CHAPTER THREE
MATERIAL AND METHODS

The present researches was carried out at the Agricultural Botany Department, Biotechnology and the Tissue Culture Laboratory, Faculty of Agriculture, Saba Bash, Alexandria University and Plant pathology Department (Genetics branch), Faculty of Agriculture, Damanhour University, Egypt. The study was conducted during 2010 up to 2014 to detect the most suitable concentration of growth regulators for callus induction from immature embryo explants of the two inbreed maize lines namely, SD7 & SD34 and callus for transformation, of COMT gene by particle bombardment to callus and finally, screening the genetic transformation the gene.

3.1. Plant materials

Two inbreed lines of *Zea mays* L; namely, SD7and SD34 were used in the current study. Seeds of the lines were grown in experimental field (Faculty of Agriculture Saba Basha, Farm Research Station); ears were harvested between 16 and 20 days after pollination then transferred to the laboratory. In this experiment immature embryos were used. Size of immature embryos was 1-4 mm. The ears were surface sterilized for 5 min in 70% ethanol and then for 20 min in 10% Chlorox, followed by three times rinse in sterile distilled water in order to remove excess of the chemical .

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3.2. Extract of immature embryos

According to **Chu *et al*., (1975)** immature embryos were aseptically isolated by cutting the tips of the kernels with a scalpel without touching the embryo.

3.2.1. Initiation medium

The embryos were placed with embryo axis in contact with callus initiation medium using the following medium N6 (**Chu et al., 1975**). N6 medium supplemented with N6 salts, 3 % sucrose, 2.76 g proline, 2 mg/l 2,4-D, 0.1 g/l Casein hydrolysate, 10 mg N6 vitamins, and 8g/l Agar. The medium was adjusted to pH 5.8 using HCl/NaOH prior to sterilization and autoclaved.

Ten embryos were placed in each Petri dish. Cultures were incubated in the dark at 28 ºC. Percentage of immature embryos forming primary callus was recorded two weeks after culture. The developing callus was sub-cultured after

21 days into the callus maintenance medium as for callus initiation. Type of induced callus (embryogenic Type II, non-embryogenic Type I and organogenic) Embryogenic callus was transferred into embryo maturation medium containing N6 medium. Cultures were incubated in the dark at temperature of 28 ºC.

3.2.2. Regulation/Second medium

We used the regulation medium containing N6 salts and vitamins, 2 mg/L 2,4-dichlorophenoxyacetic acid, 3% sucrose,100 mg/L myoinositol, 2.76 g proline, 100 mg/L casein hydrolysate, and 8 g/L Agar, 2% PEG, pH 5.8, and filter sterilized silver nitrate (25 μM) was added after autoclaving according to **Armstrong(1994).** The PEG treatment was carried out by placing ten pieces of callus, each weighing about 100 mg, on the growth medium containing different concentrations of PEG (3,350 molecular weight (MW), Sigma, St. Louis, MO), usually 2%, 5%, 10%,15%, and 20% unless otherwise specified for 21 days.

3.2.3. Callus Induction

The live pieces were transferred to the N6E medium for 21 days two more times each. Dead calluses were brownish and showed no growth. The calluses were weighed before and after each transfer and at the end of the 63-d treatment. The tested media were adjusted to pH 5.8 and autoclaved for 20 minute at 121°C.

Three different concentration of hormones and treatments were used in the present study to select the most suitable concentration. Ten embryos were isolated and cultured on each Petri dish for each treatment of the different cultivars. Petri dishes were sealed with polyethylene film and were placed in incubator 26 – 27 °C.

3 Molecular studies

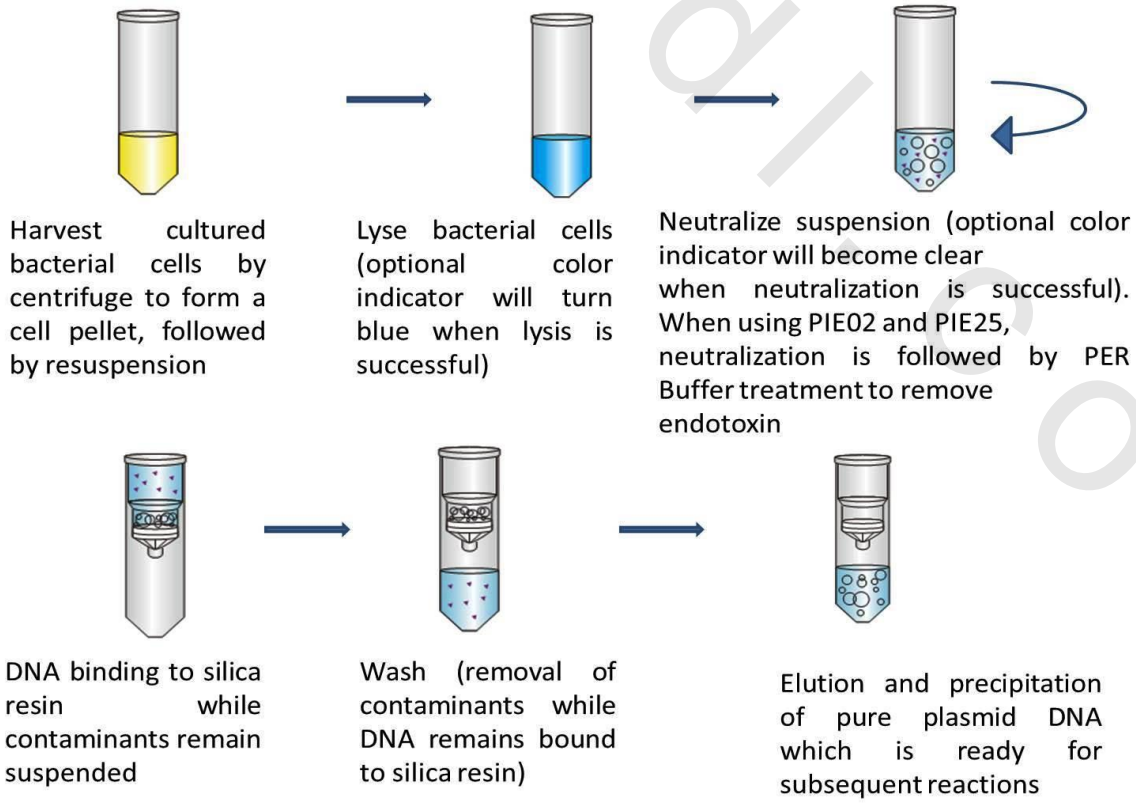
3.3.1. Plasmids component

The following are plasmids component as recommended by **Geneaid Biotech Ltd, company, (www.geneaid.com)**

Component	P1002/piE02	P1025/piE25
PM1Buffer[1]	10 ml	110 ml
PM2Buffer[2]	10 ml	110 ml
PM3Buffer[3]	10 ml	110 ml
PER Buffer (PIE02,PIE25Only)	4 ml	40 ml
PEQ Buffer	12 ml	130 ml
PW Buffer	30 ml	360 ml
PEL Buffer	25 ml	220 ml
RNase A(50mg/l)	Added	200 µl
Plasmid Midi Columns	2	25
True Blue Lysis Buffer	150 µl	1.5 ml

3.3.2. Protocol of the experiment

The following figure includes the different stages to obtain pure plasmid DNA



3.3.3 Transforming plasmids.

Plasmid ZMAS-COMT (Illinois University) as shown in Figure 3 which includes a selectable marker (bar gene) COMT anti -sense gene -decrease lignin content) under the control 35 S promoter was used.

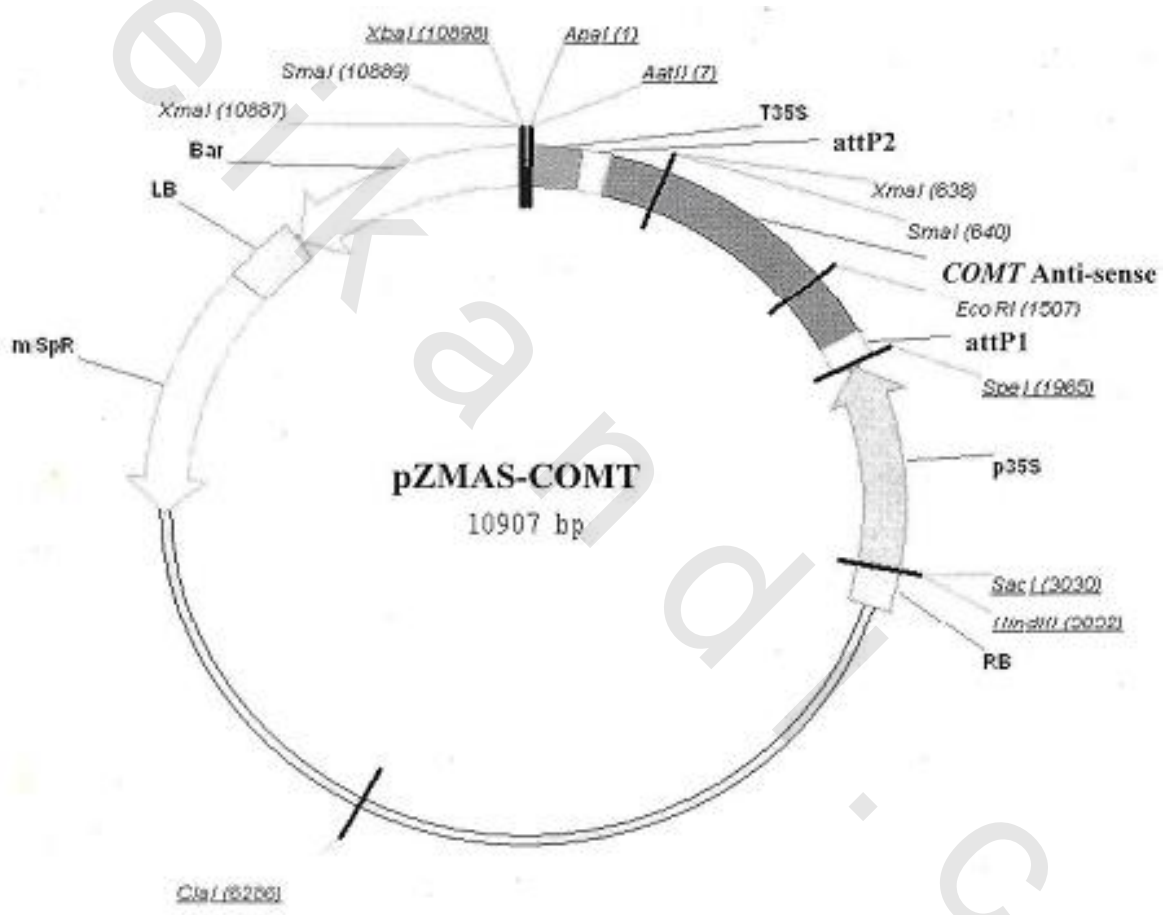


Figure (3) Schematic drawing of the plasmid pZMAS COMT (10.907 bp)

3.3.4. Particle Delivery System (The Biolistic System)

The Biolistic PDS-1000/He instrument uses pressurized helium to accelerate sub-cellular sized micro projectiles coated with DNA (or other biological material) over a range of velocities necessary to optimally transform many different cell types. The system consists of the bombardment chamber (main unit), connective tubing for attachment to vacuum source, and all components necessary for attachment and delivery of high pressure helium to the main unit (helium regulator, solenoid valve, and connective tubing).

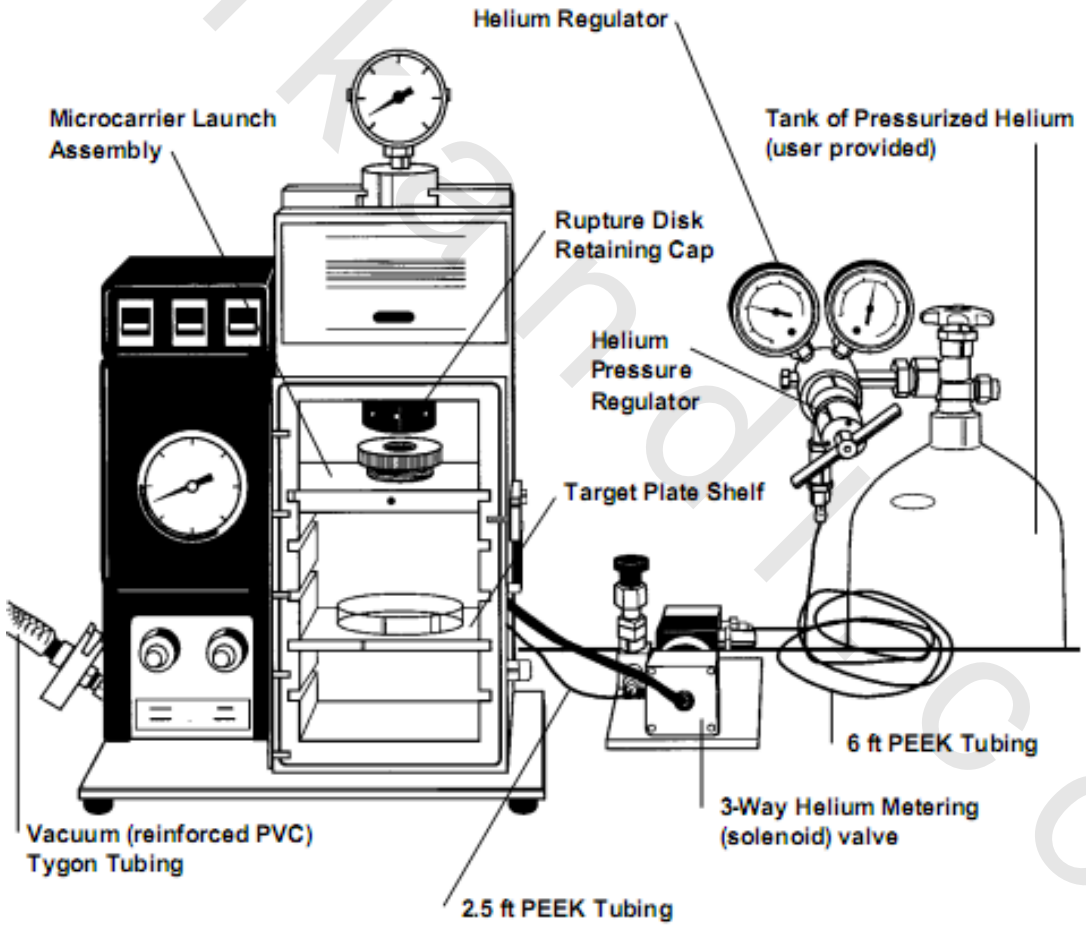


Figure (4). The structure of the Biolistic (Gene Gun)

3.3.5. The Biolistic Process

The Biolistic PDS-1000/He system uses high pressure helium, released by a rupture disk and partial vacuum to propel a macro carrier sheet loaded with millions of microscopic tungsten or gold micro carriers toward target cells at high velocity. The micro carriers are coated with DNA or other biological material for transformation. The macro carrier is halted after a short distance by a stopping screen. The DNA-coated micro carriers continue traveling toward the target to penetrate and transform the cells. The launch velocity of micro carriers for each bombardment is dependent upon the helium pressure (rupture disk selection), the amount of vacuum in the bombardment chamber, the distance from the rupture disk to the macro carrier (A), the macro carrier travel distance to the stopping screen (B), and the distance between the stopping screen and target cells (C).

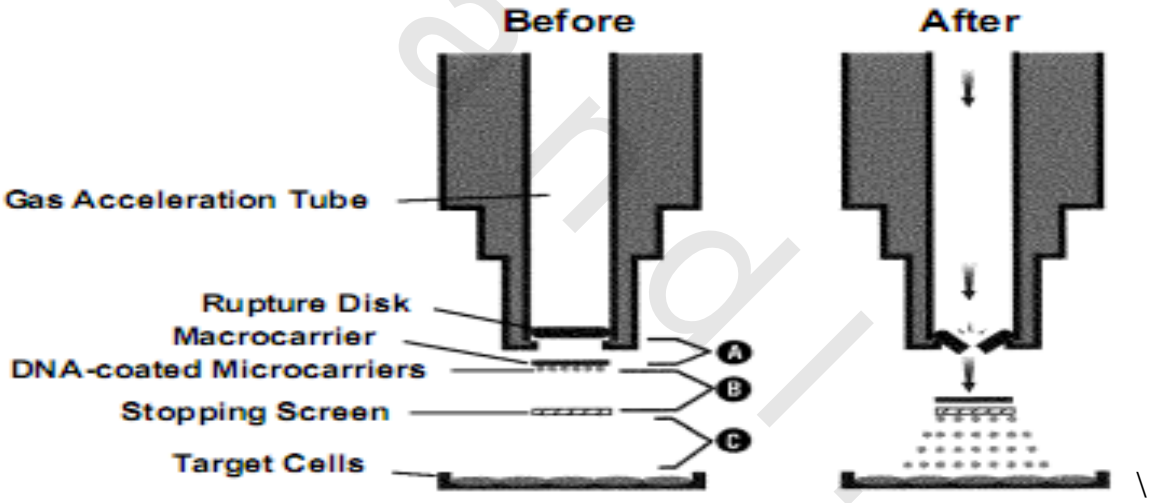


Figure (5). The Process of the Biolistic (Gene Gun)

3.3.6. Transformation with particle gun

Explants were arranged aseptically in a circle with diameter of 25mm on same media just before the bombardment. Plasmid **pZMAS COMT** was isolated by using plasmid Mini prep kit (**Geneaid Biotech Ltd, company, (www.geneaid.com),** following manufacturer's protocol. Transformation conditions were determined using the plasmid **pZMAS COMT** , which harbours the gus reporter gene and the selectable hptII gene, both controlled by the cauliflower mosaic virus (CaMV) 35S promoter.

3.3.7. Preparation of micro carriers

Micro carriers (0.5mg gold) coated with 1g of plasmid DNA and suspended in 50l absolute ethanol, were used as a standard for each bombardment. Gold micro particles were suspended in 1ml 70% ethanol (v/v) by vigorous vortexing for 3–5min followed by soaking for 15min. Micro particles were washed 3 times with 1ml sterile water by spinning for 30 s in a microfuge. After third wash, micro particles were suspended in sterile 50% glycerol and coated with plasmid DNA (**pZMAS COMT**) using CaCl2 (2.5M) and spermidine (0.1M) precipitation method.

After 10min incubation on ice, the supernatant was removed and pellet was washed with 70% (v/v) ethanol followed by washing with absolute ethanol .After washing, the particle DNA pellet was re-suspended in absolute ethanol for bombardments. Care was taken to ensure uniform particle distribution and minimize agglomeration.

3.3.8. Micro projectile bombardment

Embryogenic callus from variety sd7 and sd34 was produced from selections of using two Inbred lines, It was used for particle bombardment. Four hours prior to bombardment, callus was transferred to osmoticum medium N6 medium **(Chu *et al* , . 1975)** 36.4g/l mannitol. Plasmid DNA was absorbed on gold particles (1.0ȝ m, BioRad) according to **Wan *et al*. (1995).**

Bombardments were done with biolistic gene gun (PDS1000/He, Bio-

Rad) under a vacuum of 27 in. of Hg, a 25mm distance from rupture disc to macro carrier and a 10mmmacrocarrierflight distance for all bombardments. The variables to be optimized included five rupture disc pressures (650, 900, 1100, 1350and 1550 psi), four micro projectile travel distances (3, 6, 9, and12 cm) and micro carrier size (gold particle size 0.6, 1.0 and 1.6m).Non-bombarded embryo axes and embryo axes bombarded with uncoated micro carriers were used as controls.

3.3.9. Selection and transformants

After bombardment, callus were cultured on callus induction medium for7-10 days recovery and then transferred to the same culture medium with three concentrations of phosphinothricin (PPT) 3, 4 and 5 mg/l for selection of resistant callus) complete darkness, 28^{o}C .(About 30 pieces of callus were cultured per Petri dish and subcultured every 21 days

3.3.10. Polymerase chain reaction (PCR)

Genomic DNA was purified from callus for PCR analysis by using CTAB method (**Doyle and Doyle, 1990).** All of the putative 50 transgenic plants were analyzed by PCR for the presence of the bar gene as selectable marker using specific primers (5 'TGCACCATCGTCAACCACTA3') and (5 ' ACAGCGACCACGCTCTTGAA3').

The PCR reactions were carried out in a total volume of 20µl, containing 100 ng of each transgenic lines or wild type control maize genomic DNA, 2 µl (10×) buffer,10mM dNTP, 2 units of Taq DNA polymerase and 1.0 µl 10 mM of each primer.

PCR amplification utilized an initial melting step of 94oC f or 5 m in, followed by 30 cycles at 94^{o}C f or 45 sec., 60^{o}C f or 45 sec .and 72^{o}C f or 1 min.

The final products were annealed f or 7.0 min at 72^{o}C, and stored at 4^{o}C Reactions were carried out in 200 µl eppendorf tubes and amplifications were performed in a AB applied Biosystems 2720 Thermal cycler. PCR products were analyzed by electrophoresis through 1.0 % agarose/TAE gels where the expected size was approximately 311 bp f or bar.