Environment and Ecology 42 (3) : 1020—1027, July—September 2024
Article DOI: https://doi.org/10.60151/envec/BNIW3016
ISSN 0970-0420

Virus Infected Papaya Plants Differentially Affects Oxidative Stress and Survival in *Caenorhabditis elegans*

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Received 2 January 2024, Accepted 10 May 2024, Published on 15 July 2024

ABSTRACT

Carica papaya (papaya) leaves has significant nutraceutical properties. However, it is speculated that papaya leaf curl virus (PaLCuV) and papaya ring spot virus (PRSV) can severely affect the therapeutic properties and alter the overall nutraceutical potential of papaya leaves. Therefore, we evaluated the bioactivity of the virus infected papaya leaves extract (PLE) compared to healthy PLE in *C. elegans*. Viruses infection were confirmed by visual inspection and PCR based detection. Anatomical changes and elemental analysis were analyzed through SEM-EDS. PLE were tested for life span extension and ROS scavenging activity on *C. elegans*. Infected leaves displayed typical symptoms of curling, distortion and anatomical analysis revealed closed and less number of stomata. Healthy PLE extended (24%) the life span of *C. elegans* with no significant change in ROS generation while in PaLCuV and PRSV infected PLE, were observed to have significant reduction in the mean life span of 15.4% and 17% with 1.2-fold and 1.9-fold increase in ROS generation.

Keywords *Caenorhabditis elegans*, *Carica papaya*, Life span, ROS.

Abbreviations Caenorhabditis elegans: C. elegans; Papaya leaf curl virus: PaLCuV; Papaya ring spot virus: PRSV; Papaya leaves extract: PLE; Reactive oxygen species: ROS.

INTRODUCTION

Around 80% of the world's population including the scientific and medical communities believes that traditional herbal therapies are safe, effective and used as primary health care. Number of evidence highlighting the fact that plant based diet protects against malnutrition as well as various chronic diseases by providing many health benefits coupled with a low risk of side effects (Karunamoorthi *et al.* 2014). According to the World Wildlife Fund for Nature and the International Union for Conservation of Nature around 50,000 to 80,000 plant species are used worldwide for medicinal purposes. *Carica papaya* L, a well known tropical plant belongs to the Caricaceae family. India along with Brazil is the leading producer of papaya and accounts for more than 50% of the global papaya production. It is a commercially important crop and frequently used in different culinary and medicinal

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practices. Papaya contains high level of carotenoids, minerals (potassium and magnesium), fiber, folic acid (convert homocysteine into less harmful amino acids), phytochemicals, antioxidants like vitamin A, vitamin C, vitamin E, proteolytic enzymes like papain and chymopapain which are used in medicines, industries (as tenderizing ingredient, brewing industries), pharmaceuticals and cosmetics (Karunamoorthi *et al.* 2014). Not only the fruit but other parts of the plant such as roots, leaves, peels, latex, flowers and seeds also have nutraceutical properties (Dwivedi *et al.* 2020, Karunamoorthi *et al.* 2014). Papaya leaves have high contents of polyphenolic compounds (apigenin, catechin, deoxyquercetin, hesperitin, isorhamnetin, kaempferol, myricetin, naringenin, protocatechuic acid, quercetin, and rutin), alkaloid, saponins, pro-anthocyanins, tocopherol and benzyl isothiocyanate. The phenolic compound of papaya leaves is reported to have antibacterial, antiviral and antifungal potential and other active components such as papain, cystatin, tocopherol, ascorbic acid were found to improve the total antioxidant activity and reduce lipid peroxidation (Baskaran *et al.* 2012, Otsuki *et al.* 2010). Papaya leaves also contain high level of vitamins, minerals and are frequently consumed as teas, extracts, tablets, and juices to treat illnesses and believed to have various health benefits such as in the cure of dengue (Teh *et al.* 2022). Traditionally, leaves of papaya plant were used in diseases conditions like complications related to the heart and kidney, diabetes mellitus, amoebic dysentery, fertility, hyperglycemia, cancer. Papaya leaves and seeds are also used in cosmetics to make hair dyes and face masks while different pharmaceutical companies conducting various studies to formulate papaya-based products (Hariono *et al.* 2021).

Consistent development in the field of medical and pharmaceutical science provides evidence that papaya leaves are not only used as an informal medicine but also have scientifically proven pharmacological activities against various chronic diseases, which enhances its formal usages in professional health care system as well as develop its formulations in nutraceuticals and cosmeceuticals field. PaLCuV, a circular single-stranded DNA based virus belonging to the genus Begomovirus, is transmitted to plants through the whitefly (*Bemisia tabaci*) vector. Leaves infected with PaLCuV are characterized by small size, thickened veins, twisted and thickened petioles, curled and wrinkled leaves that roll inward and appear as an inverted cup (Soni *et al.* 2022). Similarly, PRSV, a positive-strand RNA virus belonging to the genus Potyvirus, is transmitted through mechanical activities like pruning of the infected and healthy plant by using the same garden tools or through the feeding of aphids. Characteristics of PRSV infection on papaya plant shows a yellow and stunt papaya plant, mottled foliage, shoe-stringing of new leaves, water-soaked streaking of the petioles and fruit with visible small darkened rings on the surface. Moreover, PaLCuV and PRSV infected plants also show defoliation, fail to produce flowers and fruits and exhibit constrained growth during the advanced stages of infection. Papaya leaves are rich in valuable nutrients, minerals, bioactive compounds and antioxidants and it is well established that these compounds have a cumulative role in increasing life expectancy. While infection with PaLCuV and PRSV may affect the nutraceutical properties of papaya leaves. Therefore, further studies need to perform to investigate the bioactivity of the healthy papaya leaves extract compared to PaLCuV and PRSV virus infected leaves extract through life span and ROS assays in *Caenorhabditis elegans*. *C. elegans*, is a free living nematode that lives in temperate soil environments and feeds on microorganisms. It is majorly preferred for such studies due to its short life span, easy culture in laboratories, genetic and pharmacological similarities with the human (Gammon 2017). Further, *C. elegans* is the first multicellular organism with completely mapped genome, connectome and cell lineage (Devi 2021, Rapti 2020). In *C. elegans*, major signaling pathways that regulate longevity and stress resistance are well conserved so it has simple cell as well as multicellular complexity. Literature highlighted that plants extract and their active compound exhibit lifespan extension in *C. elegans* (Ergen *et al.* 2018, Okoro *et al.* 2021).

MATERIALS AND METHODS

Collection of *C. papaya* leaves sample and extract preparation : Papaya leaves were collected from a healthy plant as well as virus infected plants that were showed disease symptoms.

To prepare PLE, leaves were thoroughly washed,

Table 1. Primer sequence used for the molecular detection of PaLCuV and PRSV infection in papaya leaves.

Gene Name	Nucleotide sequence 5'-3'
PAL1V1978	Forward- GATTTCTGCAGTTDATRT-TYTCRTCCAA
PAR1c715	Reverse- GCATCTGCAGGCCCA-CATYGTCTTYCCN
MJ1	Forward- ATGGTHTGGTGYATHGA RAAYGG
MJ2	Reverse-TGCTGCKGCYTTCATYTG

wiped and then crushed in motor pastel. Chloroform and methanol were added in a 1:1 ratio. The crushed leaves were further homogenized and sonicated to ensure that the majority of the cells break and release most of their phytocompounds. The samples were centrifuged at 4000 rpm for 10 min. The supernatant was collected in a separate tube and lyophilized. Lyophilized samples were dissolved in DMSO to prepare a stock concentration of 50 mg/mL of healthy, PaLCuV and PRSV infected leaf extract.

Confirmation of begomovirus and potyvirus infection using PCR : Infection of begomovirus and potyvirus in papaya leaves was confirmed by PCR using virus specific (Marie-Jeanne *et al.* 2000, Rojas 2019) PCR primers (Table 1). Genomic DNA was extracted using cetyltrimethylammonium bromide (CTAB) method for healthy and PaLCuV papaya leaves from 100 mg sample. To confirm the presence of papaya leaf curl virus PCR has been performed using begomovirus degenerate primers PAL1v1978 and PAR1c715 under the following conditions: 94ºC- 5min, 94ºC- 50s, 52ºC- 45s, 72ºC- 90s (35 cycles); 72ºC- 5 min. Similarly, total RNA was extracted to confirm the presence of PRSV using TRIzol reagent (Thermo Fisher Scientific) and cDNA was synthesized by Verso cDNA synthesis kit. Degenerate primers MJ1 and MJ2 specific for potyvirus were used for detection. Master mix for the amplification of cDNA during PCR contains 2.5 pmol of primers under the following standardized RT-PCR (Reverse transcription PCR) conditions: 94°C- 2 min, 35 cycles of 94°C- 30s, 50°C- 1min, 72°C- 1min and a final extension at 72°C- 10 min. The amplicons were loaded on agarose gel (1%) to confirm the presence of PaLCuV and PRSV causing leaf curl disease and ring spot diseases in papaya, respectively.

Elemental as well as leaf structure analysis of healthy and infected leaves through SEM : For scanning electron microscopy and elemental analysis, leaves were washed in running water and cut into small pieces. 2.5% glutaraldehyde fixative was used for 4-5 h at 4ºC to fix the leaves pieces. After the primary fixation, the samples were washed three times for 15 min with 0.5 M phosphate buffer. For secondary fixation, leaf samples were treated with 1% osmium tetroxide for 4 h and then washed three times with phosphate buffer for 15 min. Then, the samples were dehydrated in 30%, 50%, 70%, 90%, 95% and 100% acetone subsequently for 30 min at 4ºC. Leaves were mounted with conductive double sided adhesive carbon tape on aluminium stubs and sputter-coated with Platinum coater (JFC 1600, Auto Fine Coater JEOL, Japan) for 1min at 20 mA voltage. For SEM and EDS analysis, different magnifications and accelerating voltages were used to scan samples individually [JEOL JSM 6490 LV (Tokyo, Japan)].

***C. elegans* culture and maintenance :** Bristol N_2, a wild-type strain of *C. elegans* was grown and maintained on nematode growth medium (NGM) plates at 20ºC. NGM agar plates were seeded with a tiny spot of OP50, a strain of *Escherichia coli*. Standard bleach method was used to get synchronized L_4 young adults (Brenner 1974). An age-synchronized culture of young adults was used in all the experiments.

Life span assay : Life span assay was performed as reported in Sutphin and Kaeberlein (2009). Briefly, after the 24h exposure of healthy and infected PLE at 0.5 mg/mL, 30 worms were transferred on fresh NGM plates containing 50 µM 5-fluoro-2'-deoxyuridine (FUdR). Worms were observed under the microscope every day for their survival and periodically moved to fresh plates to restrict mold growth. Animals were scored as dead if a gentle touch of Pt-wire did not make them respond. The percent effect was determined by taking control (unexposed worms) as 100% and evaluating effects in treatment groups.

ROS assay : Generation of reactive oxygen species (ROS) among control (unexposed worms), healthy and infected PLE exposed worms was determined

as reported in Sonane *et al. (*2017). Briefly, after the exposure of healthy and infected PLE at 0.5 mg/mL, about 1000 worms from control and treated groups were separately transferred into each well of a transparent bottom black 96-well plate in triplicates to which 0.05 mM H_2DCF-DA dye (Sigma, USA) was added and incubated for 30 min on an orbital shaker. ROS was measured at 485/535 nm in Spectrophotometer Spectramax (Molecular Devices, UK). Worms exposed to a non lethal concentration (1mM) of H_2O_2 (Sigma-Aldrich, MO) were used as positive controls.

RESULTS

Comparative morphological analysis and PCR based confirmation of symptomatic papaya leaves : Virus infected plants showed morphological differences such as wrinkled and twisted leaves, yellow veins and petioles that were short in size thick and twisted in shape as shown in Fig. 1.

PCR based identification of PaLCuV and PRSV infection was performed on healthy as well as symptomatic plant leaves using specific primers. Amplicons of ~1.6 kb and 0.33 kb were observed from PaLCuV and PRSV infected plant leaves respectively, on agarose gel (1%), while no amplification was observed in healthy ones. These results confirm that leaves that were taken for the study were PaLCuV and PRSV infected (Fig. 2).

Virus infection alters the stomata structure and elemental composition in papaya leaves : Visual inspection of leaves showed several symptoms of PaLCuV (leaf curling, thickening of veins crowing) and PRSV (yellow and stunting of the leaves, darken color) infection compared to healthy leaves (Figs. 3 A-C). Scanning electron microscopy analysis of the abaxial surface of leaves revealed that healthy leaves have normal anatomical structure of leaf tissue with open stomata (Fig. 3D), while infected leaves were found to have closed or slightly open stomata with reduced density (Figs. 3 E, F). At the higher magnification i.e. 2500X, normal stomata opening and tissue were observed in healthy leaves compared to infected leaves where distorted leaf tissue was observed with closed or slightly open stomata (Figs. 3 G-I).

Elemental analysis of healthy versus PaLCuV and PRSV infected papaya leaves was performed and the results are illustrated in Figs. 3 J-L. With the available elemental standards of EDS instrument no significant difference was observed in the cases of O, S, C and Pt. While in case of Ca weight (3.38%) and atomic weight (1.26%) of healthy leaves was found to show significant decrease in case of PRSV (0.5% and 0.19%) infected papaya leaved and increase in case of PaLCuV (5.73% and 2.89%). The Mg (weight 3.5% and atomic weight 1.3%) of healthy papaya was significantly higher (5.24% and 4.36%) in PaLCuV infected papaya leaves (Figs. 3 J-L).

Fig. 1. Morphology of (A) Healthy (B) PaLCuV (C) PRSV infected papaya leaves.

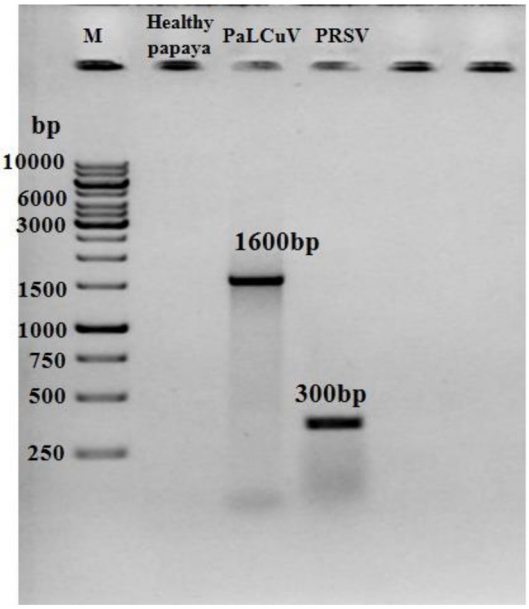

Fig. 2. PCR based detection of papaya leaf curl virus and papaya ring spot virus through degenerate primes. The amplicons of 1600 bp and 330 bp on 1% agarose gel were used to confirm the presence of Begomovirus causing leaf curl diseases in papaya and Potyvirus causing ring spot diseases in papaya.

Virus infection accelerates aging in *C. elegans* : To study whether the PLE can influence longevity of the wild-type (N_2) worms, a life span assay was performed. Compared to the control, a significant increase ($p<0.05$) of 23% was observed in the mean lifespan of worms exposed to healthy PLE (0.5 mg/mL). While a significant reduction ($p<0.05$) of 15.4% and 17% was observed in the mean life span of worms exposed to PaLCuV and PRSV infected PLE (0.5 mg/mL), compared to control (Fig. 4). These data indicated that the healthy leaves contain vital phytochemicals that may influence the longevity of *C. elegans*, which were adversely altered by the viral infection in papaya leaves.

Virus infection induces free radical generation in *C. elegans* : ROS generation was observed significantly high in the worms exposed to the virus infected PLE. We observed a 1.2 and 1.9-fold increase in ROS production in PaLCuV and PRSV infected PLE compared to the control, respectively. While, compared to the control, in healthy PLE exposed organisms ROS generation was not significantly altered. H_2O_2 (0.5 mM) was used as a positive control (Fig. 5).

DISCUSSION

Papaya leaves are rich in vitamins, antioxidants, minerals and highly explored for their therapeutic potential as an antiviral, antitumor, antibacterial, anti-inflammatory and hypoglycemic activity (Singh *et al.* 2020). Infection of viruses such as PaLCuV, PRSV is very common and major threats in the papaya plants. Although, PaLCuV and PRSV virus infection adversely affect the physiology, morphology and biochemical properties of papaya plant (Soni *et al.* 2022, Vincent *et al.* 2018). However, none of the report suggesting that it may result in the cumulative loss of nutraceutical properties including antioxidant properties and can indirectly harmful or comparatively less effective as the healthy plant in human. Lack of awareness in common people about these viral infections increases the chances of consumption of infected leaves in diverse home remedies. Therefore, this study is important and can provide a comparative difference in the therapeutic and antioxidant potential of healthy and viruses infected papaya leaves extract in *C. elegans*. *C. elegans* is a power fool for rapid screening of many compounds and chemical and shares key genetic and physiological similarities with humans (Ha *et al.* 2022).Virus infected papaya leaves were selected based on the symptomatic characteristics such as for leaf curl virus- small size, twisted and thickened vein with yellow colored, twisted and thickened petioles and for Potyvirus - mosaic, mottling, puckering, distortion of leaves (Mishra *et al.* 2018, Soni *et al.* 2022). Further, PCR-based detections were performed using degenerate primers to confirm the presence of the PaLCuV and PRSV viruses in the infected leaves. The physiological and morphological differences in the healthy and virus infected leaves were observed at the microscopic level through SEM-EDX electron microscopy. These results corroborate with previous reports (Mishra *et al.* 2018). We have also observed significant changes in the level of Ca and Mg in elemental analysis of the healthy and virus infected papaya leaves. Ca^{2+} is a major constitute of the middle lamellae of the leaf and play an important role in maintaining membrane integrity and permeability (Mishra *et al.* 2018). Increased level of Ca^{2+} in the PaLCuV infected leaves might be the cause of

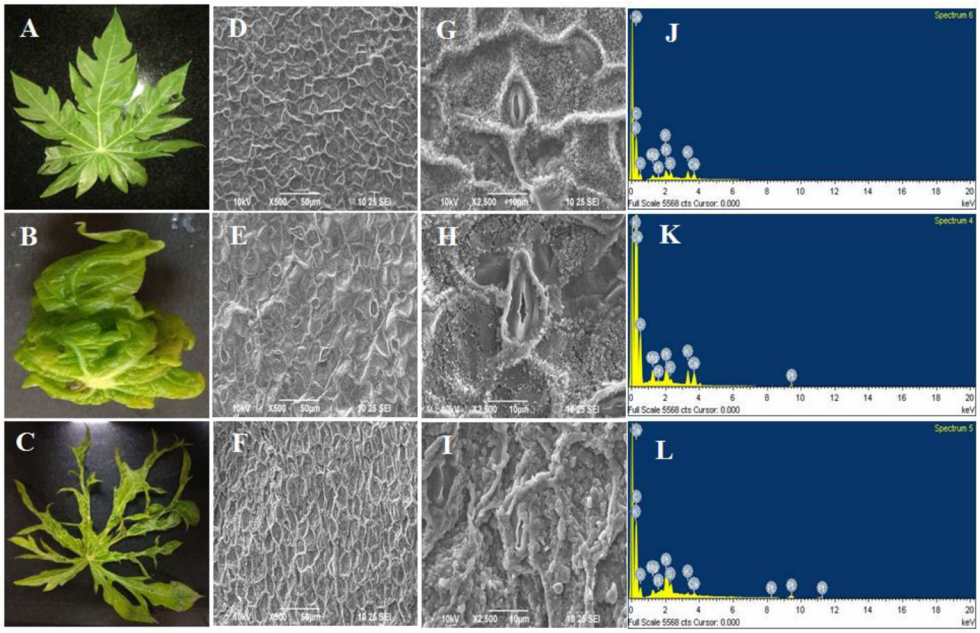

Fig. 3. Scanning electron microscopy and Energy-Dispersive X-ray spectroscopy (EDS), Comparative scanning electron microscopy analysis of healthy papaya leaf with leaf curl and ring spot disease infected papaya leaf (A-C), Abaxial surface of healthy papaya leaf showing high stomatal density with normal stomatal aperture while infected papaya leaf showing comparative less stomatal density with abnormal stomatal aperture (500X) (D-F), Abaxial surface of healthy papaya leaf showing open stomata in healthy papaya leaf with compact surrounding tissue while showing closed stomata and distorted surrounding tissue in leaf curl and ring spot infected papaya leaf (2500X) (G-I), Elemental analysis of healthy papaya leaf, PaLCuV infected and PRSV infected papaya leaves (J-L).

hypertrophy and thickening of veins while in case of PRSV infection decreased level of Ca^{2}+ might be associated with reduced height, fewer nodes, and less leaf area. Mg level was found higher of in PaLCuV infected papaya leaves. Mg is essential nutrients that majorly involves in chlorophyll synthesis, enzyme activation, protein synthesis, transportation, regulation of vacuolar ion channels and affect stomatal density in variety of plants (Hasanah *et al.* 2020).

Healthy papaya as well as PaLCuV infected

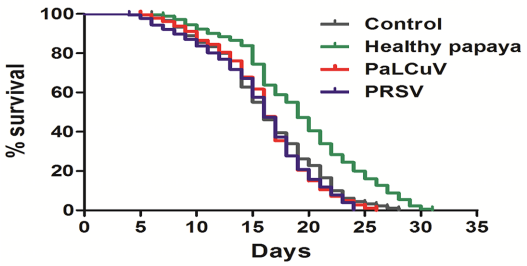

Fig. 4. Longevity of N_2 worms treated with healthy, PaLCuV and PRSV infected papaya leaves extract. Life span extension of *C. elegans* at 0.5 mg/ml concentration of healthy papaya leaves extract. n = 3 (~90 individuals per group).

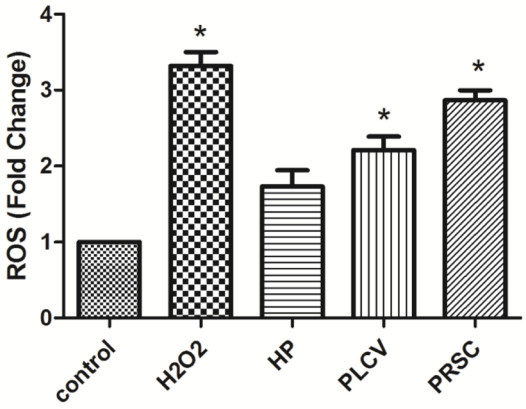

Fig. 5. Free radical generation in *C. elegans* exposed to healthy, PaLCuV and PRSV infected papaya leaves extract. The data is normalized relative to the mean control value (Control=1). Bar represent mean ± SE of three experiments and *p < 0.05. n=3.

leaves extract were reported to extend the life span of *C. elagans* at 100µg/mL concentration (Soni *et al.* 2022). We also observed similar results in the worms exposed to healthy PLE however virus infected PLE were found to reduced the life span of worms at 0.5 mg/mL. It indicates that, at higher concentration virus infected PLE excert their adverse effect on survival of the organism. We further found a significantly increased free radical generation in all the treated groups except healthy PLE exposed worms where ROS generation was slightly higher compared to the control. This amount of ROS generated in worms exposed to healthy PLE may not be able to induce any damage to the organism but may trigger the antistress response pathways that led to the extended life span of organisms. Whereas in virus infected PLE exposed worms, ROS generation was significantly high and may have been able to induce extreme cellular damage to the organism and eventually leading to early death.

Bioactive compounds of the PLE are responsible for the nutraceutical properties of the papaya plant and play key roles in ameliorating as well as treating many medical conditions. Each part of the papaya plant specially the leaves and fruits is rich in different phytochemicals and has its therapeutic values (Singh *et al.* 2022). Viral infections alter the anatomical, physiological and important bioactive compounds that reduce the nutraceutical potential of the healthy papaya plant (Soni *et al.* 2022). So this is very important as common people should know and be able to differentiate healthy papaya from virus infected papaya based on symptoms. Hence, our results suggest that while recommending home remedies efforts should be made to educate and inform the public or patient to critically observe the papaya plant and choose only healthy leaves for consumption as therapeutics.

Papaya (*Carica papaya* L.) is one of the most extensively studied plants due to its commonly known nutritional and medicinal value. The present study provides evidence that virus infection can alter the nutraceutical properties of healthy PLE. Hence, only healthy papaya leaves should be used by commercial companies for large scale production of therapeutics. This study spreads awareness among the common people about the health benefits of healthy papaya leaves extract versus virus infected papaya leaves extract and emphasizes consuming only healthy leaves for therapeutic needs.

ACKNOWLEDGMENT

All the authors are thankful to Babasaheb Bhimrao Ambedkar University for providing necessary infrastructure and USIC facility for carrying out the SEM-EDS of papaya leaves. We also acknowledge research fellowships from University Grant commission to MS and SK, Department of Science and Technology to AR, Council of Scientific and Industrial Research to P.

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