# CHAPTER THREE
# MATERIAL AND METHODS

The present experiments were carried out at the Agricultural Botany Department, Biotechnology and the Tissue Culture Laboratory, Faculty of Agriculture, Saba Basha, Alexandria University, Egypt and Plant pathology Department (Genetics branch), Faculty of Agriculture, Damanhour University, Egypt. These studies were conducted during 2010 up to 2014 to evaluate the most suitable concentration of growth regulators for callus induction from mature and immature embryo explants of the maize hybrids, regenerate and high throughput callus for transformation, of *Anthranilate synthase* genes by particle bombardment to callus and finally, screening the genetic transformation of these genes on the end products.

### 3.1. Plant materials

Tow Egyptian single cross maize (*Zea mays* L.) hybrids; namely, SC168, SC10 were used in the current study. Seeds of the hybrids 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 we used mature embryos for SC 168 and immature embryos for both hybrids. 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 40% SAVO. This was followed by three times rinse in sterile distilled water.

### 3.2. Mature seeds and immature embryo

Mature seeds were used as explant source. Whereas, seeds of each cultivar were washed under running tap water for 10-15 mint and then surface-sterilized sequentially with 70% ethanol for 3-5 minutes followed by three rinses with autoclaved distilled water in the laminar flow cabinet. The mature seeds were sterilized with 10% commercial bleach Clorox (5.5% sodium hypochlorite, v/v) supplemented with 1–2 drops of Tween 80 for 20 min followed by three rinses with sterilized water ddH2O.

Sterilized seeds were rinsed five times with sterilized water in order to remove excess of the chemical and imbibed in sterile water 20-22 hours, at

room temperature in complete darkness according to **Yin, *et al.*, (2011) and Ashraf and Osman, (2004).**

### 3.2.1. Extract of the mature and immature embryos

The mature embryos of each cultivar were aseptically isolated from the freshly imbibed seeds with a sharp knife and ten mature embryos were cultured with scutellum side by scalpel and forceps in petri dish with 10 cm diameter containing about 25-30 milliliters N6 medium salts **(Chu *et al.*, 1975).** Immature embryos were aseptically isolated by cutting the tips of the kernels with a scalpel without touching the embryo.

### 3.2.2. 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.dm 2,4-D, 0.1 mg.dm Casein hydrolysate, 10 mg N6 vitamins, and 8g Agar. The media was adjusted to pH 5.8. pH of the media was adjusted to 5.8 using HCl/NaOH prior to sterilization and autoclaved.

Twenty 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 14 days into the callus maintenance medium as for callus initiation but without silver nitrate. Type of induced callus (embryogenic, non-embryogenic and organogenic) Embryogenic callus was transferred into embryo maturation medium containing N6 medium supplemented with N6 vitamins, 3% sucrose, 8 g Agar for embryo maturation. Cultures were incubated in the dark at temperature of 28 ºC.

### 3.2.3. 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 caseinhydrolysate, and 8 g/L Agar, 2% PEG, pH 5.8, and filter sterilizedsilver 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.

### 3.2.4. Calli 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. 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.

### 3.3. Molecular studies

### 3.3.1. Plasmids component

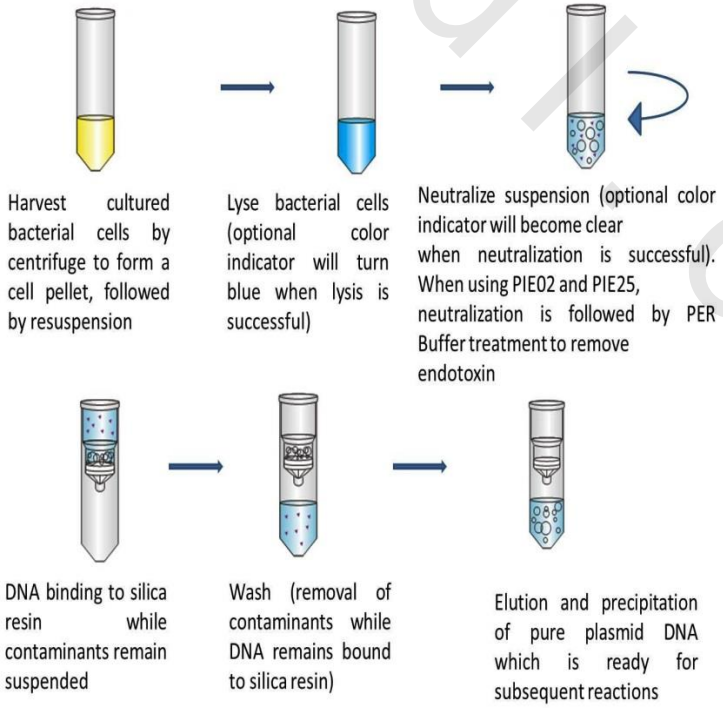
The following are plasmids component as recommended by **Geneaid Biotech Ltd, company, (www.geneaid.com)**

**Table (1) plasmids component which used in the current study**

| 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. Plasmid extraction

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



### 3.3.3. Plasmid Structure

The plasmid used in this work, pC2ASA2-NOS-ASB 16.6 kb (Illinois University), (fig, 1) contains a selectable marker (ASA2 α & β) the coding region of kanamycin, gusA, under control of the cauliflower mosaic virus (CAMV). ANOS polyA (nopaline synthase) terminator sequence

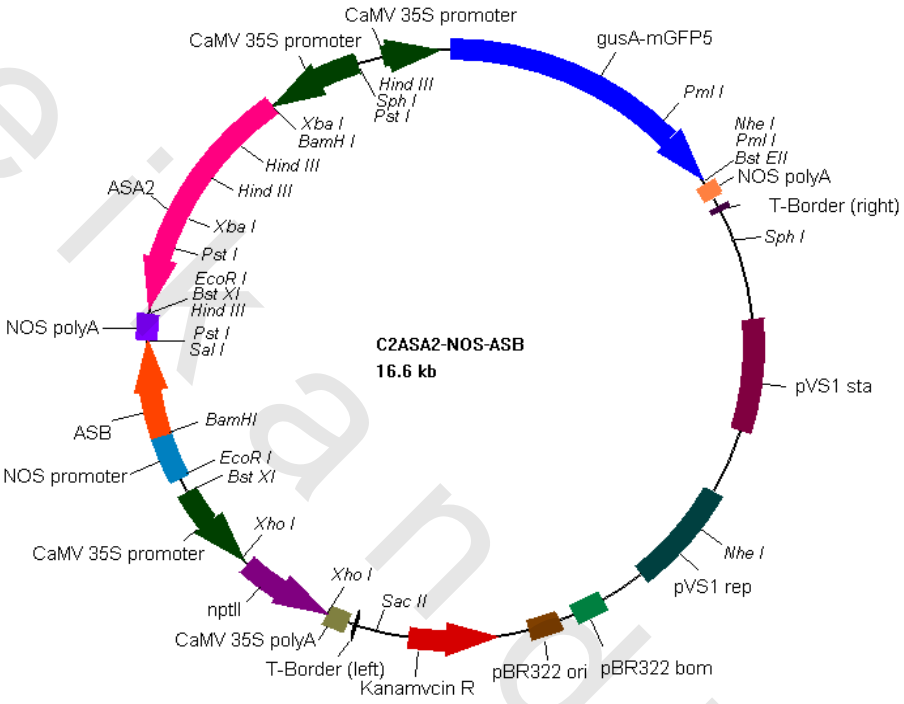



**Figure (1). Schematic drawing of the plasmid C2ASA2-NOS-ASB.The plasmid 16.6 kb consists of ASA2,ASB, CaMV35S : cauliflower mosaic virus 35 S promoter and 3'NOS : the polyadenylation signal of nopaline synthase.**

### 3.3.4. Maize transformation

Friable callus was used for particle bombardment. Callus was transferred to osmoticum medium (N6 medium + 36.4 g/L sorbitol and 36.4 g/L mannitol) for 4 hours prior to bombardment.

The gold particles (Bio-Rad) (1µg) were used to precipitate DNA onto the microparticles 1µL plasmid DNA (stock 1µg $\mu L^{-1}$). Then, 220µL(stock 2.5 M) and 50µL spermidine (stock 0.1M) were added and homogenized. The mixture was kept on ice for 5 min and vortexed for 5 min, micro centrifuged at 5000 rpm for 1 min, rinsed carefully with 250 µl of ethanol and suspended in 40 µL 100% ethanol.

10 µl of the DNA –coated particles were pipetted onto each macrocarrier (washed in absolute EtOH, dried before uses).Bombardments were performed on Petri dishes containing friable callus clump in the middle.

Different treatments were designed to test the pressure of the accelerating helium pulse (900 and 1100 psi single and double shout).Selection of putative transformed callus began 10 days after bombardment when the callus were cultured every 21 days on N6S selection medium which was similar to callus initiation medium but without proline and casein hydrolysate, filter sterilized silver nitrate (5µM) was added after autoclaving, 6- Methyl-DL-tryptophan (6MT) 100 µM was added also.

### 3.3.5. PCR analysis

For the molecular characterization of the transgenic plants generated, total genomic DNA was isolated from leaf tissue of primary leaves using a CTAB protocol described by (**Doyle and Doyle 1990**) and submitted to PCR

For the presence of ASB, the primers 5' TGTCCAAGATCCCATGACGATTCC3'and5'CAGAAATCCACAGAACCG GGAGAT 3', which amplify 800 bp were used.

For the ASA2 (α&β), the primers 5'TCTGTACACTTCAAATGGGTCAGC3'and5'CTAAAAGCGGGAACTTG ATTCCGC3' which amplify 815 bp were used and for nptII the primers 5' ATCTCACCTTGCTCCTGC3' and 5'ATACCGTAAAGCACGAGG 3'which amplify 200bp were used.

Each 20 µl amplification reactions mixture consisted of 100ng of template DNA,2 µl of Taq buffer 10X, 0.5 µl of dNTPS 10 mM, 1 µl of each

primer, and 0.3 µl of Taq polymerase. The reactions were carried out using a thermal conditions: 1cycle at 94$^{o}$c for 5 min, 35 cycles at 94$^{o}$cfor 45s,annealing at 54.6$^{o}$cfor 45s ,72$^{o}$cfor 60s and a final extension at 72$^{o}$c for 7 min for GUS, 1cycle at 94$^{o}$c for 5 min, 35cycles at 94$^{o}$cfor 30s,annealing at 57$^{o}$cfor 45s ,72$^{o}$cfor 60s and a final extension at 72$^{o}$c for 7 min for ASA2, and1cycle at 94$^{o}$c for 5 min, 30cycles at 94$^{o}$cfor 45s,annealing at 55$^{o}$cfor 30s ,72$^{o}$cfor45s and a final extension at 72$^{o}$c for 7 min. The amplified products were separated by electrophoresis on 1% agarose gel and visualized with ethidium bromide stain under UV light.

### 3.3.6. 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).

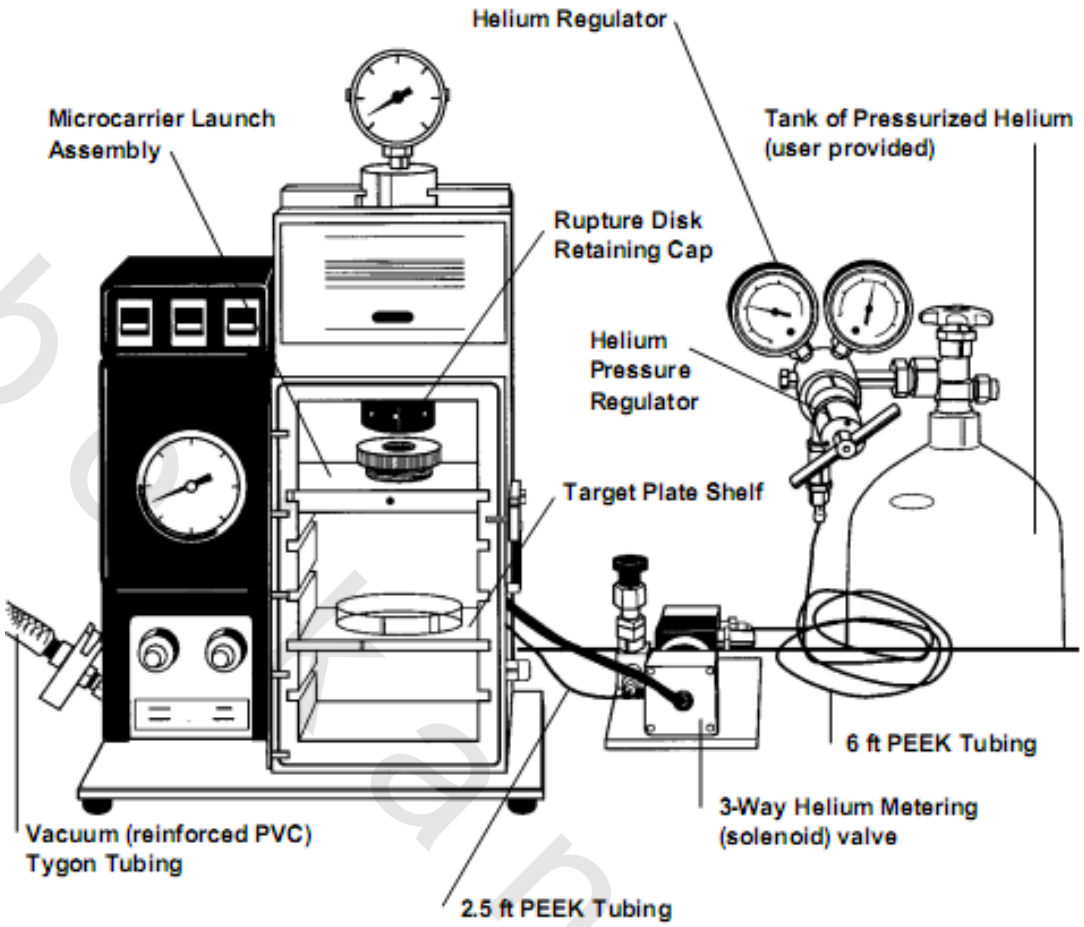



**Figure (2). The structure of the Biolistic (Gene Gun)**

### 3.3.7. 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).

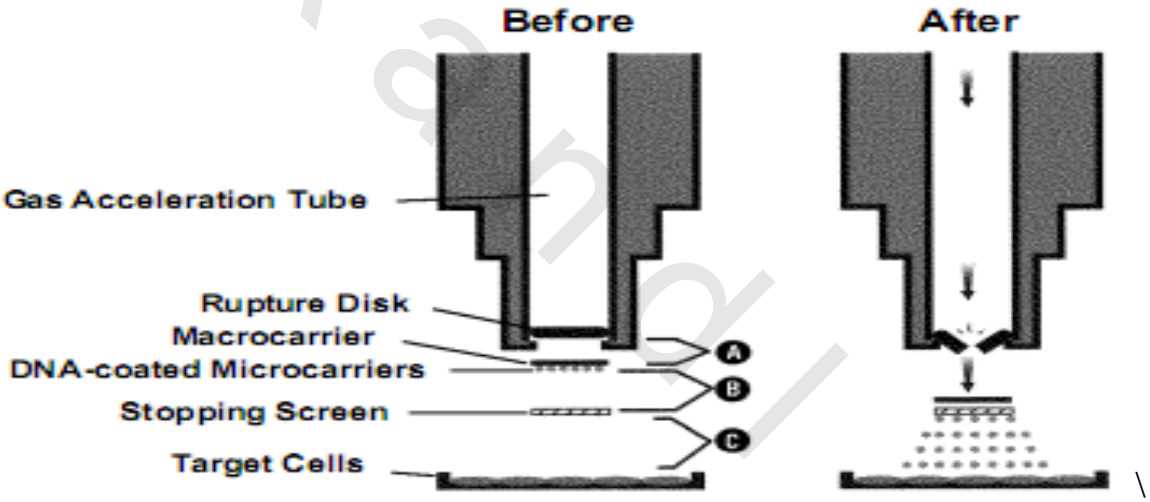



**Figure (3). The Process of the Biolistic (Gene Gun)**

### 3.3.8. Transformation with particle gun

Explants were arranged aseptically in a circle with diameter of 25mm on same media just before the bombardment. Plasmid **C2ASA2-NOS-ASB** was isolated by using plasmid Mini prep kit (Qiagen,Germany) following manufacturer's protocol. Transformation conditions were determined using the plasmid **C2ASA2-NOS-ASB**, which harbours the gus reporter gene and the selectable hptII gene, both controlled by the cauliflower mosaic virus (CaMV) 35S promoter.

### 3.3.9. 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 (**C2ASA2-NOS-ASB**) 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.

### 3.3.10. Micro projectile bombardment

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.

### 3.3.11. Selection and transformants

After bombardment, explants were kept in dark at 25 ◦C for24 h and then transferred to shoot induction medium (SIM), i.e.MS medium containing 3% sucrose, 0.8% agar and plant growth regulators 2.22M BA+ 2.27M TDZ + 0.49M IBA.

After 15 days the explants were transferred to selection medium (same as above) containing 5mg L−1 hygromycin. For effective selection, the explants were transferred to shoot regeneration medium (SRM;MS + 3% sucrose + 0.8% agar + 2.22M BA+ 0.49M IBA+ 1.45MGA3)with increasing concentration (6 and 7mg L−1) of hygromycin.