Final Thesis

Impact of Arbuscular Mycorrhiza symbiosis on photosynthesis in *Medicago truncatula*

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1. Abstract:

The Arbuscular mycorrhiza (AM) symbiosis is a mutual association formed by plant roots and soil fungi. Most vascular flowering plants have the ability to form AM associations, which show significant impact on ecosystem function and plant health. This association is based on the mutual exchange of nutrients between plant and fungus. Therefore, AM association leads to increased demands for photosynthesis. The main aim of this study was to investigate the pathway used by plants during AM to increase the photosynthetic performance. To achieve this aim, we used the model legume Medicago truncatula. We have found out that AM symbiosis develops in roots, where AM fungi colonize the roots, leading to better plant growth and more biomass. Furthermore, AM symbiosis increases chlorophyll content and photosynthetic electron transport rate in leaves. Based on these results we suggest that AM symbiosis increases both efficiency and capacity of photosynthetic apparatus in Medicago truncatula.

Keywords:  Arbuscular symbiosis (AM), Medicago truncatula, electron transport rate (ETR), chlorophyll, and photosynthesis.

2. Lists of Abbreviations

Gi - Glomus intraradices
AM - arbuscular mycorrhiza
NAM - non arbuscular mycorrhiza
ETR - electron transport rate
Fil - filtrate
Chl - chlorophyll
NPQ - non photochemical quenching
F₀ - minimum Chl fluorescence yield
Fₐ/Fₐ - maximum quantum yield of PSII
Fₚ - photosynthetic active radiation
Fₚ - maximum Chl fluorescence yield
Dw - dry weight
ANOVA - analysis of variance
Po - organic phosphorus
FW - fresh weight
KH₂PO₄ - potassium dihydrogen phosphate
Pi - inorganic phosphorus
GL - growth light (300μmol photons m⁻¹s⁻¹)
NaHCO₃ - Sodium bicarbonate
3. Introduction

3.1 Arbuscular mycorrhiza (AM) symbiosis

Plants are the only higher organisms that can convert sun’s light energy into chemical energy to synthesize carbohydrates. These photosynthetic products become food to animals and human beings. Arbuscular mycorrhiza (AM) is a root endosymbiont association formed between the fungi of ancient phylum Glomeromycota and terrestrial plants. More than 70-90% of land plant species form AM symbiosis, improving mineral nutrients and water uptake [1].

AM symbiosis develops in the plant roots, where AM fungi forms extensively branched hyphae called arbuscules in the cortical cell of plant root. In addition, the fungus develops a network of extra- radical hyphae. Inorganic phosphate (Pi) and nitrogen (N) acquired by the extra- radical hyphae are translocated to the arbuscules and released to the plant. In return plant provides carbohydrates (carbon) to fungus for their growth and maintenance [2]. The additional function of the AM symbiosis is based on its influence on plant responses to their biotic and abiotic conditions. In most cases, mycorrhizal roots show enhanced resistance to pathogens [3] and AM fungi confer tolerance to the plant under adverse soil conditions such as heavy metal contamination [4] or drought [5].

3.2 Photosynthesis and AM symbiosis

AM symbiosis leads to increased chlorophyll and carotenoid content as well as higher ETR in Solanum tuberosum. This depicts that AM symbiosis increased the photosynthetic activity [6]. Studies in cucumber have shown that AM plants have higher biomass when compared to NAM ones [7]. According to Paradi et al., [8] there was no difference in Chl content between AM and NAM plants.

3.3 Medicago truncatula as model legume

Medicago truncatula was chosen as a model plant to study the legume biology; it has the smallest genome size among legumes of 500–550 million base pair (Mbp) [9]. It has simple diploid genome, short germination time, self- fertility and higher transformation efficiency compared to Arabidopsis thaliana. It is a model for studying pathogenic and microbial interactions, especially in bacterial and fungal symbiosis [10].

3.4 Relevance

AM formation commits many advantages to both the plant and fungal at the level of organism and soil [11]. AM aids to reduce the need of phosphate fertilizer, therefore AM formation leads to increased guarantee for plant productivity and quality in emerging systems of sustainable agriculture.
3.5 Aim

The general aim corresponds to acquire the knowledge on the photosynthetic mechanisms during mycorrhization, with the preliminary goal to reduce the demand for P fertilizers. Specific aim is to investigate the pathway used by plants during AM to increase the photosynthetic performance.

3.6 Hypothesis

Based on the aim of the project we hypothesize that mycorrhization increases in the amount of chlorophyll as well as in the efficiency of the photosynthetic activity in Medicago.

4. Materials and Method

4.1 Biological material and plant growth conditions

*Medicago truncatula* cultivar Jemalog wild type (J5) seeds (originally provided by the laboratory of Prof. B.Schoefs, Dijon, France) were released from their seedpods, disinfected and treated for 5 min in 3% bleach. After germination on 7% bacto-agar for 2 or 3 days at 25°C, seedlings were transferred into pots filled with soil and inoculums of *Glomus intraradices* (Gi). Sand, slit and farm manure were used as soil. Plants grown in a growth chamber under 16h light with 300µmol m⁻² s⁻¹ and 8h dark at 22-25°C. Humidity was kept constant at 50%. Twice a week plants were supplemented with nutrition solution medium containing 2 mM of phosphate (provided as KH₂PO₄) and nitrate (provided as KNO₃) (dilution of stock solution 2X).

*M.truncatula* seedlings were grown in soil with six treatments to examine the effect of arbuscular symbioses. (1) *M. truncatula* seedlings grown without Gi and without Pi act as negative control are referred NAM, (2) *M.truncatula* seedlings grown without Gi but were watered at the beginning with water containing inoculums, in order to see the effect of bacteria on the plant growth are referred as filtrate (FIL), (3) *M.truncatula* seedlings grown without Gi but KH₂PO₄ were given at 2 mM concentration Pi, act as positive control for the effect of AM are referred as 2mM Pi. (4) *M. truncatula* seedlings grown with fungus Gi are referred as AM, (5) *M. truncatula* seedlings grown with fungus Gi and supplemented with 2 mM Pi are referred as AM+2 mM Pi,(6) *M. truncatula* seedlings grown with fungus Gi and supplemented with 0.2 mM Pi are referred as AM+0.2 mM Pi.

4.2 Root staining

During the development of AM symbiosis, the degree of root colonization was observed regularly once per a week in *M. truncatula*. Gi inoculums for medicago are prepared by growing Gi treated leak seeds and checking for degree of root colonization. The degree of root colonization was measured from weeks 1-6 in leak plant roots to confirm the presence of fungal inoculums. In leak roots the degree of colonization was measured by *in situ* root staining method as described earlier by Vierheiling *et al* [12]. In the similar way, degree of root colonization was measured in 4 week old Medicago plants groups (NAM, Fil, Pi (2 mM), AM, AM+ Pi (2 mM)
and AM+Pi (0.2 mM). The rate of root colonization was estimated by microscopy observation method described by Trouvelot et al. [13] and computer software MYCOCALC was used. It is considered a good mycorrhization if the minimum of 60% of mycorrhiza is observed. If a higher degree of mycorrhization is found, the *M.truncatula* plant roots, stems and leaves were harvested.

**4.3 Biomass analysis**

After four weeks, three plants were harvested from each group and weighed as earlier described by Javot *et al* [14]. Initially, fresh weight (g) of roots, stems and leaves was measured. Then to dry the water contents in plants, fresh roots, stems and leaves were incubated at 65°C for 48 hours and weighed.

**4.4 Chlorophyll fluorescence measurements**

Chlorophyll fluorescence was recorded on detached leaves using a fluorometer (PAM-210, Walz, Germany) at room temperature as earlier described by Rohacek *et al* [15]. Photosynthetic parameters such as minimum fluorescence signal (F_o), and maximum fluorescence signal (F_v) were recorded and the maximum quantum yield of PSII photochemistry (F_v/F_m) was calculated using equation (F_m-F_o)/F_m.

For the determination of ETR, four weeks old *M.truncatula* plant leaves were dark adapted over night (treated and untreated groups). The leaves were detached and exposed to the photosynthetic active radiation (PAR) of various intensities to measure Chl fluorescence for 5 min and then calculated ETR (0.84×R×PAR×Y’) for 5 min. First and second leaves of 1st branch of each group were selected for measurements as described by Moreau *et al*. [16].

For the determination of non photochemical quenching parameter (NPQ), four weeks old *M. truncatula* leaves were used, slow kinetics were recorded in leaves (treated and untreated) grown under GL. Plants were dark adapted, leaves were detached and exposed for 15 min to PAR of 1250 μmol.m⁻².s⁻¹. Saturation pulse of actinic light was given every 20 s and 3rd leaf was selected for recording from each plant group. NPQ was calculated using the equation using the equation (Fm-Fm’)/Fm’

**4.5 Pigment analysis**

Leaves from all treated and untreated groups of four week old *M.truncatula* plants were collected, washed in distilled water, 50 mg leaf tissue was weighed and extracted in 1 ml of 95% (v/v) ethanol by incubating at 90°C for 5 min. Chl content was measured according to reference [17]. The Chl content was expressed as mg Chl per gram leaf. The chlorophyll a/b ratio, amount of Chl a, Chl b and carotenoids were also determined.
4.6 Statistical analysis

Data were analyzed by using ANOVA to test the effects of AM. Graph pad prism (V5.0) was used to compare the effect of AM, with NAM, FIL, 2 mM Pi, AM+2 mM Pi and AM+0.2 mM Pi. All data are presented means ± standard deviation.

5. Results

5.1 AM symbiosis affects the growth of *Medicago truncatula*

*Medicago truncatula* plants were treated without Gi (NAM), with inoculum’s Filtrate (Fil), with 2 mM KH$_2$PO$_4$ (2 mM Pi), with Gi fungus (AM), with fungus Gi and with 2 mM KH$_2$PO$_4$ (AM+2 mM Pi) and with fungus Gi and with 0.2 mM KH$_2$PO$_4$ (AM+0.2 mM Pi). Variation in growth was observed between groups during development and photos were taken after one, two, three and four weeks. Plants appearance revealed that 2 mM Pi, AM+0.2 mM Pi and AM+2 mM Pi types of plants were similar to each other but healthier than NAM and Fil plants. AM treated plants look greener than NAM and smaller in size compared to Pi treated groups.

*Fig. 1. Morphological study of Medicago truncatula plants that were grown under growth light (16 h light with 300µmol m$^{-2}$ s$^{-1}$ and 8 h dark at 22-25$^\circ$ C.) with inoculum’s Fil (Fil), without Gi (NAM) with 2 mM Pi KH$_2$PO$_4$ (2 mM Pi), with Gi fungus (AM), with fungus Gi and with 2 mM Pi KH$_2$PO$_4$ (AM+2 mM Pi) and with fungus Gi and with 0.2 mM Pi KH$_2$PO$_4$ (AM+0.2 mM Pi).*
(1A) side view of three weeks old plants. (1B) Side view of four weeks old plants. Note: Order of plants in picture A and B are not similar

5.2 Establishment and degree of mychorrhization

To monitor the level of development and establishment of the colonization, roots of all plant groups were treated with black ink solution. Fungal structures indicating symbiosis was found intensely in AM group and slightly in AM+0.2mM (Pi) group. Observation consisted of arbuscules, extra-radical hyphae and vesicles (Fig.2A, 2B). No sign of fungal structure inside the roots were seen in the other groups (NAM, Fil, 2mM (Pi) and AM+2mM (Pi)) (Fig. 2C-2F).
Fig. 2. *M. truncatula* root staining with black ink solution. (A) Arbuscular mycorrhized (AM) (B) AM+0.2 mM (Pi) are showing fungal colonization and it is easily differentiated from other Non mycorrhized (c) NAM, (D) FIL (E) 2 mM (Pi) and (F) AM+2 mM (Pi) roots. Circles, arrows and stars indicate arbuscules, extra- radical hyphae and vesicles respectively.

5.3 Effect of AM symbiosis on plant biomass

After four weeks, all groups of plants were harvested. Fresh roots, fresh stems and fresh leaves were weighed for biomass. Our results showed that AM+2 mM Pi plants have more biomass of roots, stem and leaves and followed by 2 mM Pi plants. Overall, AM+2 mM Pi plants show significant increase in biomass as compared to NAM and Fil (an average increase of 3 fold). We have found no difference between AM and AM+0.2 mM Pi plants, and NAM and Fil plants did not differ significantly in biomass between each other (Fig. 3A).

All the respective roots, stems and leaves were dried and dry weight was measured. Our results showed that AM +2 mM Pi plants have more dry weight of roots, stem and leaves. Overall, AM+2 mM Pi shows significant increase in dry weight (an average increase of 2 fold) as compared to NAM and Fil. There is significant difference between total plant weight of NAM and AM (p<0.05), 2 mM Pi (p<0.01), AM+2 mM Pi (p<0.0001). Significant difference is observed in the dry mass between NAM and AM (p<0.01), 2 mM Pi(p<0.05), AM+0.2 mM Pi (p<0.05), AM+2 mM Pi (p<0.0001) groups. No significant difference is observed in the dry mass between the NAM and Fil and between AM and AM+2 mM Pi, AM+0.2 mM Pi and 2 mM Pi.
Fig. 3. A) Freshweight (g) of roots, stems, leaves and total weight of four weeks old M. truncatula, ±SD, n=3. B) M. truncatula biomass dry weight analysis of roots, stems, leaves and total dry weight (g) of four weeks old plants. (*=p<0.05),(**=p<0.01) (***=p<0.0001). ±SD, n=3.

5.4 Effect of AM symbiosis on Chlorophyll contents

All plants groups (NAM, Fil, 2 mM Pi, AM, AM+0.2 mM Pi and AM+2 mM Pi) were analyzed for Chl a, Chl b, and carotenoids per gram leaf. Our results showed that total chlorophyll content was higher in AM plants when compared to NAM. There is no significant difference observed between the levels of carotenoids and chl b. Lower amounts of chl a was reported in NAM and higher amounts in AM, AM+2mM (Pi) and AM+0.2mM (Pi). A significant difference in chl a was found in AM and AM+2mM (Pi) groups when compared to NAM and a significant difference in total chlorophyll was observed in the groups of AM (p<0.05), AM+2 mM Pi (p<0.01), 2 mM Pi (p<0.05) when compared to NAM. No significant difference was observed in AM+0.2mM (Pi) and Fil when compared to NAM.
Fig. 4. Chlorophyll content was analyzed for leaves from four weeks old Medicago truncatula ±SD, n=4. . (*=p<0.05),(**=p<0.01).

5.5 Effect of AM symbiosis on PSII activity

Photosynthetic performance was measured in mycorrhized and Pi treated plants grown under GL. ETR was recorded by using PAM-210 from overnight dark adapted plant leaves for 5 minutes. Our results showed that electron transport rate (ETR) increased in AM plants followed by 2mM Pi, AM+ 0.2mM, AM+2mM Pi and Fil. An average of 4 fold increase was observed in (Fig. 5) as compared to NAM plants. A two fold increase was observed in Fil when compared to NAM. Higher ETR was reported in groups supplied with Pi, when compared to NAM.

To understand affect of mycorrhization on non photochemical quenching (NPQ), the slow kinetics induction of NPQ and recovery time were recorded for 15 minutes at photosynthetic active radiation (PAR) intensity of 1250 μ mol m$^{-2}$ s$^{-1}$ in overnight dark adapted NAM, Fil, 2 mM Pi, AM, AM+0.2 mM Pi and AM+2 mM Pi. Our results showed no significant difference among respective plant groups. Photochemical quenching in M. truncatula plants that were grown under growth light (GL=300 μmol m$^{-2}$ s$^{-1}$). Lower NPQ was observed in Am+ 2 mM Pi but, this observation could not be considered significant as the data was obtained from two measurements.
Fig. 5. Measurement of electron transport rate (ETR) in overnight dark adapted plant leaves. Maximum ETR was observed in AM whereas minimum is observed in NAM. Figure 5 ETR was measured from overnight dark adapted leaves of four weeks old M. truncatula plants. First and second leaves of 1st branch were selected for ETR analysis. Data represent means ± SD, n = 8
Fig. 6. The slow kinetics induction of NPQ and recovery time were recorded for 15 minutes at photosynthetic active radiation (PAR) intensity (1250 μmol m$^{-2}$ s$^{-1}$) in overnight dark adapted of four weeks old M. truncatula. 3$^{rd}$ leaf was selected from each plant group. Data represent means ± SD, n= 2

6. Discussion

Previous studies reported that AM symbiosis results in higher chlorophyll content, biomass and electron transport rate (ETR) of photosynthesis [18]. In this study, we accomplished systematic photosynthetic studies in model plant M. truncatula in six plant groups. We have performed some experiments which includes combination of biochemical, physiological and microscopic approaches to investigate the impact of colonization and plant growth.

6.1 AM symbiosis stimulates growth of Medicago truncatula

AM symbiosis shows significant effect on M. truncatula plant in physical appearance and size. The data present here indicate that AM- and Pi- type plants looks similar to each other but healthier than NAM- and Fil -type plants. The reason could be availability of Pi made via either soil fertilization or AM symbiosis. Furthermore, the presence of arbuscules, extra- radical hyphae and vesicles was detected in AM plants, indicating that establishment of AM symbiosis.
6.2 AM symbiosis increases plant biomass

We have found higher biomass in AM-type when compared to NAM-type and Fil type. This correlates with the establishment of AM symbiosis in *Medicago truncatula*. Study conducted by Valentine *et al.*, [7] have reported that higher biomass (dry weight) in AM plants when compared to NAM. Based on results from biomass analysis, plants groups supplied with Pi and plant groups treated with Arbuscular Mycorrhiza showed higher biomass compared to NAM and FIL. The increased biomass (fresh weight and dry weight) in Pi treated group explains the abundance of inorganic phosphate to plant for performing photosynthetic activity, resulting in more biomass. The plant groups untreated with Pi (AM, NAM, FIL) show comparatively less biomass. But, the AM treated plant group has higher fresh weight and dry weight as compared to NAM and FIL implicating AM association leads to increased efficiency and capacity of photosynthetic due to the availability of inorganic phosphate (Pi) from symbiosis. Total plant weight after drying the tissue showed a significant difference in the biomass between NAM and AM.

6.3 AM symbiosis increases chlorophyll content

We have found increased chlorophyll content in AM-type plants when compared to NAM and Fil type plants. Content of Chl a has been high in AM and Pi treated plant groups followed by Fil and NAM. A decreased amount of carotenoids was observed in the NAM. Previously Rabie [19] reported a higher amount of chlorophyll contents found in AM than the NAM plants. We suggest low Chl a and carotenoids can be implicated to slow NPQ (in initial phase of 500 sec) and delayed acidification of lumen in the NAM when compared to AM groups. The higher amount of chlorophyll may indicate an impact of AM on photosynthetic capacity of *Medicago truncatula*.

6.4 AM symbiosis increases photosynthetic PSII electron transport rate

The photosynthetic electron transport rate (ETR) in AM plants was significantly higher than in NAM type plants by more than 4 fold, while no significant difference was observed in slow kinetics of induction for non photochemical quenching (NPQ). We suggest that increased NPQ in the initial Phase (0-300 sec) in AM and Fil could be an important mechanism of heat dissipation to avoid photo-inhibitive damage. Higher ETR in the AM and Pi groups can be inferred to increased photosynthetic activity due to availability of phosphate compared to NAM. Fil showed a dramatic increase in the ETR when compared to NAM, even though it is not supplemented with Pi, we speculate that initial supply of bacterial filtrate to Fil group might have influenced the photosynthetic activity. Louche-Tessandier *et al.*, [6] previously reported that, higher electron transport rate in AM plants than the NAM in support to our data where, AM had 4 fold increase compared to NAM. Increased ETR in pi treated groups explains the variation of photosynthetic activity under availability of Pi. Based on our results from different experiments (biomass analysis, chlorophyll measurements, fluorescence measurements), we suggest an increase in the efficiency of photosynthesis in *M. truncatula* during the AM symbiosis.
7. Conclusion

As an extension of previous study from our group by Ateeq Ur Rahman, we have added Pi to our AM treated plant groups (AM+2 mM, AM+0.2 mM). As extended study using the combination of AM+Pi resulted in finding out the necessity of inorganic phosphate to AM. Addition of excess Pi to AM (AM+2 mM Pi) resulted in lack of colonization. By supplying inadequate (AM+0.2 mM Pi) amount of Pi resulted in reduced colonization. In the Absence of Pi AM Symbiosis occurred which resulted in accessing the phosphate from the soil. Based on results presented and discussed here, we suggest that Arbuscular mycorrhiza symbiosis increases both efficiency and capacity of photosynthetic apparatus in model plant Medicago truncatula.

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9. References


