
10	2045	111.948	29	119
11	2045	114.936	112	119
12	2045	112.092	23	119
13	2045	92.123	47	117
14	2045	114.199	63	121
15	2045	78.14	26	108
16	2045	93.794	19	118
17	2045	109.272	30	117
18	2045	99.175	36	117
19	2045	102.367	24	117
20	2045	114.954	110	121
		94.9106		

Figure 10: GFP fluorescence intensity was measured by ImageJ (Version 1.52s 10). For each measurement, 10-20 points were taken randomly and the area of each circle was maintained the same for each circle. Mean values were used for statistical analysis.

3.7. Chlorophyll fluorescence measurement

Chlorophyll fluorescence parameters (maximum PSII quantum yield-QY_max) of treated and control plants were measured according to Heyer et al. (2018a) using FluoroCam FC 800-C (Photon Systems Instruments, Czech Republic) (Figure 11) with minor modifications. Fourteen days old seedlings grown on MGRL / 7 mM MGRL were transferred on no nitrate medium without (control) or with *M. hyalina*. Each plate contained 7-8 plants. NRT2.4, nrt2.4 and Col-0 plants were measured in this experiment.

QY_max of the same plants were measured in different time intervals starting at 1, 4, 6, 8 and 10 days of co-cultivation. Closed plates were kept in the dark for 20 min and then placed into the FluoroCam to measure QY_max using the same settings described in Heyer et al. (2018a). Col-0 was used as wild-type control. Measurements were performed with the following program, Act 1:50%, Act 2:50% Super: 100%. QY_max (Maximum PSII quantum yield) = *Fv*/*Fm*. *Fv* and *Fm* are variable and maximum fluorescence in the dark-adapted state respectively (Manual n.d.).

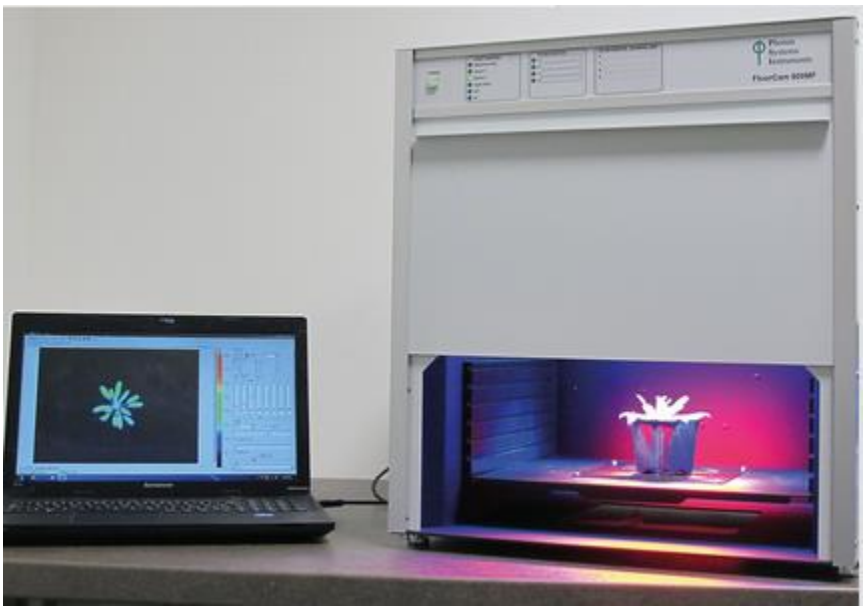


Figure 11: Closed FluorCam FC 800-C (Photon Systems Instruments). It was used to quantify chlorophyll fluorescence kinetics. It's equipped with CCD camera with standard LED panels (Manual n.d.).

3.8. Phytohormone quantification

Arabidopsis samples (both control and treatment) were collected in 10 day time intervals to analyze phytohormones according to Heyer et al. (2018b). Around 30-50 mg of fresh weight roots and shoots were collected separately in a 2 ml Geno Grinder tube with two metal balls. The samples were finely grounded and extracted with 1 ml methanol (100%) with internal standard (IS; 4µl/ml) containing 60 ng D_6-JA (HPC Standards GmbH, Germany), 60 ng D_4-SA (Santa Cruz Biotechnology, USA), 60 ng D_6-ABA (Santa Cruz Biotechnology, USA), 12 ng D_6-JA-Ile (HPC Standards GmbH, Germany) and D_5-indoleacetic acid (IAA, Olomouc Czech Republic). After short mixing with vortex (Vortex-2 Genie GmbH), samples shook at 4°C with 30 min by Starlab shaker (STARLAB GmbH, Hamburg, Germany) with using parameter 100 rpm for 15 s, 75° for 16 s and 3° for 5 s. Afterward, samples were collected and centrifuged at 13000 rpm at 4°C for 20 min. The supernatant was carefully transferred into a new 2 ml Eppendorf tube and rest of the pellet was resuspended with an additional 500 µl of methanol without IS. Next, resuspended pellet was shaken and centrifuged as like described before. The supernatant was gently transferred into the previous tubes. Later, the supernatant was concentrated at 22°C (RT) for 2-3 h using the Eppendorf concentrator plus (Eppendorf AG, Hamburg, Germany). Completely concentrated samples were resuspended (vortexed gently) with 200 µl of fresh methanol without IS and then again centrifuged at 4°C at 16000 rpm for 5 min. In the end, 200µl of supernatant was transferred into HPLC vials for Phytohormone measurement by LC-MS/MS. From this final supernatant small amount was transferred for amino acid analysis.

Phytohormone was measured by using HPLC-(Agilent 1260 Infinity II HPLC system) coupled with a tandem mass spectrometer (SCIXEX QTRAP 6500) with negatively charged turbo spray ion source. The experiment was performed according to Heyer et al. (2018b). Since it observed that both D_6 JA and D_6 JA-Ile standards contained 40 % of the corresponding D_5-labeled compound, the sum of peak areas of D_6 and D_5 was used for quantification (Figure 12).

IAA was quantified using the same LC-MS/MS system with chromatographic conditions but using ion spray voltage at 5500 eV with positive mode ionization. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion => product ion fragmentations as follow: m/z 176 =>130 (collision energy-CE) 19 V; declustering potential (DP) 31 V for IAA; m/z 181 =>133 + m/z 181

=>134 + m/z 181 =>135 (CE 19 V; DP 31 V) for D_5 IAA. More details was mentioned in Appendix 2.

Figure 12: HPLC-(Agilent 1260 Infinity II HPLC system)

https://www.srainstruments.com/p/lc-agilent-1260/

3.9. Amino acid quantification

Amino acid concentration of 10 day co-cultivated Arabidopsis plants was measured using HPLC-MS. The amino acid standard was prepared with 1:1000 dilution containing $^{13}C^{15}N$ of 10mg/ml (Isotec, Miamisburg, OH, USA). To measure amino acids final samples were prepared by 1:10 dilution with amino acid standard. For example, 30µl of sample and 270 µl of amino acid standard into HPLC vials for analysis. The analysis was performed according to Crocoll et al. 2016. Chromatography was performed by Agilent 1200 HPLC system (Böblingen, Germany) using Zorbax Eclipse XDB-C18 column with 50 × 4.6 mm, 1.8 µm (Agilent Technologies). Mobile phase A and B were prepared with 0.05% formic acid in water and acetonitrile respectively. Temperature of the column was maintained at 25ºC. LC-(Agilent 1200 Infinity II HPLC system) coupled with a tandem mass spectrometer (SCIXEX QTRAP 6500; Applied Biosystems, Darmstadt, Germany) with positively charged turbo spray ion source. The elution profile was: 0-1min 3% B in A; 1-2.7 min 3-100% B in A; 2.7-3 min 100% B and 3.1-6 min 3% B in A; with flow rate of 1.1 ml/min. The temperature of turbo gas was set to 700ºC and at 5500 eV was maintained for ion spray. Nebulizing gas, curtain gas and heating gas was set at 70 psi, 40 psi and 70 psi respectively. Details of multiple reaction monitoring (MRM) was mentioned in Appendix 3. For data analysis analyst 1.5 software was used (Applied Biosystems).

3.10. Statistical analysis:

Statistical analyses of co-cultivation and JA / IAA experiments, phytohormones and amino acid quantification were performed by Mann-Whitney U test by using SigmaPlot 12 (Systat Software GmbH, Erkrath, Germany). Graphs were plotted by using OriginPro 2019 software (Originlab Corporation, USA).

4. Results

4.1 Fluorescence expression of NRT2.1 and NRT2.4 in response to N starvation

The expression pattern of nitrate transporters is different under nitrate starvation. The expression level of *NRT2.1* and *NRT2.4* genes of *A. thaliana* depends on nitrate availability and previous studies already showed different expression pattern of *NRT2.1* and *NRT2.4* (Kiba et al. 2012). In order to repeat this result under my conditions, transgenic plants grown on MGRL / 7mM nitrate medium were transferred onto no nitrate MGRL plates and inoculated for 3 days. GFP fluorescence of NRT2.1 was observed in main roots not in lateral roots (side roots) while under the same condition GFP fluorescence was observed in lateral roots and root tip only (Figure 13). There was neither ProNRT2.4: GFP nor ProNRT2.1: GFP fluorescence observed in the shoots. These results confirmed that *NRT2.1* and *NRT2.4* are expressed only in roots, not in shoots. Furthermore, it was important to select the perfect target nitrate transporter. *NRT2.1* was expressed on both nitrate and no nitrate conditions while *NRT2.4* was observed only under nitrate starvation. As consequence of this unique expression pattern, *NRT2.4* was selected as target transporter gene for further experiments. Nevertheless, an additional experiment had to be carried out on *NRT2.1* in different nitrate medium to verify the dose depended expression pattern.

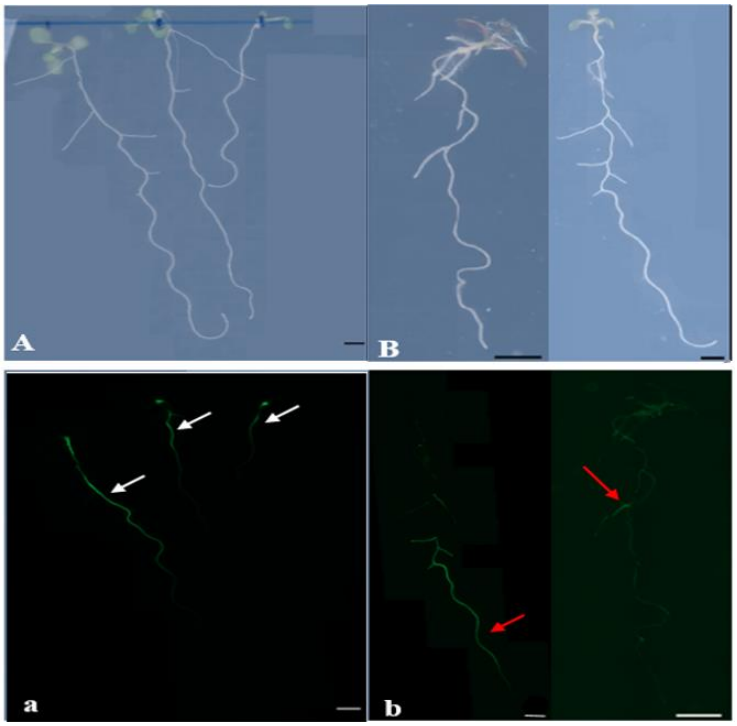


Figure 13: Fluorescence analysis of transgenic plants expressing ProNRT2.1: GFP and ProNRT2.4: GFP.

GFP fluorescence (a, b) and bright field (A, B) images of both ProNRT2.1:GFP (a, A) and ProNRT2.4:GFP (b, B). Photos were taken after 3 days growth on no nitrate medium. White arrowheads show the expression of NRT2.1 transporter whereas red arrowheads show the expression of NRT2.4 transporter.

Scale bars = 2000µm.

4.2. Regulation of *NRT2.1* gene in response to N level

Expression of *NRT2.1* is regulated by nitrogen level in the medium. ProNRT2.1: GFP transgenic plants were grown on MGRL / 7 mM nitrate medium for 14 days under standard conditons. Later, those transgenic plants were transferred onto 0.1 mM and 10 mM of nitrate containg medium and allowed to grow under same conditions. Each petri dish contained 5 transgenic plants. *NRT2.1* response of the same plant was observed at 1, 2, 3 and 6 d of inoculation. Therefore, expreesion of *NRT2.1* was observed by Auxio zoom fluorescence microscopy.

GFP fluorescence was higher in 0.1 mM than 10 mM nitrate medium after 1d of incubation. GFP intensity increases along with time. After 6 d of incubation on 10 mM nitrate, fluorescence was increased gradually while on 0.1 mM fluroscence was observed stronger (Figure 14 & 15). This ProNRT2.1: GFP fluorescence confirmed the nitrate dose and time dependent expression pattern of *NRT2.1*. However, this broad expression pattern was not suitable for co-cultivation experiments to identify the influence of fungal infections.

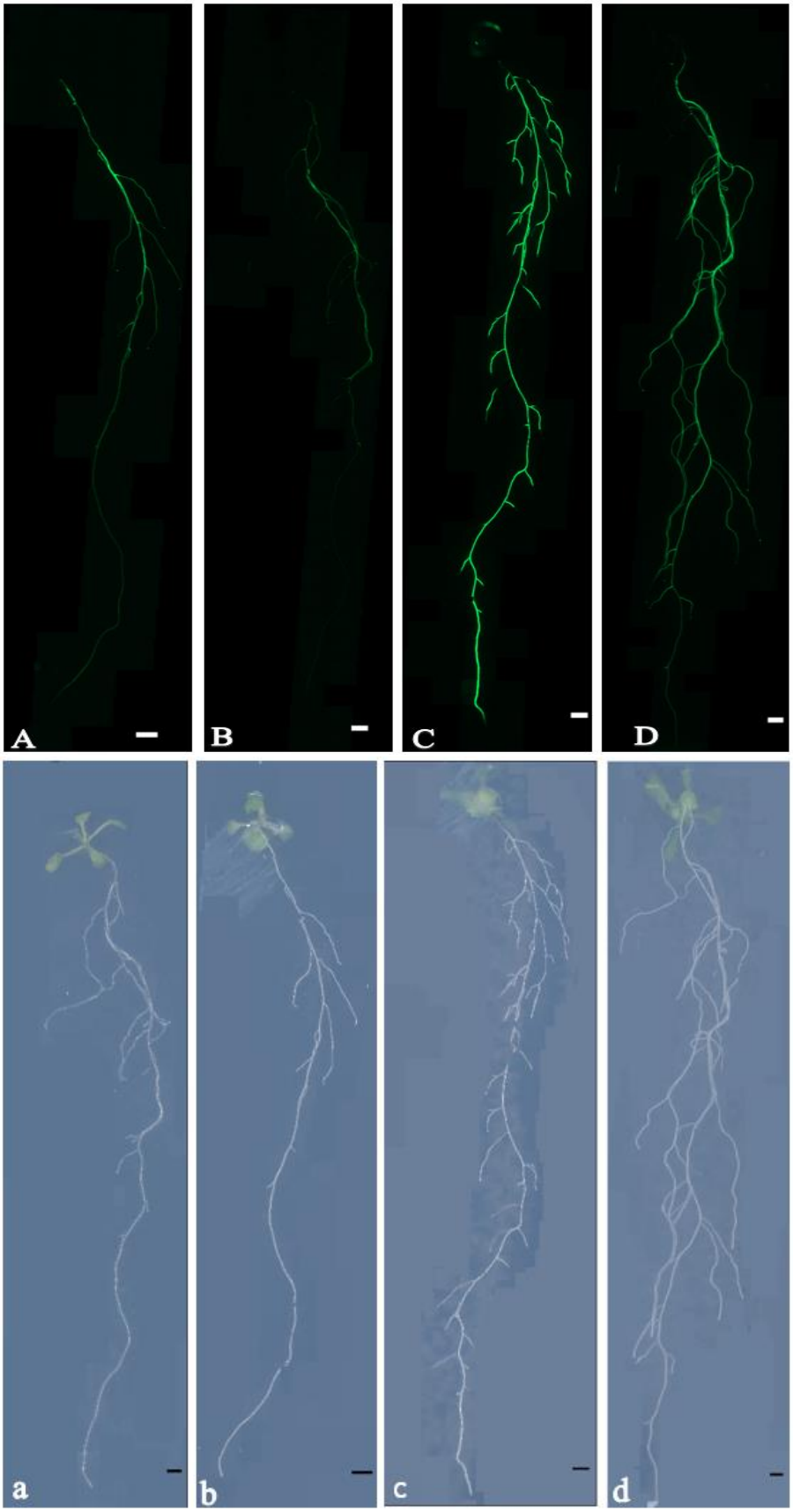


Figure 14: Image of ProNRT2.1: GFP expressing plants. A, B, C, D GFP fluorescence and a, b, c, d bright field image. 14 days old Pro:NRT2.1: GFP grown on MGRL / 7mM nitrate medium then further incubated with 0.1 mM (A, C) or 10 mM (B, D) nitrate containing medium. Photos were taken after 1 day incubated on 0.1 mM (A) and 10 mM (B) and plants incubated for 10 days on 0.1 mM (C) and 10 mM (D) nitrate containing MGRL.

Scale bar = 2000µm.

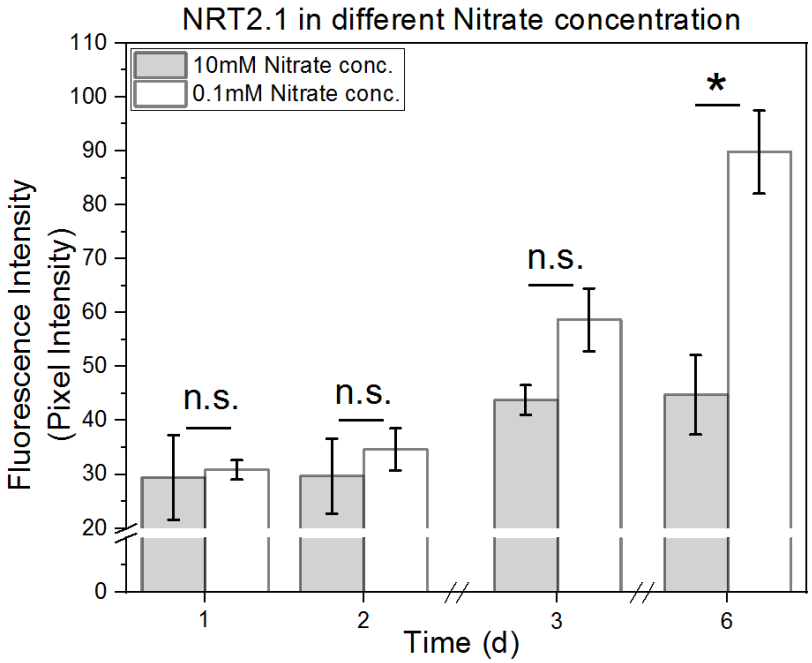


Figure 15: Statistical analysis of fluorescence images of ProNRT2.1: GFP at different time intervals (n = 8). Fluorescence intensity was obtained from Microscopic image (figure). Asterisks indicated significant difference between plants grown on 0.1mM and 10mM nitrate containing medium after 6 days of incubation.

Values represent mean ± SE (n = 8). n.s. = Not significant, Asterisks indicate significant differences, Mann Whitney test, ($p < 0.05$).

4.3. *P. indica* influence on the fluorescence expression of ProNRT2.4: GFP under nitrate starving conditions

Co-cultivation experiments were performed with the benefical fungus *P. indica* to confirm the previous experimental results and also select the perfect candidate fungus for innoculation experiments. *P. indica* is one of the most useful beneficial fungi with a broad host spectrum with positively affects on plant growth and stress management. In this experiment 14 days old Arabidopsis seedlings (ProNRT2.4: GFP) grown on MGRL / 7 mM nitrate medium were tranferred into no nitrate MGRL and inoculated with *P. indica* (Figure 16). A PDA plug (0.5 mm) was used as control. GFP fluorescence expression was observed at 1, 2, 4, 6 and 8 days of innoculation. No singificant difference was observed in main roots and root tips. Significant difference in fluorescence expression was observed in lateral roots /side roots (Figure 17 B). The mycelium position of *P. indica,* on no nitrate medium (Appendix 4 B, indicated with arrow) was also observed showing that the less fluorescence intensity in treated plants was intrinsic and not due to mycelium that covered the whole root, thereby reduced the expression of *NRT2.4*. This inoculation experiment confirmed that *P. indica* reduces the expression of NRT2.4 under nitrate starvation.

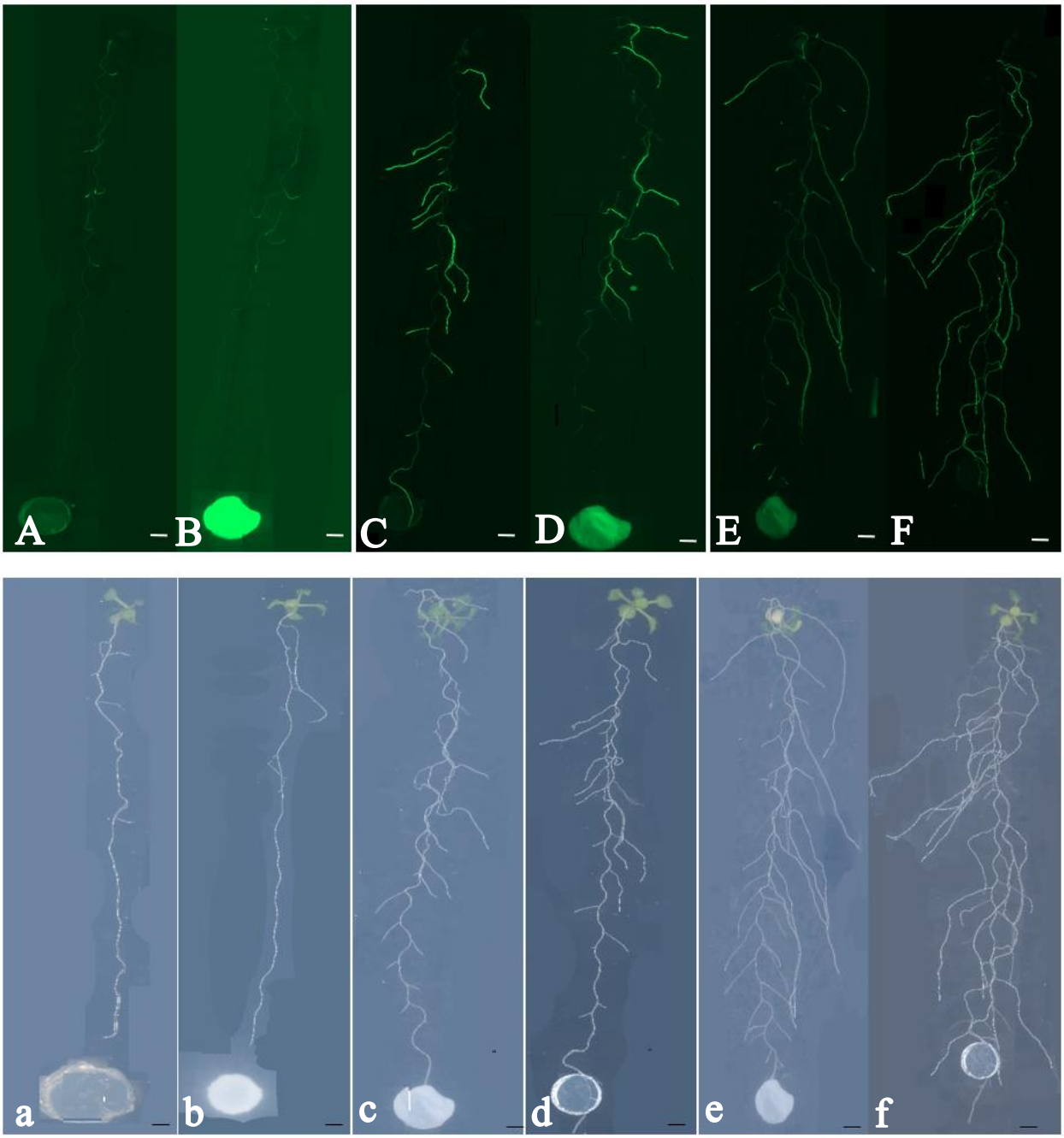


Figure 16: Image of ProNRT2.4: GFP transgenic plant colonized with *P. indica*. A, B, C, D, E, F GFP fluorescence and a, b, c, d, e, f corresponding bright field images of these respective plants. 14 days old ProNRT2.4: GFP grown on MGRL / 7 mM nitrate medium were further incubated with *P. indica* on no nitrate medium. Plants incubated at 1, 4, 8 days were observed. Image A, C, E GFP images of control plants at 1, 4 and 8 days of innoculation. Image B, D, F GFP images of *P. indica* treated plants at 1, 4 and 8 days of innoculation.

Scale bar = 2000µm.

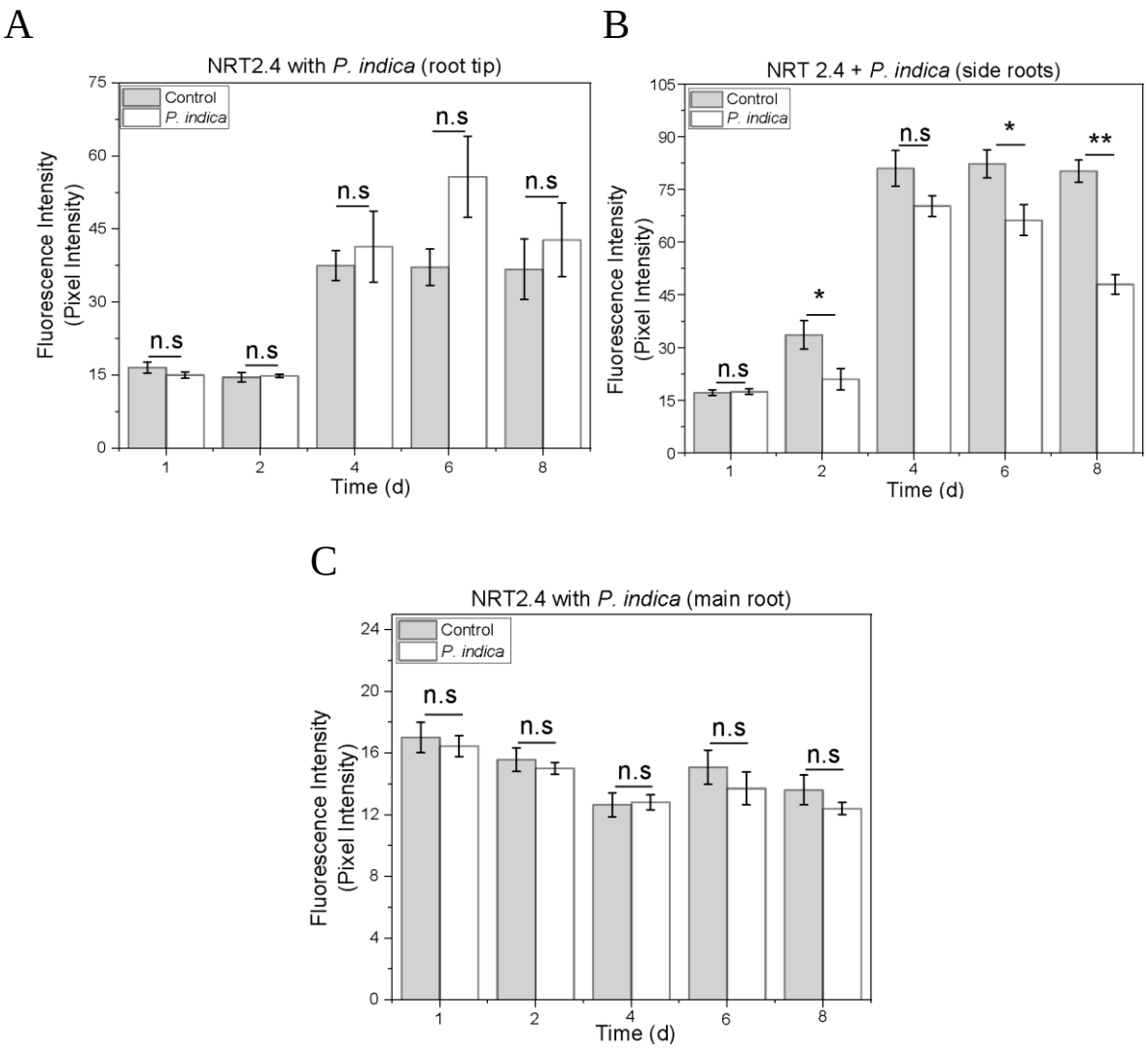


Figure 17: Statistical analysis of fluorescence image of ProNRT2.4: GFP in different time intervals (n = 8). Fluorescence intensity was obtained from Microscopic image (Figure 16). Asterisks indicated significant difference between control and *P. indica* treated plants at 1, 2, 4, 6 and 8 days of incubation. A, B and C represent the results of root tips, side roots and main roots respectively. **A.** *A. thaliana* ProNRT2.4: GFP line (NRT2.4) with *P. indica* incubation, showing no significant difference in root tip between control and *P. indica* treated plant. **B.** Significant difference in side roots at 6 days of inoculation and afterward. **C.** Main root showing no significant difference between control and *P. indica* treated plant. Control plant (dark bar), treated plant (open bar).

Values represent mean ± SE (n = 8); Experiment was repeated 3 times independently. n.s. = Not significant, Asterisks indicate significant differences, Mann Whitney U test, $P < 0.05$.

4.4. *M. hyalina* influence on the fluorescence expression of ProNRT2.4: GFP under nitrate starving condition

Next, 14 days old seedlings grown on MGRL / 7 mM nitrate medium were transferred to no nitrate medium with co-cultivation with / without *M. hyalina*. Fluorescence expression of proNRT2.4: GFP was observed by fluorescence microscopy after 1, 2, 4, 6 and 8 d of inoculation (Figure 18 & 19). Control plants were co-cultivated with only PDA plug, while treated plants were co-cultivated with *M. hyalina* plug. After transfer to no nitrate medium, *NRT2.4* expression in both controlled and treated were observed at day 2. GFP expression of controlled plant was increased after 2 days and highly increased afterwards. In *M. hyalina* treated plants, GFP expression was also observed at day 2 and expressed in very low level afterwards (Figure 18). No expression was observed in the main. Fluorescence expression of control plant was higher than in the treated plant. Highly significant difference between controlled and treated plant was observed in root tips than side roots. This observation revealed that *NRT2.4* expression decreases in the presence of beneficial fungi *M. hyalina.* Gradually reduced induction of *NRT2.4* gene under nitrate starvation condition with *M. hyalina* indicated no nitrate stress (nutrient) in the medium (Figure 20). The mycelium position of *M. hyalina,* on no nitrate medium (Appendix 4 A, indicated with arrow) was also observed which confirmed that the less fluorescence intensity in treated plants was again an intrinsic property, not due to mycelium that covered the whole root and reduce the GFP fluorescence.

These results forced me to further investigate the role of *NRT2.4* on plant growth under stressed condition.

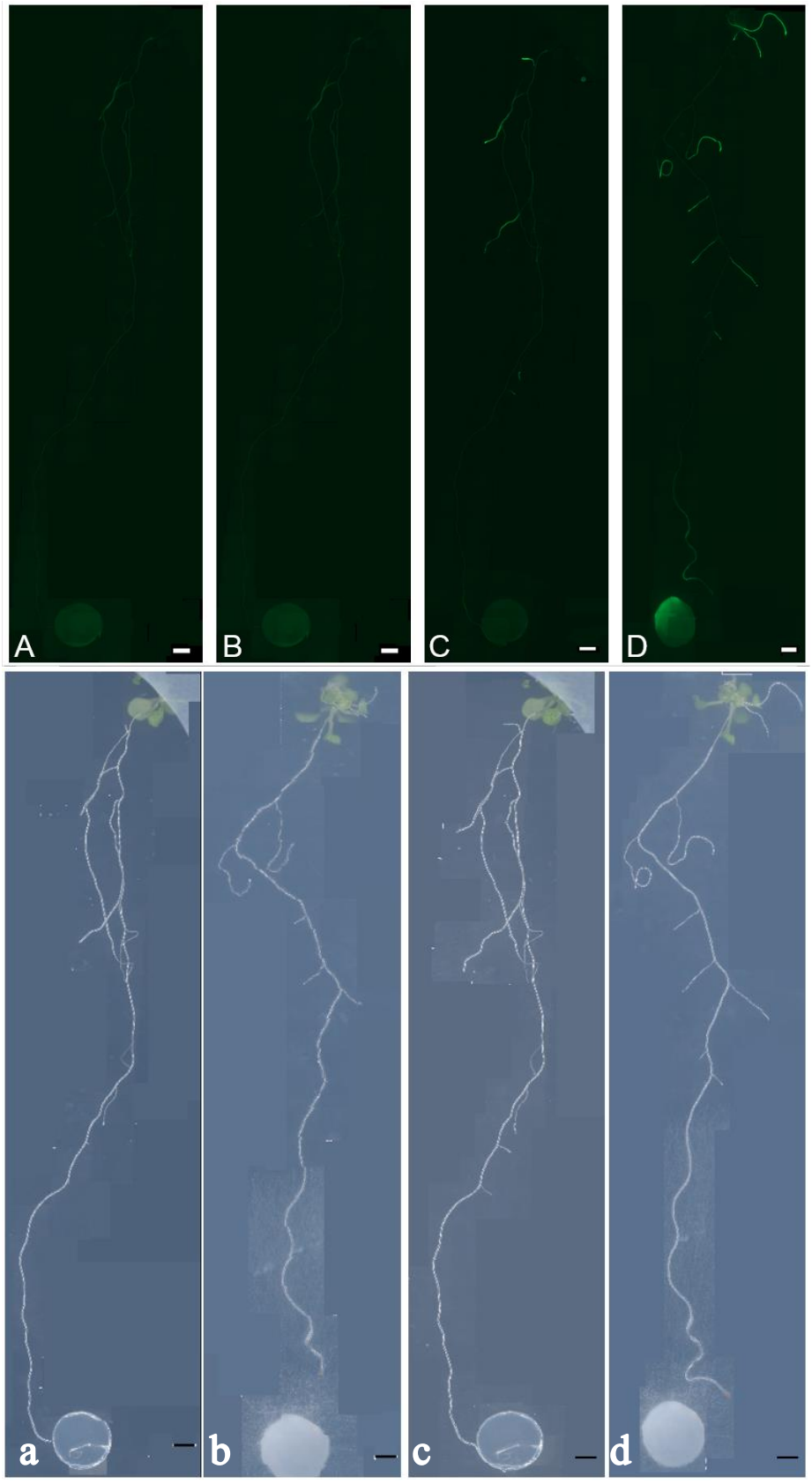


Figure 18: Image of ProNRT2.4: GFP transgenic plants colonized with *M. hyalina.*

A, B, C, D GFP fluorescence and a, b, c, d bright field images of these respective plants. 14 days old ProNRT2.4: GFP grown on MGRL / 7 mM nitrate medium were further incubated with *M. hyalina* on no nitrate medium. Plants were observed after 1 (A, a, B, b) and 2 (C, c, D, d) days of co-cultivaation. A, a, C, c Images of control plants. B, d, D, d images of *M. hyalina* treated plants.

Scale bar = 2000µm.

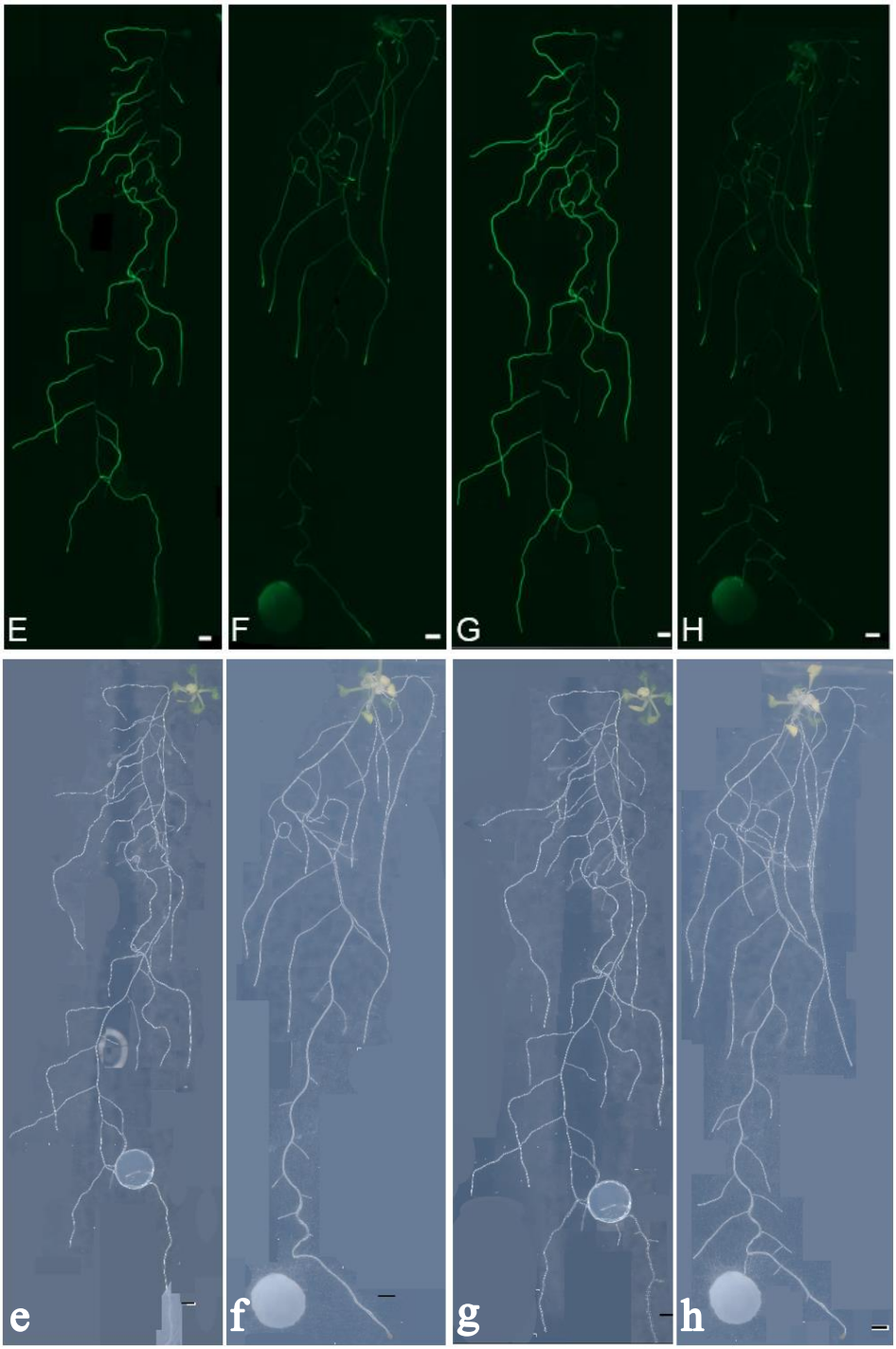
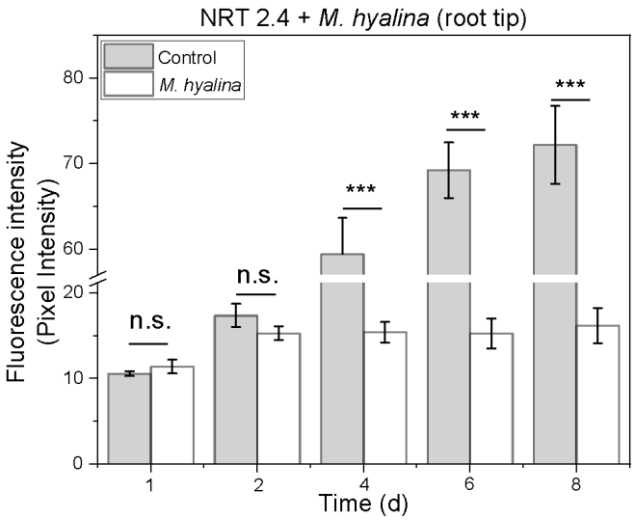
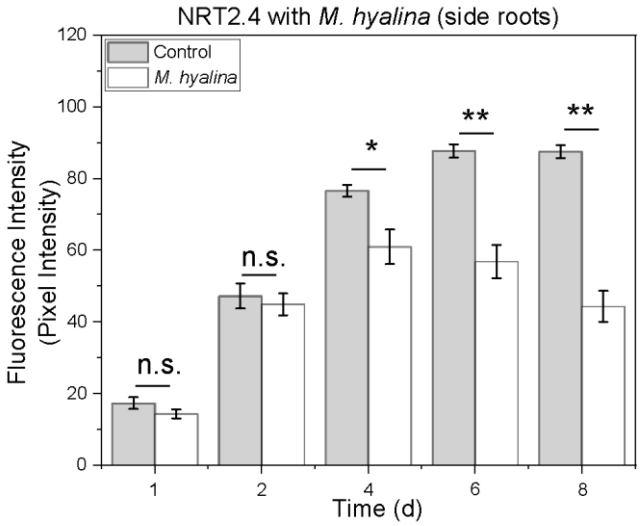


Figure 19: Image of ProNRT2.4: GFP transgenic plants colonized with *M. hyalina*. E, F, G, H GFP fluorescence and e, f, g, h bright field images of this respective plants. 14 days old ProNRT2.4: GFP grown on MGRL / 7 mM nitrate medium were further incubated with *M. hyalina* on no nitrate medium. Plants after at 6 (E, e, G, g) and 8 (F, f, H, h) day were observed. Images E, e, G, g: control plants; Images F, f, H, h: *M. hyalina* treated plants at 6 and 8 day of innoculation. Scale bar = 2000µm.

A



B



C

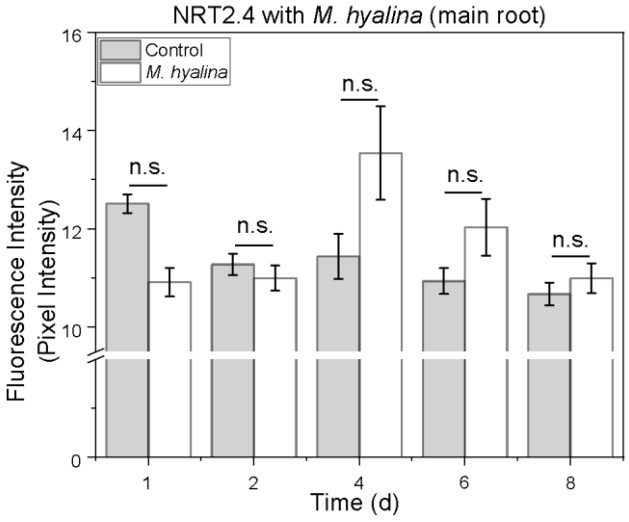


Figure 20: Fluorescence intensity of ProNRT2.4: GFP lines co-cultivated with / without *M. hylina* on no nitrate medium.

A; Fluorescence intensity was analyzed in root tips at different days.

B; Fluorescence intensity was analyzed in side roots (lateral roots) at different days.

C; Fluorescence intensity was analyzed in main root.

Control plant (dark bar), treated plant (open bar). Number of plants in each treatment = 8; this experiment was repeated independently two times.

Values represent mean ± *SE* (n = 8), Mann Whitney U test, $P \leq 0.05$.

4.5. ProNRT2.4: GFP shows wild type like phenotypes under nitrate starvation

My previous results described the growth response of plants colonized with *M. hyalina*. To further investigate the role of NRT2.4 on plant growth under nitrate starvation, I co-cultivated NRT2.4 expressing and knockout (ko) plants with beneficial endophytic fungus *M. hyalina*. Fourteen days old Arabidopsis wild type (Col-0), ProNRT2.4: GFP and nrt2-4 plants were inoculated with *M. hyalina* or PDA plug. There was no obvious phenotypical difference in aerial size and formation between ProNRT2.4: GFP and nrt2.4 lines observed (Figure 21). However, detailed analysis showed a clear trend that the size of the shoot area was affected in nrt2.4 compared with wild type (Figure 22 C). In side roots (lateral root) formation, a significant difference between ProNRT2.4: GFP and nrt2.4 lines was detected (Figure 22 A). Interestingly, number of side roots of ProNRT2.4: GFP lines was higher than in nrt2.4 line. In main root length, up to four days the nrt2.4 roots showed significant less growth bit at day 6 and afterwards this phenomenon vanished (Figure 22 B). As photosynthesis is one of the important step for plant growth, chlorophyll fluorescence parameter (PSII-photo system II) was analyzed in both *M. hyalina* treated and control plants at 1, 4, 6, 8 and 10 days post inoculation. There were significant differences detected in PSII quantum yield in all tested lines at later time points (days 4-6) upon inoculation with the fungus (Figure 23).

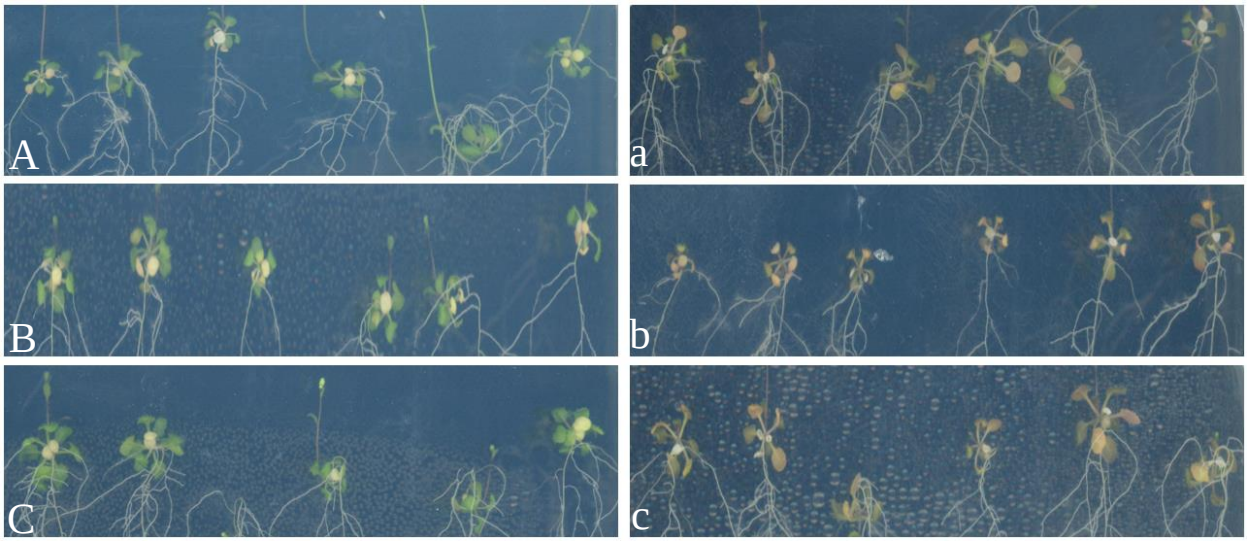


Figure 21: Response of *A. thaliana* wild type and transgenic plants inoculated with *M. hyalina* under nitrate starvation. A, B and C image of control plant. a, b and c image of treated plants. 14 days old *A. thaliana* wild type (Col-0) and transgenic lines grown on MGRL / 7 mM nitrate medium were further incubated with *M. hyalina* on no nitrate medium. Plants growth was observed at 10^{th} day of inoculation. *M. hyalina* treated plant was yellowish. Image A and a represent wild type (Col-0) control and treatment respectively. Image B and b represent ProNRT2.4: GFP control and treated respectively. Image C and c represent nrt2.4 control plant and treated respectively.

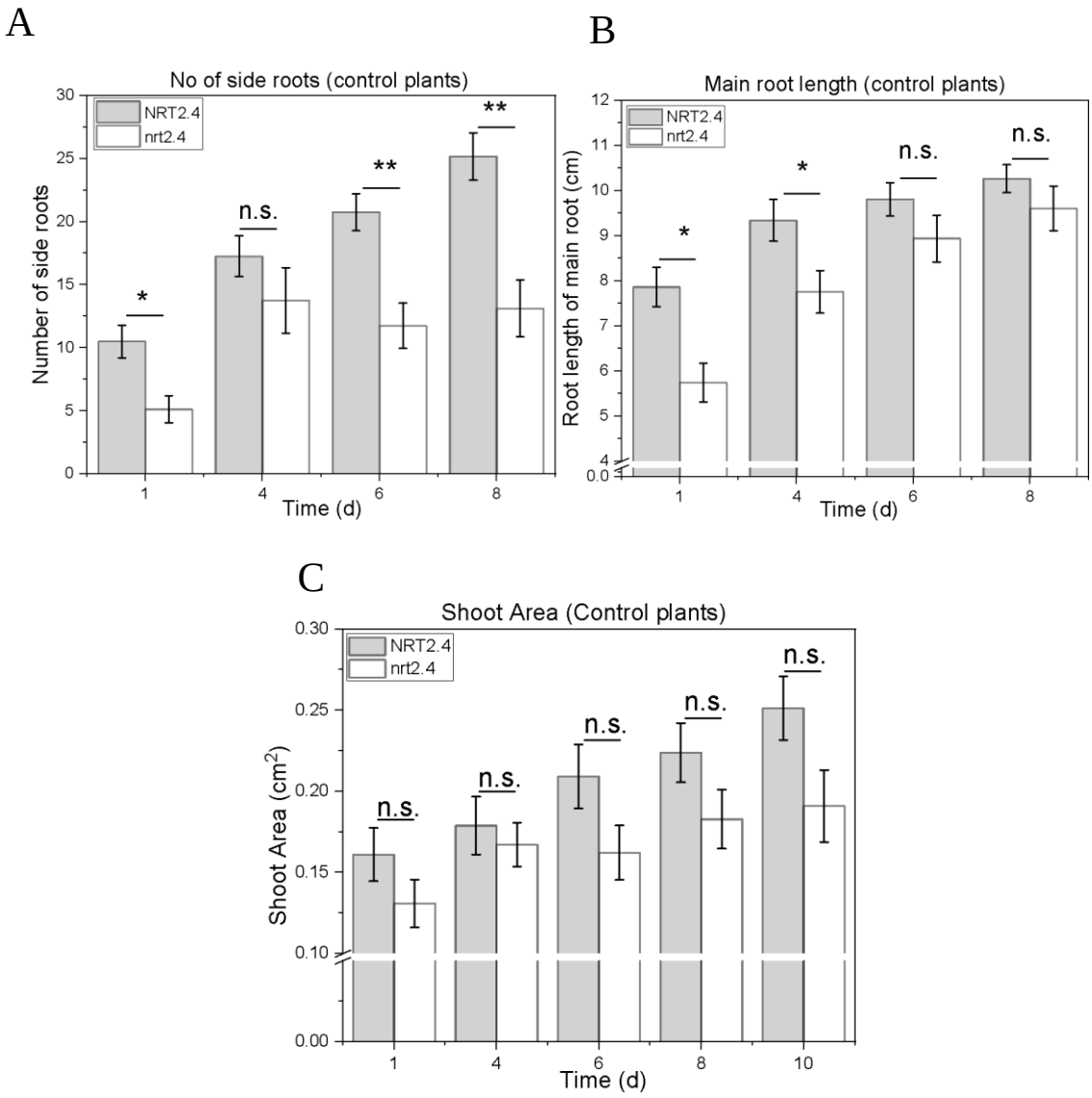


Figure 22: Plant growth analysis under nitrate stress. A. Number of side roots (lateral roots) in ProNRT2.4: GFP and nrt2.4 lines. **B.** Main root length in ProNRT2.4: GFP and nrt2.4 lines under nitrate starvation. **C.** Aerial growth of ProNRT2.4: GFP and nrt2.4 lines under nitrate starvation. ProNRT2.4: GFP plant (dark bar), nrt2.4 plant (open bar).

Values represent mean ± SE (n = 10). Experiment was repeated 2 times. n.s. = Not significant, Asterisks indicate significant differences, Mann Whitney U test, $P < 0.05$.

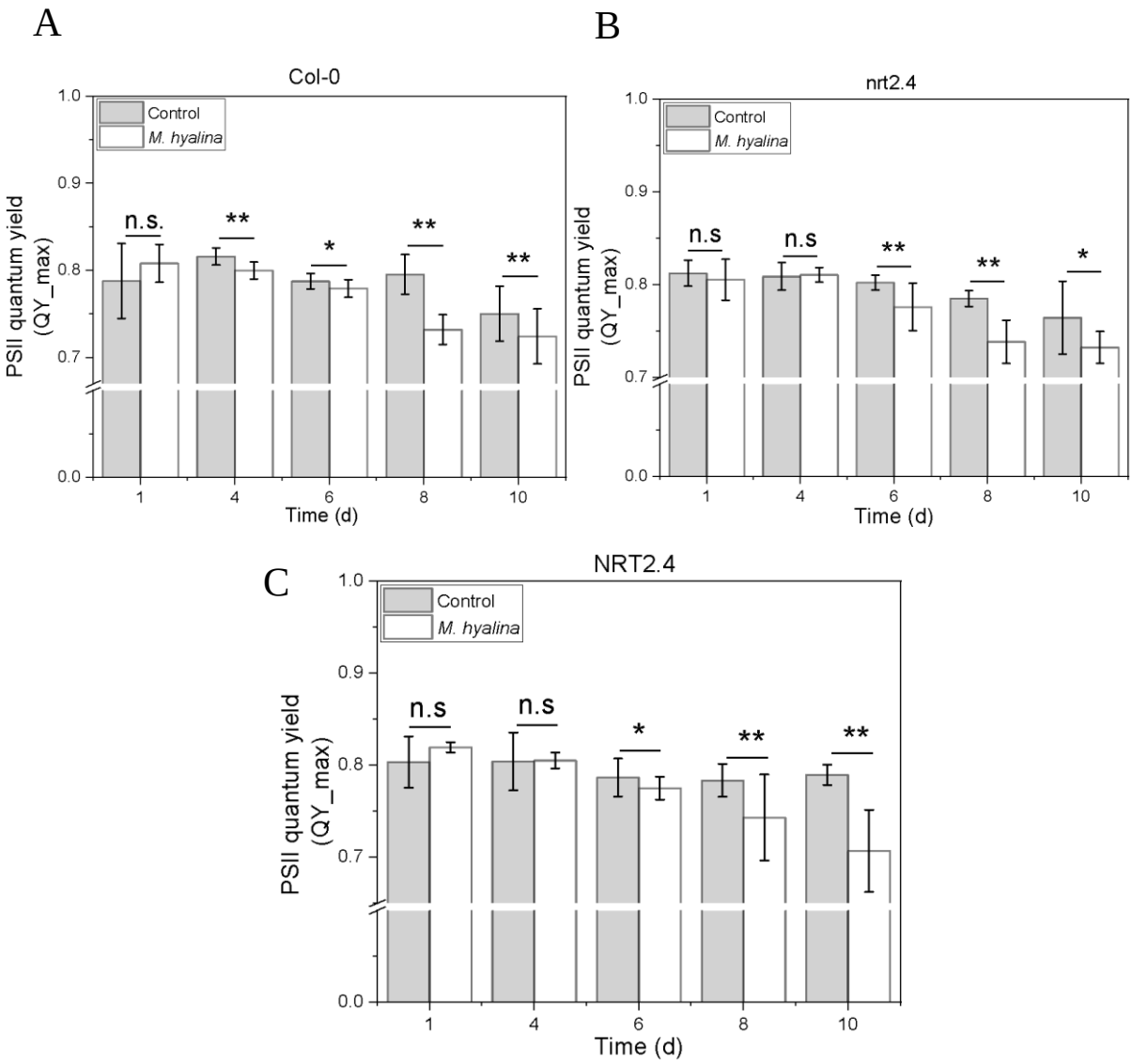


Figure 23: Statistical analysis of PSII quantum yield under nitrate stress. A. PSII quantum yield of Col-0 lines under nitrate starvation inoculated with *M. hyalina* or control. **B.** PSII quantum yield of nrt2.4 line under nitrate starvation inoculated with *M. hyalina* or control. **C.** PSII quantum yield of ProNRT2.4: GFP line under nitrate starvation inoculated with *M. hyalina* or control. Control plant (dark bar), treated plant (open bar).

Values represent mean ± SE (n = 10). Experiment was repeated 3 times. n.s. = Not significant, Asterisks indicate significant differences, Mann Whitney U test, $P < 0.05$.

4.6. Phytohormone production and influence of ProNRT2.4: GFP under nitrate starvation

To further investigate the influence of NRT2.4 on plant growth; I tried to check defense related phytohormone levels and the effects of NRT2.4 on those levels. It is already revealed that *M. hyalina* induced JA (Meents et al. 2019). Thus, it was interesting to investigate the level of defense related phytohormones in plants inoculated with *M. hyalina* under nitrate starvation. For the phytohormone measurement; ProNRT2.4: GFP, nrt2.4 and Col-0 line grown on MGRL / 7 mM nitrate medium were transferred onto no nitrate MGRL medium inoculated with *M. hyalina* or PDA plug. Col-0 line was used as control. Samples were collected after 10 days of incubation. SA concentration was significantly higher in shoots of all *M. hyalina* treated lines (Figure 24 A). Also, JA was found to be higher in shoots of Col-0 and ProNRT2.4: GFP but not in nrt2.4 (Figure 24 B). For ABA significant differences were observed in all three lines with higher level in treated plants. In roots, more ABA was detected in nrt2.4 and ProNRT2.4: GFP but not in Col-0 (Figure 24 C). In shoots ABA was detected higher in all three lines. There were no significant difference of IAA level found in both roots and shoots (Figure 25 D). A significant difference in cis OPDA was detected only in shoots of Col-0 and nrt2.4. No significant difference was observed in roots (Figure 25 E). JA Ile showed significant difference in only shoots of wild type plants but not in roots (Figure 25 F).

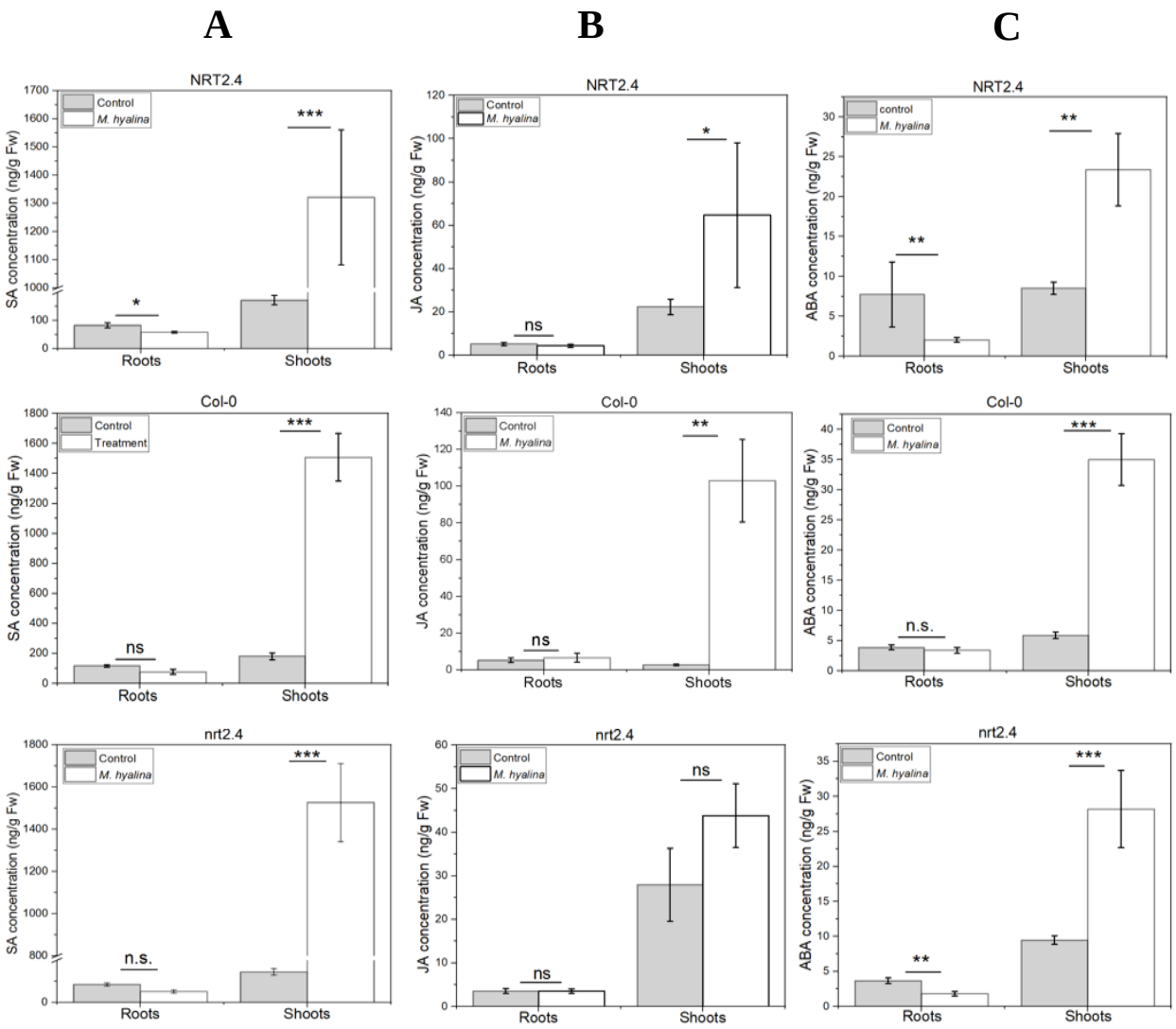


Figure 24: Phytohormone level of *Arabidopsis thaliana* wild type and transgenic lines incubated with *M. hyalina*. Column A, B and C represent SA, JA and ABA level respectively. Phytohormone level of Col-0 and transgenic lines (ProNRT2.4: GFP, nrt2.4) were measured by HPLC-MS. Asterisks indicated significant difference between control and *M. hyalina* treated plants at 10 days of incubation. Control treatment PDA plug was used as mock. Control plant (dark bar), treated plant (open bar).

Values represent mean ± SE (n = 8). Experiment was repeated 3 times. n.s. = Not significant, Asterisks indicate significant differences, Mann Whitney U test, $P < 0.05$.

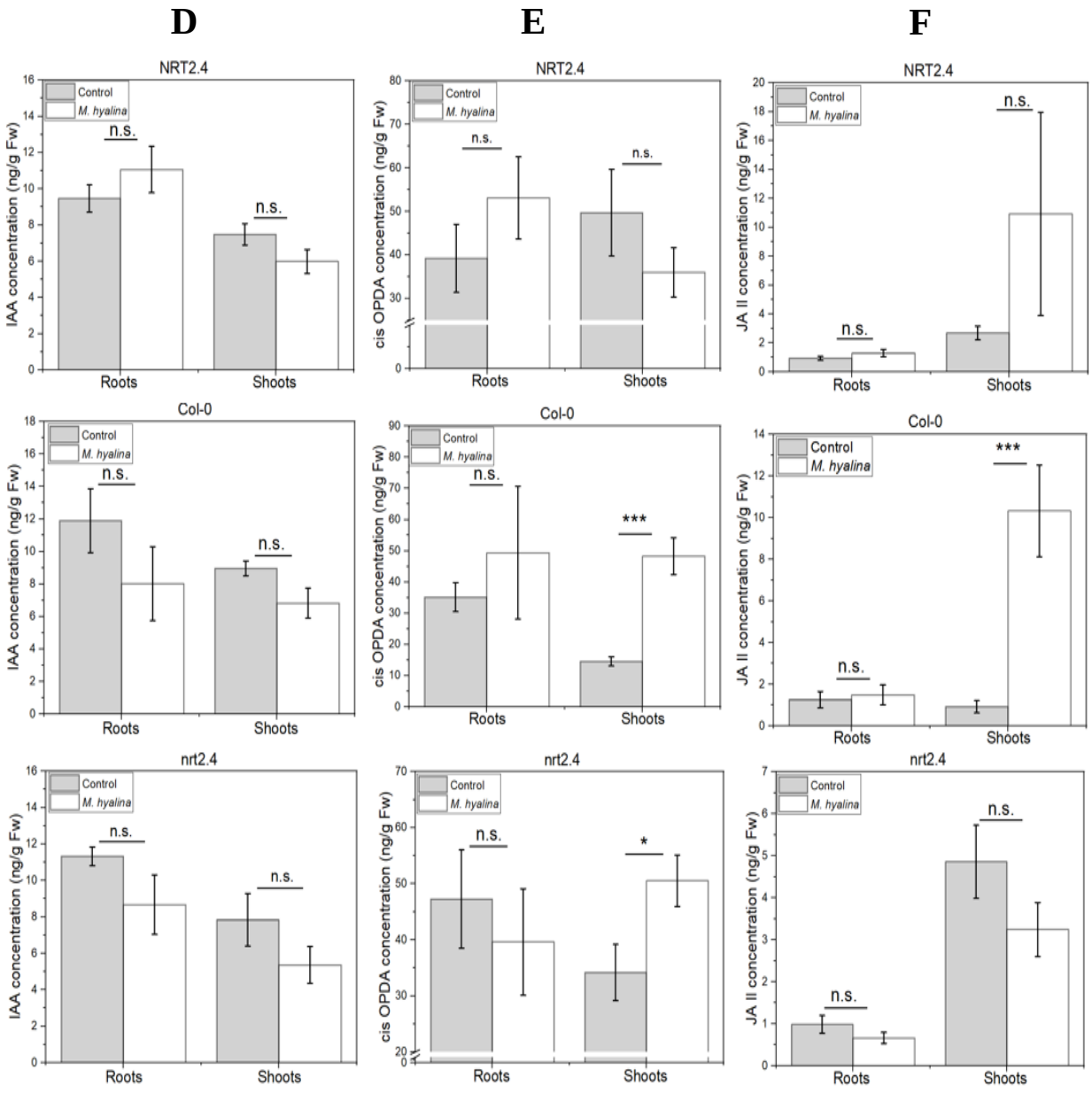


Figure 25: Phytohormone level of *Arabidopsis thaliana* wild type and transgenic lines incubated with *M. hyalina*. Column D, E and F represent IAA; cis OPDA (12-oxophytodienoic acid) and JA Il (jasmonoyl-isoleucine) level respectively. Phytohormone level of Col-0 and transgenic lines (ProNRT2.4: GFP, nrt2.4) were measured by HPLC-MS. Asterisks indicated significant difference between control and *M. hyalina* treated plants at 10 days of incubation. Control treatment PDA plug was used as mock. Control plant (dark bar), treated plant (open bar).

Values represent mean ± SE (n = 8). Experiment was repeated 3 times. n.s. = Not significant, Asterisks indicate significant differences, Mann Whitney U test, $P < 0.05$.

4.7. Amino acid level and influence of NRT2.4 under nitrate starvation

Along with phytohormones, stress affects amino acid metabolism in plants. It was important to quantify amino acid induction in colonized stressed plants. Therefore, wild-type (Col-0), ProNRT2.4: GFP and nrt2.4 lines (14 days old) were grown on MGRL / 7 mM nitrate medium and transferred to no nitrate medium with / without *M. hyalina*-colonization. Quantification of amino acids was performed after 10 days of inoculation. The results obtained showed that *M. hyalina*-colonized plants had lower level of amino acids compared with non-colonized plants in all lines. In details, nine amino acids were quantified, those were alanine (Figure 26 A), proline (Figure 26 B), lysine (Figure 26 C), glutamine (Figure 27 D), tryptophan (Figure 27 E), Valine (Figure 27 F), methionine (Figure 28 G), aspartic acid (Figure 28 H) and glutamate (Figure 28 I). Almost all the results showed significant difference in *M. hyalina*-colonized plants.

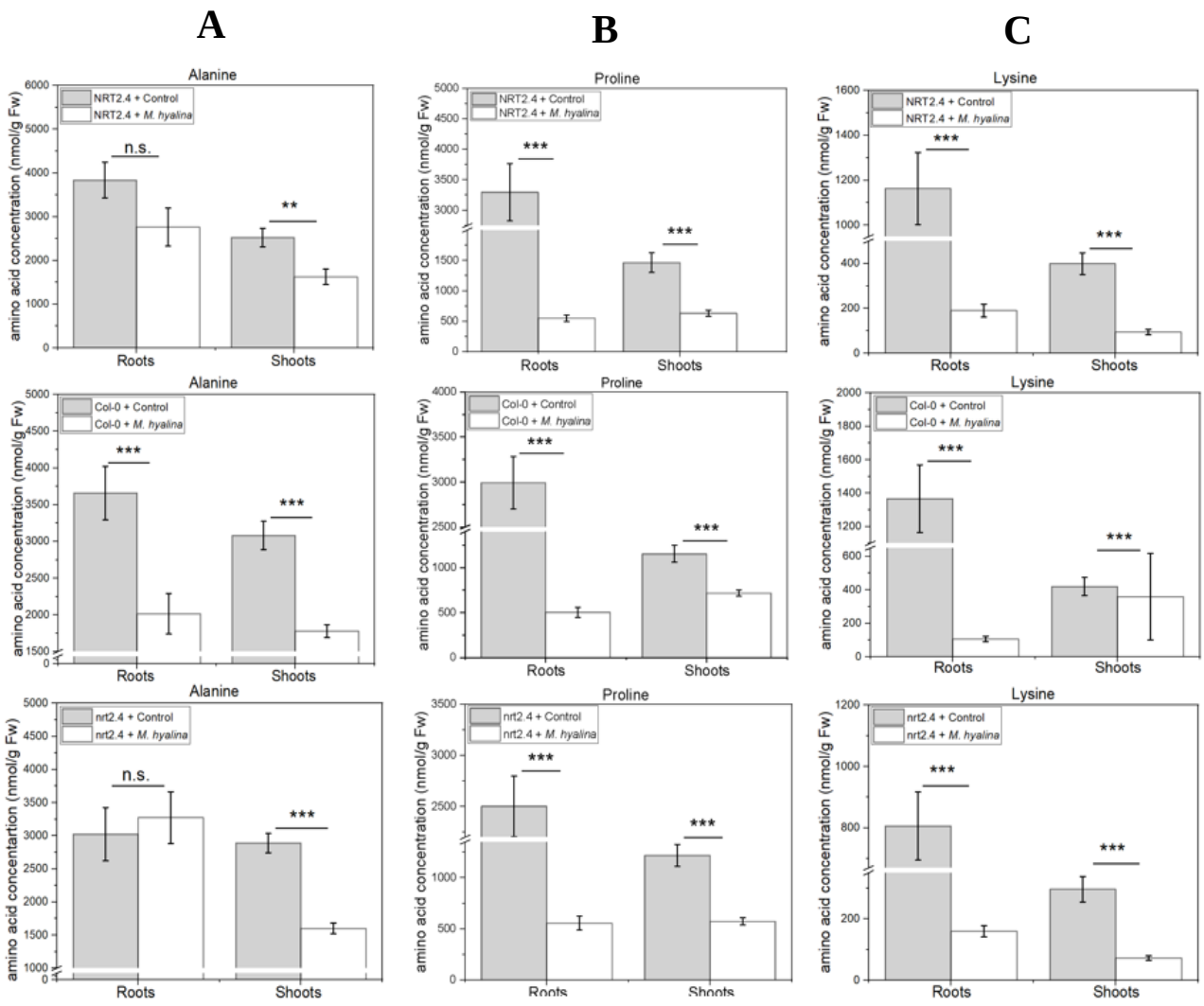


Figure 26: Amino acid level of *Arabidopsis thaliana* wild type and transgenic lines incubated with *M. hyalina*. Column A, B and C represent Alanine, Proline and Lysine level respectively. Amino acid level of Col-0 and transgenic lines (ProNRT2.4: GFP, nrt2.4) were measured by HPLC-MS. Asterisks indicated significant difference between control and *M. hyalina* treated plants at 10 days of incubation. Control treatment PDA plug was used as mock. Control plant (dark bar), treated plant (open bar).

Values represent mean ± SE (n = 14). Experiment was repeated 2 times. n.s. = Not significant, Asterisks indicate significant differences, Mann Whitney U test, $P < 0.05$.

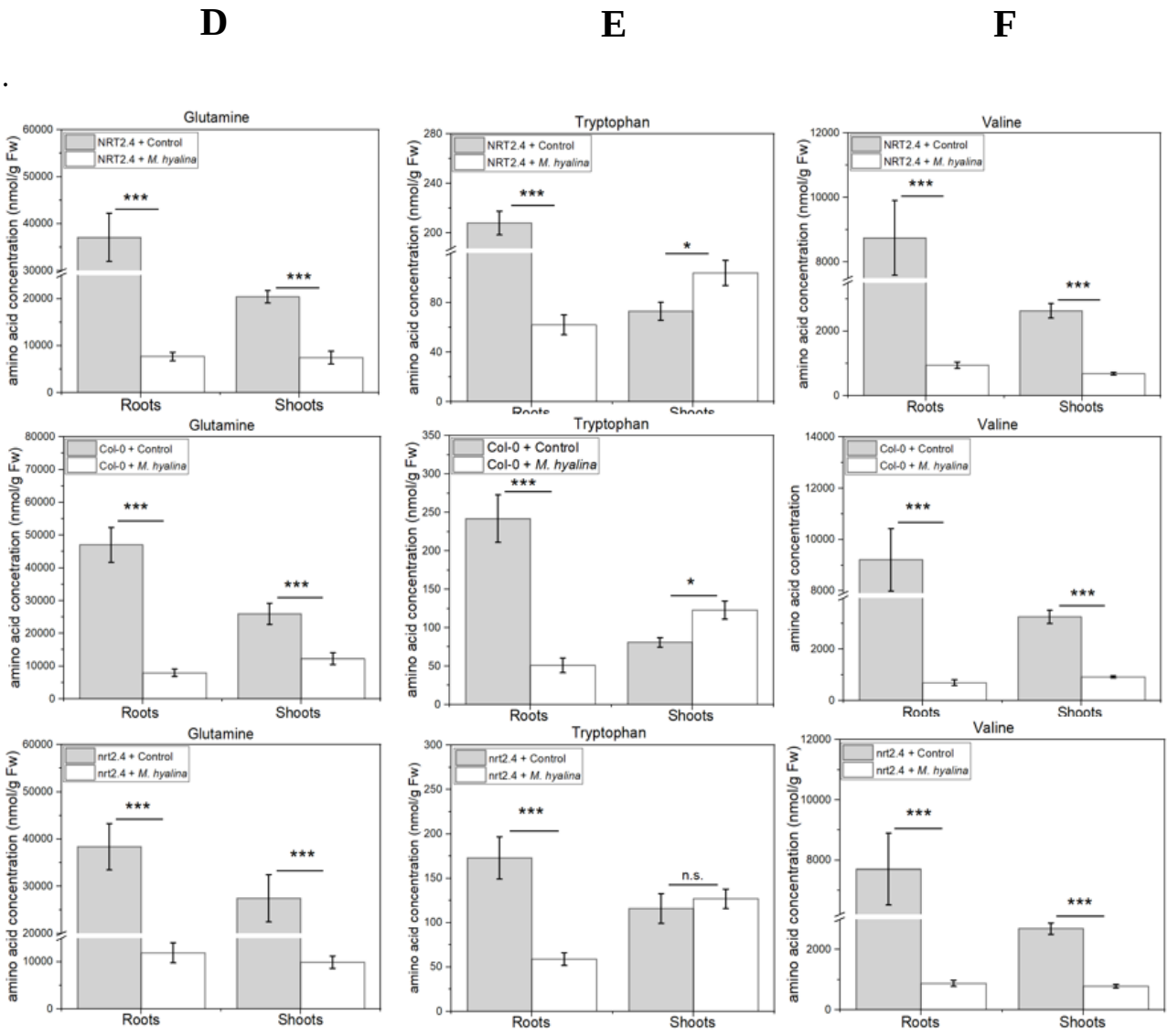


Figure 27: Amino acid level of *Arabidopsis thaliana* wild type and transgenic lines incubated with *M. hyalina.* Column D, E and F represent Glutamine, Tryptophan and Valine level respectively. Amino acid level of Col-0 and transgenic lines (ProNRT2.4: GFP, nrt2.4) were measured by HPLC-MS. Asterisks indicated significant difference between control and *M. hyalina* treated plants at 10 days of incubation. Control treatment PDA plug was used as mock. Control plant (dark bar), treated plant (open bar).

Values represent mean ± SE (n = 14). Experiment was repeated 2 times. n.s. = Not significant, Asterisks indicate significant differences, Mann Whitney U test, $P < 0.05$.

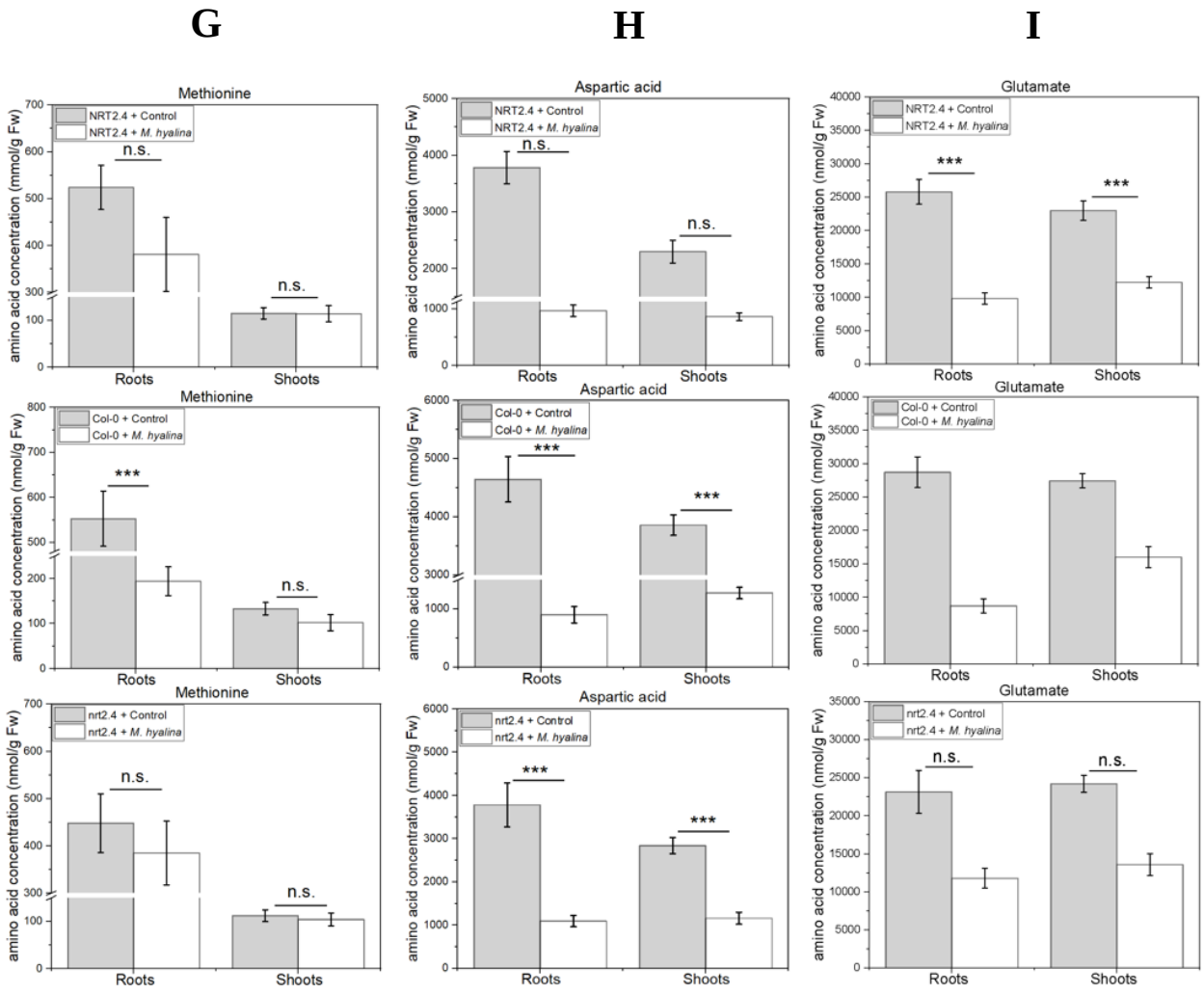


Figure 28: Amino acid level of *Arabidopsis thaliana* wild type and transgenic lines incubated colonized *M. hyalina.* Column G, H and I represent Methionine, Aspartic acid and Glutamate level respectively. Amino acid level of Col-0 and transgenic lines (ProNRT2.4: GFP, nrt2.4) were measured by HPLC-MS. Asterisks indicated significant difference between control and *M. hyalina* treated plants at 10 days of incubation. Control treatment PDA plug was used as mock. Control plant (dark bar), treated plant (open bar).

Values represent mean ± SE (n = 14). Experiment was repeated 2 times. n.s. = Not significant, Asterisks indicate significant differences, Mann Whitney U test, $P < 0.05$.

4.8. Effect of exogenously applied IAA on the expression of ProNRT2.4: GFP construct

IAA is one of the most important phytohormones for plant growth. To test whether IAA is involved in the induction of NRT2.4, we treated our plants with IAA (1 µM) in root and shoot separetley. To text the expression of ProNRT2.4: GFP to exogenously applied IAA, 14 days old Arabidopsis plants grown on MGRL / 0.25 mM nitrate medium, were observed at different time intervals. Therefore, before addition of IAA (0-IAA), after addition of IAA (0+IAA), 1, 2, 3 and 6 h of incubation was monitored. About 15 min were needed to monitore and scanned the whole experiment at one time interval (Time gap 45 min). Exogenously added water was used as a control treatment. Root and shoot were treated separately. In shoot treatment, leaves were sprayed with 1 µM IAA and GFP fluorescence was monitored under fluorescence microscope. Observation was performed before spray as well in order to verify the effect of exogenously applied IAA on NRT2.4. A significant difference has been observed in main root tips and side roots upon shoot treatment immediately after application of IAA (0+IAA), but not in main roots. Fluorescence intensity was higher (almost twice) in the IAA treated plants and maintained at a stable plateau (Figures 29 & 30).

In root treatments, 1 µM of 10 µl IAA was exogenously added to the root tips and monitored at previously mentioned time points (Figure 31 & 32). Significant intensity of the fluorescence signals was observed only in side roots. Interestingly, no significant difference was observed in root tips. In both treatments there was no significent difference in the main root detectable. However, in both root and shoot treated plants revealed that exogenously applied IAA treatment altered the expression of nitrate transporter NRT2.4 in root tissues. This results led us to investigate the expression of jasmonic acid treatment as well.

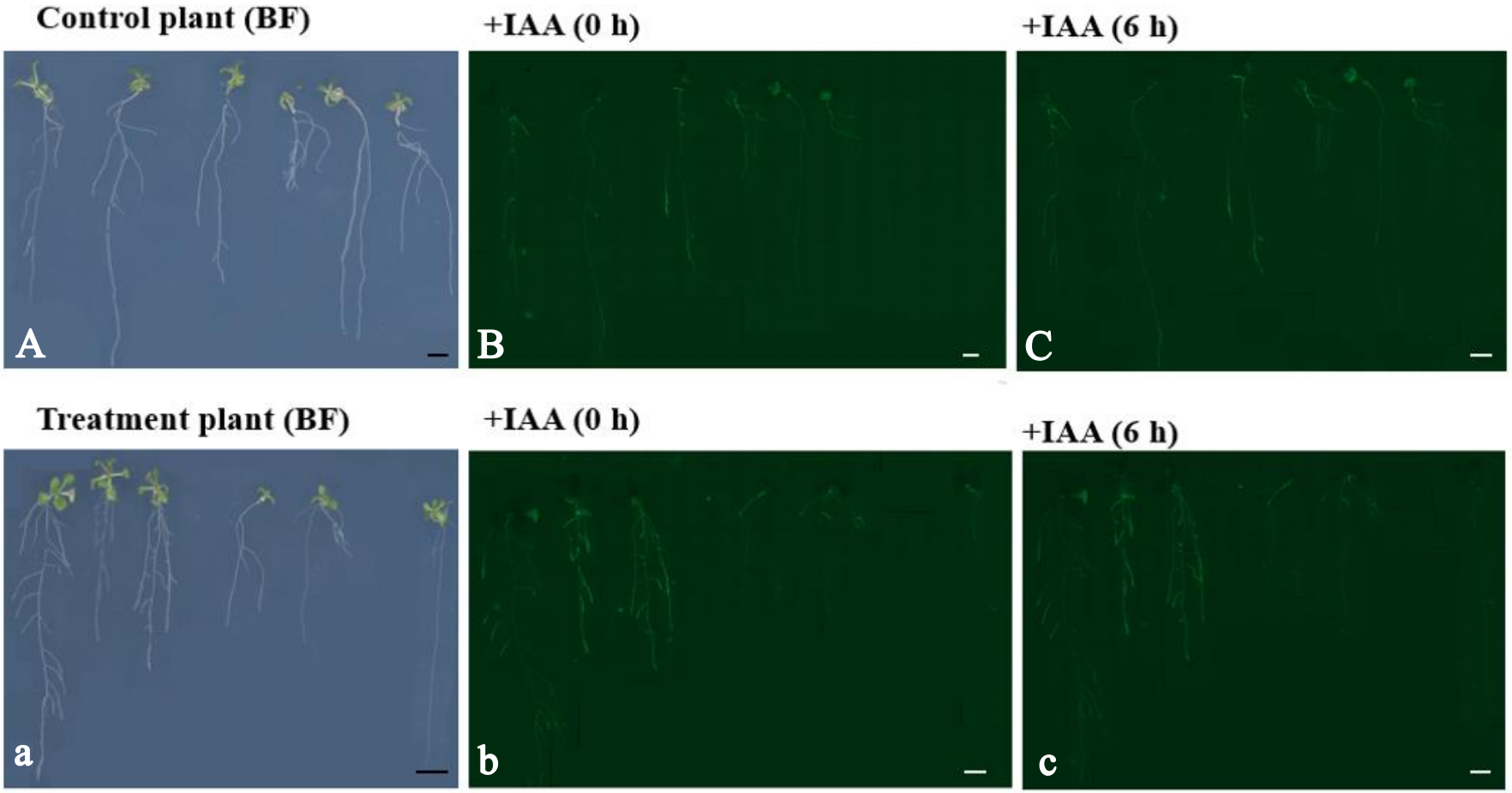


Figure 29: Image of ProNRT2.4-GFP transgenic plant, shoot sprayed with IAA. B, C and b, c GFP fluorescence and A, a bright field image of this respective plants. 14 days old Pro:NRT2.4 grown on 0.25 mM nitrate MGRL medium then further sprayed with 1 µM IAA. Plants were observed at different time interval. Image B and C GFP image of control plants at just after treatment and 6 h respectively. Image b and c GFP image of treatment plants just after treatment and 6 hr respectively. In control treatment, sterile water was used as control. Values represent mean ± SE (n = 8); n.s. = Not significant, Asterisks indicate significant differences, Mann Whitney U test,$P < 0.05$. Scale = 2000 µm.

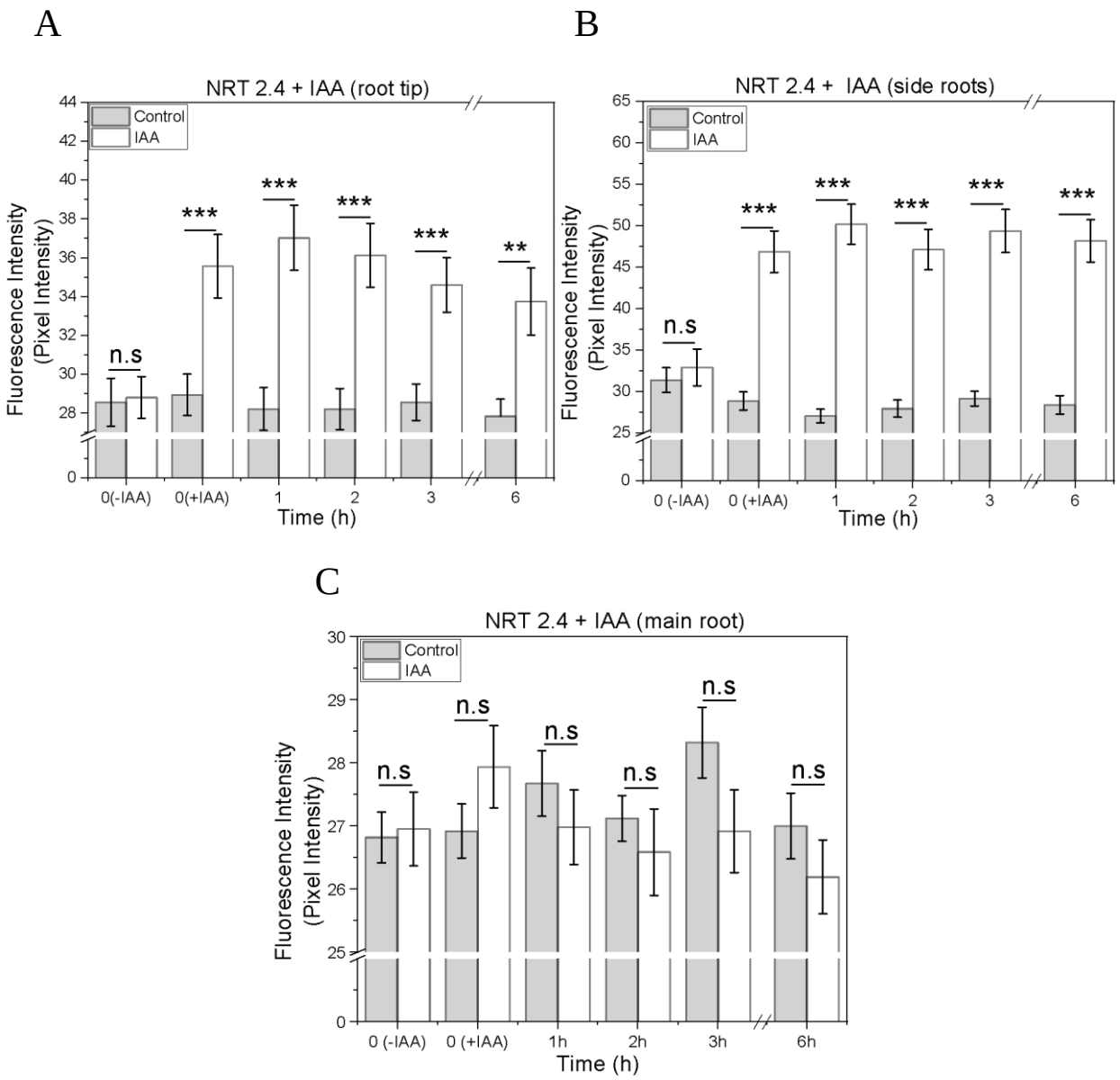


Figure 30: Statistical analysis of fluorescence image of ProNRT2.4: GFP in shoot treatment. Fluorescence intensity was obtained from Microscopic image (Figure 29). Asterisks indicated significant difference between control and IAA treated plants. A, B and C represent the results of root tips, side roots and main roots respectively. Control plant (dark bar), treated plant (open bar).

Values represent mean ± SE (n = 8). n.s. = Not significant, Asterisks indicate significant differences, Mann Whitney U test, $P < 0.05$.

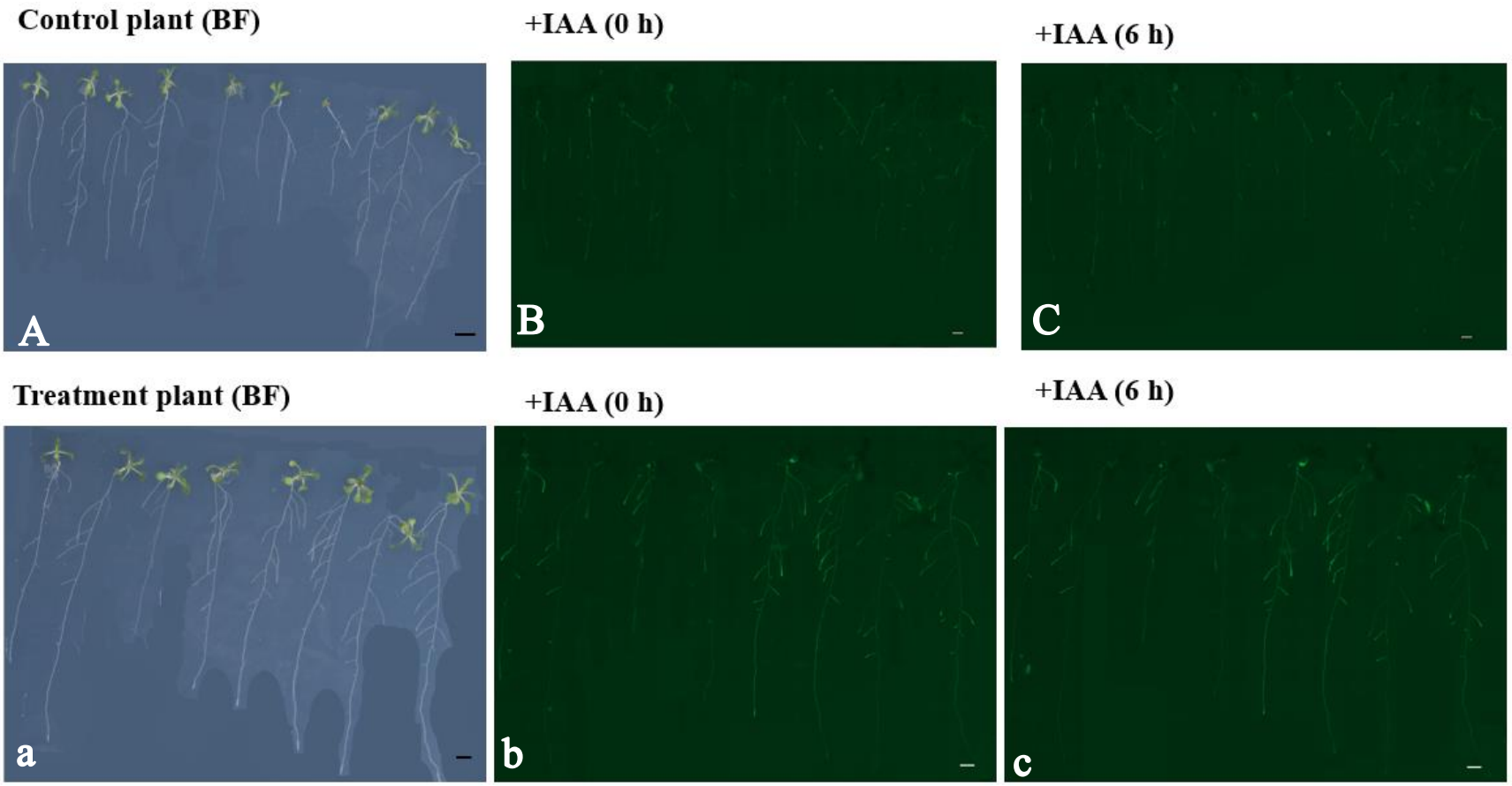


Figure 31: Image of ProNRT2.4-GFP transgenic plant. Root treated with 1µM IAA (10 µl). B, C and b, c GFP fluorescence and A, a bright field image of this respective plants. 14 days old Pro:NRT2.4 grown on 0.25 mM nitrate MGRL medium then further 10µl of IAA (1 µM) was added to the root tip of the plant. Plants were observed at different time interval. Image B and C GFP image of control plants at 0 hr (just after treatment) and 6 h respectively. Image b and c GFP image of IAA treated plants at 0 hr (just after IAA treatment) and 6 hr respectively. In control treatment, sterile water was used as control. Values represent mean ± SE (n = 8); n.s. = Not significant, Asterisks indicate significant differences, Mann Whitney U test, $P < 0.05$. Scale bar = 2000 µm.

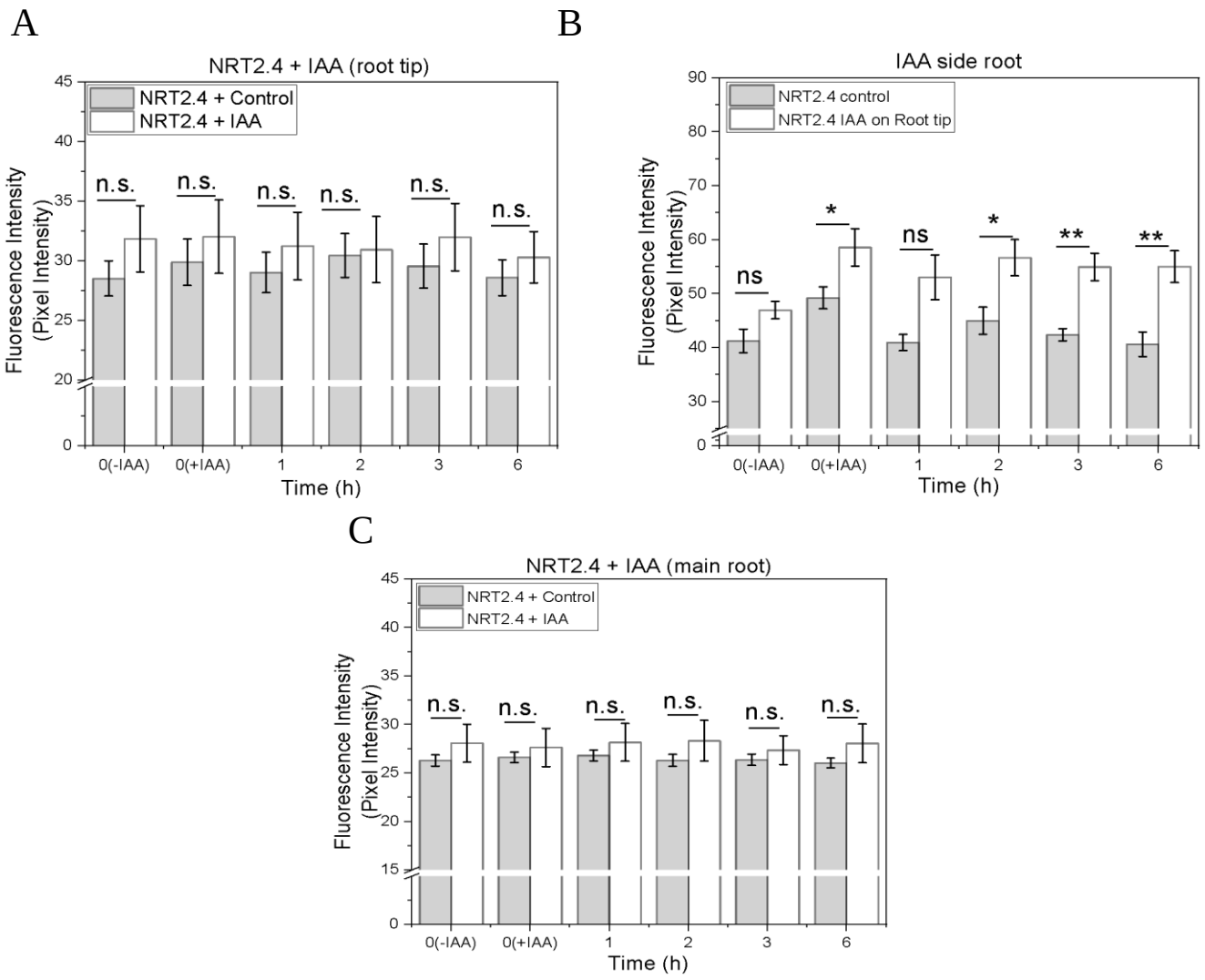


Figure 32: Statistical analysis of fluorescence images of ProNRT2.4: GFP upon root treatment. Fluorescence intensity was obtained from Microscopic image (Figure 31). Asterisks indicated significant differences between control and IAA treated plants. A, B and C represent the results of root tips, side roots and main roots respectively. Control plant (dark bar), treated plant (open bar).

Values represent mean ± SE (n = 8). n.s. = Not significant, Asterisks indicate significant differences, Mann Whitney U test, $P < 0.05$.

4.9. Effect of exogenously applied jasmonic acid (JA) on the expression of ProNRT2.4: GFP construct

M. hyalina cocultivation induced JA in host plant (Meents et al. 2019). Therefore it was interesting to investigate the effect of exogenously applied JA on ProNRT2.4:GFP construct. For this experiment, 14 days old plant grown on MGRL / 0.25 mM nitrate medium, were monitored at different time intervals like before. Each experiment (each time point) took 15 minutes to monitor the fluorescence signals. GFP fluorescence was monitored just before addition of JA (0-JA), After addition of JA (0+JA), 1, 2, 3 and 6 h of incubation. 15 min was needed to monitore and scanned the whole experiment at one time interval (Time gap 45 min). Water was used as control. Root and shoot were treated separately. In shoot treatment, leaves were sprayed with 1 mM JA and GFP fluorescence was monitored under fluorescence microscope. Observation was performed before spray as well in order to verify the effect of exogenously applied JA on NRT2.4. A significant difference has been observed only in side roots upon shoot treatment. Here, fluorescence intensity was lower in the JA treated plants (side roots) (Figures 33 & 34).

In root treatment, 1 mM of 10 µl JA was exogenously added to the root tip and monitored at previously mentioned time points. Significant intensity of the fluorescence signals was observed only in side roots after 3 h of incubation and afterward. JA treated plants showed low fluorescence intensity in comparing with control. No significant difference was observed in root tip. In both treatment there was no significent difference in the main root. However in both root and shoot treated plants, revealed that exogenously applied JA treatment altered the expression of nitrate transporter NRT2.4 in root tissues (side roots) (Figures 35 & 36).

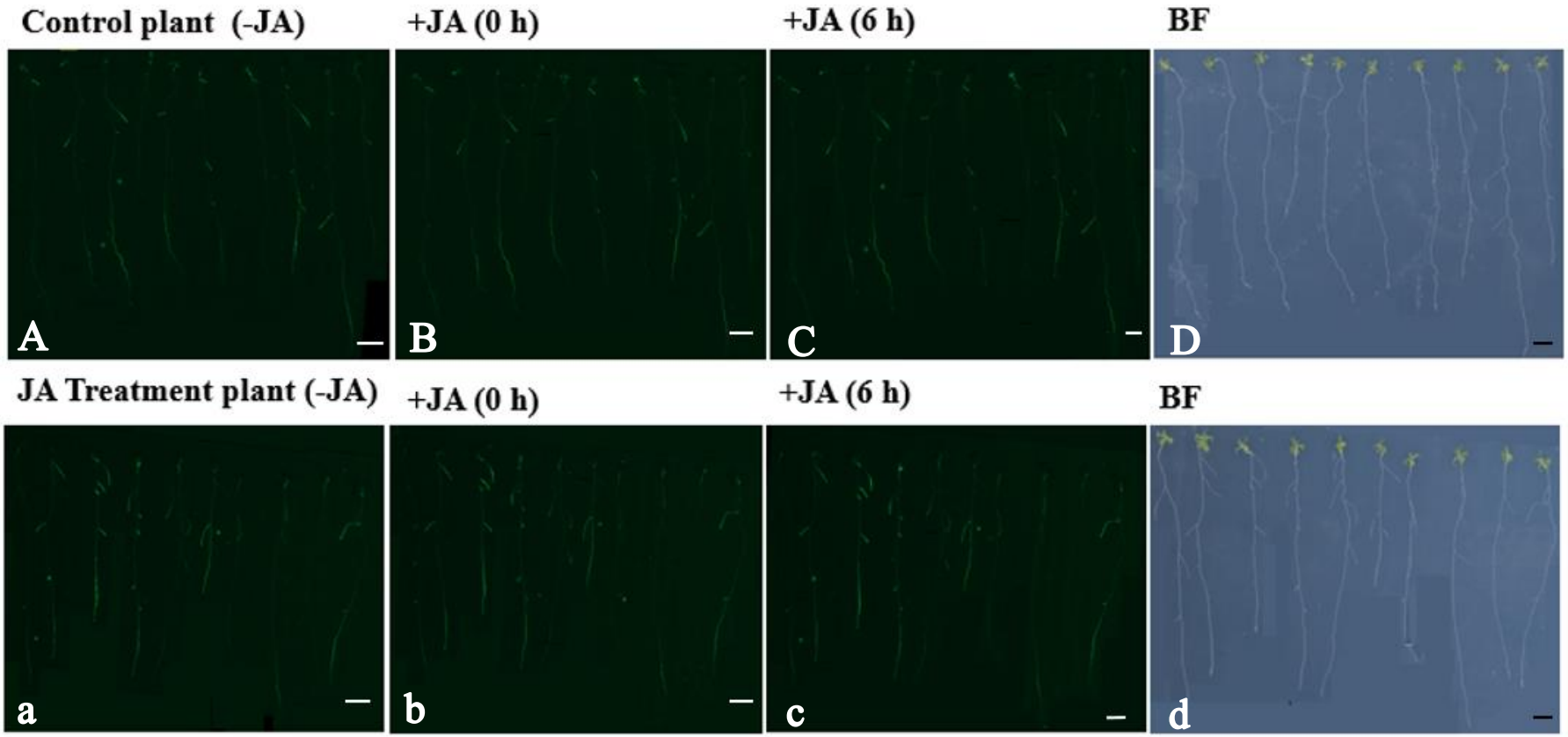


Figure 33: Image of ProNRT2.4-GFP transgenic plant, Shoot sprayed with JA. A, B, C and a, b, c GFP fluorescence and D, d bright field image of this respective plants. 14 days old Pro:NRT2.4 grown on MGRL / 0.25 mM nitrate medium then further sprayed with 1 mM JA. Plants were observed at different time interval. Image A, B and C GFP image of control plants at before treatment, after treatment and 6 hr respectively. Image a, b and c GFP image of treatment plants at before treatment, after treatment and 6 hr respectively. In control treatment, sterile water was used as control.

Scale bar = 2000µm.

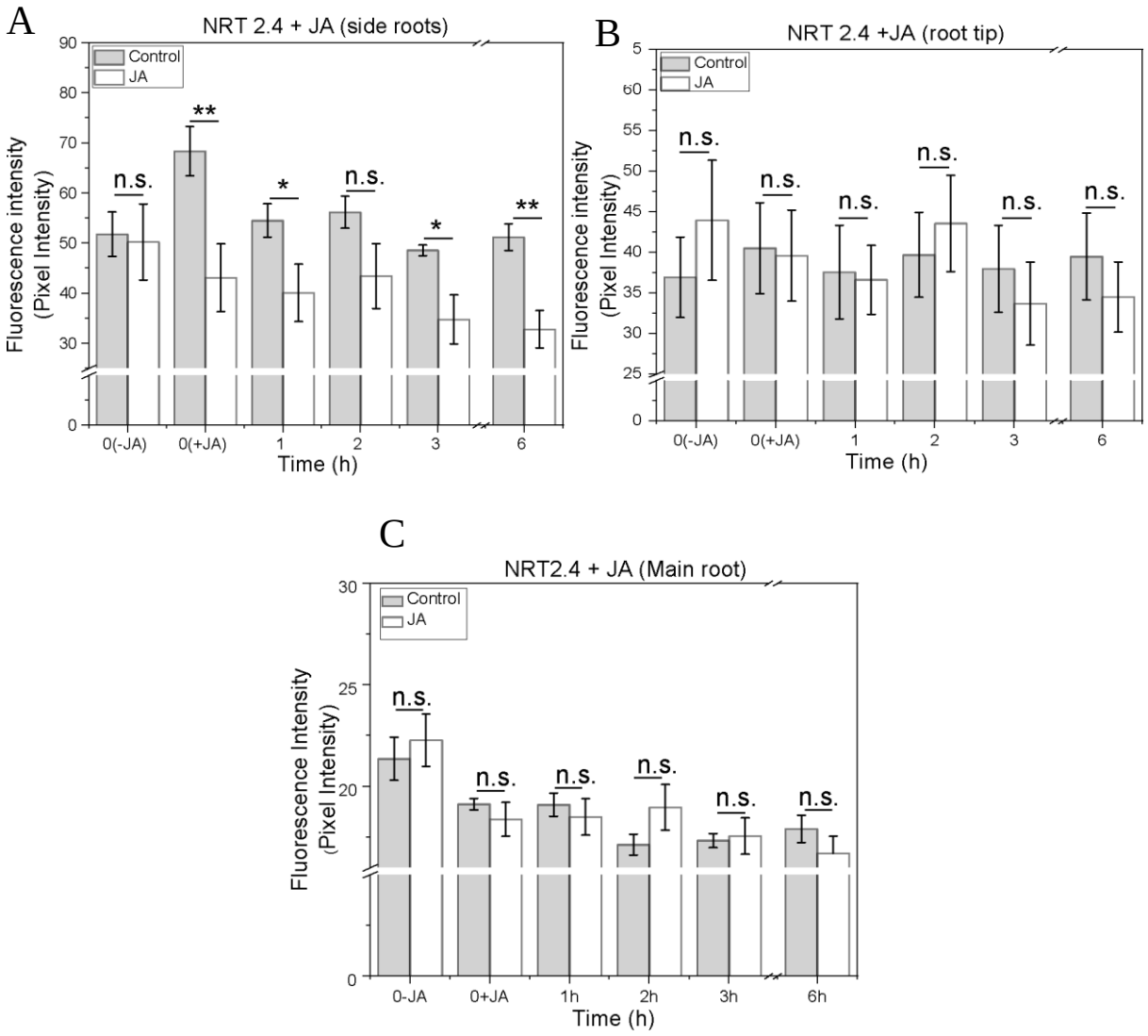


Figure 34: Statistical analysis of fluorescence images of ProNRT2.4: GFP upon JA shoot treatment. Fluorescence intensity was obtained from Microscopic image (Figure 33). Asterisks indicated significant difference between control and JA (1 mM) treated plants. A, B and C represent the results of side roots, root tips and main roots respectively. Control plant (dark bar), treated plant (open bar).

Values represent mean ± SE (n = 8). n.s. = Not significant, Asterisks indicate significant differences, Mann Whitney U test, $P < 0.05$.

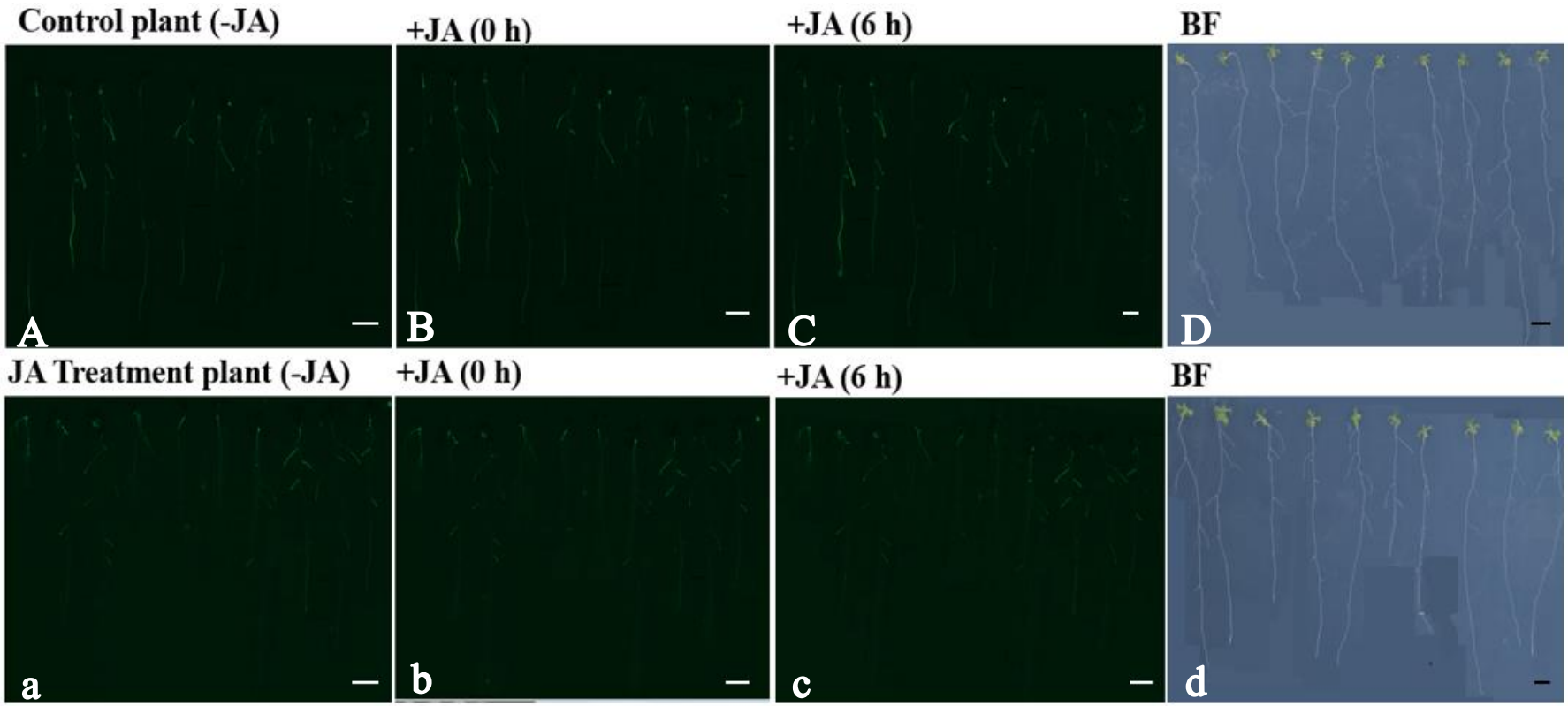


Figure 35: Image of ProNRT2.4-GFP transgenic plant, root treatment with JA. A, B, C and a, b, c GFP fluorescence and D, d bright field image of this respective plants. 14 days old Pro:NRT2.4 grown on 0.25 mM nitrate MGRL medium thentreated 10 µl of 1 mM JA. Plants were observed at different time interval. Image A, B and C GFP image of control plants at before treatment, after treatment and 6 hr respectively. Image a, b and c GFP image of treatment plants at before treatment, after treatment and 6 hr respectively. In control treatment, sterile water was used as control.

Scale bar = 2000µm.

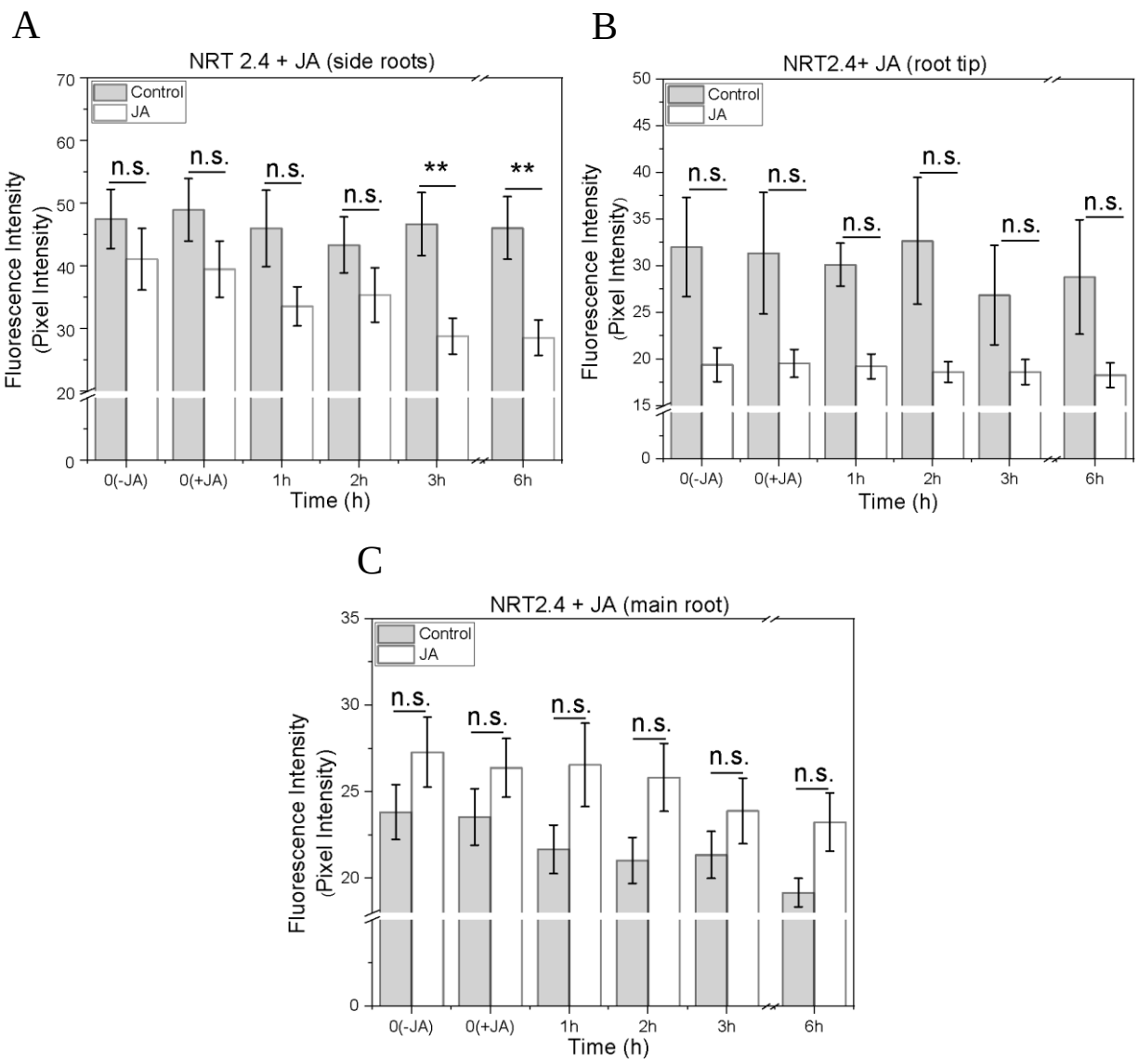


Figure 36: Statistical analysis of fluorescence image of ProNRT2.4: GFP upon JA root treatment. Fluorescence intensity was obtained from Microscopic image (Figure 35). Asterisks indicated significant difference between control and JA treated plants. Control treatment sterile water was used as control. A, B and C represent the results of side root, root tip and main root respectively.

Values represent mean ± SE (n = 8). n.s. = Not significant, Asterisks indicate significant differences, Mann Whitney U test, $P < 0.05$.

5. Discussion

Nitrate transporter 2 family genes have versatile mechanisms to adapt with different nitrate concentrations. During the nitrate uptake, the plant root is the first organ to be confronted with the environment and most of the genes of nitrate transporter family 2 are expressed in roots. In this thesis, I first verified the previous results of Kiba et al. (2012) on NRT2s gene expression. NRT2.1 and NRT2.4 both have unique mechanisms, NRT2.1 is induced both during nitrate starvation and limitation while NRT2.4 is induced only during nitrate starvation condition. NRT2.4 is located in the epidermis while NRT2.1 is in the root cortex (Kiba et al. 2012). NRT2.1 and NRT2.4 are also expressed in a tissue specific pattern. NRT2.1 is expressed in older part of the main root, whereas NRT2.4 expression is observed in younger part of the lateral root (side roots) and young part of the main root (few mm from rot tip) (Figure 13). There is no expression of NRT2.1 and NRT2.4 in shoots. Using the equipment in the lab, my first experiments confirmed the previously reported results on NRT2.1 and NRT2.4 expression pattern, providing the basis for additional NRT2.1 and NRT2.4 related studies.

NRT2.1 and NRT2.4 genes were first chosen for experiments, because of their versatile expression pattern under different nitrate concentrations. NRT2.1 expression level changed significantly between 0.1 mM and 10mM nitrate medium (Figure 14, 15). This finding indicates, the dose depended expression pattern of NRT2.1 due to different nitrate concentrations, which differentiate the NRT2.1 transporter from other nitrate transporter. On other hand Kiba et al. (2012) showed the unique expression pattern of NRT2.4. Therefore, this unique expression pattern of NRT2.4 ideally suited for co-cultivation experiments when available nitrate becomes scarce.

NRT2.4 is located at soil / root interface which would be ideal to interact with soil microbes under various nitrate condition. So it was really interesting to check NRT2.4 expression in the presence of beneficial fungi-colonized plants. Previous studies revealed that root colonizing *P. indica*, promotes the fresh biomass production of colonized plants (Lee et al. 2011; Varma et al., 2012). Kumar et al. (2012), reported that beneficial fungi like *P. indica* not only induce plant growth but also play a role in nutrient transport to the plant. For example, *P. indica* is able to transfer inorganic phosphate to plants under P limitation. Along with beneficial fungi, arbuscular mycorrhizal fungi are also able to regulate the phosphate transporter under stress conditions. My results clearly show that *NRT2.4* expression was strongly down-regulated in *P. indica*-colonized

plants under nitrate starvation (Figure 16). This effect was mainly observed in side roots (lateral roots). *NRT2.4* expression was higher in control treatment which indicated the nitrate stress in medium. Statistical analysis showed significant effects of *P. indica* on NRT2.4 after 6 days of inoculation under nitrate starvation (Figure 17). It might be that *P. indica* reduced the plants stress by transferring nitrate to the roots, which led us to investigate the co-cultivation with other beneficial fungi *M. hyalina*.

Potential role of NRT2.4 was also verified with another beneficial fungus, *M. hyalina*. *M. hyalina*, an endophyte soil fungus with root colonizing ability ideally suited with inoculation experiments. Therefore, it was really interesting to investigate whether the growth promoting activity of *M. hyalina* is just an intrinsic property of fungus or dependent on the availability of nitrogen source in environment. However, I first investigated the time-dependent effect of *M. hyalina* on NRT2.4 nitrate transporter expression. The fluorescence of the NRT2.4 representing reporter was lower upon *M. hyalina* treatment than in controls, which suggested nitrate transport ability of *M. hyalina* (Figures 18 & 19). Data analysis confirmed that *M. hyalina* down-regulated the NRT2.4 gene under nitrate limitation whereas control plant shows up-regulation of this gene (Figure 20). Low induction or down-regulation of this gene, might indicate no nitrate deficiency stress. Significant difference was observed in both side roots and root tip after 4 days of colonization and afterward (Figure 20). Up to now mechanisms concerned for the nitrate transport from *M. hyalina* to plants are still unknown. From this thesis, it assumed that nitrate could be transferred to the plant via hyphae of the fungus or it might be that *M. hyalina* converts the other component of the medium (Agar) to form nitrogen containing and usable compounds.

The *NRT2.4* expression results led me deeper investigate the effect of NRT2.4 on plant growth under nitrate limitation. In case of fungal-colonization and control no significant difference on growth phenotype was observed at 10^{th} day of inoculation in Col-0, NRT2.4 and nrt2.4 lines (Figure 21). Data analysis of nrt2.4 and NRT2.4: GFP lines (control treatment) showed no differences in various parameters, which confirmed results of Figure 21 (Figure 22). This was also verified by statistical analysis of main root length of NRT2.4: GFP and nrt2.4 lines (control treatment). No significant difference has been observed in these two lines (Figure 22, B), whereas in side roots (lateral roots) of NRT2.4: GFP expression was higher than in *nrt2.4* after 6 days of nitrate starvation (Figure 22, A). However, no significant growth effect has been observed in *nrt2.4*

mutant (only number of side roots) indicating that *nrt2.4* mutation has no effect on shoot growth. It might be that other nitrate transporters are also involved, rescuing the plant. This growth phenotype results confirmed the previous studies of Kiba et al. (2012) on sand culture condition.

Translocated nitrate takes a role in photosynthesis system via long distance root to shoot transport process referred to as nitrate photo assimilation. Around 75% of the assimilated nitrate is located into the green parts of the plant (Chloroplasts). Therefore, nitrate uptake and it's assimilation into plant cells are directly related to photosynthesis system (Kiba et al. 2012; Wu et al. 2018). That led me to investigate photosynthesis (PSII) of Col-0, NRT2.4: GFP and nrt2.4 lines. In this experiment, significant difference was observed between control and *M. hyalina*-colonized plants (Figures 23 A, B & C). The *nrt2.4* mutant showed a similar trend like wild-type (Col-0) and NRT2.4: GFP lines. PSII quantum yield was significantly down-regulated in *M. hyalina*-colonized plants at different inoculation time. This results suggested the reason behind the yellowish color of *M. hyalina*-colonized plant after 10 days of co-cultivation (Figures 21 a, b & c). It also might be happen because of the down-regulation of chlorophyll synthesis proteins like small subunit peptide Rubisco, light-harvesting complex or antenna complex protein in *M. hyalina* treated plant. However no consequences for plant growth have been observed in those three lines under control or *M. hyalina*-colonized condition.

In plant-microbes symbiosis, plant induces different phytohormones in response to microbes. This phytohormone induction or accumulation sometimes is microbe specific. Previous studies revealed that Arabidopsis co-cultivation with the endophytic fungus *P. indica* often induces SA while *M. hyalina* induces JA (Meents et al. 2019). Furthermore, It was reported that *P. indica* in Arabidopsis, *M. alpine* in *Crocus sativus* L. and *M. elongata* in maize roots showed upraised IAA concentration (Li et al. 2018; Meents et al. 2019; Wani et al. 2017). Pathogenic fungi such as *Alternaria brassicicola* and *Verticillium dahlia* produced phytotoxins, which affects the host tissues as well as the hormones production (Fradin and Thomma, 2006; Oka et al. 2005). Some bacterial pathogens like *Agrobacterium tumefaciens*, *Pseudomonas syringae*, *Azotobacter spec.* are able to accumulate IAA (Xu et al. 2018). Therefore, to analyze whether or not NRT2.4 plays a role in phytohormone accumulation, we measured the phytohormone concentration in both roots and shoots separately after 10 days of co-cultivation. Significant JA accumulation was observed in *M. hyalina*-colonized NRT2.4 and wild-type (Col-0) lines (10 days inoculation), compared to

control. Strikingly, JA accumulation was detected only in shoots of both Col-0 and NRT2.4-colonized roots, but not in nrt2.4 (Figure 24 B). In contrast, *nrt2.4* mutant showed no significant JA level difference between control and *M. hyalina* colonized plant. NRT2.4: GFP showed the same trend like wild-type (Col-0), this result suggests that NRT2.4 plays a role in JA accumulation in infected plants and it also affects JA responsive genes, this explains why the *nrt2.4* mutant showed only low JA accumulation. Interestingly, there was no significant JA accumulation in roots for all those lines (Figure 24 B).

Additionally, IAA is one of the most abundant phytohormone in nature, responsible for plant development as well as plant defense response. Therefore it is obvious that beneficial soil fungi interfere with IAA accumulation and signaling in order to manipulate the plants' development (Xu et al. 2018). IAA data analysis showed similar trends between control and *M. hyalina*-colonized plants. No significant differences have been observed among Col-0, NRT2.4: GFP and nrt2.4 lines in control and *M. hyalina*-colonized plant (Figure 25 D).

In addition, ABA, another classical stress hormone is upregulated in different stresses like drought, cold, freezing tolerance, salt and heat stress. ABA acts as plant immune responses by closing the stomata during drought stress or pathogen infection to prevent the pathogen entry. Sometimes pathogens also produce ABA to suppress or encounter the response of host immune system during the infection (Lievens et al. 2017). In plant-microbe interaction ABA is often used as stress sensor. ABA accumulates only when host or any of the associates are exposed to stress specially drought or salt stress. Studies showed that *P. indica* and Arabidopsis co-cultivation initiated moderate stress because plants need some time to recognize and establish the way to response either in symbiotic nature or in a way treating a pathogen and induce defense response (Vahabi et al. 2015, Lievens et al. 2017). Wild-type (Col-0), NRT2.4: GFP and nrt2.4 lines under nitrate starvation showed a similar trend of ABA upregulation and significant difference was observed between *M. hyalina*-colonized and control plants (Figure 25 C). The *nrt2.4* mutant showed similar effects like wild-type and NRT2.4: GFP lines. These results revealed that NRT2.4: GFP has no specific effects on ABA accumulation and signaling. SA is another important plant stress hormone that is induced against biotrophic microbes while JA is induced against necrotrophic microbes in plant-microbe interactions (Meents et al. 2019). Data analysis of SA showed similar results for all three lines in colonized or controlled plants with a significant

induction in shoots of colonized plants (Figure 24 A). JA-Ile and cis-OPDA also showed clear significant differences only in Col-0 roots and somehow support the results obtained for JA, although the picture is led clear (Figure 25 E & F).

Along with phytohormones, amino acids play an important role in plant development as well as stress tolerance against biotic or abiotic stresses. Therefore, amino acid concentration changes in *M. hyalina*-colonized NRT2.4 plants under nitrate starvation are important to investigate. Proline is one of the most important water soluble amino acids that accumulate under salinity, cold, heat, drought abiotic stresses. It plays an important role in plant development and stress tolerance. It was revealed that under stressed conditions proline concentration can be increased higher that non-colonized plants (Ali et al. 2019). Proline is used as a marker for environmental stress, it doesn't interfere with non-stressed plants condition but help plants to survive under stress condition. It had been studied that under drought condition proline increased 4 fold in maize (Ali et al. 2019; Shekari & Javanmardi, 2017). Similar results have been obtained in rice where proline concentration increased dramatically in *P. indica*-colonized plant under drought (Saddique et al, 2018) and salt stress (Jogawat et al, 2013) (Figure 26 B). The reason behind the proline accumulation in plant is not clear. We speculate that low accumulation of proline in colonized plants (both roots and shoots) indicated less stress condition. The *nrt2.4* mutant showed a similar trend like wild-type (Col-0) and NRT2.4: GFP lines in both colonized and non-colonized control plant, which revealed that NRT2.4 had no significant role in proline induction or accumulation. It might be that *M. hyalina* regulates the proline accumulation or proline responsive genes which results in low accumulation of proline under stress condition. Furthermore, alanine accumulation was observed in various plants under hypoxia or anoxia stress. Alanine accumulation is specific for hypoxia or anoxia stress. In hypoxia alanine accumulation was higher in *M. hyalina*-colonized plant than non-colonized (Limami et al, 2008; Houssein & Limami, 2016). Alanine concentration was measured in both roots and shoots. In shoots, nrt2.4 showed similar trend like NRT2.4: GFP and wild-type. In roots, significant difference was observed only in wild-type (Col-0) *M. hyalina*-colonized plant. The nrt2.4 and NRT2.4: GFP lines showed no significant difference neither in colonized nor in non-colonized plants (Figure 26 A). This result revealed that NRT2.4 may not play any role in alanine production. Another important amino acid is lysine, produced by aspartate metabolic pathway, the same like for methionine. It was shown that lysine accumulation enhanced under salt stress in wheat cultivars, maize, safflower (Ali et al, 2019). Lysine accumulation was lower in *M.*

hyalina-colonized plant in both roots and shoots (Figure 26 C). Previous studies also showed cotton under drought, soya bean under salinity, wheat under salt stress enhanced accumulation of methionine (Ali et al, 2019). I demonstrated that methionine accumulation was significant only in roots but not in shoots. In roots, NRT2.4: GFP and nrt2.4 lines showed similar trend (no significant difference) whereas Col-0 showed significant difference (Figure 26 G). It revealed that NRT2.4 gene has no specific role in lysine or methionine accumulation. It might be because of *M. hyalina* that regulates aspartate metabolic pathway and causes reduced lysine or methionine accumulation in plants. Glutamine and valine concentration was also measured. Glutamine plays an important role in plant metabolism and development, concerning chlorophyll content, root and shoot growth. Previous literature revealed that proline accumulation under stress subjected to valine accumulation (Cai et al. 2009; Huang & Jander 2017; Kan et al. 2015). Reported results showed that valine and glutamine accumulation was lower in *M. hyalina*-colonized plants than non-colonized (Figure 27 D & F). Line nrt2.4 showed no significant difference compared with NRT: GFP or Col-0 line in accumulation of valine or glutamine. Therefore, NRT2.4 very likely has no role in valine or glutamine accumulation. Aspartic acid which is the precursor of many amino acids was also measured along with tryptophan and glutamate (Figure 28 H, I and 27 E). For all three amino acid, concentration was higher in roots than in shoots. The nrt2.4 mutant line showed the same like Col-0 and NRT2.4: GFP which revealed that NRT2.4 gene has no role in accumulation of those amino acids and regulation of those responsive genes. However, no consequences for amino acid level in plants have been observed for the *nrt2.4* mutant, which revealed that NRT2.4 can't regulate the amino acid production. Therefore, low level of amino acids in colonized plant might be the intrinsic effects of *M. hyalina*.

Previous studies and our results showed that *M. hyalina* induced JA signaling in colonized plants. Jasmonate signaling plays an important role in plant defense. Exogenous MeJA application regulates the level of different photosynthesis related proteins or genes in rice (Wu et al. 2018). Exogenous applied JA also regulated the growth and intracellular pH in maize (Irving et al, 1999). Maize root growth was also regulated by exogenous IAA application (Pilet & Saugy, 1987). Therefore it was interesting to observe the effects of exogenously applied IAA or JA on the expression of endogenous NRT2.4 nitrate transporter. For this experiment, exogenously applied IAA to shoots (sprayed) or root tip showed no effects on NRT2.4: GFP fluorescence expression in main roots (Figure 30 C & 32 C). Exogenously applied IAA to shoot led to significant difference

in endogenous *NRT2.4* expression in side roots and root tips, in comparison with controlled and treated plants. In root tips, fluorescence intensity or fluorescence expression was increased just after the application to shoot, while application to root led to no significant difference between treated and controlled plants (Figure 30 A & 32 A). In side roots (lateral roots), fluorescence intensity was higher in both shoot- or root- treated (IAA) plants, in comparison with non-treated ones (Figure 30 B & 32 B). These results suggest that exogenously applied IAA in both shoot and root can alter the expression of NRT2.4 transporter. IAA application might be enhanced by other components, which regulates NRT2.4 transporter gene. Remobilization of endogenous nitrate in plants might be another reason. To verify different nitrate contents in both shoot and shoot after exogenous IAA application requires further experiments. Furthermore, exogenous application of JA to both root and shoot showed significant difference in NRT2.4 fluorescence expression in comparison with non-treated plants (Figure 33 and 35). It was important to investigate the role of exogenous JA on NRT2.4 expression in plants. Data analysis revealed that in both shoot and root treatment there was no significant difference in root tip and main root after exogenously applied JA in plants. Side roots (lateral roots) in both treatment showed lower NRT2.4 fluorescence expression in comparison with non-treated plants. It can be assumed that shoot treatment (JA) led to remobilization of endogenous nitrate from shoot to roots. Because of this mobilization fluorescence expression of NRT2.4 was lower in side roots. This expression trend was also observed in root treatment as well. It might be that exogenously applied JA to root tip led the nitrate mobilization from root to shoot. Therefore low fluorescence expression was observed in side roots only not in root tips. However, it was shown that exogenously applied JA affects the expression of endogenous NRT2.4 transporter gene. JA treatment in rice also showed the same nitrate accumulation pattern. Wu et al. (2018) mentioned the nitrate mobilization from shoot to root during JA application; Gomez et al. (2010) also showed this in tomato. These results confirm our assumption of the influence of biotic or abiotic stresses on NRT2.4.

In summary, our result showed that *M. hyalina* might be able to transfer nitrate to the plant, which reduced the NRT2.4 expression in comparison with control. Therefore it helps plants to reduce the stress during nitrate starvation. However, this co-cultivation might influence the nitrate economy in plants root, which is termed as stress-initiated nitrate allocation to roots (SINAR) (Zhang et al. 2014). JA signalling induced by *M. hyalina* also affects the NRT2.4 expression via

nitrate remobilization. Furthermore, it was also shown that NRT2.4 expression can't influence the plant development and photosynthesis.

For future aspects, there are still more questions to answer. NRT2.4 expression pattern in different environments, various fungal colonizations and upon other biotic and abiotic challenges should be investigated. Most important, nitrate measurement in colonized vs non-colonized plants, would be an important task to investigate.

Summary

Plants evolved versatile mechanisms to adopt or cope with nitrate limitation or starvation. Nitrate uptake efficiency is important for plant growth and development, therefore plants induce nitrate transporter genes and proteins like NRT2.4, which is expressed only under low nitrate concentration or nitrate starvation. Besides nitrate transporters upregulation, beneficial fungi play another key role in nitrate to uptake by influencing respective plant transporter expression. Only few details are known, about how beneficial fungi affect the nitrate transporter or influence the nitrate influx in plants under nitrate starvation. Here, growth promotional activity and regulation of NRT2.4 expression in *M. hyalina*-colonized plants under nitrate starvation was investigated. I used a ProNRT2.4: GFP reporter gene and fluorescence microscopy to monitor expression patterns of NRT2.4 in *M. hyalina*-colonized and non-colonized Arabidopsis plants under nitrate starvation. Further investigations of phytohormone and amino acids concentrations, as well as several growth parameters were determined. Exogenous jasmonic acid and indole-3 acetic acid were applied to both root and shoot separately to monitor the influence on the nitrate transporter NRT2.4. *M. hyalina*-colonized plants led to significant reduced NRT2.4 fluorescence expression under nitrate starvation compared with non-colonized plants. It is assumed that fungal hyphae release nitrate to plant or convert other component to nitrogen containing compounds. The mutant nrt2.4 showed no significant difference compared with NRT2.4 in shoot area and root length, however number of side roots (lateral roots) was higher in NRT2.4 line. Phytohormone analysis revealed the JA induction of *M. hyalina*-colonized plants under nitrate starvation, which is impaired in nrt2.4 line. Reduced amino acid levels, e.g. low concentration of proline, revealed that *M. hyalina* might be able to reduce the stress. NRT2.4 has no effects on photosynthesis system (PSII). Additionally, exogenous JA application to shoot or root showed that exogenous JA effectively reduced endogenous NRT2.4 expression in side roots while IAA can increase the expression. Together, our results demonstrate that *M. hyalina* can influence the NRT2.4 expression likely via JA signaling or provide nitrate to plants under nitrate starvation.

Zusammenfassung

Pflanzen entwickelten vielseitige Mechanismen, um sich an Nitratbegrenzung oder Hungersituationen anzupassen oder sie zu bewältigen. Die Effizienz der Nitrataufnahme ist wichtig für das Wachstum und die Entwicklung der Pflanzen. Daher haben die Pflanzen Nitrattransporter wie NRT2.4 entwickelt, die nur bei geringer Nitratkonzentration oder Nitratmangel exprimiert werden. Neben dem Transporter spielen Nutzpilze eine weitere Schlüsselrolle bei der Aufnahme von Pflanzennährstoffen durch Beeinflussung des jeweiligen Pflanzentransporters. Es sind nur wenige Details bekannt, wie die Nützlinge den Nitrattransporter selbst oder den Nitrateintrag in Pflanzen unter Nitratmangel beeinflussen; das gilt auch für die wachstumsfördernde Aktivität und die Regulation von NRT2.4 in *M. hyalina*-besiedelten Pflanzen unter Nitratmangel. Fluoreszenzmikroskopie von *M. hyalina*-besiedelten Pflanzen, die das Reportergen ProNRT2.4: GFP exprimieren, wurde unter Nitratmangel wurde durchgeführt, um die Expression von NRT2.4 zu untersuchen. Zur weiteren Analyse wurden Phytohormon- und Aminosäurekonzentration und verschiedene Wachtumsparameter bestimmt. Zusammen mit exogener Jasmonsäure und Indol-3-Essigsäure wurden Wurzel und Spross getrennt behandelt, um den Einfluss auf den Nitrattransporter 2.4 zu untersuchen. *M. hyalina*-besiedelte Pflanzen führten zu einer signifikant reduzierten NRT2.4-Fluoreszenz-Expression unter Nitratmangel im Vergleich zu nicht kolonisierten Pflanzen. Es wird vermutet, dass die Pilzhyphen Nitrat an die Pflanze abgeben oder andere Komponenten in verfügbaren Stickstoff umwandeln. Die Mutante nrt2.4 zeigte keinen signifikanten Unterschied zu NRT2.4 in der Sprossfläche und der Wurzellänge, jedoch war die Anzahl der Seitenwurzeln in der NRT2.4-Linie höher. Die Phytohormonanalyse zeigte die JA-Induktion von *M. hyalina*-besiedelten Pflanzen unter Nitratmangel: dies ist in *nrt2.4* Pflanzen stark vermindert. Die Messung der Aminosäuren, insbesondere die niedrige Konzentration von Prolin, zeigte, dass *M. hyalina* den Stress reduzieren kann. NRT2.4 hat keine Auswirkungen auf die Photosynthese (PSII). Zusätzlich zeigte die exogene JA-Applikation im Spross oder in der Wurzel, dass exogene JA die endogene NRT2.4-Expression in den Seitenwurzeln wirksam reduziert, wogegen IAA sie erhöht. Zusammen zeigen meine Ergebnisse, dass *M. hyalina* die NRT2.4-Expression über JA-Signale beeinflussen oder Pflanzen unter Nitratmangel mit Nitrat versorgen kann.

Acknowledgements

This research work was conducted with join collaboration of FSU and the Max Planck Institute for Chemical Ecology, Jena. I would like to thank Prof. Anne Krapp, Versailles, France for the gift of ProNRT2.4: GFP, ProNRT2.1:GFP, nrt2.4 insertion mutant. I thank Prof Ralf Oelmüller (FSU, Matthias Schleiden Institute) for the gift of *P. indica* and *M. hyalina*. I thank Dr Veit Grabe (MPI, Jena) for Microscopic support and Dr Michael Reichelt for chemical analysis (HPLC-MS/MS). I thank Andrea Lehr for technical support. I would like to thank Anja K. Meents for her experimental help and valuable advice. At the end, I would like to thank PD Dr. Axel Mithöfer for his guidance and providing friendly atmosphere.

Declaration

Herewith I declare that I prepared this thesis by my own. I did not use any of published sources and data than those that are specified as reference.

I also confirm that this Master's thesis has not been use as part of an earlier course achievement or examination procedure.

Date and Place Anindya Majumder

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Appendix:

Appendix 1: table of Chemicals and its companies

Chemicals name	Company Name
Triton™ X-100	Sigma-Aldrich
Sodium hypochlorite (13% active chlorine)	ACROS Organics™
Eppendorf concentrator plus	Eppendorf AG, Hamburg, Germany
Starlab Shaker	STARLAB GmbH, Hamburg, Germany
Sterile Bench (safety benches)	Herasafe™ KS, Thermo Scientific™
Pipettes	eppendorf, neoLab Migge GmbH, Germany
Square Petri plate	ThermoFisher Scientific, Germany
All Media components	Carl Roth GmbH, Germany; Fluka Analytical, Germany; Sigma Aldrich Germany
Water Purification System	Thermo Scientific™ Barnstead™ GenPure™
Axio Zoom.V16	ZEISS Microscopy, Germany
FluoroCam FC 800-C	Photon Systems Instruments, Czech Republic
Vortex-2 Genie	Labexchange - Die Laborgerätebörse GmbH
3M Micropore™ (medical tape)	3M Deutschland GmbH, Germany
Rotiprotect-LATEX (gloves)	Carl Roth GmbH, Germany
Disposal bag	SARSTEDT
Stirring Hot plate	RCT basic safety control IKAMAG®
Genogrinder	SPEX SamplePrep, USA

Appendix 2: Details of phytohormone analysis by LC-MS/MS (negative ionization-mode)

Q1	Q3	RT(min)	compound	IS	RF	DP	EP	CE	CXP
136.93	93	3.3	SA	D_4-SA	1.0	-20	**-8**	**-24**	**-7**
263	153.2	3.4	ABA	D_6-ABA	1.0	-20	**-12**	**-22**	**-2**
209.07	59	3.6	JA	D_6-JA	1.0	-20	**-9**	**-24**	**-2**
322.19	130.1	3.9	JA-Ile	D_6-JA-Ile	1.0	-50	**-4.5**	**-30**	**-4**
290.9	165.1	4.6	OPDA	D_6-JA	1.0	-20	**-12**	**-24**	**-2**
338.1	130.1	3	OH-JA-Ile	D_6-JA-Ile	1.0	-50	**-4.5**	**-30**	**-4**
225.1	59	2.6	OH-JA	D_6-JA	1.0	-20	**-9**	**-24**	**-2**
352.1	130.1	3	COOH-JA-Ile	D6-JA-Ile	1.0	-50	**-4.5**	**-30**	**-4**
140.93	97	3.3	D_4 SA			-20	**-8**	**-24**	**-7**
269	159.2	3.4	D6-ABA			-20	**-12**	**-22**	**-2**
215	59	3.6	D6-JA			-20	**-9**	**-24**	**-2**
214	59	3.6	D5-JA			-20	**-9**	**-24**	**-2**
328.19	130.1	3.9	D6-JA-lle			-50	**-4.5**	**-30**	**-4**
327.19	130.1	3.9	D5-JA-Ile			-50	**-4.5**	**-30**	**-4**

Appendix 3: Details of amino acid analysis by LC-MS/MS (positive ionization mode). LC condition-flow rate 1100 µl/min,formic acid 0.05% (A), acetonitrile (B):97% A (1 min), 97-0% A (1.7min), 0% A (0.3min), 0-97% A (0.1 min), 97% A (2.9 min).

Compound	Q1	Q3	RT(min)	IS	IS Q1	IS Q3	DP	CE
Ala	90.1	44.1	0.5	13C,15N-Ala	94.1	47.1	20	17
Ser	106	60.1	0.5	13C,15N-Ser	110	63.1	20	15
Pro	116.1	70	0.5	13C,15N-Pro	122.1	75	20	19
Val	118.1	72.2	0.5	13C,15N-Val	124.1	77.2	20	13
Thr	120.1	74.2	0.5	13C,15N-Thr	125.1	78.2	20	13
Ile	132.2	86.1	1.1	13C,15N-Ile	139.2	92.1	20	13
Leu	132.2	86.1	1.3	13C,15N-Leu	139.2	92.1	20	13
Asp	134.1	74.1	0.5	13C,15N-Asp	139.1	77.1	20	19
Glu	148.1	102.1	0.5	13C,15N-Glu	154.1	107.1	20	15
Met	150.2	104.1	0.7	13C,15N-Met	156.2	109.1	20	13
His	156.2	110.1	0.4	13C,15N-His	165.2	118.1	20	17
Phe	166.2	120.2	2.6	13C,15N-Phe	176.2	129.2	20	17
Arg	175.1	70.1	0.4	13C,15N-Arg	185.1	75.1	20	31
Tyr	182.1	136.2	1.4	13C,15N-Tyr	192.1	145.2	20	17
Asn	133.1	74.1	0.5	13C,15N-Asn				
Gln	147.1	130	0.5	13C,15N-Gln	154.1	136	20	13
Trp	205.2	188.1	3.2	13C,15N-Trp				
Lys	147.1	84.1	0.4	13C,15N-Lys	155.1	90.1	20	23

A. D8 Treatment (*M. hyalina*)

B. d8 Treatment (*P. indica*)

Appendix 4: *M. hyalina* and *P. indica*-colonized plants under nitrate starvation. It showed that *M. hyalina* (A) and *P. indica* (B) hyphae didn't cover the whole roots, which revealed that low fluorescence intensity or expression was because of intrinsic property of *M. hyalina* and *P.indica* effects, not for covered hyphae. Orange arrows indicates the area of hyphae in colonized plants.