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TISSUE CULTURE STUDIES ON SOME IMPORTANT BIODIESEL PLANTS: A REVIEW

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The present review aims at various *in vitro* studies that have been conducted on some important biodiesel plants. Various tissue culture based protocols are discussed in details for three important and potential biodiesel plants viz., *Jatropha curcas*, *Pongamia pinnata* and ***Balanites aegyptiaca***.

Keywords : Biodiesel plants; *In vitro;* Tissue culture.

Abbreviations : BAP – 6-Benzylaminopurine; GA_3 – Gibberellic acid; IAA – Indole-3-acetic acid; IBA – Indole-3-butyric acid; Kn – Kinetin; MS – Murashige and Skoog medium; NAA – Naphthalene acetic acid; TDZ – Thidiazuron

Introduction

Biofuel is defined as solid, liquid or gaseous fuel obtained from relatively recently lifeless or living biological material. It is different from fossil fuels, which are derived from long back dead biological material. Bio-diesel, considered an equal replacement of petro-diesel (with about 5% less efficiency), can be made after transesterification from virgin or used vegetable oils (both edible and non-edible). It is meant to be produced in India mainly from *Jatropha curcas* and, to a lower extent, from other non-edible virgin oils (in particular *Pongamia pinnata*). It requires little or no engine modification up to 20% blend and minor modification at higher percentage blends.

Tissue culture is an important and a useful tool to mass multiply quality planting material at a high frequency. Moreover, tissue culture plays an important role in development of a base for genetic transformation system. If properly developed and standardized, an *in vitro* mass multiplication protocol can come up handy in establishment of a large scale plantations for commercial exploitation. The three important and potential biodiesel plants taken up in the present review are *Jatropha curcas*, *Pongamia pinnata* and *Balanites aegyptiaca.*

Micropropagation protocols for *Balanites aegyptiaca* and *Jatropha curcas* have already been standardized by Arid Forest Research Institute, Jodhpur. The field trial of tissue culture raised plants for *B. aegyptiaca* is being evaluated; which has been established in 2006. Acclimatization experiments for *Jatropha curcas* and *Pongamia pinnata* are underway. Thee efforts will help in technology transfer from laboratory to land.

Jatropha curcas - It belongs to family Euphorbiaceae. This plant is considered resistant to drought and pests and it produces seeds containing 27-40% (average: 34.4%) oil[1,2]. The remaining press cake of jatropha seeds after oil extraction could also be considered for energy production[3]. Several reports have been published on ***in vitro*** studies of *Jatropha*. The research work carried out in this genus is as follows:

Somatic embryogenesis - Spera *et al.*[4] cultured immature embryos of *Jatropha podagerica* (0.2 – 0.5 cm long) on solid MS medium alone or amended with GA_3 (0.029-2.90 µM), with or without 0.5, 1.0 or 2.0 g activated charcoal per liter and maintained at 20°C under a 16 h photoperiod. The number of days to germination was highest with 1.0 gm activated charcoal and GA_3 (0.29 µM), whilst the number of days to germination was lowest in the absence of charcoal and with GA_3 (0.29 µM) in the absence of activated charcoal germination percentage was highest (70 - 75 percent) with GA_3 (0 - 2.9 µM) and lowest with intermediate concentration of GA_3. The longest number of roots occurred in medium supplemented with 0.5 g activated charcoal per liter and GA_3 at a concentration of (2.0 µM)[4].

Sardana *et al.*[5] standardized an efficient 2-stage method for plant regeneration from leaf explants of *Jatropha curcas*. In stage I they developed globular

embryogenesis callus on MS-Gamborg's medium containing MS basal salts and Gamborg's vitamins, supplemented with BAP (13.31μM) + IAA (17.12 μM). In stage II plantlets were developed from callus on MS medium supplemented with gibberellic acid (8.66μM), IAA (5.71 μM) and sucrose. Somatic embryos developed into normal plantlets on full strength MS medium + 3% (w/v) sucrose. Upon transfer to soil, the plantlets grew well and appeared normal[5].

Jha *et al.*[6] reported embryogenic calli were obtained from leaf explants on MS basal medium supplemented with only Kinetin (9.3 μM). Induction of globular somatic embryos from 58% of the cultures was achieved on MS medium with different concentrations of Kinetin, IBA. Addition of Adenine Sulphate (13.6 μM) stimulated the process of development or somatic embryos. Mature somatic embryos were converted to plantlets on half strength MS basal medium with 90% survival rate in field conditions[6].

Micropropagation- Sardana *et al.*[7] observed regeneration from shoot tips of *Jatropha curcas* on MS medium supplemented with GA_3 (8.66 μM) and IAA (17.12 μM). Plantlets were acclimatized, and successfully transferred to pots and finally to the field[7].

Rajore *et al.*[8] developed a method for production of clonal plants of *Jatropha curcas* by *in vitro* production of multiple shoots from nodal segments. The nodal segments were cultured on MS medium supplemented with cytokinins viz., Kinetin (Kn), BAP and auxins viz., IAA, IBA, and NAA. Multiple shoots were obtained on MS medium fortified with Kinetin (at 9.29 μM) and IBA (7.38 μM). Addition of various additives viz., Ascorbic acid (56.77 μM) + Citric acid (260.25 μM) + Adenine Sulfate (67.87 μM) + Glutamine (684.22 μM) showed a synergistic effect in shoot proliferation and its development. *In vitro* produced shoots were transferred on the rooting medium which comprised of full, half or quarter strength MS medium supplemented with various auxins viz., IAA, IBA, NAA and 2,4-D (5.71 - 34.24, 4.90 - 29.52, 5.37 - 32.22 and 4.52 - 27.14 μM).Best rooting was observed on half strength MS medium supplemented with NAA (26.85 μM). The plantlets were hardened on sterilized mixture of soil and vermiculite (3:1) and were established in soil with a survival rate of 70 ± 10% [8].

Rajore and Batra[9] obtained prolific shoots from shoot tips of *Jatropha curcas (L)* inoculated on MS medium containing cytokinin singly as well as in combination. Combination of BAP (8.87 μM) and IAA (2.85 μM) proved optimal for maximum shoot proliferation along with Adenine Sulphate (67.87 μM), Glutamine (684.22 μM) and Activated charcoal (0.2%). *In vitro* produced shoots induced roots on IBA (2.46 - 24.60 μM) added to half strength MS medium. The highest frequency of root induction was on the medium with IBA (14.76 μM). Regenerated plantlets were successfully transferred to field after initial acclimatization[9].

Compos *et al.*[10] treated the nodal segments of *Jatropha elliptica* with doses of IAA (0.1 – 0.5 μM) and BAP (5.0, 10.0, 15.0 μM) for multiplication. For rooting, micro-cuttings were submitted to IBA and NAA (1.0 and 5.0 μM) treatments. The best rooting was carried out with 5.0 μM NAA. Acclimatization was carried out successfully[10].

Datta *et al.*[11] reported axillary shoot bud proliferation which was best initiated on MS basal medium supplemented with BAP (22.2 μM) and Adenine Sulphate (55.6 μM). Root induction at a frequency of 52% occurred in MS basal medium supplemented IBA (1.0 μM). The plantlets were successfully acclimatized in soil with 87% survival frequency[11].

Shrivastava and Banerjee[12] achieved regeneration via axillary buds on MS medium with BAP (13.31 μM), IBA (4.90 μM), Adenine Sulphate (678 μM), Glutamine (342.11 μM), L-arginine (86.10 μM) and Citric acid (130.12 μM) within 3-4 weeks of inoculation. The highest frequency of root induction was on the medium with IBA (14.76 μM)[12].

Organogenesis - Sujatha and Dhingra[13] observed prolific adventitious shoots on combination of BAP (2.2 or 4.4 μM) and IBA (4.9 μM) in *Jatropha integerrima.* On reduction of IBA concentration (2.5μM) development of shoots was promoted. Regenerated shoots were rooted readily on hormone free MS medium. Plantlets were acclimatized and successfully transferred to pots[13].

Sujatha and Mukta[14] cultured various explants viz; hypocotyl, petiole and leaf on medium supplemented with Zeatin, Kinetin and BAP either singly or in combination of IBA. Higher regeneration from hypocotyl and petiole explants was obtained on BA with IBA than on Zeatin or Kinetin supplemented media. Leaf disc from the third expanding leaf exhibited higher regeneration potential than those from the fourth leaf. Independent of explant type, maximum adventitious shoots were recorded on MS medium supplemented with BA (2.22 μM) and IBA (4.9 μM). Although BA concentration with reduced IBA concentration (0.49 μM) proved effective in callus development from hypocotyl and leaf explants. The callus of petioles required further lower concentration of the two growth regulator (i.e. BA 0.44 μM and IBA 0.49 μM). Regenerated shoots could be rooted on hormone free full

strength MS medium. Following simple hardening procedures, the *in vitro* raised plants were transferred to soil and grown to maturity in the field[14].

Spera *et al.*[15] cultivated of *Jatropha podagrica* on MS medium supplemented with all possible combination of growth regulator 2, 4-D (0, 4.5, 9.1 or 18.1 µM) and Kinetin (0, 0.46 or 4.6 µM). Cultures were maintained at 26 ± 1°C with a 16 hours photoperiod. Interaction between 2, 4-D and Kinetin was significant and maximum callus was on Kinetin (4.6 µM) + 2, 4-D (9.1 µM) treatment. Regardless of the kinetin concentration callus weight was high at 4.5 and 9.1 µM concentration of 2, 4-D[15].

Sujatha and Reddy[16] inoculated hypocotyls, stem, peduncle and leaf explants on MS medium containing BAP, Kinetin and Zeatin (0.44 - 8.87, 0.46 - 9.29 and 0.46 - 9.12 µM) in combination with IBA (4.90 µM) for shoot induction in *Jatropha integrrima*. BA was the most effective cytokinin for promoting shoot induction. Shoot regeneration frequency varied between 19.6 to 100% depending on explants. The highest shoot regeneration was observed in stem and leaf explants. However, leaf explant failed to regenerate shoots on the medium supplements with Kinetin and Zeatin. Frequency of callus formation and shoot induction were significantly effected by explants, cytokinin type and cytokinin concentration. Histological sections revealed early stages of embryoid development in leaf and peduncle explants; however, regeneration from all explants was predominantly through organogenesis[16].

Qin *et al.*[17] reported direct induction of adventitious buds from the surface of epicotyl explants with the combination of IBA (0.49 µM) and BAP (0.88 - 3.11 µM). The highest regeneration frequency induced on IBA (0.49 µM) and BAP (2.22 µM). These shoots rooted on growth regulator free MS media. Plantlets were successfully transferred to greenhouse[17].

Sujatha *et al.*[18] reported axillary bud proliferation and direct adventitious shoot bud regeneration from leaf segment. Shoot bud proliferation was obtained on an initial basal MS salt medium supplemented with different concentration of BAP, Kinetin and TDZ. Shoot multiplication rate was optimum on medium supplemented with TDZ (2.3 to 4.5 µM). Efficient adventitious shoot regeneration from leaf tissues was achieved with culture on medium supplemented with BAP (8.9 - 44.4 µM) + IBA (4.9 µM). Maximum shoot regeneration was achieved on medium with BAP (8.9 µM) + IBA (2.5 µM)[18]. Similar results were reported by Deora and Johnson[19]. According to them MS medium supplemented with TDZ (2.27 µM), BAP (2.22 µM) and IBA (0.49 µM) resulted in induction of adventitious shoot buds. Induced shoot buds were multiplied and elongated into shoots following transfer to MS medium supplemented with BAP (4.44 µM), Kn (2.33 µM), IAA (1.43 µM) and GA_3 (0.72 µM). Well developed shoots were rooted on MS medium supplemented with IBA (0.5 µM)[19].

Pongamia pinnata- It belongs to family Fabaceae. It is commonly known as honge or pinnata, as well as Neeni and Mahua. Recently several reports on micropropagation of *Pongamia pinnata* have been published. Micropropagation of *Pongamia pinnata* through enhanced axillary branching has been reported[20]. Multiple shoots were induced *in vitro* from nodal segments through forced axillary branching. Murashige and Skoog (MS)[21] medium supplemented with 7.5 µM benzylaminopurine (BAP) induced up to 6.8 shoots per node with an average shoot length of 0.67 cm in 12 days. Incorporation of 2.5 µM gibberellic acid (GA_3) in the medium during the first subculture after establishment and initiation of shoot buds significantly improved the shoot elongation. Single use of GA_3 during the first subculture eliminated the need for prolonged culturing on BAP medium. Further use of GA_3 in the medium was not useful. Shoot culture was maintained for at least two subcultures without loss of vigor by repeatedly subculturing the original cotyledonary node on shoot multiplication medium followed by shoot elongation medium after each harvest of the newly formed shoots. Thus, from a single cotyledonary node, about 16–18 shoots were obtained in 60 days. Shoots formed *in vitro* were rooted on full-strength MS medium supplemented with 1.0 µM indole butyric acid (IBA). Plantlets were successfully acclimated, established in soil, and transferred to the nursery.

Recently, *De novo* organogenesis and plant regeneration in *Pongamia pinnata* has been reported[22]. Role of Thidiazuron (TDZ) in inducing adventitious organogenesis in *Pongamia* was studied. TDZ at different concentrations (0, 0.45, 2.27, 4.54, 6.71, 9.08, 11.35, 13.12 and 22.71 µM) were used for induction of caulogenic bud formation in de-embryonated cotyledon explants. Each cotyledon was cut into three segments and identified as proximal, middle and distal. Duration of TDZ exposure, influence of the segment and orientation of the explant were studied. TDZ at 11.35 µM concentration was optimum for the induction of shoots and rapid elongation. Shoots induced at higher concentration elongated after several passages in growth regulator free medium, thereby extending the period of differentiation. Exposure of the explant for 20 days yielded more number of buds than 10 days. Proximal segment of the cotyledon was more

responsive. Contact of abaxial surface in the medium was more effective and generated more buds than the adaxial side. Buds differentiated and elongated on transfer to MS basal medium for 8–12 passages of 15 days each. Rooting and elongation of shoots was achieved in charcoal supplemented half-strength MS medium. Rooted plantlets survived on transfer to sand soil mixture. The plants were hardened and transferred to green house. This is the first report on *in vitro* regeneration of *Pongamia pinnata* via adventitious organogenesis using TDZ. This protocol may find application in studies in genetic transformation, isolation of somaclonal variants and in induction of mutants. It also provides a system to study the inhibitory role of TDZ on shoot differentiation.

Balanites Aegyptiaca - Balanites aegyptiaca L (Del.) is another potential biodiesel plant of dry land. Seed weights of 500-1500 seeds/kg were reported for cleaned, extracted and air dried (15% moisture content) seeds[23]. The seeds are reported contain oil from 35-60%. Oil is edible and can be used for cooking purpose. Oil cake is used as an animal feed. Heating value of oil is 40. 8 kJ/g and that of diesel is 45.6 kJ/g so the oil can be used as bio-fuel also[24]. Characterization of this Desert date plant material showed that using proper cultivation practices with emphasis on low quality irrigation water trees can be extremely well developed in hyper-arid conditions of the Israeli Arava desert and yield oil-rich fruits. Best selected trees can yield date fruits up to 52 kg/trees. Desert date kernels oil content may reach up to 46.7% (based on dry weight) [25].

Micropropagation of *Balanites aegyptiaca* using axillary bud explants obtained from mature trees of *Balanites aegyptiaca* have been reported[26-30]. Cultures were established in Murashige and Skoog (MS) medium supplemented with 11.1 µM 6-benzylaminopurine (BAP) and 0.54 µM naphthalene acetic acid (NAA). The effects of kinetin on shoot growth and proliferation *in vitro* was also investigated. Results show that shoot multiplication required 11.1 µM of BAP. Shoot length was significantly affected by the presence of BAP or 6-furfurylaminopurine (Kin). Rooting of shoots *in vitro* was achieved on MS medium containing 98.4 µM of the auxin, indolebutyric acid (IBA). Rooted shoots acclimated and were successfully transferred into soil, with 48% of the plantlets surviving[26].

With the aim of developing high frequency fast micropropagation protocol for *Balanites aegyptiaca in vitro* germinated seeds were used as source of juvenile seedling root explants[27]. They reported that the root segments, 1-2 cm in length, were inoculated on B5 medium supplemented with low concentrations of different auxins, shoot morphogenesis was observed. The peculiarity of this shoot formation was that these did not arise from the cut end but from the middle region of the segment. Little callus induction also occurred from the cut end of the explant segments.

The callus mediated regeneration system for *Balanites aegyptiaca* (L) Del has been reported[28]. Different explants like apical buds, young thorns and cotyledon pieces from mature tree and root segments from *in vitro* raised seedlings were used for callus induction on MS medium supplemented with 2.23 µM 2,4-Dichlorophenoxyacetic acid. Seven to eight weeks old calli were transferred on hormone free MS medium in order to get regeneration. Shoot morphogenesis was achieved only from cotyledon-derived callus. The shoots so produced rooted well, when cultured on B5 medium supplemented with 9.84 µM Indole-3-butyric acid. Plantlets have been transferred to the field after two-phase hardening and are performing well.

Gour *et al.*[29] evaluated the suitability of stomatal frequency, size of stomata and chlorophyll content as a set of marker traits for predicting the correct stage of hardening for - *Balanites aegyptiaca*. It was found that when number of stomata on lamina of *in vitro* raised plantlet reaches up to that of 60% of naturally growing plant, stomata size attains that of 90% of naturally growing plant and the chlorophyll content reaches up to 95% of the naturally growing plants then plantlets can be considered as enough hardened to survive in field. On the basis of the same criterion 80% success in field has been ensured for this potential biodiesel plant[29].

Gour[30] reported shoot morphogenesis from cotyledon-derived callus in *B. aegyptiaca*. The shoots so produced rooted well, when cultured on B5 medium supplemented with 9.84 µM Indole-3-butyric acid. Plantlets were transferred to the field after two-phase hardening and performing well[30].

Nodal segments including axillary bud from mature tree were used as an explant and their morphogenetic potential was tested on MS media with various concentrations (2.5–15.0 µM) of 6-benzyladenine (BA), Kinetin, and Thidiazuron alone or in combination with different concentrations (0.5–2.5 µM) of α-naphthalene acetic acid (NAA). Nodal segments showed axillary bud proliferation in almost all media tried. MS medium containing 12.5 µM BA alone was effective for inducing multiple shoots. A better shoot differentiation and elongation was achieved in a combined treatment of BA (12.5 µM) and NAA (1.0 µM). Half strength MS medium supplemented with Indole-3-butyric acid (IBA)

gave the best result for rooting. The maximum frequency of root formation (68%), number of roots (5.3 ± 0.32) and root length (4.1 ± 0.38 cm) was obtained on half strength MS medium containing 1.0 µM IBA. The regenerated plantlets were potted and acclimatized successfully in a growth chamber and then moved to the greenhouse[31].

Conclusion - A lot of work ranging from preliminary *in vitro* morphohesesis studies to regeneration of complete plantlets through tissue culture techniques of biodiesel potential has been carried out. However except for a few, most studies did not carry out field evaluation of the regenerated plants after hardening. The most crucial stage that can ensure large-scale success of a tissue culture based protocol is efficient hardening and successful filed trials. Data from field trials can reinforce the efforts that go in a tissue culture laboratory. Moreover, with the availability of a good *in vitro* regeneration system, research on genetic modification for higher oil yield, biotic and abiotic stress tolerance and much more can give encouraging results.

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