Chapter 3

Materials & Methods
3. MATERIALS AND METHODS

3.1 PLANT MATERIAL

The plant material selected for the present study was *Cosmos bipinnatus*. Seeds were obtained from Gujarat Agricultural University, Anand.

Botanical Name: *Cosmos bipinnatus* Cav.  
Family: Compositae (Asteraceae)  
Common Name: Cosmos, Lace Cosmos, Mexican Aster, Cosmea.

It is native to Mexico and southern USA. It is a common glabrous or somewhat pubescent annual plant having 7-10 feet height. It has fine feathery foliage. Leaves are 2-4 inches long, opposite bipinnately cut into remote linear to filiform entire lobes. It has large, showy, wide-open long-peduncled, solitary flowers (heads) having 8 ray florets (Plate-1A). Ray florets are truncate or somewhat toothed and are available in many colours (from white, pink to crimson) (Plate-1B). The disc florets are all yellow. It is propagated by seed. It should be planted out only when all danger of frost has past. It should be planted in full sun with moist, well-drained soil. Plants grow best under full day light (Post, 1949; Armitage, 1993). Overfeeding of the plants may make them top-heavy. They may need staking anyway. *Cosmos* is considered to be a facultative SD plant and seedlings should be kept under LD until six pairs of leaves have formed. Molder and Owens (1985) classified *C. bipinnatus* as a facultative SD plant in that mature plants will eventually initiate flowers under LD, but floral development is promoted by SD. GA₃ sprays (100 ppm) can substitute for SD for *C. bipinnatus*, as treated plants flowered under an 18 - hour photoperiod (Wittwer and Bukovac, 1957). *Cosmos* is grown as cut and garden flower. Long stems bearing several flowers, buds and leaves are cut and made into bunches of a dozen for the market.
PLATE - 1A  Showing the flower twig of *Cosmos bipinnatus* Cav.
PLATE - 1B Shows the flowering twigs of various cultivar varieties of *Cosmos bipinnatus* 

**PLATE-1B**

*Cosmos bipinnatus* Cvar. Sonata Series, ‘Sonata Pink’

*Cosmos bipinnatus* Cvar. Sonata Series, ‘Sonata Carmine’

*Cosmos bipinnatus* Cvar. Sonata Series, ‘Sonata White’
3.2 METHODOLOGY

The seeds were grown in the experimental plots of the botanical garden of the Department. The experiments were divided into following main sections:

(A) Field Study and Growth Analysis.

(B) Post-Harvest study.

(C) Study of physiological and biochemical changes.
   
   I. During uncut conditions (senescence).
   
   II. During cut conditions (after harvest- post harvest shelf life).

   III. Comparison of the two conditions.

3.2.A Field Study and Growth Analysis:

The seeds of *Cosmos bipinnatus* were sown in the month of October directly in the plots in five rows and the seedlings were raised. Cow & goat dung were used as manure. After a month of sowing, growth data were recorded regularly at an interval of 15 days till the end of the season. For this, ten plants were selected at random from the plots and were carefully uprooted in the morning. They were immediately placed in the beakers filled with water to avoid drying. The roots were washed properly under running tap water to remove the soil completely. These were then dried with blotting paper. Each of the plant part was then separated i.e. root, shoot, leaves, flower and buds were separated. Root length and shoot length of each plant was recorded separately (in cms). Similarly number of leaves, flower buds and flowers from each plant were also noted. The fresh weight of root, shoot and leaves of each plant was measured separately by a single pan electrical balance and kept in an oven at 80°C for drying till attainment of constant dry weight. Then the dry weight of each of the part was taken. During this field work, meteorological data i.e. Relative Humidity, Maximum and Minimum Temperature and Photoperiod were also recorded daily. The same methodology was repeated in the next seasons to study, analyze and confirm the pattern and findings.

For each of the data taken statistical analysis was also done. The growth data was categorized into shoot length, root length, number of leaves, number of buds and number of flowers. Then, for each of the parameter standard deviation and standard error was
calculated. Same calculation was done separately for the fresh weight of each of the parameter i.e. shoot root, leaves, buds and flowers for each data. Each of the dissociated part of the plant which was packed separately and kept in an oven at 80°C for drying till attainment of constant dry weight, was weighed and the values noted for each of the parameter were further analyzed for the calculation of standard deviation and standard error.

Dry weight was also used to calculate various growth parameters like Relative Growth Rate (RGR), Net Assimilation Rate (NAR) and Leaf Weight Ratio (LWR). The significance of growth characters such as Net Assimilation Rate (NAR) and Leaf Weight Ratio (LWR) in the study of plant growth using the data of dry weight growth indices namely Relative Growth Rate (RGR) (Blackman, 1919), Net Assimilation Rate (NAR) (Gregory, 1926) & Leaf Weight Ratio (LWR) was suggested by Williams (1946) and Coombe (1960). The RGR, LWR & NAR were calculated using the following formulas :-

1. **R.G.R. (Relative Growth Rate)**:
   R.G.R. is determined as difference between Naperian Logarithms of dry weights of successive samples as given by Blackman (1919).

   \[
   \text{R.G.R.} = \frac{\log_e W_1 - \log_e W_0}{T}
   \]

2. **N.A.R. (Net Assimilation Rate)**:
   N.A.R. was calculated using Gregory’s formula (1926) from the data of dry matter product of whole plant and leaf.

   \[
   \text{N.A.R.} = \frac{(W_1 - W_0) \log_e W_1 - \log_e W_0}{(L_1 - L_0)}
   \]

3. **L.W.R. (Leaf Weight Ratio)**:
   It is calculated as follows:

   \[
   \text{L.W.R.} = \frac{(L_1 - L_0) \log_e W_1 - \log_e W_0}{(\log_e L_1 - \log_e L_0) (W_1 - W_0)}
   \]
Where,

\[ W_0 = \text{initial dry weight of whole plant.} \]
\[ W_1 = \text{dry weight of the whole plant after a period of time.} \]
\[ L_0 = \text{initial dry weight of leaves of the plant.} \]
\[ L_1 = \text{dry weight of the leaves of the plant.} \]
\[ T = \text{period of time.} \]

3.2 B: **Post-Harvest Study:**

Post-harvest study was also conducted during the flowering season of *Cosmos bipinnatus*. Fresh flowers of *Cosmos bipinnatus* grown in the botanical garden of the department were used for the experimental work. The flowers that had just opened were cut diagonally from the plant in the morning. They were immediately placed in the beaker containing water and were brought to the laboratory. Leaves, if any, were removed from the flowering twig, were recut again diagonally and were immediately placed in a definite volume of different preservative solutions (Joshi, 1993). The length of the twig was kept 10 cms to overcome the influence of flower stalk length on vase life (Sangama and Singh, 1999). The twigs were placed in a cool place in the laboratory at room temperature. The tubes containing solutions were covered with transparent polythene pieces to prevent loss by evaporation. The weight of each tube containing solutions with and without flower was recorded every day. Water uptake, transpiration loss and water balance were calculated everyday (Venkatayarappa et al., 1980). The overall appearance of the flower was also taken into consideration. The sets were maintained in triplicate and the average values were taken.

The difference between the measurements of the weight of the tube + solution + flower and the weight of the tube + solution gives the fresh weight of the flower on that particular day and was expressed as % of initial fresh weight. The difference between consecutive measurements of the tube + solution (without flower) represents water uptake expressed as gm/flower/day whereas the difference between consecutive measurements of the tube + solution + flower gives the transpiration water loss expressed...
as gm/flower/day. The sets were maintained in triplicate. The experiments were repeated and the average values were taken.

(i) **Fresh Weight on a day** = \( \text{(weight of tube + solution + flower)} - \text{(weight of tube + solution)} \) expressed as gm/flower/day.

(ii) \( \% \text{ Fresh weight} = \frac{\text{Fresh weight on a day}}{\text{Original weight}} \times 100 \)

(iii) **Transpiration Water Loss** = \( \text{(weight of tube + solution + flower on day 1)} - \text{(weight of tube + solution + flower on day 2)} \) expressed as gm/flower/day.

(iv) **Water Uptake** = \( \text{(weight of tube + solution on day 1)} - \text{(weight of tube + solution on day 2)} \) expressed as gm/flower/day.

(v) **Water Balance** = Water uptake – Water Loss for every value expressed as gm/flower/day.

(vi) The **Shelf life** of these cut flowers was hence counted in terms of days from the harvest till the day they were appeared useful in the preservative solutions.

### 3.2 C: Study of physiological and biochemical changes:

As the research also deals with revealing the physiological status of the flower during senescent period, biochemical estimations were done from 1 gram fresh or dry material of petals from the flower petals under both uncut and cut conditions.

In order to carry out the estimations from dry material, the petals were collected from the plant during the season. Thus, to study the changes occurring within the flower which leads it to the senescence, petals from every stage (every 24 hours) of flower were collected starting from the day it opened till its senescence. Every day the field was surveyed in the morning and the flowers which had just opened were tagged. These flowers were considered as Stage 1 (0 hr.) flowers. Petals from some of the Stage 1 flowers were collected and packed separately with proper labels. Similarly, petals for
Stage 2 (24 hrs), Stage 3 (48 hrs), Stage 4 (96 hrs), Stage 5 (120 hrs) and Stage 6 (144 hrs) (senescent stage) [Plate-2] flowers were also collected. These petals were then placed in the oven (Toshinwal, Sr No. 1078) at 80°C for drying. Enough material was collected since these dried petals served as the source for various biochemical estimations. These show the physiological status of the flower before harvest i.e. on the plant under uncut conditions.

• Stages for Uncut Conditions:
The uncut flowers of all the three varieties of *Cosmos bipinnatus* remained on the plant for 5 days with 6th day as the senescent day at which the petals started abscising. Thus, 6 stages in case of uncut flowers (that is the flowers attached to the plant) were defined as follows (Plates-2A, 2B and 2C)

  Stage 1 : Flowers that had just opened (Day 1)
  Stage 2 : After 24 hours (Day 2)
  Stage 3 : After 48 hours (Day 3)
  Stage 4 : After 96 hours (Day 4)
  Stage 5 : After 120 hours (Day 5)
  Stage 6 (Senescent stage) : After 144 hours (Day 6)

Like wise for the cut conditions, the fresh flowers of Stage 1 were harvested and placed in DW as mentioned in the section 3.2 B. Once the flowers that had just opened were placed in the test tubes, they were labeled as and considered at Stage 1 (0 hr.). After 24 hours, they were considered as Stage 2 (24 hrs) flowers; after 48 hours, they were considered as Stage 3 (96 hrs) flowers and so on till the end of their shelf life as shown below.

• Stages for Cut Conditions:
In case of cut flowers of *Cosmos*, a shelf life of 7 days was observed. The flowers were completely unacceptable on 8th day with the petals completely wilted and dried. Hence, in case of cut flowers 7 stages were defined as follows (Plates – 3A, 3B and 3C)

  Stage 1 : Day when the flowers were cut and placed in DW as holding solution (Day 1)
PLATE – 2A Shows various stages of *Cosmos bipinnatus* Cvar. Sonata Series, ‘Sonata White’ Uncut flowers
PLATE-2 A
SONATA WHITE UNCut FLOWERS

Stage 1 (0 hr)          Stage 2 (24 hrs)

Stage 3 (48 hrs)       Stage 4 (72 hrs)

Stage 5 (96 hrs)       Stage 6 (120 hrs)
PLATE-2B  
SONATA PINK UNCHUT FLOWERS

Stage 1 (0 hr)

Stage 2 (24 hrs)

Stage 3 (48 hrs)

Stage 4 (72 hrs)

Stage 5 (96 hrs)

Stage 6 (120 hrs)
PLATE – 2C Shows various stages of *Cosmos bipinnatus* Cvar. Sonata Series, ‘Sonata Carmine’ (Crimson) Uncut flowers
PLATE- 2 C
SONATA CARMINE (CRIMSON)
UNCUT FLOWERS

Stage 1 (0 hr)
Stage 2 (24 hrs)
Stage 3 (48 hrs)
Stage 4 (72 hrs)
Stage 5 (96 hrs)
Stage 6 (120 hrs)
PLATE – 3A Shows various stages of *Cosmos bipinnatus* Cvar. Sonata Series, ‘Sonata White’ Cut flowers
PLATE- 3A
SONATA WHITE CUT FLOWERS

Stage 1 (6 hrs)

Stage 2 (24 hrs)

Stage 3 (48 hrs)

Stage 4 (72 hrs)

Stage 5 (96 hrs)

Stage 6 (120 hrs)

Stage 7 (144 hrs)
PLATE – 3B Shows various stages of *Cosmos bipinnatus* Cvar. Sonata Series, ‘Sonata Pink’ Cut flowers
PLATE- 3B
SONATA PINK CUT FLOWERS

Stage 1 (0 hr)

Stage 2 (24 hrs)

Stage 3 (48 hrs)

Stage 4 (72 hrs)

Stage 5 (96 hrs)

Stage 6 (120 hrs)

Stage 7 (144 hrs)
PLATE – 3C Shows various stages of *Cosmos bipinnatus* Cvar. Sonata Series, ‘Sonata Carmine’ (Crimson) Cut flowers
Stage 2: After 24 hours (Day 2)
Stage 3: After 48 hours (Day 3)
Stage 4: After 96 hours (Day 4)
Stage 5: After 120 hours (Day 5)
Stage 6: After 144 hours (Day 6)
Stage 7 (Senescent stage): After 168 hours (Day 7)

The dry petals of each stage were collected and dried as described above for the flower petals from the uncut flowers for carrying out some biochemical estimations.

Various biochemical estimations carried out from all the stages of flower petals for both the conditions – uncut and cut are as mentioned below:

In order to start for the estimations, the fresh and dry plant materials were subjected to following procedures:

**FOR FRESH MATERIAL**

FRESH MATERIAL (100 mg petals)

\[\text{Crushed in cold DW – Made final volume up to 10 ml}\]

\[\text{Centrifuge at 5,000 to 10,000 rpm [4° C, 20 min.]}\]

\[\text{Supernatant} \quad \text{Residue}\]

\[\text{Volume made up to 10 ml with cold DW}\]

\[\text{Used as Enzyme Extract}\]
FOR DRY MATERIAL

Preparation of Reagents:

1. 80% Ethanol: 80 ml absolute alcohol (Ethanol) was diluted up to 100 ml

DRY MATERIAL (100 mg petals)

Crushed in 80% ethanol – Made final volume up to 10 ml

Centrifuge at 5,000 to 10,000 rpm [25°C, 15 min.]

Supernatant-1

Residue

Wash with 80% ethanol
[Make total vol. 10 ml]

Centrifuge

Supernatant-2

RESIDUE

SUPERNATANTS

Made final volume 20 ml with 80% ethanol

Used as aliquot for estimations from dry petals
3.2 C I  **ESTIMATIONS FOR CARBOHYDRATE METABOLISM**

It includes the estimation of following metabolites and enzymes:

1. Total Sugars
2. Reducing Sugars
3. Invertase Activity
4. Starch
5. Amylase Activity

The biochemical methods for estimation of above mentioned parameters is as follows:

**1. REDUCING AND TOTAL SUGARS**

(Nelson-Somogyi Method, 1944)

- **Preparation of Reagents:**

  1. 1N H\textsubscript{2}SO\textsubscript{4} : 2.77 ml Concentrated sulphuric acid was diluted up to 100 ml with DW.
  2. 1N NaOH : 4 g NaOH dissolved up to 100 ml with DW.
  3. Nelson A :
     - 12.5 g Na\textsubscript{2}CO\textsubscript{3} (Sodium carbonate)
     - 12.5 g KO\textsubscript{2}C\textsubscript{6}H\textsubscript{4}(OH).CH(OH).COONa.4H\textsubscript{2}O (Potassium sodium tartarate)
     - 10 g Na\textsubscript{2}HCO\textsubscript{3}
     - 100 g Na\textsubscript{2}SO\textsubscript{4}
     - These were diluted one by one and the final volume was made up to 500 ml with DW.
  4. Nelson B :
     - 15 g CuSO\textsubscript{4}.7H\textsubscript{2}O dissolved up to 100 ml with DW.
  5. Nelson C or Nelson Reagent :
     - 50 ml Nelson A + 2 ml Nelson B.
  6. Arsinomolybdate reagent (AMR) :
     - 25 g Ammonium molybdate was dissolved in 450 ml DW to which 21 ml Conc. H\textsubscript{2}SO\textsubscript{4} was added.
     - 3 g sodium arsenate was dissolved in 25 ml DW and both solutions were mixed.
     - This solution was kept overnight at 37°C before use.
Procedure Flow-chart:

1 ml aliquot + 1 ml Nelson C
20 minutes boiling water bath
Cool the tubes
1 ml Arsinomolybdate (AMR) reagent
Make volume up to 20 ml with DW
O.D. at 540 nm

Blank was also prepared following the same procedure using 1 ml DW instead of aliquot.

For calculations of both the sugars regression formula of glucose was used

\[ X = 204.78Y + 3.27 \]

Results expressed as mg glucose reduced / g plant material

Non-reducing sugars were calculated by subtracting Reducing sugars from the Total sugars.
2. INVERTASE ACTIVITY
(Hatch and Glasziou, 1963)

❖ Preparation of Reagents:

1. Citrate Buffer (0.1M, pH=5.4) : 16 ml Citrate A + 34 ml Citrate B were mixed together.
   
   Citrate A : 0.1 M Citric Acid - 21.01 g citric acid was dissolved up to 1000 ml with DW.

   Citrate B : 0.1 M Sodium Citrate – 29.41 g sodium citrate was dissolved up to 1000 ml with DW.

2. Nelson C or Nelson Reagent:
   
   50 ml Nelson A + 2 ml Nelson B.

   Nelson A:
   
   12.5 g Na₂CO₃ (Sodium carbonate)

   12.5 g COOK.CH(OH).CH(OH).COONa.4H₂O (Potassium sodium tartarate)

   10 g Na₂HCO₃

   100 g Na₂SO₄

   These were diluted one by one and the final volume was made up to 500 ml with DW.

   Nelson B:

   15 g CuSO₄.7H₂O dissolved up to 100 ml with DW.

3. Arsinomolybdate reagent (AMR):

   25 g Ammonium molybdate was dissolved in 450 ml DW to which 21 ml Conc. H₂SO₄ was added.

   3 g sodium arsenate was dissolved in 25 ml DW and both solutions were mixed.

   This solution was kept overnight at 37°C before use.

4. 0.1 M Sucrose in Citrate Buffer : 3.42 g sucrose was dissolved up to 1000 ml with Citrate Buffer. (0.1M, pH=5.4)

5. Sodium Sulphate (Na₂SO₄) (5%) : 5 g Na₂SO₄ was dissolved in 100 ml DW.

75
**Procedure Flow-chart:**

1. **Test**
   - 1 ml Enzyme extract
   - 1 ml Citrate buffer (0.1 M, pH=5.4)
   - 1 ml Sucrose
   - Incubate for 1 hour
   - 2 ml Absolute Alcohol
   - 2 ml 5% sodium sulphate (Na₂SO₄)
   - Incubate in boiling water bath for removal or evaporation of alcohol (10-20 minutes)
   - Impurities filtered or centrifuged
   - 1 ml solution
   - 1 ml Nelson Reagent
   - Incubation in boiling water bath for 20 minutes
   - 1 ml AMR
   - Shake well for complete evolution of CO₂
   - Make final volume up to 20 ml with DW
   - O. D. read at 540 nm
   - Calculation using the regression formula of glucose
   \[ X = 204.78Y + 3.27 \]
   - Results expressed as glucose reduced / g plant material

2. **Control**
   - 1 ml DW
   - 1 ml Citrate buffer (0.1 M, pH=5.4)
   - 1 ml Sucrose

3. **Blank**
   - 1 ml DW
   - 1 ml Citrate buffer (0.1 M, pH=5.4)
3. STARCH
(Chinoy, J. J.; 1939)

- **Preparation of Reagents:**

  1. 0.7% KOH: 700 mg KOH was diluted and volume made up to 100 ml with DW.
  2. 20% Acetic Acid: 20 ml glacial acetic acid was diluted up to 100 ml with DW.
  3. I₂KI Solution: 200 mg Iodine crystals and 2 gm KI (Potassium iodide) were dissolved up to 100 ml with DW.
  4. Citrate Buffer (0.05M, pH=5): 20.5 ml Citrate A + 29.5 ml Citrate B were diluted up to 100 ml with DW.

    Citrate A: 0.1 M Citric Acid – 21.01 g of citric acid dissolved in water and volume made up to 1000 ml
    Citrate B: 0.1M Sodium Citrate – 29.41 g of sodium citrate dissolved in water and volume made up to 1000 ml.
Procedure Flow-chart:

1. Residue
2. Dissolved in 20 ml 0.7% KOH
3. Boil it for 40 minutes for gelatinization
4. Cool
5. Centrifuge at 5000-10000 rpm for 10 minutes

Supernatant

1 ml aliquot
+ 0.5 ml 20% acetic acid
+ 1 ml Citrate Buffer (0.05M, pH=5)
+ 1ml I₂KI

Incubate for 10 minutes

O.D. at 600 nm

Calculation using the regression formula of Starch

\[ X = 7000Y + 5546 \]

Results expressed as mg / g plant material

Blank was also prepared following the same procedure using 1ml DW instead of aliquot.
4. AMYLASE ACTIVITY
(Sumner and Howell, 1935)

- Preparation of Reagents:

1. 0.1 % Starch Solution: 100 mg Starch was dissolved in some amount of hot DW and volume was made up to 100 ml.

2. $I_2$KI Solution: 200 mg Iodine crystals were dissolved in 2 gm KI (Potassium iodide) and final volume was made up to 100 ml with DW.

3. Citrate Buffer (0.05M, pH=5): 20.5 ml Citrate A + 29.5 ml Citrate B were diluted up to 100 ml with DW.
   - Citrate A: 0.1 M Citric Acid – 21.01 g of citric acid dissolved in water and volume made up to 1000 ml
   - Citrate B: 0.1M Sodium Citrate – 29.41 g of sodium citrate dissolved in water and volume made up to 1000 ml.
Procedure Flow-chart:

Enzymic extract

**TOTAL**

**α - Amylase**

1 ml Enzyme extract

Heat in boiling water bath for 20 mins

Test

1 ml Enzyme extract + 1 ml DW + 1 ml Citrate buffer (0.05 M, pH=5) + 1 ml 0.1% Starch

Incubate for 10 minutes at Room Temperature

Add 1 ml I₂KI solution

Incubate at Room Temperature for 10 minutes

Final volume was made up to 20 ml with DW.

O. D. at 600 nm

Calculation using the regression formula of Starch

\[ X = 7000Y + 5546 \]

Results expressed as mg starch reduced / g plant material
3.2 C II  ESTIMATIONS FOR PROTEIN METABOLISM

It includes the estimation of following metabolites and enzymes:

1. Total Protein
2. Enzyme Protein
3. Protease
4. Free Amino Acids

1. TOTAL PROTEIN

(Lowry et al., 1951)

❖ Preparation of Reagents:

1. 10% 2,3,5-Trichloroacetic acid (TCA) : 10 g TCA was dissolved in 100 ml DW.

2. 0.1 N Sodium Hydroxide (NaOH) : 4 g NaOH was dissolved and volume was make up to 100 ml with DW.

3. Lowry’s Reagent or Lowry C : 50 ml Lowry A + 1 ml Lowry B.
   Lowry A : 2% Na₂CO₃ in 0.1N NaOH
   Lowry B : 0.5% CuSO₄ in 1% of Na-K tartarate.

4. Folin-ciocalteau’s reagent (IN) : Commercially available (2N) reagent was diluted with an equal volume of DW.
Procedure Flow-chart:

100 mg plant material

Homogenated with 2 ml cold DW

Precipitated with 4 ml cold 10% TCA

Centrifuge at 5000-10000 for 10 minutes

Supernatant

10 ml 0.1 N NaOH

1 ml aliquot

5 ml Lowry C

10 minutes incubation

0.5 ml Folin

10 minutes incubation

O.D. read at 600 nm

Calculation using the regression formula of Casein

\[ X = 302Y + 11.28 \]

Results expressed as mg / g plant material
2. ENZYME PROTEIN

(Lowry et al., 1951)

**Preparation of Reagents:**

1. Lowry's Reagent or Lowry C: 50 ml Lowry A + 1 ml Lowry B.
   - Lowry A: 2% Na₂CO₃ in 0.1N NaOH
   - Lowry B: 0.5% CuSO₄ in 1% of Na-K tartarate.
2. Folin-ciocalteau's reagent (1N): Commercially available. (2N) reagent was diluted with an equal volume of DW.

**Procedure Flow-chart:**

```
Enzymic extract
   ↓
  Test
   ↓
  1 ml Enzyme extract
      +
  5 ml Lowry C
   ↓
Incubated for 10 minutes at Room Temperature
   ↓
  0.5 ml Folin Phenol Reagent
   ↓
  O. D. at 600 nm
   ↓
Calculation using the regression formula of Casein
  X = 302Y + 11.28
Results expressed as mg / g plant material
```

Results expressed as mg / g plant material
3. PROTEASE
(Penner and Ashton, 1967; modified by Cruz et al., 1970)

Preparation of Reagents:

1. Phosphate Buffer (0.2M, pH=7) : 39 ml Phosphate A + 61 ml Phosphate B were mixed together.
   Phosphate A : 0.2 M Monobasic sodium phosphate
   Phosphate B : 0.2 M Dibasic sodium phosphate
2. 1% Casein Solution : 1 g Casein was dissolved in 5 ml 1N NaOH and final volume was made up to 100 ml with DW.
3. 20% Trichloroacetic acid (TCA) : 20 g TCA was dissolved in 100 ml DW.
4. Lowry’s Reagent or Lowry C : 50 ml Lowry A + 1 ml Lowry B.
   Lowry A : 2% Na₂CO₃ in 0.1N NaOH
   Lowry B : 0.5% CuSO₄ in 1% of Na-K tartarate.
5. Folin-ciocalteau’s reagent (1N) : Commercially available (2N) reagent was diluted with an equal volume of DW.
Procedure Flow-chart:

**Test**
1 ml Enzyme extract +
1 ml Phosphate buffer (0.2 M, pH=7) +
1 ml 1% Casein
Incubate for 1 hour
1 ml 20% TCA (Trichloro acetic acid)
Incubate for 1 hour
Centrifuge for 15-20 min at 5000-10000 rpm
Supernatant
1 ml aliquot +
5 ml Lowry reagent
Incubated for 10 minutes
Add 0.5 ml Folin reagent
Incubated for 10 minutes
O.D. read at 600 nm

**Standard**
1 ml DW +
1 ml Phosphate buffer (0.2 M, pH=7) +
1 ml 1% Casein
Incubate for 1 hour
1 ml 20% TCA (Trichloro acetic acid)
Incubate for 1 hour
Centrifuge for 15-20 min at 5000-10000 rpm
Supernatant +
5 ml Lowry reagent
Incubated for 10 minutes
Add 0.5 ml Folin reagent
Incubated for 10 minutes
O.D. read at 600 nm

**Blank**
2 ml DW +
1 ml Phosphate buffer (0.2 M, pH=7)
Incubate for 1 hour
1 ml 20% TCA (Trichloro acetic acid)
Incubate for 1 hour
Centrifuge for 15-20 min at 5000-10000 rpm
Residue (Discarded)

Calculation using the regression formula of Tyrosine
\[ X = 205.5Y - 9.75 \]
Results expressed as mg protein reduced / g plant material
4. FREE AMINO ACIDS
(Lee and Takahashi, 1966)

• Preparation of Reagents:

1. 2% Ninhydrin Reagent: It was a mixture of A, B & C in the ratio of 5:12:2
   A. 1% Ninhydrin in Citrate Buffer: (1 g Ninhydrin was dissolved in 100 ml citrate buffer)
   B. 100% Glycerol.
   C. Citrate Buffer (0.05M, pH=5.5)
2. Citrate Buffer (0.05M, pH=5.5): 14.9 ml Citrate A + 35.1 ml Citrate B were diluted up to 100 ml with DW.
   Citrate A: 0.1 M Citric Acid
   Citrate B: 0.1M Sodium Citrate
3. 10% Pyridine: 10 ml Pyridine was diluted up to 100 ml with DW.

• Procedure Flow-chart:

Supernatant
  ↓
  Test
  ↓
  1 ml aliquot
  +
  1 ml 10% Pyridine
  +
  1 ml 2% Ninhydrin
  Incubate for 30 minutes in boiling water bath
  Make final volume 20 ml with DW
  O.D. at 570 nm
  Calculation using the regression formula of Glycine (10mg / 100ml)
  \[X = 413.42Y + 19.23\]
  Results expressed as mg amino acid / g plant material
3.2 C III  ESTIMATIONS FOR OXIDISING ENZYME & PHENOLIC COMPOUND

It includes the estimation of following metabolite and enzyme:

1. IAA Oxidase
2. Total Phenols

1. IAA OXIDASE
(Mahadevan, 1964)

❖ Preparation of Reagents:

1. 0.5 mM Manganese chloride (MnCl₂·4H₂O) : 9.9 mg MnCl₂·4H₂O was dissolved up to 100 ml with DW.
2. Indole 3-acetic acid (IAA) stock solution (100μg/ml) : 10 mg IAA was dissolved up to 100 ml with MnCl₂ solution.
3. 2, 4 Dichlorophenol (DCP) (0.1 mM) : 8.15 mg DCP was dissolved up to 100 ml with DW.
4. Phosphate Buffer (0.05M, pH=6.5) : 34.25 ml Phosphate A + 15.75 ml Phosphate B were diluted up to 200 ml with DW.
   Phosphate A : 0.2 M Monobasic sodium phosphate
   Phosphate B : 0.2 M Dibasic sodium phosphate
5. 1% Casein Solution : 1 g Casein was dissolved in 5 ml 1N NaOH and final volume was made up to 100 ml with DW.
6. SWR (Salkowski’s Reagent) : 2 ml 0.05M FeCl₃ was mixed with 100 ml 35% perchloric acid.
   35% perchloric acid : 50 ml Perchloric acid (70%) was diluted up to 100 ml with DW.
   0.05M Ferric chloride (FeCl₃) : 4.0552g FeCl₃ was dissolved and final volume was made up to 50 ml with DW.
**Procedure Flow-chart:**

- **Enzymic extract**
  - **Test**
    - 1 ml Enzyme extract
    - 1 ml IAA
    - 1 ml DCP
    - 1 ml MnCl₂
    - 1 ml Phosphate buffer (0.05 M, pH=6.5)
    - 1 ml 1% Casein
    - Incubate for 1 hour in Dark
    - 2 ml SWR (Salkowski’s reagent)
    - Incubate for 30 minutes in Dark
    - O. D. read at 530 nm
    - Calculation using the regression formula of IAA
      \[ X = 188.54Y + 2.759 \]
      Results expressed as mg IAA oxidized / g plant material

- **Control**
  - 1 ml DW
  - 1 ml IAA
  - 1 ml DCP
  - 1 ml MnCl₂
  - 1 ml Phosphate buffer (0.05 M, pH=6.5)

- **Blank**
  - 1 ml DW
  - 1 ml IAA
  - 1 ml DCP
  - 1 ml MnCl₂
  - 1 ml Phosphate buffer (0.05 M, pH=6.5)
2. **TOTAL PHENOLS**

(Bray and Thorpe, 1954)

- **Preparation of Reagents:**
  1. 20% Sodium Carbonate (Na$_2$CO$_3$): 20 g Na$_2$CO$_3$ was dissolved and volume made up to 100 ml with DW.
  2. Folin-ciocalteau’s reagent (1N): Commercially available (2N) reagent was diluted with an equal volume of DW.

- **Procedure Flow-chart:**

```
Supernatant
  ↓
1 ml aliquot
  +
1 ml 20% Na$_2$CO$_3$
  +
0.5 ml Folin-ciocalteau’s reagent
  ↓
Test tubes stoppered
  ↓
Placed in boiling water bath (10 minutes)
  ↓
Cool the tubes
  ↓
Volume made up to 20 ml with DW
  ↓
Kept undisturbed for some time
  ↓
O.D. at 660 nm
  ↓
Calculation using the regression formula of Tannic acid (10mg / 100ml)
  X = 400Y - 0.001
Results expressed as mg phenols released / g plant material
```

Blank was also prepared following the same procedure using 1ml DW instead of aliquot.
3.2 C IV  ESTIMATIONS FOR ANTIOXIDANT ENZYMES

It includes the estimation of following enzymes:

1. Peroxidase
2. Polyphenol oxidase
3. Catalase

1. PEROXIDASE

(George, 1953)

 Preparation of Reagents:

1. Phosphate Buffer (0.1 M, pH=6.4): 73.5 ml Phosphate A + 26.5 ml Phosphate B were diluted up to 200 ml with DW.
   Phosphate A: 0.2 M Monobasic sodium phosphate
   Phosphate B: 0.2 M Dibasic sodium phosphate
2. 20 mM Guaiacol: 0.22 ml Guaiacol was diluted up to 100 ml with DW.

 Procedure Flow-chart:

\[
\begin{align*}
\text{Enzymic extract} & \\
& \downarrow \\
\text{Test} & \\
1 \text{ml Enzyme extract} & + \\
& + \\
1 \text{ml Phosphate buffer (0.1 M, pH=6.4)} & + \\
& + \\
1 \text{ml Guaiacol (20 mM)} & \\
& \downarrow \\
\text{O. D. at 420 nm} & \\
& + \\
0.5 \text{ml } H_2O_2 & \\
& + \\
\text{O. D. read every 30 seconds till 2 minutes} & \\
& \downarrow \\
\text{Results expressed as } \Delta \text{ OD increased / minute / gm plant material} & \\
\end{align*}
\]
2. POLYPHENOL OXIDASE
(Kar and Mishra, 1976)

- Preparation of Reagents:

1. Phosphate Buffer (0.02M, pH=7) : 7.35 ml Phosphate A + 2.65 ml Phosphate B were diluted up to 100 ml with DW.
   - Phosphate A : 0.2 M Monobasic sodium phosphate
   - Phosphate B : 0.2 M Dibasic sodium phosphate
2. 50 mM Pyrogallol : 12.6 mg Pyrogallol was dissolved in some DW and final volume was made up to 100 ml with DW.

- Procedure Flow-chart:

- Test
  - 1 ml Enzyme extract
  - + 2 ml Phosphate buffer (0.02 M, pH=7)
  - Incubate at Room Temperature for 2 minutes
  - O. D. at 420 nm
  - 2 ml Pyrogallol (0.05 M or 50 mM)
  - Incubate at Room Temperature for 2 minutes
  - O. D. read at 420 nm
  - Results expressed as Δ OD / 2 minutes / gm plant material

- Standard
  - 1 ml DW
  - + 2 ml Phosphate buffer (0.02 M, pH=7)
  - O. D. at 420 nm

- Blank
  - 1 ml DW
  - + 2 ml Phosphate buffer (0.02 M, pH=7)
  - O. D. at 420 nm
3. CATALASE
(Chance and Maehly, 1955)

❖ Preparation of Reagents:

1. Phosphate Buffer (0.1 M, pH=6.8) : 51 ml Phosphate A + 49 ml Phosphate B were diluted up to 200 ml with DW.
   Phosphate A : 0.2 M Monobasic sodium phosphate – 27.8 g NaH₂PO₄·2H₂O dissolved up to 1 liter.
   Phosphate B : 0.2 M Dibasic sodium phosphate – 53.65 g Na₂HPO₄·7H₂O dissolved up to 1 liter.
2. 2% Sulphuric Acid (H₂SO₄) : 2 ml concentrated H₂SO₄ was diluted up to 100 ml with DW.
3. 0.01 N Potassium Permanganate (KMnO₄) : 31.61 mg KMnO₄ was dissolved and volume made up to 100 ml with DW.
4. 0.1 M Hydrogen Peroxide (H₂O₂) : 3.401 ml H₂O₂ (100V, 30%) was diluted up to 1000 ml with DW.

❖ Procedure Flow-chart:

1 ml Enzyme aliquot
+ 3 ml Phosphate buffer (0.1 M, pH=6.8)
+ 1 ml 0.1 M H₂O₂
   Incubated at Room Temperature for 1 minute
   Added 10 ml 2% H₂SO₄
   Titrated against 0.01N KMnO₄ to estimate the residual H₂O₂
   Until a faint pink colour persisted for atleast 15 sec.
   Expressed enzyme activity as amount of ml enzyme broke down by H₂O₂ / minute/g plant material
3.2 C V  ESTIMATIONS FOR ELECTROLYTE LEAKAGE (\%)  
(Serek et al., 1995)

- **Preparation of Reagents**:  
  1. 0.11 M Mannitol: 20.0387 g D-Mannitol pure was dissolved and volume was made up to 1000 ml with DW.

- **Procedure Flow-chart**:  
  1. 10 petal discs  
     2. Incubated in 20 ml of 0.11 M Mannitol with agitation for 3 hour  
     3. Conductivity of the mannitol solution then measured with a conductivity meter  
     4. Discs were then frozen by maintaining at -20°C for 24 hour and then heated in an autoclave at 121°C for 30 minutes  
     5. Conductivity of the mannitol solution was measured again and considered as 100% ion leakage  
     6. Rate of ion leakage expressed as % of total conductance following petal destruction