Compatible arbuscular mycorrhizal fungi of *Jatropha curcas* and spore multiplication using cereal crops

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Jatropha curcas is being considered as a biofuel crop for Thailand. Seedlings of *J. curcas* were used as bait plants to trap compatible arbuscular mycorrhizal fungi (AMF) in field soils in northern Thailand. Of the ten species of AMF that were trapped, two species, *Scutellospora heterogama* (CMU33) and *Entrophospora colombiana* (CMU05) produced abundant spores (>50 spores/100 g soil) and heavily colonized the roots of the trap plant. In a second experiment, the two AMF species were used to assess the effectiveness of four annual cereal crop plants (job's tears, *Coix lacryma-jobi*; rice, *Oryza sativa*; sorghum, *Sorghum bicolor*; maize, *Zea mays*) as suitable nurse plants for AMF spore multiplication. Higher mycorrhizal colonization and spore production were found after 120 days in sorghum than in the other crop species. Spore multiplication did not occur with corn and CMU33, nor with rice and CMU05. Except for the shoots of rice, inoculation increased the root and shoot dry weight of all four crop species. Sorghum is a suitable host for spore multiplication of *E. colombiana* but an alternative host, with the potential to produce higher spore yields, is required for *S. heterogama*.

Key words – *Entrophospora* sp. – host plant – *Scutellospora* sp. – spore production, spore trapping

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Introduction

Arbuscular mycorrhizal fungi (AMF) are natural plant growth stimulants (Wood & Cummings 1992). Most terrestrial plants form mycorrhizas (Smith & Read 1997) and many mycorrhizae have been shown to enhance plant survival and fitness through mechanisms such as increasing water and nutrient uptake (Marschner & Dell 1994, Peterson et al. 2004, Pasqualini et al. 2007, Plassard & Dell 2010) and reducing the impact of plant pathogens (Mukeji & Ciancio 2007). Most of the research has focused on food and forest crops, and less attention has been paid to oil-yielding plants such as fennel (*Foeniculum vulgare* Mill.) (Kapoor et al. 2004), oil plam (*Elaeis guineensis* Jacq.) (Corley & Tinker 2003, Phosri et al. 2010) and menthol mint (*Mentha arvensis* L.) (Gupta et al. 2002). Physic nut (*Jatropha curcas* L.) is a drought-tolerant

shrub that is now being cultivated in industrial plantations in many countries, for example, Brazil, India, Mexico, Nicaragua and Thailand (Foidl et al. 1996, Heller 1996, David et al. 2009, Prueksakorn et al. 2006). The seeds contain 31–37% oil content suitable for bio-diesel production (Heller 1996).

Although it is often reported that physic nut can grow rapidly and produce commercial oil yields on poor agricultural lands (Openshaw 2000, Pramanik 2003, Rao et al. 2008, Narenda et al. 2009), growth is likely to be slow unless there is appropriate application of beneficial organisms and balanced fertilizer. In a previous study, we showed that there is a high diversity of AMF associated with field-grown plants in northern Thailand (Charoenpakdee et al. 2010). Before these fungi can be recommended for commercial application, compatible species must be identified and procedures for inoculum production developed (Silva et al. 2005, Feldmann & Shneider 2008, Feldmann et al. 2008).

Therefore, this study has two objectives: firstly, to use physic nut seedlings to trap compatible AMF species, and secondly to determine whether cereal crop species are suitable for spore multiplication.

Methods

Source of AMF inocula and trap culture

Physic nut was used as a bait plant to trap compatible AMF in field soils collected from the rhizosphere of physic nut at ten sites in north and north-eastern Thailand. Samples were stored over ice for transportation, then at 4˚C for 1 week. Five hundred gram of soil from each sample was placed in the middle of 1.5 kg sterilized substrate in black plastic pots (15 cm top diameter). The substrate was an infertile soil from Mae Hea Agricultural Research Station and Training Center mixed with river sand (2:1, w/w). The soil was air dried and passed through a 4 mm mesh sieve, and the substrate was sterilized twice in an autoclave at 121˚C/15 psi for 30 minutes. The substrate had the following analysis: available P 4.9 $mg.kg^{-1}$, total N 0.06 $g.100\ g^{-1}$, extractable K 104.6 $mg.kg^{-1}$, organic matter 1.55%, and pH_{H2O} 6.1.

Physic nut seeds were disinfected with 0.5% sodium hypochloride for 5 minutes and washed with sterilized water. Seeds were planted in autoclaved river sand and were watered with sterilized water until seed germination (2 weeks). Two uniform seedlings were transplanted into each pot and there were 3 replicate pots per soil collection. Seedlings were thinned to one per pot after 2 weeks and grown for 90 days in a randomized complete block design. Tap water was used to irrigate seedlings and watering stopped 7 days before harvesting to allow the substrate to gradually dry out. After harvest, the soil was maintained at 4ºC except when it was transferred to room temperature for spore assessment. Wet sieving and sucrose centrifugation methods were used to extract spores (Brundrett et al. 1996). The percent root colonization method was determined using 10% (w/v) potassium hydroxide clearing and 0.05% trypan blue staining at 121˚C for 15 minutes. Thirty root segments (each about 1 cm long) were assessed using the intercept method under a compound microscope (Brundrett et al. 1996). The criteria of AMF species selection for the next experiment was based on the number of spore multiplications (mean >50 spores per 100 g soil) and colonization (mean >70% of 30 root segments). Spores were identified using spore morphology (Trapp & Schenck 1982, Charoenpakdee et al. 2010, International Culture Collection of Vesicular and Arbuscular Endomycorrhizal Fungi [http://invam.caf.wvu.edu/Myc_Info/Taxonomy/species.htm]). The AMF were allocated Chiang Mai University culture collection accession numbers (Table 1) (Charoenpakdee et al. 2010).

AMF sporulation in the rhizosphere of cereals

Treatment combinations were performed in a factorial of 4 host plants × 3 AMF treatments × 3 replicates laid out in a completely randomized block design. Seeds of corn (*Zea mays* L.), job's tears (*Coix lacryma-jobi* L.), rice (*Oryza sativa* L.) and sorghum (*Sorghum bicolor* L.) were obtained from the Faculty of Agriculture, Chiang Mai University. These species were chosen because they are easy to grow in containers, are not known to require specific AMF, and are reported to be good hosts and trap plants for AMF due to characteristics such as fast growth, abundant

Table 1 AMF species, spore density (SD) per 100 gram of soil and percent colonization (C) from trap culture with physic nut seedlings harvested at 90 days.

AMF	Site*									
	CR1	**CR2**	**CM1**	**CM2**	**CM3**	**CM4**	**LO1**	**LP1**	**KK1**	**NK1**
Code*	CMU31	CMU14	CMU33	CMU06	CMU05	CMU14	CMU03	CMU12	CMU23	CMU02
Species	*S. pellucida*	*A. lacunosa*	*S. heterogama*	*A. scrobiculata*	*E. colombiana*	*A. lacunosa*	*A. tuberculata*	*A. excavata*	*Glomus* sp.4	*A. foveata*
SD	3±2.7a**	2±1.4a	57±8.0b	2±2.0a	52±3.9b	3±4.3a	6±4.4a	2±3.2a	9±4.2a	5±8.7a
C	11±5.2a	22±4.6a	97±4.6b	14±9.8a	89±5.2b	20±12.7a	30±4.6a	17±0.0a	25±8.0a	16±14.4a

*Chiang Rai site1 (CR1), Chiang Rai site2 (CR2), Chiang Mai site1 (CM1), Chiang Mai site2 (CM2), Chiang Mai site3 (CM3), Chiang Mai site4 (CM4), Loei (LO1), Lamphun (LP1), Khon Kaen (KK1), Nong Khai (NK1).

**The same letters in each row indicate that there are no significant differences at $P \leq 0.05$ using Tukey test. Values are mean (n=3) ± standard deviation.

Table 2 Comparison of spore number before (B) and after (A) trapping with physic nut seedlings.

AMF	Site*																			
	CR1		**CR2**		**CM1**		**CM2**		**CM3**		**CM4**		**LO1**		**LP1**		**KK1**		**NK1**	
SD	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**
	7	3	14	2	3	57	5	2	74	52	31	3	31	6	6	2	17	9	73	5
Code*	CMU31		CMU14		CMU33		CMU06		CMU05		CMU14		CMU03		CMU12		CMU23		CMU02	
Species	*S. pellucida*		*A. lacunosa*		*S. heterogama*		*A. scrobiculata*		*E. colombiana*		*A. lacunosa*		*A. tuberculata*		*A. excavata*		*Glomus* sp.4		*A. foveata*	

*Chiang Rai site1 (CR1), Chiang Rai site2 (CR2), Chiang Mai site1 (CM1), Chiang Mai site2 (CM2), Chiang Mai site3 (CM3), Chiang Mai site4 (CM4), Loei (LO1), Lamphun (LP1), Khon Kaen (KK1), Nong Khai (NK1).

**The same letters in each row indicate that there are no significant differences at $P \leq 0.05$ using Tukey test.

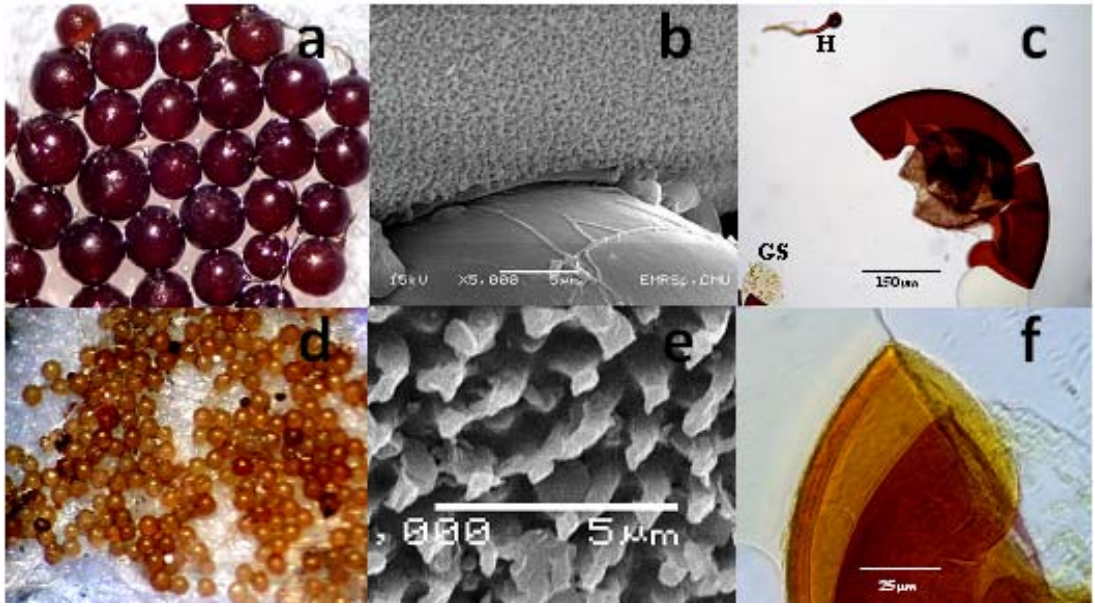

Fig. 1 – AMF species trapped with physic nut as the bait plant viewed under stereomicroscope, scanning electron microscope and compound microscope (left to right). (a, b, c) Spores of *Scutellospora heterogama* (CMU33) showing bulbous subtending hyphae (H) and germination shield (GS) and (d, e, f) spores of *Entrophospora colombiana* (CMU05) showing spore ornamentation and stained with Melzer's reagent.

fine and hairy roots and tolerance to adverse conditions (Simson & Daft 1990, Brundrett et al. 1996, Habte & Osorio 2001). One hundred healthy seeds of each plant species were disinfected with 0.05% sodium hypocloride (NaOCl) for 5 minute and washed in sterile water. Then, all seeds were grown in sterile coarse river sand. After 7 days, 108 healthy and uniform seedlings of each host plant were randomly selected and planted, three seedlings per pot in each treatment. *Scutellospora heterogama* (CMU33) and *Entrophospora colombiana* (CMU05) were chosen for testing because they produced abundant spores (>50 spores/100 g soil) and heavily colonized the roots of the trap plants. Fifty spores of each fungus, taken from the trap culture experiment, were placed on sterile filter paper (Whatman No.1) and placed below the seed in each pot. The spores were washed with sterilized water prior to placement on the filter paper. The control treatment received filter paper without spores. The pots were black plastic pots, 25 cm in top diameter containing 2.5 kg of autoclaved sandy soil (prepared as above). Modified Hoagland solution (Gambrog & Wetter 1975) was prepared with low phosphorus (0.01 M, pH 6.5) and 100 ml applied to each pot once per week. The pots were watered every other day with filtered tap water from which chlorine was evaporated for 24 hours in a 100 liter black tank before use. The pots were maintained in a greenhouse with natural photoperiod, temperature range of 25–38ºC, and relative humidity between 44 and 88%, which is in the range for the wet season in Thailand (May–July 2008). Watering was stopped 7 days prior to harvesting at 120 days (all host plants had mature grain), to allow the soil and plants to dry slowly. Spore density per gram soil, percent colonization, height, and dry weight of shoots and roots were determined.

Statistical analysis

The data in percentage were transformed into arcsin for analysis. Univariate analysis was employed for percentage of colonization and spore density. One-way analysis of variance (ANOVA) was used for height, and weight of fresh and dry shoots and roots. Tukey's post hoc multiple mean comparison test was used to test significant differences between treatments at $P \leq 0.05$. All statistical analyses were performed with Statistical Package for Social Sciences version 11.5 (SPSS Inc., Wacker Drive, Chicago, IL, USA).

Results

Physic nut seedlings as bait plants

Ten compatible morphospecies of AMF were trapped from the rhizosphere soil of physic nut under our experimental conditions. Surprisingly, after 3 months, the bait plants only trapped one sporulating AMF species from each site sample as follows: *Scutellospora pellucida* (T.H. Nicolson & N.C. Schenck) C. Walker & F.E. Sanders CMU31 in Chiang Rai site1 (CR1), *Acaulospora lacunosa* J.B. Morton CMU14 in Chiang Rai site2 (CR2) and Chiang Mai site4 (CM4), *S. heterogama* CMU33 in Chiang Mai site1 (CM1), *A. scrobiculata* CMU06 in Chiang Mai site2 (CM2), *Entrophospora colombiana* CMU05 in Chiang Mai site3 (CM3), *A. tuberculata* CMU03 in Loei (LO1), *A. excavata* CMU12 in Lumphun (LP1), *Glomus* sp.4 CMU23 in Khon Kean (KK1) and *A. foveata* CMU02 in Nong Khai (NK1). The percent colonization and spore density data showed similar trends. Except for two species, spore numbers and root colonization were low (Table 1). Two AMF exceeded the criteria (multiple spores, high root colonization) set for selection for the second experiment. They were *S. heterogama* (CMU33) and *E. colombiana* (CMU05) obtained from CM1 and CM3 (Fig. 1). They had high sporulation of 57 and 52 spores per 100 gram soil, and colonization of 97.3% and 89.0%, respectively. Trapping other site samples resulted in few spores and low (≤ 30%) infection rates (Table 1).

The comparison of spore numbers before and after baiting showed that most AMF were not able to increase their spore densities under the experimental conditions (Table 2).

AMF sporulation in the rhizosphere of cereals

Sorghum had a higher percent colonization (68%) and sporulation (175 spores per gram soil) for *E. colombiana* (CMU05) than other host plants, whereas rice gave higher percentage of colonization (68%) for *S. heterogama* (CMU33) but sporulation was very low. Percent colonization in sorghum was 64% and spore number averaged 26 spores per gram soil for *S. heterogama* (CMU33) (Tables 3, 4). Sorghum had the highest percent root colonization following by job's tears, corn and rice (Table 3). Spore production of *S. heterogama* (CMU33) did not occur with corn and likewise *E. colombiana* (CMU05) did not multiply with rice. However, there was some colonization of roots in corn for *E. colombiana* (CMU05) and in rice for *S. heterogama* (CMU33).

The effect of AMF species on the dry weight of shoots and roots and plant height is shown in Table 5. All the parameters for inoculated plants followed similar trends with values generally higher than the controls. Except for shoot dry weight of rice, *E. colombiana* (CMU05) and *S. heterogama* (CMU33) promoted the dry weight growth of host plants. Futhermore, *E. colombiana* (CMU05) increased the dry weight of sorghum more than *S. heterogama* (CMU33).

Discussion

Spore production using physic nut as the bait plant

Several different methods have been used to propagate AMF, which are obligate symbionts and cannot complete their life cycle without a host plant (Corkidi et al. 2008). The most widely used is pot culture, where the fungi are usually maintained and multiplied in combination with suitable host plant roots (Ferguson & Woodhead 1982). Native AMF inocula, which include spores, hyphae and root fragments colonized by AMF, can be obtained and produced from field soil using the rhizosphere soil of target plant species (Corkidi et al. 2008). Physic nut was used as trapping or bait plant for compatible AMF species as this was the target plant species of interest. In an earlier study, 34 AMF morphospecies were recorded from the rhizosphere of physic nut (Charoenpakdee et al. 2010), but given that weeds may have been hosting some of these fungi, it was unsure how many species of AMF were associated with physic nut roots in the field. In the current study, after 3 months, only 9 AMF species were reported to be sporulating and one species dominated at each sampling site.

Most AMF were not able to increase their spore densities under rhizosphere of host plant in our experimental conditions. It is likely that some of the spores in the field soil were not

Table 3 Percent root colonization of four crop species inoculated with *Entrophospora colombiana* (CMU05) or *Scutellospora heterogama* (CMU33).

Host plant	Control	CMU05	CMU33	Mean
Corn	0	54.00±18.00	32.00±18.33	36.86ab*
Job's tears	0	49.33±14.05	65.33±6.11	49.14bc
Rice	0	24.00±12.00	68.00±3.06	31.14a
Sorghum	0	68.00±6.93	64.00±6.93	56.57c
Mean	0a*	48.83b	52.50b	

*The same letters in each column and row mean that there were no significant differences at $P \leq 0.05$ using the Tukey test. Values are mean (n=3) ± standard deviation.

Table 4 Spore density in the rhizosphere of four crop species inoculated with *Entrophospora colombiana* (CMU05) or *Scutellospora hetergama* (CMU33).

Host plant	Control	CMU05	CMU33	Mean
Corn	0	51.66±3.84	0	22.14a*
Job's tears	0	3.00±0.32	15.63±2.50	7.98a
Rice	0	0	0.07±0.03	0.03a
Sorghum	0	175.02±54.29	26.33±1.25	86.29b
Mean	0a*	105.10c	17.42b	

*The same letters in each column and row mean that there were no significant differences at $P \leq 0.05$ using the Tukey test. Values are mean (n=3) ± standard deviation.

Table 5 Effect of *Entrophospora colombiana* (CMU05) or *Scutellospora heterogama* (CMU33) on growth of four host plants.

Treatment	Corn	Job's tears	Rice	Sorghum
		Shoot dry weight (g)		
Control	44.7±2.2a	50.1±0.8a	50.7±0.5a	57.2±0.9a
CMU05	48.2±0.2b	56.4±0.9b	51.5±0.5a	63.4±2.0c
CMU33	48.9±1.3b	54.5±2.0b	52.3±0.8a	60.4±5.5b
		Root dry weight (g)		
Control	30.8±6.6a	48.0±1.4a	41.3±2.1a	60.3±0.3a
CMU05	42.0±1.6b	52.8±0.5b	44.1±0.1b	66.4±0.6c
CMU33	40.9±2.4b	53.6±1.2b	45.3±0.6b	63.4±2.1b
		Height (cm)		
Control	65.7±1.3a	64.6±0.8a	61.5±0.9a	65.1±0.7a
CMU05	67.5±1.3a	66.0±1.5a	62.3±0.9a	67.3±2.0a
CMU33	66.3±1.3a	66.0±2.6a	62.7±3.0a	65.9±0.8a

*The same letters in each column mean that there were no significant differences at $P \leq 0.05$ using Tukey test. Values are mean (n=3) ± standard deviation.

viable or that some were not compatible with physic nut roots. In addition, a more aggressive AMF that germinates rapidly may dominate by speedily colonizing roots and sporulating, such as in the case of *S. heterogama*. Furthermore, there may be some specificity for AMF and plant species concerning AMF development and sporulation (Liu & Wang 2003). The mycorrhizal dependency of a host is genetically fixed and the degree of mycorrhizal dependency can be expressed at the level of an individual, or as a gradient within the ecological niche and relevant environmental conditions of the host (Feldmann et al. 2008). Clearly, as shown in other studies (van der Heijden et al. 1998, Douds & Millner 1999, Cardoso & Kuyper 2006, Wang et al. 2009), the combination of a suitable environment and host allowed *E. colombiana* (CMU05) and *S. heterogama* (CMU33) to proliferate in the pot study. The preliminary data suggest that physic nut may only form functional associations with

a limited range of AMF taxa. However, there are other possible explanations for the above finding. It is possible that some AMF may take longer than 3 months to produce spores or are recalcitrant under the pot conditions of the experiment. Our experimental conditions for sporulation, such as use of modified Hoagland's nutrient solution at pH 6.5 with low phosphorus level, may have constrained the development of some fungi as most of the initial soil inocula had a low pH. Carrenho et al. (2001) reported that the number of spores produced was influenced by soil chemical and physical properties and sporulation of *Glomus macrocarpum* was reduced at pH below 5.0. In the future, monosporic cultures will be required to evaluate the true extent of any host specificity with AMF and physic nut.

Spore multiplication

The evaluation of suitable pot hosts for AMF propagation was examined because the host type is one of the most important factors in optimizing spore production and multiplication (Safi & Khan 1997, Ryan & Graham 2002). In this study there was no cross contamination of the AMF species in any treatments, including the controls in the greenhouse, probably largely due to protection from rain splash. Spores can disperse by rain and germinate under optimal humidity (Brundrett et al. 1996). The present data indicated that AMF species can differ in their ability to infect different host plants. *Entrophospora* sp. (CMU05) had low colonization in rice and job's tears and *Scutellospora* sp. (CMU33) had low colonization in corn. Differences in spore production under the different plant species may be due to characteristics of the host plant and environment interactions (Smith & Read 1997, Mukerji et al. 2002, Ryan & Graham 2002).

This study found that the best growth promoting AMF species for sorghum and rice were *Entrophospora* sp. (CMU05) and *Scutellospora* sp. (CMU33), respectively. For corn and job's tears, both AMF species could be suitable because there was no significant difference in promoting growth by the two species when inoculated with either AMF, though growth of both species was greater than the controls.

Sorghum was more compatible as a host plant for spore multiplication, and thus similar to *Glomus intraradices* (Dabire et al. 2007) because their roots are fast-growing with extensive root systems and they are tolerant to fluctuating environmental conditions. Sorghum is also easy to grow in many parts of the world. Therefore, it is a good host plant for AMF propagation (Brundrett et al. 1996, Habte & Osorio 2001). Inoculum production on a commercial scale has always used widespread host plants such as *Allium cepa* L., *Cenchrus ciliaris* L., *Panicum maximum* Jacq., *Paspalum notatum* Flueggé, *Sorghum halepense*, *Trifolium subterraneum* L. and *Zea mays* in which spores develop within 3-4 months (Chellappan et al. 2001, Tahat et al. 2008).

Future research will focus on *Scutellospora heterogama* (CMU33) and *Entrophospora colombiana* (CMU05) to determine their effectiveness in promoting growth and oil yield of physic nut in the field. The fungi may also prove to be useful for the management of mixed plantings, including some cereal and perennial cops.

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