SYNOPSIS

HOLISTIC ASSESSMENT OF BURL DISEASE OF MANGO GERMPLASM

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Introduction

Mango (*Mangifera indica* L., Anacardiaceae), is the most economically important fruit crop, popularly known as 'King of Fruits'. It is a rich source of vitamins (A, C), amino acids, carbohydrates, fatty acids, water content, minerals, glucose, fructose, and sucrose (Bally, 2006; Banerjee, 2011). It is anti-diabetic, anti-oxidant, anti-viral, cardiotonic, hypotensive, anti-inflammatory properties (Shah *et al*., 2010). Therefore, it is used in Ayurvedic and indigenous medicinal systems since time immemorable.

The mango is a national fruit of India, Pakistan, the Philippines and the national tree of Bangladesh (Mehta, 2017). It is under cultivation for many years in the Indian sub-continent, which is estimated to be more than 4000 to 6000 years ago (Singh *et al.*, 2016; Mehta, 2017). According to De Candolle (1884), mango is native to South Asia or Malay while Hooker (1876), believe that it has been naturalized in India. Based on fossil records, Seward (1912) concluded that mango is originated in Assam. It is estimated that more than 1000 varieties of mango exist and each one differs from the other in size, shape, colour, texture and taste of the fruit or tree size (Litz, 2009; Singh, 2019).

Despite its economic significance and nutritional value, the mango crop is suffering from numerous diseases including Powdery mildew, Anthracnose, Dieback, Phoma blight, Bacterial canker, Red-rust and Sooty mould (Prakash and Srivastava, 1987; Prakash, 2004; Litz, 2009) while some of the disorders/ diseases are neglected by earlier researchers. One of the diseases named "Mango burl" is also an important disease that is poorly investigated and even there is no confirmed information on the aetiology of the disease.

Chand and Rao (1954) reported burl disease on a variety *Mahmud Vikarabad* from India. Subsequently, the disease was also observed on other varieties of mango including *Langra, Pairi* and *Gulab Jamun* as woody galls of different sizes (Prakash and Srivastava, 1987). It has also been recorded from USA (Malaguti and de Reyes, 1964; Cook *et al.*, 1971; Ploetz and Prakash, 1997), Mexico (Angulo and Villapudua, 1982) Ploetz *et al.* (1996b) from Venezuela and Pakistan (Jiskani *et al*., 2007).

Saran *et al*. (2011) worked on the mango burl from India and reported yield loss in three mango varieties. Several researchers have isolated and identified various

pathogen including insect, fungi and bacteria (Malaguti and de Reyes, 1964; Angulo and Villapudua, 1982) and Ploetz *et al.* (1996b) reported that the burl disease in mango is caused by *Fusarium decemcellulare* C. Brick (*synonym*: *Fusarium rigidiuscula* (Brick) Snyd. and Hans.). Cook (1975) isolated bacterial pathogen but after re-inoculating it in the host species; he failed to recover it from inoculated plants. In contrast, Hafiz (1986) and Jiskani *et al*. (2007) reported that burl causes due to the infection of *Agrobacterium tumefaciens*. Thus, available literature indicates ambiguity about the causal organism for this disorder and no affirmed studies are available on its aetiology.

Therefore, the main aim of the present study was to confirm the causal organism, disease development, its impact on yield loss, alterations in the nutritional value of the fruits in affected trees and its comparison with healthy individuals of different varieties of *M. indica*. The present study also intends to study the changes induced in morphology, the biochemical composition of fruits and structural alterations brought by the pathogen in the infected portions, transition zone and a healthy portion of the secondary xylem of stems.

The objectives of this study were the following:

- Survey and screening of different mango germplasm against burl disease
- Identification of the causal organism
- To study the effect of burl disease on fruit yield
- Biochemical changes in fruit and stem bark
- Histopathological study of burl and alterations induced in response to infection

Materials and Methods

Details of materials and methods are illustrated in-depth in the Material and Methods chapter of the thesis and the present writeup provides brief information about the methodology followed to achieve proposed objectives.

***Survey of mango orchards*:** The study was conducted from 2017-2019 at the Department of Botany, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, (Gujarat) and the field survey was carried out throughout India. Nearly 473 germplasms/varieties were screened throughout the country, from the two varieties *viz*. *Rajapuri* and *Langra* were selected for further study. The reason for the selection of these two species is that both of them are potential varieties. They are popular and the most preferred varieties for cultivation due to their utility in pickles and

taste of the fruits respectively. The collection of samples was mainly done from Gujarat state to study the burl disease, incidence and symptoms. From the state four regions *viz*. Anand, Banaskantha (station: Dantiwada), Junagarh and Valsad (station: Pariya) were selected to investigate the incidence of the burl disease. Term germplasm/varieties are used here to include different varieties, cultivars or variants within a variety.

***Collection of samples*:** Wood samples from healthy trees, burl samples from infected stem and fruits (from both healthy and infected trees) were collected from the mango orchards of the above-mentioned locations. For comparison, samples were collected from the same location to avoid the effect of microclimate. The woodblocks from the healthy trees were excised with the help of a chisel and hammer while burl samples were collected with the help of an electric saw due to their hardness and large size. Collected samples were packed in sterile polyethene bags and brought to the laboratory. These blocks were trimmed further and fixed immediately in FAA (Formaldehyde-Acetic Acid-Alcohol) for anatomical examination (Berlyn and Miksche, 1976). Some of the unfixed samples were used for the isolation of pathogen. Fruits samples collected from both normal and infected trees were used to investigate the nutritional value and other biochemical analysis of fruits such as total soluble solids (TSS), total soluble sugar, reducing sugar, non-reducing sugar, ascorbic acid and acidity.

***Disease incidence and characteristics*:** Important cultivated varieties of mango were surveyed throughout the country for the presence of burl disease and disease incidence was calculated by using the following formula:

Disease incidence per cent = Total number of plant screened/Diseased plants×100

Morphological observations were recorded for burl shape, colour, surface feature, the position of burl formation on the stem and the presence of gummosis. Quantitative data was also recorded for burl size, total no of burl per plant and height of burl from ground level. These parameters were applied as per the methodology described by Saran *et al.* (2011). The incidence of burl disease is also correlated with the climate and age groups of the mango trees.

***Isolation of micro-organisms*:** Additional samples that were packed in polythene bags were used for the isolation of unknown pathogens (bacteria or fungi) to obtain pure cultures. For fungi as a pathogen, surface sterilised wood chips were inoculated on PDA media while for bacteria as a pathogen samples were inoculated in MGY, NASA and

MacConkey media. For fungal isolation, small pieces of wood were cut with the help of a sterile surgical blade and sterilised by 0.1% $HgCl_2$ for 40-45 seconds and the samples were washed thoroughly with distilled water followed by 70 % ethanol for few seconds and inoculated on PDA media and observed for 4-6 weeks. For bacterial isolation, small pieces of burls were surface sterilised and incubated overnight in sterile distilled water at 28-30 ^oC for 24 hrs. Subsequently, growth media were incubated with 1 µl of this water and kept at 28-30 ^oC for 34-48 hrs. We have also followed a similar protocol for control wood samples looking normal and collected from healthy trees. For further confirmation of *Agrobacterium tumefaciens*, a carrot disk bioassay was used for the induction of tumours as described by Ali *et al*. (2016).

***Molecular identification of microorganisms*:** One ml of 24 hrs old bacterial culture was centrifuged at 13,000-16,000 x g for 2 minutes to obtain pellets of bacteria. Genomic DNA was extracted using Wizard® Genomic DNA Purification Kit (Cat# TM050) and subsequently, PCR reactions were carried out using Veriti® thermal cycler (Applied BioSystems) under the following conditions: 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 52 °C for 30 sec and extension at 72 °C for 1:30 min., with a final extension at 72°C for 10 min. The PCR reactions were performed in a 20µl volume containing 1x final concentration of Dream Taq Green PCR Master Mix (Cat# K1081), 50 ng of genomic DNA and 10 pmol of both primers 27F and 1492R (Lane *et al*. 1991). The PCR product was visualized on 2% agarose gel and the amplified PCR product was purified using Pure link TM Quick PCR Purification kit (Cat# K310001). Successful PCR purified products were sent for sequencing of 16S rRNA (by using the same primers) to Eurofins Genomics India Pvt Ltd., Bangalore. Obtained sequences were compared with sequences available in the NCBI database using the Basic Local Alignment Search Tool in the GenBank database (www.ncbi.nlm.nih.gov) for confirmation of the identity of the bacterial species. Identification was done by 99% base pair match of the sequence obtained to the closest available reference sequences (Leung, 1991) and the bacterial colonies were identified as *Agrobacterium tumefaciens* Smith and Townend. These sequences have been submitted to NCBI GenBank with the accession number MK835677.

***Pathogenicity test*:** A pathogenicity test was also employed to check whether the formation of burl is associated with the isolated culture of *Agrobacterium tumefaciens*

from the samples. Two methods were employed to check the pathogenicity: i) Carrot disk essay and ii) Koch's postulates. Detailed methods employed for both Carrot disk essay and Koch's postulated is provided in the thesis chapter on 'Materials and Methods. A bacterial suspension containing approximately 10^8 CFU ml^{-1} inoculated in injuries made with a sterile surgical blade on the stem region and the inoculated saplings were allowed to grow under normal conditions. After 2-3 months of inoculation, plants were examined, which showed small measurable outgrowth on both mango (*Mangifera indica* L.) and tomato (*Solanum lycopersicum* L.) plants while no such symptoms were observed on control plants.

***Correlation between climate and burl disease*:** Relationship between the effect of climatic factors (temperature, relative humidity and rainfall), on disease incidence, burl size, the total number of burls per plant and fruits yield loss were examined. The climatic data of three years was collected from meteorology site of respective location and used for the interpretation of the results (Saran *et al*., 2020 a, b).

***Correlation between tree age and burl disease*:** During the present study, the relationship between four different age groups of trees such as 10-20, 21-30, 31-40 years and more than 40-year-old were investigated with the burl disease for different parameters including disease incidence, burl size, number of the burl per individual tree and fruit yield loss. To study this parameter, the method described by Saran *et al*., (2011, 2020 a, b) is followed as such without any modifications.

***Fruit yield loss*:** Fruit yield loss was studied in three plots in each selected orchard; from which ten individuals of burl infected trees and ten trees free from burl disease were selected from each plot. 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:

$$\text{Fruit yield loss \%} = \frac{\text{Weight of fruit harvested from disease plant}}{\text{Weight of fruits harvested from healthy plant}} \text{ X } 100$$

***Biochemical analysis of fruits and stem wood*:** A biochemical analysis of fruits collected from the burl infected and non-infected trees for variety *Rajapuri* and *Langra* was also carried out to check the alterations induced in various parameters including, total soluble solids (TSS), total soluble sugar, reducing sugar, non-reducing sugar,

ascorbic acid and acidity. A hand refractometer (Erma Tokyo Aº32) was used for observation of TSS of fruit pulp with five replicates. Total soluble sugar, reducing sugar, non-reducing sugar estimation was estimated by using modified methods as described by Nelson (1944) Somogyi (1952) and Saran *et al*. (2020b). Ascorbic acid and Acidity content was estimated as per the method given by Ranganna (1979), Garner *et al*. (2008) and Saran *et al*. (2020b) respectively.

The biochemical analysis of wood obtained from the infected and normal stems of both varieties was also carried out. Wood samples from the burl region, the transition between burl (i.e., tumour and normal looking portion) and healthy (i.e., free from burl incidence) trees were collected freshly and dried under aseptic conditions. The dried wood was powdered with the help of a grinder, from which 0.5 g dry wood powder of each sample was used for further 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 to study ascorbic acid content. 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 for each parameter. Obtained results are mentioned in detail and discussed with relevant literature.

***Histological studies*:** Wood samples of the healthy trees and burl wood collected from the infected trees were microtomed to obtain transverse, radial and tangential longitudinal sections of 15–20 μm thickness by using a Leica SM2010R sliding microtome. A total of 25 samples of varying sizes belonging to the different development stages were sectioned for both the varieties (*Langra* and *Rajapuri*). Sections were stained with Safranin-Astra blue combination (Srebotnik and Messner, 1994), and slides were mounted in Dibutyl Phthalate Xylene (DPX) after dehydration through ethanol-xylene series. Permanent slides were observed with a Leica DME 2000 trinocular fluorescence microscope and photographed with a Canon DC 150 Digital Camera.

Results and Discussion

***Disease incidence and characteristics*:** 473 mango germplasm/varieties were screened against burl disease, from which 34 germplasms/varieties were found affected and resulting disease incidence was recorded at 7.18 % throughout India. In Gujarat state,

approximately 167 mango germplasms/varieties were screened, from these nearly 20 varieties/germplasms were found susceptible to burl disease and showed 12 % disease incidence. The highest disease incidence was recorded in *Arka Aruna*, *Seedling*, *Mahmood Vikarabad*, *Mahuvas*, *Tree 253* and *Krishna bhog* while it was lowest in *Desi*, *Khodi* and *Kesar*. Saran *et al*. (2011) and Kumar and Saran (2018) reported the highest incidence of this disease in *Langra* variety followed by *Chausa* and *Gulab Jamun* from Dehradun.

Morphological parameters of burl in different germplasms of mango were also studied for their shape, colour, the surface of burl, side of burl formation and presence of gummosis. The burl shape was recorded globose, globose to elongated and globose to semi elongated in different varieties of mango. The colour of burl was brownish-black, blackish-brown, brownish-grey to brownish-black and greyish black. In fully grown (i.e., large-sized) burls, gummosis was a common feature for most varieties. Similar burl morphological parameters were also recorded in different varieties in earlier studies (Saran *et al*., 2011, 2020a; Kumar and Saran, 2018; Prakash and Shrivastava, 1987; Ploetz *et al.,* 1996b).

***Fruit yield loss*:** The highest fruit yield loss was recorded in *Mahmood Vikarabad* and *Langra* followed by *Hybrid* and *Sukul* varieties whereas it was found lowest in *Olour*, *Alphanso* × *Baneshan* and *Alphanso* × *Sabja*, germplasms respectively. As mentioned earlier, only two varieties viz., *Langra* and *Rajapuri* were selected from Gujarat for further studies on fruit yield loss. The maximum fruit yield loss was recorded in *the Langra* variety and it was minimum in the *Rajapuri* variety. Saran *et al*. (2011) reported (162.3 kg in normal vs. 121.4 kg tree^{-1} in burl affected trees) fruit yields losses in '*Langra'* and (129.1 kg in normal vs. 109.5 kg tree^{-1} in affected trees), in '*Chausa'* variety respectively growing at Dehra Dun. However, the variety '*Gulab Jamun*' they found an increase in yield (97.7 kg in normal vs. 100.2 kg tree^{-1} affected). Kumar and Saran, (2018) reported that *Langra* variety is severely affected by burl and yield loss was more as compared to other varieties under cultivation in Uttarakhand.

***Pathogen isolation and identification*:** The surface-sterilized burl piece inoculated on different selective media (MGY, MacConcay, Hofer's and NASA), after 24 hours of incubation bacterial colonies appeared on the growth media. These colonies were white and creamy on Hofer's media while the red colour on NASA media while shiny and

translucent in an appearance on MacConkey media. Microscopically, slides stained with gram stain showed rod-shaped bacillus form and appeared pink coloured. Based on these initial morphological characteristics, the isolated bacterial colonies were tentatively identified as *Agrobacterium*. For further confirmation, NASA media (Chen *et al.*, 1999) was used and the isolated colonies showed typical red colour specific to *Agrobacterium tumefaciens*. Holt *et al.* (1994) and Bopp *et al.* (1999) reported that *Agrobacterium tumefaciens* is a gram-negative bacteria that showed pink to brick-red colour with smooth taxture, circular, micoud, translucent and shiny appearance on MacConkey agar media. Based on molecular characterization of the isolated pathogen, it was identified as *Agrobacterium tumefaciens* using phylogenetic analysis. The sequence showed 100% sequence homology and the causal organism was identified as *Agrobacterium tumefaciens*. Available literature indicates that *Agrobacterium* was identified using molecular methods i.e., 16s RNA gene sequencing (Mougel *et al.*, 2006; Puopolo *et al.*, 2007).

Pathogenicity test was conducted on three-month-old healthy saplings of *Langra* variety of *Mangifera indica* L., and two months old tomato (*Solanum lycopersicum* L.) plants. After 2-3 months of inoculation, plants showed small measurable outgrowth on both mango and tomato saplings. All the inoculated plant of mango (variety *Langra*) used for regeneration of burl showed 33 % incidence while tomato plants showed 20 % incidence of burl initiation. The pathogen was re-isolated from the inoculated saplings of mango and tomato and they were subjected on bacterial specific (Hofers) media. After 24 hrs colonies on the media showed similar results as it was recorded from the burl samples. Regarding control saplings, no symptoms were observed and no pathogen could be isolated.

***Biochemical analysis*:** Biochemical analysis was aimed to find out chemical alterations in the quality of fruits and for their nutritional value and, biochemical changes in the stem. The fruits of burl infected plants of *Langra* and *Rajapuri* varieties have maximum content of TSS (18.8 °brix), total soluble sugar, reducing sugar, non-reducing sugar and ascorbic acid while the acidity was found minimum. Marmit and Sharma (2008) investigated leaf gall of mango and recorded that compound like total sugars, starch, α-amylase, invertase enzymes activity and reducing sugar were found significantly high as compared to normal/healthy mango plants.

For wood samples, moisture, ash, cellulose, fibre, lignin, total soluble sugar, reducing sugar, non-reducing sugar, total phenol, starch and ascorbic acid were examined and compared between normal and burl portions of wood of *Langra* and *Rajapuri* varieties. The moisture, cellulose, lignin, total sugar, reducing sugar, non-reducing sugar, phenol and ascorbic acid content was found maximum in burl infected woods and minimum in fresh (healthy/normal) wood of both the mango varieties. As compared to burl wood, starch, ash and fibre contents were found maximum in wood collected from healthy trees of both varieties. The moisture content is mostly found higher where the microorganism activity is high. Gelbrich *et al.* (2008) reported that wood portion infected with bacteria have low ash content while fresh stem wood has high ash content. In the present study, the wood portion of burl showed high content of lignin as compared to normal/healthy wood. An increase in the concentration of lignin in burl wood may be associated with the composition of wood. In normal wood, vessels are the widest elements that occupy a considerable area of the wood, axial parenchyma is relatively thin-walled while fibres are the longest cells with 12-15 µm in lumen diameter. In contrast, burl wood was composed of relatively few vessels, more tracheids, axial parenchyma and several cells that are more or less isodiametric and thick-walled. Increased lignin content may be associated with such composition of cell types which consisted of more thick-walled elements. Similar results have also been recorded Gelbrich *et al.* (2008) in forest wood (Pinus and Picea) which associated with bacterial activity. Dsouza and Avishankar (2014) reported that total soluble sugar and reducing sugar increase considerably in gall wood (diseased part) as compared to healthy wood. Similar results in the alterations in the sugar parameters are also investigated in infected wood of *Langra* variety by Saleem *et al.* (2017) where sugars were found increased in diseased parts. Lattanzio *et al.* (2006) reported an increase in the amount of phenolic compound due to the interaction of host and pathogens (insects and fungal pathogen), whereas Gelbrich *et al.* (2008) reported no changes in phenol, ash and sugar content due to bacterial infection in the woods.

***Anatomical examination*:** Histological investigation showed drastic alterations in the burl secondary xylem. All the axial elements lost their vertical alignment and look like wound tissue or pith flecks. Vessel elements, xylem fibres, axial and ray parenchyma cells were deformed, irregular in shape and showed significant variation in their size and shape as compared to the secondary xylem of healthy/normal trees. Formation of tyloses

was observed in narrow vessels and ray cells. Tyloses in these elements were measured from 22 to 59 (33 ± 3.238) µm in diameter. Saran *et al*. (2011) examined the anatomy of the burl infected stem and our results are in agreement with their report. A similar study was also conducted by Saran *et al*. (2020a) and documented significant variations in the structure of the wood cells of normal and burl samples. A detailed description of alterations in the wood structure is explained and showed with photographic evidence in the result chapter.

In conclusion, several mango germplasm/varieties showed susceptibility towards burl disease, from which *Mahmood Vikarabad*, *Arka Aruna*, *Arka Punit* and *Langra* are severely affected while *Amrapali*, *Kesar*, *Hapus*, *Vanraj*, *Mallika* and *Totapuri* is free from the disease. Burl size and their number increase with the increase in the age of the trees. The disease is caused by the infection of *Agrobacterium tumefaciens*. Physical injuries during agricultural practices may be the source of infection. Pathogenicity test of the isolated pathogen from burl also showed positive results on young saplings of mango and tomato giving the confirmation that *A. tumefaciens* is the causal organism for the disease. Affected trees of *Langra* and *Rajapuri* variety showed a reduction in fruit yield. The biochemical analysis also showed increased concentration of total soluble sugar, reducing sugar, non-reducing sugar and ascorbic acid while the acidity was found minimum. Wood samples from the healthy stem and burl wood also showed alterations in their biochemical components. The anatomy of burl wood is also adversely affected and showed complete loss of polarity of the axial elements that lead to the formation of deformed xylem tissues appearing like wound tissue characteristic to tumours.

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