Journal of Environmental Science and Management 16(2): 63-76 (December 2013)
ISSN 0119-1144

Survival, Growth and Cu Accumulation by Non-Mycorrhizal and Mycorrhizal *Jatropha curcas* L. Seedlings or Cuttings in a Grassland and in Mine Tailing Soils

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ABSTRACT

Jatropha curcas has been identified as an alternate source for biofuel, and thus requires immediate establishment of plantations in areas not utilized for food production such as in mine tailings sites. Screenhouse experiments were conducted to determine the survival, growth and copper (Cu) accumulation of non-mycorrhizal or mycorrhizal J. curcas seedlings or cuttings grown in oven sterilized grassland soil from Caliraya, Laguna and in mine tailing soils from Paracale, Camarines Norte and Mogpog, Marinduque. Grassland soil was sterilized in an oven for three days at 100˚C prior to use. Seedlings or cuttings were either uninoculated or inoculated with Mykovam or MineVAM mycorrhizal inoculants. Results show that Paracale soil supported the highest survival and best growth of seedlings or cuttings. In Mogpog soil, all seedlings died before two months except those inoculated with Mykovam while all cuttings died on the third month. Non-mycorrhizal seedlings did not survive in Caliraya soil while mycorrhizal inoculation increased seedling survival and growth. Cuttings did not respond to inoculation, probably due to low mycorrhizal infection (≤14 %) as compared to seedlings (≤100 %). Mykovam promoted higher survival and better plant growth than MineVAM because of its higher root colonization. Cu accumulation was higher in the roots of mycorrhizal plants and the lowest was in the leaves especially in the Mykovam-inoculated plants. In conclusion, Mykovam inoculated J. curcas grew better with higher survival rate than the control thus this imply that rehabilitation of grasslands in Caliraya, Laguna and mine tailing areas in Paracale, Camarines Norte can be done for its potential for biofuel production. However, field trials should be conducted.

Key words: *arbuscular mycorrhizal fungi, nutrient deficient soil, heavy metal accumulation*

INTRODUCTION

Jatropha curcas has been identified as an alternate source for biofuel, and thus require immediate establishment of plantations (*Ndong et al. 2009, Nahar and Sunny 2011*). Targeted areas are the unproductive grasslands and the mine tailing areas. Mine tailings are wastes from mining activities containing considerable amount of heavy metals. For example, the mine tailings in Mogpog, Marinduque, the top (in terms of content in the soil) four heavy metals present are copper (Cu), cadmium (Cd), lead (Pb) and zinc (Zn) (*Raymundo et al. 2006*), thus, these area are normally devoid of plants. The ability of *J. curcas* to tolerate such extreme environment is a basis for its potential as an agent in alleviating heavy metal pollution in mine areas (*Patil 1998*).

Jatropa curcas is a shrub that belongs to the family Euphorbiaceae which has 300 genera and around 7,500 species (*Charoenpakdee et al. 2010*). It is grown in many parts of the world, for example, in Brazil, India, Mexico, Nicaragua and Thailand, mainly because of its multiple potentials for medicinal and industrial purposes such as biodiesel (*Foildl et al. 1996, Heller 1996, David, Joerg and Alberte 2009*). The oil extracted from the seeds is now being formulated in the Philippines as a potential pesticide, molluscide, illuminant, and most importantly, as a safer diesel fuel substitute (*Solsoloy and Duldulao 2004*).

The hulls of its seed are pressed to form a cake believed to be a valuable source of organic manure (*Heller 1996*). Local people are known to utilize the remaining vegetative parts of this plant, such as its roots, barks, and leaves as medicines for a wide range of ailments (*Quisumbing 1978*).

With all these benefits that can be derived from the different parts of *J. curcas*, the determination of the extent by which heavy metals such as Cu, Fe, Zn and Mn accumulation within the plant will greatly aid in the assessment of the quality and safety of these products. The establishment of *J. curcas* plantations will also produce an alternate source for biodiesel and restore the productivity of marginal grasslands and abandoned mine tailing sites. Unfortunately, there are no protocols available yet for *J. curcas* for a successful plantation establishment on grasslands and much more in mine tailing soil.

Grasslands in Caliraya, Laguna contain very low concentration of essential nutrients but with high concentration of iron (Fe) (*Aggangan et al. 2012*). Being very acidic, with pH ranging from 4.6 to 5.1 (*Orig 2004*), growth of plants is stunted and exhibit nutrient deficiency symptoms (e.g. purple leaves indicating phosphorus deficiency) and toxicities (whole plant in yellow color which indicates either

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nitrogen deficiency or Fe toxicity) (*Marschner 1995*). On the other hand, mine tailing areas are piled up wastes from mining activities. These areas are devoid of plants because of high concentration of heavy metals (*Raymundo et al. 2006*). Heavy metal contamination in soils is one of the major environmental problems in many parts of the world (*Gremion et al. 2004*) and are due to anthropogenic activities such as mining and smelting of metalliferous ores (*Garbisu and Alkorta 2003*). Accumulation of heavy metals such as Cu resulting from these activities, exerts toxic effects to plants rendering grasslands and mine areas infertile and uninhabitable. Moreover, these sites are exposed to excessive drought and high temperature aside from the high concentration of soluble heavy metals that inhibit normal growth of plants.

Through the years, there have been increased efforts in rehabilitating these areas. However, most conventional rehabilitation methods are either extremely costly or provide only short-term solutions. This scenario has lead researchers to look for safer and cheaper technologies for ecological rehabilitation. *J. curcas* are naturally associated with arbuscular mycorrhiza (AM) fungi. *J. curcas* plantations were found to be associated with mycorrhizal species belonging to the genera *Acaulospora*, *Entrophosphora*, *Gigaspora*, *Glomus* and *Scutellospora* (*Charoenpakdee et al. 2010*). AM fungi colonized roots of *J. curcas* in soils with varying pH from acidic to calcareous, low to moderate organic matter and low to high available P (*Charoenpakdee et al. 2010*). AM fungi are integral functioning part of plant roots and are widely recognized as enhancing plant growth on severely disturbed sites. AM fungi prevent root infection by pathogens, increase plant tolerance to drought and play an important role in metal tolerance and accumulation in heavy metal contaminated soils (*Yucel 1997, Gaur and Adholeya 2004*). Thus, this study was conducted to determine the survival, growth and Cu accumulation of non-mycorrhizal and mycorrhizal associated *J. curcas* seedlings or cuttings grown in soils collected from grasslands in Caliraya, Laguna and in mine tailing areas in Paracale, Camarines Norte and Mogpog, Marinduque.

METHODOLOGY

Experimental design

Two concurrent experiments were conducted (one for seedlings and one for cuttings) following a two factor in Randomized Complete Block Design with three blocks and with 12 subsamples per block. For Factor A, three soils, i.e. grassland soil from Caliraya, Laguna and mine tailing soils from Paracale, Camarines Norte and Mogpog, Marinduque, were used as the growing media. For Factor B, inoculation treatments included those plants with Mykovam™ or MineVAM. As control, uninoculated plants were used resulting, a nine treatment combinations. The experiments were conducted in a screenhouse of the Mycorrhiza Laboratory, National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB), College, Laguna.

Description of soil collection sites

Soils were collected in a grassland in Caliraya, Lumban, Laguna and in mine tailing areas in Paracale, Camarines Norte and Mogpog, Marinduque (**Figure 1**). Vegetation in the grassland soil in Caliraya, Laguna is dominated by cogon [*Imperata cylindrica* (L.) Raeuschel], the soil is therefore very acidic and deficient in essential nutrients. Such soil conditions were reported to be very responsive to inoculation with mycorrhizal fungi (*Aggangan 1996, White, Tallaksen and Charat 2008*).

Soils from the Paracale mine tailing areas are dominated by a mixture of cogon (*I. cylindrical*) and talahib (*Saccharum spontaneoum* L.) while soils in Mogpog, Marinduque were devoid of plants, but the neighboring areas within the mine tailings have few patches of talahib and Acacia auriculiformis of about five years old.

Collection and preparation of soil

Bulk soil (0-15 cm depth) collected from the study sites were brought to BIOTECH, UPLB, air-dried for one week, pulverized and sieved through 0.5 cm-pore diameter screen wire to remove undecomposed organic materials and stones, and finally oven-dried for 3 d at 100˚C. This was done to reduce growth-limiting pathogens and other microorganisms present in the soil. Two hundred fifty grams of dry soil were then dispensed into each plastic bag (8.8 cm x 17.6 cm) with two holes at the bottom for drainage.

Physico-chemical analyses of the soils were done at the Bureau of Soils and Water Management in Diliman, Quezon City. Nitrogen was analyzed using Modified Kjeldahl Method (*Black 1965*) while available P and exchangeable K using Bray No. 2 (*Dewis and Freitas 1970*). Analysis of Cu in the soils was done at the Philippine Institute of Pure and Applied Chemistry (PIPAC) using atomic absorption spectrophotometry.

The soil from Caliraya is red with clay texture, Paracale soil is yellow with silt loam texture and Mogpog is white with silt loam texture (**Table 1**). The pH of the three soils ranged from very acidic (pH 4.4, Caliraya soil), slightly acidic (pH 5.4, Paracale soil) to acidic (pH 5.0, Mogpog soil). All the three soils have normal CEC concentration but the N, P and Ca were low. Mg concentrations in Caliraya and Mogpog soils are low while that of Paracale has medium Mg level. Cu concentrations of Caliraya, Paracale and Mogpog are 100, 45 and 160 $\mu g\ g^{-1}$, respectively, which are beyond

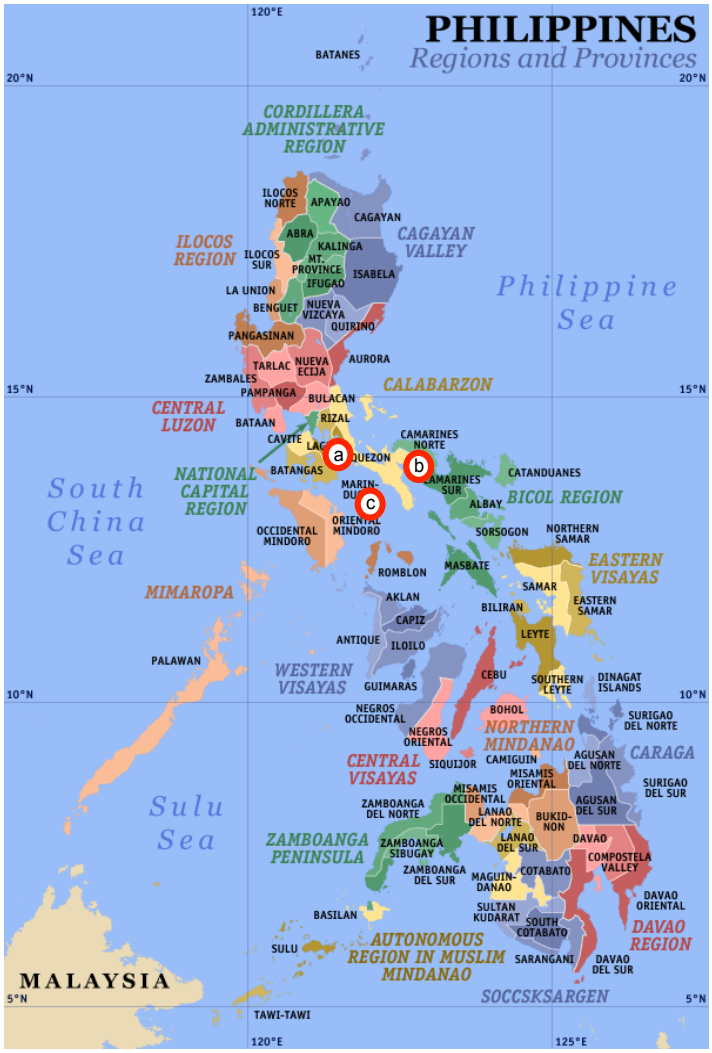

Figure 1. Map showing the geographic location (red circles) of Caliraya, Lumban, Laguna (a) Paracale, Camarines Norte (b) and Mogpog, Marinduque (c), in the island of Luzon where grassland (a) and mine tailing soils (b and c), respectively, were collected. (Source: www.mapcentral.ph)

the allowable limits of Cu: 30 µg g^{-1} to 36 µg Cu g^{-1} by the Dutch standard for soil contamination (*Ministry of Housing 1994*).

Collection, cultivation and mycorrhizal inoculation of *J. curcas* seeds and cuttings

Mature seeds were collected from a five-year old *J. curcas* and were de-husked and air dried for one week. Prior to sowing, the seeds were surface sterilized with 10 % chlorox, rinsed several times with sterile water, and sown in individual polybags. Stem cuttings (2.2 cm stem diameter x 12 cm length) were taken one foot away from the shoot tip.

Two seeds were then directly planted in black (18 cm x 22 cm) polyethylene bag filled with either two kilo grassland or mine soils (36 bags per soil sample). The soil samples were watered to field capacity (20 mL) with tap water. Thinning into one seedling per bag was done one month after seed sowing (ASS). Cuttings (1.5-2.0 cm diameter and 13 cm in length) were planted in plastic pots filled with either grassland or mine tailing soils (two kg per pot). Pots were watered at filled capacity (200 mL). The soil was oven sterilized for three days at 100˚C.

Five grams of Mykovam™ or MineVAM was placed in one-inch deep holes at the center of soil filled polybags during transplanting or seeding. Five grams of the inoculant consist of 50 to 100 spores of mycorrhizal fungi, the recommended inoculum potential per plant. The seeds were placed directly above the mycorrhizal inoculants. This mycorrhizal inoculum potential (50-100 spores per plant) was verified by isolating the spores from the soil following the wet sieving and decanting technique (*Giovanetti and Mosse 1986*).

Table 1. Physico-chemical characteristics of soil used in this study and reference levels as to the interpretation of soil-nutrient concentration of surface soil samples.

Soil characteristics	Caliraya grassland soil	Paracale mine tailing soil	Mogpog mine tailing soil	Reference level		
				Normal range	Low	Medium
pH (1:1, H_2O)	4.4 (Very acidic)	5.4 (Slightly Acidic)	5.0 (Acidic)			
Nitrogen (%)	0.07 (Low)	0.13 (Low)	0.14 (Low)	2.5-3.5	<1.8	1.8-5.50
Available P (ppm)	8.8 (Low)	10.27 (Low)	10.57 (Low)	36-50	<26	26-50
K (cmol(P+)/100 g soil)	0.32 (Medium)	0.38 (Medium)	0.41 (Medium)	0.3-0.5	<0.3	0.3-0.6
Ca (cmol(P+)/100 g soil)	3.26 (Low)	3.59 (Low)	3.87 (Low)	3-20	<5	5-10
Mg (cmol(P+)/100 g soil)	0.09 (Low)	1.10 (Medium)	0.09 (Low)	0.5-4	<0.5	0.5-1.5
CEC conc(cmol(P+)/100 g soil)	22.17 (Normal)	20.57 (Normal)	11.22 (Normal)			
CEC Clay and clay loam (cmol(P+)/100 g soil)				20-50		
CEC Loam and silt loam (cmol(P+)/100 g soil)				10-25		
Cu concentration (µg g^{-1})	100	45	160			
Color	Red	Yellow	Brown			
Texture	clay	Silt loam	Silt loam			

Care and maintenance

Each plant received 5 mL ¼ strength Hoagland's solution (*Bonner and Galston 1952*) once a week, beginning 7 weeks after seed sowing in the case of seed or 7 weeks after transplanting in the case of cuttings until the end of three months. Plants were watered when needed. Plants were re-randomized once a week in order to avoid a possible lack of homogeneity of light in the screenhouse that may alter plant responses to the treatments.

Parameters gathered

a. **Plant height and stem diameter**. Height and stem diameter of seedlings were monitored once a month after seed sowing (ASS) while height and diameter of cuttings were measured once a month after transplanting (AT) for 4 months. Plant height (using a ruler) was measured once inch above the soil surface up to the tip of the main stem. Stem diameter was measured once inch above the soil surface using a digital vernier caliper. For cuttings, height was measured from the base to the tip of the sprout and stem diameter was taken one inch above the base of the sprout.
b. **Number of leaves and leaf area**. The number of leaves per plant was counted, detached and the leaf area was measured using an Automatic leaf area meter (Hayashi Denkoh, Japan Model AAM 7) available at the Institute of Plant Breeding (IPB), UPLB, College, Laguna.
c. **Root length**. Root length was measured upon harvest from the root collar to the tip of the root. Only the longest root of each plant was measured.
d. **Plant dry weight**. Plants were harvested after four months. The above portion (stem and leaves) were cut one inch above the soil surface, washed in running water to remove soil or dirt, air dried, wrapped separately in paper towels, placed inside brown paper bags, and oven dried at 70˚C for three days. The roots were carefully separated from the soil under running water. Fine (≤0.02 mm diameter) roots were separated from the coarse roots by macerating the roots by hand in water and the detached fine roots were collected in a wire mesh screen (325 µm). Fine roots were blot dried to remove excess water then samples (0.2 g) were taken for the assessment of mycorrhizal root colonization. The fine and coarse roots were separately wrapped with paper towels, placed in paper bags and also oven dried similar to that of the stem and leaves. Dry weights were taken using an analytical balance. For cuttings, only the roots and the sprouts were considered for biomass.
e. **Mycorrhizal root colonization**. Assessment of root colonization by Mykovam and MineVAM was done at harvest. Fine roots subsample (0.1 g fresh weight) was cut into 2-3 mm lengths and fixed in 50 % ethanol. The roots were placed in individual test tubes with 10 % KOH (w/w), cleared in a water bath at 75˚C, stained with 0.05 % trypan blue in lactic acid: glycerol:water (1:1:1) solution, and finally destained with lactic acid:glycerol:water solution (*Brundrett et al. 1996*). Heavily pigmented roots were bleached with 3 % hydrogen peroxide and 2 % hydrogen chloride solution for 5 min before staining. The rate of root colonization was evaluated by the gridline intersect method (*Giovanetti and Mosse 1986*). Root colonization was assessed by counting all roots that crossed the grid lines (15 lines corresponding to field views under the stereomicroscope) and infection was scored on stained blue mycelia, vesicles or arbuscules inside the roots. Roots with attached mycelia were also considered as mycorrhiza infected roots. Mycorrhizal root colonization was computed as: total roots that intersected the grid lines minus the total number of mycorrhiza infected roots that intersect the grid lines over the total roots counted and the dividend multiplied by 100 (*Giovanetti and Mosse 1986*).
f. **Cu concentration and uptake**. Plants grown in Caliraya and Paracale soils were ground separately in a Wiley Mill at the Mycorrhiza Laboratory of BIOTECH and were sent to the Bureau of Soils and Water Management, Diliman, Quezon City. Cu concentration was analyzed in an atomic absorption spectrophotometer (AAS) and Cu uptake was computed as the product of Cu concentration and plant dry weight.

Plant parts of *Acacia auriculiformis*, *Leucaena leucocephala* and *J. curcas* growing in the mine tailing collection site in Mogpog, Marinduque were also collected and analyzed for Cu concentration to compare the Cu concentrations obtained in this study.

Statistical analyses

All data collected were analyzed using analysis of variance (ANOVA) of a two factor in RCBD and means were compared using DMRT at $p<0.05$ if ANOVA showed significant effects (*Duncan 1955*). Percent mycorrhizal root data were arcsine transformed (*Gomez and Gomez 1984*) before subjecting to ANOVA analysis. Statistical analyses were done using MSTAT-C statistical computer program (*Michigan State University 1989*).

RESULTS

Survival, growth response and Cu accumulation in seedlings

Survival of J. curcas seedlings

In Caliraya soil, no seedlings emerged one month after sowing (**Figure 2a**). At two months, 30 % seedling survival was obtained but all seedlings died on the third month. Those inoculated with Mykovam exhibited 100 % survival. In Paracale soil, all mycorrhizal seedlings survived from the first to the fourth month, while 90 % survival was obtained from the non-mycorrhizal counterpart from

the second to fourth month (**Figure 2b**). In Mogpog soil, however, all seedlings died four months after planting (**Figure 2c**). Those inoculated with Mykovam survived until the third month while the other treatments survived until the second month only. Moreover, in Mogpog soil, seedling emergence in the unbinoculated treatment occurred one month after seed sowing. Thus, the subsequent measurements were done only on plants grown in Caliraya and Paracale soils because of the death of seedlings grown in Mogpog soil.

Periodic height and stem diameter of J. curcas seedlings

Monthly height growth of seedlings was highly significantly affected by the interaction between soil type and mycorrhiza inoculation (**Table 2**). In Caliraya soil, Mykovam inoculated seedlings consistently were taller than those inoculated with MineVAM throughout the four months period. In Paracale soil, both Mykovam and MineVAM comparatively promoted the tallest height throughout the four months duration of the experiments. Height growth due to mycorrhizal inoculation was higher in Paracale soil than in Caliraya soil. The uninoculated seedlings had the shortest height.

Stem diameter of seedlings grown in Caliraya soil was generally smaller than in Paracale soil (**Table 3**). In Paracale soil, plants inoculated with Mykovam and MineVAM gave similar stem diameter from the second to the fourth month experimental period. In Caliraya soil, Mykovam was more effective in promoting larger stem diameter than by MineVAM. The uninoculated seedlings had the smallest stem diameter.

Leaf and root characteristics in J. curcas seedlings

Leaf and root characteristics of *J. curcas* seedlings were generally highly significantly smaller in Caliraya than in Paracale soil (**Table 4**). Mykovam and MineVAM comparatively promoted the highest number of leaves, largest leaf area and longest root length of *J. curcas* seedlings grown in Paracale soil. In Caliraya soil, mycorrhizal inoculants promoted more leaf number (2 leaves per plant), larger leaf area (12 cm^2) and longer (11 cm) root length of *J. curcas* seedlings than the uninoculated counterpart. All the leaves of the uninoculated seedlings defoliated at four months after seed sowing. Moreover, uninoculated cuttings exhibited significantly higher mean leaf area and higher root growths

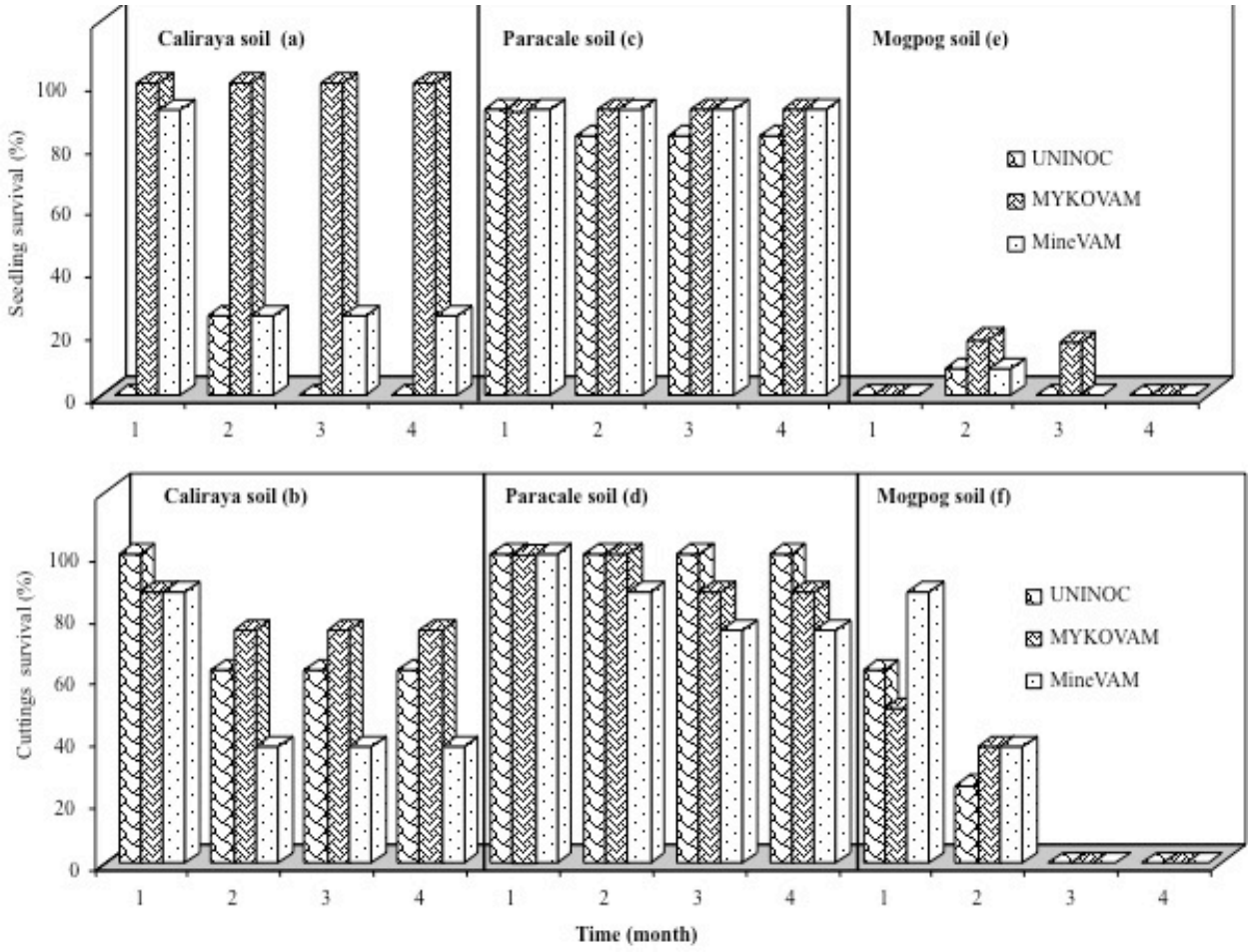

Figure 2. Periodic survival rate of non-mycorrhizal and mycorrhizal J. curcas seedling (a, c and e) and cuttings (b, d and f) grown in grassland soil from Caliraya , Laguna (a and b), mine tailing soils from Paracale, Camarines Norte (c and d) and from Mogpog, Marinduque (e and f).

Table 2. Interaction effect of soil type and mycorrhiza inoculation treatment on monthly height of *J. curcas* seedlings grown in Caliraya grassland soil and Paracale mine tailing soil and inoculated with mycorrhizal fungi.

Soil type	Inoculation treatment	1-mo ASS	2-mo ASS	3-mo ASS	4-mo ASS
Caliraya	Uninoculated	0 d***	5.00 d***	0 d***	0 d***
	Mykovam	15.35 b	15.94 b	16.21 b	16.61 b
	MineVAM	8.76 c	13.67 c	14.20 c	14.40 c
Paracale	Uninoculated	14.92 b	16.82 b	17.31 b	17.63 b
	Mykovam	17.03 a	21.37 a	21.80 a	22.01 a
	MineVAM	18.88 a	22.23 a	22.70 a	22.81 a

ASS = After seed sowing
ns=Not significant
***= Very highly significant at $p<0.001$
In each column, treatment means with the same letters are not significantly different from each other using DMRT at $p<0.05$.

Table 3. Interaction effect of soil type and mycorrhiza inoculation treatment on the monthly stem diameter of *J. curcas* seedlings grown in Caliraya grassland soil and Paracale mine tailing soil and inoculated with mycorrhizal fungi.

Soil type	Inoculation treatment	1-mo ASS	3-mo ASS	4-mo ASS
Caliraya	Uninoculated	0.27 d***	0 d***	0 d***
	Mykovam	0.43 b	0.46 b	0.51 b
	MineVAM	0.37 c	0.35 c	0.44 c
Paracale	Uninoculated	0.55 b	0.65 b	0.71 b
	Mykovam	0.60 a	0.71 a	0.80 a
	MineVAM	0.56 a	0.69 a	0.77 a

ASS = After seed sowing
ns=Not significant
***=Very highly significant at $p<0.001$
In each column, treatment means with the same letters are not significantly different from each other using DMRT at $p<0.05$.

Table 4. Interaction effect of soil type and mycorrhiza inoculation treatment on leaf and root characteristics of *J. curcas* seedlings grown in Caliraya grassland soil and Paracale mine tailing soil and inoculated with mycorrhizal fungi.

Soil type	Inoculation treatment	No. of leaves per plant	Leaf area (cm^2)	Root length (cm)
Caliraya	Uninoculated	0 d***	0 d***	5.863 d***
	Mykovam	2.33 bc	12.05 c	10.63 c
	MineVAM	1.67 c	11.95 c	11.00 bc
Paracale	Uninoculated	2.58 b	16.45 b	11.74 bc
	Mykovam	3.86 a	25.80 a	13.39 a
	MineVAM	4.13 a	26.91 a	12.25 ab

***= Significant at $p<0.001$.
In each column, treatment means with the same letters are not significantly different from each other using DMRT at $p<0.05$.

than inoculated ones.

Plant dry weight in J. curcas seedlings

Root, leaf, stem and total dry weights of *J. curcas* seedlings were heavier in Paracale than in Caliraya soil (**Table 5**). Mycorrhizal seedlings were heavier than the non-mycorrhizal counterpart. Mykovam promoted ($p<0.001$) the highest (0.41 g plant^{-1}) root dry weight while MineVAM promoted ($p<0.001$) the heaviest (0.30 g plant^{-1}) leaf dry weight. The highest stem and total plant dry weight was obtained by inoculating seedlings with MineVAM or Mykovam and grown in Paracale soil. In Caliraya soil, the two mycorrhizal inoculants comparatively promoted heavier roots, leaves, stem and consequently total dry weight than the uninoculated ones.

Mycorrhizal root colonization in seedlings

Root colonization in *J. curcas* seedlings ranged between 5 % and 100 % (**Table 10**). Percentage root colonization was higher in seedlings grown in Paracale soil (5 – 100 %) as compared to those grown in Caliraya soil (0 – 32 %). Moreover, seedlings inoculated with Mykovam exhibited the highest (32 % in Caliraya soil and 100 % in Paracale soil) percentage mycorrhizal root colonization. The uninoculated plants became mycorrhizal but the level of colonization was very low (0 and 5 % in Caliraya and Paracale soils, respectively).

Cu concentration in plant parts of J. curcas seedlings

Generally, mycorrhizal plants generally took more Cu

Table 5. Interaction effect of soil type and mycorrhiza inoculation treatment on dry weight of *J. curcas* seedlings grown in Caliraya grassland soil and Paracale mine tailing soil and inoculated with mycorrhizal fungi.

Soil type	Inoculation treatment	Root dry weight	Leaf dry weight	Stem dry weight	Total dry weight
		g plant^{-1}			
Caliraya	Uninoculated	0.02 e***	0 c***	0.03 d*	0.05 c**
	Mykovam	0.12d	0.04 c	0.24 c	0.40 b
	MineVAM	0.17 cd	0.02 c	0.14 cd	0.33 b
Paracale	Uninoculated	0.22 c	0.12 b	0.03 d*	1.03 ab
	Mykovam	0.41 a	0.14 b	0.24 c	1.54 a
	MineVAM	0.33 b	0.21 a	0.14 cd	1.65 a

*, **, ***= Significant at $p<0.05$, $p<0.01$ and $p<0.001$, respectively.
In each column, treatment means with the same letters are not significantly different from each other using DMRT at $p<0.05$.

from the soil than non-mycorrhizal ones Cu concentration in mycorrhizal seedlings grown in Caliraya soil ranged from 20 to 29 µg g^{-1} (**Figure 3a**). The highest ($p<0.001$) concentration was obtained from the roots of Mykovam-inoculated seedlings which was followed by those in the roots of seedlings inoculated with MineVAM (23 µg g^{-1}). The lowest Cu concentration was in the stem. In Paracale soil, Cu accumulation by mycorrhizal seedlings ranged from 12 to 28 µg g^{-1} as compared to 9 to 16 µg g^{-1} in non-mycorrhizal counterpart (**Figure 3b**). The roots retained more ($p<0.001$; 18 µg g^{-1} in the uninoculated, 23 µg g^{-1} in Mykovam-inoculated an d 20 µg g^{-1} in the MineVAM-inoculated seedlings) Cu than in the leaves (13 µg g^{-1} in the uninoculated, 27 µg g^{-1} in Mykovam-inoculated and 18 µg g^{-1} in the MineVAM-inoculated seedlings) and the lowest was obtained in the stem (9 µg g^{-1} in the uninoculated, 14 µg g^{-1} in Mykovam-inoculated and 12 µg g^{-1} in the MineVAM-inoculated seedlings). Cu accumulation in plants grown in Mogpog soil was not determined because, all the plants died after the third month. Moreover, plant samples from those that survived at two and three months were not enough for Cu analysis thus, Cu accumulation was measured only in plants grown in Caliraya and Paracale soils.

Survival, growth response and Cu accumulation in cuttings

Survival of J. curcas cuttings

Survival rate of cuttings was generally lower (40-100 %) in Caliraya soil (**Figure 2d**) than in Paracale soil (80-100 % (**Figure 2e**) and the lowest (20-80 %) was in Mogpog soil (**Figure 2d-f**). In Caliraya soil, the uninoculated cuttings had a 100% survival rate during the first month while the

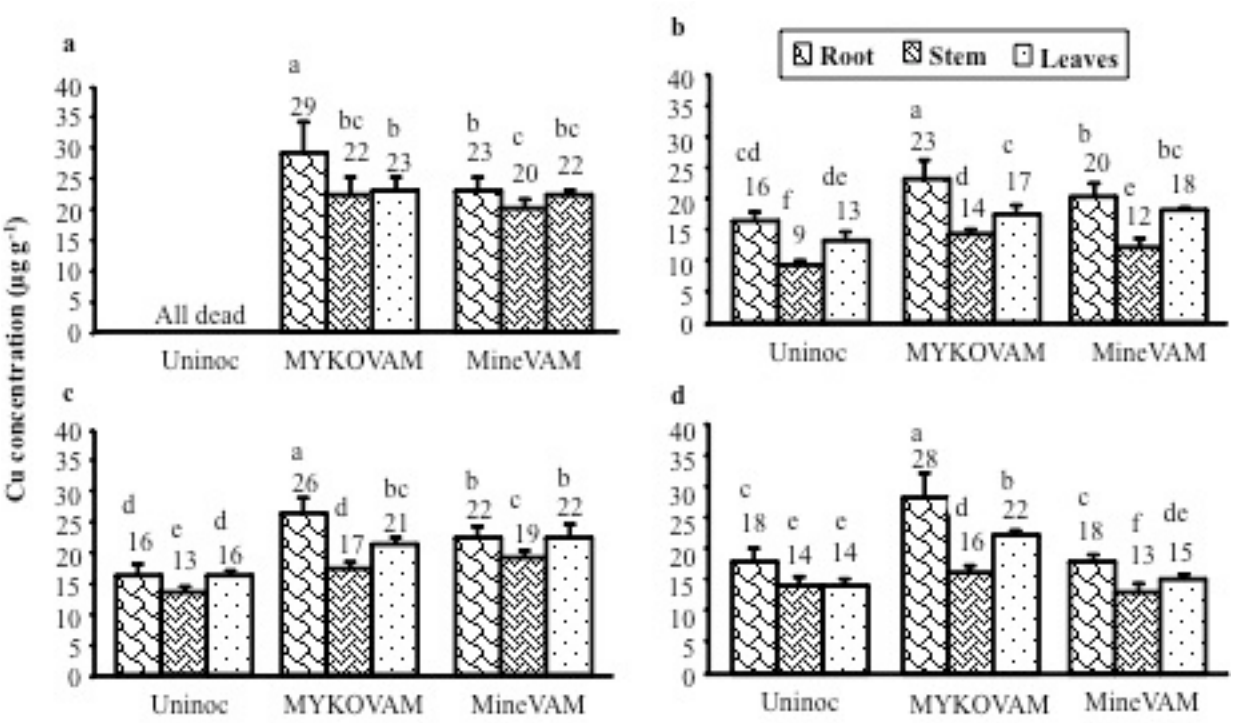

Figure 3. Cu concentration in the roots, stem and leaves of four-mo old *J. curcas* seedlings (a and b) and cuttings (c and d) grown in Caliraya (a and c) and in Paracale (b and d) mine tailing soils. Bars (±SE) with the same letters within the same plant part at $p<0.05$. n=4.

mycorrhizal counterpart had 90 % only. Survival rate of uninoculated cuttings dropped from 100 % during the first month to 60 % on the second month which was maintained until fourth month. Survival rate of Mykovam and MineVAM inoculated cuttings also declined from 90 % to 80% for the former and from 90 % to 40 % for the latter (**Figure 2d**) which was even lower than the survival rate (60 %) of the uninoculated cuttings. In Paracale soil, all the uninoculated and mycorrhizal cuttings survived during the first month (**Figure 2e**). On the second month onward, only the uninoculated treatment gave 100 % survival rate. At two months, all Mykovam inoculated cuttings survived but on the third month, survival rate declined to 90 %. Survival rate of MineVAM was 90 % on the second month and 80 % on the third and fourth month. In Mogpog mine soil, no cuttings survived three months after planting (**Figure 2f**).

Height and stem diameter of J. curcas cuttings

Height growth of cuttings at one to three months was not affected by soil type nor mycorrhizal inoculation and their interaction (**Table 6**). Significant interaction effect was observed only at fourth month where MineVAM inoculation gave significantly taller (10.36 cm, $p<0.05$) than the Mykovam inoculated cuttings (8.61 cm) grown in Paracale soil. In Caliraya soil, mycorrhizal inoculation did not affect height growth of *J. curcas* cuttings.

Stem diameter of cuttings was significantly affected at three and four months after transplanting (**Table 7**). In Paracale soil, MineVAM promoted bigger ($p<0.05$) stem diameter than by Mykovam when grown in Paracale soil. Stem diameter of Mykovam inoculated cuttings was comparable with the uninoculated counterpart at three and four months after transplanting. In Caliraya soil, stem diameter of mycorrhizal cuttings did not significantly differ with that of uninoculated counterpart.

Leaf and root characteristics in J. curcas cuttings

Mykovam and MineVAM significantly increased the number of leaves (4.6 and 4.0 pieces per plant, respectively) of cuttings grown in Caliraya soil which promoted still much higher leaf number when grown in Paracale soil (**Table 8**). Moreover, leaf area was highest ($p<0.05$) in MineVAM inoculated cuttings grown in Paracale soil. Whereas, the longest (24.04 cm, $p<0,05$) root was obtained from cuttings inoculated with Mykovam and grown in Paracale soil. This was significant as compared with the MineVAM inoculated cuttings (18.50 cm). In Caliraya soil, the effects of Mykovam and MineVAM inoculation on the number of leaves, leaf area and root length of cuttings were not significant from each other but significantly higher as compared with the uninoculated treatment.

Table 6. Interaction effect of soil type and mycorrhiza inoculation treatment on the monthly height of *J. curcas* cuttings grown in Caliraya grassland soil and Paracale mine tailing soil and inoculated with mycorrhizal fungi.

Soil type	Inoculation treatment	1-mo AP	2-mo AP	3-mo AP	4-mo AP
Caliraya	Uninoculated	4.73 a^{ns}	6.34 a^{ns}	6.62 a^{ns}	7.26 c*
	Mykovam	6.40 a	6.45 a	6.78 a	7.07 c
	MineVAM	5.27 a	5.87 a	6.10 a	6.23 c
Paracale	Uninoculated	4.91 a	7.14 a	7.79 a	8.30 b
	Mykovam	4.91 a	7.26 a	8.16 a	8.61 b
	MineVAM	5.11 a	7.51 a	8.83 a	10.35 a

AT = After transplanting
ns=Not significant
*= Highly significant at $p<0.05$
In each column, treatment means with the same letters are not significantly different from each other using DMRT at $p<0.05$.

Table 7. Interaction effect of soil type and mycorrhiza inoculation treatment on the monthly stem diameter of *J. curcas* cuttings grown in Caliraya grassland soil and Paracale mine tailing soil and inoculated with mycorrhizal fungi.

Soil type	Inoculation treatment	2-mo AT	3-mo AT	4-mo AT
Caliraya	Uninoculated	0.45 a^{ns}	0.48 b*	0.60 b*
	Mykovam	0.54 a	0.55 ab	0.63 ab
	MineVAM	0.48 a	0.49 b	0.61 ab
Paracale	Uninoculated	0.48 a	0.52 ab	0.60 b*
	Mykovam	0.52 a	0.55 ab	0.63 ab
	MineVAM	0.46 a	0.57 a	0.61 ab

AT = after transplanting
ns= Not significant
*= Significant at $p<0.05$
In each column, treatment means with the same letters are not significantly different from each other using DMRT at $p<0.05$.

Plant dry weight in J. curcas cuttings

Leaf dry weight was the only plant part of cuttings grown in Caliraya soil that was significantly increased by mycorrhizal inoculation wherein the two mycorrhizal inoculants exhibited similar plant growth promoting effect (**Table 9**). In this soil, there was no effect of inoculation on root, stem and total dry weight. In Paracale soil, Mykovam promoted the heaviest root and leaf dry weight whereas MineVAM promoted the heaviest stem and total dry weight.

Mycorrhizal root colonization in cuttings

Mycorrhizal infection in the cuttings ranged from 0 % to 14 % (**Table 3**). Root colonization in cuttings grown in Caliraya (7 %) and in Paracale soils (9 %) was not significantly different from each other. Those inoculated with Mykovam also exhibited a higher percentage of root colonization than that by MineVAM and in the uninoculated treatment.

Cu accumulation in plant parts of J. curcas cuttings

Cu accumulation by mycorrhizal cuttings grown in Caliraya soil ranged from 14 to 23 μg g^{-1} while the non-mycorrhizal counterpart ranged from 13 to 16 μg g^{-1} (**Figure 3c**). The roots of Mykovam-inoculated cutting gave the highest (23 μg g^{-1}, p<0.001) Cu concentration and the lowest (16 μg g^{-1}) was in the roots of uninoculated ones. Cu concentration in the leaves (16-22 μg g^{-1}) was intermediate and the least (13 - 19 μg g^{-1}) was in the stem. Cu accumulation by cuttings (**Figure 3d**) grown in Paracale soil followed the same trend with that in Caliraya soil (**Figure 3d**). The roots retained more Cu than in the leaves and the lowest was obtained in the stem. Mycorrhizal plants generally took more Cu from the soil than non-mycorrhizal ones. Cu concentrations in the roots of cuttings mycorrhizal with Mykovam retained the highest (p<0.01, 28 μg g^{-1}) and the lowest was observed in the stem of MineVAM inoculated cuttings (9 μg g^{-1}) (**Figure 3d**).

Cu concentration in plant parts of selected plants growing in Mogpog mine tailing soil

Cu concentrations in the different plant parts (roots, stem, leaves , pods, fruit pulp and seeds) of *A. auriculiformis*, *L. leucocephala* and *J. curcas* growing in Mogpog mine tailing soil were within the safe level (≤ 36 μg g^{-1}) for Cu (**Table 11**). Irrespective of plant species, Cu concentration was greatest (32 μg g^{-1}) in the roots, then in the leaves, stem, pod or pulp and the lowest was in the seeds (12 μg g^{-1}). Cu concentration in *A. auriculiformis* ranged from 12 - 32 μg g^{-1}, 18 – 23 μg g^{-1} in *L. leucocephala* and 20 to 28 μg g^{-1}) in *J. curcas*. Seeds of *J. curcas* and *L. leucocephala* contained 20 μg g^{-1} as compared with that of *A. auriculiformis* which was 12 μg g^{-1}.

Table 8. Interaction effect of soil type and mycorrhiza inoculation treatment on leaf and root characteristics of *J. curcas* cuttings grown in Caliraya grassland soil and Paracale mine tailing soil and inoculated with mycorrhizal fungi.

Soil type	Inoculation treatment	No. of leaves per plant	Leaf area (cm^2)	Root length (cm)
Caliraya	Uninoculated	2.67 c*	13.62 c*	12.05 c*
	Mykovam	4.60 b	15.71 c	18.10 b
	MineVAM	4.00 b	16.24 c	15.80 bc
Paracale	Uninoculated	4.75 b	23.66 b	17.00 bc
	Mykovam	6.46 a	25.51 b	24.04 a
	MineVAM	6.00 a	33.62 a	18.50 b

*= Significant at p<0.05

In each column, treatment means with the same letters are not significantly different from each other using DMRT at p<0.05.

Table 9. Interaction effect of soil type and mycorrhiza inoculation treatment on dry weight of *J. curcas* cuttings grown in Caliraya grassland soil and Paracale mine tailing soil and inoculated with mycorrhizal fungi.

Soil type	Inoculation treatment	Root dry weight	Leaf dry weight	Stem dry weight	Total dry weight
		----------g plant^{-1}----------			
Caliraya	Uninoculated	0.09 c*	0.11 c**	0.78 b*	0.98 b*
	Mykovam	0.12 bc	0.26 a	1.18 ab	1.56 ab
	MineVAM	0.18 abc	0.27 a	1.29 ab	1.74 ab
Paracale	Uninoculated	0.12 bc	0.18 b	1.21 ab	1.51 ab
	Mykovam	0.24 a	0.30a	1.20 ab	1.74 ab
	MineVAM	0.20 ab	0.21 ab	1.79 a	2.20 a

*, **= Significant at p<0.05 at p<0.01, respectively.

In each column, treatment means with the same letters are not significantly different from each other using DMRT at p<0.05.

Table 10. Percentage of roots colonized by mycorrhizal fungi in four-month old *J. curcas* seedlings and cuttings grown in Caliraya grassland soil and Paracale mine tailing soil.

Inoculation treatment	Caliraya	Paracale	Mean
Seedlings			
Uninoculated	0	5.21	2.61 c***
Mykovam	32.09	100	66.05 a
MineVAM	12.58	15.81	14.20 b
Mean	14.89b***	40.34 a	
Cuttings			
Uninoculated	0	2	1.0 b***
Mykovam	12	12	12 a
MineVAM	10	14	12 a
MEAN	7.33 a^{ns}	9.33 a	

ns=Not significant
***= Highly significant at $p<0.001$
Treatment means with the same letters are not significantly different from each other using DMRT at $p<0.05$.

Table 11. Cu concentrations of various plant parts of selected plants presently growing in an abandoned mine area in Mogpog, Marinduque. n = 3.

Plant species	Plant organ analyzed	Cu concentration (µg g^{-1})
A. auriculiformis	Roots	32 ± 5
	Leaves	21 ± 2
	Stem	18 ± 3
	Seeds	12 ± 2
L. leucocephala	Roots	23 ± 4
	Pods	20 ± 3
	Seeds	18 ± 2
J. curcas	Roots	28 ± 4
	Stem	20 ± 2
	Pulp	23 ± 2
	Seeds	20 ± 2

DISCUSSION

The present results confirmed previous studies that *J. curcas* are capable of surviving and growing in marginal grasslands (*Ultra 2010*) and in Cu-contaminated soils with minimal nutrients (*Mendoza et al. 2006, Adiova et al. 2012*). In the present study, plant survival and growth were better in Paracale mine tailing soil than in grassland soil and the worst was in Mogpog mine soil. This implies that the Cu contents in the soil affected the growth and survival of *J. curcas*. Cu analysis of the three soils used in this study revealed that mine soil from Mogpog, Marinduque contained the highest (160 µg g^{-1}) concentration while the mine soil from Paracale, Camarines Norte contained the lowest (45 µg g^{-1}). Cu concentration in Caliraya was 100 µg g^{-1}. This indicates that Cu level of the three soils exceeded the allowable limit of Cu concentration which is 36 µg g^{-1} soil (*Ministry of Housing 1994*), apparently indicating that bioremediation of these soils is highly recommended. Growth of seedlings and cuttings in Mogpog soil ceased on the third-month of the experiments. *Fontanilla and Cuevas* (*2010*) reported that *J. curcas* seedlings grown in Cu contaminated Mogpog soil containing (212 µg g^{-1}) died after 1 mo in the control and in the *T. pseudokoningii* treatment (with or without compost). In the Mogpog soil with 42 ppm Cu, seedlings in the control and in the *T. pseudokoningii*-amended soil performed very poorly compared with those grown in soil amended with compost. The Cu content (42 to 212 µg g^{-1}) of the Mogpog soil used by *Fontanilla and Cuevas* (*2010*) is very low as compared with the Cu content in the Mogpog soil (160 µg g^{-1}) used in this study. This is further supported by previous investigations (*Wong and Bradshaw 1982, Onac and Trifu 2005*) on the growth of seedlings in soils directly obtained from mine spoils with Cu contents similar to Mogpog soil. *Black* (*2006*) showed that soils with Cu levels above 100 µg g^{-1} are considered very toxic to plants. Examination of the seeds sown in the Caliraya soil with 100 µg g^{-1} Cu content had killed the embryo inside the seeds thus seedlings did not even had the chance to emerge from the seed. The kernel or the embryo became black and could have affected the processes occurring inside the seed (*Onac and Trifu 2005*). Moreover, *Onac and Trifu* (*2005*) reported that high amount of Cu in the soil when taken up by a plant impedes photosynthesis, translocation of photosynthetic products, uptake of water and cell division and, excessive levels of Cu can damage plant cell membranes, increase free radicals and peroxidase activity in plants that impair respiration (*Mohanapriya et al. 2006*). Aside from Cu, there might be other metals present in Mogpog mine tailing soil that may have inhibited seedling emergence. It is unfortunate that other heavy metals were not analyzed.

The pH of the soils is relatively very acidic to slightly acidic. High acidity in soil decreases the solubility of essential soil nutrients making them less available for plant uptake. Low soil pH also promotes leaching of nutrient cations such as Ca, Mg, and K from the soil surface (*Smith and Smith 2000*). Heavy metals, such as Cu, become more soluble and available for uptake by plants below pH 5.0, their availability can be excessive and toxic in more acidic conditions. The N, P, K, Ca, and Mg contents

of the three soils generally fell into the low soil test level further indicating the low nutrient availability in the soils.

Mycorrhizal plants grew better and gave higher survival rate than without inoculation. This may be attributed to the extraradical hyphae produced by mycorrhizal fungi ramifying beyond the root hair zone. These extraradical hyphae are effective in increasing the root absorptive area of plants (*Gaur and Adholeya 2004*). The diameter of mycorrhizal mycelia is smaller, thus the transport of nutrients from the soil to the plant is faster through these structures than nutrient transport via root hairs (*Raymundo et al. 2006*). Thus, it is possible that inoculated seedlings were better supplied with nutrients taken up by the extraradical hyphae, resulting in improved growth and survival.

The results of the present study further support previous studies (*Mendoza et al. 2006, Mercado and Reyes 2006, Adiova et al. 2006, Ultra 2010*) on the growth-promoting effects of mycorrhiza on *J. curcas*. However, the present study differed from the previous ones in terms of mycorrhizal inoculants used. For instance, *Ultra* (*2010*) inoculated *J. curcas* with AM fungi (collected in the grasslands) and grown in Luisiana and Libertad marginal upland soils with pH 4.4 and pH 5.2, respectively. He reported that AM fungi significantly increased the total dry matter yield of 6 weeks *J. curcas* seedlings in acidic marginal upland soils. The Mykovam mycorrhizal inoculant used in this study is a commercial inoculant, composed of cocktail of eight mycorrhizal species which had originated in a grasslands, mine sites and other stressed conditions. The MineVAM on the other hand was also a mixture of mycorrhizal fungi collected in mine tailings. These mycorrhizal fungi are mass produced routinely at BIOTECH.

The two mycorrhizal inoculants Mykovam and MineVAM used in the study exhibited statistically comparable effectiveness in promoting the overall plant growth. However, Mykovam showed relatively better positive effect on stem and root growth while MineVAM was more effective than Mykovam in promoting leaf growth. Previous studies (*Yucel 1997, Aggangan and Aggangan 2012, Aggangan et al. 2006*), have also shown that Mykovam generally increases root length and effective root surface area, improves dry matter production and overall growth of plants (*Mendoza et al. 2006, Aggangan and Aggangan 2012, Aggangan et al. 2008*). Mykovam inoculated plants not only had the longest roots but had numerous adventitious roots that may have contributed high efficiency in cycling and absorbing essential nutrients and ions (*Mulkey et al. 1996*). MineVAM-inoculated plants grown in Paracale soil produced more leaves and greater leaf area than the Mykovam-inoculated counterpart. Mycorrhizal fungi in the MineVAM (contains a consortium of different species of *Glomus*, *Scutellospora*, *Entrophosphora* and *Gigaspora*) were isolated from a mine ecosystem in Paracale, thus the fungal species are presumably naturally tolerant to Cu and perhaps to other metals such as Zn, Cd and Pb inherent in mine tailing soils. Mykovam contains eight plant growth promoting mycorrhizal species from mine sites and from grassland areas. The greater diversity of mycorrhizal fungi present in the Mykovam could be one reason why this inoculant was more effective than MineVAM in promoting better growth, survival and nutrient status of inoculated plants.

Seedlings and cuttings accumulated significantly higher Cu in Caliraya soil than in Paracale soil. This was expected, since Caliraya soil had lower pH and higher soil Cu concentration which means more Cu is available for plant uptake. Most of the absorbed Cu, was retained in the roots and less amount was translocated into the stems and leaves. The accumulation of Cu in the roots is due to the fact that Cu has very low mobility and is speculated to be capable of complexing with the proteins composing the root cell membranes (*Mohanapriya et al. 2006*). Although Cu accumulated into the stem and leaves, their concentrations passed the safe limit for human use which is 36 µg g^{-1} soil. Thus, these parts can be utilized for medicinal and industrial purposes.

Mycorrhiza inoculated seedlings and cuttings accumulated higher amount of Cu compared to the non-mycorrhizal ones. This is because mycorrhiza are capable of enhancing uptake of metals such as Cu. G*ildon and Tinker* (*1981*) speculated that heavy metals such as Cu, Zn and Mn are adsorbed in the mycelium for the growth of mycorrhizal fungi. The heavy metals adsorbed in the mycelial wall are then released either passively or through local decay of hyphae within the plant root cells (*Streit and Stumm 1993*) which may ultimately result in the enhanced accumulation of Cu in the plant. Plants grown in Mykovam-inoculated Caliraya soil accumulated the highest concentration of Cu and that highest accumulation occurred in the roots. This is attributed to the higher percent infection by Mykovam in both seedlings and cuttings grown in this soil. Caliraya soil is more acidic than Paracale soil. Acidic environments are more favorable for the growth and colonization of mycorrhizal fungi (*Gildon and Tinker 1981*). Desirable mycorrhizal inoculants should have high root colonization level that translate into greater ability to sequester heavy metals in their hyphal wall which renders increased Cu concentration in the roots but lower Cu concentration translocated into the aerial and more sensitive parts of the plant (*Read et al. 1992*). Mykovam had 100% root colonization and was effective in removing more Cu from the soil and retaining most of the absorbed Cu in the roots, thereby effectively lessen Cu accumulation in the aerial parts of the plant.

For biodiesel purposes, the Cu concentration in the seeds of *J. curcas* growing in Mogpog mine tailing is 20 ± 2 μg g^{-1} which is far below the allowable limit (30-36 μg g^{-1}) for Cu. In an earlier study, Cadiz et al. (2010) also reported that Cu concentration in the oil extracted from the seeds of *J. curcas* growing in a mine tailing site in Mogpog, Marinduque, is below the allowable Cu concentration. The *J. curcas* plantation presently growing in this mine tailing site was established four years ago employing inoculation with Mykovam and MineVAM with or without compost or lime (*Raymundo et al. 2006*). Initial results from this field trial suggest that, it is feasible to establish *J. curcas* plantation in Mogpog, Marinduque as long as soil amendments such as mycorrhiza, lime and compost should be added. *Fontanilla and Cuevas* (*2010*) reported that 20 % compost, reduced Cu concentrations from 169.6 to 28 ppm in high-Cu-contaminated samples and from 33.6 to 4.0 ppm in low-Cu-contaminated samples. Growth improvement with compost amendment in Mogpog soil, was due to increased soil pH and reduction of Cu content in the potting media because the organic matter in the compost form complexes with heavy metals (*Fontanilla and Cuevas 2010*).

AM fungi are well-known to excrete substances that influence the immediate environment. Example are amino acids and whole proteins that can have a direct selective effect on the microbial community in the rhizosphere (*Marschner and Baumann 2003*). AM fungi can also induce changes in plant physiology such as root exudation (*Graham et al. 1981*) and carbohydrate metabolism of the plant (*Buwalda and Goh 1982*) which can indirectly affect the microbial community. Either way, the impact of AM fungi on plant growth and rhizosphere microbial populations, as a consequence of microbial groups supported by a particular AM fungi inoculum, may be decisive for the successful establishment of plants under limiting soil conditions such as in mine tailings (*Medina et al. 2003*).

CONCLUSION

Planting of *J. curcas* seedlings or cuttings coupled with mycorrhiza inoculation offers great potential in rehabilitating grasslands in Caliraya, Laguna and in abandoned mine tailing sites in Paracale, Camarines Norte and possibly in sites with similar soil conditions. Commercial mycorrhizal inoculant Mykovam ™, was more effective than the indigenous fungi in MineVAM in terms of alleviating Cu toxicity in *J. curcas* plants by retaining more metals in the roots. This suggests that Mykovam™ inoculation is important to ensure that only trace amounts of metals would accumulate in the aerial parts such as in the seeds which are tapped as one source of oil for biodiesel or in vegetative parts which are sources of different useful medicinal products. It is recommended that more soil amendment studies are urgently needed for Mogpog mine tailing soil, although positive results from initial efforts using lime, compost and mycorrhizal inoculants Mykovam™ and MineVAM have been observed (*Cadiz et al. 2012*).

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