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Original Research Article

AGROBACTERIUM-MEDIATED TRANSFORMATION OF COTTON (*GOSSYPIUM HIRSUTUM* L.) USING DMO GENE FOR ENHANCED TOLERANCE AGAINST DICAMBA PESTICIDE

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Abstract: *The agrobacterium based transformation of herbicide-resistant crops has modernized weed management in crops by producing cost-effective and ecosystem friendly transgenic plants. Cotton is one of the major crops which are grown worldwide due to its great economic value in textile industries. Dicamba is a commonly used herbicide in broadleaf plants to kill a wide range of weeds in many dicotyledonous crop fields since the 20th century. In this study, Eagle 2 cotton variety was transformed with the DMO gene which is responsible for the synthesis of the Dicamba monooxygenase enzyme that exhibits tolerance against the Dicamba herbicide. This entire study was conducted at Four Brothers Genetics Lab, Lahore. Transformed cultures of Agrobacterium Tumefaciens with the DMO gene were acquired. Cotton embryos were isolated and co-cultivated with transformed Agrobacterium cultures under sterile conditions. Transformed embryos were grown on an artificial growth medium and acclimatized under favorable conditions. Healthy and stable plants were shifted infield where they were grown into a mature plant. Leaf samples of these plants were collected and DNA was successfully isolated by the CTAB method. Transformed plants were confirmed by Polymerase chain reaction and gel electrophoresis. Variations in different traits among transformed cotton plants were found which indicated that the transgenic plant 4 showed higher plant height, monopodial and sympodial branches, leaf length, leaf width, number of bolls, and bolls weight. The better performance of plant 4 indicated that the yield potential of the transgenic plant was improved as compared with other transgenic cotton plants.*

Keywords: *Gossypium hirsutum*, *Agrobacterium tumefaciens*, Dicamba, genetic engineering, herbicide

Introduction

Gossypium is the genus of flowering plants that belongs to mallow or Malvaceae family similar to hibiscus and okra which are natural producer of pure cellulose fiber known as cotton used in the production of fabric products. Fruit of Cotton is like a capsule which is called "boll" that contains seeds surrounded by layers of soft and staple fiber. Cotton plant can grow in both tropical and subtropical regions. *Gossypium* is the largest genus of Gossypieae tribe because it contains 50 species and even more that are yet to be discovered (Jonathan *et al*., 2009). These 50 species are distributed on the basis of their ploidy levels like some of them are diploid (2n=26) and tetraploid (3n=52) (Wendel, 1989; Wendel and Cronn, 2003). The word *Gossypium* is derived from an Arabic word "goz" that literally means soft substances (Gledhill, 2008). Cotton is a cash crop majorly produces in Africa, Australia, America, Pakistan, Turkey and India due its great economical, commercial and industrial importance (Liu *et al*., 2005; Hari, 2007). Seeds of cotton are also used to feed cattle and poultry animals as cottonseed meal. It is also used in paper manufacturing as 75% of paper money is made up of cotton in United States. Income of one billion people depend on the Production of cotton regardless of technology adopted in the field and in the factories (Heinicke and Grove, 2005; Humphrey, 2006). Insects attack is one of the major causes for yield loss in the whole world. Intensive use of herbicides and pesticides has been carried out in pest management to solve this problem in past but it also dangerously affects the non-target organisms in numerous ways. Most common pests of cotton are the species of Lepidopteron such as fall armyworm (*Spodoptera frugiperda*), cotton bollworm (*Helicoverpa zea*), pink bollworm (*Pectinophora gossypiella*), cotton leaf worm (*Alabama argillacea*), tobacco budworm (*Heliothis virescens*) and old-world bollworm (*Helicoverpa armigera*) (Torres *et al*., 2009). Environmental pollution and pest resistance against insecticides are the major problems that are caused by intensive use of these chemicals which leads towards low profit. Many scientists are working on the modification of plants to reduce the usage of

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herbicides and pesticides. Therefore, the economic and environmental needs support the alternative strategies to develop the pest resistant cotton crop. Many farmers used genetically modified plants to increase the yield and enhance the quality of crop worldwide (Pretty, 2001; Uzogara, 2000; Davies, 2007). In Agriculture, primary interest is to bring major improvements in agronomic traits of cotton through conventional methods such as mutation, hybridization and multiline backcrossing (Awan 1991; Opondo and Ombakho, 1997; Carvalho, 1999; Venkateswarlu and Corta, 2001; Ahloowalia *et al*., 2004; Basbag and Gencer, 2007). Multiple cultivar techniques are used separately or in combination with other methods such as single lock descendant, selection of pedigree, cultivar reselection, single lock descendant, forward crossing and back-crossing (Fehr, 1987; Bowman, 2000; Bayles *et al*., 2005).

Above conventional plant breeding techniques are laborious, time taking and low efficient (Fehr, 1987). Various genes in the genome of cotton are responsible for the insect resistant traits (Fehr 1987). Genetic engineering is a transformation technique in which foreign genes are introduced in the genome of host plant through transgenesis or sometimes enhance the expression of already present genes such as genes responsible for salinity and drought resistance. Genes responsible for undesirable traits are isolated from the genome of plant through the process of gene silencing for instance, genes exhibits flavor (Kinney, 2003) in soybeans, toxicity secondary compounds such as gossypol in the seeds of cotton are also injurious to humans (Kumar *et al*., 2006). In order to produce transformed varieties capable to tolerate various environmental stresses such as soil salinity and inadequacy supply of water, there is need to completely understand the regulatory mechanism of genes exhibits plant responses to climate changes (Huang and Liu, 2006; Wang *et al*., 2007). Since the work of Mendelian inheritance in 1866, genetic transformation has been considered as a major progress in the improvement of plant traits. During 21st century, additional traits are also introduced in plants to enhance the nutritional content and yield for medicinal purpose.

Genetic engineering of cotton has tremendously increased the yield and brings the desired characteristics such as resistance against pests, temperature, salinity and disease by altering its genome. Fiber quality and yield has been improved by genetic engineering that further enhance the quality of seed for feed and edible oil, it also increase the production rate of cotton and reduce the cost. In 1987, first transformed cotton was developed in USA termed as Bt cotton (Firoozabady *et al*.1987; *Umbeck et al*. 1987). Cotton based products can be produced through transformed cotton which is highly important for economy. Transgenic cotton was produced via *Agrobacterium tumefaciens* based transgenesis (Adang *et al*., 1989). Major advancements were made in plant molecular research when *A. tumefaciens* were first demonstrated to generate transgenic plants (Barton *et al*., 1983). Transformation in plants allows introduction of foreign DNA in plants cell as well as plants can be regenerated from already transformed cells. Genome of various plant species such as petunia, tobacco, sunflower and carrot were successfully engineered through *A. tumefaciens* based transgenesis. Through wide-spread research, various procedures were established for *A. tumefaciens* mediated transformation of many plant species such as cotton (Umbeck *et al*., 1987), sugarbeet (D'Halluin *et al*., 1992), maize (Ishida *et al*., 1996), wheat (Cheng *et al*., 1997), papaya (Fitch *et al*., 1993), soybean (Hinchee *et al*., 1988) and rice (Hiei *et al*., 1994). *A. tumefaciens* transformation is applied by various agronomists all over the world to bring desirable traits in various monocot species. Although, it is quite challenging while dealing with the cereal genotypes and further studies are required to bring more advancement in it. Plant varieties with certain characteristics have been developed to resist against various herbicides such as glufosinate and glyphosate tolerance plants in order to produce low cost varieties with have improved weed management (Ervin *et al*., 2010; Duke *et al*., 2008). In addition of these herbicide tolerant varieties, plant biotechnologist has developed a new variety exhibit tolerance against Dicamba herbicide (Behrens *et al*., 2007). Dicamba is a commonly used herbicide in broadleaf plants to kill a wide range of weeds in many dicotyledonous crops since the 20th century. Gene responsible for the synthesis of dicamba monooxygenase is first isolated from DI-6 strain of a bacterium *Pseudomonas maltophilia* specialized in the degradation of Dicamba. Dicamba resistant gene is altered through genetic engineering to express in broadleaf crops and provide tolerance against many times higher concentration of Dicamba than the recommended concentration that is used as herbicide in various plants (Behrens *et al*., 2007). Genetically engineered cotton and soybean plants having Dicamba resistant gene are in last phases of development and will be available in market soon.

Dicamba resistant varieties of cotton plant will offer wide range of significant characteristics such as broadleaf weeds management as well as ability of fight with weeds resistant to various herbicides and

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hence, combat against the various new types of herbicide resistant weeds (Behrens *et al*., 2007; Service *et al*., 2007). Dicamba resistant gene is responsible for the synthesis of a vital enzyme Dicamba monooxygenase also known as DMO (Herman *et al*., 2005) is isolated from the DI-6 strain of a bacterium *Pseudomonas maltophilia* (Krueger *et al*., 1989).The function of this enzyme is the elimination of an O-methyl group present in the aromatic ring of the Dicamba herbicide. Its function requires the activity of two other enzymes ferredoxin and reductase along with a reducing agent NADH (Chakraborty *et al*., 2005). Earlier studies suggested that the activity of these other enzymes are not required for the DMO based degradation of Dicamba when targeting the chloroplast of transgenic broadleaf plants such as cotton due to the presence of chloroplast ferredoxin isolated from *P. maltophilia*.

Material and methods

These studies were conducted in Four Brothers Genetics Laboratory, Lahore. The DMO gene cassette was designed by Dr. Arfan Ali from Four Brothers Genetics Lab and the research work were performed there.

Preparation of electro-competent *Agrobacterium* cells

LBA4404 strain of *Agrobacterium tumefaciens* bacterium was cultured on petri plates contains YEP media and isolated complex colonies were appeared. Single colony was collected with the help of sterilized tooth pick and inoculated into 5ml of YEP broth and 100µg/ml concentration of rifampicin was used for the selective growth of desire *Agrobacterium* cells. After that culture were placed on incubation at 28˚C for 2 days. Grown culture was transferred to 100ml of YEP broth. Culture was reserved on orbital shaker at 28˚C at 300 rpm for 48 hours incubation. Furthermore, culture was incubated on ice for 15 min and transferred into 50ml sterilized falcon tube. Cells were collected after 15 minutes centrifugation at 4˚C. Then, supernatant was removed from the solution. After that washed the pellet for two times with 40ml of HEPES solution. Re-suspend the pellet in 1.0ml glycerol solution. Make the aliquots of 80µl as a stock solution then stored at -70ºC in ULT freezer.

Confirmation of transformed Colonies

Whole work was conducted in laminar flow cabinet where YEP cultured cells were set in shaking incubator at 28°C for 60 minutes. Cultures were mixed well by shaking. Then, 100µl of cultured YEP were taken and spread on YEP plates with kanamycin selection. Sterilized spreader was used to spread the whole culture on media. These plates were Kept incubated on 28°C for 48 hours. Colonies were confirmed after incubation.

Colony PCR

Colony PCR was used to confirm the colonies of transformed cells where 3ml of bacterial culture of YEP were taken in a sterilized falcon tube. After that, 100µl of bacterial culture were taken in an Eppendorf tubes. Culture was placed on Centrifuge machine for 10 minutes centrifugation. Supernatant of the centrifuged solution was discarded and pellet was dissolved by 50ul of TE buffer. When the pellet was completely dissolved, then mixture was shifted into PCR tubes. Dissolved solutions were placed on PCR at 98◦C for 12 minutes incubation. After incubation, PCR tubes were placed on short spin for 10 minutes and Template was ready to use in colony PCR. 4ul of supernatant was picked and transferred to another PCR tubes. The master mix was prepared for colony PCR in which $mgcl_2$, buffer, dNTPs, forward primer, reverse primer, taq polymerase and water was added.

Confirmation of *DMO* gene into *Agrobacterium*

Construct of *DMO* gene transformation into *Agrobacterium* competent cells was confirmed when colony PCR was applied as confirmation step of transformation. Primers were designed related to gene construct. Amplify the product at 501 bp region. PCR product was determined at 1% agarose gel electrophoresis for 30 minutes at 120 volts. Predict the bands under UV light.

Preparation of Glycerol stock

Positive clones of PCR were inoculated into YEP broth of 50 ml falcon tubes. The cultures were incubated at 28ºC for 24 hours. They were used as a glycerol stock solution by the addition of 250µl glycerol and 750 µl of bacterial culture was also added in Eppendorf tubes. Glycerol stock was prepared and preserved at -70ºC for future use.

Preparation of competent cells

Secondary culture were prepared from first culture after 48 hours incubation and then, further incubated for 7-8 hours. When incubation was successfully performed the culture tubes were kept on ice for further preservation. The cells were accumulated by centrifugation at 13500 rpm at 4ºC for 10 minutes. Discard the supernatant solution and dissolved the pellet by using 1ml of 0.1M $CaCl_2$. Solution was centrifuged again. Pellet was re-suspended by 100µl of $CaCl_2$. Competent cells ready to use.

Selection of seed variety

Seeds of cotton variety Eagle-2 were obtained from Four Brothers biotechnology and genetics Lab. Seeds were delivered to lab and removed the lint by sulphuric acid (H_2SO_4).

De linting of Seeds

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De linting is a process used to completely eliminate fuzz from seed coat in cotton seed. It's a management technique for crop specific seed. Efficient variety of seeds was taken with high germination rate. 100ml/Kg is a concentration of sulphuric acid (H_2SO_4). When seeds were poured in a beaker 20ml of acid was added and (80ml distilled water). Whole solution was mixed by continuously stirring of spatula for 7-10 times until the lint was removed. Wash the seeds for 5-7 times with tape water to completely remove the residue of acid. Some of the seed was floated and some seeds sink. Only sinker seed was selected for further processing. This process associate the variety Eagle-2 of cotton was selected for transformation.

Screening of Seeds

Process involved screening of seeds. This process is beneficial for decontamination of seed. Damaged seed were removed. Dirt and other trash must be removed. Healthy and disease free cotton seed was taken.

Soaking of Seeds

25g of Eagle-2 variety of cotton seed was taken in a sterilized autoclaved 1L conical flask and autoclaved water was used for soaking of seeds. The seed were sterilized by adding 1ml of 10% SDS and 2ml of 5% $HgCl_2$. The seeds were steeped by putting 100ml of distilled autoclaved water. Shake it gently until the SDS is completely removed from seeds. Desirable amount of water was removed. Aluminium foil was used to cover the mouth of flask. Overall flask was covered by paper and incubated at 30˚C for overnight incubation. Next day wash the seed with 2ml of 5% $HgCl_2$.Shake it gently, until all $HgCl_2$ was removed from seeds. Again covered the flask by paper and overnight incubated at 30˚C. Next day germinated embryos were observed.

Isolation of Seed Embryo

After 48 hours incubation, germinated seeds were grown in flask. With the help of scalpel and forceps the seat coat was removed. Deal the embryo slightly with the help of forceps and pressed the testa of seed with the support of scalpel. Gently embryo was isolated from seed coat. Surgical blade was used for light cut of embryo. Mature embryo of germinating seeds was taken. All healthy embryos were kept in bacterial culture while damaged embryos were discarded.

Co-Cultivation of *Agrobacterium* Inoculum

After embryo isolation, the culture of *DMO* was taken and centrifuged the culture for 5 min at 4˚C at 5000 rpm. Supernatant solution was discarded. Then, dissolve the pellet in 10ml of broth. Further the isolated embryos were transferred to the *Agrobacterium* 4explants4 after placing a cut on them. Embryos were kept on orbital shaker at 28ºC for half an hour.

Preparation of MS medium

MS media was prepared and autoclaved. Cool the media at 50ºC. Cefotaxime (250µg/ml) was added into media. 15ml tubes were taken and 10ml of YEP broth was added that possess 50µg/ml kanamycin for selection. Further addition of glycerol stock of 10µl for *Agrobacterium* retained the *DMO* gene construct. Tubes were kept in culture room.

Shifting of embryos in plates

After co-cultivation, embryos were dried on sterilized filter paper and transferred on MS media plates with the help of forceps. Kept the plate at room temperature for suitable growth conditions where embryos were incubated and grew well.

Plantlets shifting in tubes

Plantlets were produced after 3 days (72h) of co-cultivation. The plantlets that present in plate of MS were taken. The plantlets were shifted in tubes by the selection of kanamycin (500µl) and ceftriaxone (250µl) drug. Standard plantlets were preserved on MS medium. After that, the tubes were covered with pre-sterilized cotton plugs and kept the tubes on plant growth room at suitable conditions.

Time period of 2 months were required for its growth in tubes.

Shifting of plants into pots and acclimatized

Plantlets were mature after 8-10 weeks in tubes, and were shifted into pots containing sterilized autoclaved soil. Fungicide was added in soil to prevent the growth of fungus. MS media was removed from roots by washing with water and dried with tissue paper. The Growth hormone such as indole-3-butyric acid (IBA) was added in roots for better growth of transgenic cotton plants. Plants were covered by plastic bags. To maintain the photoperiod time 16h light and 8h dark, kept the plants at room temperature at 25±2°C. Plants were acclimatized at this stage.

Plants shifting into field

Transgenic cotton plants that were shifted into pots from tubes were acclimated. Plastic bag is removed for 15min interval of light and then time period increases15min onward on daily basis for one month. Plants were daily watered and opened at 10:00 AM onward for 4 weeks. During 1st week plants showed minimal droop due to dehydration, this was retrieved by passing of time. However, the loss of 5% transgenic cotton plants was detected for *DMO* gene after 10-15 days. The healthy plants were acclimated and stabilized were shifted to field (Behria farm) of four bothers.

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DNA Extraction

Transgenic cotton plant with *DMO* gene was examined through molecular analysis. DNA extraction from plants sample can be done by young germinated leaves of the plants that were sited into liquid nitrogen and grinded into a fine powder. The powder was transferred into Eppendorf tubes and 500µl of extraction buffer was added. 400µl of CTAB was added. 10µl of *β mercaptoethanol* was added. Vortex the Eppendorf tubes thoroughly. Incubated Eppendorf tubes in a water bath at 65°C for 90 minutes. After that, 500µl of equal volume of chloroforlm:isoamylalcohol (24:1) were added. Centrifuged the solution at 13500 rpm for 15 min. 500µl supernatant solution was taken, and the pellet was discarded. Repeated the above step until solution is not clear. Again 500µl of supernatant was taken and transferred to a new Eppendorf. 60% of chilled isopropanol was added. Kept the Eppendorf tubes at -20°C for overnight incubation until DNA was clustered. Next day, centrifuged the sample and harvested pellet. Discarded the supernatant solution, pellet was remained in Eppendorf tube. 1ml of washing solution with (70% ethanol) was added to wash the DNA pellet. Again Centrifuged the DNA After electrophoresis, samples are absorb in gel medium and photographed in ultraviolet light genomic DNA.

Polymerase Chain Reaction (PCR) of transgenic cotton plant

PCR (polymerase chain reaction) was used to determine the *DMO* gene in cotton plants. Assembled the mixture with 2µl of DNA, 10x PCR buffer(2ml), 2.5mM Mgcl2 (2.5) 1mM dNtps (2ml) one picomole each primer (2ml) and 2.5µl taq DNA polymerase for a total volume of 20 ml was prepared with gene pellet for 10 min at 13500 rpm. The supernatant solution was discarded, air dried the pellet for 1hour at room temperature. Eluted the DNA pellet with 15µl of nuclease free water. 7µl of Rnase was added. Kept the genomic DNA Eppendorf tubes on water bath at 37°C for 30 minutes to determine the genomic DNA extraction of transgenic cotton plant used agarose gel electrophoresis.

Gel Electrophoresis

Gel electrophoresis is used for analysis of the DNA molecules. It separated the fragments of genomic DNA according to their size and charge, consisted of one glass caster plate and comb which form wells in the gel used to load DNA samples. DNA is a negatively charge, and the movement of charge occurs towards the positive electrode due to electric current.

1% concentrated gel was prepared by adding 200ml of 1x buffer into 1.6g of agarose in a gar. Heat the solution mixture at oven for 2-3 minutes. After that, kept the solution mixture at room temperature for 5 minutes. 5µl of Ethidium bromide was added. Gel was cast into a caster plate.

Gel Documentation

(254nm) with a red filter on the camera. Its ultraviolet light shows the fragments (bands) of specific primer. The process were performed thermocycler PCR machine depends on following conditions, initial denaturation at 95°C for 3 min, then further denaturation at 95°C for 30 sec of 35 cycles, annealing at 60°C for 45 sec for DMO gene, followed by extension at 72°C for 10 min. amplification were completed then products was determined by 1% agarose gel and visualized under UV light.

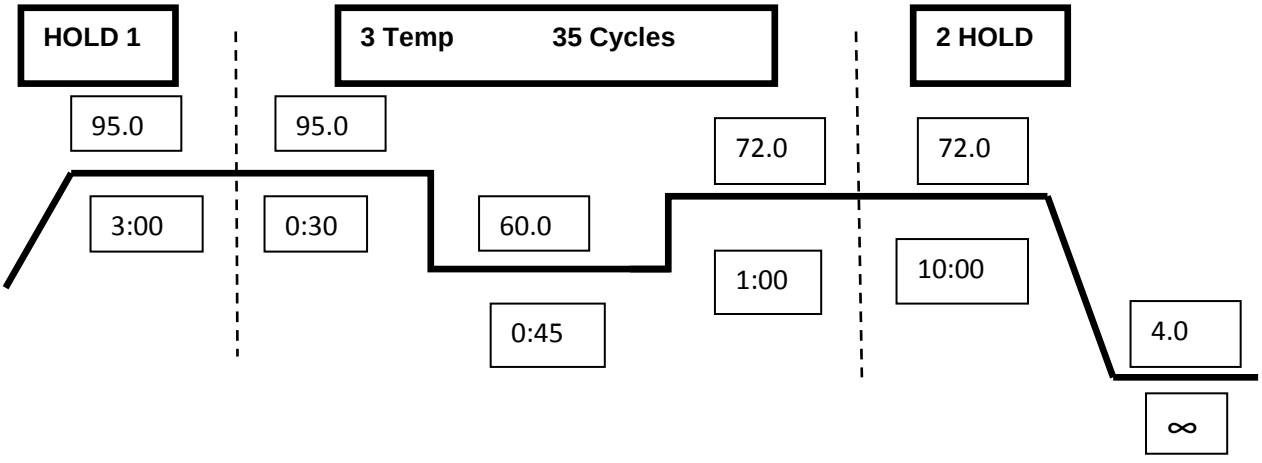

Figure 1: Temperature conditions of PCR for *DMO* gene

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Results

Transformation and confirmation of DMO gene construct

DMO-gene construct was positively transformed into *Agrobacterium tumefaciens* and colonies were used to determine of gene through colony PCR. PCR amplification of 501 bp was done by using primers. PCR resolute the existence of *DMO* gene construct in *Agrobacterium.* PCR products were resolved on 1% agarose gel. DNA ladder of 1 kb plus was used to determine the size of amplified fragments. Positive colonies were formed after 48 hours grown culture as a glycerol stock. Glycerol stocks were preserved at -70°C. Positive clones are used for embryonic transformation.

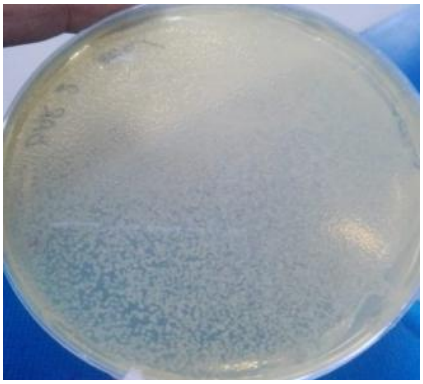

Figure 1: Transformed Isolated colonies

Fuzzy seeds

Fuzzy seeds were covered with lint and it was removed through sulphuric acid concentration. Continuously stirring with spatula was used to completely remove lint from seeds.

Fuzzy seeds **De lint seeds**

Figure 2: De linting of Seeds

Germinated soaked seeds

After 48 hours incubation, the flask having germinated sterilized soaked seeds as embryos germinated. The white embryonic roots were appeared from seed coat.

Figure 3: Germinated soaked seeds

Co-cultivation Isolated embryos

After germination, embryos were isolated and co-cultivated in a culture medium. Then, kept the culture at orbital shaker at 28°C for half an hour. Embryos were washed with MS media until solution was not clear. Embryos were dried on filter paper to transform on MS plates.

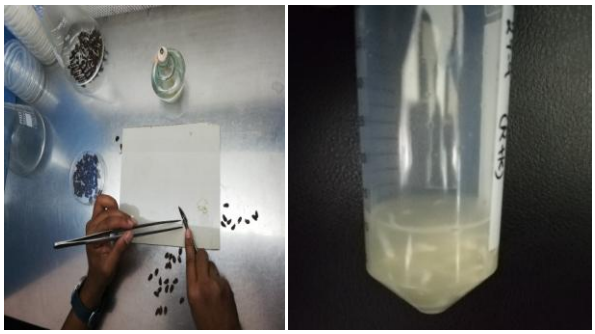

Figure 4: Embryos isolation (Left)
Figure 5: Co-cultivation of Embryos (Right)

Transformation of embryos on MS medium plates

After the co-cultivation of embryos. They were transferred to MS medium plates for the growth of plantlets at specific growth conditions for 3 days. Three days stages are following:

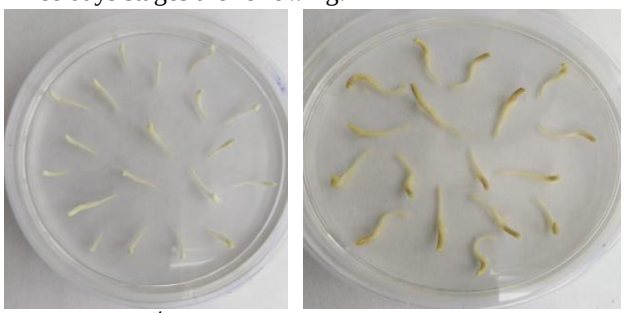

Figure 6.1: 1st Stage of embryo transformation on MS plates (Left)
Figure 6.2: 2nd stage of embryo transformation (Right)

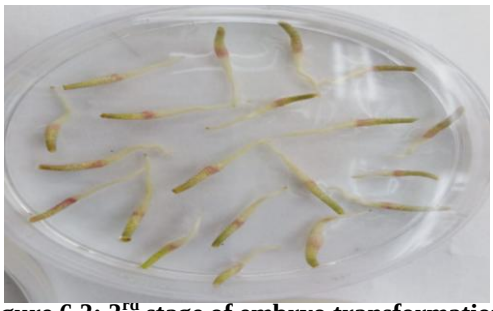

Figure 6.3: 3ru stage of embryo transformation

Transformation of cotton along with Eagle-2 variety and shifted to MS tubes

Transformation of *Gossypium hirsutum* var. Eagle-2 was completed through trials. Full grown embryos were taken as a source of 7xplants and were shifted into MS medium of tubes. Total 10,000 embryos were deal with the DMO construct. Out of 10,000 embryos only 85 plants were persist of following 2 months of time period with the selection of drug as kanamycin hold within MS media tubes. Only 1% transformation efficiency was conducted by the selection of kanamycin. Embryo isolation and incubation was performed according to materials and methods. Different stages were performed and *Agrobacterium* treated show one by one stage of incubation in plates and tubes already mentioned in material and methods. The results indicated that 85 embryos were treated well with selection on MS medium tubes.

Figure 7: Transformed plantlets shifting in test tube of MS media

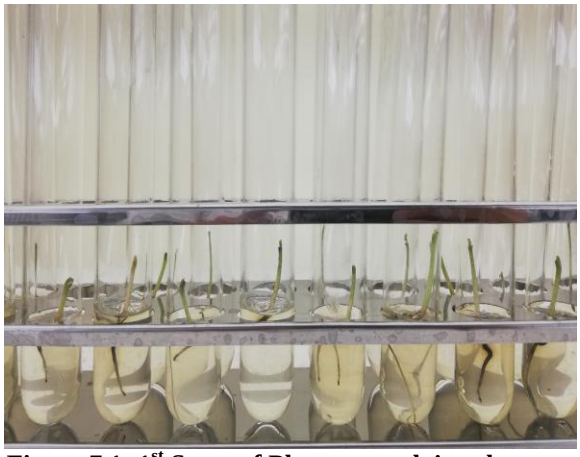

Figure 7.1: 1st Stage of Plants growth in tubes

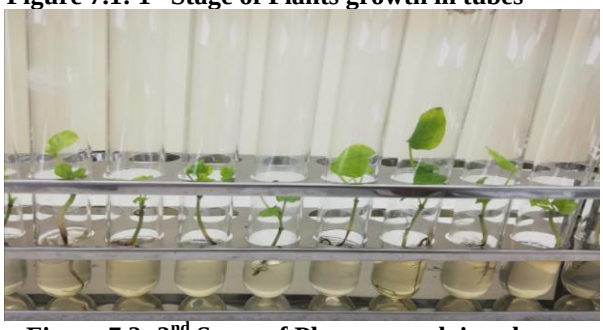

Figure 7.2: 2nd Stage of Plants growth in tubes

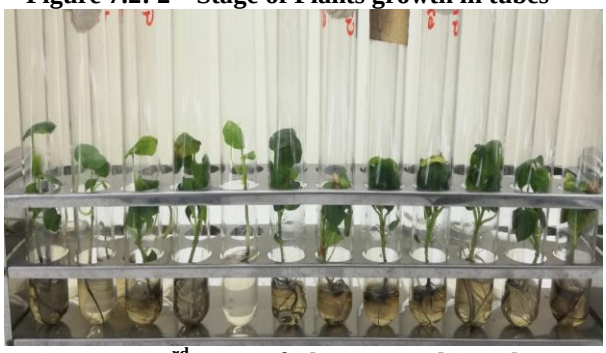

Figure 7.3: 3rd Stage of Plants growth in tubes

Shifting of plants to soil pots

After 7-8 weeks plantlets were mostly grown on MS medium tubes. The result show that these plantlets were further transferred to soil pots.

Figure 8: plants shifting in soil pots

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Plants shifted to field

Plants were developed in pots than at this stage were shifted to field. Herbicides spray was applicable to resist the plants. Some plants were remained after field conditions and gave high amount of productivity. Some were dying at this stage and showing the negative effects for *DMO* gene.

Molecular analysis of Transgenic cotton Plant

***DMO* confirmed through PCR**

DNA extraction of 50 plants were done stated to procedure. PCR was performed regards to *DMO*. PCR product size (501 bp) were amplified by 1 kb plus DNA ladder and resolved on 1% agarose gel. Results indicated that 9 plants out of 21 were shown the positive effect for *DMO*.

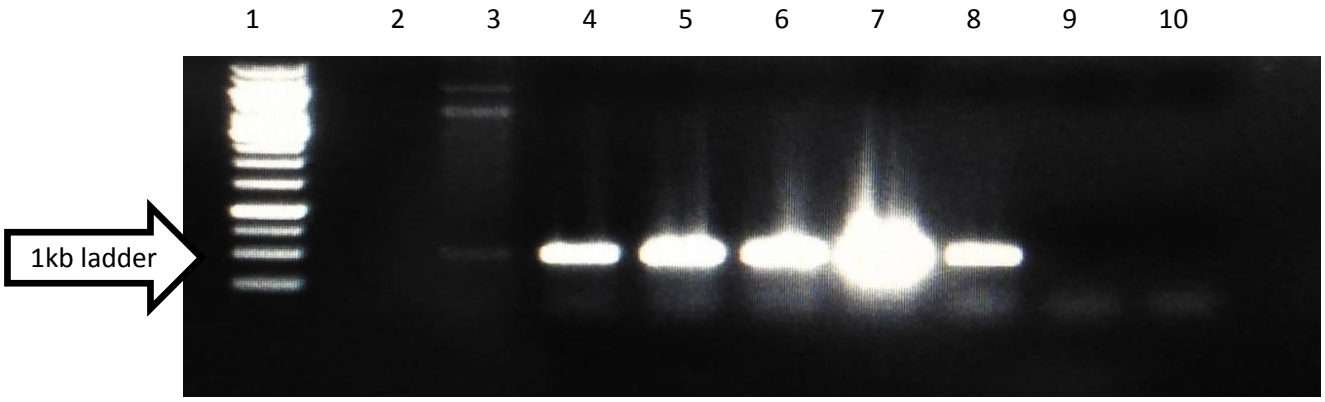

Figure 9 Lane 1: 1Kb ladder, lane 2: negative control, lane 3: positive control, lane 4, 5, 6, 7and 8: positive transformation of DMO gene (PCR product size is 501 bp) and lane 9, 10: negative transformation

Table 1. Analysis of transgenic cotton plants

Plant no.	Height (cm)	Leaf length (cm)	Leaf width (cm)	No. of bolls	Bolls weight (g)	Monopodial branches	Sympodial branches
Control	69	9.5	6.4	31	5.6	8	23
Plant 1	56	9.6	6.2	12	5.8	7	15
Plant 2	54	9	5.1	13	4.4	8	8
Plant 3	63	9.1	5.3	22	5.6	6	20
Plant 4	68	10.4	6.9	39	6.2	13	34
Plant 5	63	8.7	5	32	6	5	20
Plant 6	54	9.2	4.9	34	5.4	9	34
Plant 7	39	7.8	5.8	18	4.8	10	29
Plant 8	46	8.1	5.3	31	5.1	13	27
Plant 9	64	10.3	5.7	18	4	8	20

The plant height was found higher for plant 4 (68cm) while lowest of plant 7 (39cm), leaf length was found higher for plant 4 (10.4cm) while lowest for plant 7 (7.8cm), leaf width was higher for (6.9cm) while lower for plant 2 (5.1cm). The higher number of bolls was found for plant 4 (39) while lower for plant 1 (12), the higher boll weight was for plant 4 (6.2g) while lower for plant 9 (4g), the higher number of monopodial and sympodial branches were for plant 4 (13) and (34) respectively while lower monopodial branches were for plant 5 (5) while lower sympodial branches were found for plant 2 (8).

Discussion

Pakistan's major cash-cow crop is maize, sugarcane, wheat and cotton. Farmers earn their living by selling these crops. Cotton is responsible for providing 80% of raw material to the industries. This crop plant offers maximum yield. The main hindrance in cotton production is viruses, insects, weeds and pests. Weed is responsible for 25% loss of the cotton crop whereas insects are responsible for 20% loss (Liu *et al*., (2005). In our recent research, an attempt on *Agrobacterium mediated transformation* method was done to alter *DMO* gene in cotton. The main goal was not only to transform cotton but also to increase the transformation efficiency and to provide maximum yield. Cotton cultivators found effectiveness in transformation at optimized conditions reliant upon genotype. In order to withstand severe environmental conditions; healthy seed embryo was taken. Related

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transformation efficiency was observed by Puspito *et al*., (2015) Strengthening of 501 bp product through colony PCR for DMO was clarified the productive transformation in cotton plant. Hence, the results proved that the cotton production can be improved by inserting DMO in cotton.

In our current research, by *Agrobacterium tumefaciens* Eagle-2 variety was used for transformation in cotton. Eagle-2 variety of cotton was screened because it is capable to germinate and form mature embryos. The germinated embryos of Eagle-2 variety of cotton were placed in culture medium for co-cultivation for half an hour at shaking incubation at 28◦C. it is compulsory to cut the embryo with the help of scalpel then these embryos are transferred on plates of MS medium. This is done before placing it in culture medium (Davies, 2007; Hari, 2007). After 48 hour plantlets produced on MS medium plates, these plantlets were further transferred to MS medium tubes already having selection of drugs. The results obtained from this showed that some plants survived of MS medium tubes and some died at this stage after 3-4 weeks. Hence, those plants that died could not produce roots on tubes medium. While some of the other plants that survived developed roots and grew well on MS medium after 3-4 weeks. The plants that grew from Eagle-2 variety of cotton were then shifted to soil pots and adapted at growth room at optimum conditions. Some plants that survived were then ready to shift in the field. For the genetic transformation of cotton, we were able to obtain 20 plants from 1000 embryos. After that DNA extraction was performed. After this transformation 9 plants were positive for *DMO* gene and the other remaining 9 showed the negative control for amplification of product 501 bp through colony PCR. After all these processes, the Eagle-2 variety of cotton showed best results (Ahloowalia *et al*., 2004; Jonathan *et al*., 2009). Therefore, Eagle-2 variety was selected for transformation in cotton via *Agrobacterium tumefaciens.* In field condition plants showed variation in different traits among transformed cotton plants were found which indicated that the transgenic plant 4 showed higher plant height, monopodial and sympodial branches, leaf length, leaf width, number of bolls and bolls weight. The better performance of plant 4 indicated that the yield potential of transgenic plant was improved as compared with other transgenic cotton plants.

Conclusion

Genetic Engineering is breading strategy that allows the number of foreign genes (from any source) at a time to be introduced into a plant. Cotton is grown in all parts of the world. The main reason is that it is a good source of economy. It has a good economic ratio and it is also widely used at industrial scale for the manufacturing of household materials and clothing apparel. Due to selective breeding, commercially available cotton have white colour. White cotton is further dyed into different colors with the help of fabric dyes for the manufacturing of clothes. The conducted study was evaluated to examine the positive influence of *DMO* gene on transgenic cotton plant. The samples were collected at Four Brothers Genetics Lab. The *DMO* gene has good potential to come over the problems which are being caused by insects and weeds. The embryos isolation and co-cultivation was conducted in lab under sterile conditions. And hence the plants obtained from following transformation method were then transferred in acclimatized room and adapted on growth room at optimum conditions, and after that shifted to field growth conditions. DNA extraction was carried out using CTAB method on fresh leaves of 21 plants of transgenic cotton which were obtained from the field. PCR was conducted on these plants to confirm the positive influence and successful integration of DMO gene in cotton. After PCR confirmation, some variations were observed in parameters like some plants have high height rate than others. Therefore, leaf height, leaf width, leaf length, Number of bolls per plants, weight of each bolls, bolls of each plant, sympodial branches and Monopodial branches were took into consideration and compared to other plants.

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