

## Studies on anatomical behaviour of PaLCuV infected papaya (*Carica papaya* L.)

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### Abstract

Papaya leaf curl virus (PaLCuV) of geminiviridae family is a major threat to papaya plants in the world. The major visual characteristics of PaLCuV infected plants are downward and inward rolling and curling of leaves in the form of an inverted cup and thickening of veins. Microscopic observation showed that in the healthy papaya leaf, stomata guard cell size was 19-20  $\mu\text{m}$ . However, it increased significantly in infected plant leaves up to 29-30  $\mu\text{m}$ . This observation suggested that stomatal density and guard cell size were changed due to puckered anatomy of leaf. SEM analysis revealed that subsidiary or accessory cells of guard cells were less turgid and the arrangement of starch grains was disturbed as compared to healthy plant leaves. Light microscopic, scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS) analyses of symptomatic leaves showed the puckering leaf lamina due to presence of loosened cells in its tissues and hyper-accumulation of  $\text{Ca}^{2+}$  ions. High accumulation of  $\text{Ca}^{2+}$  in PaLCuV infected leaves as compared to healthy leaves which might be the cause of hypertrophy and thickening of veins of infected papaya leaves. Uniform trichomes/hairs/nodular structures were present in midrib of healthy leaf but were missing in infected leaf. The infected midrib showed scantily distributed floret-like structures instead of a smooth trichomes/hairs/nodular structures in midrib of the healthy leaf.

**Key words:** EDS, PaLCuV, papaya, SEM, viral disease.

### Introduction

Papaya (*Carica papaya* L.) is a giant herbaceous plant native to tropical America. Papaya has a long history of cultivation and use in tropical and subtropical regions between 32° latitude, North and South of the Equator. The main papaya producing countries are Brazil, Nigeria, Congo, Indonesia, Malaysia and India. Papaya has become a high-ranking fruit crop for its great nutritive and commercial value (Van Droogenbroeck *et al.*, 2004). Papaya (*Carica papaya* L.) is grown commercially in India, USA, Brazil, Indonesia, Mexico, Philippines, Nigeria, Jamaica, China, Taiwan, Peru, and Thailand (Jayavalli *et al.*, 2011). Papaya Leaf Curl Virus (PaLCuV) disease has been outlined from different parts of the world such as India (Singh *et al.*, 2006), Pakistan (Nadeem *et al.*, 1997), Taiwan (Chang *et al.*, 2003) and China (Wang *et al.*, 2004; Zhang *et al.*, 2005; Huang and Zhou, 2006). PaLCuV disease has been reported from different parts of India *viz.* Tamilnadu (Thomas and Krishnaswamy, 1939) Bihar; Karnataka (Govindu, 1964) and Uttar Pradesh (Saxena *et al.*, 1998a, 1998b). In India, papaya leaf curl disease has been reported from different states *viz.* Tamil Nadu (Thomas and Krishnaswami, 1939), Bihar, Karnataka (RC and Govindu, 1964) and Uttar Pradesh (Saxena *et al.*, 1998). PaLCuV disease is characterized by severe curling, crinkling and distortion of leaves accompanied by vein thickening and reduced leaf size. Morphologically the leaf margins are rolled downwards and inwards to form inverted cup followed by thickening of veins. The affected leaves become leathery, brittle and petioles get twisted in a zigzag manner. The affected leaf interveinal areas are raised on the upper surface which gives

wrinkles to leaves due to hypertrophy. The affected plants fail to flower or bear fruits. In advance stage, defoliation takes place and plant growth is arrested (Varun *et al.*, 2017).

Earlier studies showed different environmental factors such as cold shock, drought stress, fungal and bacterial infection affect the crop yield of papaya globally. Along with these factors, few viral diseases are also strongly hampering the growth and production of papaya. Two listed viral diseases, PRSV (papaya ring spot virus) and PaLCuV (papaya leaf curl virus) are the major threats for papaya yield in the world. Papaya cultivation is curbed severely due to PaLCuV which belongs to geminiviridae family. The disease is known to be transmitted by whitefly (*Bemisia tabaci*) in a persistent manner and a begomovirus was detected from papaya by nucleic acid hybridization tests (Hamilton *et al.*, 1983; Saxena *et al.*, 1998). Begomoviruses affect a large number of horticultural crops including papaya (Kumari and Mishra, 2017). Begomoviruses show rolling circle mechanism of replication as they consist of mainly closed, circular, ss-DNA components namely DNA-A, DNA-B or single stranded circular satellite DNA. The rolling circle mechanism results in double stranded intermediate which acts as a template for initiation of transcription of various viral mRNAs and thus acts as a trigger for viral infection in host plants (Lazarowitz *et al.*, 1992). It is interesting to note that the whitefly is unable to feed continuously on papaya to complete the acquisition, latency and effective inoculation periods. The first report on association of ToLCNDV with leaf curl disease of papaya in India predicts that the disease is characterized by severe curling, crinkling and distortion of leaves

Table 1. Primer sequence using for molecular characterization of PaLCuV infected leaf with PCR analysis

Gene name	Accession number	Fwd/Rev	Nucleotide sequence	Database
<i>PALIV1979</i>	Degenerate primer	Fwd	GCATCTGCAGGCCACATYGTCTTYCCNGT	(Bela-ong and Bajet, 2007)
<i>PARIC715</i>	Degenerate primer	Rev.	GATTCTGCAGTTDATRT TYTCRTCC ATCCA	Bela-ong and Bajet, 2007

accompanied by vein thickening and reduction in leaf size. The leaf margins are rolled downwards and inwards to form inverted cup followed by thickening of veins. The affected leaves become cup shaped, leathery, and brittle; and petioles get twisted in zigzag manner. The interveinal areas are raised on the upper surface due to hypertrophy which gives rugosity to leaves. The affected plants fail to flower or bear fruits. In advance stage, defoliation occurs and plant growth is arrested (Saxena *et al.*, 1998a). The association of Tomato leaf curl New Delhi virus with leaf curl disease of papaya was detected by using begomovirus-specific primers for polymerase chain reaction (PCR) and confirmed by highest sequence similarities and close phylogenetic relationships (Raj *et al.*, 2008).

The morphological studies have apparently not been investigated yet in PaLCuV infected papaya leaves. Hence, the current study examines the different basic steps involved at mild stage as well as advance stage of PaLCuV infection.

## Materials and methods

**Sample collection and identification of PaLCuV infected papaya:** Four months old potted *Carica papaya* plants which showed leaf curl symptoms after challenge inoculation with carborundum at ICAR-Central Institute for Subtropical Horticulture, Lucknow, India were taken for study. For molecular detection of papaya leaf curl virus, total DNA was isolated by plant genomic DNA miniprep kit (Qiagen) at Baba Bhimrao Ambedkar University, Lucknow, India. PCR was performed using a set of degenerate primers- PALIV-1978 and PARIC-715 to detect PaLCuV (Table 1). The PCR conditions were: 94 °C-5 min; 94 °C-30 s; 56 °C-90 s; 72 °C-120 s (35 cycles); 72 °C-5 min. The amplification of a 1.5-1.6 kb band on 0.8 % agarose gel confirmed the presence of a geminivirus causing leaf curl disease in papaya.

**Leaf stomatal density and guard cell size:** Stomatal density was determined by using the impression approach method and was expressed as the number of stomata per unit leaf area (Radoglou and Jarvis, 1990). The lower surface epidermis of the leaf was cleaned and smeared with wax in the mid-area between central vein and leaf edge for 15-20 min. The thin film was peeled off from the leaf surface, mounted on a glass slide, immediately covered with a cover slip and then lightly pressured with forceps. Numbers of stomata for each film strip were counted under a light microscope system with a computer attachment (MPS 60, Leica, Wetzlar, Germany). Impressions were taken from 10 healthy and 10 infected papaya leaves. The leaf stomatal density was estimated using the following formula:

$$\text{Stomatal density} = \text{Numbers of stomata} / \text{Area (0.072463 mm}^2\text{)}$$

The stomatal size was defined as the length in  $\mu\text{m}$  between the junctions of the guard cells at each end of the stoma and was taken to indicate the maximum potential which reveals the maximum potential opening of stomatal pore (Xu and Zhou, 2008).

**Scanning electron microscopy and energy-dispersive X-ray spectroscopy:** For scanning electron microscopy and elemental

analysis, the leaf samples were washed and fixed in 2.5 % glutaraldehyde fixative for 2–6 hours at 4 °C. After primary fixation, the samples were washed with 0.1 M phosphate buffer with 3 changes each for 15 min and fixed in 1 % Osmium tetroxide for post-fixation for 2 hours. It was then washed with 0.1 M phosphate buffer with 3 changes each of 15 min. Thereafter, the samples were dehydrated in acetone (30, 50, 70, 90 and 95%) and finally kept in 100 % dry acetone for 30 min. All steps were carried out at 4 °C. Leaves were mounted with double-sided carbon tape on aluminium stubs and sputter-coated with Palladium coater (Auto Fine Coater JFC- 1600 JEOL, Japan). Each sample was examined by JEOL JSM 6490 LV (Tokyo, Japan) scanning electron microscope at different magnifications and accelerating voltages for SEM and EDS analysis. Different anatomical differences were detected in the apical leaf and phloem tissues of both healthy and severely infected papaya leaves.

**Statistical analyses:** All experimental data obtained are the means of more than 5 independent biological replicates and the results with standard deviation (Mean $\pm$ SD) or standard error (Mean $\pm$ SE). The levels of significance were compared between healthy and leaf curl infected *C. papaya* plant leaves by 't' test using SPSS software version 16.0 (SPSS Inc./IBM Corp. Chicago, USA). The results were represented graphically using Microsoft office excel 2007.

## Results and discussion

**Morphological observation and PCR analysis of symptomatic papaya leaves:** The PaLCuV affected papaya plant leaves showed twisted petiole, downward curling of leaf lamina with yellowing veins and puckered shape (Fig. 1a). Moreover, at advance stage, fresh leaves had highly reduced leaf area due to significant curling with vein clearing. The leaf petiole was also thick, twisted and short in length (Fig. 1b). Additionally, we observed that the PaLCuV infected papaya plants showed arrested growth sometimes and fruit size was highly reduced with distorted shape. For PaLCuV confirmation in papaya plant, we isolated the DNA on the basis of initial symptoms of leaf curling. PCR analysis clearly showed ~1.5 kb amplicon from 5 symptomatic leaf samples out of 15 samples collected, while no amplification was obtained in healthy one (Fig. 2).

**Light microscopic observation of stomata density and guard cell size:** Distorted morphology of leaf lamina suggested that PaLCuV could influence the physiological performance in papaya plants. Therefore, we estimated stomata density and also measured guard cell length in PaLCuV infected leaves. Light microscopic observations showed that the healthy plant leaves had stomatal density approximately 1000/mm<sup>2</sup> (Fig. 1c, e). However, it was reduced significantly in all symptomatic papaya leaves, wherein at advance stage of infection, the stomatal density was found to be less than 200/mm<sup>2</sup> (Fig. 1d, e). Microscopic observation showed that in the healthy papaya leaf, stomata guard cell size was 19-20  $\mu\text{m}$  (Fig. 1c, f). However, it increased

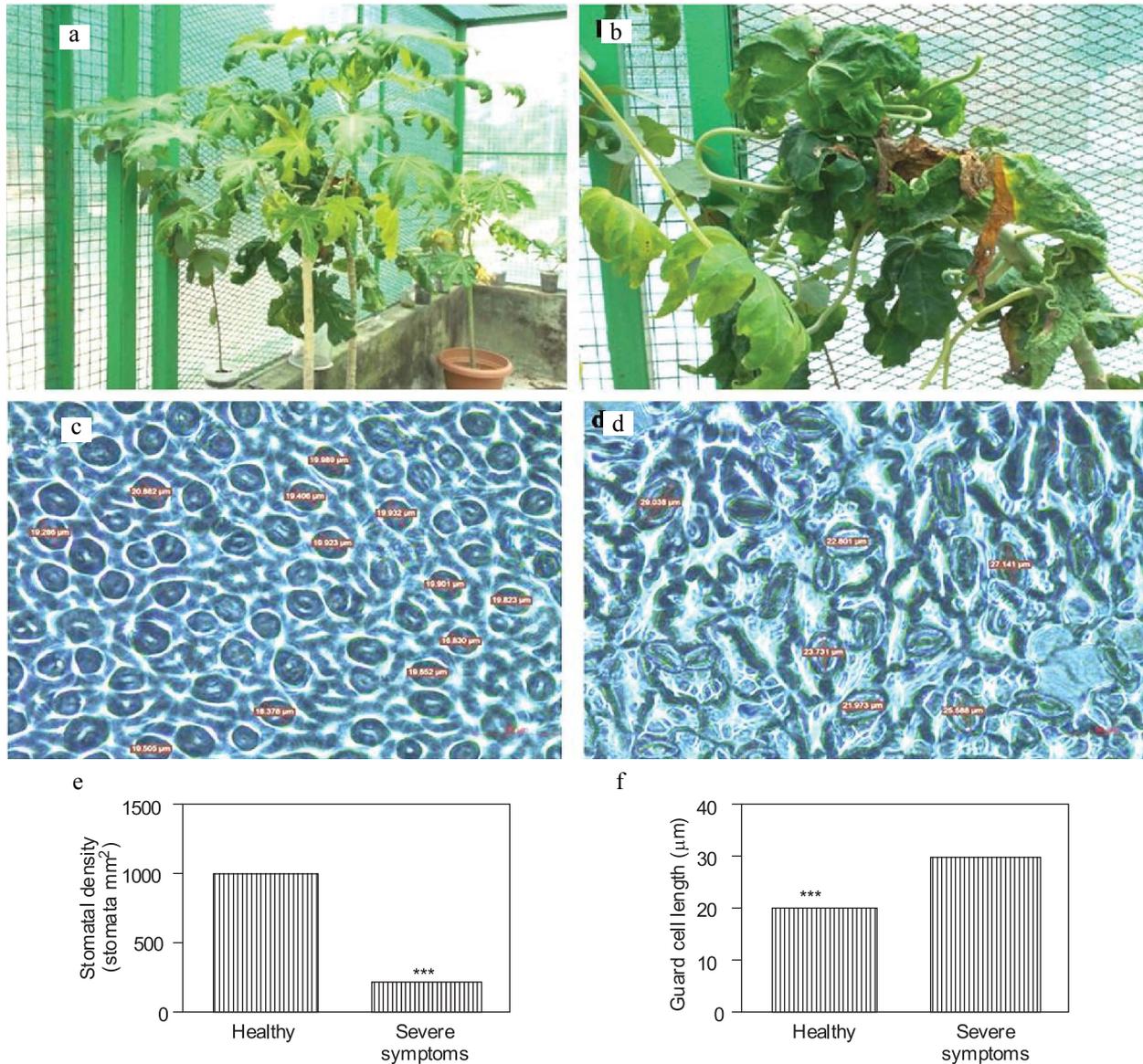


Fig. 1. Morphological characterization of PaLCuV diseased *Carica papaya* and estimation of stomatal density and guard cell size in healthy and leaf curl infected leaves (a), PaLCuV-symptomatic papaya plants in net house (b), PaLCuV infected papaya leaves at advanced stage (c), Light microscopic analysis of healthy papaya leaf (d), Light microscopic analysis of PaLCuV affected papaya leaf. Note: Each value represents the mean±S.E. of 10 leaves. Statistical analysis was done by Student t-test. Each value represents the mean±SD (n = 12) (\*) for  $P \leq 0.05$ , (\*\*) for  $P \leq 0.01$ , (\*\*\*) for  $P \leq 0.001$  or  $0.005$ , significantly different from the control (t-test).

significantly in infected plant leaves up to 29-30 µm (Fig. 1d, f). This observation suggested that stomatal density and guard cell size were changed due to puckered anatomy of leaf. Since the guard cells of healthy papaya leaves were surrounded by higher number of starch grains and thicker or thinner zones of epithelial cells, these differences influenced the change in shape of the guard cells in response to shifts in turgor pressure (Shai *et al.*, 1986) which affected the opening and closing of the aperture.

**Scanning electron microscopic analysis of leaf lamina:**

Visual observation showed that the infected leaves had severe leaf curling, thickening of veins, puckering of leaf lamina and leaf colour darkening as compared to healthy leaves (Fig. 3a-i, 3a-v). SEM analysis at abaxial leaf surface showed difference regarding the normal anatomy of leaf tissue with open stomata in case of healthy leaf (Fig. 3a-ii). However, infected leaf showed reduced stomatal density with closed or slightly open stomata (Fig. 3a-vi). When the same side was viewed

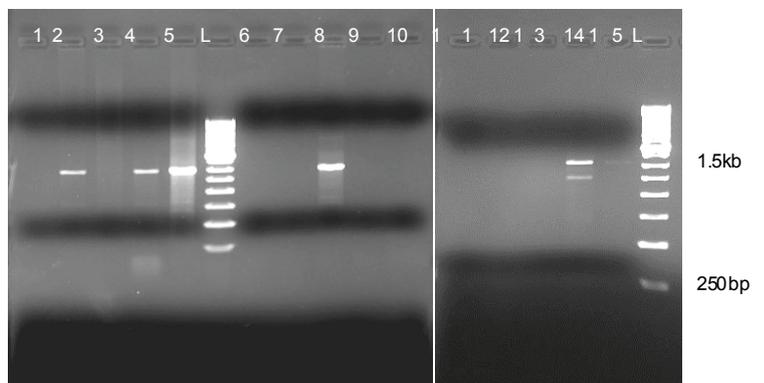


Fig. 2. PCR analysis for detection of papaya leaf curl virus through degenerate primers. The amplification of a 1.5-1.6 kb band on 0.8% agarose gel was used to confirm the presence of a geminivirus causing leaf curl disease in papaya.

at higher magnification i.e. 1000X, a fine stomatal opening in normal tissue could be observed in the case of the healthy leaf (Fig. 3a-iii) as compared to the infected leaf which showed slightly open or closed stomata due to distortion of leaf tissues (Fig. 3a-vii). Additionally,

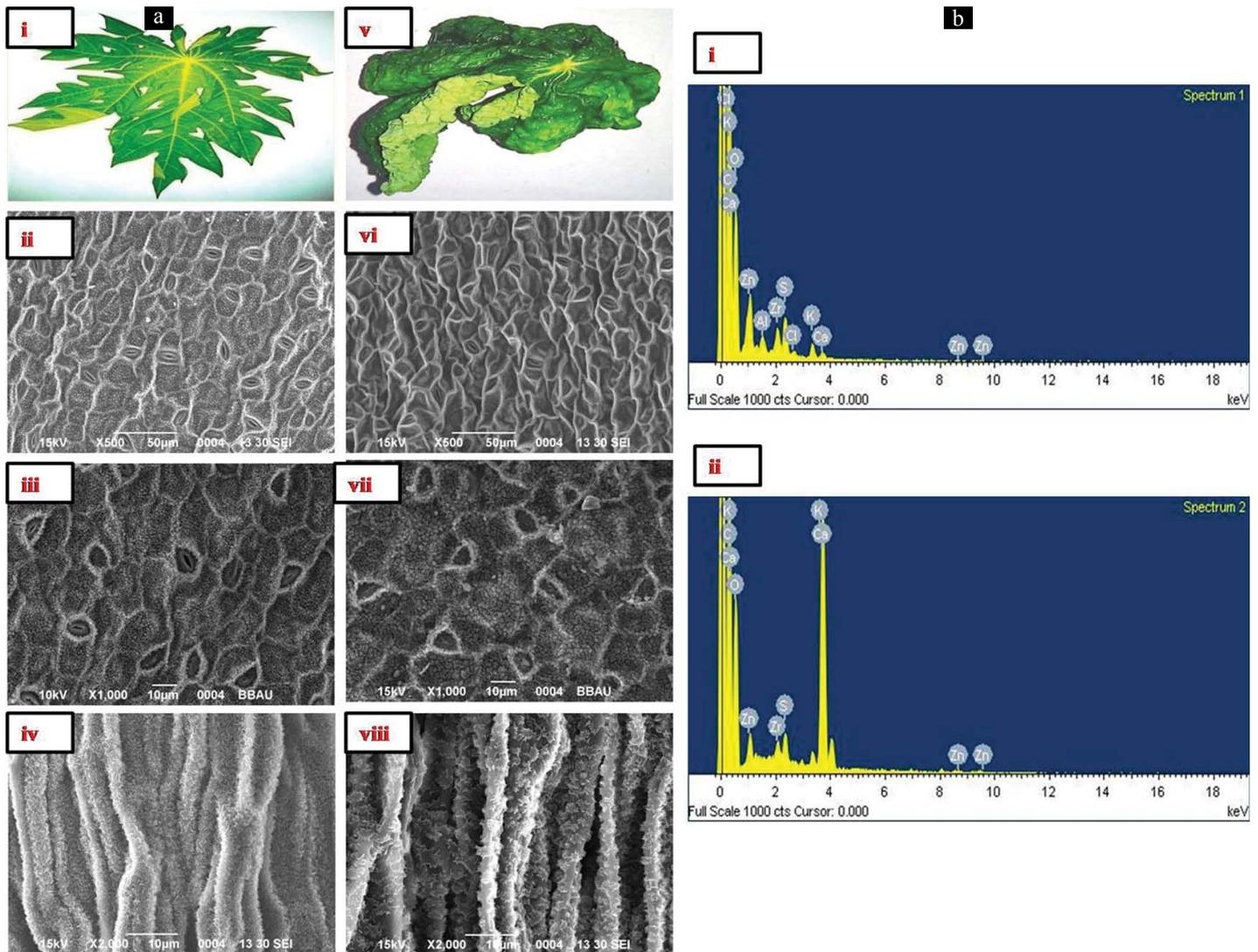


Fig. 3. Scanning electron microscopy and Energy-dispersive X-ray spectroscopy (EDS) (a), Comparative scanning electron microscopy analysis of controlled healthy papaya leaf with leaf curl disease infected papaya leaf (i), Leaf lamina of healthy papaya plant (ii), Abaxial surface of healthy papaya leaf showing high stomatal density with normal stomatal aperture (500X) (iii), Abaxial surface of healthy papaya leaf showing open stomata in healthy papaya leaf with compact surrounding tissue (1000X) (iv), Midrib (2000X) of healthy papaya leaf having compact tissues (v), Leaf lamina of PaLCuV infected papaya plant (vi), Ventral leaf surface (500X) of infected papaya leaf showing comparative less stomatal density with abnormal stomatal aperture (vii), Ventral leaf surface (1000X) showing closed stomata in leaf curl infected papaya leaf (viii), Midrib (2000X) of infected papaya leaf having coarse structure with loosened tissues and gap (ix), EDS on SEM (i), Elemental analysis of healthy papaya leaf midrib (ii), Elemental analysis of PaLCuV infected papaya leaf midrib showing high peak of  $\text{Ca}^{2+}$ .

SEM analysis also revealed that subsidiary or accessory cells of guard cells were less turgid and the arrangement of starch grains was disturbed as compared to healthy plant leaves (Fig. 3a-vii). Since PaLCuV is a geminivirus which is a phloem-based virus, we analysed the leaf midrib at 2000X magnification and found again that in healthy leaf, the cells/tissue were normal in appearance with smooth, flaccid and intact veinal structure (Fig. 3a-iv) as compared to infected leaf midrib which showed a coarse structure with loosened tissue and gaps in the veinal area (Fig. 3a-viii). Interestingly, at a higher magnification, it was observed that uniform trichomes/hairs/nodular structures were present in midrib of healthy leaf but were missing in infected leaf. The infected midrib showed scantily distributed floret-like structures instead of a smooth trichomes/hairs/nodular structures in midrib of the healthy leaf.

Earlier ecophysiology report of papaya plant revealed that stomatal density on abaxial surface of leaves translated to an increase in gaseous exchange and greater photosynthetic

rate under the high light condition and vice versa. Moreover, anatomical study of healthy papaya leaf showed stoma formed by two sunken guard cells and surrounded by several epidermal cells. These guard cells were found to be anomocytic, including higher number of starch grains in the surrounding walls. The rate of gas exchange (stomatal conductance) and transpiration through the leaf stomata were determined by the density, size and degree of stomatal aperture. In the present study, light microscopic observation showed that the healthy plant leaves had maximum stomata density with fine opening at lower surface, which was better able to regulate  $\text{CO}_2$  uptake for photosynthetic carbon assimilation and water loss through transpiration. Besides this, PaLCuV infected leaves had comparatively less stomatal density with large size of guard cell. However, PaLCuV infection in papaya caused distortion in the shape of leaves. Hence, the anatomy of leaf cell was wrinkled and arrangement of surrounding epithelial cell was disturbed, resulting in open guard cells. This cell arrangement pattern might have influenced the size and turgidity of guard cells in PaLCuV symptomatic leaf. SEM

analysis with both low and high resolution also supported our findings, PaLCuV infected plant leaves had puckered anatomy, slightly open or closed stomata with loosened guard cell tissue. Therefore, essential pressure was not generated resulting in impaired movement of sugar from the surrounding cell and its accumulation (Balachandran *et al.*, 1997; Herbers *et al.*, 2000; Shai *et al.*, 1986). Virus infection results in the movement of virus colonies via symplastic cell-to-cell connectivity and the phloem cell. Transport of assimilates in the phloem occurred by pressure flow arising from osmotically generated turgor gradients. SEM analysis at higher magnification showed that in midrib and vein of PaLCuV affected papaya leaves phloem cells with loosened tissue and gaps were present. These changes suggested that the virus infected leaves functioned as sinks (Lemoine *et al.*, 2013) leading to increase in the sugar content. Restricted gaseous exchange or CO<sub>2</sub> diffusion in leaves affects the carbohydrate content of the leaf with consequences for crop yield as compared to healthy plants (Lawson and Blatt, 2014). It had been seen that the accumulation of carbohydrate suppressed the photosynthetic activity through feedback inhibition of carbohydrate synthesis, thereby decreasing the stomatal availability of orthophosphate and causing physical impediment of CO<sub>2</sub> diffusion. Therefore, massive carbohydrate accumulation in chloroplasts physically disrupted the thylakoid structure and decompartmentalized the photosynthetic membranes (Araya *et al.*, 2006; Sagaram and Burns, 2009).

**Energy-dispersive X-ray spectroscopy on SEM:** Elemental analysis of both healthy and PaLCuV infected papaya leaf midrib was done and the results are illustrated as Fig. 3b-i (Goldstein *et al.*, 2017). Within the limitations of EDS instrument i.e. the available elemental standards, the major difference recorded was in the case of calcium, while no significant change was observed in the case of C, S, K and Zn. The Ca weight (0.32%) and atomic weight (0.11%) of healthy leaf was found to show significant increase in the infected leaf where weight percentage of Ca was 11.2 and atomic weight was 4.2% (Fig. 3b-ii).

Energy-dispersive X-ray spectroscopy in SEM suggested high accumulation of Ca<sup>2+</sup> in PaLCuV infected leaves as compared to healthy leaves which might be the cause of hypertrophy and thickening of veins of infected papaya leaves. It is very well known that calcium ion is involved in maintaining cell integrity, membrane permeability and is the major constituent of the middle lamellae of the leaf (Devlin and Witham, 1983). It was also reported that higher concentration of Ca<sup>2+</sup> interferes with a variety of important processes, including microstructural dynamics, Ca<sup>2+</sup>-dependent signalling and phosphate-based energy metabolism (Webb, 1999). Hence, it is considerable to note that due to PaLCuV infection, metal ion concentration might have changed which could be a reason for leaf curling.

A case study of PaLCuV infection in papaya plants suggested puckered anatomy of leaves due to major gaps between the adjacent cells with coarse structure of midrib and high accumulation of Ca<sup>2+</sup> ion. As papaya is a major horticultural crop with enriched nutrient content, so papaya leaf curl virus remains a big threat for cultivating papaya globally. This study is imperative to gain an insight to how plants modulate their anatomical structure during papaya leaf curl virus infection. More in-depth studies are needed for complete understanding of structural modifications in PaLCuV affected crops.

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