Modification of Lignin Polymers with COMT Anti-Sense Gene in the *Jatropha curcas* L. by Green Biotechnology Tools

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ABSTRACT

Lignin, a complex aromatic polymer, is considered a part of the secondary cell wall. Also, it's called a "cell adhesive" based on provides strength and rigidity to plant tissues and fibers through strong cross-linking with cell wall components. Moreover, lignin acts as a line of defense for plants against biotic and abiotic stresses. In the field of plant biotechnology, particle bombardment remains the most important technique to directly transfer DNA from source to target organ and the most widely used method to generate transgenic plants for commercial purposes. Down-regulation of *Jatropha curcas's* lignin-producing caffeic acid 3-O-methyltransferase (COMT) gene production pathway led the transgenic plants to develop cell walls. The transgene COMT antisense is used as a selection marker pZMAS-COMT 10,907 bp plasmid (University of Illinois) to confer resistance to glufosinate herbicides under the 35S promoter. Irradiation of embryonic callus with bullet particles introduced the COMT antisense gene and reduced the amount of lignin. Transgenic green callus was used to extract DNA, RNA, and PCR & RT-PCR were performed to detect the presence of genes. A positive sharp band appeared in the green callus, indicating that the gene expression level was downregulated compared to the control. Transgenic shoots and green callus had lower lignin content compared to controls.

Key words: *Jatropha curcas*, lignin, genetic engineering, tissue culture, PCR, particle bombardment.

INTRODUCTION

To satisfy the worlds projected energy needs, the development of renewable energy essential (Guerrero *et al*., 2021 and Attari *et al*., 2022). As the global population and economy continue to rise, the world's energy consumption is rising quickly (Zhang *et al*., 2022).

All vascular plants deposit lignin, an intricate aromatic polymer, in their secondary cell walls (Kim *et al.*, 2021). Modifying the expression of the enzymes involved for lignin production by genetic engineering is a popular strategy (Furtado *et al.*, 2014). As a result of its close cross-linking to other cell wall constituents, it is sometimes referred to as the "cellular glue" since it gives plant tissues and fibers strength and stiffness (Chandel and Singh, 2011).

Lignin makes up most "lignocellulose" plant components, together with the carbohydrate polymers cellulose and hemicellulose. As a result, lignin makes up a sizeable part of all organic carbon in the biosphere, only being surpassed by cellulose (Zhao and Dixon, 2011). Lignocelluloses have recently discovered to be a plentiful supply of feed stock for the generation of bioenergy (Frei, 2013). Because biofuels are marketed as a way to combat climate change and improve the world's energy supply, (Arshad *et al.,* 2023).

The Euphorbiaceous family includes the species of little flowering plant known as *Jatropha curcas*, plant has been identified as a potential biofuel crop due to its remarkable adaptability. There are around 300 genera and 8,000 species in the enormous and diverse family Euphorbiaceous (EL-Torky *et al.,* 2021 and Njoya *et al.,* 2021).

Over 175 native species of Jatropha, which are found in South to Central America, which is a member of the Crotonoideae tribe Joannesieae (Mukherje *et al.,* 2011).

The development and application of this strategy, which results in the acquisition of desired qualities including increased crop yield, enhanced nutritional content in plants, and resistance to biotic and abiotic stress, has revolutionized agricultural biotechnology and lower costs and increase energy input needs in the manufacture of *J. curcas* ethanol (Sanagala *et al.,* 2017).

The use of somatic embryogenesis as a more effective alternative to propagate plants produced encouraging outcomes of two perspectives: initially, regenerates originated from a two things happened: first, many plantlets were produced, and single embryonic cells were developed just as intended quickly (Borpuzari and Kachari, 2022).

A good substitute method for plant genetic modification is particle bombardment (Matsumoto & Gonsalves, 2012 and Baltes *et al.*, 2017).

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COMT is principally in charge of assisting in the O-methylation of 5-hydroxyconiferaldehyde's 5-hydroxyl group to generate snap aldehyde during the lignin formation process (Maury *et al.*, 2010). The phenolic profiles of *J. curcas*, tobacco, and switch grass have been demonstrated to be impacted by the inhibition of COMT enzyme activity (Quentin *et al.*, 2009).

While the down-regulation of According to their placement in the pathway, C3H or HCT considerably raised the proportion of H units, while the decrease in F5H or COMT expression led to lower levels of S units. Later research using the same transgenic lines revealed a direct correlation between the amount of lignin in the biomass and its resistance to both acid pretreatment and enzymatic digestion (Chen and Dixon, 2007).

Genetic engineering has produced a new trend in the field of lignin incorporation of atypical phenolic monomers to produce a more easily degradable lignin, since drastic reductions in lignin levels have negative effects on plant growth and development, even though large shifts in lignin composition are still tolerable (Vanholme *et al.*, 2012). In *J. curcas*, gene silencing an additional crucial method for gene validation—has also been used (Patade *et al.*, 2014).

The final this study's purpose is to get transformed plants with COMT antisense gene was inserted into embryonic calluses to lower the content of lignin through biolistic particle bombardment used bar gene is a selected marker imparts resilience to the pesticide glufosinate under the 35S promoter in the plasmid pZMAS-COMT.

MATERIALS AND METHODS

Preparation of the culture:-

The used *J.* curcas medium is a Skoog and Murashige medium (Murashige and Skoog, 1962). Agar and sugar (30 g/L) were added to all of the study's mediums as gelling agent (7g /L). Using l N NaOH/HCl, the mediums pH was brought to 5.8. Once the pH was adjusted, the medium were autoclaved for 20 minutes at 121 °C. Culture was kept in petri dishes at 28 °C and grown in the dark for callus ignition and maintenance.

Plasmid structure:-

PZMAS-COMT (Illinois University) which includes (COMT anti-sense gene, selectable marker bar gene) under the control 35 S promoter was used (Fig. 1).

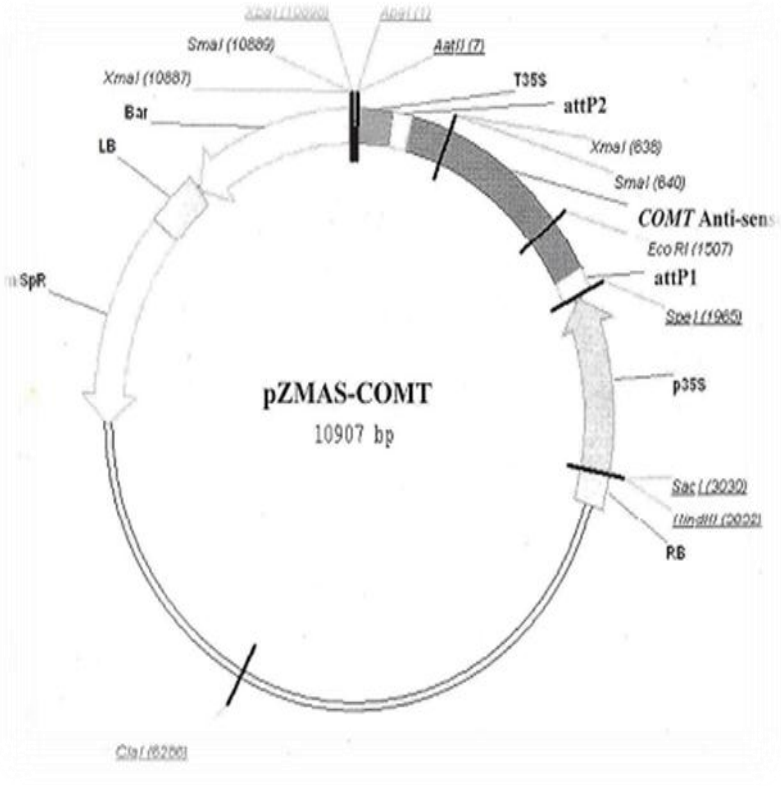


Figure 1. Plasmid schematic drawing pZMAS COMT (10907 bp) consists of bar gene is an adjustable marker that imparts herbicide resistance to glufosinate under cauliflower mosaic virus 35S catalyst.

***Jatropha curcas* L. transformation and regeneration:**

Embryo genic calluses from *jatropha* were employed in bombardment. Callus was moved to osmotic medium (MS medium + 36.4 g/L sorbitol, 36.4g/L mannitol) for 4 hrs. prior to bombardment. The gold particle (Bio-Rad) (1µg) was used to precipitate DNA onto the micro particles 1µl plasmid DNA (stock 1µg µl-¹). then, 220µl (stock 2.5 M) and 50 µl spermidine (stock 0.1 M) were added and homogenized. The was kept on ice for 5 min and vortexes for 5 min, micro centrifuged at 5000 rpm for 1 min rinsed carefully with 250µl of ethanol and suspended in 40 µl 100% ethanol. 10 µl of the DNA coated particles were pipetted onto each macro carrier (washed in absolute EtOH, dried before uses). Non bombardment calluses were used as a positive and negative control.

The embryo genic *J. curcase* calluses were divided into 2 groups; control and treatment which included transformed (shot) calluses. The transformed calluses were transferred to selection medium with three concentrations of inhibitor (PPT) 150, 200 and 250 ppm then to regeneration medium. The control group was divided in to 2 sub-groups both positive and negative control. Negative control includes non-transformed calluses on callus induction medium followed by regeneration medium, not treated with selection medium. While positive control included non-transformed calluses on selection medium. The calluses were transferred on *J. curcase* regeneration medium containing, the cultures were cultured at 25±2 °C under white light with 2.22 µM/L BA and 4.9 µM/L IBA (2500- 3000 lux) 16 hrs of light and 8 hrs of dark. Regeneration rate% = Number of regenerated calluses/ Number of embryogenic calluses x 100.

PCR evaluation:-

Whole genome DNA was taken out of green calluses of *J. curcase* using Doyle (1991) described the cetyl trimethyl ammonium bromide (CTAB) technique. Genomic DNA was amplified through Polymerase Chain Reaction by thermal cycler (Bio Rad). PCR assays were conducted in 20 µl reaction volumes according to instruction supporting with REDTaq® Ready Mix ™ PCR Reaction Mix. Specific primers were used. Primers were designed based on sequencing data of bar gene on the website of National Center for Biotechnology Information (NCBI). Total genomic DNA was amplified through (Bio Rad) Polymerase Chain Reaction (PCR) system cycler Thermo Scientific (Dream Taq Green) master mix (2x) with some important modification, reaction consists of denaturation step at 95 °C for 5min, 35 cycles at 95 °C for 45 sec., annealing temperature at 51.8 °C During 30 seconds, with a 72 °C extension for 30 sec. then, ultimate prolongation at 72 °C for 7 minutes. The amplification products were retrieved in electrophoretic in 1 percent agarose gel with ethidium bromide stains additionally PCR products were imagined by using gel documentation system (Bio-Rad).

RESULTS AND DISCUSSION

Effect of callus morphology on transformation efficiency:-

In the current study, *J. curcase* was used as an experimental explant. The leaves were sterilized and inoculated into MS medium. Addition of 3% (w/v) sucrose to MS medium was followed by solidification using 0.6% agar, and in this experiment two hormones, 2, 4-D (0.5 mg/L) and BA (0.1 mg/L) were added to calli. After 3 weeks, embryogenic callus (yellowish, friable, and easy to separate) was generated. Calli was raised in a similar subculture fresh callus induction medium every 21 days. After each subculture, the total percentage of live calli for BA, and 2, 4-D was calculated as shown in Fig. (2,3). The highest values of live callus were 88 % in callus induction medium supplemented with BA and 85 % in augmented callus induction medium with 2, 4-D, and, respectively. Successful somatic embryogenesis was caused by a number of variables, including the selection of growth regulators and explants as reported by Singh *et al.* (2010). Any plant species' tissue culture process begins with the formation of a callus with strong morphogenetic potential micro-propagation using Murashige and Skoog's (1962) basal media supplemented with various growth regulator formulations, such as 2, 4-D, callus cultures were started from leaves isolated from 4-day-old *Jatropha curcas* L. seedlings and BA (Fig. 4). The medium treated with 0.5 mg/L 2, 4-D produced excellent callus development alone After 7 to 30 days of culture, the callus generated from explants expanded more quickly (Al-Khayri *et al*., 2022). While Ali *et al.* (2015) declare that Callus formation Callus was hard, compact, and yellow with loose, yellowish, and off-white calluses in *Jatropha curcas* use of 0.1 mg/L BA.

Efficient gene transfer *in vitro* plant by particle bombardment in *Jatropha curcas*:-

2, 4-D, and BA somatic embryo genic calli were exposed to particle bombardment. Sixty-four plates were used for experiments on biolistic transformation of target tissues upon entry of micro-particles into cells. The calli were moved to osmotic medium supplemented alongside high amounts of mannitol and sorbitol for 4 h before shooting to reduce cell damage (Fig. 5). Calli were then transformed with the COMT antisense gene.

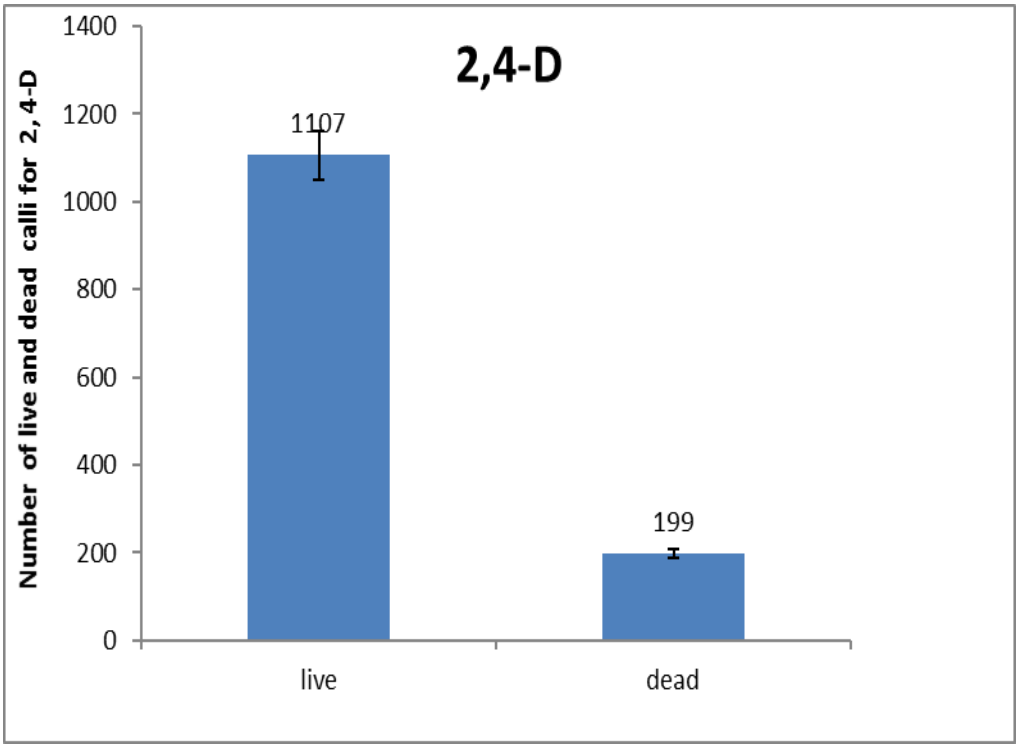


Figure 2. Number of alive and dead calli *Jatropha* callus induction medium utilizing 2, 4-D hormone.

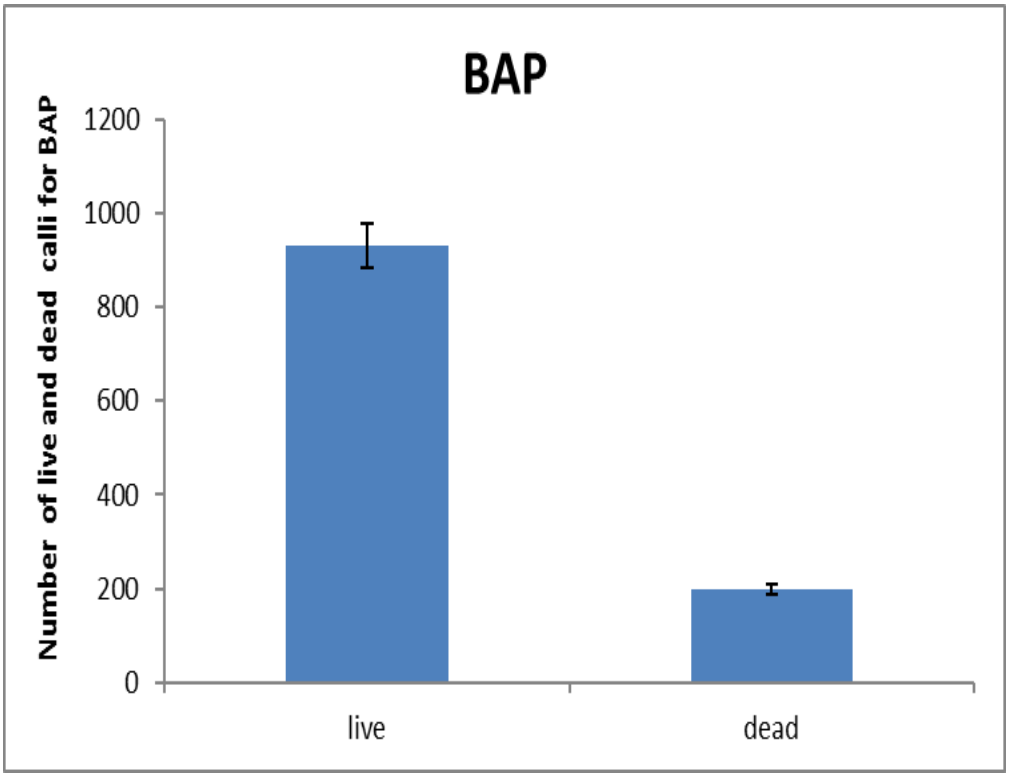
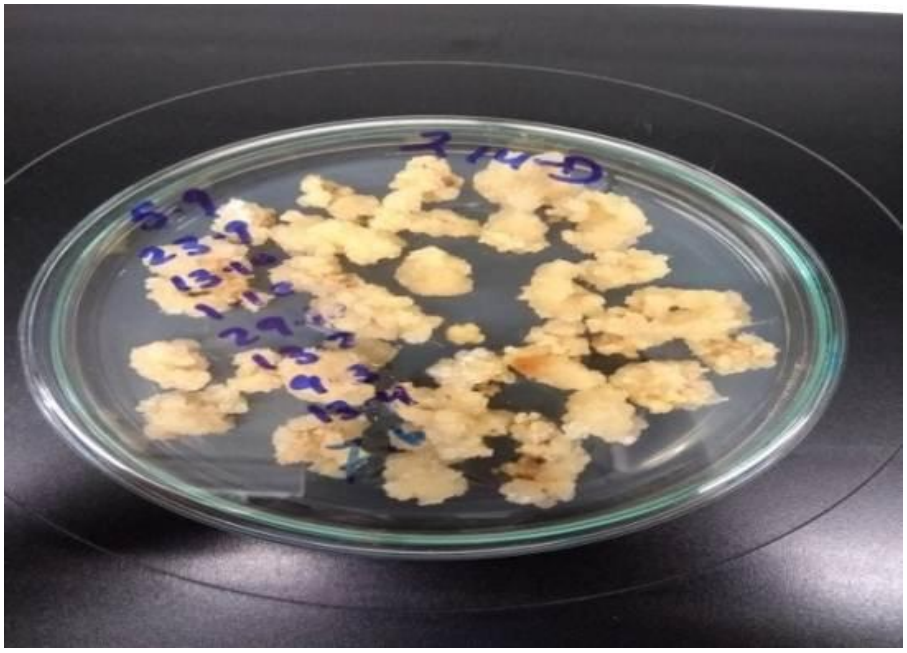
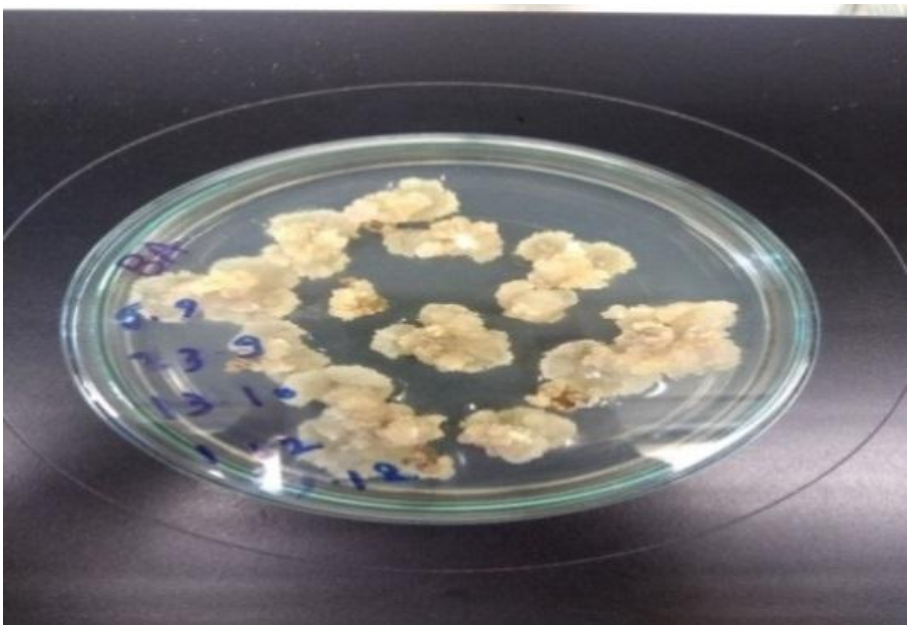


Figure 3. Number of alive and dead calli *Jatropha* on callus induction medium with BAP hormone.



2, 4-D



BA

Figure 4. Growth of *Jatropha Curcase* plant on MS medium (callus induction) using two hormones BA and 2, 4-D.

Three sizes (psi) of rupture discs were used in this experiment: single shot 650 psi, single shot 900 psi, single shot, and double shot 1100 psi for embryogenic callus. Although Gene transformation is a common use of biolistic delivery inconsistencies between irradiated samples often hinder quantitative analysis in transient gene expression analysis. A new combination of impact devices and analytical methods permits simultaneous parameter adjustment and comparison in bullet delivery.

The platform created at this location is broadly applicable to all target samples using biolistic, including animal cells and tissues (Miller *et al.*, 2021). Gene guns are effective devices for introducing molecules into plants, but Reproducibility and consistency can differ significantly between samples. Many Variables have demonstrated influence the effectiveness of the delivery procedure (Sanford 1990; Parveez *et al.*, 1997 and Hamada *et al.*, 2017).

Anti-senses under the dual 35S promoter of the cauliflower mosaic virus (CaMV) (Ko *et al.,* 2020) were applied in each experiment. Techniques to alter lignin profiles have used potent constitutive promoters like CaMV 35S in an effort to change lignification by genetic modification of lignin biosynthesis enzymes successfully induced transgenic expression as well (Li and Zheng, 2017).

The plant's achievement genetics primarily based on creation of genotype-independent, dependable, and effective plant transformation methods. Micro-projectile in the 1980s, bombardment was created as a direct gene transfer technique for the creation of genetically altered plants. For many years, this method and transformation mediated by Agrobacterium have been very popular and used in a number of agriculturally significant crop species for transgenic delivery (Christou, 1995 and Breitler *et al*., 2002).

Transformants' selection and regeneration:

Transformed calli were transferred to selective media containing different concentrations of the inhibitor L-phosphinothricin acetyltransferase (PPT) for three cycles (21 days each cycle) (Joshi *et al.,* 2011). Explants were moved to the aforesaid selective medium with 5 mg/L (PPT) after 15 days. Explants were placed in regeneration media (SRM; MS + 3% sucrose + 0.8% agar + 2.22 μM BA + 0.49 μM IBA) in order to facilitate successful selection at concentration 1 (PPT). After three selection cycles, the putatively transformed calli were transferred to 2.22 μM BA, 0.8% agar, 3% sucrose, and SEM were used for approximately 40–60 days to obtain further green calli (Fig. 6). Data analysis revealed that the optimal rupture disk used for Jatropha transformation was 900 psi, followed by 1100 psi single shot, 650 psi, and lowest was 1100 psi double shot. Regarding PPT concentration, the results show that for Rupture Disc 900, 200 mg/L PPT is the most suitable for selecting between transformed and non-transformed, followed by 150 mg/L PPT and finally 250 mg/L Indicated that it is a PPT. For Rupture Disc 650, the highest concentration was 250 mg/L PPT, then 200 mg/L PPT, and the lowest concentration was 150 mg/L. For Rupture Disc 1100 single shots, all densities are similar and equal. For Rupture Disc, Double Shot 1100 had the lowest cell average (Fig. 7).

Effectively choosing remodeled cells and tissues is a critical a stage in the genetic modification of plants protocols. Most of the time experiments, resistance to antibiotics genes, namely Neomycin phosphor-transferees I and resistance to hygromycin B gene were used in addition to antibiotics as recognizable indicators. This is also true for previous reports on transformation Neomycin phosphotransferase II was employed as a selection marker in the sesame study (Yadav *et al.,* 2010 and Chowdhury *et al.,* 2014b). Bhattacharyya *et al.* (2015) succeeded in creating transgenic sesame plants using the Bar gene, which provides -phosphinothricin resistance, and the broad-spectrum herbicide's active component Bialaphos. Although useful, particle bombardment techniques have multiple shortcomings, including incorporation of redundant DNA sequences ligated into a plasmid vector as well as integration of multiple transgene copies. This might be resolved by inserting the intended coding region into the plant genome's target cells together with only its regulatory elements (Lowe *et al*., 2009). The amount of copies of transgene can also be ascertained similarly to by Chowdhury *et al.* (2014b).

Detection of molecular markers by PCR screening:-

Successfully transformed calli were selected based on resistance and grown after three selection cycles in selection medium containing three different concentrations (150, 200, and 250 mg/L) of PPT. PCR analysis was performed using bar gene primers according to the NCBI and Primer 3 websites. A sharp band was found at 311 bp and was isolated in transformed green callus on regeneration medium. Most transformed calli showed a positive sharp band resolved at 311 bp of DNA using the bar primer. In the case of rupture discs, a sharp 650 psi band appeared on callus transferred from selective medium containing 150 mg/L PPT. On the other hand, a sharp band of rupture disc (900 psi) was found in callus transferred from selective medium containing 250 mg/L PPT. Finally, sharp bands were found at all His PPT concentrations used in this experiment (150, 200 psi). And 250 mg/L were detected These finding with in a line with Bhattacharyya *et al*. (2015), the majority of the non-transformed shoots may be removed with a selection of higher stringency at 2.5 mg/L of PPT. Four distinct series of biological bombardment tests were conducted the 1100 double, single and 650,900 summarize the results of these. Successful transformed calli were selected based on their tolerance which grown after three selection cycles on selection medium containing PPT with three different concentration (150, 200, 250 mg/L). PCR analysis was performed using bar gene primer according to NCBI and primer 3 website.

After rigorous screening, PCR-positive transgenic green calli were formed from resistant calli. Such a rigorous screen was made possible by using the synthetic bar gene, which was reconstituted containing codons tuned for maximal expression in plants. For the representative transgenic events analyzed, we were able to demonstrate steady change in the host and transfer from its artificial bar transgene to its calli.

Sharp bands were found at 311bp were resolve in transformed green calli on regeneration medium. Most

of transformed calli showed a resolved positive sharp band at 311bp for DNA using bar primer. For rupture disc 650 Psi sharp bands was appeared at calli transferred from selection medium with 150 mg/L PPT. On the other hand, rupture disc (900 Psi) sharp band was found at calli transferred from selection medium with 250 mg/L PPT.

While rupture disc 1100 Psi were appeared sharp bands at calli transferred from selection medium 150 mg/L PPT and 200 mg/L PPT. Finally, sharp band was declared at calli transferred from all concentration of PPT used in this experiment (150, 200 and 250 mg/L) (Fig. 8).

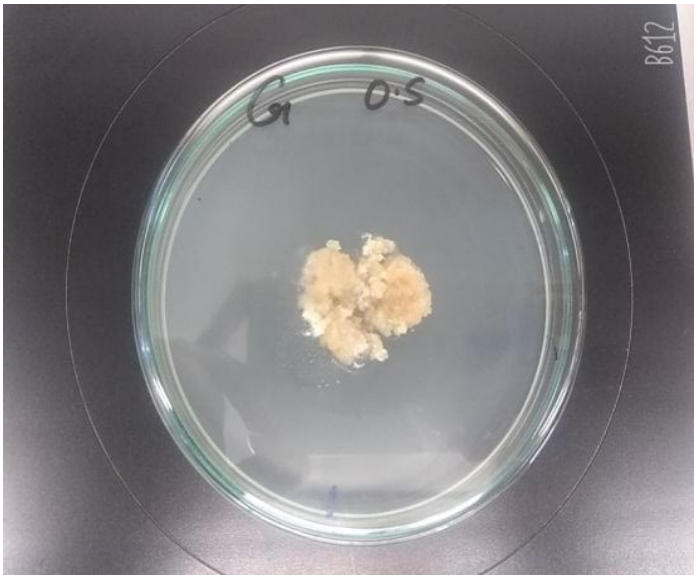


Figure 5. Callus on osmotic medium.

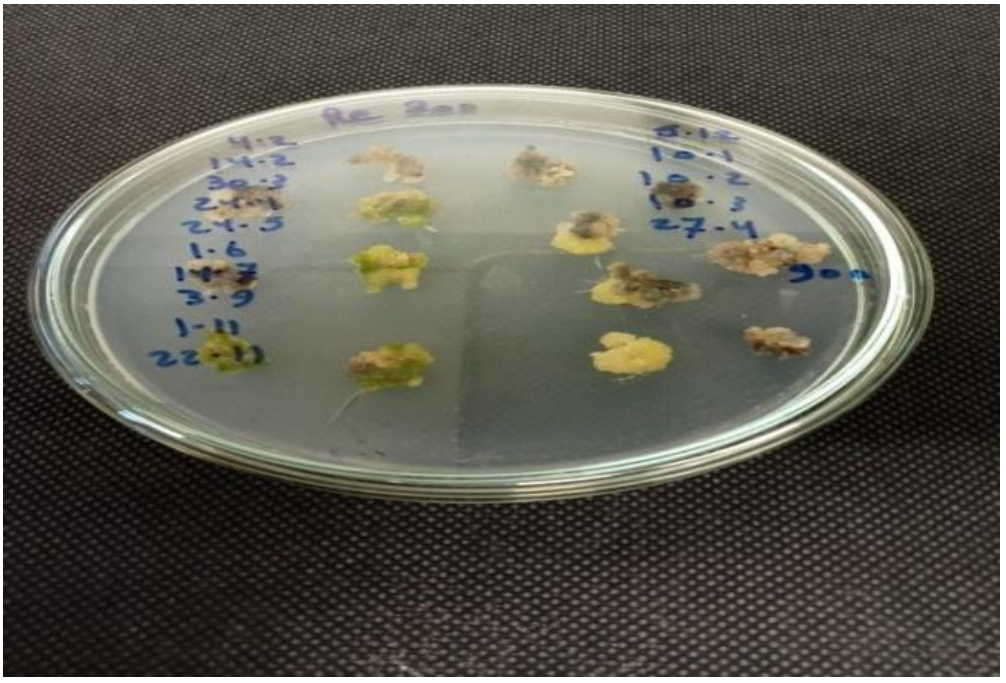


Figure 6. *J. Curcase* calli on regeneration medium with single shot 900 Psi and 200 mg/L ppt.

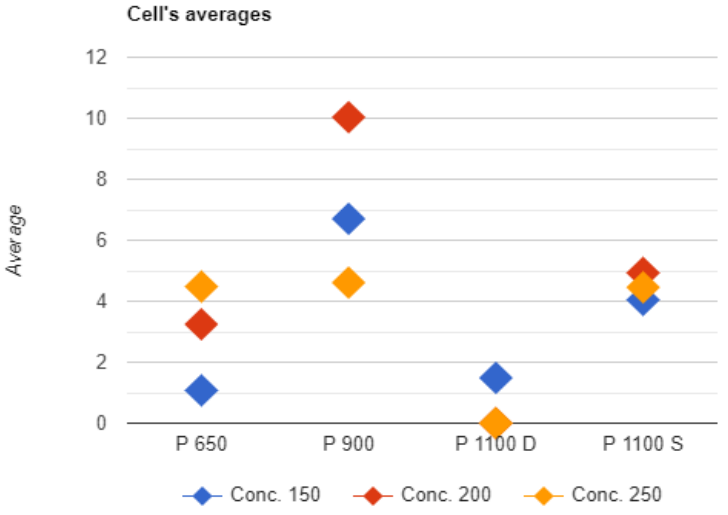


Figure 7. Average *J. Curcase* calli on regeneration medium with single shot 900 psi and 200 mg/L ppt.

M	1	2	3	4	5	6	7	8	9	10	11	12
	+	-	150	200	250	150	200	250	150	200	250	150
			650 psi			900 psi			100 s psi			

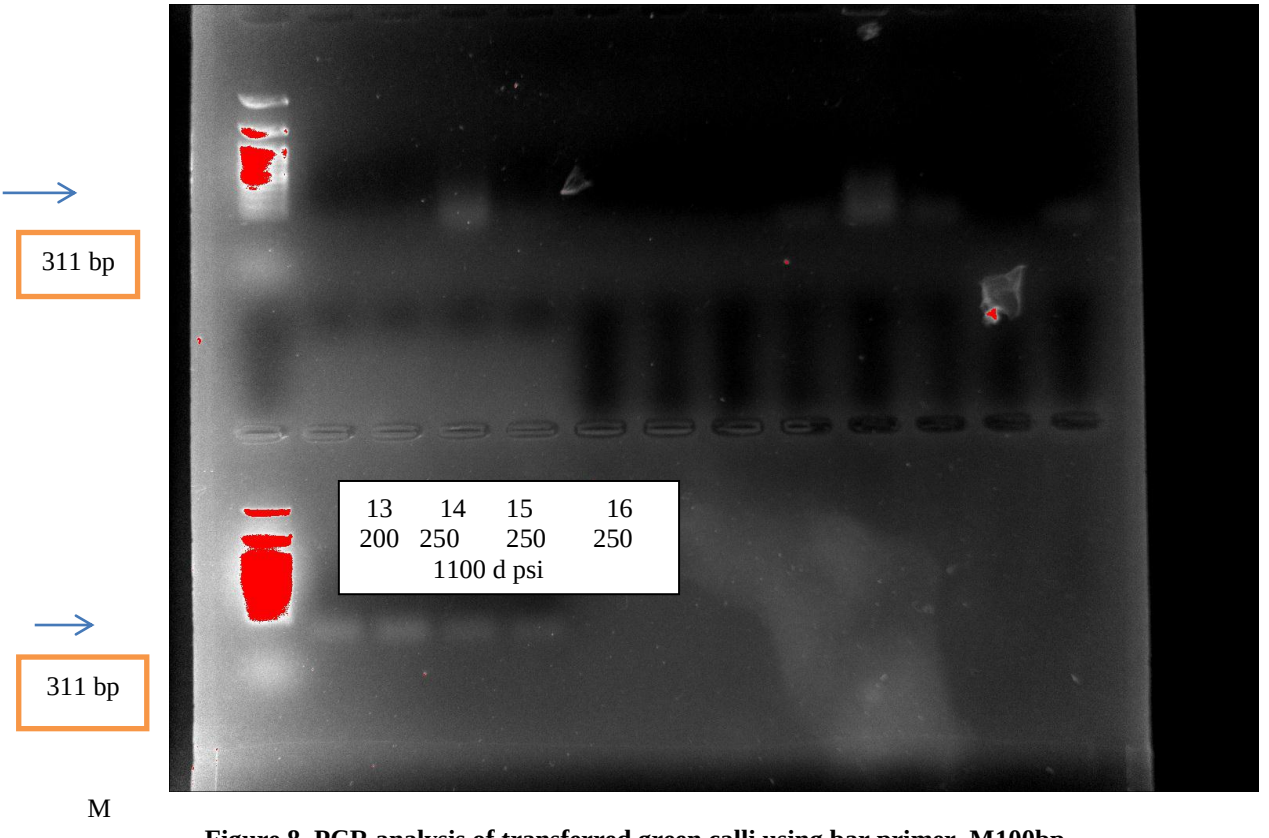


Figure 8. PCR analysis of transferred green calli using bar primer, M100bp.

Real Time q PCR analysis:-

Moderate to elevated levels target gene expression are the most crucial properties in genetically modified approaches in order to accomplish the desired agricultural or business benefits. Therefore, proof of the transgene's steady inheritance and genetic transformation offspring need to be complemented by an analysis of expression levels that allows selecting the transgenic event best suited to the goal. In the current investigation, a thorough expression analysis of COMT transgene was performed quantitatively using (qPCR) RNA levels. All there was consistency between the results. Of the genetically altered calli tested, showed lower levels of transgene expression than compared to control. Incidentally, all transgenic calli showing lower levels transgene expression demonstrated transgene integration on a single location (Fig. 9). This was consistent with several other previous reports (DeBuck *et al.*, 2001, 2004).

Measurement of lignin content in green calli:-

Plant cell walls are made up primarily of lignin. An essential part of the cell walls of plants is lignin. The ethanol production process's hydrolytic enzymes are physically blocked by the structural cross-linking of these polymers convers ion method, restricting its effective application for the synthesis of bioethanol. The results were shown that lignin concentration in exceedingly erratic with in comparing the transgenic family to the control there is an inverse proportion between rupture disc and lignin content by increasing rupture disc used in this experiment the lignin content in control 313 while the lignin content in transformed calli shot with rupture disc (650) was 294.8 on the other hand, the lignin content in transformed calli shot with rupture disc (900) was 123.8. Finally, lignin content in transformed calli shot with rupture disc single 1100 was 97.4 and with rupture disc double 1100 was 52.9. The results indicated that, by increasing the rupture disc, decreasing the lignin content in transformed calli (Fig. 10).

CONCLUSION

Through rapid and high rates of plant leaf growth, the developed protocol is crucial for the mass propagation of elite strains and the genetic improvement of this valuable plant with desirable characteristics. Gene transfer to lower the lignin content of *Jatropha* calli will be very helpful in the future to express genes of interest into biofuel crops for trait improvement, making *Jatropha* ideal as a raw material for biofuels.

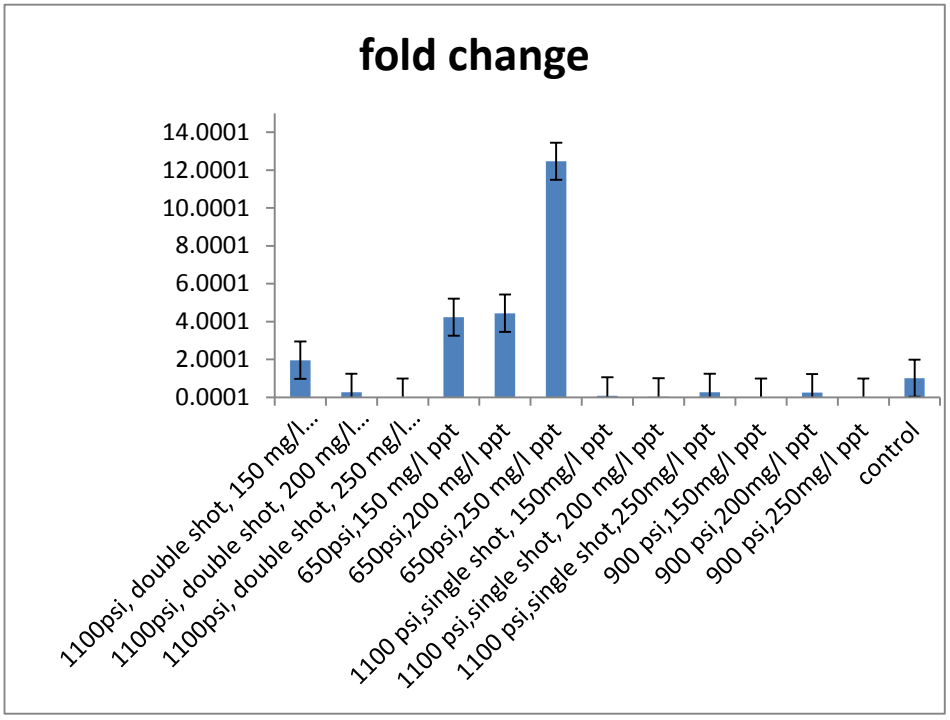


Figure 9. Down-regulation in lignin content for the expression of COMT gene when compared with the control line.

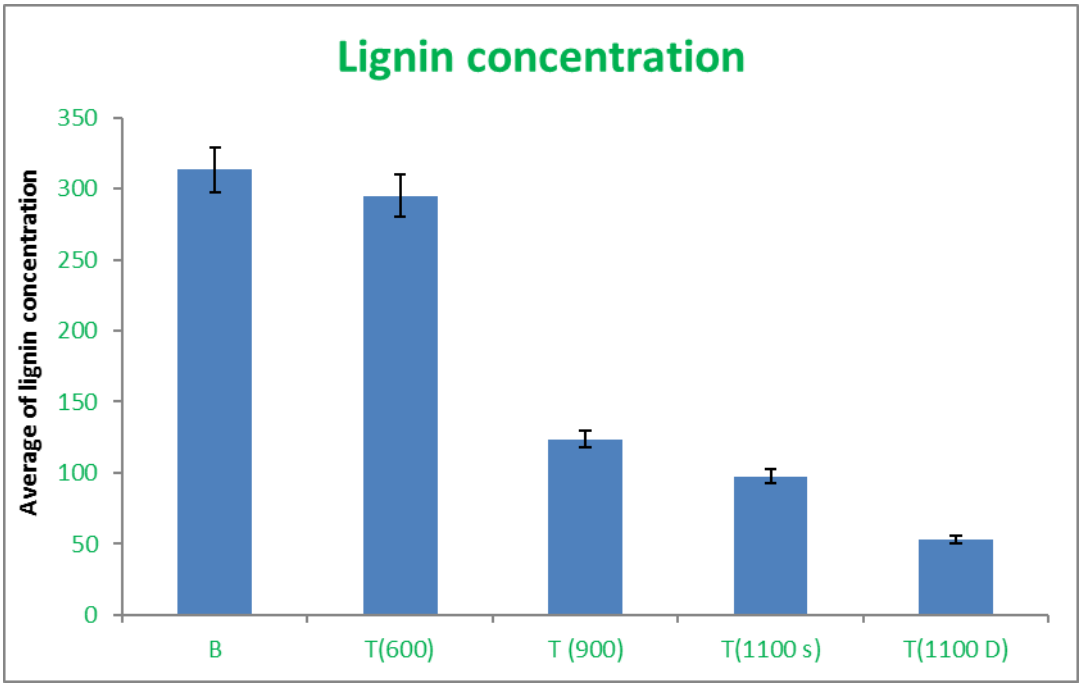


Figure 10. Lignin concentration in exceedingly erratic with in comparing the transgenic family to the control there is an inversely proportion between rupture disc and lignin.

A STATEMENT OF COMPETING INTERESTS

The authors state that they are not aware of any conflicting personal or financial interests that might seem to have an impact on the research presented in this article.

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الملخص العربي

تعديل بوليمرات اللجنين مع الجين المضاد للإحساس COMT في نبات الجاتروفا *Jatropha curcas* L. باستخدام أدوات التكنولوجيا الحيوية الخضراء

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يعتبر اللجنين، وهو بوليمر عطري معقد، جزءًا من جدار الخلية يوفر القوة والصلابة للأنسجة والألياف النباتية من خلال الارتباط المتبادل القوي مع مكونات جدار الخلية. بالاضافه الى ذلك ، يعمل اللجنين كخط دفاع للنباتات ضد الاجهادات الحيوية وغير الحيوية.في مجال التكنولوجيا الحيوية النباتية، يستخدم قاذف الجينات هو الأسلوب الأكثر أهمية لنقل الحمض النووي مباشرة من المصدر إلى العضو المستهدف والطريقة الأكثر استخدامًا لانتاج نباتات محورة وراثيا. أدى استخدام تقنيه تعارض الحمض النووى لمسار إنتاج جينات حمض الكافيين ٣-O-ميثيل ترانسفيراز (COMT) في الجاتروفا إلى قيام النباتات المعدلة وراثيًا بتطوير جدران الخلايا.يتم استخدام مضاد COMT المعدل كعلامة اختيار pZMAS-COMT 10,907 bp plasmid (جامعة إلينوي) لمنح مقاومة لمبيدات الأعشاب الجلوفوسينات تحت بادئ S٣٥ أدى إلى إدخال الجين باستخدام جهاز قاذف الجينات مستعينا بتغطيه الجين COMT بجزيئات الذهب لتقليل كمية اللجنين. تم استخدام الكالس الأخضر المعدل وراثيا لاستخراج DNA و RNA و PCR و RT-PCR للكشف عن وجود الجينات. ظهرت شريط حاد إيجابي في الكالس الأخضر، مما يشير إلى أن مستوى التعبير الجيني تم ضبطه مقارنة بالتحكم، تحتوي البراعم المعدلة وراثيا والكالس الأخضر على محتوى أقل من اللجنين مقارنة بعناصر التحكم.