Isolation and Selection of Effective Arbuscular Mycorrhizal Fungus for Bermuda Grass Growth Promotion under Neutral and Acidic Soil Conditions

Niramon Lakkason*, Watcharin Yuttavanichakul, Nantakorn Boonkerd, Neung Teaumroong and Panlada Tittabutr

School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Thailand.
*Corresponding author, e-mail: niramonlakk@gmail.com

Abstract

The objective of this research was to isolate and select an effective Arbuscular Mycorrhiza (AM) fungus as biofertilizer to be used with Bermuda grass (*Cynodon dactylon*) growing on the golf course. The rhizospheric soil samples were collected from four golf courses in Thailand and subjected to observe the presence of indigenous AM fungi and their ability to recolonize Bermuda grass. The result showed that AM fungi were presented in all soil samples and the highest efficiency of re-colonization was observed in soil from Toscana valley golf club. Abundance of AM species in this soil sample was investigated. The spore morphology together with single spore DNA sequencing and phylogenetic tree analyses revealed that most AM fungi belonged to the genera *Claroideoglomus* and *Acaulospora*. The spore of *Claroideoglomus* sp. isolate Tos1, which is a dominant specie was selected to propagate by using different host plants. The highest spore number of 74 spores/100 g soil was obtained when propagated in maize (*Zea mays*). AM spores derived from the propagation were used to test the effectiveness on Bermuda grass growing under pH 4.5 and pH 6.5 in comparison with the commercial AM (*Rhizophagus irrigularis*). The result showed that higher chlorophyll content was found in plant inoculated with *R. irrigularis*. However, the isolate Tos1 could be able to promote plant biomass in similar level to *R. irrigularis* under both neutral and acidic soil conditions. Therefore, the isolate Tos1 could be used as biofertilizer to promote Bermuda grass. The application method and field performance should be further examined.

Keywords**:** *Bermuda grass, golf course, Arbuscular mycorrhiza, phosphorus, biofertilizer*

1. Introduction

Grass on golf courses is grown under high maintenance condition for the good quality of golf course. The maintenance included a large quantity of chemical substances such as herbicides, insecticides, fungicides, and chemical fertilizers. Chemical fertilizers are mainly applied on golf course. However, these applications increase the accumulation of mineral in soil and water. Phosphorus (P) is an essential nutrient required by plant. Although P in soil may appear in large amount, it could be available for plant only 0.1% of average P content in the natural soil (0.5% w/w) (Illmer & Schinner, 1995). Since P has low solubility in water and normally was fixed in the soil by binding with other ions, such as iron, aluminum, and calcium, thus P was transformed into insoluble form and unavailable for plant (Sharma, Sayyed, Trivedi, & Gobi, 2013). P availability also occurs in narrow range of soil pH. The P availability is quite low in alkaline soils (pH > 7.3) and in acidic soils (pH < 5.5) (Potash Development Association, 2017; Valsami-Jones, 2004). Therefore, golf course located on inappropriate soil pH may have direct effect on grass growth due to lack of P availability for plant. In this case, the high amount application of P-chemical fertilizer may not efficiently promote grass growth, while it may cause pollution to the environment. The questions are how to increase the phosphorus availability for grass and reduce the utilization of chemical phosphorus fertilizer in the golf course.

Currently, arbuscular mycorrhiza (AM) fungi are known as the beneficial fungi that can provide many benefits to host plants. The AM symbiosis could enhance nutrient acquisition efficiency in the field, especially the phosphorus (Toro, Azcon, & Barae, 1997; Khan, Zaidi, & Ahmad, 2014; Valsami-Jones, 2004). Therefore, application of AM inoculum may be a useful tool to facilitate an effective P utilization and support grass growth on golf course. Bermuda grass (*Cynodon dactylon*) is mostly used for growing on the golf green (McCarty & Miller, 2002). Although the commercial AM inoculum is available and can be imported from abroad, it may not be appropriate for a specific usage with Bermuda grass growing in Thailand. Since several factors, such as plant species, soil condition, or climate in each location may influence the colonization and symbiosis efficiency (Massenssini et al., 2014), the effective AM fungi strain

should be selected for specific use as biofertilizer for the growth promotion of Bermuda grass on golf course in Thailand.

This study focused on the selection and identification of AM fungus to be used with Bermuda grass. Experiments to determine the effectiveness of this selected AM fungus in comparison with a commercial AM inoculum on supporting Bermuda grass growing under normal and acidic soil conditions were also performed.

2. Objectives

To select an effective AM fungus as biofertilizer that can promote the growth of Bermuda grass for golf course application.

3. Materials and methods

3.1 Soil samples and source of collection

Soil samples were obtained from four golf courses in Thailand including (i) Toscana valley golf club, Nakhon Ratchasima, Thailand (14°30'25.8"N 101°30'12.3"E), (ii) Pattana golf club & resort Chon Buri, Thailand (13°05'12.6"N 101°08'41.2"E), (iii) Suranaree golf club, Nakhon Ratchasima, Thailand (14°57'36.5"N 102°05'50.9"E) and (iv) Tiger golf club, Nakhon Ratchasima, Thailand (14°56'28.5"N 102°04'11.7"E). The soil samples were collected from rhizosphere of grass grown on each golf course by removing shoots, while the remaining roots in the soil were chopped and mix thoroughly before using in further experiments.

3.2 Observation of AM re-colonization by trap culture technique

Each rhizospheric soil sample was mixed 1:1 (v/v) with autoclaved coarse sand and transferred into a 15-cm (diameter) plastic pot. Seeds of Bermuda grass (obtained from Westar seeds international Inc. El Centro, California) were planted in the soil around 80-100 seeds/pot. Plants were grown in a growth room under the controlled environment condition following, 28 ± 2˚C with 16 h light/ 8 h dark cycle at light intensities 300 µE/m^2S and with 50% humidity for four months to allow the re-colonization of indigenous AM fungi in each soil sample. Fertilization is kept to a minimum level with ½ Hoagland's solution (Hoagland & Arnon, 1938) containing with 50 µM of Phosphate (Pi), and being applied only when plants show signs of phosphorus (P) deficiency (purpling of leaf sheaths), or nitrogen deficiency (chlorosis of young leaves) (West Virginia University, 2017a). The re-colonization ability of AM fungi presented in each soil sample was observed by determination of AM root colonization efficiency.

3.3 Determination of root colonization efficiency

To assess the grass root colonized by AM fungi from each location, roots of grass were stained with 0.05% Trypan blue and de-stained with 50% glycerol. Mycorrhiza colonization was determined in three replicates by a grid intersect method (Giovannetti & Mosse, 1980). For frequency of mycorrhiza in the root system (F%), intensity of the mycorrhizal colonization in the root system (M%), intensity of the mycorrhizal colonization in the root fragments (m%), arbuscule abundance in the root system (A%) and arbuscule abundance in mycorrhizal parts of root fragments (a%) using the MYCOCALC computer program (https://www2.dijon.inra.fr/ /mychintec/Protocole/WorKshop_Procedures.html#1.5). The soil sample showing highest ability of AM re-colonization on grass was selected for further experiments.

3.4 Isolation of AM fungal spore from soils

The AM fungal spores were extracted from the selected rhizospheric soil sample. The spores were collected by wet sieving and following by 60% sucrose gradient centrifugation method (Dandan & Zhiwei, 2007). The process was started by re-suspending the soil sample in 200 ml of tap water and stirred for 10 min. The suspension was allowed to settle for 10 sec and then the fraction between 38 µm and 500 µm were collected using Sieve Shaker model EML 200 Premium Remote machine (OMEGA SCIENTIFIC (THAILAND) Co., LTD). Samples were re-suspended in 200 ml of tap water to repeat the procedure. The filtrate was transferred to 50 ml centrifuge tubes with a fine stream of water, centrifuged first in tap water for 10 min at 6000 x g to remove floating organic debris from rhizospheric soil, and re-suspended in 60% sucrose for 8 min, after shaking vigorously to separate the spores from denser soil particles. The sucrose supernatant was poured through a 38 µm sieve, and the trapped spores were washed with tap water to

remove the sucrose. Then, the collected spores were rinsed into a small Petri dish for further characterization.

3.5 Spore morphology characterization

AM fungal spores were grouped and counted each population under a stereo microscope according to their morphological characteristics (West Virginia University, 2017b). Criteria for morphological spore characterization were mainly based on spore size, spore color, and wall structure. The number of AM fungal spore in each population was counted from 20 g soil sample in triplicate. The AM fungal isolates showing high number of population were used to confirm their genus by small subunit ribosomal ribonucleic acid (SSU rRNA) gene sequencing method.

3.6 DNA extraction and sequencing

DNA from single AM spore was extracted by crushing spores in a PCR tube using a needle, and used directly as template for PCR. Partial SSU rRNA (18S rRNA) gene fragments were amplified using nested PCR with the universal eukaryotic primers NS1 (5′-GTA GTC ATA TGC TTG GTC TC-3′) and NS4 (5′-CTT CCG TCA ATT TCC TTT AAG -3′). The first PCR product (1,100 bp) was diluted 1/100 with 1X Tris-EDTA (TE) buffer. The dilution was used as DNA template in second PCR reaction performed using primer AML1 (5′-ATC AAC TTT CGA TGG TAG GAT AGA-3′) and AML2 (5′-GAA CCC AAA CAC TTT GGT TTC C-3′) which gave a product size of 795 bp (Lee, Lee, & Young, 2008). PCR products derived from each AM fungal isolate were directly cloned into the pTG-19 cloning vector (Vivantis, USA) and transformed into *Escherichia coli* (DH5α; using as the competent cell). The positive transformed cells were screened by X–gal (white colonies were selected only) and inserted clone was examined using M13f, M13r primers amplification. The insert DNA in plasmid was sequenced by Macrogen Inc. The DNA sequence was analyzed by GENDOC program. All sequences were submitted to a BLAST search using the GenBank database (http://www.ncbi.nlm.nih.gov).

3.7 Phylogenetic tree construction

Sequences obtained from this study were aligned using Molecular Evolutionary Genetics Analysis version 6.0 (MEGA6) along with the representative 16 AM fungal sequences from GenBank. The alignment of SSU rRNA (18S rRNA) gene dataset was trimmed to the primer terminal ends (*c*.800 bp). Neighbour-joining (NJ) was constructed using MEGA6. Distances for the NJ tree were computed using the Kimura 2-parameter model with 1000 bootstraps. (Lee et al., 2008). A consensus phylogenetic tree was computed using *Archaeospora trappei* (accession no.Y17634.3), *Paraglomus brasilianum* (accession no. AJ301862.1), *Appendicispora fennica* (accession no. AM26819.4) and *Archaeospora leptoticha* (accession no. AJ301861.1) sequences as out group.

3.8 Propagation of selected AM spore fungus

Fifty AM spores were mixed with sterilized coarse sand and transferred into a 15-cm (diameter) plastic pot (50 spores/pot). Surface sterilized seeds (using 3% sodium hypochlorite) from each host plant were germinated and planted in the pot (100 seeds for Bermuda grass (*Cynodon dactylon*), 1 seed for maize (*Zea mays*) and *Lotus japonicus*). Plants were grown in a greenhouse for four months to allow the colonization and spore production. Fertilization is kept to a minimum level as described above. Production ability of dominant AM fungi presented in each host plant was observed by spore isolation method and count spore number under stereo microscope. The spore number was calculated in triplicate of 100 g soil from each sample pot.

3.9 Effectiveness of selected AM spore fungus on Burmuda grass growth promotion

To test the effectiveness of isolated AM, plants were grown under acidic (pH 4.5) and neutral (pH 6.5) conditions. Fifty seeds of Bermuda grass were sterilized with 70% Ethanol for 5 min, 3% sodium hypochlorite for 90 min (Stephenson, 1942) and rinsed with sterilized water for 10 times. Next, Bermuda grass seeds were sawn into nursery tray size 2.3×2.3 cm^2 and watered with sterilized water by mist spray. At 3 days after seed germination, fifty spores of isolated AM fungus in comparison with fifty spores of *Rhizophagus irrigularis* (Product from Premier Tech Biotechnologies Mycorise® ASP Arbuscular Mycorrhizal Fungal Inoculant) were inoculated into nursery tray containing germinated seeds. Then, plants

were transferred into new pot containing sterilized river rock after 2 weeks of planting. Plants were watered with ½ Hoagland's solution (Hoagland & Arnon, 1938) with 100 µM of Pi twice a week. The data of plant dry weight and chlorophyll contents (measured by chlorophyll meter (Ling, Huang, & Jarvis, 2011)) were collected at one month after spore inoculation.

4. Results and Discussion

4.1 Re-colonization efficiency on Bermuda grass by indigenous AM fungi presented in different golf courses

To evaluate the re-colonization efficiency of indigenous AM fungi from rhizospheric soil collected from different golf courses, Bermuda grass (*C. dactylon*) was used as host plant for trap culture test. The result indicated that soil from all four golf courses contained indigenous AM fungi which could re-colonize Bermuda grass roots, but with the differences in ability of re-colonization (Table 1). Indigenous AM fungi from Toscana valley golf club soil showed highest frequency of mycorrhiza presented in the root system (F%) of Burmuda grass. Whereas, the highest intensity of the mycorrhizal colonization in the root system (M%) and in the root fragments (m%) were detected when using soil sampling from Pattana golf club, and the highest value of arbuscule abundance in the root system (A%) and in the root fragments (a%) were detected from Suranaree golf course soil sample. Differences in re-colonization efficiency of AM on Bermuda grass could be due to their host-fungus specificity (Leake et al., 2004). Host plant species may have an important role in AM development, sporulation, diversity and also patterns of fungal species composition in the rhizosperic soil. In this experiment, Toscana golf club and Pattana golf club used Bermuda grass as a turf grass, while Zoysia grass (*Zoysia* spp.) and Paspalum grass (*Paspalum* sp.) were used as turf grass in Suranaree golf club and Tiger golf club, respectively. It could be implied that different grass species causes an effect on specific signal between plant-indigenous AM interaction (Gadkar, David-Schwartz, Kunik, & Kapulnik, 2001) and influence on the re-colonization with Bermuda grass. Therefore, soil sample from Toscana valley golf club which provided highest F% was selected for further experiment since there is high possibility to obtain AM fungal specie specific to Bermuda grass.

Table 1 Re-colonization ability on Bermuda grass by AM fungi presented in each soil sample

Sampling site	F%	M%	m%	A%	a%
Suranaree golf club	53.33 ^{c}	2.03 ^{a}	3.01 ^{a}	0.62 ^{a}	30.33 ^{a}
Tiger golf club	30.00 ^{d}	0.43 ^{c}	1.44^{b}	0.10 ^{b}	22.31 ^{b}
Toscana valley golf club	80.00 ^{a}	1.07 ^{b}	1.33^{b}	0.03 ^{c}	2.50 ^{d}
Pattana golf club	66.67 ^{b}	2.57 ^{a}	3.85^{a}	0.24 ^{a}	9.22 ^{c}

Note; F%, Frequency of mycorrhiza in the root system; M%, Intensity of the mycorrhizal colonization in the root system; m%, Intensity of the mycorrhizal colonization in the root fragments; A%, Arbuscule abundance in the root system; a%, Arbuscule abundance in mycorrhizal parts of root fragments. Data represents mean of three replicates. Different letters in the same column indicate statistical difference by the Duncan test (α = 0.05).

4.2 Isolation and identification of AM fungi from Toscana valley golf club

Indigenous AM fungal spores presented in the rhizospheric soil of Toscana valley golf club were isolated. All spores were grouped and counted the spore number in each group based on spore morphological characteristics. Five different spore morphologies were found in the sample and labeled as Tos1, Tos2, Tos3, Tos4 and Tos5 (Figure 1). Based on spore morphology index (http://fungi.invam.wvu.edu/the-fungi/species-descriptions.html), Tos1 may be classified as *Claroideoglomus lamellosum* (REF No.ON393) with cream to pale yellow color, globose to subglobose shape, and size distribution of spores was 80-140 µm (average 104 µm). Tos2 may be classified as *Claroideoglomus etunicatum* (REF No.NE108A) with orange to red brown color, globose to subglobose shape, and size distribution of spores was 60-160 µm (average 129 µm). Tos3 may be classified as *Acaulospora foveata* (REF No. BR861) with red-orange to dark red-brown color (immature spores initially are cream-colored and gradually acquire an orange tint as the spore wall begins to differentiate), globose to subglobose shape (sometimes irregular), and size distribution of spores was 240-360 µm (average 289 µm). Tos4 may be classified as *Cetraspora pellucida* (REF NoFL966) with hyaline/white color in most recently

formed spores to yellow-brown in older spores, globose to subglobose shape, and size distribution of spores was 120-240 µm (average 189 µm). Tos5 may be classified as *Gigaspora gigantea* (REF No.MA435A) with bright greenish yellow to bright yellow green color, globose to subglobose shape (rarely irregular), and size distribution of spores was 240-400 µm (average 324 µm). The number of spore were found at 8, 6, 3, 2 and 1 spore/g soil for Tos1 to Tos5, respectively (Figure 1).

The top three dominant population, Tos1, Tos2 and Tos3 were collected for identification by sequencing the 18S rRNA gene. BLAST search analysis showed 96 - 98% similarity to sequences belonging to the phylum *Glomeromycota*. The phylogenetic tree analysis on 18S rRNA gene of these three dominant isolates was constructed along with the representative 16 AM fungal sequences from GenBank. The result showed that the spore of AM isolates Tos1 and Tos2 were belonging to *Claroidioglomus* group, and Tos3 was located in *Acaulospora* group with 95% of similarity (Figure 2). This result confirmed the identification of AM by morphological observation at the level of genus. However, the specie of these AM isolates could not be verified due to the PCR product of 18S rRNA gene sequences in this study was only 795 bp long. The longer fragment of gene should be amplified for further identification in the level of AM specie.

4.3 AM fungal spore propagation

To continue testing the grass growth promoting property of AM, the selected AM fungal spore must be propagated before used. The most dominant specie form Toscana valley golf club *Claroideoglomus* sp. Isolate Tos1 were selected for propagation in different host plants including Bermuda grass (*C. dactylon*), maize (*Zea mays*) and *Lotus japonicus* to compare the host preference for spore production in greenhouse. The result showed that using maize as a host plant significantly produced higher number of AM spore (74.3 spores/100 g soil) than that of *L. japonicus* (62.7 spores/100 g soil) and Bermuda grass (51.7 spores/100 g soil) (Figure 3). It has been reported that the monocotyledon host plant could have high percentage of AM root colonization due to the density of root system (Rahim, Jais, & Hassan, 2016). However, different ability of spore production may be due to the preference of AM fungus and host plant. Since AM fungi use strigolactones as signal molecules in the initial communication with host plants for symbiosis (Yoneyama et al., 2008), it may be possible that different plant hosts released different types or concentrations of strigolactones and affected the symbiosis interaction and lead to affect the spore production. Therefore, the selected AM spore (Tos1) was propagated in maize and used for further experiment.

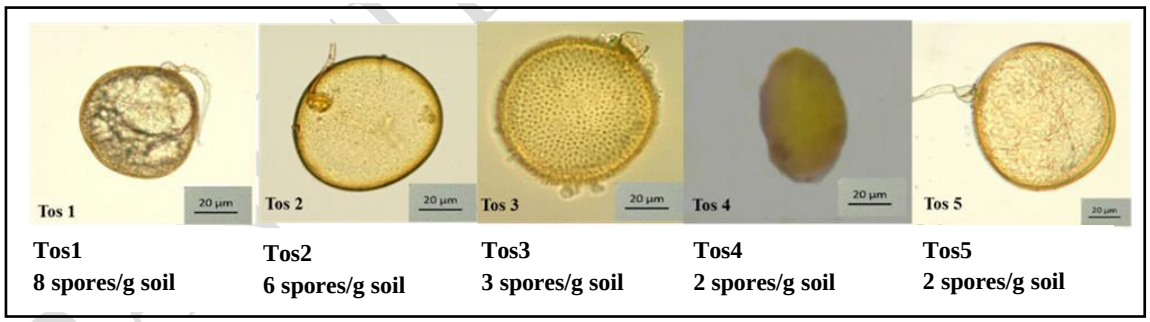

Figure 1 Spore morphology of AM fungi isolated from Toscana valley golf club (Tos1, Tos2, Tos3, Tos4, and Tos5) observed under light microscope with objective lens 40x. The average spore number of each AM was indicated

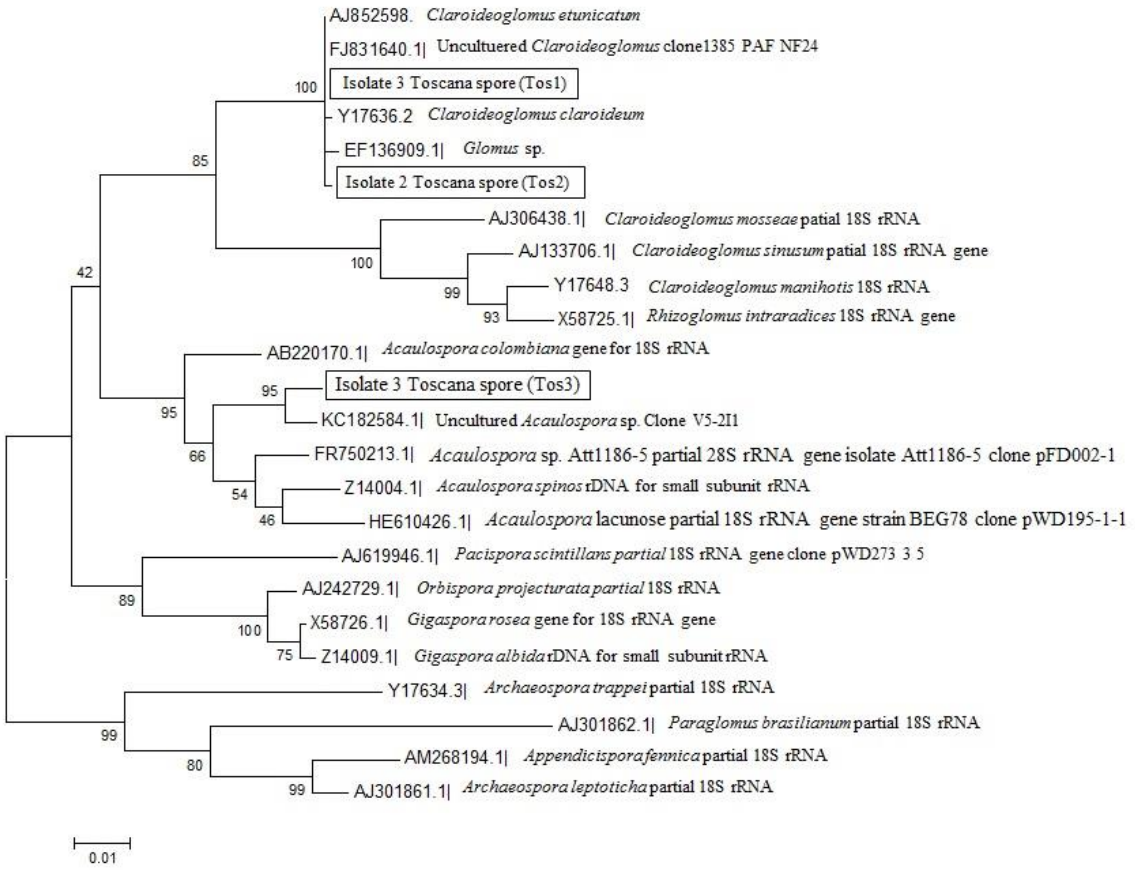

Figure 2 Phylogenetic tree based on sequences of 18S rRNA genes showing classification of the arbuscular mycorrhiza fungi isolated from soil sample. Bootstrap values are expressed as percentages of 1,000 replications. The bar represents 1 estimated substitution per 100 nucleotide positions

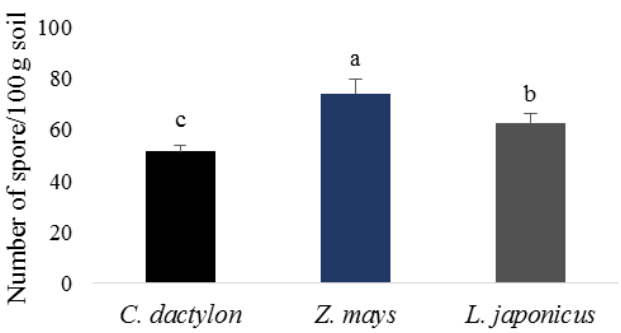

Figure 3 Number of AM spore (Tos1) production when propagated with different host plants growing under greenhouse condition. Data represents mean of three replicates ± standard error of the mean. Different letters indicate statistical difference by the Duncan multiple range test (α = 0.05)

4.4 Effectiveness of selected AM spore fungus on promotion of grass growth

To evaluate the efficiency of plant growth promotion by the selected AM, the AM spore (Tos1) from Toscana valley golf club was inoculated into Bermuda grass root under neutral and acidic soil conditions in comparison with non-inoculated plant (control treatment) and plant inoculated with commercial AM (*R. irregularis*). The result showed that both Tos1 and *R. irregularis* could significantly increase Bermuda grass dry weight greater than that of non-inoculated plant. *R. irregularis* and Tos1 could promote plant biomass around 1.7-1.9 folds and 1.5-1.6 fold when compared with control under pH 4.5 and pH 6.5 conditions, respectively (Figure 4a). The chlorophyll content in plant leaves was also significantly increased when inoculated with *R. irregularis* or Tos1. Plant inoculated with Tos1 and *R. irregularis* contained chlorophyll content around 4.6-5.4 folds and 2.4-3.1 folds when compared with control under pH 4.5 and pH 6.5 conditions (Figure 4b). The overall plant phenotypes were shown in Figure 4c and Figure 4d. It seems that the overall growth of Bermuda grass was affected from acid condition. The external pH cause an effect on plant growth and nutrient absorption response due to unavailable of some plant nutrients, such as N, P, K, Ca, Mg, Mo and S (Ruan, Gerendás, Härdter, & Sattelmacher, 2007; www.pda.org.uk). However, the significant increase of plant biomass or chlorophyll content was influenced by inoculation of AM fungi. Colonization of plant roots by AM would increase more possibility of nutrients acquisition through the fungal mycelia (Rillig & Mummey, 2006). Moreover, it has been reported that AM colonization on plant root could increase mineral content accumulation in plant under acidic condition (Clark & Zeto, 2000). Therefore, the access of unavailable nutrients under acidic condition would be increased when plant was colonized by AM. However, this efficiency is dependent on the AM specie and its host plant (Clark, 1997).

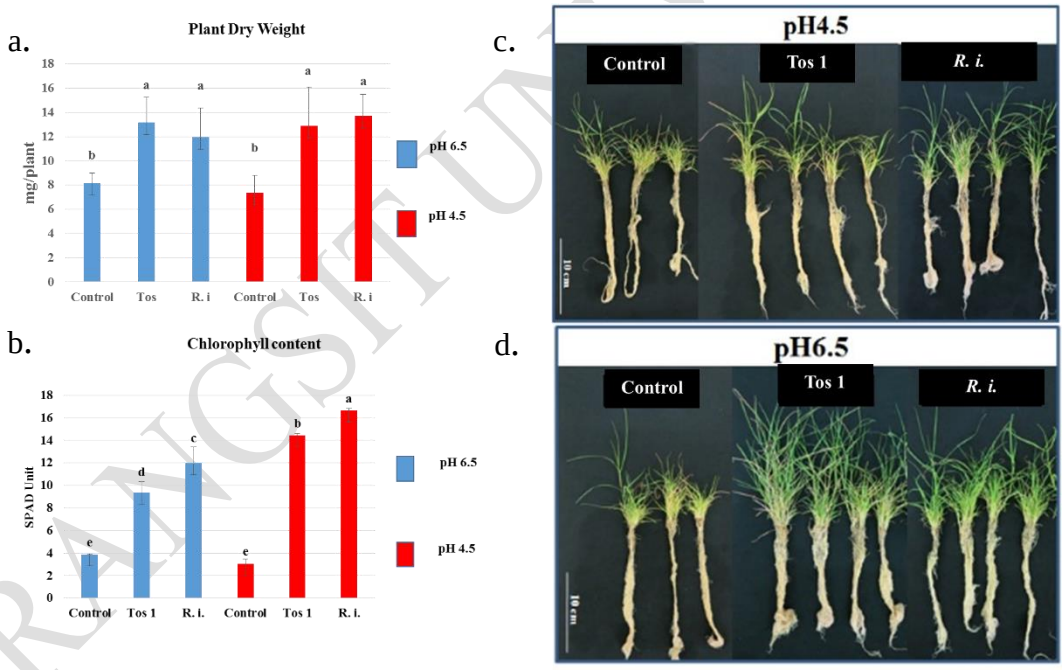

Figure 4 Effect of Tos1 and *R. irregularis* inoculation on (a.) plant growth promotion, and (b.) chlorophyll content of Bermuda grass growing under (c.) acidic (pH 4.5) and (d.) neutral (pH 6.5) conditions

5. Conclusion

This research explained about the isolation and selection of an arbuscular mycorrhiza to be used with Bermuda grass and the plant growth promotion efficiency under acidic and neutral soil conditions. An AM spore isolate Tos1 belonging to the genus *Claroidioglomus* was selected and this AM fungus could promote Bermuda grass growth similar to the commercial AM strain (*R. irregularis*). Therefore, AM isolate Tos1 may be used as a biofertilizer for the golf course. The application of Tos1 by co-inoculation with the

commercial AM strain or with the plant growth promoting rhizobacteria (PGPR) may increase higher efficiency on the plant growth promotion. These experiments will be further performed.

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