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# The effects of arbuscular mycorrhizal inoculations and cotyledon removal on early seedling growth of *Pongamia pinnata*

**Anuradha JHA, Madhvi KAMALVANSHI, Anil KUMAR, Neha CHAKRAVARTY, Ashok SHUKLA*, Shiv Kumar DHYANI**
National Research Centre for Agroforestry, Jhansi, India

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**Abstract:** *Pongamia pinnata* (L.) Pierre, a fast-growing oil-seed-producing tree legume, has the ability to grow on wastelands. It can be utilized for biofuel plantation on such lands. The preconditioning of young seedlings during the early stage of development with efficient arbuscular mycorrhizal fungi (AMF) confers several benefits enhancing the possibility of their establishment in fields after outplanting from nurseries. However, before recommending these fungi for inoculation, the suitable AMF species must be identified. Therefore, to determine the potential AMF species, 9 purified fungi (isolated from different sites) were tested for their growth promotion activity. The inoculated fungi were not able to increase seedling growth. Some fungi reduced growth, which was attributed to more utilization of carbon by fungal symbiont. Development of fine roots was delayed up to 30 days after sowing and hence their colonization by AMF inoculants. This could be due to the large amount of nutrients stored in cotyledons of large *P. pinnata* seeds. Removal of cotyledons significantly reduced seedling growth and plants showed some extent of dependency on AMF inoculants. Thus, it was concluded that AMF inoculations should enhance biomass of *P. pinnata* only after depletion of metabolic reserves in its cotyledons and such mycorrhizal seedlings can be utilized for biofuel plantation.

**Key words:** Arbuscular mycorrhizae, biofuel, cotyledon removal, mycorrhizal dependency

## 1. Introduction

India consumes about 127 million tons of petroleum products annually, of which only 40 million tons are produced in the country (Kesari and Rangan, 2010). In order to achieve self-dependency and to fulfill the energy deficit, the best option is the use of biodiesel (Bhojvaid, 2008). It is produced from a wide range of edible oils (corn, rapeseed oil, and unconventional used cooking oils) in Europe, the USA, and other countries (Naik et al., 2008). For a country like India, however, which is already short of edible oils, use of nonedible oils only seems to be a suitable option (Mukta and Sreevalli, 2010). In Brazil, the USA, and Canada, food grains (corn and wheat) are being diverted for ethanol production (Focacci, 2005). India cannot afford this option owing to the limited area for food production to feed a population of about 1.21 billion (Zhou and Thomson, 2009). In this context, planners have rightly focused on some tree-borne oil (TBO) seed species. Amongst the TBOs, *Pongamia pinnata* (L.) Pierre has been found as a suitable option for biodiesel production (Karmee and Chadha, 2005). It is a fast-growing nitrogen-fixing tree legume with the potential for high oil seed production (seed contains 30% to 35% oil). *Pongamia pinnata* has the ability to grow on wastelands (Scott et al., 2008) and hence can be utilized for biofuel plantation on such lands (47.22 million ha; National Remote Sensing Centre, 2010).

Mukta and Sreevalli (2010) suggested that survival of any plant in fields depends upon the supply of quality planting materials. In forestry, the transplantation of seedlings is generally carried out after propagation in nurseries. Sometimes such seedlings face transplantation shocks in fields (Hartmann and Kester, 1986) and consequently plants become weaker and poorly established. It has been reported that preconditioning of young seedlings with efficient arbuscular mycorrhizal fungi (AMF) makes plants stronger and helps in their establishment in fields (Navarro Garcia et al., 2011; Çekiç et al., 2012; Shi et al., 2012). The early inoculation of AMF can be beneficial for plant cultivation in 2 ways: superior and stronger growth of seedlings and improved performance at outplanting. However, before utilizing these fungi for inoculation, the suitable AMF species must be identified. Caravaca et al. (2003) suggested that native AMF species produce more

* Correspondence: ashokshukla06@gmail.com

vigorous seedlings than nonnative species. Therefore, prior to exploitation of the potential of AMF, it seemed necessary to examine its occurrence in *P. pinnata* rhizosphere. Hence, the present work was carried out with the hypothesis that AMF species inhabiting the rhizosphere of *P. pinnata* would perform well in terms of seedling growth.

To determine the efficient AMF inoculants, purified fungi were tested for their growth promotion activity. Earlier it has been reported in our laboratory that native AMF substantially increased the growth of various crops/grasses/trees (Shukla et al., 2009; Hashmi et al., 2010; Jha et al., 2012; Shukla et al., 2012a), but in the present study the inoculated AMF species were not able to increase seedling growth during the early stage. It was assumed that this might be due to the availability of stored food materials in large *P. pinnata* seeds, which prompted us to assess the effects of cotyledon removal immediately after seed germination on growth and mycorrhizal dependency (MD) of *P. pinnata*. We presumed that removal of food materials stored in cotyledons would make plants dependent on AMF. Thus, our study was carried out with the following aims: identifying common AMF species in the rhizosphere of *P. pinnata*, screening AMF species for good growth of pongamia seedlings, and establishing the colonization potential of AMF in *P. pinnata* during the early stage of seedling growth and the effect of cotyledon removal on growth and MD of *P. pinnata* seedlings.

## 2. Materials and methods

The present study was carried out at the National Research Centre for Agroforestry (NRCAF), Jhansi (78°17′E, 24°11′N), Uttar Pradesh, India. Mean annual rainfall of the region is 960 mm with an average of 52 rainy days per year. Mean maximum temperature ranges from 47.4 °C (June) to 23.5 °C (January) and mean minimum temperature from 27.2 °C (June) to 4.1 °C (December). May and June are the hottest months. The maximum recorded temperature on a particular day often touches 47–48 °C during summer.

### 2.1. Description of selected sites

Different arid and semiarid regions of India, namely Hissar (Haryana; Western Plain, hot arid with desert and saline soils N8E1), Hyderabad (Andhra Pradesh; Deccan Plateau and Eastern Ghats, hot semiarid with red and black soils K6D2), Jhansi (Uttar Pradesh; Northern Plain and Central Highlands, hot semiarid with alluvium-derived soils N8D2) and Jodhpur (Rajasthan; Western Plain, hot arid with desert and saline soils M9E1), were selected as *P. pinnata* growing regions. Pantnagar (Uttrakhand; Western Himalayas, warm subhumid (inclusion humid) ecoregion with brown forest and podzoice soils AL1C4) was also included in the study, as the occurrence of *P. pinnata* in lower hills is quite common. The soil properties and other details of the selected sites were as follows: Hissar, soil order: entisol, type of plantation: pure, pH: 8.4 (1:2.5 $H_2O$), EC: 120.0 μS $cm^{-1}$, organic C: 0.47%, Olsen P: 12.0 kg $ha^{-1}$; Hyderabad, soil order: vertisol, type of plantation: pure, pH: 6.6 (1:2.5 $H_2O$), EC: 110.0 μS $cm^{-1}$, organic C: 0.48%, Olsen P: 13.5 kg $ha^{-1}$; Jhansi, soil order: vertisol, type of plantation: agroforestry, pH: 6.5 (1:2.5 $H_2O$), EC: 189.4 μS $cm^{-1}$, organic C: 0.74%, Olsen P: 23.4 kg $ha^{-1}$; Jodhpur, soil order: aridisol, type of plantation: pure, pH: 8.1 (1:2.5 $H_2O$), EC: 200.0 μS $cm^{-1}$, organic C: 0.15%, Olsen P: 10.0 kg $ha^{-1}$; and Pantnagar, soil order: mollisol, type of plantation: pure, pH: 7.1 (1:2.5 $H_2O$), EC: 365.0 μS $cm^{-1}$, organic C: 0.81%, Olsen P: 27.9 kg $ha^{-1}$.

### 2.2. Culturing, purification, and identification of AMF

The trap cultures were set using rhizosphere soils of *P. pinnata*, collected from the aforementioned sites. At each site, 10 plants 7–8 years old were marked and their rhizospheres were sampled. From each of the 10 plants, 4 soil core samples (500 g each) were taken from a depth of up to 30 cm with the help of a corer, after removing the litter/top soil. Collected samples were kept in polyethylene bags, sealed, labeled, and brought to the laboratory. All the 4 rooting-zone soil samples along with fine roots were mixed thoroughly to make a composite sample (2 kg approximately). To set trap cultures, rhizosphere soils were mixed in 1:1 ratio (w/w) with sterilized coarse sand, separately. Then mixtures (3 kg) were transferred to plastic pots (size: 24 × 16 cm) and seeded with *Zea mays* L. and *Phaseolus mungo* L. Thus, 10 pots per site (1 per plant) were established, which were kept in a greenhouse for 4 months. The rest of the samples (500 g) were analyzed for AMF diversity and some soil chemical properties [pH (1:2.5 $H_2O$), EC (μS $cm^{-1}$), organic C (%), and Olsen P (kg $ha^{-1}$)].

For isolation and purification of AMF species, briefly 100-g soil samples, collected from fields and trap cultures, were taken in a substantial amount of water (1 L) and decanted through a series of sieves (mesh size 32–420 μm). Sievings were individually collected and transferred onto a gridded petri dish (11 cm) for microscopic observations. Spores were isolated following the procedure of Gerdemann and Nicolson (1963). These were used to inoculate the pre-geminated seedlings of *Sorghum bicolor* L. in sterilized sand, which were kept in the greenhouse. After 3 months, AMF spores were extracted from these pots and observations required to distinguish the genera were made with a stereomicroscope (Nikon SMZ 800). Spores were properly rolled in water under reflected and/or transmitted light to observe hyphal attachments. Isolated spores were mounted on glass slides in polyvinyl-lactoglycerol (PVLG) or Melzer's reagent, for microscopic examinations. Then spores were identified on the basis of their morphological characteristics (shape, size, color and content of spores, surface ornamentations, width of

hyphal attachment, pore size, and wall structures) under a compound microscope (Nikon Eclipse E 400) at ×100 magnification, and were compared with morphological descriptions of species given on the INVAM webpage (http://invam.caf.wvu.edu). The latest taxonomic classification of AMF (Oehl et al., 2011) was followed to place the identified species under the correct genera. Number of AMF species (richness) in field/trap cultures and frequency of occurrence (%) were also calculated. Then the purified AMF species were utilized for testing their growth promotion activity in *P. pinnata*.

**2.3. Effect of AMF inoculations on seedling growth**

The efficiency of purified AMF species was tested in a completely randomized design. The trial consisted of 10 treatments, viz., *Acaulospora mellea* Spain and Schenck, *A. scrobiculata* Trappe, *Glomus arborense* McGee, *G. cerebriforme* McGee, *G. fasciculatum* Thaxter, *Glomus* 1, *Rhizophagus intraradices* (Blaszk., Wubet, Renker and Buscot) Walker and Schubler, *Simiglomus hoi* Berch and Trappe, *Paraglomus occultum* (Walker) Morton and Redecker, and a control. Each treatment was replicated 4 times. Red soil (alfisol; pH: 6.29, EC: 134 μS $cm^{-1}$, organic C: 0.27%, Olsen P: 2.5 ppm) was used as potting substrate. The soil was sieved (2 mm) and sterilized (15 psi for 3 consecutive days) to eliminate naturally occurring AMF propagules and other microbes. The sterilized soil was filled in plastic pots (4 kg capacity). The next day, 50 g of AMF inocula consisting of sand with chopped root bits, spores, and extramatrical mycelium were applied 4–5 cm below the seed. The control pots received an equal amount of autoclaved inoculum to provide general microbial populations free of AMF propagules. Pre-germinated healthy seedlings of similar sizes were transplanted to each pot; then thinning was carried out leaving 1 plant/pot, after a few days. The pots were transferred to the greenhouse, and watered as and when required. Seedlings (1 per replicate; 40 seedlings) were harvested after 4 months and analyzed for shoot length (cm), collar diameter (mm), and root and shoot dry weights (g) by standard methods. Phosphorus content of plants was determined using the vanadomolybdophosphoric yellow color method (Jackson, 1973) with a UV-VIS spectrophotometer (Halo DB 20, Double Beam, Australia) at 420 nm and expressed in mg $plant^{-1}$ on the basis of dry weight $plant^{-1}$. Mycorrhizal dependency was calculated in terms of plant growth as [(M – NM)/M] × 100, using dry weights of individual mycorrhizal plants (M) and mean dry weight of corresponding nonmycorrhizal (NM) plants (Plenchette et al., 1983).

**2.4. Colonization potential of AMF**

Colonization potential of the above-mentioned AMF species was tested with *P. pinnata* in sterilized sand (4 kg). Inoculation with different AMF was done by inoculating 50 g of inocula 4–5 cm below the seed, as per treatments. The control pots received an equal amount of autoclaved inoculum (free of AMF propagules). Each treatment was replicated 4 times, with 6 plants maintained in each pot. All the pots were kept in the greenhouse. The plants were irrigated with filtered water and half-strength Hoagland's solution was applied twice a week. The composition of the solution was 0.51 g/L $KNO_3$, 0.246 g/L $Ca(NO_3)_2$, 0.245 g/L $MgSO_4.7H_2O$, 1.43 g/L $H_3BO_3$, 0.91 g/L $MnCl_2.7H_2O$, 0.11 g/L $ZnSO_4.5H_2O$, 0.04 g/L $CuSO_4.5H_2O$, and 0.04 g/L $H_2MoO_4.H_2O$. Soil/root samples were taken at different intervals, i.e. 1, 2, and 3 months after sowing (MAS). Presence of arbuscules and vesicles, and the formation of sporocarps were monitored; then the root colonization index, i.e. percentage of root length colonized by AMF, and spore population in 100 g of sand were calculated. Fine roots were cleared with 10% KOH and stained with acid fuchsin (0.01% in lacto-glycerol) as reported by Kormanik et al. (1980) and observations on arbuscules and vesicles were recorded. Colonization index was determined by the gridline intersect method of Giovannetti and Mosse (1980). Sporocarps and spores were isolated according to Gerdemann and Nicolson (1963) and counted. Observations on formation of spores on extramatrical hyphae and inside the fine roots were also recorded. The better performing AMF species were selected for further study.

**2.5. Effect of cotyledon removal on growth and mycorrhizal dependency**

A factorial experiment in a completely randomized design was conducted to study the effect of cotyledon removal on growth and MD of *P. pinnata*. It consisted of 2 factors, viz., cotyledon removal (2 levels: with and without cotyledon) and AMF inoculations (4 levels: *A. scrobiculata, G. cerebriforme*, *R. intraradices*, and control). Thus, a total of 8 treatment combinations were employed and each treatment was replicated 4 times (i.e. 32 pots). The experiment was conducted in sterilized alfisol (4 kg). The pots were inoculated with AMF (as mentioned above) and sowing was done. All the pots were transferred to the greenhouse and watered. Fifteen days after sowing, cotyledons were removed without disturbing the embryonic axis with the help of a scalpel in half of the pots (i.e. 16 pots). Then only 1 plant was maintained in each pot. Observations on shoot length (cm), root length (cm), collar diameter (mm), dry weight (g $plant^{-1}$), MD, P concentration, and P uptake (mg $plant^{-1}$) were recorded at harvesting, i.e. after 3 months.

**2.6. Statistical analysis**

The data were analyzed statistically using a general linear model for analysis of variance in a completely randomized design. Least significant difference ($LSD_{0.05}$) was used to compare treatment differences. ANOVA (1 way: effect of

AMF inoculations on seedling growth; 2 way: effect of cotyledon removal on growth and MD) was performed by using the statistical package SYSTAT version 12.

## 3. Results and discussion

### 3.1. Diversity of AMF in field and trap cultures

Different AMF species were recovered from selected sites as well as from trap culture pots (Table 1). Six species were identified directly from field samples and 9 additional ones were isolated from trap cultures. Thus, a total of 15 species belonging to 6 different genera were identified. *Glomus* was the predominant genus (7 species), followed by *Acaulospora* (4 species), *Paraglomus* (1 species), *Rhizophagus* (1 species), *Sclerocystis* (1 species), and *Simiglomus* (1 species). Venkatesh et al. (2009) have also reported the predominant occurrence of *Glomus* in *P. pinnata* rhizosphere. Such wider occurrence of *Glomus* can be explained on the basis of variations in types of infective propagules (Biermann and Linderman, 1983; Shukla et al., 2013) because all parts of *Glomus* (hyphal fragments, spores etc.) are totipotent in initializing mycorrhization (Jasper et al., 1989). Two AMF spores (*Glomus* 1 and *Sclerocystis* 1) could not or not clearly be attributed to species level so far. Further, the frequency of occurrence of AMF varied from 20% to 80%. Its maximum value was recorded for *R. intraradices* (80%), followed by *G. arborense* (60%) and *A. scrobiculata* (60%). Oehl et al. (2003) called such AMF species "AMF generalists". Comparatively high frequencies of these indicate their adaptability to various soil conditions. Out of 15 identified species, only 9 could be purified.

Among the surveyed locations, maximum AMF diversity was recorded in vertisol at Jhansi (pH: 6.5), aridisol at Jodhpur (pH: 8.1), and entisol at Hissar (pH: 8.4). Among vertisols, it was higher at Jhansi (pH: 6.5) than at Hyderabad (pH: 6.6). The samples from Jhansi were taken from an agroforestry plot, which could be the reason for the higher AMF diversity. The results are in good accord with an existing report on the stimulatory effect of intercropping on AMF diversity in tree rhizosphere under agroforestry systems (Shukla et al., 2012b). Further, AMF showed good adaptations to the light-colored base-rich soils of Jodhpur (pH: 8.1) and Hissar (pH: 8.4). Recently, we recorded good AMF diversity in aridisol at Jodhpur

**Table 1.** Arbuscular mycorrhizal fungi (AMF) isolated from field and trap cultures, established by rhizosphere soils of *Pongamia pinnata*.

| AMF species | Soil sites | | | | | Frequency of occurrence (%) |
|---|---|---|---|---|---|---|
| | Hissar | Hyderabad | Jhansi | Jodhpur | Pantnagar | |
| *Acaulospora dilatata*# | +[a] | | | | | 20 |
| *A. longula*# | + | | | | | 20 |
| *A. mellea** | | | + | + | | 40 |
| *A. scrobiculata** | + | | | + | + | 60 |
| *Glomus arborense** | + | | + | + | | 60 |
| *G. cerebriforme** | | | + | + | | 40 |
| *G. fasciculatum** | | | + | + | | 40 |
| *G. invermayanum*# | | + | + | | | 40 |
| *G. pulvinatum*# | | + | + | | | 40 |
| *G. viscosum*# | | | + | | | 20 |
| *Glomus* 1* | | | | + | | 20 |
| *Paraglomus occultum** | | | + | | | 20 |
| *Rhizophagus intraradices** | + | | + | + | + | 80 |
| *Sclerocystis* 1# | + | | | | | 20 |
| *Simiglomus hoi** | | | + | + | | 40 |
| Species richness | 6 | 2 | 10 | 8 | 2 | |

*AMF species isolated from field as well as from trap culture pots, #AMF species directly isolated from field samples, [a] + Present

and entisol at Hissar from the rhizosphere of *Jatropha curcas* (Kamalvanshi et al., 2012). Fewer AMF species was isolated from mollisol at Pantnagar, which could be due to the higher level of P present in the soil. Higher soil P concentration reduces AMF spore production (de Miranda and Harris, 1994; Shukla et al., 2012a), abundance, and diversity (Bhadalung et al., 2005; Antunes et al., 2012).

### 3.2. Effect of AMF inoculations on seedling growth

*P. pinnata* showed negligible growth response towards AMF inoculations (Table 2). The growth parameters (shoot length, collar diameter, dry weights, P content of plant, and P uptake plant$^{-1}$) were comparable in different treatments. Most of the MD values were in a negative range, except in *A. scrobiculata*-inoculated plants. Thus, the results showed that *P. pinnata* seedlings did not respond to AMF inoculations at an early stage. This was not the only case; similar results have also been reported with *J. curcas* in our laboratory (results not published). Grace et al. (2009) termed such plants "non-responsive species" that exhibit lack of positive response towards AMF inoculants. Li et al. (2005) suggested that the plant's responsiveness to AMF inoculations changes with the passage of time (plant life span). Further, the total dry weight of AMF inoculated *P. pinnata* seedlings was lower at most of the places than the control, although the differences were not significant. Such growth reduction resulting from AMF inoculations are attributed to carbon loss to the fungal symbiont (Graham and Abbott, 2000). According to Jones and Smith (2004), whenever AM symbiosis results in plant growth depression the fungi can no longer be assumed as a simple root parasite. Under such conditions, the fungus acts as a "cheater" (Johnson et al., 1997). In the literature, there are several reports in which AMF inoculations decreased plant biomass but this decrease has often been found to be transient and reversed later, being followed by a positive growth response (Jones and Smith, 2004; Correa et al., 2006; Li et al., 2008). Results also suggested that the extent of growth depression or reduction in *P. pinnata* varied with inoculated AMF species, which can be related to the variations in carbon demand of different AMF species. Li et al. (2008) suggested that such differences in growth response towards different AMF inoculants are directly related to the balance between benefits and costs of the symbioses. Thus, results clearly showed that inoculated AMF species were not able to promote growth, P content, or MD of *P. pinnata* at an early stage. Therefore,

**Table 2.** Effect of arbuscular mycorrhizal fungi (AMF) inoculations on growth, mycorrhizal dependency (MD), and phosphorus (P) uptake of *Pongamia pinnata* during the early stage of development.

| AMF inoculants | Shoot length (cm) | Collar diameter (mm) | Dry weight (g) | | Total dry weight (g plant$^{-1}$) | Root: shoot | MD (%) | P concentration of host tissue (μg g$^{-1}$) | P uptake plant$^{-1}$ (mg) |
|---|---|---|---|---|---|---|---|---|---|
| | | | Shoot | Root | | | | | |
| *Acaulospora mellea* | 62.0 ± 11.9 | 12.4 ± 1.4 | 14.8 ± 5.1 | 8.0 ± 4.2 | 22.8 ± 9.2 | 0.51 ± 0.14 | -46.8 ± 70.6 | 994.62 ± 287.70 | 30.377 ± 11.105 |
| *A. scrobiculata* | 66.4 ± 2.13 | 12.6 ± 2.2 | 18.3 ± 2.4 | 11.8 ± 1.3 | 30.1 ± 3.5 | 0.65 ± 0.05 | 3.3 ± 10.5 | 1595.27 ± 528.76 | 38.493 ± 25.632 |
| *Glomus arborense* | 70.1 ± 9.6 | 11.9 ± 1.1 | 16.5 ± 1.4 | 9.2 ± 0.6 | 25.7 ± 2.0 | 0.56 ± 0.02 | –12.5 ± 8.7 | 2127.11 ± 121.45 | 54.606 ± 2.698 |
| *G. cerebriforme* | 77.4 ± 7.3 | 12.2 ± 0.8 | 18.2 ± 4.9 | 6.9 ± 1.0 | 25.1 ± 5.7 | 0.39 ± 0.06 | –19.0 ± 23.1 | 1764.86 ± 1110.72 | 45.294 ± 27.933 |
| *G. fasciculatum* | 63.6 ± 7.8 | 11.5 ± 0.9 | 14.0 ± 1.5 | 9.6 ± 2.6 | 23.6 ± 4.1 | 0.68 ± 0.14 | –25.2 ± 23.6 | 1621.02 ± 161.35 | 37.880 ± 4.705 |
| *Glomus* 1 | 65.5 ± 14.4 | 11.4 ± 0.2 | 15.8 ± 5.3 | 8.1 ± 1.7 | 23.9 ± 6.5 | 0.55 ± 0.20 | –27.2 ± 32.7 | 2097.61 ± 414.90 | 49.330 ± 12.616 |
| *Paraglomus occultum* | 66.6 ± 9.7 | 11.8 ± 0.9 | 14.2 ± 2.9 | 8.7 ± 1.6 | 22.8 ± 3.3 | 0.63 ± 0.17 | –28.4 ± 18.6 | 1221.09 ± 939.63 | 27.673 ± 20.934 |
| *Rhizophagus intraradices* | 76.0 ± 8.2 | 11.5 ± 1.7 | 16.0 ± 2.0 | 9.3 ± 1.2 | 25.2 ± 2.3 | 0.59 ± 0.12 | –15.1 ± 11.0 | 1345.51 ± 695.71 | 33.374 ± 16.640 |
| *Simiglomus hoi* | 71.9 ± 14.6 | 12.7 ± 1.1 | 16.5 ± 2.9 | 9.7 ± 2.0 | 26.2 ± 2.7 | 0.60 ± 0.19 | –11.0 ± 11.3 | 1422.753 ± 228.02 | 37.134 ± 5.821 |
| Control | 73.4 ± 18.4 | 13.3 ± 1.7 | 20.1 ± 6.5 | 8.8 ± 2.2 | 28.8 ± 7.9 | 0.46 ± 0.12 | - | 1102.76 ± 462.78 | 29.205 ± 4.918 |
| F | 0.872 | 0.873 | 1.009 | 1.550 | 0.853 | 1.760 | 0.892 | 1.752 | 1.270 |
| P value | 0.560 | 0.559 | 0.455 | 0.176 | 0.575 | 0.118 | 0.536 | 0.120 | 0.293 |
| $LSD_{0.05}$ | NS | NS | NS | NS | NS | NS | NS | NS | NS |

the colonization potential of all tested AMF species was assessed for successful mycorrhization in *P. pinnata* rhizosphere/roots in sterilized sand.

### 3.3. Colonization potential of AMF

Formations of arbuscules and vesicles were obscure in *P. pinnata* roots, while spore formation in roots was recorded in *G. arborense*-, *G. cerebriforme*-, and *Simi. hoi*-inoculated seedlings (Table 3). Occurrence of extramatrical mycelium was observed in all inoculated pots. Formation of spores on mycelium was recorded only in *G. arborense*- and *G. cerebriforme*-inoculated pots. Moderate levels of sporocarps were recorded in *G. arborense*, *G. cerebriforme*, and *Glomus* 1. Spore population was highest in *G. cerebriforme* (160/100 g sand), followed by *A. scrobiculata* (58/100 g sand). Percent root colonization was maximum in *R. intraradices* (30.6%), followed by *G. cerebriforme*

**Table 3.** Colonization potential of arbuscular mycorrhizal fungi (AMF) in rhizosphere/roots of *Pongamia pinnata* during the early stage of plant growth.

| Characteristics | MAS[1] | AMF inoculants | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Am[2] | As | Gar | Gc | Gf | G1 | Po | Ri | Simh |
| Formation of arbuscules | 1 | –[3] | – | – | – | – | – | – | – | – |
| | 2 | – | – | – | – | – | – | – | – | – |
| | 3 | + | + | – | + | + | – | + | – | – |
| Formation of vesicles | 1 | – | – | – | – | – | – | – | – | – |
| | 2 | – | – | + | + | – | – | – | + | + |
| | 3 | – | – | + | + | – | + | + | + | + |
| Extramatrical mycelium | 1 | – | + | – | – | – | – | – | – | – |
| | 2 | + | ++ | + | + | – | – | + | + | – |
| | 3 | + | +++ | ++ | + | + | + | + | + | + |
| Extramatrical mycelium with spores | 1 | – | – | – | – | – | – | – | – | – |
| | 2 | – | – | + | + | – | – | – | – | – |
| | 3 | – | – | + | + | – | – | – | – | – |
| Spores in root | 1 | – | – | – | – | – | – | – | – | – |
| | 2 | – | – | – | + | – | – | – | – | – |
| | 3 | – | – | + | – | – | – | – | – | + |
| Formation of sporocarp/loose clusters | 1 | – | – | – | – | – | – | – | – | – |
| | 2 | – | – | + | – | – | – | – | – | – |
| | 3 | – | – | ++ | ++ | – | ++ | – | – | – |
| Spore count/100 g sand | 1 | – | – | – | – | – | – | – | – | – |
| | 2 | – | – | – | – | – | – | – | – | – |
| | 3 | 6 | 58 | – | 160 | – | – | – | – | – |
| Colonization index | 1 | – | – | – | – | – | – | – | – | – |
| | 2 | 3.1 | 8.0 | 2.8 | 10.0 | – | – | – | 6.6 | 1.7 |
| | 3 | – | – | 2.5 | 20.6 | – | – | 3.1 | 30.6 | 6.0 |

[1]: Months after sowing

[2]: Am, *Acaulospora mellea*; As, *A. scrobiculata*; Gar, *Glomus arborense*; Gc, *G. cerebriforme*; Gf, *G. fasciculatum*; G1, *Glomus* 1; Po, *Paraglomus occultum*; Ri, *Rhizophagus intratradices*; Simh, *Simiglomus hoi*

[3]: – Absent; + Good; ++ Very good; +++ Excellent

**Table 4.** Response of *Pongamia pinnata* to arbuscular mycorrhizal fungi (AMF) inoculations and cotyledon removal during early stage of development.

| Treatments | AMF inoculants | | | | Mean |
|---|---|---|---|---|---|
| | *Acaulospora scrobiculata* | *Glomus cerebriforme* | *Rhizophagus intraradices* | Control | |
| Shoot length (cm) | | | | | |
| + Cotyledons | 52.8 ± 5.8 | 42.8 ± 7.3 | 41.9 ± 7.5 | 44.1 ± 3.0 | 45.4 ± 7.1 |
| – Cotyledons | 38.0 ± 4.0 | 35.5 ± 5.7 | 38.6 ± 4.0 | 33.8 ± 1.5 | 36.5 ± 4.2 |
| Mean | 45.4 ± 9.1 | 39.1 ± 7.2 | 40.3 ± 5.8 | 38.9 ± 6.0 | |
| Root length (cm) | | | | | |
| + Cotyledons | 47.3 ± 12.2 | 35.5 ± 4.4 | 36.5 ± 7.5 | 42.5 ± 7.0 | 40.4 ± 8.9 |
| – Cotyledons | 29.0 ± 5.8 | 27.8 ± 2.9 | 28.3 ± 5.2 | 23.3 ± 4.3 | 27.1 ± 4.8 |
| Mean | 38.1 ± 13.2 | 31.6 ± 5.4 | 32.4 ± 7.4 | 32.9 ± 11.6 | |
| Number of leaves | | | | | |
| + Cotyledons | 6.8 ± 1.0 | 5.5 ± 0.6 | 7.0 ± 1.2 | 5.8 ± 1.3 | 6.3 ± 1.1 |
| – Cotyledons | 5.8 ± 0.5 | 5.3 ± 1.0 | 6.8 ± 0.5 | 5.3 ± 0.5 | 5.8 ± 0.9 |
| Mean | 6.3 ± 0.9 | 5.4 ± 0.7 | 6.9 ± 0.8 | 5.5 ± 0.9 | |
| Total fresh weight (g) | | | | | |
| + Cotyledons | 52.9 ± 6.0 | 45.5 ± 9.2 | 48.9 ± 6.6 | 40.1 ± 5.1 | 46.9 ± 7.8 |
| – Cotyledons | 26.9 ± 2.6 | 18.6 ± 1.1 | 19.7 ± 1.4 | 11.8 ± 2.6 | 19.3 ± 7.2 |
| Mean | 39.9 ± 14.5 | 32.1 ± 15.6 | 34.3 ± 21.1 | 26.0 ± 15.6 | |
| Total dry weight (g) | | | | | |
| + Cotyledons | 18.1 ± 1.6 | 16.6 ± 4.6 | 14.2 ± 1.9 | 14.9 ± 3.4 | 15.9 ± 3.2 |
| – Cotyledons | 15.1 ± 3.2 | 9.1 ± 0.6 | 10.6 ± 2.9 | 6.3 ± 2.0 | 10.3 ± 3.9 |
| Mean | 16.6 ± 2.8 | 12.9 ± 5.0 | 12.4 ± 3.0 | 10.6 ± 5.3 | |
| Mycorrhizal dependency (%) | | | | | |
| + Cotyledons | 17.32 ± 7.49 | 3.72 ± 32.54 | –6.16 ± 14.11 | - | 4.96 ± 21.43 |
| – Cotyledons | 57.24 ± 8.16 | 30.92 ± 4.60 | 38.04 ± 14.32 | - | 42.07 ± 14.65 |
| Mean | 37.28 ± 22.54 | 17.32 ± 25.96 | 15.94 ± 27.04 | - | |

| | Treatment | | | AMF | | | Treatment × AMF | | |
|---|---|---|---|---|---|---|---|---|---|
| | F | P value | $LSD_{0.05}$ | F | P value | $LSD_{0.05}$ | F | P value | $LSD_{0.05}$ |
| Shoot length | 23.187 | <0.001 | 1.3 | 2.675 | 0.070 | NS | 1.731 | 0.187 | NS |
| Root length | 31.671 | <0.001 | 4.9 | 1.553 | 0.227 | NS | 1.714 | 0.191 | NS |
| Number of leaves | 2.743 | 0.111 | NS | 5.371 | 0.006 | 0.9 | 0.343 | 0.795 | NS |
| Total fresh weight | 275.081 | <0.001 | 3.7 | 11.055 | <0.001 | 5.3 | 2.627 | 0.073 | NS |
| Total dry weight | 33.347 | <0.001 | 2.00 | 6.687 | 0.002 | 2.9 | 2.041 | 0.135 | NS |
| Mycorrhizal dependency | 30.850 | <0.001 | 12.15 | 4.262 | 0.031 | 17.19 | 0.584 | 0.568 | NS |

+ Cotyledons, with cotyledons; – Cotyledons, without cotyledons

(20.6%). Thus, the results clearly suggested that, among the tested AMF inoculants, *A. scrobiculata*, *G. cerebriforme*, and *R. intraradices* colonized the rhizosphere of *P. pinnata* more efficiently. Helgason et al. (2002) suggested that some AMF species can colonize the same host plant more efficiently than others. As per the results, the level of mycorrhization was low during the early stage, which could be attributed to the problems associated with plant–fungus recognition (Ivanov et al., 2010). Recently, we examined the mycorrhization level of some important regional plants (*Azadirachta indica*, *Dalbergia sissoo, Dendrocalamus strictus*, *J. curcas*, *Leucaena leucocephala, Madhuca latifolia*, and *P. pinnata*), which suggested that it was minimum in *P. pinnata* (Jha et al., 2011). This could be due to the large seeds of *P. pinnata*. Jin et al. (2009) pointed out that plants with large seeds generally exhibit lower AMF densities. Furthermore, *P. pinnata* seedlings did not produce fine roots up to 30 days after sowing; it only formed relatively thick roots, which were not colonized by AMF. This can also be explained on the basis of the large amounts of nutrients stored in cotyledons of *P. pinnata* seeds. It was presumed that in the presence of stored food materials the growing seedlings did not require fine roots during the early stage of development. Our results were consistent with those reported by Gehring and Connell (2006). They suggested that plants with large seeds did not need symbiotic association with AMF, because in the early growth stage they utilize their photosynthates for growth of their own tissues. According to Allsopp and Stock (1995), the seedlings germinated from large seeds are able to establish themselves independently of mycorrhizae. Hence, plants delayed the development of fine roots, and thus their colonization was also delayed.

Therefore, on the basis of the results obtained from inoculation of purified AMF in the above experiments, the present hypothesis, we were working for, was rejected. Cuenca et al. (1990) suggested that cotyledons and their persistence after germination prevent plants from becoming dependent on roots for nutrients and water, and cause plants to respond to a lesser degree to AMF. This prompted us to assess the effect of removal of food materials/metabolic reserves through cotyledon excision on growth and dependency of *P. pinnata* on better performing AMF inoculants.

### 3.4. Effect of cotyledon removal on growth and mycorrhizal dependency

Removal of cotyledons immediately after germination from *P. pinnata* seeds significantly reduced most of the studied parameters, like shoot length, root length, and total dry weight of seedlings after 3 months (Table 4). Our results were consistent with the findings of Kitajima (2003) and Jin et al. (2009). Seedling growth reductions as a consequence of removal of cotyledons have been reported in soybean (Chin et al., 1977) and *Quercus robur* L. (Garcia-Cebrian et al., 2003). According to Ba et al. (1994), the removal of cotyledons not only reduces plant growth but also decreases the concentration of soluble carbohydrate in plant roots. On the other hand, AMF inoculations significantly ($P < 0.05$) increased the number of leaves, total fresh weight, and dry weights of the seedlings. Mycorrhizal dependency was significantly increased by the removal of cotyledons. Furthermore, among the tested inoculants, *A. scrobiculata* showed greater ability and gave maximum MD value, which was significantly higher than that of the other 2 inoculants. This was probably due to the increase in the soil volume explored for nutrients and water uptake by *A. scrobiculata* as compared to the other inoculants. Muthukumar and Udaiyan (2000) suggested that plants with large seeds generally exhibit less dependency on AMF, because such seeds offer large amounts of metabolic reserve to the growing seedlings. However, as plant growth proceeds, the metabolic reserve of the seed declines rapidly and under these conditions plants becomes dependent upon AMF for their survival and establishment.

Thus, in conclusion it can be stated that AMF inoculations should enhance the biomass productivity of *P. pinnata* only after depletion of the metabolic reserves in its cotyledons and preconditioning of *P. pinnata* seedlings with efficient AMF could be utilized for biofuel plantation on wastelands.

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