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Research Article

Efficient *in vitro* regeneration of biodiesel plant *Jatropha curcas* .L

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Abstract

In the present investigation, in vitro propagation of Jatropha curcas L. was achieved employing nodal explants. Axillary shoot bud proliferation was best initiated on Murashige and Skoog's (MS) medium supplemented with 20 µM N6-benzyl- adenine (BA) and 50 µ M adenine sulphate, in which cultures produced 8.2 ± 0.56 shoots per nodal explant with 3.0 cm length after 3-5 weeks. The rate of shoot multiplication was significantly enhanced after transfer to MS medium supplemented with 2.5 µ M 6-furfuryl amino purine (Kn), 0.5 µ M indole- 3-butyric acid (IBA) and 25 µ M adenine sulphate for 4 weeks. Internode explant segments of *Jatropha curcas* plants responded *in vitro* and formed callus tissue when cultured on Murashige-Skoog (MS) full strength nutrient medium supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D - 4 mg/L) and N6-Benzyl adenine (BA- 4 mg/L). The internode-derived callus tissues were found non-embryogenic and hence did not regenerate into shoot and root, respectively. The internode segments when cultured on MS (full strength) media supplemented with BA - 5 mg/L) were found to grow forming two to three buds. However, these shooting explants did not form roots upon hormonal regulation. On the contrary, endosperm tissue cultured on full strength MS media supplemented with 3 mg/L BA and 1 mg/L Indole-3-butyric acid (IBA) along with activated charcoal (100 mg/L) and ascorbic acid (50 mg/L) yielded simultaneous shooting and rooting response after four weeks of incubation.

Keywords: Callus, internode explant, *Jatropha curcas* L, Murashige-Skoog medium.

Introduction:

Jatropha curcas L. species belonging to the family *Euphorbiaceae* is multipurpose shrub or small tree of significant importance because of its several industrial and medicinal uses. Jatropha grows in a number of climatic zones in trophical and subtrophical regions and can be grown in area of low rain fall and problematic soil. Its oil is a potential source of biofuel in countries where the resource to import fossil fuel is poor. Jatropha has requisite potential of providing a promising and commercially viable alternative to diesel as it has the desirable physico- chemical and characteristics comparable to diesel to facilitate continuing to run the machine without much change design. There is a growing interest in *Jatropha curcas* L. (Jatropha) as a biodiesel feedstock plant. Variations in its morphology and seed productivity have been well documented. However, there is the lack of systematic comparative evaluation of distinct collections under same climate and agronomic practices. With the several reports on low genetic diversity in *Jatropha* collections, there is uncertainty on genetic contribution to *Jatropha* morphology [1].

Since the petroleum crisis in 1970 s and the recognition of limited world fossil energy resources, plant oils which can replace fossil oil have been given more attention [2]. Special interest has been shown in the cultivation of *Jatropha curcas* (jatropha, a member of Euphorbiaceae family) for its drought and poor soil tolerance. It can be cultivated on marginal land and does not compete with food production crops. Jatropha, also known as physic nut, is a small tree or large shrub which can reach a height of 5 m [3]. The ever growing demand of energy and its consistent impact on impart bill for fossil fuel has necessitated the search for alternative and complementary sources of energy. The various alternative fuel options explored include biogas, biodiesel. Of these biodiesel is gaining world wide acceptance as a solution to environmental problems, energy security, reducing imports and improving agricultural economy.

Biodiesel is made from fresh and used from vegetable oil of edible and non edible nature as well as from animal fat. It is commercially available in the world market. Biodiesel from Jatropha is an important alternative towards energy independence to our country. *Jatropha curcas* oil the potential of providing a promising and commercially viable alternate to diesel, since it has desirable characteristics comparable to diesel [4]. When compared to with diesel oil for exhaust gas tests, differences in smoke and carbon monoxide content were not only negligible but the values were also lower than the accepted value as per the standard specification of environmental board [5]. No sulphur dioxide emission was found in the exhaust fumes of the engine run or Jatropha oil as against 125ppm sulphur dioxide found in the exhaust gas of the engine, run on diesel oil.

Jatropha grows well under subtropical and tropical climate. It can tolerate extremes of temperature but not the frost. It is grown in wide range of soils. Recently awareness of the depletion of traditional energy resources has evoked an intense interest in alternative sources of energy. Jatropha curcas L is one such plant that has oil that may be substitute energy oil [6].The active principle in its latex Jatropha, has shown anticancer properties. The present study established that multiple shoots can be obtained directly from inter nodal segments of the plant.

Materials and methods:

Plant materials:

The material used [explants] for investigation was obtained from plants grown in botanical garden, Madurai Kamaraj University, Madurai. Explants were taken from disease free *Jatropha curcas* internadal segments as the 3rd to the 6th node from the apex were excised and washed throughly with tap water and surface sterilized in 20% sodium hypo chloride with 3-4 drops of 20 for 15 minutes followed by $HgCl_2$ for 2-3 minutes and rinsed 4-5 times with sterile double distilled H_2O these explants then transferred to solid MS medium [7]. The medium was supplemented with various combinations and concentrations of different growth regulations namely 6-Benzyl amino purine(BAP), kinetin, 1-Napthalene acetic acid (NAA),2,4-Dichlorophenoxy acetic acid (2,4-D) and adenine sulphate were used for regeneration studies. The pH was adjusted to 5.8 before autoclaving at 115 IbSfor 15min $^{-1}$. All the cultures were maintained at 26-28^{o}C with 24 hours light and 32% relative humidity. The media were congealed with agar (0.8%) and sucrose 3% were used as a source of carbohydrate. All the experiments were repeated thrice and had 5 replicate with single explants.

J. curcas L. seeds were surface sterilized with 0.1% $HgCl_2$ solution for 2 minutes, washed two times with sterile distilled water. The seeds were imbibed at 26±1°C for 8 hours in sterile water. Thereafter coats were removed followed by endosperm of seeds were aseptically transferred to empty 100 ml sterilized conical flasks. All organs and tissues in culture (initial and sub/re-cultures) were incubated at 26±1°C temperature in controlled culture room conditions.

Tissues for Callus Induction and Shoot regeneration were provided a 16hr light and 8hr dark photoperiod while tissues kept for rooting were provided an 8hr light and 16hr dark photoperiod.

Results and Discussion:

In vitro multiple shoot regeneration of *J.curcas* via nodal explants cultured on MS medium for fortified with various auxins viz, NAA, IAA in varying concentrations (0.1-2.0 $mg1^{-1}$ and cytokinin (BAP/kinetin singly as well as in combination showed varied response with respect to number of shoot buds obtained per explants. However, BAP 2.0 $mg1^{-1}$ was most effective in shoot bud induction. Combination of BAP (2.0 $mg1^{-1}$) and IAA (0.1mg 1^{-1}) were found to be effective for maximum shoot proliferation. Addition of adenine sulphate (10 $mg1^{-1}$) Glutamine (100 mg 1^{-1}) and activated charcoal (0.1% 0 to the above medium has promoted high frequency of multiple shoot proliferation within 30 days [8]. However plantlets with well developed roots were taken out from culture vessels, washed thoroughly with distilled H_2O and transferred to pot containing vermiculture and soil (1:3). Glutathione mainly play a role to prevent immature leaf fall in multiple shoots. Activated charcoal has the able to control the excess release of latex and phenolic substances there by stimulated callus formation as well as shoot elongation. Of all the growth hormones tried in this experiment fast rooting was resulted with IAA (0.5mg 1^{-1}) in the medium, the roots developed with no intervening callus [Figure 1].

Figure1: Callus induction from Explants

Legend: Callusing observed on combination of 2,4-D with BA (4 mg/L each) in MS medium after 15 days. Left image shows initiation of callus, Middle image shows callusing without activated charcoal and ascorbic acid while right image shows set-up supplemented with activated charcoal and ascorbic acid. Enhanced callusing was observed on media supplemented with activated charcoal (100 mg/L) and ascorbic acid (50 mg/L).

Figure 2: Regenerated shoots from endosperm cultured on MS medium

Legend: Different stages of emerged shoot and root were regenerated from endosperm cultured on MS medium + 100 mg/l activated charcoal, 3 mg/l BA and 1 mg/l IBA for 4 weeks.

Effects of supplements on Callus induction from apical stem bud derived internodal explants: Internode explants of *J. curcas* L. have the potential to regenerate plantlets by indirect organogenesis [Figure 3]. Growth regulators added exogenously are essential for this to occur; acting as factors that selectively influence the genes to first generate undifferentiated cellular mass (callus) which later, upon constant influence of plant growth regulators get differentiated to yield the shoot and root systems. MS media containing 2,4-D showed response within one week. 4 mg/l of 2,4-D produced 95% induction of explants. The other combinations of auxins and cytokinins were found to be ineffective for explants proliferation. Our results had developed a protocol for profuse development of green, embryogenic callus in MS basal medium supplemented with BA (4.0 mg/L) and 2,4-D (1.0 mg/L) obtained by 3rd to 4th week of culture.

Figure 3: ***In vitro* shoots from nodal explants**

Legend: Mutiple shoots were regenerated from nodal Explants cultured on MS medium. Complete plantlet (8-10 week) Nodal cultured on medium with different concentrations of growth hormones. Shooting observed MS media + 5mg/l BA. Left image shooting observed at the end of 2 weeks and right image shows shooting observed at the end of 4 weeks.

Explants for shoot induction on MS medium supplemented with various concentrations and combinations of hormones. Results were recorded at a regular interval and final data were scored after four weeks of culture to observe the effect of auxin and cytokinin and in combinations with adenine sulphate to induce multiple shoots were analyzed to study the role of auxins, cytokinin alone, same number of explants were transferred to MS basal medium and treated as control

Hardening:

After three weeks of cultures on shoot and rooting medium the plantlets were transferred to pot for hardening and acclimatization [Figure4]. Micropropagated plantlets were gently washed under running tap H_2O and transferred to polypropylene boxes containing autoclaved garden soil. The plants were covered with polythene bags with fine holes and kept in a culture room at 26+ 2 ^{o}C and 60-70% relative humidity for two weeks. After 15days the polythene covers were removed and transplanted to the field for further growth. *Jatropha* is mainly planted on marginalized soil i.e. unused public areas and school areas for fencing.Since Jatropha has a deep reaching taproot, it is able to “pump” minerals from the depth of the soil to the surface. This leads to a rehabilitation of degraded land. The *Jatropha* System creates a positive reciprocity between raw material/energy production and environment/food production.

Figure 4: *Jatropha curcas* grown under field condition

Legend: The pot culture showing regenerated responses in the number of shoots per explants were recorded. Plants grown observed after 10-12 weeks old of hardening.

An efficient regeneration of *J. curcas* L were obtained via nodal segment as explants which were medicinally as well as economically important plant. Moreover the plant is very reliable in curing several diseases like rheumatism, leprosy, scabies, eczema, ringworm, chronic dysentery, urinary discharges, abdominal complaints, anaemia and diseases of the heart. Besides its medicinal potentialities it was an excellent species for agroforestry programme. It can be used in control of floods, nutrient leaching, soil erosion and shifting of sand dunes. Most of the plant parts of Jatropha have medicinal value tannins as their main components are astringent in nature and are used for treating intestinal disorders such as diarrhea and dysentery [10]. Tannins are known to react with proteins to provide the typical tanning effect which is important for the treatment of inflamed or ulcerated tissues reported by [9]. These observations therefore support the use of *J. curcas* in herbal cure remedies. The bio-logical activities of tannins and observed that tannins have anticancer activity and can be used in cancer prevention, thus suggesting that *J. curcas* has potential as a source of important bioactive molecules for the treatment and prevention of cancer [11] also confirmed the antiviral property of steroids. Flavonoids, another constituent of *J. curcas* stem bark extracts exhibited a wide range of biological activities like antimicrobial, anti-inflammatory, anti-angionic, analgesic, anti-allergic, cytostatic and antioxidant properties.

In tropical and subtropical countries, *Jatropha* has high potential as a biofuel crop. Among the oil-bearing tree species, *Jatropha* is desired due to its multiple positive attributes including drought resistant, rapid growth, high oil content,small gestation period wide adaptation [12] phenotypic variation and seed biochemical composition have been widely reported. The oil of *J. curcas* non-edible and was extensively used in various industries. The semi-dried oil called bio-diesel used as one of the efficient fuel substituted for diesel engines [13] trans-esterified oil has quality equivalent to the Euro II diesel. *Jatropha* oil cake could be used as a organic fertilizer. It is a hardy shrub that can grow on poor soils and areas of low rainfall (from 250 mm a year) hence its being promoted as the ideal plant for small farmers. Since *Jatropha* can grow relatively well in marginal areas compared to other traditional crops, it may help to reclaim degraded land and protecting the soil from soil erosion. Despite its multiferous potentiality, there were some limitations in propagation of this potent plant species as it is a latex containing shrub that makes it recalcitrant for tissue culture. We have developed an efficient, reliable in vitro system for regeneration of *Jatropha curcasvia* nadal segment.

Conclusion:

Present study describes the developed micropropagation protocol of *J. curcas* L. from internode explants and endosperm tissue. Attempts to develop *J. curcas* L. plantlets developed from callus tissue overcome high level of phenolics released causing embryogenesis. Activated charcoal used as adsorbent of phenolics and antioxidant ascorbic acid were effective in case of internode explants and endosperm tissue but proved effective induction of plantlets from callus tissue. Future studies will be focus to develop transgenic incorporated with engineered genome essential for high yield of oil content derived from callus tissue can be tailored to achieve *J. curcas* L. varieties having higher oil-yield and other qualities as desired. Biodiesel, an environmental friendly diesel fuel similar to petro-diesel in combustion properties, has received considerable attention in the recent past worldwide. Biodiesel is a methyl or ethyl ester of fatty acid made from renewable biological resources such as vegetable oils (both edible and nonedible), recycled waste vegetable oil and animal fats.The Jatropha System is an integrated rural development approach. By planting *Jatropha* hedges to protect gardens and fields against roaming animals, the oil from the seeds can be used for soap production, for lighting and cooking and as fuel in special diesel engines. The *Jatropha* System the more seeds/oil Jatropha hedges produce, the more food crops are protected from animals and erosion also additional income is created, mainly for women. It may still take some time, until the *Jatroph*a system will contribute economically to the rural development in a large scale. But it seems that the activities of many different organizations more and more support the *Jatropha* approach.

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