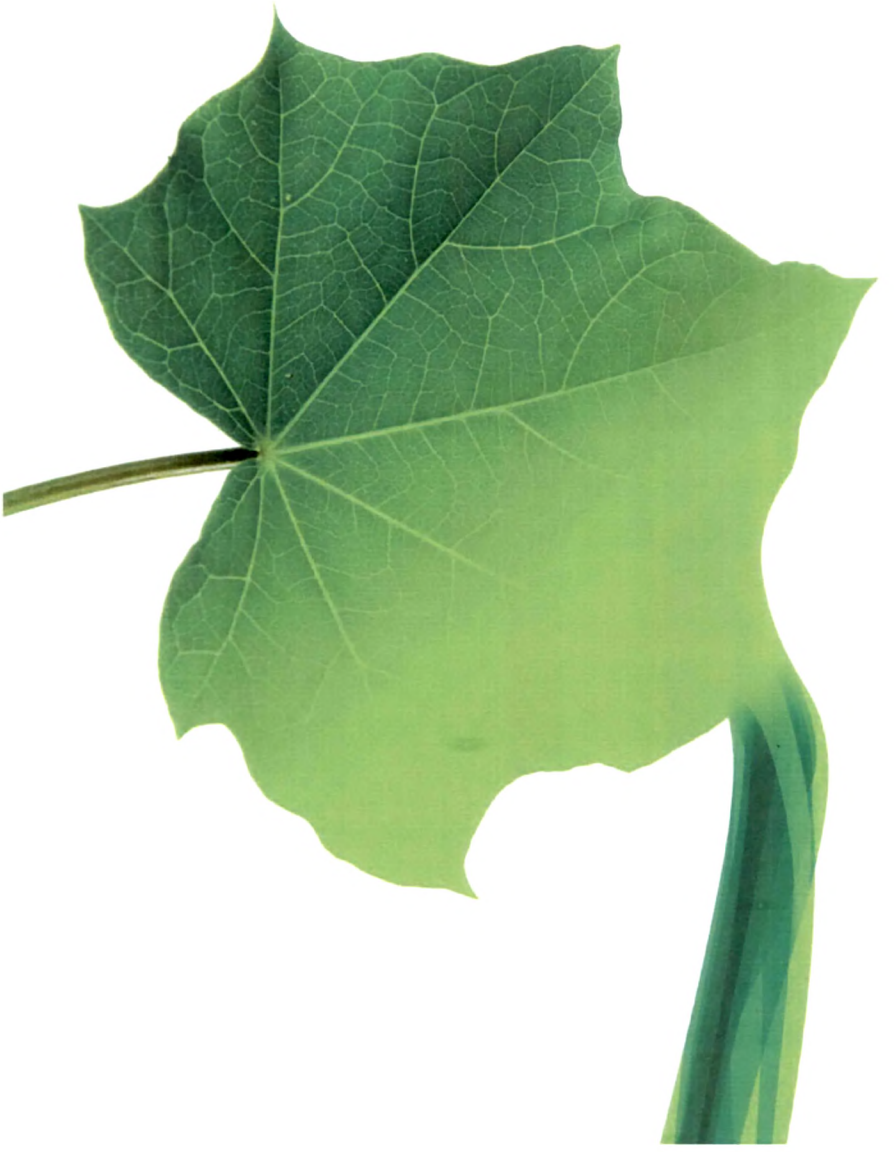
CHAPTER 2



Materials and Methods

CHEMICALS

Ascorbic acid, 4-Aminoantipyrine, Betamercaptoethanol (BME), 2-Chloroethylphosphonic acid (Ethephon), 2, 4-Dichlorophenoxyacetic acid (2, 4-D), 4',6-Diamidino-2-phenylindole (DAPI), Dimethyl formamide, Dithiothreitol (DTT), Evans blue stain, Gibberellic acid, Hexadecyltrimethylammonium bromide (CTAB), Hydrogen peroxide, Indole-3-acetic acid (IAA), Mercury chloride, *N*-(2-hydroxyethyl)piperazine-*N*-3-propanesulfonic acid (EPPS), Peroxidase, Pyridoxyl 5-phosphate (PLP), Polyvinylpyrrolidone (PVP), Phenylmethanesulfonyl fluoride (PMSF), Pyrogallol, Sodium thiosulfate, Sodium dodecyl sulphate (SDS), Sodium hypochlorite, Silver diethyldithiocarbamate, Silver nitrate, Tween 20, Trypan blue, Trichloroacetic acid (TCA), Agarose, All reagents were of analytical grade.

Experimental plants

Fifteen month old, *Jatropha curcas* plants of Vishwamitri Railway Colony plantation and Padra region plantation, Vadodara, India, were selected for experiments. Phytohormones such as Gibberellic acid (GA), 2, 4-Dichlorophenoxyacetic acid (2, 4-D), 2-Chloroethylphosphonic acid (Ethrel) and Silver thiosulfate at different concentrations alone and in combination were used. Solutions were prepared by dissolving phytohormones in small volume of isopropyl alcohol and final volume was made up with demineralized water after adjusting pH to 7.5–7.8. A few drops of surfactant (Tween 20) were added to the solution. Plants sprayed with demineralized water containing only surfactant were considered as control. The selected time of spray was early morning hours and spraying was initiated from the time foliar bud emerged. Each inflorescence received three sprays of equal volume of solution at an interval of 5 days. Total number of flowers and sex ratio were calculated 1 week after the last spray whereas fruit yield was measured 1 month after the last spray. Test and control plants were tagged with appropriate labels to follow flower development until about one and half months. Fruit yield was observed for 3 months at an interval of 1 month. After each treatment, dried seeds were collected and weighed.

Table 2.1: Groups of phytohormones treated plants are shown in the table as below.

Treatment (ppm)	No of Dose (5 day interval) 1	2	3
Control	H_2O + Tween 20	H_2O + Tween 20	H_2O + Tween 20
GA (10 ppm)	GA 10 + H_2O + Tween 20	GA 10 + H_2O + Tween 20	GA 10 + H_2O + Tween 20
GA (50 ppm)	GA 50 + H_2O + Tween 20	GA 50 + H_2O + Tween 20	GA 50 + H_2O + Tween 20
GA (100 ppm)	GA 100 + H_2O + Tween 20	GA 100 + H_2O + Tween 20	GA 100 + H_2O + Tween 20
GA (1000 ppm)	GA 1000 + H_2O + Tween 20	GA 1000 + H_2O + Tween 20	GA 1000 + H_2O + Tween 20
2, 4-D (50 ppm)	2, 4-D 50 + H_2O + Tween 20	2,4-D 50 + H_2O + Tween 20	2 ,4-D 50 + H_2O + Tween 20
2, 4-D (100 ppm)	2, 4-D 100 + H_2O + Tween 20	2,4-D 100 + H_2O + Tween 20	2 ,4-D 100 + H_2O + Tween 20
GA + 2, 4-D (50 ppm)	GA + 2, 4-D 50 + H_2O + Tween 20	GA + 2, 4-D 50 + H_2O + Tween 20	GA + 2, 4-D 50 + H_2O + Tween 20
GA + 2, 4-D (100 ppm)	GA + 2, 4-D 100 + H_2O + Tween 20	GA + 2, 4-D 100 + H_2O + Tween 20	GA + 2, 4-D 100 + H_2O + Tween 20
Ethrel (5ppm)	Ethrel 5 + H_2O + Tween 20	Ethrel 5 + H_2O + Tween 20	Ethrel 5 + H_2O + Tween 20
Ethrel (15ppm)	Ethrel 15 + H_2O + Tween 20	Ethrel 15 + H_2O + Tween 20	Ethrel 15 + H_2O + Tween 20
Ethrel (25ppm)	Ethrel 25 + H_2O + Tween 20	Ethrel 25 + H_2O + Tween 20	Ethrel 25 + H_2O + Tween 20
$Ag_2S_2O_3$ (25mM)	$Ag_2S_2O_3$ + H_2O + Tween 20	$Ag_2S_2O_3$ + H_2O + Tween 20	$Ag_2S_2O_3$ + H_2O+ Tween 20
GA (1000 ppm) - $Ag_2S_2O_3$ (25mM)	GA 1000 + H_2O + Tween 20	GA 1000 + H_2O + Tween 20	$Ag_2S_2O_3$ + H_2O+ Tween 20

Oil extraction from dry seed

Dry mature seeds after weighing by fine balance (Sartorius) were analyzed for oil content using the method of Bligh and Dyer, 1959.

1 gm of seed after removing seed coat was taken and ground in a pestle and mortar with 2.5 ml distilled water and one pinch of glass powder. Chloroform: methanol mixture (1:2 v/v) was added to it. The samples were mixed and vortex it. This mixture was then centrifuged at 2500 rpm for 10 min. Take lower phase and add 1 ml of chloroform, vortex it and then centrifuged at 2,500 rpm for 10 min. Take lower phase and evaporate them overnight. Next day record the weight of tube and determine the amount of oil in the sample by subtracting the weight of empty tube. Calculate the results in terms of gram % oil in the given sample of the seed.

Fatty acid analysis of oil (Kates, 1986; Sawhney and Singh, 2002)

The quantitative composition of fatty acids in oil can be evaluated by identification of fatty acids present in it. Quantification of fatty acid esters can be identified by gas chromatography (GC). For this, fatty acids are first converted into their respective volatile esters by transesterification i.e. by converting them from glycerol esters to methyl esters. The esters are identified and quantified by injecting the processed sample into GC and comparing with chromatographic patterns of a set of standard esters. Take 1-30 mg of oil in a centrifuge tube and add small volume of 0.4 N sodium methylate. Cover the mouth of the tubes with aluminium foil, heat in water bath at 65 °C and shake it on vortex. Appearance of homogenous solution will indicate the completion of esterification. Inject an aliquot (1-2 µl) into preconditioned GC in which the temperature of injection port is 280 °C and column temperature 165 °C. After all the components of the sample have come out of the column, inject the standard methyl esters separately and note the retention time of esters of different fatty acids. Gas Chromatography equipped with SS packed column and flame ionization detector. Identify the individual peaks of the samples by comparing their relative position or retention time with the standards. GC automatically calculates the peak area of each acid sample and compares with that of standard.

Determination of GA and Auxin

GA levels were determined by the method of Hollbrook et al., 1961 as mentioned in Jones, 1968. Tissue of specific stage of inflorescence (0.5 gm) was ground to a homogenized paste in a mortar with pestle, along with 10 ml Phosphate buffer. This mixture was then centrifuged at 5,000 g for 20 min at 4°C. The supernatant was recovered and its pH was adjusted to 1 by 1N Hydrochloric acid. GA was extracted from supernatant by ethyl acetate (Berrios et al., 2004). 3.75 N HCl was added to the extracted GA and absorbance at 254 nm was recorded at 20s intervals for 2 min. Temperature was kept at 20 ± 0.5°C during the process.

Auxin was determined by Gorden and Weber method (Gordon et al., 1951). Tissue of specific stages (0.5 gm) was ground in a mortar with pestle, together with 0.01M EDTA and 0.02M Sodium diethyldithiocarbamide in 80% ethanol. This was transfered to test tube, kept in ice bath for 15-20 min, shaking intermittently (Sinha et al., 1981). This mixture was centrifuged at 5,000 g for 20 min at 4°C. Auxin was estimated in the supernatant at 530 nm.

Standardization and preparation of ethylene gas

The Ethephon stock solution (1 M) was prepared in ethanol and diluted to desired concentrations. Disodium hydrogen phosphate buffer (5 mM) was prepared for the decomposition of ethephon. For ethylene gas production, 5µl ethephon solution was mixed with 5 ml disodium hydrogen phosphate buffer (5 mM) or water and measured the gas formation by Gas Chromatography as described by Zhang and Wen, 2010.

Preparation of samples for the estimation of ethylene production

To estimate ethylene produced by plants directly from samples is difficult as its concentration is very low. For this reason it is necessary to find a right reaction vessel; it must be suitable for gas sampling and it should keep the volume as small as possible and prevent the escape of gas released. Under normal conditions, plant is cultivated in vessels, which can be sealed either with caps with a plastic septum. The basic precondition of successful cultivation of plants is to maintain a required humidity in vessels. Excised stage-II (Distinct appearance of floral bud) floral bud from phytohormone treated plants were taken and placed in 15 ml airtight tube sealed with cap. Tubes were kept in dark condition at 25 °C for 1-2 hours. The empty vials (without floral bud) were sealed for measuring ethylene gas from air. After incubation, gas samples were collected with a syringe with the volume of 2 ml and 25 gauge needle. Ethylene gas was manually delivered to a gas chromatograph and detected using a flame ionization detector (manufacture Chemito, India; 15 m capillary column Al_2O_3 "S" 15 µm, ID 0.53 mm), spray, column and detector temperature 230 °C, 40 °C and 200 °C, respectively (Fiserova et al., 2001). The rate of ethylene production was expressed as nmol C_2H_4 /h /g FW.

Extraction and assay of 1-aminocyclopropane-1-carboxylic acid (ACC)

0.2 gm floral bud (stage II) from phytohormone treated plants were taken and homogenized in 2 ml of extraction buffer consisting of 0.1 M Tris-HCl, pH 7.2, 5 mM dithiothreitol, 30 mM Na-ascorbate, and 10% glycerol (v/w) at 2°C. The homogenate was centrifuged at 14,000 g for 20 min at 4°C (Kato et al., 2000). The supernatant was used for assay of ACC. ACC content was assayed by the method of Lizada and Yang (1979). After preparing the mixture as described by Lizada and Yang, ethylene that was formed, assayed on a gas chromatograph equipped with a 3 mm x 1 m alumina column and a flame ionization detector. The temperature of the column was maintained at 80 °C. The efficiency of the conversion of ACC to ethylene in plant extracts was determined as described by Lizada and Yang (1979). ACC activity was expressed as nmol ethylene formed per h per g FW.

Cell viability test by Trypan blue exclusion technique (Kosta et al., 2001)

This is a vital staining procedure, where Trypan blue stains dead cells exclusively as live cells can pump out the dye. The stained dead tissue then appears blue, while viable tissue remains colourless. Thin section of stem (peduncle part) is taken and 10 µl of 0.1% Trypan blue solution is added and observed under 40X objective of the light microscope. A cell count is carried out in each image objective. The percentage viability is then calculated using the following formula

Number of dead cells X 100 =% Dead cells
Total number of cells

Note: Trypan blue is commercially available as 0.4% stock solution. It was diluted 4 times with 1X Phosphate buffer to make 0.1% working solution.

Determination of H_2O_2

H_2O_2 extraction and estimation from plant tissues was carried out as described by Zhou et al., (2006). Fresh tissue from abscission zone region (0.5 gm) were frozen in liquid nitrogen and ground to powder in a mortar with pestle, along with 5 ml of 5% TCA and 0.15 gm activated charcoal. This mixture was then centrifuged at 10,000 g for 20 min at 4 °C. The supernatant was recovered and its pH was adjusted to 8.4 with 17 M ammonia solution before subjecting to filtration. The resultant filtrate was used to determine H_2O_2 levels. The reaction mixture containing 1 ml filtrate and 1 ml colorimetric reagent was incubated for 10 min at 30°C. In a similar way, 1 ml of distilled water was used as a blank instead of filtrate. Absorbance at 505 nm was determined spectrophotometrically (Aebi et al., 1974 and Patterson et al., 1984).

Catalase activity assay

For enzyme activity assays, plant tissue from abscission zone region (0.5 gm) were homogenized in 50 mM potassium phosphate buffer (pH 7.2) containing 2% PVP at 4°C. Homogenate was filtered through Nylon net and centrifuged (12,000 g for 10 minutes). The supernatant was used to determine catalase activity. Five plants per treatment were analysed at each sampling time (Fernandez-Garcia et al., 2004). 100 µl of 500 mM H_2O_2 solution in 50mM (800 µl) potassium phosphate buffer, pH 7.8 was used as assay solution and 100 µl enzyme extract was added. Catalase activity was assayed by monitoring the degradation of H_2O_2 at 240 nm every 10 seconds for 2 minutes (Aebi, 1984). Catalase specific activity was expressed as µmoles of H_2O_2 degraded per min per mg protein. Protein concentration was determined using Folin Lowry method (Lowry et al., 1951).

Peroxidase activity assay

The method of assay measures the oxidation of pyrogallol to purpurogallin by peroxidase when catalysed by peroxidase at 420 nm and at 20°C.

$$\text{Pyrogallol} + H_2O_2 \xrightarrow{\text{Peroxidase}} \text{Purpurogallin}$$

Peroxidase breaks H_2O_2 to H_2O and O_2. Pyrogallol in the presence of O_2 is oxidized into purpurogallin, which produces the colour change of the test solution and thus read in spectrophotometer. One unit of peroxidase is defined as the amount of enzyme required to catalyse the production of 1mg of purpurogallin from pyrogallol in 20 seconds at 20°C.
For enzyme activity assays, plant tissue from abscission zone region (0.5 gm) was homogenized in 50 mM potassium phosphate buffer (pH 7.2) containing 2% PVP at 4°C. Homogenate was filtered through Nylon net and centrifuged (12,000 g for 10 minutes). The supernatant was used to determine peroxidase activity. Five plants per treatment were analysed at each sampling time (Fernandez-Garcia et al., 2004). 100 µl of 5% Pyrogallol solution and 50 µl of 10 mM H_2O_2 solution in 750 µl of 100 mM potassium phosphate buffer, pH 6.0 was used as assay solution and 100 µl enzyme extract was added. Peroxidase activity was assayed by monitoring the degradation of Pyrogallol into

Purpurogallin at 420 nm in 20 seconds per mg of protein at 20°C (Chance and Maehly, 1955). Protein concentration was determined using Folin Lowry method (Lowry et al., 1951).

Superoxide dismutase activity (SOD) assay

For SOD activity assay, known amount of plant tissue (0.5 gm) was homogenized in 3-5 ml buffer pH 7.0, composed of 50 mM potassium phosphate, 0.1 mM EDTA and 1 mM DTT as described by Dixit et al., 2001. Homogenate was filtered through Nylon net and centrifuged (12,000 g for 10 minutes). The supernatant was used to determine SOD activity. Five plants per treatment were analysed per sample. 200 µl supernatant was added in a quartz cuvette containing 800 µl of 75 mM Tris Hcl buffer, pH 8.0 followed by 1 mg/ml of pyrogallol (8 mM). Auto-oxidation of pyrogallol was monitored by recording the absorbance at 420 nm every 10 sec for 3 minutes. Specific activity of SOD was estimated by units of enzyme required for the 50% inhibition of auto-oxidation of pyrogallol per mg protein (Marklund and Marklund, 1974). Protein concentration was determined using Folin Lowry method (Lowry et al., 1951).

Isolation of DNA from plant tissue to monitoring DNA fragmentation by agarose gel electrophoresis (Keb - Llanes et al., 2002)

Weigh 0.3gm tissue from abscission zone region. Place tissue on a clean glass slide. Chop the tissue into a paste using a clean single edge razor blade. Immediately transfer tissue to a 2.0 ml microcentrifuge tube and further grind tissue with a tube pestle. Once the sample is prepared, 300µl Extraction buffer A (EBA) was added. EBA contained 2% (w/v) hexadecyltrimethylammonium bromide (CTAB); 100mM Tris (pH 8.0); 20mM EDTA; 1.4M NaCl; 4% (w/v) polyvinylpyrrolidone; 0.1% (w/v) ascorbic acid and 10mM betamercaptoethanol. This was followed by addition of 900µl Extraction buffer B (EBB) containing 100 mM Tris Hcl (pH 8.0); 50mM EDTA; 100 mM Nacl and 10 mM betamercaptoethanol (BME). To this, add 100µl SDS. Vortex and incubate at 65 °C for 10 min. Place tube on ice and add 410µl cold potassium acetate. Mix by inversion and place

tube back on ice for 3 min. Centrifuge at 13,200 rpm for 15 min. Transfer 1ml of the supernatant to a new 1.5ml microcentrifuge tube, add 540µl of ice cold absolute isopropanol, and incubate in ice for 20min. Centrifuge at 10,200 rpm for 10min. Discard the supernatant. Wash the pellet once in 500µl 70% ethanol and let dry. Resuspend the dry pellet in 600µl of TE (10mM Tris (pH 8.0) with 1mM EDTA). Add 60µl 3M sodium acetate (pH 5.2) and 360µl ice cold absolute isopropanol. Incubate on ice for 20min. Repeat the above steps twice. Resuspend the pellet in 50µl TE. The amount of DNA was estimated spectrophotometrically at 260 nm. To monitor internucleosomal fragmentation, equal amounts of total DNA (3 µg) were immediately electrophoresed in a gel containing 2% agarose- Tris-borate-EDTA and visualized with ethidium bromide (Yamada et al., 2007). The gel pattern was photographed, using an electronic UV transilluminator system.

Nuclear morphology; chromatin condensation

The abscission zone region of phytohormone treated plant tissue were collected and incubated for some time in phosphate buffer (pH 7.2) containing 4% paraformaldehyde, 0.3 % glutarldehyde, 50 mM EDTA (fixative solution which maintains the cells in iso-osmotic condition). The tissues were chopped with razor blade in nuclear extraction buffer which was a low pH solution containing Triton X-100. The extract was filtered through 50-µm nylon mesh. The medium with the isolated cell or nuclei was collected, the buffer with fluorescent stain 4, 6-diamidino-2-phenylindole (DAPI 1µg/ml) from a standard reagent set (Sigma) was added, and the solution was vortexes as described by Yamada et al., 2006. The stained cells or nuclei were analysed using fluorescent and bright field microscope.