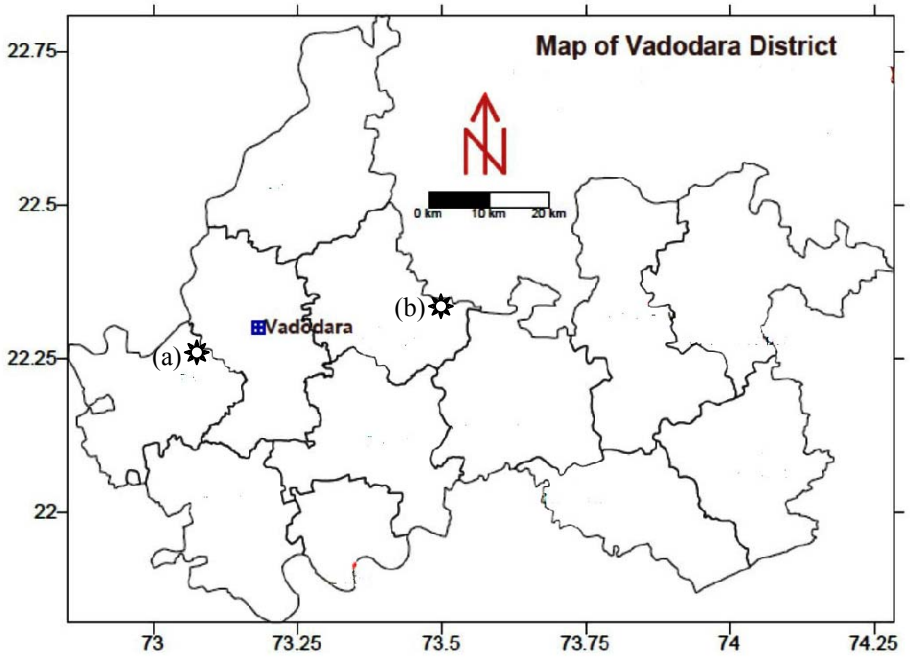
CHAPTER-II

MATERIALS AND METHODS

2.1. Field survey and site selection

Field survey was carried out near Vadodara, Gujarat, India. Geographically the area of Vadodara lies at 22.3° North latitude and 73.2° East longitude. It is located in the western part of Gujarat at an elevation of 39 meters (123 feet) above sea level. Vadodara lies on the banks of a river, the Vishwamitri. The vegetation type prevailing at Vadodara is tropical deciduous type. The area is characterized by three main seasons, summer, monsoon and winter. Apart from monsoon the weather is dry. Different locations near Vadodara were surveyed. These spread across the study area. Survey was carried out to observe variation in vegetation characteristics. The environmental factors like temperature, light and wind flow pattern at all the locations were almost similar across the study area. The maximum and minimum mean temperatures recorded at these sites by the Meteorological Department, M.S. University of Baroda for the entire study duration (2008-2012) were 38°C and 12°C. The winds are generally light with occasional strengthening in force during late summer and early monsoon. Monsoons period ranges from July-September. Average annual precipitation recorded by the Meteorological Department, M.S. University of Baroda for the years 2008-2012 was 93cm. Type of soil at these sites was observed to be sandy-loam and light brown colored. For analyzing the amount of precipitation, Rain gauge measurements were carried out at the sites for 15 days (during monsoon). The measurements were repeated several times during the entire study duration (2008-2012). The average precipitation at all the locations (under survey) was almost similar (93cm), except for one site, where the amount of precipitation was observed to be different. This site showed a lowered value of precipitation (20%) as compared to other sites. This site was considered as experimental site (Figure 3). A site (from the set of other sites) having vegetation distribution akin to experimental site was considered as control site (Figure 3).

Figure 3 Location of (a) control and (b) experimental site, and (c) the two selected Convolvulaceae species.



(c) ***Ipomoea campanulata*** ***Jacquemontia pentantha***

2.2. Analyses of soil water status

Soil water status was analyzed by determining the soil water content at three depths i.e. surface, 20-30cm and 30-40cm at both control and experimental site. Soil water content was measured by following the protocol suggested by Granier et al., (2006). The procedure is,

a) With the help of a trowel, soil samples were collected (at each depth) from control and experimental site and stored in an air tight container. The containers were labeled and brought to the laboratory. Ten samples at each depth (for both the sites) were collected for the analysis.
b) 5 g of soil from each sample was weighed immediately. This was recorded as the *in situ* weight of the soil (initial weight, g).
c) Weighed soil samples were kept for oven drying at 180°C for 4d to evaporate the water (Granier et al., 2006). It causes removal of all the soil moisture present in the forms of capillary and hygroscopic water. These samples were weighted to record the final weight (g).
d) Soil water content was calculated by using the following equation.
   Soil water content (g H_2O g^{-1} dry soil) = Initial weight (g) – Final weight (g)/Final weight (g)
e) The values for soil water content (n=10) at each depth were averaged. Differences in the soil water content (at each depth) between the two sites were analyzed for the entire study duration.

2.3. General vegetation survey

Vegetation at these two sites was observed to be almost similar. It consists of naturally growing wild plants and few of the cultivated species growing due to anthropogenic interference. Tree species observed at these sites largely consist of *Ficus* species (*Ficus benghalensis* and *Ficus religiosa*), *Azadirachta indica, Polyalthia longifolia, Pithecellobium dulce, Terminalia arjuna* and *Ailanthus excelsa* belonging to family Moraceae, Meliaceae, Annonaceae, Fabaceae, Combretaceae and Simaroubaceae respectively. Shrubs and herbaceous vegetation that dominated these sites consist of *Accasia nilotica, Datura stramonium, Calotropis procera, Cassia tora, Cassia occidentalis, Xanthium strumarium, Tephrosia purpurea, Cynodon dactylon, Indigofera cordifolia, Hedyotis corymbosa, Achyranthes aspera, Dactyloctenium*

aegyptium, Tridax procumbens, Gossipium herbaceum, Commelina nudiflora, Helianthus annuus, Croton bonplandianum, Ocimum gratissimum, Ipomoea pes-tigridis, Ipomoea campanulata, Sida acuta, Tylophora indica, Parthenium hysterophorus, Euphorbia neriifolia, Jacquemontia pentantha, Withania somnifera and Clitoria ternatea belonging to families Fabaceae, Solanaceae, Poaceae, Euphorbiaceae Aesteraceae, Commelinaceae Malvaceae, Apocynaceae, Lamiaceae and Convolvulaceae. Morphological observations were made at regular intervals on these species to examine their growth at the two sites. Many of the wild plant species such as *Accasia nilotica, Datura stramonium, Tephrosia purpuria, Parthenium hysterophorus, Calotropis procera* and *Ipomoea campanulata* showed almost no change in the analysed morphological parameters at the two sites, indicative of their adaptability to variations in water availability. Few of the wild and most of the cultivated species such as *Cassia tora, Cassia occidentalis, Indigofera cordifolia, Achyranthes aspera, Gossipium herbaceum, Commelina nudiflora, Helianthus annuus, Ipomoea pes-tigridis, Sida acuta, Tylophora indica, Jacquemontia pentantha, Withania somnifera and Clitoria ternatea* showed differences in the analyzed morphological parameters at the two sites. Based on these observations few wild and cultivated species were selected for studying variations in leaf traits.

2.3.1. Observing phenotypic variations in few selected plant species

Based on commonality and distribution pattern, few of the wild and cultivated species were identified for the study. This includes six wild and six cultivated species, each belonging to six different families (Solanaceae, Fabaceae, Aesteraceae, Malvaceae, Apocynaceae and Convolvulaceae). Wild species belonging to these families included *Datura stramonium, Tephrosia purpuria, Parthenium hysterophorus, Sida acutifolia, Calotropis procera* and *Ipomoea campanulata.* Cultivated species belonging to these families included *Withania somnifera, Clitoria ternatea, Helianthus annuus, Gossipium herbaceum, Tylophora indica* and *Jacquemontia pentantha.* They were analyzed to examine variations in their phenotypic traits in response to water deficit prevailing at the experimental site. The analyzed phenotypic traits include leaf area, length, width, perimeter, Specific leaf area (SLA) and Leaf dry matter content (LDMC). For determination of these leaf traits, leaf samples were collected from five well-growing plants of each species (under study). Fully mature and expanded leaves

free from herbivore or pathogen damage (3 replicate leaves/plant species) were chosen randomly. Leaf area, width, length and perimeter were measured using a self-contained and hand-held Leaf area meter (CI-203, CID, Inc.). For measuring specific leaf area (SLA) and leaf dry matter content (LDMC), these leaf samples were weighed immediately after sampling. These data was recorded as saturated fresh mass of leaves. These leaf samples were oven-dried at 60°C for at least 2 d, and the dry mass was measured. The SLA was calculated using the formula SLA = Leaf area (cm^2)/Leaf dry weight (g). The LDMC, a proportion of the leaf matter content without water related to the mass of the leaf with the maximum water content was determined using the formula LDMC= leaf dry mass (g)/saturated leaf mass (g). For all these parameters, mean values were calculated. Based on this data, two species were considered as experimental material for the study.

2.3.2. Rationale for selecting *I. campanulata* and *J. pentantha*

Amongst the above described plant species belonging to different families, wild and cultivated species belonging to family Convolvulaceae were selected for a detail study (Figure 3c). It includes wild plant *I. campanulata* and cultivated plant *J. pentantha* showing differential response to water deficit. Following are the major reasons for selecting these two species.

1) Both the species belong to the same family and flower almost throughout the year. Comparing these species helps in understanding variations in stress responsive mechanisms of wild and cultivated ones.
2) Both the species propagate easily through seeds and cuttings helping in their continuous availability.
3) *Ipomoea campanulata* (wild) commonly known as morning glory, grows naturally in dry soil, soil with optimal water content and near aquatic bodies. This is indicative of the inherent adaptive nature of the species.
4) *Jacquemontia pentantha* commonly referred as sky blue cluster vine is a cultivated species mostly found growing in gardens and other areas with optimal soil water content. It is barely found growing in dry soils.
5) Both the species showed distinct variation in their leaf traits (like leaf area, width, length, perimeter, SLA and LDMC) and reproductive traits (corolla length, length of stamen and length of stigma) at experimental and control site.

2.3.3. Foliar sampling

For analysis of selected biochemical parameters and miRNA expression levels in response to water deficit, mature leaves (sixth from the tip) were collected from both the species growing under,

I. *In situ* conditions (control and experimental site)

II. *Ex situ* conditions (control and drought stressed pots in green house)

I. *In situ* conditions

Leaf samples (replicate) were collected from plants (of both the species) showing similarity in physical characteristics such as height and spread. After collecting the samples they were immediately frozen in liquid Nitrogen (N_2) and brought to the laboratory. The samples were used for analyzing selected physiological and biochemical parameters and miRNA expression by small RNA high throughput sequencing and miRNA blotting. However, sequences of small RNAs from these samples could not be analyzed due to RNA quality control issues at LC Sciences (Houston, TX, USA).

II. *Ex situ* conditions

Under *ex situ* conditions, both the species were propagated clonally though stem cuttings. Clonal propagation was opted as plants grown from cuttings are identical to parent plant growing in the field. The procedure adopted for propagating is,

a) Healthy plants free of insect and disease were collected from control site for making 4-10 inches long tip cuttings. These cuttings were made from side branches which were in vegetative stage. Cuttings were not made from shoots with flower buds or flowers as it influences timing of rooting. Care was taken to select cuttings of similar length and girth.

b) Cuttings were trimmed from bottom to remove most of the leaves. 2-3 leaves near the tip were kept intact. This minimizes loss of water due to transpiration and helps in faster establishment.

c) A slant cut was made at the bottom of the cuttings to increase surface area for rooting. Bottom end of the cuttings were dipped in rooting hormone ‘Rootone’ containing indolebutyric acid (IBA). Concentration used was as

per the recommendations of Horticulture division of Agricultural University, Anand, Gujarat.

d) Cuttings were carefully transferred to standard pots (32.8cm diameter top, 25.5cm diameter base and 25.8cm depth) containing steam sterilized homogeneous garden soil. For both the species ~25 plants were cloned (one in each pot).

e) The pots were kept in a green house with no artificial day length control. The temperature and humidity are regulated automatically. Potted plants for each species were watered daily to the soil capacity and allowed to grow for 2 months in the green house so as to ensure uniformity in growth and morphological characteristics. Morphologically both the plant species were healthy and fast growing with lush green leaves. *I. campanulata* plants were bearing 13-15 leaves plant^{-1} and *J. pentantha* were bearing 35-37 leaves plant $^{-1}$.

f) Three replicate pots for control and drought stress were arranged in a complete randomized block design. One set of plants were subjected to drought stress by not watering them for 48h. These pots were considered as drought stressed pots. Watering cycle was the same in the other set of plants. This set was considered as control set.

g) Morphological features were observed in both the sets (control and experimental) after 48h. After 48h of water deficit cultivated species showed leaf dropping while no effect was seen in wild species. Mature leaves from control and drought exposed pots of both the species were collected to measure the leaf relative water content (RWC) and specific leaf area (SLA).

h) These leaf samples were frozen in liquid Nitrogen (N_2) and brought to the laboratory for analyzing selected physiological and biochemical parameters and miRNA expression by small RNA high through put sequencing and miRNA blotting.

2.4. Analysis of Physiological and biochemical parameters

2.4.1. Estimation of chlorophyll content

Leaf chlorophyll content was determined using a chlorophyll meter (SPAD-502, Minolta, Japan). SPAD-502 chlorophyll meter provided an easy alternative for non-destructive measurement of relative chlorophyll levels than compared to traditional method for determining leaf chlorophyll concentration (e.g. Arnon, 1949; Porra et al., 1989). The meter consists of two light-emitting diodes and a silicon photodiode receptor that measures the transmittance of red (OD_{650}) and infrared (OD_{940}) radiation through the leaf. Based on these transmittance values the meter calculates relative SPAD meter value. These measured units less SPAD values were correlated with chlorophyll values coming from Acetone based extraction method (Arnon, 1949). Following is the outline of the Acetone based extraction method.

a) Leaf samples (0.2 g) were crushed in a pre-cooled mortar and pestle with liquid N_2. The powdered leaf tissue was ground with 5ml of cold 80% acetone until suspension becomes homogeneous.

b) The samples were centrifuged at 2000*g* for 10min in a cooling centrifuge at 4°C and the supernatant was transferred to a clean tube.

c) Absorbance was read at OD_{652} against a blank using the spectrophotometer and concentration of total chlorophyll was determined by following equation.
Total chlorophyll content (mg/g) = 27.8 OD_{652} (Volume of extract/weight of leaf tissue x 100)

The chlorophyll concentration determined by Acetone extraction was used to prepare a standard curve as reported previously (Li et al. 2006). This standard curve was used for converting the SPAD meter values into units of chlorophyll concentration.

2.4.2. Estimation of Anthocyanin content

Extraction of anthocyanin was carried out as reported previously (Rabino and Mancinelli, 1986; Sunkar et al., 2006).

a) Leaf tissue (0.2 g) was quickly ground in a pre-cooled mortar and pestle with liquid N_2.

b) Grinding was continued after adding 5ml of 99:1 methanol: HCl (v/v) to the mortar which was kept at 4°C.

c) Samples were incubated at 4°C overnight. The samples were centrifuged at 2000*g* for 15 min at 4°C. The supernatant was collected in a clean tube for measuring the absorbance.

d) Absorbance was read at OD_{530} and OD_{657} against a blank using Perkin-Elmer spectrophotometer.

e) Relative anthocyanin content was determined by using the equation suggested by Sunkar et al., (2006).
Relative units of anthocyanin x g^{-1} fresh weight of tissue = $(0.25 \times OD_{657}) \times$ extraction volume (mL) × 1/fresh weight of leaf tissue (g)

2.4.3. Lipid Peroxidation

In order to measure the cellular lipid injury due to water deficit, assay for lipid peroxidation was carried out. Level of lipid peroxidation was measure in terms of malondialdehyde (MDA) concentration. Concentration of MDA was determined by thiobarbituric acid-reactive-substances (TBARS) assay following the protocol of Taulavuori et al., (2001) and Fazeli et al., (2007). It is a modified protocol derived from the original protocol discovered by Heath and Packer, (1968). The original protocol deals with measuring the MDA concentration based on the absorbance at OD_{532} only. The problem with this protocol is that at OD_{532} certain other compounds such as anthocyanins and carbohydrates also showed absorption causing error in calculating MDA concentration. Using the modified protocol this problem is overcomed by deducting the non-specific absorbance at OD_{600} from the absorbance at OD_{532}. Following is the outline of the protocol adopted in the current study (Taulavuori et al., 2001; Fazeli et al., 2007).

a) Frozen leaf samples (0.2g) were ground in liquid N_2 with a pre-cooled mortar and pestle. Four ml of 0.1% trichloroacetic acid (TCA) solution was added to the powdered tissue in the mortar and the samples were homogenized.

b) The homogenate was centrifuged at 11,000*g* for 10 min at 4°C, and the supernatant was collected. 0.5 ml of this supernatant was transferred to a clean test tube and one ml of 20% TCA (containing 0.5% TBA) was added. Sample

was shaken thoroughly and placed in boiling water bath for 30 min. The sample was then cooled in an ice bath.

c) Cooled samples were centrifuged at 11,000*g* for 15 min and supernatants were collected.

d) Their absorbance was measured at OD_{532} and OD_{600} against the blank using a Perkin-Elmer spectrophotometer. MDA concentration was calculated by using the extinction coefficient 155 mM^{-1} cm^{-1}.

2.4.4. SOD enzyme extraction

SOD enzyme extraction was carried out as suggested by Wendel and Weeden, (1989). Amongst the three kinds of enzyme extraction buffer suggested; "extraction buffer with moderate levels of interfering substances (polysaccharides and phenolics)" was standardized for both the plant species (*I. campanulata* and *J. pentantha*). Following are the steps used for the extraction.

a) 0.5g of frozen leaf tissue was crushed in liquid N_2 with a pre-cooled mortar and pestle. The powdered leaf tissue was immediately transferred to a test tube containing 5ml extraction buffer. The extraction buffer consists of 75mM Tris-HCl buffer (pH 7.5) containing 5% Glycerol (w/v), 5% PVP-40 (w/v), 14mM Mercaptoethanol (0.1% v/v), 50mM Na-salt, 10mM Dithiothreitol (DTT) and 0.1% Bovine serum albumin (w/v). The homogenate was thoroughly mixed by vigorous shaking.

b) The homogenized samples were centrifuged at 10,000*g* for 20 min at 4°C and the supernatant containing the crude enzyme mixture was collected and preserved at -20°C for in-gel resolution of different SOD isoforms.

2.4.4.1. Gel electrophoresis (native PAGE) and activity staining of SOD isoforms

Gel electrophoresis technique was useful for separating and analyzing the extracted enzyme mixtures. Enzymes were resolved through native polyacrylamide gel electrophoresis (PAGE), a multiphasic buffer system that has two kinds of gel in one run; analyzing-lower gel and stacking-upper gel. pH of the electrophoresis buffer was manipulated for optimum band resolution. Gel running was carried out in a cold room to avoid the impact of heat (generated during electrophoresis) on enzyme degradation.

Following is the procedure adopted for preparation and running of native PAGE (Davis, 1964; Walker, 2002).

a) Gel plates were cleaned with 70% ethanol and set up for preparing the gel. 10% separating gel was prepared by mixing 10 ml of stock acrylamide solution (30% acrylamide and 0.8% *bis*-acrylamide), 10 ml of separating gel buffer (1.5 *M* Tris-HCl pH- 8.8), 9.95 ml water, 150 µl of 10% ammonium persulfate (APS) and 15 µl of TEMED. The solution was mixed gently to avoid formation of bubbles and immediately poured into the gel cassette until it reaches a position 1 cm from the bottom of the sample loading comb. 1-2ml of distill water was gently spread over the gel surface in order to ensure that the gel sets with a smooth surface. The gel was allowed to polymerize for 45 min.

b) While the separating gel was setting, solution for 10% stacking gel was prepared by mixing 2 ml of stock acrylamide solution (30% acrylamide and 0.8% *bis*-acrylamide), 4 ml of stacking gel buffer (0.5 M Tris-HCl, pH- 6.8), 5.8 ml water, 100 µl of 10% APS and 15 µl of TEMED. The solution was mixed gently and poured over the separating gel. Care was taken to avoid bubble formation.

c) A well-forming comb was inserted into the solution and allowed to set for 45min. Subsequently the comb was carefully removed and the wells were cleaned with gel running buffer (3.0 g of Tris base and 14.4 g of glycine in 1l of water; pH-8.3).

d) Samples were prepared by mixing the enzyme extract with 5X sample buffer (15.5 ml of 1 M Tris-HCl pH 6.8; 2.5 ml of a 1% solution of bromophenol blue; 7 ml of water; and 25 ml of glycerol) in such a manner that the final concentration of sample buffer was 1X.

e) Samples were loaded onto the gel and allowed to run at constant 100V at 4°C until the dye front reaches the bottom of the gel.

f) Once the running was over the gel was removed from the cassette and subjected for staining.

The activity staining for SOD isoenzymes was performed as reported by Beauchamp and Fridovich, (1971) and Fazeli et al., (2007). The protocol followed is,

a) For activity staining, the gel was incubated in two solutions consecutively. The gel was first kept in 2.5mM nitro-blue tetrazolium (NBT) for 30 min with gentle shaking and then washed thoroughly.

b) Later the gel was transferred to a tray containing solution of 50mM K-phosphate buffer (pH 7.8) and 28mM riboflavin and kept in darkness for 30 min (with occasional shaking). This gel was again washed thoroughly and exposed to light in a light box for 30 min during which enzyme isoforms appeared as colorless bands in a purple background.

c) For identification and characterization of these isoenzyme bands. The gel was treated with 50mM K-phosphate buffer (pH 7.8) containing either 3mM KCN or 5mM H_2O_2 for 20-30min before activity staining.

d) It helped in the identification and characterization of isoenzymes such as CuZnSOD, FeSOD and MnSOD bands as they showed differential sensitivity to KCN and H_2O_2. CuZnSOD bands were sensitive to both KCN and H_2O_2. MnSOD bands were resistant to both KCN and H_2O_2. FeSOD bands were inhibited by H_2O_2 but were resistant to KCN (Fazeli et al., 2007).

2.4.5. Protein extraction for detection of CuZnSOD

Protein extraction and subsequent immunoblot analysis was carried out in order to validate the results of CuZnSODs observed in-gel activity assay. High quality protein samples were extracted from both the species following the TCA/acetone protocol suggested by Isaacson et al., (2006). The extraction procedure followed is,

a) Frozen leaf tissue (0.5g) was crushed in pre-chilled mortar and pestle with liquid N_2. Sterilized glass wool was added for proper grinding and fracturing the cell wall.

b) The powder was then suspended in 5ml of TCA extraction buffer (10% v/v TCA in ice cold acetone; 2% v/v b-mercaptoethanol added immediately before use) and thoroughly homogenized. Homogenates were stored at -20°C overnight during which proteins precipitate as white flakes.

c) Later the samples were centrifuged at 5,000*g* at 4°C for 30 min. The supernatant was carefully pipette out and discarded.

d) The pellet was washed with 5ml of ice cold acetone and centrifuged at 5,000g at 4°C for 10 mins. The supernatant was again discarded. This step was repeated 2-3 times.

e) The white pellet was air dried and resuspended in SDS gel loading buffer [50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% (w/v) SDS, 0.1% (w/v) bromphenol blue, 10% (v/v) glycerol] which dissolves the proteins. These protein samples were separated by one dimensional gel electrophoresis.

2.4.5.1. SDS-PAGE and immunoblot for detection of CuZnSOD

Immunoblotting also called as western blotting or protein blotting was used to identify CuZnSOD (CSD2) using an antibody. Specificity of the antibody-antigen interaction enables a target protein to be identified amongst the protein mixture. Following were the steps carried out,

a) Protein samples were separated by their size using denaturing gel electrophoresis (sodium dodecyl sulfate polyacrylamide gel electrophoresis or SDS PAGE) described by Laemmli, (1970) and Walker, (2002).

b) The internal surface of gel plates were cleaned with 70% ethanol and the cassette was clamped in vertical position.

g) 10% separating gel was prepared by mixing 8ml of 1.875 *M* Tris-HCl pH 8.8, 18.1ml of water, 13.3ml of stock acrylamide (30% acrylamide, 0.8% *bis*-acrylamide), 0.4ml of 10% SDS, 0.2 ml of 10% APS and 14 µl of TEMED. The solution was mixed gently and poured immediately into the gel cassette until it reaches a position 1 cm from the bottom of the comb that will form the loading wells. 1-2ml of distilled water was gently spread over the gel surface in order to ensure that the gel sets with a smooth surface. The gel was allowed to polymerize for 45 min.

c) While the separating gel was setting, 10% stacking gel was prepared by mixing 1.0 ml of 0.6 *M* Tris-HCl pH- 6.8, 1.35 ml of stock acrylamide, 7.5 ml of water, 0.1 ml 10% SDS, 0.05 ml of 10% APS and 14 µl of TEMED. This

solution was mixed and poured over the surface of separating gel. A well-forming comb was inserted into the solution and allowed to set for 45min.

d) Subsequently the comb was carefully removed and the wells were cleaned with gel running buffer (12 g Tris, 57.6 g glycine and 2.0 g SDS in 2 l of water).

e) Samples were denatured in sample buffer (5.0 ml 0.6 M Tris-HCl pH- 6.8, 0.5 g SDS, 5.0 g Sucrose, 0.25 ml β-Mercaptoethanol, 5.0 ml of 0.5% Bromophenol blue) by heating at 95°C for 5 min.

These samples were cooled and loaded in the wells. Gel was allowed to run at constant 100V until the dye front reaches the bottom of the gel. Care was taken to avoid heating of the gel during the running process. Later the gel was removed from the cassette and subjected for blot generation.

The separated proteins on SDS PAGE were transferred onto a solid support matrix; polyvinylidene difluoride (PVDF) membrane (Bio-rad). Transfer was carried out using a wet (tank) transfer apparatus following the manufactures instructions (Thermo Scientific).

a) The gel was placed in a tray containing 25-50ml of transfer buffer for 10-15 minutes with gentle agitation on a shaker.

b) Tris-glycine buffer with methanol (25mM Tris, 192mM glycine pH- 8.3, 20% methanol) was used as the transfer buffer (as it is the most common choice for wet transfer). The transfer buffer was stored at 4°C after preparation.

c) For preparation of gel “sandwich”, two sheets of filter paper, the PVDF transfer membrane and the transfer-cassette pads were wet with transfer buffer by dipping them briefly. As PVDF membranes are highly hydrophobic, they were pre-wetted with 100% methanol for 30 sec prior to submersion in transfer buffer.

d) The gel “sandwich” was constructed in the transfer cassette where the gel faced the cathode (red electrode) side relative to the membrane; while the membrane was placed on the anode (black electrode) side relative to the gel. Care was taken to avoid air bubbles between the gel and PVDF

membrane. Later the cassette was loaded into wet (tank) transfer apparatus. Overnight transfer was allowed to take place at constant power supply of 30V or 100mA at 4°C.

e) The membrane was removed from the transfer unit and rinsed briefly with nanopure water. In order to confirm the efficiency of protein transfer, the membrane was stained with reversible Ponceau S stain. This stains the membrane rapidly (5mins) for detecting protein bands. It is subsequently destained with 0.1 M NaOH for 1 min; without affecting the transferred proteins. In order to ensure complete transfer of proteins from the gel to the membrane, the gel was even stained with CBB dye (Coomassie brilliant blue).

f) The blot was placed in a tray containing TBST (Tris Base Saline Tween prepared just before use as Tween is known to precipitate out in 2-3 days) and 5%NFDM (non-fat dry milk) with mild shaking at RT for 2h. It blocks the non-specific sites of the blot.

g) The blocking solution was replaced by primary antibody working dilution, where the blot was incubated overnight at 2-8°C with very mild shaking. The primary antibody working solution used in the current study was antiserum generated against CuZnSOD (Klebenstein et al., 1998). This primary antibody was used as 1:2000 dilutions.

h) The membrane was briefly rinsed in TBST 3-4 times for 10min so as to ensure the removal of unbounded primary antibody. This step helps to minimize the background signals.

i) The blot was incubated with secondary antibody HRP-conjugate (horseradish peroxidase) (Thermo scientific) working solution (1:25,000 dilution) for 1h at RT. Later step ‘h’ was repeated 3-4 times to remove unbounded HRP-conjugate.

j) The blot was developed using Pierce ECL2 Western Blotting Substrate. It is highly sensitive, nonradioactive, enhanced acridan based chemiluminescent/chemifluorescent substrate for detecting HRP on immunoblot. It enables picogram detection of antigen by converting the substrate to acridinium ester intermediates by HRP and peroxide.

k) The ECL2 substrate working solution was prepared by mixing substrate A and substrate B in a ratio 40:1 (as per the manufactures instructions). 0.125ml of working solution per cm^2 of blot was used.

l) The blot was incubated with working solution for 5mins at RT.

m) The blot was removed and placed in a plastic wrap. All the excess liquid and air bubbles were removed using an absorbent tissue.

n) The blot was then placed in a film cassette with the protein side facing up. An X-ray film was placed on the blot in a dark room.

o) The exposure time for the X-ray film was 60 seconds. Later the film was developed using appropriate developing solution and fixative.

p) Alternatively, images of the blots were taken using storage phosphor imaging device (Molecular Imager System from Bio-Rad). As the light emission/signal detection is maximum during first 5-30 min after substrate incubation, images were capture within 30min.

2.5. Statistical analysis

Analysis of above parameters (including soil water content, chlorophyll content, anthocyanin content, MDA levels and SOD) was performed for triplicate samples (excepting for soil water content wherein n=10). The values were averaged. One way analysis of variance (ANOVA) was carried out to test whether the differences seen in the measured values were statistically significant or not.

2.6. Total RNA extraction and quality analysis

Total RNA was extracted from both the plant species under control and water deficit using Trizol reagent (Invitrogen). As both the plants had considerably higher amount of secondary metabolites and polysaccharides, little modification was made in the protocol described by the manufacturer. Following is the outline of the RNA extraction protocol followed for *I. campanulata* and *J. pentantha*,

a) Frozen leaf tissue (0.5g) was crushed in liquid N_2 with a mortar and pestle. The powder was transferred to a tube containing Trizol reagent in a ratio 50mg tissue/ml of Trizol. Care was taken to avoid tissue thawing.

b) Homogenized samples in the tube were thoroughly mixed and incubated for 5 min at RT.

c) 0.2 mL of chloroform per 1 mL of Trizol Reagent was added and shaked vigorously. Samples were incubated for 2-3 min at RT.

d) Samples were centrifuged at 12,000*g* for 20 min at 4°C.The mixture separates into a lower red phenol chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.

e) The aqueous layer was carefully pipetted out and 0.5 ml of 100% isopropanol was added per ml of the Trizol reagent for precipitating RNA.

f) In order to acquire high quality RNA samples another step was incorporated in the protocol as suggested by Invitrogen. 1.5M Ammonium acetate was added which helps in dissolving the polysaccharides thereby avoiding their interference.

g) These samples were incubated at -80°C for 1h to ensure complete precipitation of RNA. Samples were centrifuged at 12,000*g* for 10 min at 4°C.

h) Supernatant was discarded and the RNA pellet was washed with 75% ethanol and centrifuged at 7500*g* for 5 min at 4°C.

i) The RNA pellet was air dried and dissolved in DEPC treated water.

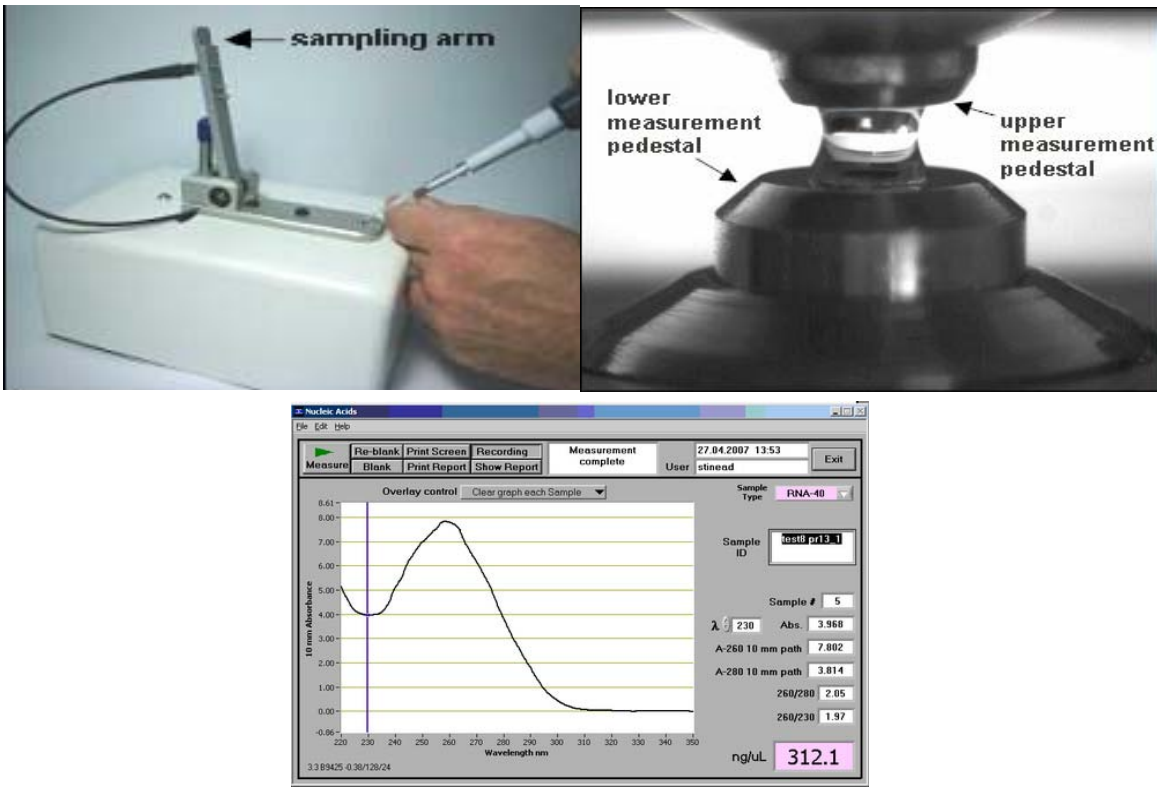
j) RNA quality was checked by running 1% agarose gel (i.e. by detection of 5S, 18S and 28S rRNA bands).

k) Total RNA concentration and quality were analyzed by Nano drop (Thermo Scientific). The Thermo Scientific NanoDrop™ 1000 Spectrophotometer can measure RNA quality and concentration from only 1µl of RNA sample with high accuracy and reproducibility. It has the capability to measure highly concentrated samples without dilution (50X higher concentration than the samples measured by a standard cuvette spectrophotometer).

l) For measuring the quality and concentration of RNA samples, the 'Nucleic Acid' application module in the software was used (Figure 4). Concentrations of the RNA samples were measured in ng/µl unit. Quality of RNA sample was accessed by measuring the ratios of OD_{260}/OD_{280} and OD_{260}/OD_{230}. For both

the ratios a value ranging from 1.8-2.0 indicated high purity of RNA sample. Ratio lower than that may indicate the presence of protein, phenol or other organic contaminants that absorb strongly at or near OD_{280} and OD_{230}. High quality total RNA samples were used for downstream process such as miRNA sequencing and blotting.

Figure 4 NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) and the 'Nucleic Acid' application module in the software.



2.6.1. miRNA high through-put sequencing

High quality small RNA extracted from control and drought-stressed libraries of *I. campanulata* and *J. pentantha* are subjected to high through-put sequencing at LC Sciences (http://www.lcsciences.com, Houston, TX, USA). LC Sciences provides a miRNA discovery service using the Illumina high-throughput sequencing technology which enables comprehensive coverage, highly sensitive and specific discovery and profiling of all miRNAs. miRNAs from model and non-model plants (lacking genome information) can be sequenced using these platform. After sequencing, small RNA sequence reads were selected in accordance with standard quality control of LC Sciences. Raw small RNA sequences containing clear adaptors were further processed. Using in-house written software, the adaptor sequences were trimmed of the raw sequenced data. The clean small RNA reads of 18-30nt were obtained (Jagadeeswaran et al., 2012). These reads were considered as unique reads. After removal of non-coding RNAs such as rRNAs, tRNAs, snRNAs and snoRNAs from the set of unique reads, small RNA reads were BLASTn searched against the known plant miRNAs in the database (miRBase version 21 available at http://microrna.sanger.ac.uk/sequences/). miRBase is an online database repository for known miRNAs that provides an integrated web interface to analyze miRNA sequence data and to predict gene targets. Small RNA sequences that align to the known plant miRNAs in the miRBase were identified as conserved miRNAs. The set of conserved miRNAs were identified from control and drought libraries of *I. campanulata* and *J. pentantha.* In order to compare the differential expression of miRNAs in control versus drought stressed libraries of both the species, the Normalized miRNA expression levels were calaculated. Normalized miRNA expression levels = actual miRNA reads/ Total count of clean reads*1000000

2.6.2. miRNA blot analysis

miRNA northern protocol was carried out as suggested by Sunkar and Zhu, (2004) and Sunkar and Jagadeeswran, (2008). Following is the outline of protocol followed.

a) Gel setup for large vertical gel electrophoresis (Thermo Scientific)

- The entire gel electrophoresis unit along with the glass plates and the spacers were cleaned with nanopure water and wiped with 70% ethanol to remove all the contaminants.

- The gel apparatus was set up to have a thick gel using 1.5 mm spacers.
- 15% denaturing Urea-PAGE was prepared using 40% Acrylamide:Bis (18.75ml), 5x TBE Buffer (5 ml), Nanaopure H_2O (10 ml), Urea (21 g), 10% APS (400 µl), TEMED (40 µl).
- The gel was allowed to polymerize for 1 hour.
- Subsequently, gel apparatus were assembled and the running buffer (1X TBE) was added.
- The wells were rinsed thoroughly to ensure removal of Urea, before loading sample.

b) RNA Preparation and Gel Running

- Approximately 40µg of total RNA was loaded per lane. All the samples were made to equal volume by adding specific volume of DEPC H_2O.
- RNA sample loading dye (Sigma-aldrich) ~10µl (equal volume) was added to the RNA sample and mixed thoroughly.
- Samples were kept in water bath (65ºC) for 10 min and chilled for 1 min by placing in ice.
- Samples were loaded into the clean well along with 10bp DNA ladder. They were run at 180V until the dye reaches the bottom of the gel.

c) Gel Transfer and blot generation

- Nylon membrane was cut according to the size of gel.
- Transfer sandwich was built on the black side of cassette by arranging the components in the following order: sponge, 3 pieces of pre-wet Whatman filter paper, gel, membrane, 3 pieces of pre-wet Whatman filter paper and sponge. All the components were pre-wet in 1X TBE.
- Hybond-N+ nylon membrane was wetted for 2min before placing it on gel.
- The bubbles between the membrane and gel were rolled out with a pipette and the sandwich was assembled.

- The cassette was transferred to BioRad wet trans-blot cell which was filled with pre-chilled 1X TBE.
- Overnight transfer was carried out at 18V in cold room.
- After the transfer, the cassette was removed from the buffer tank and disassembled. The blot was marked for the position of the wells and rinsed in 1X TBE.
- The wet membrane with RNA-side facing upwards, was placed on a dry piece of Whatman filter paper no.1 and UV cross-linked for 1min. The gel was stained with EtBr to assess the transfer efficiency.
- The membrane (after UV crosslinking) was kept in oven at 50-60°C for 2h. Subsequently it was wrapped in a plastic sheet and preserved in freezer.

d) Protocol for end Labeling DNA oligo

- Following reagents were mixed in a single RNase-free micro centrifuge tube and incubated at 37°C for 1 h: Nuclease free water (to make a final volume of 20 µl), DNA oligo (100 pmol DNA), 25 pmol [γ-^{32}P]ATP (7000 Ci/mmol), 2 µl 10X Kinase Buffer (NEB), 1 µl 10X T4 PNK (Polynucleotide Kinase) (New England Biolabs).
- After incubation, the probe was purified using G-25 column. The labeled and purified probe is collected in several micro centrifuge tubes from the lower end of the column and the column is discarded in the radioactive waste.
- The micro centrifuge tubes having the maximum activity (cpm count) as measure by scintillation counter were considered as highly purified and concentrated ^{32}P labeled probes, which were used for hybridization.

e) Pre-hybridization of blots

- Blots preserved in deep freeze were removed from the plastic sheet and rolled with the transfer side inside and kept in the hybridization bottle. The blots were then wetted with 1xTBE. All the air bubbles between glass bottle and blot were removed and excess of TBE was removed.

- Pre-hybridization was carried out using Perefect hybridization buffer (Sigma) (~5-10ml) pre-heated at 65°C (which dissolves any precipitated material) in a hybridization bottle. Pre-hybridization was performed in a rotating hybridization chamber for 2h at 38°C.

f) Hybridization and washing of blots

- The probe was added to the bottom of the hybridization bottle containing the pre-hybridization buffer, which ensures uniform probe distribution.
- Hybridization was carried out overnight at 38°C in the hybridization chamber.
- The blots were washed for 15min (two times with 2 × SSC + 1% SDS and one time with 1 × SSC + 0.5% SDS) at 50°C in the rotating hybridization chamber. The liquid was disposed into the radioactive waste.
- The blots were removed with a clean forceps and placed in saran wrap. All the air bubbles were removed and excess liquid was squeezed out.
- The blot were kept in a cassette and exposed to a phosphor screen (Kodak). The cassette was closed and kept in dark overnight.

g) Images were acquired by scanning the films with a Typhoon scanner

h) Subsequently each blot was labeled with U6 (small nuclear RNA) adopting the above described protocol. Their intensity of the U6 band was used as loading control and to analyze the relative accumulation of miRNAs.

i) Stripping a blot

The blots were stripped with 0.5% SDS (boiled in the microwave oven) in the hybridization chamber for 20mins at 50°C. The blots were removed from the hybridization bottle and sealed in a saran wrap. They were exposed to phosphor screen to ensure complete removal of labeled probe. These stripped blots were used for detecting other miRNAs. Stripped blots were rarely used and if used were largely for re-confirming specific miRNA expression.