EXECUTIVE SUMMARY

UGC SPONSORED MAJOR RESEARCH PROJECT

01/07/2012 to 30/06/2015

Title: Improvement of *Jatropha curcas* biodiesel stability through transgenic approach

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SUMMARY OF THE FINDINGS:

Jatropha curcas oil contains 34.3- 45.8% oleic acid and 29.0- 44.2% linoleic acid (Gubitz GM et., 1999). The esterification of *Jatropha curcas* oil leads to biodiesel with higher amount of linoleic esters. The oxidation rates of methyl esters of oleic (18:1), Linoleic (18:2) and Linolenic (18:3) acid increases in a ratio of 1: 41: 98 (Knothe G, 2005). Higher amount of Linoleic esters cause low oxidative stability of *Jatropha curcas* biodiesel. Hence, *Jatropha curcas* biodiesel meets all the specification of ASTM/ EU/ IS except its oxidative stability. According to Biodiesel European standard EN-14214 and Indian standard IS-15607, oxidative stability of biodiesel should not be less than 6 hrs at 110˚C by as calculated by Rancimat method. However, oxidative stability of *Jatropha curcas* biodiesel is nearly 3.23 hrs (Rakeshsarin et al., 2006). Increasing the oleic acid content will result in high methyl oleate leading to oxidative stability and increased shelf life of biodiesel. Present project aim to develop transgenic *Jatropha curcas* containing high levels of oleic acid and low level of linoleic acid by down regulating *FAD*2 gene. *FAD2* gene is responsible for conversion of oleic acid into linoleic acid. Down regulation of *FAD2* gene will increase oleic acid content in oil.

Regeneration of *Jatropha curcas*

The published protocol (meiru et al., 2007, Plant Cell Tiss Organ Cult, 92, 173-181, 2008) did not work in our laboratory conditions. We standardized protocol as per our lab condition.

Regeneration was initiated from cotyledonary leaves of in-vitro grown 10-12 days old seedlings.Cotyledonary leaves were cut into small pieces and inoculated in various callus induction media. Four different combinations of benzyl amino purine (BAP) and indole butyric acid (IBA) were used for callus induction. In general, callus was of two types: white colored

non-regenerative and yellow colored regenerative callus. The compact yellowish calluses with shoot primodia were further sub-cultured in shoot regeneration media. The best response of 78.3 ± 2.64 percent regenerative calluses was obtained in ½ MS + 2mg/l BAP + 0.03 mg/l IBA media.

Two different media were tested for frequency of shoot regeneration. Maximum shoot regeneration frequency was 2.1±0.3 shoots/callus obtained with ½ MS media supplemented with 2 mg/l BAP, 0.03 mg/l IBA and 1mg/l GA3. There have been various reports in *J. curcas* wherein IBA was used for root induction. Meiru et al. 2007 has reported 85% rooting but we could not achieve more than 15% rooting using their protocol. To improve rooting percentage we tested various IBA concentrations. A maximum of 26% rooting was achieved with MS media supplemented with 0.5 mg/l IBA.

After development of roots, plantlets were allowed to grow for a few weeks and transferred to growth chamber for acclimatization. The plantlets were washed with tap water to remove agar attached with the roots and transferred to pots containing soil rite and soil in a ratio of 1:1. Pots were covered with plastic bags for maintain high humidity. Initially plants were grown in growth chamber at 25 ± 1°C. After 2 weeks, the plastic bags were removed and plantlets were allowed to grow for another 2 weeks. The acclimatized plants were eventually transferred in net house.

Genetic Transformation

Transformation was standardized using a cassette having bar under 35Sde (double enhancer) promoter and Gus with intron under 35S promoter. Bar gene provides resistance against phosphinothricin allowing its use for selection while Gus provides a way to monitor transformation efficiency. To ensure expression of Gus in plant cell, we used Gus where coding region was interrupted with an intron adopted from castor bean catalase gene.

Different components of the cassette were amplified from various sources and brought together with the help of SOEing (Splicing by Overlap Extension). The complete cassette was amplified with primers having restriction sites for and cloned in pGEM-T easy vector. The cassette was excised out with the help of respective restriction enzyme and cloned in binary vector pPZP200.

Transformation of *Jatropha curcas* was performed using the protocol of Meiru et al. 2007 with modifications. *Agrobacterium* strain LBA4404 was used for genetic transformation of *Jatropha curcas*. Preparation of electrocompetent cells and transformation with binary vectors were performed as described by Mattanovich et al (1989). Primary culture of *A. tumefaciens* (strain LBA4404), harboring binary vector of choice, was grown in 10 ml of YEB liquid media supplemented with 50 mg/l rifampicin, 50 mg/l spectinomycin and 50 mg/l streptomycin for 36 hrs in dark at 28°C at 180 rpm. A secondary culture was initiated by using 200 µl of primary culture to inoculate 30 ml of YEB with requisite antibiotics. Secondary culture was allowed to grow till an O.D. $_{600}$ of 0.6, centrifuged and suspended in liquid callus induction media to a final O.D. $_{600}$ of 0.3. This cell suspension was used for infecting cotyledonary explant, precultured on callus induction media for three days. Infection was assisted through vacuum infiltration at 40kPa vacuum applied for duration of 15 minutes. Vacuum infiltration helped transformation as confirmed with Gus activity test. Explants assisted with vacuum infiltration showed higher Gus activity compared to those where vacuum was not used during *Agrobacterium* infection. Excess bacterial culture was blot dried using a sterile whatman filter paper. The infected explants were then transferred to solid callus induction media supplemented with 20mg/l acetosyrinzone and incubated in dark for 3 days. Subsequently, explants were washed with a solution of augmentin (200 mg/l) and blot dried. Finally, explants were transferred to solid callus induction media supplemented with 1 mg/l phosphinothricin (Duchefa) and 200 mg/l augmentin and incubated for 4 weeks at room temperature (25°C) in dark. Approximately 45% of total explants gave phosphinothrycin resistant callus.

Agrobacterium-mediated transformation of *Jatropha curcas* was also carried out using pAS*FAD2* and pNapin-*GUS* constructs. Four independent transformation events were performed with each of the construct. The number of explants that regenerated shoot in selection media was used to calculate transformation frequency. The transformation frequencies obtained in independent events with the construct pAS*FAD2* ranged from 1.95% to 3.72%, while 2.37% to 3.27% in case of pNapin-*GUS* construct. Approximately 38% of shoots rooted in root induction media. A total of 15 transgenics were developed with pAS-*FAD2* while 16 transgenics were obtained with pNapin-*GUS* construct. Approximately 50% of rooted plantlets were successfully acclimatized and transferred to

the net house. The morphological appearance of transgenic plants was normal when compared to those of wild type plant.

Confirmation of putative transgenics

Genomic DNA was extracted from leaves of putative transgenic plants obtained using p*GUS*, pAS-*FAD2* and pNapin-*GUS* constructs. The transformants were analyzed by PCR. A fragment of bar gene (552 bp) was amplified from putative transgenic plants raised using p*GUS* and pNapin-*GUS* constructs while for the pAS-*FAD2* construct both bar gene (552 bp) and the cassette "Napin-*FAD2* AS-35SpA" (1705 bp) were amplified to check the presence of transgene. All the transgenic plants tested showed the presence of transgene. No amplification was obtained from untransformed plants.