CHAPTER 3

3.1 *Field Screening*:

The study was conducted from 2017-2019 at the Department of Botany, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, India. The field survey was carried out throughout India to cultivate the preferable varieties and for the occurrence of burl incidence (Table 1). However, for the ease of sample availability, the collection of fruits and wood samples was mainly done from Gujarat state to study the burl disease, incidence and symptoms. Based on agro-climatic zones, four districts *viz*. Anand, Banaskantha (station: Dantiwada), Junagadh and Valsad (station: Pariya) were selected from the Gujarat state (Fig. 3). These four districts are known as potential areas for mango cultivation and production in the state. Some popular germplasm/varieties cultivated in Gujarat state include *Kesar*, *Alphanso*, *Rajapuri*, *Jamadar*, *Neelam*, *Dashehari*, *Langra*, *Vanraj*, *Totapuri etc* (Table 1).

3.2 *Collection of Samples*:

Burl samples (i.e., wooden blocks or complete burl) and fruits were collected from five individuals of each variety from different orchards during the survey study. For each location [i.e., Anand, Banaskantha (station: Dantiwada), Junagadh and Valsad (station: Pariya], 3-5 orchards were selected for the collection of samples. These orchards were either private farms or belonging to the Gujarat Agriculture Universities. The wooden blocks of burl infected and non-infected stem were cut down from the tree with the help of an electric saw for burl and from healthy stem and branches by using a sharp chisel. Some of the samples were also packed in sterile polyethylene bags for isolation of the causal organism.

In contrast, other samples (both healthy stem and burl) were fixed immediately in FAA (Formaldehyde-Acetic Acid-Alcohol) for anatomical examination (Berlyn and Miksche, 1976). After 24 to 48 hrs of fixation, these woodblocks were transferred to 70 % alcohol for further storage and histological preparations. Samples of fruits were collected to investigate the nutritional value and biochemical analysis from the burl infected and non-infected individuals from the same orchard for each location. Collected fruit samples were packed in sterile, airtight polyethylene bags to avoid contamination. All the sampled trees were of different age group ranging from 05 to 40 years, and these samples were analysed and compared with the same age group.

3.3 *Incidence and Characterization of Burl Disease*:

For the national-level survey, four different sites (North, South, East and Western India) were selected for the study, which includes major locations like Uttarakhand (Dehradun), Karnataka (Bangalore), Bihar (Samastipur), New Delhi (PUSA campus), Haryana (Hisar), Maharashtra (Akola) and Gujarat (Anand, Dantiwada, Junagadh, Pariya) were preferred for disease-related observations. The incidence of burl in various mango varieties was recorded from commercial orchards, government, semi-government organizations, state agricultural universities and farmer's private field at different agro-ecological zones of all selected sites of India.

Important cultivated varieties of mango (Table 1) were observed for the presence of burl disease. As mentioned above, different age groups of plants (from 10 to more than 40-year-old) were selected to record morphological and quantitative data, particularly in *Langra* and *Rajapuri* variety. Morphological observations were recorded for burl shape, colour, surface feature, side of burl formation and presence of gummosis. Quantitative data was also recorded for burl size, the total number of burls per plant and the height of burl from ground level. These parameters were selected based on the review of the literature and the kind of parameters chosen by the earlier researchers to follow the standard methodology (Sinclair *et al*., 1993; Mishra and Prakash 1999; Saran *et al*., 2011). Disease incidence was calculated using the following formula, which was used earlier by Kumar and Saran (2018), Tucho *et al.* (2014) and Saran *et al.* (2011, 2020b).

$$\text{Percent disease incidence} = \frac{\text{Total number of burl infected plant}}{\text{The total number of plants observed}} \text{ X } 100$$

3.4 *Burl Symptoms Expression in Young Age of Trees*:

Varieties *viz*. *Arka Aruna*, *Mahmood Vikarabad*, *Langra* and *Rajapuri* that showed high incidence and severely affected by the burl disease, were preferred for the present study. For the young age burl disease-affected trees, 5 to 10 years old plants. The age of trees was decided based on a maintained record of orchards and concern orchardist. In the orchards, age was marked on five plots for each variety, and symptom expression was recorded. Studies for burl symptoms in the young age of trees were earlier documented in many germplasms/varieties using standard methodology (Saran *et al*., 2011, 2020b, c; Kumar and Saran, 2018).

3.5 *Fruit Yield Loss*:

Loss of fruit yield was observed in ten individuals of the same aged individuals growing in the same orchard severely affected with burl. Ten individuals free from the burl disease were also selected from the same farm or orchard. Obtained data were analysed using a randomized block design method, and standard errors were calculated using SPSS software. Fruit yield losses were calculated by using the following formula:

$$\text{Yield loss} = \frac{\text{Weight of fruits harvested from disease plant}}{\text{Weight of fruits harvested from healthy plant.}} \text{ X } 100$$

3.6 *Correlation Studies Between Burl Size and Tree Age*:

In the present study, a correlation between burl size and trees age was also investigated to understand the effect of plant age on disease incidence and burl development. Trees of different age groups *viz*., 10-20, 21-30, 31-40 and more than 40 years old were selected from different varieties *viz*., *Langra*, *Bathua*, *Sukul*, *Arka Aruna*, *Arka Punit*, *Mahuvas*, *Rajapuri*, *Krishna bhog*, *Mahmood Vikarabad* etc. From which, *Rajapuri* and *Langra* varieties were chosen from the same orchards and exact location for burl study. Five plots for each variety in an orchard were marked for above-given age groups to determine the correlation between burl size and age.

3.7 *Correlation Studies Between Burl Disease and Climatic Factors*:

The correlation between burl incidence, burl size, fruit yield loss and climate were investigated for *Langra* and *Rajapuri* variety. As mentioned earlier, there were four age groups *viz.*, 10-20, 21-30, 31-40 and more than 40 years-old trees. For climatic factors *viz*., temperature, relative humidity and rainfall data were collected from the meteorological stations of the different agro-ecological zone (Anand, Valsad, Dantiwada, Junagadh) of Gujarat. Meteorological data (average) for five years was collected from other mango growing agro-ecological zone of Gujarat for the relationship studies. Five plots and ten plants were selected in an orchard from each site/location as a replication. The relationship between disease incidence, plant age and location were discussed in earlier research studies (Saran *et al*., 2011, 2020a, b; Kumar and Saran, 2018).

3.8 *Anatomical Examination*:

Burls with different diameters (ranging from 5 to 50 cm) were collected from the fully grown mango varieties *viz*., *Langra*, *Arka Aruna*, *Arka Punit*, *Rajapuri*, *Mahmood Vikarabad etc.*, from different mango orchards of Gujarat. No significant variation was observed in the histological structures within these varieties; thus, a detailed study is presented only for *Langra* and *Rajapuri* variety. Healthy stem samples were also excised from these varieties for comparison purpose. Samples were excised using a sharp chisel or electric saw based on their size, and they were packed in sterile polyethylene bags to bring into the laboratory. These samples were cut into small pieces and immediately fixed in FAA (Berlyn and Miksche, 1976), while some samples were used to investigate causal organism. Samples fixed in FAA were used to obtain transverse, radial and tangential longitudinal sections of 12–15 μm thickness. These wood samples were sectioned directly using Leica SM2010R sliding microtome and stained with Safranin-Astra blue combination (Srebotnik and Messner, 1994). Sections were mounted in DPX (Dibutyl Phthalate Xylene) after dehydration through the ethanol-xylene series. The young stem samples (for primary growth) were processed by embedding in paraffin wax as described by Johansen (1940) and stained with the above-mentioned staining combination. Permanent slides were observed under Leica DME 2000 trinocular fluorescence microscope, and important results were micro-photographed with a Cannon DC 150 Digital Camera fitted on the abovesaid microscope.

To obtain the dimensional details of the xylem elements, wood samples were also macerated using Jeffrey's fluid (Berlyn and Miksche, 1976). Small pieces of secondary xylem (2×2×2 mm) adjacent to the cambium was macerated with Jeffrey's fluid (Johansen, 1940) for 36-48 hrs at 55-60 ^{o}C. Treated samples were washed thoroughly with tap water, stained with 0.5% aqueous safranin and mounted in 0.5% glycerine to measure the length of vessel elements and fibres. In contrast, the tangential diameter of the vessel lumen was obtained directly from the transverse sections. Thirty measurements were obtained randomly to get the mean and standard deviation.

3.9 *Sample Preparation for The Isolation of Causal Organism*:

Healthy stems and burl samples of *Langra* and *Rajapuri* varieties collected from different locations selected from the Gujarat state were used to study causal organisms. Samples that were packed in polythene bags were exploited for the study of the causal organism. Samples were washed thoroughly with running tap water to remove surface

debris, soil or other unwanted material. The inner tissues of the burl were excised with a sterile surgical blade to obtain small pieces of burl tissue. The chopped burl pieces were washed with 10 % commercial bleach for 3 to 5 minutes and rinsed with sterile double distilled water for 2-3 times (Islam, 2010; Sarker *et al*., 2011). Subsequently, these burl slices were surface sterilised with 70 % ethanol for 2 to 3 minutes and again rinsed with sterile distilled water and immersed in double-distilled water for incubation for 24 hrs at room temperature.

3.10 *Preparation of Media and Their Constituents*:

Since there is an ambiguity about the causal organism of burl disease, different types of growth media were prepared to isolate the pathogen associated with burl disease. These media includes fungi and bacteria. The sterilised wood pieces were inoculated on Petri plates or in a conical flask depending on the type of media (*i.e.* solid or liquid). Then the plates were kept for incubation in an incubator at 26 ± 1 ˚C. Different media, including PDA, Hofer's media, MacConkey media, MGY media, and NASA media, were prepared to isolate the causal organism. For fungal pathogen, samples were inoculated on PDA media by routine method, while for bacteria MGY, NASA and Hofer's media and MacConkey media were used. For bacterial isolation, small wood pieces of burls were surface sterilised and incubated in sterile distilled water at 28-30 ^{o}C for 24 hrs. After that, 1 µl of water was used for streaking purpose in above said growth media. Inoculated Petri plates were incubated for 34-48 hrs. At 28-30 ^{o}C. Details of the media composition are given below:

3.10.1 *Potato Dextrose Agar (PDA) Media*:

PDA media were prepared in 1000 ml of distilled water, 200 g potato extract, 20 g sucrose and 20 g dextrose were added and mixed thoroughly. After mixing the above components, media was autoclaved for 20 min at 120 ^{o}C for sterilisation purpose. After autoclaving, 10-15 ml of PDA were poured into Patri plates and kept at room temperature for 30 min to solidify the media.

3.10.2 *MacConkey*:

As suggested by Bopp *et al*. (1999), the MacConkey media is composed of 1.5 g bile salts, 17 g pancreatic digest of gelatine, 10 g lactose monohydrate, 3 g peptones (casein and meat), 13.5 g agar, 0.001 g crystal violet, 5 g NaCl, 0.03 g neutral red and pH 7.1 MacConkey media (49 g) was weighed according to the manufacturer

instructions and dissolved in 1000 ml double distilled water. Subsequently, all these components were mixed thoroughly and autoclaved for 15 min at 121 °C. After that, media was poured into sterilised Petri plates using laminar airflow and the media was allowed to solidify at room temperature. After the inoculation of the wood slices obtained from healthy and burl samples, the Petri plates were sealed with paraffin film and kept in the incubator at 27 ^{o}C temperature for 24 hrs.

3.10.3 *MGY Media*:

MGY media was composed of 10 g L-glutamic acid, 2 g sodium salt, KH_2PO_4 0.5 g, NaCl 0.2 g, $MgSO_4$ $7H_20$ 0.2 g, yeast extract 1.0 g, agar 15 g, in which yeast extract and agar were added after adjusting the pH to 7.0. The above contents were weighed according to the manufacturer instructions and dissolved in 1000 ml double distilled water. The solution was mixed thoroughly and autoclaved for 15 min at 121 °C for sterilisation purpose. The media was poured into Petri plates and allowed to cool under the sterilised conditions. These Petri plates were inoculated with wood chips of healthy and burl wood samples and sealed with parafilm. The sealed plates were kept in the incubator at 27^{o} C temperature for 24 hrs.

3.10.4 *Hofers Media*:

Hofers media was composed of mannitol 10 g, dipotassium phosphate 0.5 g, magnesium sulphate 0.2 g, sodium chloride 0.1 g, yeast extract 1 g thymol blue 0.016 g, agar 15 g, with final pH 7 (at 25 °C). Readymade Hofer's media was weighed up to 26.8 g in 1000 ml and dissolved in distilled water (Gupta *et al*., 2012). Media was heated till all the constituents get entirely dissolved. Subsequently, the media was sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes and poured into sterile Petri plates and allowed to cool down under sterilized condition.

3.10.5 *NASA Media*:

NASA media, also known as Clark's selective medium, was modified by Brisbane and Kerr (1983) and Serfontein and Staphorst (1994). The nutrient agar supplemented with 50 ppm of sodium selenite and 250 ppm of cycloheximide (NASA) dissolved in 1000 ml of distilled water. The prepared medium was entirely dissolved by heating on a warm plate followed by it was sterilized by autoclave at 15 lbs pressure (121 °C) for 15 minutes.

3.11 *Isolation and Identification of Causal Organism*:

Wood slices obtained from the burl wood as well as healthy (normal) stem were transferred into the glass test tube containing 4 to 5 ml of double-distilled water and incubated at 28-30 °C for 24 hrs. After that, the suspension was streaked on bacterial specific media *viz*., Hofer's alkaline media and NASA media (Gupta *et al*., 2012; Serfontein and Staphorst, 1994; Brisbane and Kerr, 1983). The streaked Petry plate was incubated for 24 hours in an incubator at 28-30 °C to get a bacterial colony. Isolated bacterial colonies were identified based on the gram staining method, morphological and cultural characters. The morphological and cultural characteristics were recorded based on the growth pattern of a bacterial colony, colour, shape, size and fermentation etc. Every selective media shows different morphological characteristics of bacterial colonies. These bacterial colonies were further subcultured to get purified colonies. Similarly, some of the sterilised wood slices of burl wood and healthy wood samples were subjected to PDA media to check whether any fungal pathogen.

3.12 *Isolation of Genomic DNA from Pathogen*:

Two days old, 1ml of bacterial culture was taken in an Eppendorf tube and centrifuged at 13,000-16,000 rpm for 2-3 minutes to acquire the pellets. Obtained pellets were subjected to the isolation of genomic DNA using Wizard® genomic DNA purification kit (Cat# TM050). Obtained DNA was used for PCR amplification in Veriti® thermal cycler (Applied Biosystems) with following conditions: 95 ℃ for 5 min, followed by 35 cycles of denaturation at 95 ℃ for 30 sec., annealing at 52 ℃ for 30 sec. and extension at 72 ℃ for 1:30 min., and final extension at 72 ℃ for 10 min. Total volume for PCR reactions was 20 µl containing 10 µl final concentration of dream Taq Green PCR master mix (Cat# K1081), 1 µl of genomic DNA and 1 µl of both primers 27F and 1492R and 7 µl nuclease-free water (Lane *et al*., 1991). The PCR product was visualised on 2 % agarose gel, and the PCR amplified product was purified using PurelinkTM quick PCR purification kit (Cat# K310001). Successfully purified products were sent for sequencing to Eurofins Genomics India Pvt Ltd., Bangalore.

3.13 *Molecular Characterisation and Phylogenetic Analysis*:

The phylogenetic tree was generated using 16S rRNA sequences data obtained in the present study with *Agrobacterium tumefaciens* and other sequences of *Agrobacterium*, which were obtained from GenBank (Table 4), whereas *Mesorhizobium*

tianshanense and *M. kowhai* were taken as out-group species. The nucleotide sequences were aligned with Clustal-W (Thompson *et al*., 2002) embedded in MEGA X (Kumar *et al*., 2018). Aligned data was analysed with Partition Finder (Lanfear, 2012) for optimal partitioning strategy and evolutionary substitution model. A Maximum Likelihood (ML) analysis was employed to infer phylogenetic relationships in RAxML (Silvestro and Michalak, 2012). An ML analysis was run for 1000 bootstrap replicates under the GTR + I model to assess clade support.

Table 4. *Agrobacterium* species and their GenBank accession numbers

S. No.	Species	GenBank accession numbers
1	*Agrobacterium tumefaciens* - Present study	MK835677
2	*Agrobacterium tumefaciens*	KU955329
3	*Agrobacterium rhizogenes*	D14501
4	*Agrobacterium radiobacter*	AJ389904
5	*Agrobacterium rosae*	KY828974
6	*Agrobacterium rubi*	D14503
7	*Agrobacterium vitis*	D14502
8	*Mesorhizobium kowhaii*	KC237405
9	*Mesorhizobium tianshanense*	FJ550310

3.14 *Pathogenicity Test*:

A pathogenicity test was employed to check whether the formation of burl is associated with the isolated culture of *Agrobacterium* from the burl samples. Two methods were used to study the pathogenicity: i) Carrot disk assay and ii) Koch's postulates. For rapid testing of pathogenicity of the isolated organism, the carrot disk test as described by Islam *et al.* (2010) method is employed in the present study. Fresh and healthy carrots were procured from the market, and they were washed thoroughly with tap water to remove soil particles and surface sterilised with a commercially available bleaching solution. After surface sterilisation, the slice was obtained from the carrot with the help of a sharp cutter and washed again with distilled water and placed on Petri plates containing 1 % agar and kept in an incubator at temperature 28-30 °C for 3-4 weeks.

Koch's postulate is a worldwide accepted experiment to confirm the pathogenicity of the organism. Thus, it was conducted to verify the pathogenicity of the isolated organism. For this purpose, two months old, five individuals of mango saplings of *Langra* variety were selected to confirm Koch's postulate. Additionally, 4-weeks old tomato plants were also used to check the pathogenicity as per the method applied by New and Kerr (1972). A four to five days old culture of *Agrobacterium tumefaciens* isolated from the burls was used to culture on the Hofers media slants. Ten ml of sterile double distilled water was added and shaken to give approximately 10^8 CFU ml^{-1}. Mango saplings of *Langra* variety were wounded with a sterile and sharp blade at a different portion of the stem up to 2-3 mm depth and inoculated with five days old suspension prepared in water. Followed by wounds were covered with parafilm to avoid other environmental contamination.

Similarly, tomato plants were also inoculated with isolated bacterial suspension. The bacterial suspension was taken (1 ml) with the help of a sterilised syringe and inoculated in tomato stem by injecting. The place of inoculation was immediately wrapped with sterilised non-absorbent cotton and was examined for the presence or absence of galls after four weeks. A similar method of *Agrobacterium* transformation by artificial method has been described in tomato and tobacco (Gupta *et al*., 2012; Matthysse, 2006). A similar approach was employed for the control samples employed, except the pathogen was absent in the suspension used for inoculation.

3.15 *Biochemical Analysis of Fruits*:

Biochemical analysis of fruits collected from the burl infected and non-infected trees of a variety *Rajapuri* and *Langra* was also carried out to check the alterations induced in various parameters like ascorbic acid, acidity, reducing, non-reducing and total sugars. The evaluation was done using the pulp of fruits collected from the plants growing in the same orchard belonging to the same age group. Fruits were harvested in June (2018 and 2019) in the first week at the same stage of maturity and kept at room temperature for ripening. One gram of fruit pulp was taken from each sample for the study and dissolved in different solutions (*viz*. distilled water for acidity, oxalic acid for ascorbic acid, buffer solution [80 % ethanol] for total sugar and reducing sugar). Six parameters, including total soluble solid (TSS), total soluble sugar, reducing sugar, non-reducing sugar, ascorbic acid and acidity, were examined. A hand refractometer (Erma Tokyo A^{o}32) was used to observe the TSS of fruit pulp with five replicates.

3.15.1 *Total Sugar*:

Total sugar estimation was analysed by using modified methods as described by Nelson (1944) Somogyi (1952), and Saran *et al*. (2020b), in which the following formula was used for the estimation of total sugar:

$$\text{Total sugar (\%)} = \frac{\text{Graph factor} \times \text{reading} \times \text{total volume} \times 1}{\text{Aliquot taken for estimation} \times \text{sample weight}} \times 10^{-4}$$

One gram of fruit pulp was homogenized in 10 ml buffer solution (80 % ethanol), and each sample was centrifuged for 10 min., at 1000 rpm. From that, 50 µl supernatant was collected, in which 2.95 ml distilled water and 1 ml of phenolic solutions (5 %) were added. The mixture was incubated at room temperature for 3 min. The final volume was obtained by adding 5 ml of concentrated H_2SO_4 and kept it at room temperature for another 10 min, followed by 30 min incubation at 90 °C in a water bath. The OD (Optical density) value was measured at 490 nm in a spectrophotometer with the blank standard.

3.15.2 *Reducing Sugar*:

Reducing sugar was estimated by the modified method of Nelson (1944), Somogyi (1952) and Saran *et al*. (2020b). One gram of fruit pulp was homogenized in 10 ml solution of 80% alcohol, and each sample was centrifuged for 10 min at 1000 rpm. From it, 0.2 ml supernatant was collected, and 2 ml distilled water was added with 1 ml of alkaline copper tartrate regent to make up the final volume and incubated in a water bath for 10 min. After incubation, samples were allowed to cool at room temperature, and a final volume of 10 ml was made by adding Arsenomolybdate reagent with distilled water. After 10 min, the absorbance was recorded using a spectrophotometer at 620 nm. The following formula was used to calculate the reducing sugar:

$$\text{Reducing sugar (\%)} = \frac{\text{Graph factor} \times \text{reading} \times \text{total volume} \times 1}{\text{Aliquot taken for estimation} \times \text{sample weight}} \times 10^{-4}$$

3.15.3 *Non-reducing Sugar*:

Non reducing sugar content was determined by subtracting the reducing sugar content from total sugar content using the following formula and methodology as described by Saran *et al*. (2020b).

Per cent of non-reducing sugar = (Total sugar - Reducing sugar) × 100

3.15.4 *Ascorbic Acid*:

The ascorbic acid content of mango fruit pulp was determined as per the method given by Ranganna (1979) and Saran *et al*. (2020b). One gram of fruit pulp was taken from each sample and dissolved in 10 ml of oxalic acid (4 %), and centrifuged for 10 minutes at 1000 rpm. Out of which, 2 ml of supernatant was taken into a 100 ml conical flask, and the final volume of 10 ml was made by adding pure oxalic acid. Samples were titrated against the standardized freshly prepared dye (2, 6-dichlorophenol indophenol), and the dye factor was calculated. Titration was continued till the light pink colour persists. The following formula was used for the calculation of ascorbic acid:

$$\text{Ascorbic acid (\%)} = \frac{\text{Titrate value} \times \text{dye factor} \times \text{volume made up}}{\text{Aliquot taken for estimation} \times \text{weight of sample taken}} \times 100$$

3.15.5 *Acidity*:

The acidity of fruit pulp was estimated by following the modified method of Garner *et al*. (2008) and Saran *et al*. (2020b). One gram of the fruit pulp was taken in a volumetric flask, and the final volume of 100 ml was made by adding distilled water. The solution was centrifuged for 10 minutes at 1000 rpm, and the supernatant was taken in a beaker for titration with standard 0.1 N NaOH and phenolphthalein as an indicator. The titratable acidity was expressed in terms of percentage citric acid equivalent by adopting the following formula:

$$\text{Acidity (\%)} = \frac{\text{mlsNaOH used} \times \text{0.1 N NaOH} \times \text{milliequivalent factor}}{\text{Grams of sample}} \times 100$$

3.16 *Biochemical Analysis of Stem*:

The biochemical analysis of burl infected and normal stem obtained freshly from the *Langra* and *Rajapuri* variety and dried under aseptic conditions. The main aim was to study the chemical difference (differences between several chemical parameters in

burl infected or non-infected stem wood) in the stem wood content of the same aged plants growing in the same orchard. The dry wood powder of 0.5 g was taken from each sample for the analysis. The obtained powder was dissolved in 80% ethanol to analyse total soluble sugar, reducing sugar, non-reducing sugar and total phenol, whereas another sample from the same powder was also dissolved in 4 % oxalic acid for the study of ascorbic acid. Total eleven parameters including moisture content, ash content, cellulose, fibre, lignin, total soluble sugar, reducing sugar, non-reducing sugar, total phenol, and ascorbic acid were examined with five replicates.

3.16.1 ***Moisture Content*****:**

Moisture availability plays an important role in the process of microbial infection. Therefore, moisture content of wood from three regions of stems *viz*. healthy wood, adjoin (transition portion between healthy and burl initiation), and diseased wood (i.e., actual burl) from both the varieties were used for the study. Powdered samples were oven-dried at 105 ^{o}C for 4 hrs. Thereafter, the powdered samples were transferred in desiccators for 30 minutes to cool down and again; the weight was measured. The process was repeated until a constant weight was achieved. After drying and cooling, weight loss was measured as moisture content and calculated by following formula according to AOAC (2000) method:

$$\text{Moisture (\%)} = \frac{\text{Fresh weight - the weight of the dry sample}}{\text{Fresh weight}} \times 100$$

3.16.2 ***Estimation of Cellulose and Lignin in Mango Stem Wood*****:**

The cellulose and acid detergent lignin estimation were determined by using modified methods as described by AOAC (2000) and Soest (1963). Fibrotron FRB-6, crude fibre analyser equipment, was used for the estimation of cellulose and lignin. One gram of dried wood sample was taken for each sample in the crucible, and 10 ml of ADF (acid detergent fibre) solution was added (prepared by a mixture of CTAB and H_2SO_4) and steadily boiled for one hour with a vortex between every 10 min. The ADF solution was removed and washed three times with hot water and again washed two times with 15 ml acetone. After washing, acetone was evaporated in a water bath at 65 °C and samples were kept at 90 °C for overnight. Crucible was weighed for a dry sample and filled with 72 % H_2SO_4 and kept at 20 to 25 °C for 2-3 hrs. The acid was filtered from the crucible and washed with hot water until it was free from acid. Then

crucibles dried at 100 °C for 8 hrs and weighed. After drying, crucibles were kept in the furnace for 2 hrs., at temperature 550 °C and again weighed. The estimation of cellulose was carried out by the following formula:

$$\text{Cellulose \%} = \frac{\text{Weight of crucible before treating with 72\% } H_2So_4 - \text{weight of crucible after treating with 72\% } H_2So_4}{\text{weight of sample}} \times 100$$

The following formula was used for the estimation of acid detergent lignin:

$$\text{Lignin \%} = \frac{\text{Weight of crucible before ignition} - \text{weight of crucible after ignition}}{\text{weight of sample}} \times 100$$

3.16.3 ***Estimation of Fibre*****:**

The estimation of crude fibre was determined using a modified method of AOAC (2000) and Soest (1963). Total 2 g of moisture or fat-free powder of wood was taken for each replication and filled with 200 ml of 1.25 % H_2SO_4. These samples were boiled for 30 minutes and filtered. The remaining residue was then transferred to a beaker, and 200 ml of 1.25 % sodium hydroxide was added to it. These samples then boiled again for 30 min and washed thrice with hot water. Subsequently, the residue was transferred to the weighing dish and dried at 100 °C to get constant weight. At last, the residue was ignited to bring ash and final weight. The percentage of crude fibre was calculated as per the following given formula:

$$\text{Fibre \%} = \frac{\text{Weight of acid detergent fibre} - \text{the weight of Ash}}{\text{weight of sample}} \times 100$$

3.16.4 ***Estimation of Ash Content*****:**

The acid detergent lignin content (the residue left after acid treatment) was transferred to a muffle furnace and heated at 550 °C for 3-4 hours. Finally, the ash content was calculated by using the following formula as described by AOAC (2000)

$$\text{Ash content} = \frac{(\text{Weight of crucible} + \text{Ash}) - \text{weight of the empty crucible}}{\text{Fresh sample weight}} \times 100$$

3.16.5 ***Estimation of Phenol*****:**

Phenol content was estimated by using the method described by Malik and Singh (1980). Total 0.5 g of dried wood samples were homogenised in a 10 ml solution of 80% alcohol, and each sample was centrifuged for 10 min at 1000 rpm. From which, 0.1 ml supernatant was collected, and 2.9 ml distilled water was added with 1 ml of Folin's reagent in the ratio of 1:2 and incubated at room temperature for 3 min. After incubation, 1 ml of sodium carbonate was added, and a final volume of 10 ml was made by adding distilled water. After 10 min, the absorbance was recorded using a spectrophotometer at 620 nm. The following formula was used to calculate the reducing sugar:

$$\text{Phenol (\%)} = \frac{\text{Graph factor} \times \text{reading} \times \text{total volume} \times 1}{\text{Aliquot taken for estimation} \times \text{sample weight}} \times 10^{-4}$$

3.16.6 ***Estimation of Starch*****:**

A 100 mg of wood powder of both varieties (*Langra* and *Rajapuri*) were taken in 15 ml of 80 % alcohol and centrifuged at 8000 rpm for 5 min, and the supernatant was discarded. The above process was repeated 4 to 5 times to obtain the residues. Thereafter, 6.5 ml perchloric acid was added to the obtained residue and centrifuged for 5 min, and the supernatant was transferred to flask A. Another 3.3 ml of perchloric acid was added and kept at 37 °C for15 min and centrifuged, and the final volume of 100 ml was made in a conical flask. From it, 0.1ml sample was taken in flask A, and 2.9 ml distilled water and add 4 ml of anthrone reagent was added and keep it on boiling water bath for 8 min. After cooling, readings were taken with the help of a spectrophotometer at 630 nm OD.
The following formula was used for the calculation of starch percentage:

$$\text{Starch (\%)} = \frac{\text{Graph factor} \times \text{reading} \times \text{total volume} \times 1}{\text{Aliquot taken for estimation} \times \text{sample weight}} \times 10^{-4}$$

Note: - Methodology for the estimation of sugars, ascorbic acid and acidity of bark was used similarly as for burl infected fruit. The detailed methodology was described in the biochemical analysis of fruits section.

3.17 ***Statistical analysis*****:**

Each tree selected from different germplasm was observed/studied carefully for morphological parameters, burl incidence, burl size, fruit yield, tree age and burl height

from the ground level. The values of different field observations from sampled trees were averaged to get the mean value. The analysis of variance was done in randomized block design for various observations using statistical software SAS 9.2. The results were presented at 5 % level of significance (P=0.05). The critical difference (CD) values were calculated to compare various treatment means.