

Arbuscular mycorrhizal fungus improves the yield and quality of *Lactuca sativa* in an organic farming system

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ABSTRACT: Arbuscular mycorrhizal fungi have been reported to enhance the growth of many crop plants. This study examines the effect of *Funneliformis mosseae*, a type of arbuscular mycorrhizal fungus (AMF), on the growth of butterhead lettuce in organic cultivation. Lettuces were inoculated with 50 spores of *F. mosseae* to determine their physiological and biochemical characters compared to uninoculated lettuces in an abandoned field. After 60 days of growth, the AMF-inoculated plants had significantly more leaves (on average, 19.4) and greater leaf fresh weight (on average, 30.4 g) than for the no-AMF treatment which had 15.8 leaves with a total weight of 14.5 g. Net photosynthesis and the chlorophyll *a* content were higher in the AMF-inoculated plants than in the control plants. The rhizosphere soil of 60-day-old AMF-inoculated butterhead lettuce showed higher organic matter, available phosphorus content, and exchangeable potassium content. The plants in the AMF treatment showed higher total nitrogen, phosphorus, and potassium contents in leaf and root tissues than the control plants. Antioxidant capacity was analysed by quantifying catalase and ascorbate peroxidase activities, ascorbic acid content, and carotenoid content. All of these parameters tended to be higher in the AMF treatment than in the control. The diphenylpicrylhydrazyl radical scavenging activity in AMF-inoculated plants (54%) was twice that in the control plants (24%). These results demonstrate that applying AMF is a cost-effective way to enhance the growth of organically farmed butterhead lettuce.

KEYWORDS: butterhead lettuce, *Funneliformis mosseae*, plant nutrition, photosynthesis, soil quality

INTRODUCTION

Lettuce (*Lactuca sativa*) is popular worldwide among health-conscious consumers because of its high nutritive value¹. As lettuce is mostly consumed as fresh leaves, the organic farming system has excellent potential for lettuce cultivation in order to avoid accumulation of toxic substances derived from synthetic compounds. Lettuce cultivated in an organic system was reported to have a better nutritive value than those cultivated in conventional agricultural systems². However, it is often difficult to cultivate plants organically, especially the first crop in abandoned areas, because of poor soil quality³. Areas that have been periodically disturbed by natural events tend to have shallow, eroded, and/or degraded soils⁴. Thus appropriate soil management or amendments are required before cultivating the first crop in a new area.

One method to improve soil quality is to inocu-

late microbes commonly found in soils of successful organic farms into new areas. Arbuscular mycorrhizal fungi (AMF) are a group of fungi often used in organic farming systems, and they have many reported advantages⁵. One of the most important characteristics of AMF is to improve the absorption of nutrients, especially phosphorus, from soil. Soil nutrients are taken up into the plant root via the AMF hyphae⁶. AMF also play an important role in stabilizing soil aggregates as they secrete glomalin-like proteins from the hyphae into soil⁷. Additionally, AMF affect the number of soil microbes, particularly plant growth promoting rhizobacteria (PGPR) and bacterial and fungal plant pathogens⁸. Hence, the use of AMF which may be associated with other soil microbes in organic farming has beneficial effects not only on crops by improving soil quality and increasing microbial community in soil, but also on the AMF themselves. For example, it was reported that the AMF in soils of organic

farms were more numerous and diverse than soils of conventional farms, and that the extent of AMF colonization in plants was greater in organic farming systems⁹.

Even though the effectiveness of AMF has been recognized for many decades, limited information is available about the physiological and biochemical effects of each AMF isolate on each plant species. Specificity of the host-isolate interaction especially between butterhead and *Glomus mosseae* to promote growth in a pot experiment was demonstrated in the previous study¹⁰. Thus the objective of this study was to evaluate the effects of a *Funneliformis mosseae* (Nicolson & Gerdemann) Walker & Schuessler (recently renamed from *Glomus mosseae* Schenck & Smith by Schüßler and Walker¹¹) inoculated in a butterhead crop grown under natural field conditions in an organic farming system. The effects of an AMF on the growth, net photosynthesis, and nutrient absorption of butterhead lettuce were evaluated. The influence of the AMF on soil quality was determined. In addition, the nutritive quality of lettuce in terms of its antioxidant capacity, antioxidant enzyme activity, and the nature of non-enzymatic antioxidant compounds in lettuce tissues were analysed to determine whether the AMF affected plant qualities.

MATERIALS AND METHODS

Study area

The experiment was carried out at the Centre of Learning Network for the Region of Chulalongkorn University, Keang Koi District, Saraburi Province, Thailand. The study site was located at 14° 52' N, 101° 02' E, and altitude 47 m AMSL. This area has a tropical climate. The land was abandoned and nothing had been cultivated at the site for more than 10 years. The soil at the site was characterized as loam with a pH of 5.7–6.0. The experiment was performed in August to October 2014, when the average temperature and rainfall were 28.0°C–28.5°C and 87.4–225.0 mm per month, respectively (average annual rainfall = 1457 mm and annual temperature = 27.8°C).

Experimental design and treatments

A completely randomized design was used with four replications and six subreplications. The experiment consisted of two treatments: uninoculated butterhead lettuce (control; no-AMF) and butterhead inoculated with 50 spores of *Funneliformis mosseae* (AMF) per plant. There were four plots (1 × 8 m²)

of soil mixed with coconut fibre substrate (5 kg/plot) in each treatment with 0.5 m spacing between plots, making a total of eight plots, in random order. Seeds of butterhead lettuce (*L. sativa*) were purchased from ACK Hydrofarm Co., Ltd., Thailand, and germinated in seedling trays for 2 weeks before transplanting into each plot. Each plot contained two rows of plants with 0.35 m spacing between plants to obtain plant density at 5 plants/m². At transplantation, soil inoculum was mixed roughly with the soil at the point where each seedling was transplanted. The plants were watered at the rate 6 l per row twice daily and cultivated for 60 days. No fertilizer was added to the soil during these experiments and weeds were managed manually.

Plant growth measurement

Six plants were randomly collected from each plot, and then leaves and roots were analysed separately. For leaves, the number, fresh weight, dry weight, length, and width were determined. For roots, the AMF colonization percentage at 2nd, 4th, 6th, and 8th week was calculated as described by Phillip and Hayman¹².

Photosynthesis measurement

Net photosynthesis, transpiration rate, intracellular CO₂, and stomatal conductance were measured using a portable photosynthesis system (Li-cor 6400) on the biggest leaf (3rd or 4th leaf from outside) with a photosynthetic photon flux density of 800 μmol m⁻² s⁻¹ and a CO₂ concentration of 400 μmol/mol (obtained from a light response curve). Photosynthetic pigments, chlorophylls *a* and *b*, were extracted from the same leaf in 80% acetone in the dark for 24 h. Then, the absorbance of the acetone solution was measured using a spectrophotometer at 663.2, 646.8, and 470 nm. The concentrations of chlorophyll *a* and *b* were calculated from the absorbance values as described by Porra¹³.

Soil properties and plant nutrient analysis

Three replications of rhizosphere soils were collected for analysis according to soil group: soil before transplanting, control soil at harvest time, and AMF-inoculated soil at harvest time. The soils were kept at room temperature until analysis. The physiochemical properties of the soils including pH, organic matter (OM), available phosphorus, and exchangeable potassium, magnesium, and calcium contents were determined. For plant samples, whole leaves and roots were separately oven-dried at 60°C for 3 days. Then, samples from each

treatment were pooled and ground to powder before determining the total nitrogen, phosphorus, and potassium contents. Organic matter and available phosphorus were determined using the Walkley and Black method and the Bray II method, respectively^{14,15}. Exchangeable potassium, calcium, and magnesium were extracted in 1 N NH₄OAc (pH 7.0) and then quantified by atomic absorption spectrometry¹⁶. Total nitrogen in plant tissues was quantified by the semi-micro Kjeldahl method followed by distillation¹⁷. Total phosphorus in plant tissues was analysed using the vanadomolybdophosphoric acid yellow method. Total potassium in plant tissues was determined by atomic absorption spectrometry after digestion with concentrated nitric acid¹⁶.

Antioxidant enzyme assays and quantification of non-enzymatic antioxidants

To determine the effects of the AMF on the nutritive value of butterhead, the activity of the antioxidant enzymes catalase (CAT) and ascorbate peroxidase (APX), the contents of three non-enzymatic antioxidants (ascorbic acid (AA), phenolic compounds (PHE), and total carotenoids (CAR)), and the diphenylpicrylhydrazyl (DPPH[•]) free radical scavenging capacity in AMF-inoculated and control plants were determined. To measure the activity of enzymatic antioxidants, 1 g leaf tissue from 60-day-old butterhead plants was ground in liquid N₂ and then extracted in buffer (50 mM phosphate buffer, pH 7.0; 1% (w/v) polyvinylpyrrolidone, 1 mg/ml dithiothreitol, and 100 mM phenylmethylsulphonyl fluoride). The mixture was centrifuged at 10 000g for 10 min at 4 °C, and then the supernatant was collected and used to determine the activities of CAT and APX as described by Aebi¹⁸ and Nakano and Asada¹⁹, respectively.

To extract and quantify non-enzymatic antioxidants, AA was extracted with 6% metaphosphoric acid in 2 M acetic acid and then quantified as described by Shin et al²⁰. The absorbance of the AA assay mixture at 540 nm was determined and compared with a standard curve constructed using known concentrations of ascorbic acid. CAR was extracted from plant tissues by the same method as that used to extract total chlorophyll, and then the concentration was calculated as described by Sims and Gamon²¹. Methanol was used as the solvent to prepare the crude extract for the PHE and DPPH[•] assays. PHE was quantified using the Folin-Ciocalteu method. The absorbance of the mixture at 750 nm was used to calculate total PHE, which is expressed as mg gallic acid equivalents²².

Table 1 Growth parameters of 60-day-old butterhead lettuce cultivated in an organic farming system.

Treatment	Leaves	FW (g)	DW (g)	W (cm)	L (cm)
no-AMF	15.77	14.50	0.95	5.41	9.38
AMF	19.41	30.37	1.02	5.36	8.88
<i>p</i> value	<i>p</i> < 0.01	<i>p</i> < 0.01	NS	NS	NS

NS = No significant difference at *p* < 0.05.

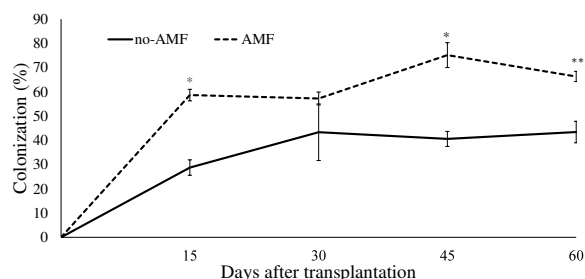


Fig. 1 Colonization percentage of 60-day-old butterhead lettuce cultivated in an organic farming system. (*, **: significant difference at *p* < 0.05, *p* < 0.01, respectively).

The change in the concentration of the free radical DPPH[•] was determined by measuring the decrease in absorbance at 515 nm. The DPPH[•] radical scavenging activity is expressed as the percentage inhibition of this reaction²³.

Statistical analysis

The data for soil properties were subjected to ANOVA and means were compared using the Least Significance Difference test using SPSS 20.0. Data for growth parameters, photosynthetic activity, antioxidant enzyme activity, and antioxidant compound content were analysed by *t*-test. All statistical analyses were tested at 95% or 99% confidence intervals.

RESULTS

Plant growth

Compared with control plants, the plants inoculated with the AMF had significantly more leaves and approximately double the leaf fresh weight at harvest (Table 1). However, the leaf dry weight, leaf width, and leaf length were not significantly different between the AMF-inoculated and control plants. The colonization percentage in the roots of AMF-inoculated plants was twice that in the roots of control plants by the end of the 60-day growth period (Fig. 1), with significant differences at weeks 2, 6, and 8.

Table 2 Photosynthetic parameters and photosynthetic pigments contents in 60-day-old butterhead lettuce cultivated in an organic farming system.

Treatment	Photosynthetic parameters				Pigments		
	net photo ^a	C _i ^b	Tr mmol ^c	Cond. ^d	Total Chl ^e	Chl a ^e	Chl b ^e
no-AMF	4.27	208.18	0.71	0.04	520.67	374.17	146.49
AMF	6.12	174.43	0.85	0.04	602.58	437.05	165.53
<i>p</i> value	<i>p</i> < 0.01	NS	<i>p</i> < 0.05	NS	<i>p</i> < 0.05	<i>p</i> < 0.05	NS

net photo = net photosynthesis; C_i = intercellular CO₂; Tr mmol = transpiration rate; Cond. = stomatal conductance; Chl = chlorophyll. NS = No significant difference at *p* < 0.05.

^a (μmol CO₂ m⁻² s⁻¹); ^b (μmol CO₂ mol⁻¹); ^c (mmol H₂O m⁻² s⁻¹); ^d (mol H₂O m⁻² s⁻¹); ^e (μg ml⁻¹ g⁻¹ FW).

Table 3 Properties of rhizosphere soil before and 60 days after transplanting butterhead lettuce in an organic farming system.

Treatments	pH	OM (%)	AvP (mg/kg)	ExK (mg/kg)	ExCa (mg/kg)	ExMg (mg/kg)
Before	5.9 ^a	1.85 ^a	2.00 ^a	181.33 ^a	1469.00 ^c	240.00 ^b
no-AMF	6.2 ^{ab}	2.40 ^{ab}	3.67 ^a	223.00 ^a	1202.67 ^a	212.67 ^a
AMF	6.4 ^b	3.07 ^b	12.33 ^b	238.67 ^a	1362.67 ^b	224.00 ^a

OM = Organic matter; AvP = Available phosphorus; ExK = Exchangeable potassium; ExCa = Exchangeable calcium; ExMg = Exchangeable magnesium. Different letters within a column represent significant difference at *p* < 0.05.

Photosynthesis measurement

Net photosynthesis and transpiration rate were higher in AMF-inoculated plants than in control plants (Table 2). The intercellular CO₂ concentration tended to be lower in AMF-inoculated plants than in control plants, but the difference was not statistically significant. The stomatal conductance was equivalent in the AMF-inoculated plants and the control plants (0.04 mol H₂O m⁻² s⁻¹).

The total chlorophyll content was significantly higher in the AMF-inoculated plants than in the control plants (Table 2). Subsequent analysis showed that only the chlorophyll *a* content, and not the chlorophyll *b* content, was significantly higher in the AMF-inoculated plants.

Soil properties and plant nutrient contents

The rhizosphere soil pH and OM percentage of both the AMF treatment and the control increased by the harvesting time, but only the AMF soil had significant differences in pH value and OM percentage compared with that before transplantation (Table 3). The OM percentage in the AMF soil was approximately 1.5-fold higher than that before transplantation and 0.5-fold higher than that of the control soil at harvest. The available phosphorus content in AMF soil at harvest was 12.33 mg/kg soil, markedly higher than that before transplantation (2 mg/kg soil) and that in the no-AMF control at harvest (3.67 mg/kg soil). No significant difference

in exchangeable potassium content was detected between the AMF treatment and the control at harvest. The exchangeable magnesium and calcium contents in soil significantly decreased in both the AMF treatment and the control during the experiment. Only the exchangeable calcium content in soil was significantly higher in the AMF treatment than in the control.

The concentrations of nitrogen, phosphorus, and potassium were significantly higher in the leaf than in the root, and higher in the leaves and roots of plants in the AMF treatment compared to the control plants (Fig. 2). The levels of nitrogen, phosphorus, and potassium in the leaf were 17%, 6%, and 5% higher, respectively, in the AMF treatment as compared to the control. In the root, the levels of nitrogen, phosphorus, and potassium were 38%, 26%, and 20%, higher, respectively, in the AMF treatment than in the control.

Antioxidant enzyme activities and antioxidant compound contents

The activities of CAT and APX increased after AMF inoculation (Table 4), but the increases were not statistically significant. The total free radical scavenging activity, expressed as the percentage inhibition of the DPPH[•] reaction, was 2-fold higher in the AMF treatment than in the control (Table 4). The levels of the non-enzymatic antioxidants AA and CAR tended to increase in plants in the AMF

Table 4 Enzymatic and non-enzymatic antioxidants in 60-day-old butterhead lettuce cultivated in an organic farming system.

Treatment	Antioxidant enzymes		Non-enzymatic antioxidants			
	CAT (units) [†]	APX (units) [†]	AA ^a	PHE ^a	CAR ^b	DPPH (%)
No-AMF	35.23	87.37	0.31	0.50	58.49	24.12
AMF	39.30	95.05	0.33	0.47	64.37	53.85
<i>p</i> value	NS	NS	NS	NS	NS	<i>p</i> < 0.01

CAT = catalase; APX = ascorbate peroxidase; AA = ascorbic acid; PHE = phenolic content; CAR = carotenoid; DPPH = DPPH[•] scavenging; NS = no significant difference at *p* < 0.05. ^a (mg g⁻¹ FW); ^b (μg ml⁻¹ g⁻¹ FW).

[†] One unit is defined as the amount of enzyme that converts 1 mol substrate per min.

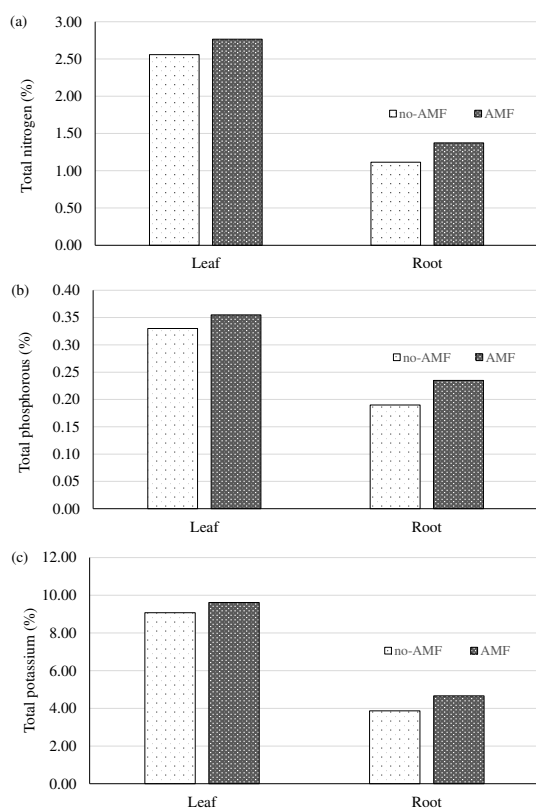


Fig. 2 (a) Total nitrogen, (b) phosphorus, and (c) potassium contents in 60-day-old butterhead lettuce inoculated with *Funneliformis mosseae* (AMF) or not inoculated (no-AMF) and cultivated in an organic farming system. (A composite sample of each treatment is presented).

treatment, but these increases were not statistically significant.

DISCUSSION

The results of this study provided evidence that AMF inoculation increased the yield of butterhead lettuce cultivated in an area that had been abandoned for

10 years. Although the benefits of AMF on plant growth have been reported since the 1980s, most studies have concluded that the effects of AMF on plant growth and development are complex, and rely on the interaction between the host and the AMF, and on the genotypes of both organisms²⁴. In the present study, specificity of butterhead lettuce inoculated with *F. mosseae* showed greater yield, which was attributed to greater fresh weight and more leaves at harvest. The greater fresh weight of AMF-inoculated butterhead may be explained by the higher water content in the leaves of the AMF plants. Allen²⁵ reported that AMF directly absorbed and transferred soil water to the root of the inoculated plant. Other studies have shown that AMF extract soil moisture via their hyphae and transport it to the root²⁶, which may result in higher leaf water content. Hence the yield improvement is necessary for organic farmers to adapt this AMF amendment in cultivation.

The mechanisms by which the AMF increased plant growth were clarified by the increase in photosynthetic activity and chlorophyll contents. The effects of AMF on photosynthesis however differ among plant species and AMF isolates. Augé reported that different species of AMF resulted in different photosynthetic responses under various abiotic stress conditions. For example, colonization with *G. fasciculatum* tended to increase stomatal conductance in many plants such as *Medicago sativa* while colonization with only *G. mosseae* had less effect on this parameter²⁷. In the present study, the AMF did not affect the stomatal conductance or intercellular CO₂ of butterhead lettuce.

Several studies have reported that AMF increase the photosynthetic rate and the chlorophyll content in various host plants^{28,29}, including several lettuce varieties in Spain³⁰. The increased chlorophyll content was suggested to result from the AMF-induced increase in the uptake of nutrients, espe-

cially magnesium, which is an important component of chlorophyll³¹ and increase the levels of core proteins of photosystem I and II and the light-harvesting complex II in Indica rice, leading to increased photosynthetic capacity^{32,33}. The higher available phosphorus content in AMF-inoculated soil was suggested as another important factor in increasing photosynthesis in lettuce, by increasing the amount of ATP available to fuel CO₂ assimilation in the Calvin cycle³⁴.

The rhizosphere soil at harvest was less acidic in the AMF treatment than in the control, compared with the pH before transplanting. AMF were reported to neutralize soil pH by consuming H⁺ or by secreting organic substances³⁵. Increase in organic matter and level of available phosphorus in soil containing *F. mosseae* facilitated the growth of butterhead lettuce in this abandon area and was useful for organic farming system. The ability of AMF to absorb nutrients from soil, and hence, promote plant growth, depends on the AMF species. For example, *G. mosseae* was shown to absorb nutrients from soil in a wide pH range, in both alkaline and calcareous soils³⁶. The ability to increase available phosphorus levels is a significant and unique characteristic of AMF, and results from the production and secretion of phosphatase, which converts phosphorus from an inactive form into the active form, phosphate³⁷. As the soil properties improved, the contents of major nutrients in the plant also increased. These increases were more evident in the root than in the leaf. This could be related to the fact that lettuce is a short-lived plant with a growth period of less than 60 days, and nutrient contents in the leaf are more strongly affected by AMF in longer-lived plants³⁸. This AMF species was also shown to associate with some PGPR such as *Pseudomonas* and *Bacillus*. These bacteria living on or inside the AMF spore wall are beneficial to plants³⁹. Such PGPR are responsible for the decomposition of both inactive and active soil organic matter⁴⁰. Thus the increase in organic matter in the AMF-inoculated soil could be due to larger populations of these PGPR. These bacteria can transform various soil nutrients into available forms that the plant root and fungus can take up.

In the present study, the activities of CAT and APX did not increase after AMF inoculation, contrary to the results of several other studies. Blilou et al⁴¹ showed that *G. mosseae* inoculation increased the activities of CAT and APX in tobacco roots, but these increases were transient. In that study, the highest activity of both enzymes coincided with the

entry of the AMF appressoria into the plant root, after which the activity of both enzymes decreased over time. Increased activities of these antioxidant enzymes reflected the defence mechanism of the plant to infection by the fungus⁴². The overall antioxidant capacity can be measured as the percentage inhibition of the DPPH• free radical reaction, representing radical quenching kinetics⁴³. Butterhead lettuce inoculated with *F. mosseae* contained high DPPH• scavenging activity which increases value of lettuce and is beneficial to consumers. It has been reported recently that AMF can increase the DPPH• scavenging activity of many plants, including cyclamen and onion^{44,45}. In the present study, the levels of the non-enzymatic antioxidants AA and CAR were similar between the control and the AMF treatment. However, because the water content was higher in the leaves of the AMF-inoculated butterhead lettuces, probably there was some dilution of these compounds. Moreover, the AMF may have led to changes in the levels of other antioxidant substances that increased the DPPH• scavenging activity such as superoxide dismutase⁴⁶. Further studies should be conducted to clarify the mechanisms by which the AMF increased the total antioxidant activity of butterhead lettuce.

The results of the present study represented the specificity between *F. mosseae* as inoculum and butterhead lettuce to increase both the quantity and quality of the crop in organic cultivation system. After an AMF is introduced into a planting area, it can continuously reproduce as long as it has a compatible plant host. Thus the presence of the AMF isolate in soil not only benefited the first crop, but can also be expected to improve the growth of subsequent crops at this site. The AMF isolate can be developed to be a bio-fertilizer that farmers can propagate the spores and consequently reduce their cost for the next crop⁴⁷.

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