Chapter 13
Arbuscular Mycorrhizal Fungi for Jatropha Production

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Abbreviations

AMF	Arbuscular mycorrhizal fungi
dS m^{-1}	Deci Siemens per meter
EC	Electrical conductivity
ha	Hectare
K	Potassium
kg	Kilogram
N	Nitrogen
NaCl	Sodium chloride
P	Phosphorus

Introduction

Mutualistic fungi depend on plants for photosynthetic carbon while host plants receive inorganic nutrients and water captured from the soil through fungal hyphae. Most *arbuscular mycorrhizal fungi* (AMF) are obligate symbionts within the monophyletic phylum, Glomeromycota. The AMF form highly branched exchange

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surfaces, known as arbuscules, within the cortical tissue of roots during periods of active root growth. Many AMF also form vesicles, which are lipid-filled structures produced in intercellular spaces. Their function is food storage, but can also serve as reproductive propagules for the fungus in some species. Other structures are auxiliary cells and asexual spores, which are formed in the soil (Fisher and Jayachandran 1999; Sylvia et al. 2005; Trappe 2005; Schüßler et al. 2009). These fungi cannot finish their life cycle or multiply on artificial media without their plant host (Sieverding 1991). The majority of vascular plants develop mycorrhizal associations (Smith and Read 1997; Öpik et al. 2008; Parniske 2008; Siddiqui et al. 2008; Verma 2008) that play important roles in plant fitness including suppression of soilborne pathogens (Brundrett et al. 1996; van der Heijden and Sanders 2003; Siddiqui et al. 2008), nutrient cycling and soil conservation in natural as well as agricultural ecosystems (Boomsma and Vyn 2008). These fungi are sensitive to some agricultural chemicals; their diversity and abundance are also reduced by fertilizers, especially those containing high amounts of phosphorus (P). Many AMF produce large spores; a fact that facilitates their extraction from soil and observation under microscope (Brundrett et al. 1996; van der Heijden and Sanders 2003; Sylvia et al. 2005; Verma 2008). Hence, field surveys can readily be undertaken since the results are only limited by the capacity to identify AMF using molecular tools. Lastly, inoculation with target mycorrhizal fungi can lead to an improvement in growth and reproduction in many crops (Brundrett et al. 1996; Smith and Read 1997; van der Heijden and Sanders 2003; Öpik et al. 2008; Parniske 2008; Verma 2008) depending on the prevailing edaphic factors.

There is strong interest in bringing marginal lands, which cannot be used for food production, into cultivation for physic nut (*Jatropha curcas* L. hereafter referred to as Jatropha). However, in order for this plant to perform well on these marginal sites, inoculation with symbiotic microorganisms, such as AMF, is likely to be essential due to the loss of soil microflora resulting from past land management and other practices. This report explores the association of AMF with Jatropha including AMF diversity, compatible AMF species and known benefits of AMF for Jatropha growth and development. It draws heavily on work undertaken in northern Thailand, supplemented with studies elsewhere. Knowledge gaps were identified and served as guidelines to investigate future directions for research and management.

The Mycorrhizal Status of Jatropha

Investigations of Jatropha seedlings or cuttings from the field indicate that their roots are generally associated with AMF (see for example Leye et al. 2009; Kamalvanshi et al. 2011; Singh and Jamaluddin 2011). No systematic study has been undertaken on AMF associated with Jatropha in the wild, thus comparisons can only be drawn between Jatropha under cultivation. The analyses by Charoenpakdee et al. (2010a) revealed a strong relationship between Jatropha and AMF in the rhizosphere of Jatropha trees in six provinces of Thailand (Fig. 13.1). Thirty-four AMF morphospecies were identified among which the predominant

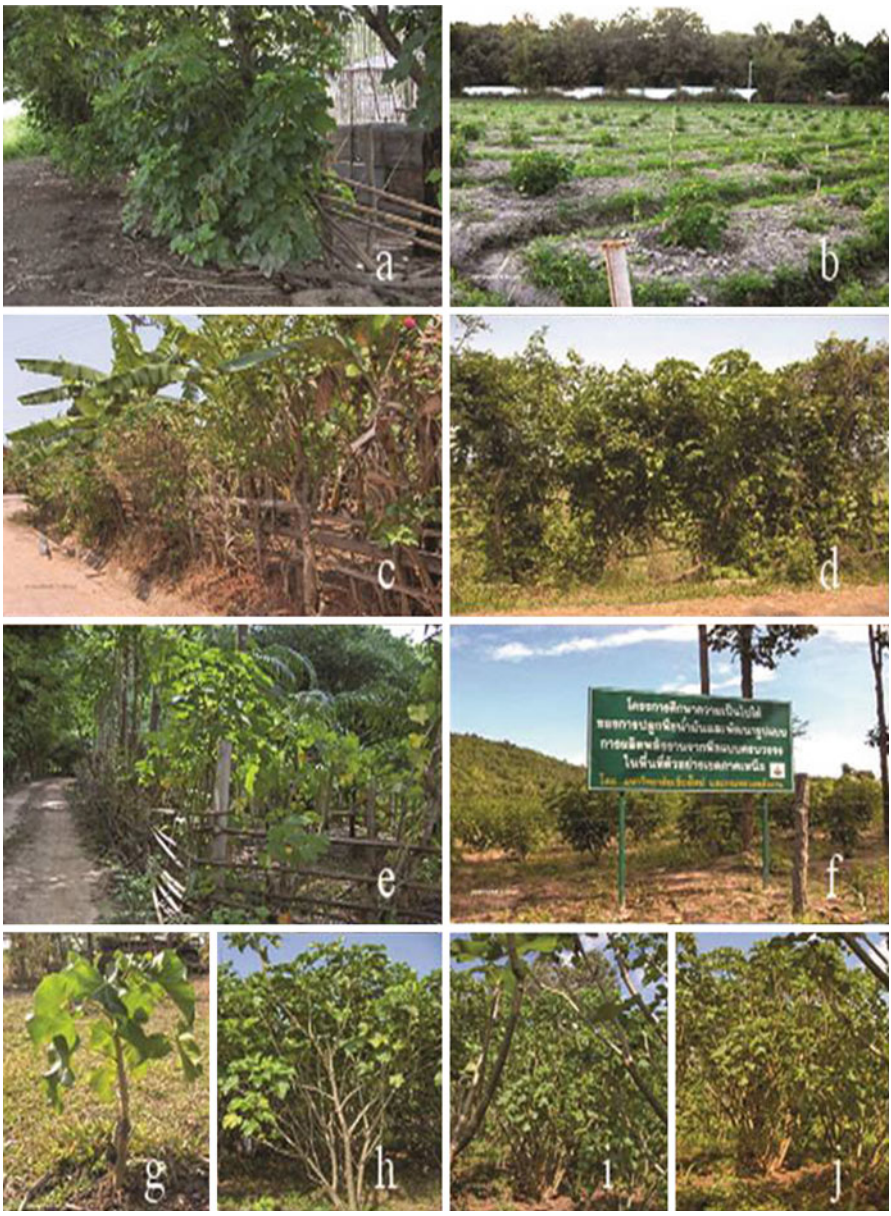

Fig. 13.1 Collection sites for AMF associated with Jatropha in six provinces of Thailand. (**a**) CR2: Chiang Rai, (**b**) CM1: Chiang Mai, (**c**) CM2: Chiang Mai, (**d**) CM3: Chiang Mai, (**e**) CM4: Chiang Mai, (**f**) LP1: Lumphun, (**g**) CR1: Chiang Rai, (**h**) KK1: Khon Kean, (**i**) LO1: Loei, (**j**) NK1: Nong Khai

genus, in terms of spore density and species diversity, was *Acaulospora* followed by *Glomus*, *Scutellospora*, *Gigaspora* and *Entrophospora*. Furthermore, seven species dominated the AMF spore populations in many sites (Fig. 13.2). Jatropha roots were heavily colonized in all the field sites sampled (Fig. 13.3). Thus, in both acid

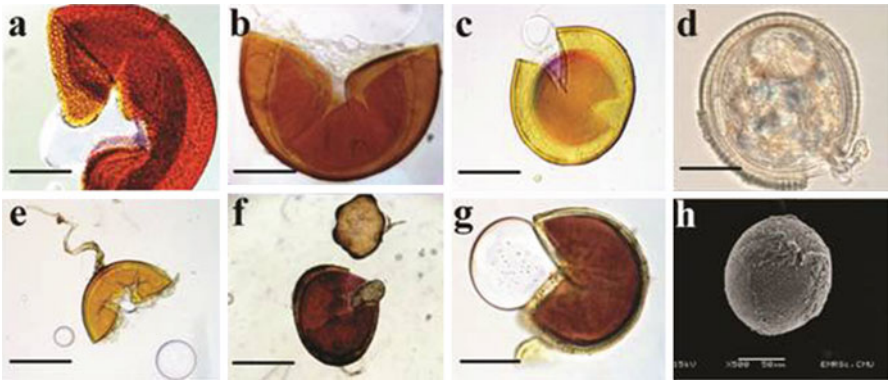

Fig. 13.2 Spore traits of the dominant AMF collected from Jatropha rhizosphere under light (**a**–**c**, **e**–**g**) and scanning electron (**h**) microscopes. *Acaulospora foveata* (CMU02) (**a**), *Entrophospora colombiana* (CMU05) (**b** and **h**), *A. dilatata* (CMU09) (**c**), *A. lacunosa* (CMU14) (**d**), *Gigaspora rosea* (CMU29) (**e**), *Gigaspora* sp.01 (CMU28) (**f**), and *A. scrobiculata* (CMU06) (**g**) with Melzer's reagent. Bars: **a**, **b**, **c**, **d**, **g** = 38 μm (40×); e, f = 150 μm (10×); h = 50 μm

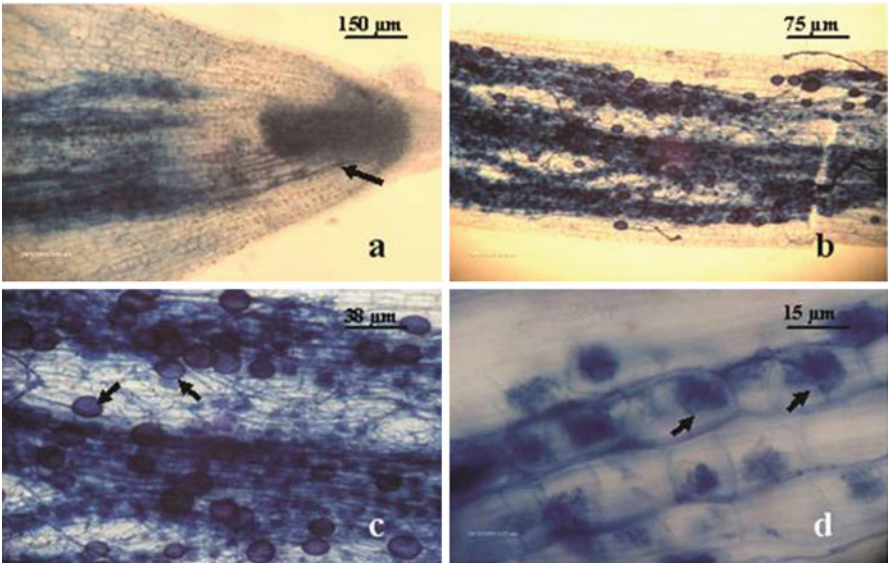

Fig. 13.3 AMF colonization in Jatropha roots. (**a**) AMF structures near the root tip (*arrow*), (**b**) highly infected root, (**c**) vesicles (*arrows*), and (**d**) arbuscules (*arrows*). Bars are 150 μm (**a**), 75 μm (**b**), 38 μm (**c**), 15 μm (**d**), respectively

and alkaline soils in Thailand, Jatropha appears to be readily colonized by AMF under a range of field conditions, including acidic to alkaline soils, soils of low to moderate organic matter content and soils differing greatly in the concentration of available P.

Spore Production of AMF Species Compatible with Jatropha

Jatropha has been introduced into many countries from its natural distribution in the American tropics. The soil microbiota and soil conditions of these new sites can differ markedly from those of its natural habitat. Under these situations, especially where soil microbial diversity has been depleted from soil by disturbance in the past, it may be desirable to introduce compatible beneficial rhizosphere bacteria (Johansson et al. 2004; Desai et al. 2007; Khan 2008; Nehra and Saharan 2011) and fungi. This necessitates the production of inocula for commercial application. In the case of AMF, which cannot complete their life cycle without a host plant (Corkidi et al. 2008), spore inoculum has to be produced in the rhizosphere of compatible host plants (Liu and Wang 2003). A range of techniques are available for producing inoculum including growing plants in sterile sand or in more controlled environments, such as aeroponic and nutrient film systems (Setiadi 2002). Alternatively, when good facilities are available (Pratap and Potty 2011), cultures of transformed roots are preferred for multiplying AMF spores to ensure that only the desirable species are present in the inoculum (Ishii et al. 1997).

Many mycorrhizal fungi have been distributed around the world with the movement of plants prior to the establishment of quarantine services and also because some AMF are compatible with a broad range of hosts. Thus, it is a good practice to first evaluate whether local AMF are effective for Jatropha or not. According to this approach, Jatropha was used as a bait plant for AMF in soils taken from the field in northern and north-eastern Thailand. Although these soils contained over 34 species of AMF, only one species of *Entrophospora* and one species of *Scutellospora* were able to multiply their spores (Fig. 13.4) in the rhizosphere of pot-grown Jatropha, suggesting that the majority of AMF species may be preferentially associated with the roots of weeds and not Jatropha. Each species of AMF was identified on the basis of its spore morphology under microscope. Spore shape, size and ornamentation were used for genus classification. For example, based on light microscopy alone, the AMFs of Fig. 13.4a can be from *Gigaspora* or *Scutellospora* and those of Fig. 13.4d from either *Acaulospora* or *Entrophospora*. Internal structures of spores, such as (1) the *germination shield* can then be used to confirm *Scutellospora* (Fig. 13.4b) and (2) the presence of two *scars* on the outer spore wall in addition to a red to purple reaction of the inner wall layer to Melzer's reagent suggest *Entrophospora* (Fig. 13.4e). Electron microscopy provides additional traits such as spore ornamentation that can be used to confirm some species (Fig. 13.4c, f). Molecular tools, such as the sequence of small (18S) and large subunits (28S) of rDNA genes can be used for final species identification (Charoenpakdee 2009). These two compatible AMFs were then multiplied further using a range of cereals (Fig. 13.5) of which sorghum was the best host plant for AMF sporulation (Charoenpakdee et al. 2010b). Plants such as grasses, which grow fast and have extensive systems of fine hairy roots are ideal for producing AMF spores in sterile sand in pots or in raised beds in the greenhouse. Once compatible species of AMF have been multiplied in sufficient quantity their effectiveness must be

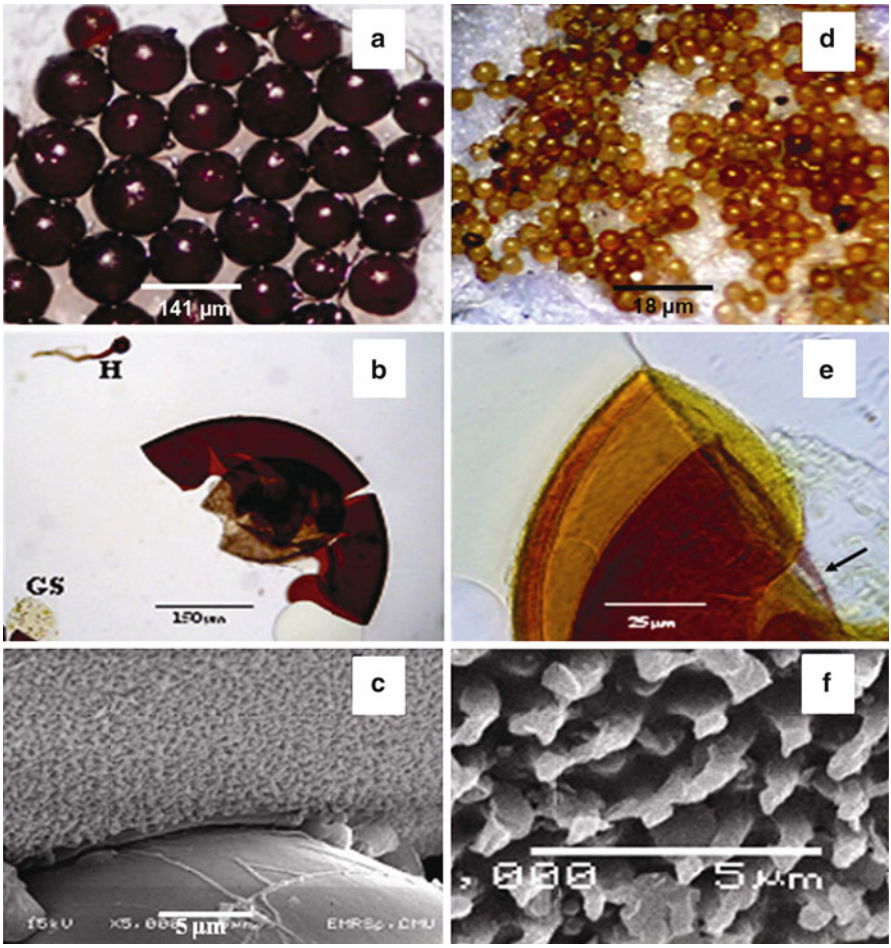

Fig. 13.4 Examples of AMF species trapped with Jatropha viewed under stereomicroscope (**a, d**), compound microscope (**b, e**) and scanning electron microscope (**c, f**). Spores of *Scutellospora* sp. (**a, b, c**) are ~130 µm (**a**). A general view of bulbous subtending hyphae (*H*) and germination shield (*GS*) is given in (**b**). The attachment region of bulbous subtending hyphae is a character that allows species classification (magnification 5,000) (**c**). Spore of *Entrophospora* sp. (**d, e, f**) are ~3 µm (**d**). The spore wall layer appears red to purple in Melzer's reagent (arrow) (**d, e**) and spore ornamentation is evident at magnification 10,000 (**f**). Bars are 141 µm (**a**), 150 µm (**b**), 5 µm (**c**), 18 µm (**d**), 25 µm (**e**) and 5 µm (**f**), respectively

evaluated in field trials before deployment in commercial plantations. Only a few species of AMF have been assessed so far in field trials (e.g., Behera et al. 2010). By contrast, a wider range of AMF species have been evaluated in containers (Feldmann et al. 2008; Leye et al. 2009; Kumar et al. 2010; Ultra 2010) for many food and fiber crops.

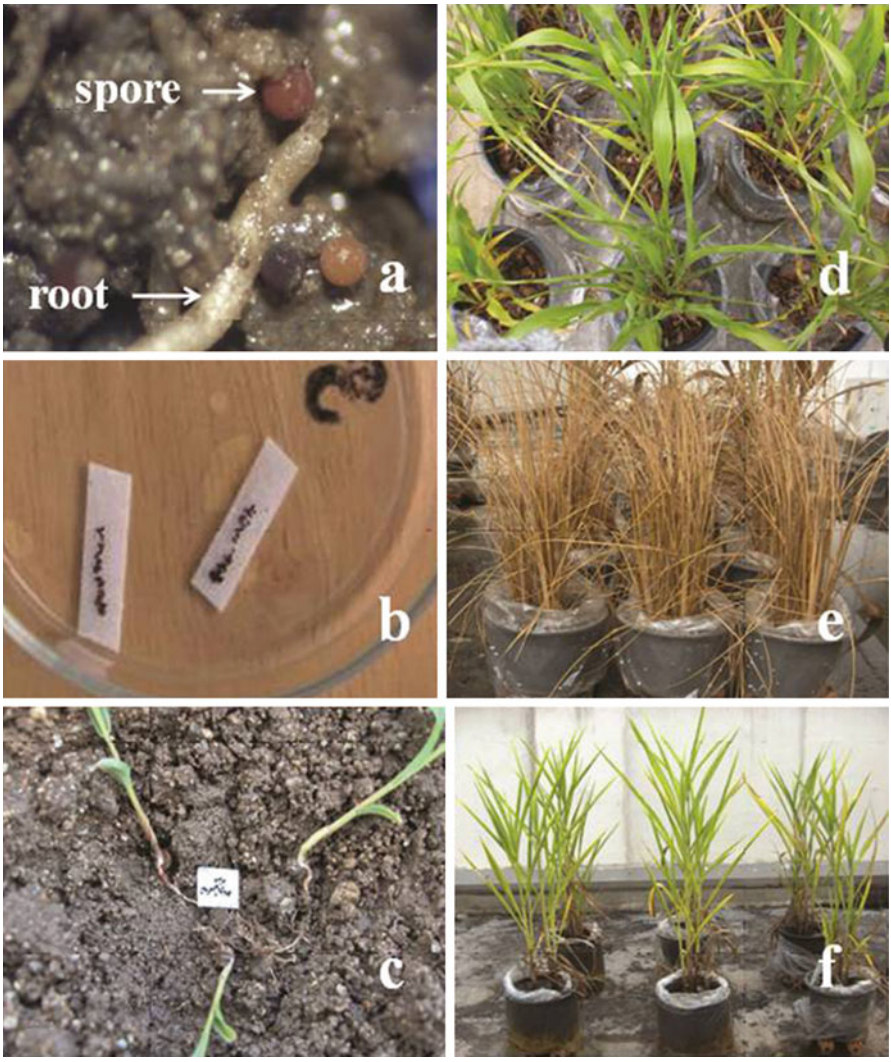

Fig. 13.5 Process of AMF spore multiplication in pot culture. Spore inoculum of *Scutellospora* sp. in soil (**a**) and on strips of sterilized paper used as starter inoculum (**b**). Sorghum seedlings are being planted in sterilized soil pre-inoculated with spores (see paper strip) (**c**). The surface of potting mix is kept moist with sterilized coir (host is *Coix lacryma-jobi*) (**d**) and external contamination is controlled by soil cover with a plastic sheet and by pots elevation on bricks (job's tears) (**e**). A case experiment with rice kept on water sheet before harvesting (**f**)

Benefits to be Gained from Symbiosis with AMF

Studies on a wide range of crop species have shown considerable advantages to be gained from roots being associated with compatible AMF. As Jatropha is a relatively

new crop for biodiesel production, studies are incomplete. However, it is reasonable to assume that similar benefits as found for many other crops will also apply for this new crop. The following sections describe the main benefits of AMF symbiosis for Jatropha as they are documented in the literature.

Enhancing Nutrient Uptake

AMF play an important role in the mineral nutrient uptake by host plants (Marschner and Dell 1994), which facilitate plant growth and yield (Monzon and Azcon 1996; Hartwig et al. 2002; van der Heijden and Sanders 2003; Davies et al. 2005; Schreiner 2007; Plassard and Dell 2010). AMF produce fine hyphae, which ramify into soil providing a large absorption surface for nutrient uptake. Nutrient capture can take place in sites where root diameter precludes root access especially for elements that are strongly adsorbed to soil surfaces (Brundrett et al. 1996; Smith and Read 1997; van der Heijden and Sanders 2003; Verma 2008). Furthermore, AMF may have access to forms of nutrients that are not readily available to roots including organic forms of P (Jones 1998; van der Heijden and Sanders 2003; Plassard and Dell 2010). AMF may also have a role in the cycling of soil nitrogen (N) (Veresoglou et al. 2011).

Studies undertaken so far with Jatropha indicate that macronutrients are often limiting when this crop is grown on poor soils. Not surprisingly, inoculation with selected AMF can increase the growth of young plants in these soils. In a field trial in India, the application of *Glomus fasciculatum* and *Scutellospora calospora* spores promoted a larger growth of Jatropha compared to the unfertilized control and to plants that were given 10 g NPK fertilizer at establishment (Behera et al. 2010). Similar growth responses to AMF inoculation have been observed on soils with low available P in pot trials. For example, Leye et al. (2009) tested four species of AMF (*Glomus aggregatum, G. fasciculatum, G. interradices,* and *G. mosseae*) and found that growth was stimulated in 4-month old seedlings. However, there was a significant interaction between AMF species and Jatropha cultivars concerning growth stimulation. In the Philippines, Ultra (2010) suggested that AMF should be added in conjunction with 15–30 kg NPK fertilizer/ha to improve nutrient utilization.

In Thailand, growth stimulation of Jatropha's seedlings have been obtained with a range of AMF species (*Acaulospora* sp. no.22, *Entrophospora colombiana*, *Glomus caledonium*, *G. etunicatum*, *Scutellospora* sp. CMU33, a commercial product containing two species of *Glomus* and two species of *Acaulospora*). The values of all parameters referring to plant growth were increased after AMF inoculation (Table 13.1). The increased vigor of inoculated plants can be deduced from their two times larger diameter of their stems (Fig. 13.6, Table 13.1) and their two times larger root system (Charoenpakdee 2009). Despite their preference for specific hosts, AMFs have a wide range of hosts as reported by Zhu et al. (2000). Similarly, application of *G. aggregatum* with organic fertilizer (duck guano) and either phosphate rock or triple superphosphate (TSP) increased fruit production, seed weight and plant height in 2-year old Jatropha cuttings grown in the field in Thailand (Silpachai et al. 2009).

Fig. 13.6 Effect of inoculation with AMF on the growth of Jatropha seedlings for 30 days in 30 cm diameter pots containing 5 kg of sterilized sandy soil; T0 = control (no AMF), T1 = *Acaulospora* sp., T2 = *Entrophospora* sp., T3 = *Glomus caledonium*, T4 = *G. etunicatum*, T5 = *Scutellospora* sp, T6 = mixed species (a mixture of 20 spores of each of the above five AMF species) and T7 = commercial product (Charoenpakdee 2009)

Table 13.1 Growth of inoculated (T1-T7) and uninoculated (T0) Jatropha seedlings assessed 90 days after transplantation

Treatment	Height (cm)[a]	Stem circumference (cm)[a]	Weight (g)[a]		Colonization (%)[b]
			Fresh	Dry	
T0	11.60 ± 2.29a[c]	3.68 ± 0.56a	16.51 ± 11.10a	5.82 ± 5.40a	0 ± 0.00a
T1	13.35 ± 3.28ab	4.45 ± 0.91ab	28.13 ± 6.11b	10.49 ± 3.90b	73 ± 5.19bc
T2	13.10 ± 1.61ab	4.30 ± 0.24ab	23.79 ± 6.61ab	8.38 ± 2.53ab	75 ± 3.42bc
T3	13.58 ± 1.31ab	4.63 ± 0.75b	25.19 ± 4.83ab	9.21 ± 2.01ab	85 ± 2.64c
T4	13.43 ± 3.33ab	4.75 ± 0.33b	28.68 ± 6.64b	9.13 ± 4.24b	55 ± 5.04b
T5	15.93 ± 1.51b	5.00 ± 0.00b	33.43 ± 2.96b	13.36 ± 2.09b	80 ± 4.51c
T6	14.38 ± 2.22ab	4.93 ± 0.30b	31.85 ± 4.44b	12.74 ± 4.03b	87 ± 2.97c
T7	13.83 ± 2.29ab	4.55 ± 0.64b	30.52 ± 5.44b	11.43 ± 2.57b	77 ± 3.76bc

T0 = control, T1 = *Acaulospora* sp. no.22, T2 = *Entrophospora colombiana*, T3 = *Glomus caledonium*, T4 = *G. etunicatum*, T5 = *Scutellospora* sp., T6 = mixed species and T7 = commercial product

[a]Values are averages of four replications (mean ± standard deviation)

[b]Values are averages of 30 replications (mean ± standard deviation)

[c]Letters "a", "b" and "c" stand for the groups of individuals which variance can be considered homogeneous and which mean can be considered significantly different from one another at $P < 0.05$ using Duncan's multiple range test. One can see that group "a" appears only for the control, which implies that all the inoculated individuals are significantly different

In Brazil, Jatropha seedlings inoculated with *Gigaspora margarita* or *Glomus clarum* in pots containing 4 kg of sandy soil had higher shoot and root dry matter, plant height, leaf number and total leaf area (Balota et al. 2011). Not surprisingly, in degraded soils AMF can accelerate revegetation including with exotic species, such as Jatropha (Singh and Jamaluddin 2011). The higher success rate of revegetation was demonstrated in India using an inoculum of *G. fasiculatum* and *S. calospora* spore (Behera et al. 2010).

Enhancing Tolerance to Saline Soils

Salt stress can markedly reduce plant growth and can lead to death in sensitive species. In addition to osmotic damage due to the accumulation of salts in plant tissues and to the water deficit in shoots, salinity can also result in mineral nutrient imbalance at the whole plant level. AMF may assist some plants to withstand low levels of salinity stress by enhancing plant biomass and altering host plant physiology (Ruiz-Lozano et al. 1996). Examples of these responses have been demonstrated for food and fiber crops including cotton (Tian et al. 2004), green basil (Enteshari and Hajbagheri 2011), lotus (Sannazzaro et al. 2007), maize (Amerian and Stewart 2001; Feng et al. 2002), tomato (Fritz et al. 2006; Subramanian et al. 2006) and wheat (Daei et al. 2009). Rabie and Almadini (2005) described some of the mechanisms of AMF on ion transport in plants. Mycorrhizal plants have relatively less export of sodium (Na) and chloride (Cl) from the roots to the shoots (Scheloske et al. 2004) and this can have beneficial effects such as reducing Na in leaves as well as increasing membrane stability and concentration of N, P and K (Rinaldelli and Mancuso 1996; Kaya et al. 2009). Moreover, higher rate of K accumulation by mycorrhizal plants under NaCl stress may help in maintaining a large K/N ratio (for biochemical benefits of AMF, see Sannazzaro et al. 2007 and Borde et al. 2011).

Differences between AMF have been observed in their capacity to influence the uptake of Na and Cl (Tian et al. 2004; Daei et al. 2009). Moreover, salinity can have negative consequences on symbiosis by reducing the (1) rate of colonization, (2) rate of spore germination, (3) growth of hyphae in soil and hyphal spread after infection and (4) arbuscule number.

Jatropha is quite sensitive to saline soils. Trial in containers showed the addition of NaCl decreased tap root length and root biomass. However, plants in the same conditions (light, soil pH, soil and air humidity, etc.), but preinoculated with AMF had larger root systems, better leaf water status, less leaf membrane damage (low lipid peroxidation activity), higher solute (proline and sugars) and higher leaf chlorophyll concentrations than uninoculated plants. The authors concluded that inoculation with AMF in the nursery before transplanting in the field is useful for improving the growth of Jatropha in soils with up to 0.5% NaCl (EC of 7.2 dS m^{-1}).

Enhancing Tolerance to Heavy Metals

The plant tolerance to heavy metals such as zinc, chromium, nickel and arsenic is generally improved by mycorrhization (Subramanian et al. 2006; Wu and Xia 2006; Jankong and Visoottiviseth 2008; Turkmen et al. 2008). For example, seedling inoculation with *Glomus mosseae*, *G. intraradices* and *G. etunicatum* resulted in an improved tolerance of *Pityrogramma calomelanos*, *Tagetes erecta* and *Melastoma malabathricum* to arsenic contamination of soil in Thailand

Fig. 13.7 One year old Jatropha seedlings planted in abandoned mine tailings in Mogpog, Marinduque, Philippines. Seedlings were inoculated with a commercial mix of eight AMF species (*Enterphosphora*, *Gigasporea*, *Glomus*) before planting (**a**) or uninoculated for the control (**b**). The inoculated plants had better survival (100%), height (19 cm) and stem diameter (2.1 cm) than the uninoculated plants (70%, 13 cm, 0.8 cm, respectively). Photographs and data are a courtesy of Nelly S. Aggangan (BIOTECH, UPLB, Laguna, Philippines)

(Jankong and Visoottiviseth 2008). Two possible reasons for AMF-mediated arsenate tolerance are: (1) AMF colonization might down-regulate the high-affinity for phosphate or arsenate transport system by enhancing phosphorus uptake, but suppressing arsenic uptake and (2) AMF might increase the efflux of arsenic (as arsenite) from mycorrhizal roots (Jankong and Visoottiviseth 2008). AMF might also reduce plant exposure to metals by their preferential uptake into fungal tissues as well as into extra-cellular glycoproteins in the rhizosphere. Organic amendments in conjunction with AMF could further help to mitigate heavy metal toxicity in soil (Nanda and Abraham 2011).

In the Philippines, Jatropha has been successfully established using AMF on mine tailings contaminated with heavy metals (Fig. 13.7). Inoculation with AMF enhanced survival and growth of seedlings (Lu et al. 2007; Charoenpakdee 2009; Kumar et al. 2010). In general, sequestering of heavy metals in roots of mycorrhized plants reduces heavy metal accumulation in shoots.

Enhancing Tolerance to Water Stress

Mycorrhization strongly affects the growth and tolerance to water deficit of many plants (Borkowska 2002; Kaya et al. 2003; Pinior et al. 2005; Lu et al. 2007). Under unstressed conditions, AMF can (1) increase the relative leaf water content and transpiration rate and (2) decrease stomatal resistance to gas exchanges. Moreover, under continuous drought conditions, mycorrhized plants recovered more quickly their optimal turgor and experienced higher growth rate compared to non-mycorrhized plants (Kaya et al. 2003; Pinior et al. 2005; Lu et al. 2007). The exact mechanisms underlying these physiologic changes are not understood though changes in hormone balances have not been implicated (van Rooyen et al. 2004; Khalvati et al. 2005; Bárzana et al. 2012).

So far, there are no detailed investigations on benefits to be gained from inoculating Jatropha with AMF in water-limited environments. Since Jatropha plantations are being established in some regions with a long dry season where risks of drought are likely to be exacerbated by climate change, it is prudent to undertake research to identify AMF that may enhance survival and oil yield under water-limited conditions. Preliminary observations on rewatering wilted Jatropha seedlings showed that plants inoculated with AMF regained stomatal function more quickly than uninoculated plants (Charoenpakdee, unpublished data).

Enhancing Tolerance to Biotic Stress

The biological control of soil-borne pathogens by AMF has been suggested by many workers because fungi have some ability to produce antimicrobial substances. However, the extent of such pathogen control is likely to be overstated and often pot studies are not confirmed in the field (Sylvia et al. 2005; Li et al. 2007). Biocontrol agents can exert disease suppression by different modes of action, including competition, direct parasitism, antibiosis and the induction of plant resistance mechanisms (Li et al. 2007; Mukerji and Ciancio 2007). Moreover, AMF may enhance host fitness by impairing nematode development. The net effects vary with the environment, plant genotype, nematode species and fungal isolates (Talavera et al. 2001; Gera-Hol and Cook 2005; Oyekanmi et al. 2007). Many changes in plant physiology after AMF infection have been reported including higher chitinase activity in roots (Jothi and Sundarababu 2002).

Application of Mycorrhizal Technology for Jatropha Production

From earlier sections of this report, it is clear that Jatropha can be strongly associated with AMF in the field and that AMF can play an essential role in overall plant fitness, improving the uptake of nutrient and water by the host, protecting plants against toxic

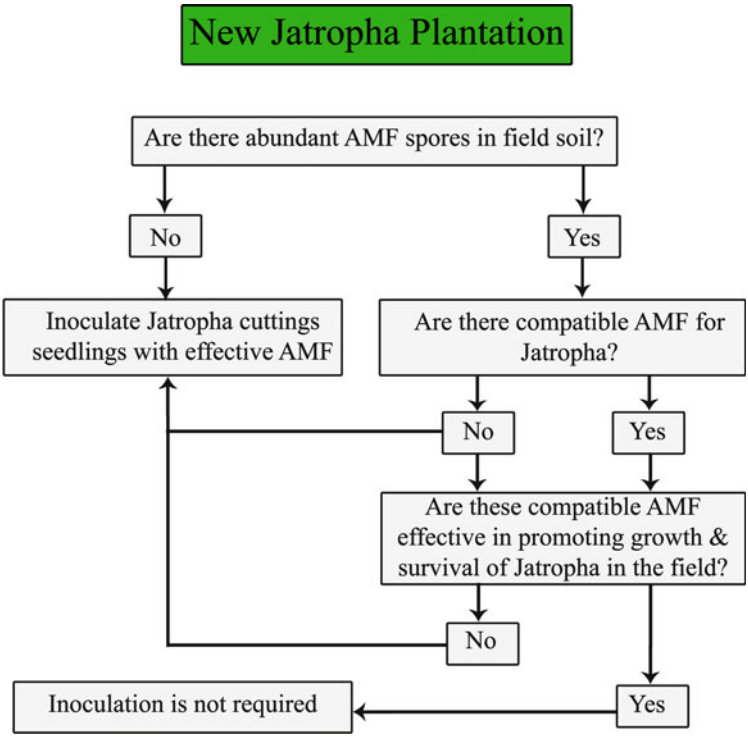

Fig. 13.8 Questions to consider when evaluating the need to inoculate Jatropha with AMF in the context of new commercial plantations

stress, etc. Therefore, managers of Jatropha plantations should request information on the AMF status of the crop being established. The flow chart in Fig. 13.8 shows a hierarchal approach that facilitates decision making as to whether inoculation is desirable or not.

If inoculation is deemed desirable, then it is important to thoroughly test the AMF inoculum and to evaluate its ability to colonize the roots of Jatropha and confer benefit to the crop. Hence, it is most desirable that specific inocula be developed for different site conditions and regions. Investment in the production of these inocula will pay dividends in the future. Managers need to be wary of deploying AMF inoculum that has not been specifically tested and developed for their plantations. It is evident that considerable research and development is still required. However, the task is not difficult since the technology exists and protocols for testing and evaluating AMF are widely available.

Although Jatropha is a robust plant and can grow in marginal environments, oil production for biodiesel will be uneconomic unless a crop with consistent vigor is achieved. Even in soils of larger fertility with less abiotic stress, AMF can be beneficial in nutrient cycling, by increasing the efficiency of fertilizer use and reducing fertilizer loss. Thus Fig. 13.8 should be a component of proper

agronomic management in order to obtain optimum yield and economic return (Francis et al. 2005; Ultra 2010).

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