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Original Research Article

Effect of Arbuscular mycorrhizae on *Jatropha curcas* further its effect on tissue culture and leaf extract for antifungal property

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Abstract: *Jatropha curcas* is significant for its biodiesel and medicinal properties. This study is to compare growth of inoculated Arbuscular Mycorrhizae Fungi (AMF) and control effects on callus development, suspension culture and antifungal activity. Two hundred *J. curcas* cuttings were grouped into four equal sets and inoculated with AMF of 5g, 10g and15g set with one set kept as control. Micropropogation conducted with MS medium supplemented supplemented sucrose in combination with IBA (Indole 3 butryic acid) and BAB (benzyl adenine). After 6 months sun dried leaves were powdered and treated with 1:1 chloroform methanol solvent. This was column chromotographed with hexane and ethyl acetate (80:20) that separated into four column bands, later analyzed for antifungal activity. In three months root length, total colonization levels and spore number was found to increase according to the amount of AMF applied. The callus growth between the control and treated did not show a significant difference. But in general hypocotyl increased in dry cell weight compared to leaves. The suspension culture growth rate of fresh weight increased till 2 weeks then there was a rapid growth decline. The 6 months AMF treated and control column extracts failed to show any antifungal properties.

Key words: Arbuscular Mycorrhizae fungi, *Jatropha curcas,* inoculation, callus, antifungal activity and suspension culture

Introduction

Jatropha curcas L., a soft wood perennial plant belongs to family Euphorbiaceae, commonly known as Jamalghota, Physic nut, Ratanjot or Purgative nut. *J. curcas* is one of the most valuable crude drugs of primitive times and is still widely used in modern medicine. In recent years this plant has received extensive attention of many scientists in view of its great economic importance, medicinal significant and for its seed oil as commercial source of fuel (Datta and Pandey, 1993). It is also recommended as a drought resistant plant suitable for erosion control and is not palatable to grazing animals due to the toxicity (Munch & Kiefer, 1989).

Arbuscular mycorrhizae are ubiquitous and obligate symbiotic with plants, it has symbiotic association with 90% of vascular plant families. It is a proven fact that it aids to improve plant water relation by increasing the P uptake and other nutrients through increase in soil moisture extraction making the plant drought resistant other effects are beneficial in biological nitrogen fixation of rhizobium and biological control of root pathogen plant cell cultures are generally desire solid medium because of higher growth rates resulting from a high medium to tissue contact. The advantages of plant cell culture have for enhancing shoot proliferation and growth is reported in several species (Ilan *et al.,* 1995; Escalona *et al.,* 1999). There was also studies carried out on the effect of environmental condition on culture induction, maintenance, somatic embryogenesis, transformation and plant regeneration (Kim *et al.,* 2003, 2005, 2005a; Rao *et al.,* 2006, Nakamura & Ishikawa, 2006). However despite their economic importance, drought resistance characteristic *J. curcas* is also known for its medicinal value. *In vitro* culture studies have been reported, including formation of callus and plant regeneration through callus (Sujatha & Mukta, 1996). Fungal pathogen has considerable yield loss in agriculture and post harvest loss. But *J. curcas* extracts had reported many antimicrobial, ant parasitical, mollucidal and insecticidal activities.

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The objective of the present studies effects of inoculated Arbuscular Mycorrhizae fungi (AMF) in *J. curcas* cutting and its impacts on callus development, suspension culture. Then column extracts of grown plants leaves tested for antifungal activity.

Materials and Methods

Plant material

Healthy and uniform stem cuttings (12-15 cm length) of *J. curcas* were obtained from the branches of 1-2 years from Selection-1 plots grown in State Forest Research Institute (SFRI), Chennai during the end of November and were screened to 200 cuttings.

Assessment of VAM fungal colonization and measurement of the root Length

Cuttings were grown in polybags containing red soil and clay (potting mixture). The bio fertilizer AMF was bought from Tamil Nadu state Agriculture University, Coimbatore. They were weighed and applied to polybags as 10g per bag replicates done in a set of fifty bags. The AMF containing 20g and 30g were repeated making a total of 150 inoculated bags and with one set kept as control grown inside moist chamber. Root colonization of VAM is identified by the tryphan blue staining. After 3 months root length, the collar disc, No. of root hair, root length and No. of leaves were measured, counted and difference in biomass increment was calculated.

Micropogation

Preparation of Medium: The medium was prepared by readily available MS broth media (Himedia). Added with filter sterilized growth regulators auxin: IBA at .002 mg L^{-1} and cytokinin: BAB .0025 mg L^{-1}.

Initiation of callus: Sterilized leaf petioles and terminal shoots of *J. curcas* are collected. Cut with sterile knife less than 1.5cm and cultured on the media. Cultured tubes were maintained in growth room at 25°C under controlled light intensity of 2000 Lux, photoperiod of 16/8 hrs L/D cycle was adjusted with the help of an electric timer fitted.

Suspension culture: Cell suspension cultures of *J. curcas* were initiated by sub culturing, 2 week old callus tissue developed from explants. One gm of friable actively growing lush green callus was excised in a Whatman No.1 filter paper callus was mashed and carefully transferred with sterilized forceps into the autoclaved cooled MS media. The flasks were agitated at 120rpm on an incubated shaker at 25ºC under continuous low light (Fang *et al.,* 2005).

Antifungal activity from J. curcas extract:

The cutting was allowed to grow and establish their rooting system and after six months there leaves were collected from the mist chamber. It is separated by a cold extraction process. The 500 g of powdered leaves were taken in Erlyen Meyer flask and methanol was added in the ratio of 1:3 (w/v). It is kept in a 60±10 RPM shaking condition for 72h. The methanol extract is filtered and dried for 72h. This is vacuum distilled to concentrate the powder. The dried weights of the dry extracts were measured and reported the % of recovery = dry weight of extract recovered / Initial dry weight of leaves x 100.

This 15g of concentrated powder is taken process is repeated with hexane solvent (S_1). Another set of 15g of concentrated powder with chloroform: methanol in 1:1 ratio (S_2). These residual extract were stored at 4° C.

Fraction of extracts

The 3 gm of the condensate powder was again diluted with their corresponding extracts (1:1) diluents. A TLC plate solvent system using hexane as stationary phase and mobile phase solvent was tested as follows with ethyl acetate (10:2), ethyl alcohol (10:1) and ethyl acetate (80: 20) the elute collected were termed as elute A,B and C respectively.

They were column chromatographed using silica gel (Mesh Range – 100-200) of 30 cm length and 2cm with column was used with various solvent system. A 0.3 g of the condensate were taken and dissolved with solvent made into 3 mL which was loaded into the column packed with silica gel chromotographed on a column of silica gel eluted. The elution resulted in various bands of compounds which were collected according to a standard time interval (3min). The collected samples were then checked for compound by finding the $A_{190\text{-}900nm}$.

The eluted fraction of 67 samples were pooled together based on their similarity in absorbance. The collected sample were then dried to remove the solvent. The dried sample

were dissolved in 100% DMSO and stored in dark at 4°C for further use.

Antimicrobial assay of extracts

In order to test the efficacy of column fractions of *J. curcas* samples were tested with six different fungal pathogens- *Rhizoctonia solani* (4634-MTCC), *Fusarium oxysporium* (3075 MTCC), *Alternaraia alternate* (149-MTCC), *Sclerotium hydrophilum* (2157- MTCC), *Curvularea lunata* (2098-MTCC). The fungi were grown on Sabouraud dextrose agar (SDA) overnight and till it reached 1OD then overlaid on NB. The fraction was dissolved in DMSO at 8% then used for antifungal activity.

The antifungal activity of the crude extracts was determined in accordance with the agar-well diffusion method. 0.1ml of culture broth was added to 0.9 ml of nutrient broth medium and the fungal isolates were allowed to grow on a SDA at 25°C (Igbinosa *et al.,* 2009). The antifungal activity of the crude extracts was determined in accordance with the agar-well diffusion method described by Irobi *et al.,* (1994). Approximately 500µl of the elute were introduced into the wells, allowed to stand at room temperature for about 2 h and then incubated at 25°C. Controls were set up in parallel using the solvents that were used to reconstitute the extract. The plates were observed for zones of inhibition after 92 h for fungal test cultures.

Statistical Analysis

The values were tested using paired student's t test and the correlation between variables was also determined using GraphPad 6 (SanDiego, Ca, USA) software.

Results and Discussion

AMF effect on plant development

Mycorrhizal association was observed in all the VAM added packets under culture conditions and throughout the growing season in the field. Intercellular hyphae and vesicles, with occasional inter radical spore formation, characterized VAM colonization. The trypan blue staining root cells, very thin root segments, the stele of the root showed positive blue colour. That was found in all VAM treatment of *J. curcas* 150 cuttings.

The observation is shown in table 1, the different parameters after interval of 3 months the plants were closely monitored and the average of 50 plants of the given concentration is taken. There was no significant increase in the first month due to the lag phase of the microbial inoculation. As there is a general lag phase form the inoculation to the physical effects observed in plant (Brandon and Shelton 1993). Stating the increase in concentration increases the root development of the plant was observed. But the 10^{th} gm and 15^{th} gm there was no significant increase of AMF due to accumulation difference of nutrients.

Table 1: Effect of vesicular-arbuscular mycorrhiza inoculation on growth performance of *Jartropha curcas* after 60 days in a mist chamber

Growth Parameter	Treatment VAM inoculation	Non Inoculated Control (cm)	Inoculated (cm)	Difference	Biomass Increment (%)
Root Length	10g	01.5±0.9	02.2±2.1	0.7±2.0	46.66
	20g		03.1±2.0	01.6±1.1	106
	30g		03.3±2.5	01.8±1.6	120
Leaf Number	10g	02.0±1.4	02.6±1.6	0.6±0.2	30
	20g		03.5±2.1	01.5±0.7	75
	30g		03.6±2.4	01.6±1.0	80
Root Number	10g	01.0±0.25	03.8±2.4	02.8±2.1	280
	20g		04.7±3.0	03.7±2.8	370
	30g		04.1±2.7	03.1±2.5	309
Shoot Height	10g	15.0±5.1	20.8±7.3	05.8±2.2	38.66
	20g		27.4±8.2	12.4±3.1	82.66
	30g		27.9±6.3	12.9±1.2	85.99

Nitrogen is also taken up from inorganic source of ammonia by AMF (Ames *et al.,* 1983). Mychorrhiza colonization is generally attributed to tree species. AMF inoculated plants increases the phosphorous that is immobile in soil uptake by 2 to 5 times (Sanders *et al.,* 1983) as it adsorbs phosphorous faster per gram of root than the non mychorrizal plants (Jacobsen *et al.,* 1992). AMF inoculation had increased the shoot and root weights in medicinal plants (Srivastava and Basu 1995). Mychorrhiza

treated plants had survival rate two to three times higher than untreated. Inoculated mychorrhiza had a 30% increase in biomass and seed production seven month after plantation in a one year old samplings (Tewari 2007).

Callus development

The callus was prominently observed from *J. curcas* petiole within 7 days. But the control (without growth regulators) of the hypocotyls had shown browning with decreased regeneration due to enzymatic polyphenol oxidase. The leaves took 12 days for callus production. The plant was established *in vitro* in 45 to 60 days. There was no significant difference in the plant parts collected from the AMF treated and the untreated samples.

Plant cell and tissue cultures provide an alternative approach to the plants which are difficult to cultivate, or has a long cultivation period, or has a low yield, product yield by cell culture may be significantly produce a higher yield that obtained from the parents (Hippolyte *et al.,* 1992; Zhong *et al.,* 1994).

Suspension Culture

Initially cell suspension culture comprised of some isolated cells and small cell aggregates of 5-8 cells. Hypocotyl cleaved suspension cultures able to grow faster and accumulated a greater biomass in media. Till 15 days there was an increase in wet weight and later there was a considerable decrease with browning of the callus.

Growth of finely dispersed homogeneous and chlorophylous cell suspension culture was obtained. The suspension cultures comprised mainly of round, dense cytoplasm starch containing cells. Sampling the culture the rate of growth had found to increase. Growth was progressive but after 15th day the rate gradually decreased in suspension cultures (Fig. 1).

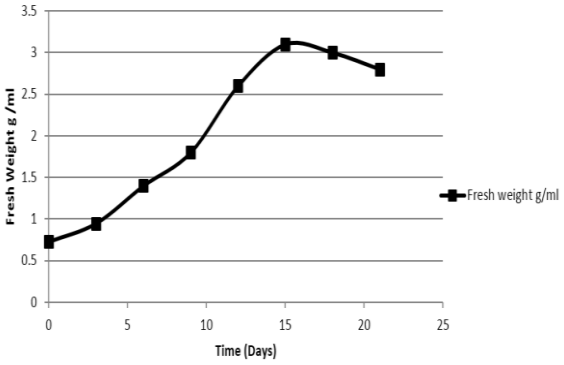

Figure 1: Fresh weight growth curve of *J. curcas* grown from hypcotyl derived callus grown in MS broth supplemented with IBA

The suspension cultures comprised mainly of round, densely cytoplasmic starch containing cells with distinct nuclei. Some cells are large elongated and highly vacuolated with sparse cytoplasm. Maximum increase in fresh weight was reached on day 21 which was about 5-6 fold over initial fresh weight similar to Mudalige & Longstreth 2006. Monacilli *et al.,* (1995) found 3 fold increase in cell suspension culture of *Taxus baccat* over a period of 40 days. The rate of growth was stable for 30 days (Stationary phase) but the rate gradually increased as the duration after the initiation of suspension cultures increased.

Hypocotyl cleaved suspension cultures were grown faster and accumulated a greater biomass in media containing 0.5mg/L 2, 4-D over a period of 30 days. Similar results were reported by Pathirana & Eason. (2006) in *Arabidopsis thaliana* suspension cultures derived from leaf callus. A majority of authors establish cell suspension culture by using 2.4-D at different concentration (Kobayashin & Esteves Vieira, 2000; Nakamura & Ishikawa, 2006; Risika *et al.,* 2006; Rao *et al.,* 2006). However other used axing with low concentration to cytokine for establishment of cell suspension cultures. After 3 weeks of growth a finely dispersed homogeneous and chlorophyllous cell suspension culture was obtained

Maximum increase in fresh weight was reached on day 21 which was about 5-6 fold over initial fresh weight. Monacilli *et al.,* (1995) found 3 fold increase in cell suspension culture of *Taxus baccat* over a period of 40 days.

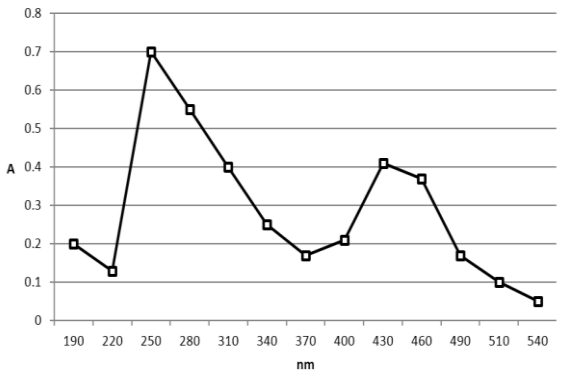

Figure 2: Adsorption profile of *J. curcas* extract in a column chromatography of elute A

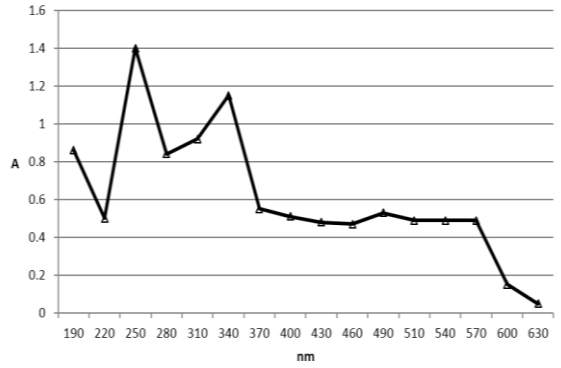

Figure 3: Adsorption profile of *J. curcas* extract in a column chromatography of elute B

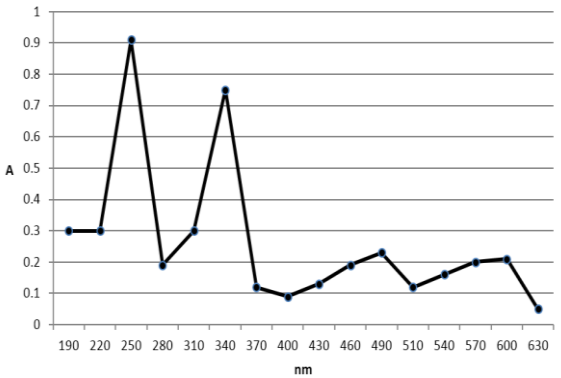

Figure 4: Adsorption profile of *J. curcas* extract in a column chromatography of elute C

Establishment of solvent system

Column chromatography: The dry weight of *J. curcas* leaves was found to be 91.5±2.7 g and recovery percentage was found to be 18.3%.

Initially the Rf values of *J. curcas* of crude extract was established in various solvent system. The solvent system that was chromotographed in TLC plating with hexane: ethyl acetate (10:2) showed 2 bands, hexane: ethyl alcohol (10:1) showed 3 bands while Hexane: ethyl acetate (80: 20) showed 9 bands with Rf vales ranging from 0.478 to 1.00 with clear banding pattern. The solvent system that has shown more bands was taken further for column chromatography.

The elute A had 1 band, elute B had 2 bands and elute C had 4 bands. These distinct band were observed were collected in 2 mL eppendorf tubes and stored in cold storage. The A_{220nm} to A_{250nm} was found to have the most absorbance elute A-.07 A_{250nm} (Fig. 2); elute B-1.4 A_{250nm} (Fig. 3) and elute C-0.9 (Fig. 4).

Determination of antimicrobial activity

These elutes did not show any distinct activity against any of the fungus.

Conclusion

The callus development was found to prominently well grown and hardened. The cuttings reported to have good effect with VAM with 15g was found to be appropriate concentration for application. Unpaired one tailed t test showed no significance between root length and leaf number but significant difference was seen with root and leaf number. The dried leaf extract shown no antifungal activity

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