4. MATERIALS AND METHODS

4.1. Collection of mulberry leaves

Fresh, healthy and disease free leaves were used for preservation purpose and were collected from three different locations of Darjeeling district, West Bengal, India on the basis of availability. The locations of leaf collection are as follows:

- Directorate of Textiles (Sericulture), Govt. of West Bengal, Matigara Sericulture Complex, Matigara, Darjeeling (26°70′40″ N and 88°35′37″ E)
- Directorate of Textiles (Sericulture), Govt. of West Bengal, Naxalbari Sericulture Complex, Naxalbari, Darjeeling (26°66′23″ N and 88°19′46″ E)
- Mulberry Germplasm Garden, Department of Botany, University of North Bengal, Darjeeling (26°50′58.3″N 88°26′26.9″E)

4.2. Preparation of voucher specimens

Healthy and disease free twigs of mulberry cultivars were collected during field visit. The collected twigs were tagged, and field characters were recorded in Field Notebook. Cleaning and processing of the collected samples were carried out following the standard protocol of Jain and Rao (1977) and Paul et al. (2020) with slight modifications, followed by drying using old news and blotting papers. Post drying, the specimens was soaked in saturated solution of mercuric chloride (4 – 6%) prepared in rectified ethanol, followed by drying and mounting against standard herbarium sheets. Prepared herbarium sheets were labelled and deposited at Herbarium of University of North Bengal against accession numbers NBU – 11722, NBU – 11723, NBU – 11724, NBU – 11725, NBU – 11726 and NBU – 11727 corresponding to the mulberry cultivar namely V1, Guangdong, BC259, S1, S1635, TR10 respectively.

4.3. Screening of mulberry cultivar for experimental setup

Six cultivars of white mulberry *viz.* S1, S1635, V1, BC259, TR10 and Guangdong were primarily selected from the locally available mulberry cultivars on the basis of their frequency of availability for screening out most suitable mulberry genotype for conducting preservative based experiments. For maintaining the fresh texture of the leaves, the samples were collected early morning in between 6:00–7:00 am.

4.3.1. Study of foliar macro-morphology

Macro-morphological characters *viz*. total leaf length (lamina + petiole), lamina length, lamina breadth, petiole length and internodal distance were measured directly from the studied cultivars during field visit and were recorded in Field Note Book.

4.3.2. Study of foliar micro-morphology

Different techniques were followed to study foliar micro-morphological parameters. The protocols used are prescribed below.

4.3.2.1. Study of Venation pattern

Method of Mishra et al. (2011) was followed to study the venation pattern of the studied cultivars. Leaves were incubated with 80% ethanol for 48 hrs leading to chlorophyll removal, followed by distilled water washing. Leaves were then treated with 3–5% sodium hydroxide and incubated for ~15 days at 25˚C. Following incubation, the leaf skeleton was acquired after careful removal of digested tissue. The leaves were then treated with lactic acid (Himedia) and boiled to obtain transparent clean tissues (Lama 2004). Finally the leaf specimens were stained with 2% safranin (Himedia, RM1315-25G, Batch#0000029519) and washed with 70% ethanol before observing under simple and compound microscope.

The primary (1˚) and secondary (2˚) veins were primarily observed by naked eyes and the detailed layout was observed using simple magnifying lens. Minor venation pattern *viz*. 3˚ and 4˚ veins, vein course, vein angle variability etc. were observed under compound microscope using 5X objective and 10X eyepiece.

4.3.2.2. Study of Indumentum

Detection of indumentums type and nature among the studied cultivars was conducted by observing the processed lamina under compound microscope using 5X, 10X and 40 X objectives.

4.3.2.3. Detection and study of stomatal parameters

Study of stomata, its type and dimension is of significant taxonomic importance as it provides identifying characters. Beside this, stomatal frequency and index are

significantly important characters for physiological and ecological studies. Detection of stomatal parameters among the studied cultivars was conducted following two separate detection protocols as stated below.

- Epidermal peeling of the leaves were taken, stained with safranin, mounted with glycerol and observed under microscope. Observed stomata and its associated cells was photograph for documentation.
- Portion of the leaf (without mid-vein) was allowed to boil 2–3 times using absolute (100%) alcohol (causing de-pigmentation), followed by lactic acid treatment (causing leaf transparency) and observed under microscope after mounting with glycerol. Safranin staining was also conducted for proper visibility.

Following the method prescribed by Salisbury (1927), stomatal frequency (SF) and index (SI) was detected using the following formula:

$$\text{Stomatal frequency} = \text{Number of stomata} / \text{milimeter square of microscopic field}$$

$$\text{Stomatal index} = \frac{\text{S}}{(\text{E} + \text{S})} \times 100$$

S = number of stomata per microscopic field

E = number of epidermal cell per microscopic field

4.3.3. Statistical data analysis of macro- and micro-morphological data

Results were expressed as Mean ± SEM where n = 50 for macro-morphological characters and 20 for micro-morphological characters. Differences between different attributes among the studied samples are indicated with different letters (a, b, c,... etc.) differs significantly at $p \leq 0.05$ by Duncan's Multiple Range Test (DMRT). The obtained data was subjected to analyze of variance (ANOVA) for determining the level of significance among the attributes under study. To determine direct or inverse relationship between different attributes under observation, correlation analysis was performed using XLSTAT 2017 software. Principle Component Analysis (PCA) of different genotypes and variables under study was conducted

using XLSTAT 2017 software. Similarity Dendrogram was constructed using XLSTAT 2017 for clustering genotypes with similar morphological features.

4.4. Experimental setup leading to post-harvest mulberry leaf preservation

The most commonly grown and used genotype of mulberry in Siliguri sub-divisional region of Darjeeling district was used as experimental material. The leaves were collected from sericulture farms, early morning, in between 6:00 – 7:00 am during April to October of four consecutive years starting from mid-2016 to late-2020. Uniform experimental condition was maintained by selecting leaves of same maturity and by eliminating unhealthy and disease leaves from the harvested lot. Post-harvest, the petiole of the leaves was immersed in preservative solutions for 7 days. For retaining moisture content, leaves were preserved inside perforated zipper bag. Prior to preservation, fine oblique section of the leaf petiole under water was made for maintaining continuous water column. The experimental units were kept under constant physical parameters maintaining at 25˚C, 260 – 270 lux light intensity and 40% humidity. During the course of preservation, leaves were evaluated at regular interval for determining preservative potentiality of used solution. Schematic representation of the steps involves in post-harvest leaf preservation was provided in Figure 11.

4.5. Screening of preservative solutions

Mulberry leaves were preserved in different preservative solutions for screening out the ideal solution and effective concentration suitable for prolonging shelf life at post-harvest stage. Leaves were allowed to preserve in different hormonal solutions *viz*. indole acetic acid (Himedia, PCT0803-5G, Batch# 0000229586), kinetin (Himedia, RM448-1G, Batch# 0000176257), benzyl adenine (Himedia, RM789-5G, Batch# 0000080236) and gibberellic acid (Himedia, RM1867-1G, Batch# 5-2154); phenolic phyto-hormone *viz*. salicylic acid (Himedia, RM1286, Batch# 3-2583); ethylene blocker *viz*. Putrescine (Himedia, RM443-1G, Batch# 00192970) and sodium nitroprusside (SRL, 13755-38-9, Batch# 4827140); silver salts *viz*. silver nitrate (Himedia, RM408-10G, Batch# 000159591) and silver thiosulphate (Himedia, PCT1308-100ML). For each solution, four different concentrations were used *viz*. indole acetic acid (IAA; 10, 5, 1, 0.1 mM), kinetin (10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M), benzyl adenine (BA; 10^{-2}, 10^{-3}, 10^{-4} M and 0.05 mM), gibberellic acid (GA; 1,

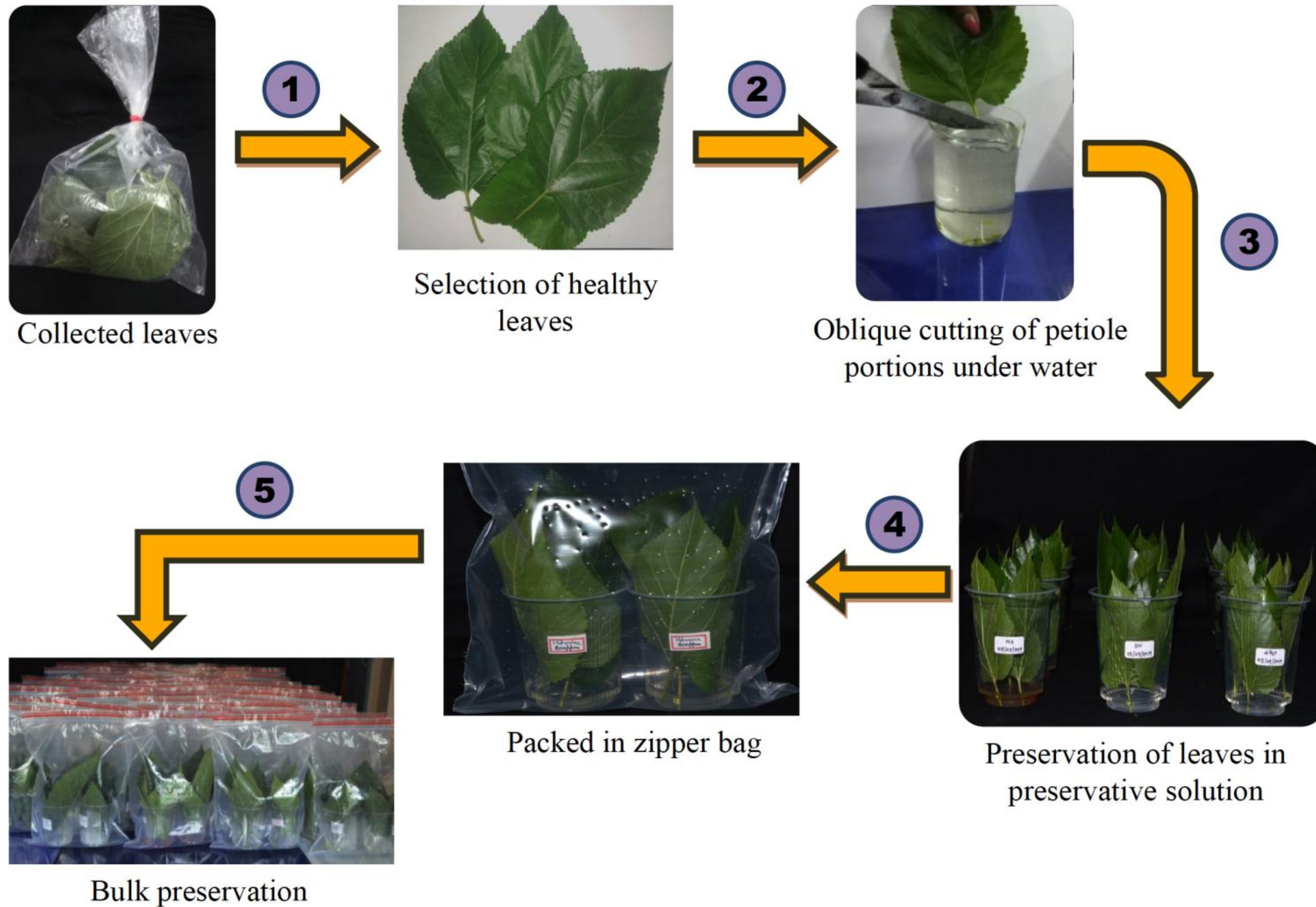


Fig. 11: (1-5) Schematic diagram representing sequential steps involve in post harvest preservation of mulberry leaves

0.5, 0.1 and 0.05 mM), salicylic acid (SA; 2, 1, 0.5 and 0.1 mM), putrescine (800, 600, 400 and 200 ppm) and sodium nitroprusside (1, 0.1, 0.05 and 0.01 mM) silver nitrate (SN; 150, 100, 50 and 10 ppm) and silver thiosulphate (STS; 300, 200, 100, 50 μM). At the end of preservation period, leaves were evaluated with respect to both qualitative and quantitative parameters for determining most suitable solution extending shelf life at post-harvest stage.

4.6. Phytosynthesis of silver nanoparticles

Silver nanoparticles were synthesized by reduction of silver salt (silver nitrate) using phyto-extract of mulberry leaves.

4.6.1. Preparation of plant extract

For preparing extract of mulberry leaves, 10 gm leaf was measured (Sartorius Lab Instruments GmbH & Co. KG, Model No. QUINTIX224–10IN), washed with distilled water (dH_2O) repeatedly for 2 – 3 times and then gauzily chopped into small pieces. To the conical flask (Borosil Make) containing 100 ml double distilled water (ddH_2O), chopped leaves were added and were allowed to reflux for 60 min at 100°C. At the end of refluxing period, pale yellowish coloured leaf extract was collected.

4.6.2. Preparation of silver nitrate ($AgNO_3$) solution

For phytosynthesis of silver nanoparticles, 10^{-3} M silver nitrate (Sigma-Aldrich v800358-25G, Batch# 0000003756, VETEC; DH7D671829, 1.9320.0027, Batch# 8907357614039; Himedia GRM409-10G, Batch# 0000384304) solution was prepared in ddH_2O.

4.6.3. Biosynthesis of nanosilver

Prepared leaf extract of mulberry was added drop-wise to the prepared silver nitrate solution, in the ratio of 9:1 (Kuppurangan et al. 2016). The resultant solution was mixed uniformly using magnetic stirrer (REMI Equipments) for 10 min. The solution was allowed to stand for ~30 – 40 min until completion of entire reduction process.

The schematic representation of the entire process leading to the synthesis of nanosilver was given in Figure 12.

4.7. Validation and characterization of phytosynthesized silver nanoparticles

Following parameters were adopted for validation and characterization of phytosynthesized silver nanoparticles.

4.7.1. Colour transformation

Initial confirmation of nanosilver formation was determined by the emergence of brown colour solution with time, which was an indicative signal for complete reduction of transparent silver nitrate to nanosilver.

4.7.2. Spectrophotometric analysis

Validation of silver (Ag^{+}) ion reduction leading to the formation of nanosilver was done using UV-Vis Spectrophotometer (SYSTRONICS-2201). Synthesized nanosilver after 12 hrs of reaction was diluted in the ratio of 1:4 and was run against wavelength range of 300 – 800 nm. The spectrum obtained by plotting absorption against the wavelength was analysed for the appearance of surface plasmon resonance (SPR).

4.7.3. Fourier transformed infrared spectroscopy (FT-IR)

Detection of functional groups for predicting the possible involvement of organic molecules present in phyto-extract as reducing and capping agents participating in reduction of silver ion was conducted using Fourier transformed Infrared Spectroscopy (THERMO NICOLET, AVATAR 370). FT-IR analysis was carried out in the wavelength range of 4000 – 500 cm^{-1} and a resolution of 4 nm. Dried plant extract and nanosilver was incorporated directly and individually on separate potassium bromide crystals to acquire the spectra in transmittance mode.

4.7.4. Scanning electron microscopy (SEM)

Scanning electron microscopic (JEOL Model JSM-6390LV) analysis was conducted for studying shape and surface morphology of synthesized nanoparticles. For analysis, drop of nanosilver was dried on a carbon-coated copper grid and then observed and imaged at different magnifications.

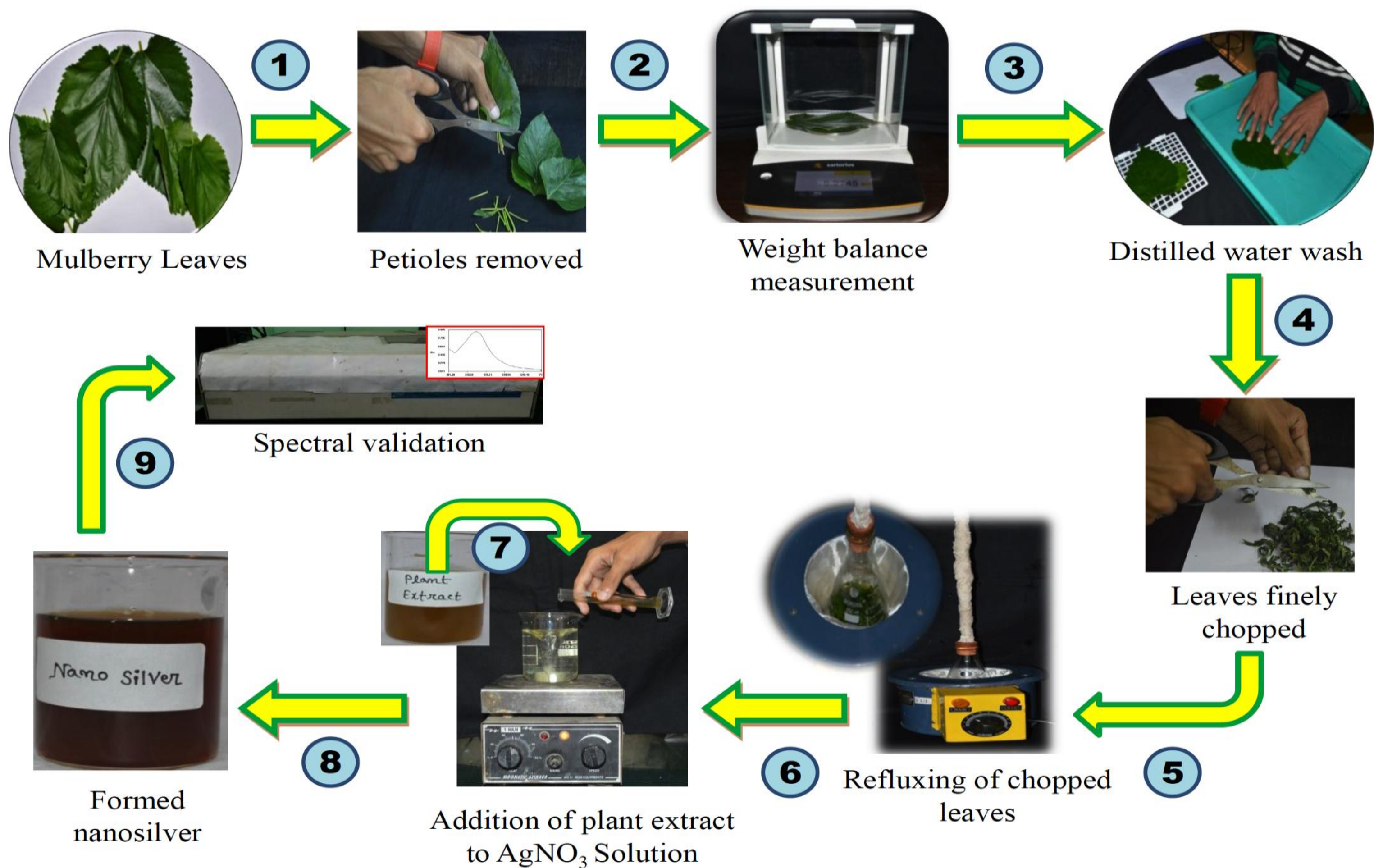


Fig. 12: (1-9) Schematic diagram representing sequential steps involve in phytosynthesis of silver nanoparticles

4.7.5. Field emission scanning electron microscope (FESEM)

Investigation of topographical features of synthesized nanoparticles was conducted using field emission scanning electron microscopy (JEOL Model JSM-7600F). The instrument was operated at an accelerated voltage of 10.0 kv and was imaged at magnification range of 33X – 100,000X.

4.7.6. Energy dispersive X-ray spectroscopy (EDX)

Profiling of elements present in biosynthesized nanoparticles was done using energy dispersive X-ray spectroscopy (Oxford-EDX system). For analysis, dry sample was used that was passed through 80 mm^2 silicon drift detector (SDD) that identified element under high resolution.

4.7.7. Transmission electron microscopy (TEM)

Transmission electron microscopy (Philips-CM 200) was conducted for measuring size and determining shape of biogenic silver nanoparticles. The micrographs were obtained at an operational voltage of 120 KV. The size distribution pattern was analysed using Image J (version 1.50b) by sampling more than 200 particles and plotted graphically using Origin statistical software (Origin Pro 2020, Origen Lab Corporation, USA). Polydispersity index of particle distribution was calculated using the following formulation:

$$\text{Percent polydispersity} = \left\{ \left({}^{\alpha}/_{\beta} \right) \times 100 \right\}$$

Where “α” was the standard deviation of particle size distribution and “β” stands for average nanoparticles size.

4.7.8. High resolution transmission electron microscopy (HR-TEM)

High resolution transmission electron microscopic analysis was done using PHILIPS MODEL CM 200 electron microscope, by loading sample on a carbon coated copper grid operated at an accelerated voltage of 120 kV. TEM analysis was done for determining the size, shape and morphology of synthesized nanosilver. Sampling of more than 350 particles per microscopic filed was conducted using Image J software (version 1.50b) for determining particle size. The particle size distribution range was obtained using Origin statistical software (version b9.5.5.409, Origen Lab

Corporation, USA). Percent polydispersity of was determined using the same formula as mentioned in 4.7.7.

4.7.9. X-ray diffraction analysis (XRD)

For determining crystalline nature of biogenic silver nanoparticles, X-ray diffraction pattern (BRUKER AXS D8 ADVANCE: BRUKER KAPPA APEX II) was used. For analysis, centrifuged dry crystals of nanoparticles were operated at 30 mA current and at operational voltage of 40 kV. The sample was Cu Kα radiated for generating 2θ data, operated at a speed of 5˚/min. The obtained result was compared with standard Joint Committee on Powder Diffraction Standards (JCPDS) library for determining the crystalline structure. The average crystalline size was calculated using Debye - Scherrer's (D) formula:

$$D = {}^{0.9\,\lambda}/_{\beta\,\mathrm{Cos}\,\theta}$$

Where "λ" denotes the X-ray wavelength, "β" represents the angular full width at half maximum (FWHM) of the XRD diffraction peak and "θ" represents Bragg angle. FWHM was calculated from Gaussian function using Origin software.

Bragg's Law was applied for measuring inter planar spacing (d), represented as follows:

$$2d\,\mathrm{Sin}\,\theta = n\lambda$$

Where "n" denotes the order of diffraction pattern and "λ" is the wavelength of the X-ray source

Lattice constant (a_0) has been derived using the following formula, $a_0 = d \times \sqrt{(h^2+K^2+l^2)}$, where d is inter-planar spacing and h, k, l are directions of planes.

4.7.10. Dynamic light scattering (DLS)

Dynamic light scattering analysis displays the graphical representation of distribution of particle size with respect to percent intensity. Size distribution was performed through DLS analyser (Zetasizer Nano ZS90 ZEN3690) operated at standard room temperature (25˚C) having viscosity of 0.8872 cP and count rate of

343.7 Kcps. For analysis, water was used as medium of dispersion having dispersion and material refractive index of 1.332 and 1.330 respectively.

4.7.11. Zeta potential

Measurement of stability of biosynthesized silver nanoparticles was determined through zeta potential analyser (Zetasizer Nano ZS90 ZEN3690). Zeta potential analysis was conducted at 25˚C having dispersant dielectric constant of 78.5, count rate of 4.5 Kcps and measurement position of 2.00 mm.

4.8. Time kinetics of nanosilver formation

Phytosynthesized nanosilver was scanned under UV-Vis Spectrophotometer in the wavelength range of 300 – 800 nm at a regular interval of 1 hr for estimating actual reaction (time) period requisite for complete reduction of silver ion (Ag^{+}) to nanosilver by phyto-extract.

4.9. Bioactivity assessment of phytosynthesized silver nanoparticles

Assessment of bioactive potential of phytosynthesized silver nanoparticles was conducted in terms of antioxidant and antimicrobial activity.

4.9.1. Antioxidant activity assessment

Antioxidant activity of prepared nanosilver and plant extract was evaluated in terms of DPPH, $ABTS^{+}$, superoxide, nitric oxide and metal chelating activity by comparing with respect to standards.

4.9.1.1. DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay

DPPH radical scavenging activity was conducted according to the method of Sidduraju et al. (2002), against ascorbic acid as standard. For assessment, 2000 µl DPPH (Himedia, RM2798-1G, Batch# 00136706) was added to 200 µl sample and absorbance was measured at 517 nm. Scavenging activity was calculated as percentage inhibition using the following equation:

$$Inhibition\ \% = \frac{(\alpha - \beta)}{\alpha} \times 100$$

Where "α" and "β" is the absorbance of the control and the sample respectively. Antioxidant activity was expressed as concentration at which 50% reduction in free radical activity occurs referred to as IC_{50} value.

4.9.1.2. ABTS$^+$ (2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) scavenging assay

Method prescribed by Li et al. (2009) was followed for ABTS$^+$ scavenging activity. Scavenging activity was estimated by reacting 1000 µl extract with 2000 µl ABTS (Himedia, RM9270-1G, Batch# 0000278630). The reaction mixture was allowed to incubate at 25˚C for 10 min. Absorbance was recorded at 734 nm and percent inhibition and antioxidant activity was calculated in the same way as stated in 4.9.1.1 against butyl-hydroxytoluene (BHT) as standard.

4.9.1.3. Nitric oxide (NO) scavenging assay

Method described by Marcocci et al. (1994) was used to measure nitric oxide scavenging activity. For estimation, 2000 µl 20 mM sodium nitroprusside, 0.5 ml phosphate buffer (pH 7.4) and 3 ml Griess reagent (mixture of 1000 µl 5% sulphanilamide in 20% glacial acetic acid and 1000 µl 0.2% N-(1-Naphthyl) ethylenediamine dihydrochloride) was mixed with 500 µl extract and incubated for 30 min. Absorbance was measured at 540 nm, and percent inhibition and antioxidant activity was measured in the same way as stated in 4.9.1.1 using gallic acid standard.

4.9.1.4. Superoxide radical (SO) scavenging assay

Standard method of Fu et al. (2010) was used for estimating superoxide scavenging activity. To 100 µl sample, 100 µl 312 µM nitroblue tetrazolium chloride (NBT) was added and incubated for 5 min. After incubation, 1000 µl 936 µM nicotinamide adenine dinucleotide (NADH) was added and reincubated for 5 min, followed by addition of 10 µl 120 µM phenazine methosulphate (PMS). The resultant solution was allowed to stand for 30 min in presence of fluorescence light and absorbance was recorded at 560 nm. Antioxidant activity was measured by comparing with tocopherol (Himedia, RM185-5G, Batch# 5-1114) as standard using the same formula as stated in 4.9.1.1.

4.9.1.5. Metal chelating activity (MC)

Method of Dinis et al. (1994) with few modifications was followed for estimating metal chelating activity. For estimating 400 µl extract was added to a reaction mixture containing 1600 µl methanol, 40 µl 2 mM ferrous chloride and 80 µl 5 mM ferrozine. Absorbance was measured at 562 nm after incubating for 10 min. Inhibition percentage was calculated as described previously in 4.9.1.1. and activity comparison was done using ascorbic acid as standard.

4.9.2. Antimicrobial activity assessment

Disk diffusion method was followed for determining the antimicrobial potentiality of phytosynthesized silver nanoparticles. Activity of nanosilver was assessed against both gram positive *viz. Bacillus megaterium* (ATCC 14581), *Staphylococcus aureus* (ATCC 11632) and *Bacillus subtilis* (ATCC 11774) and gram negative *viz. Escherichia coli* (ATCC 11229) and *Salmonella typhimurium* (ATCC 25241) bacteria. Silver nanoparticles were diluted to seven different concentrations *viz.* 25, 50, 100, 200, 300, 400, 500 µg/ml for estimating antimicrobial activity. For obtaining rapidly growing viable cells, test organisms were grown for 6 hrs on nutrient broth (Himedia, M002-500G, Batch# 0000302074) prior to their application. Freshly grown 100 µl test organism from nutrient broth was mixed uniformly with molten nutrient agar (Himedia, M001-500G, Batch# 000270060) in a sterilized culture plate and was allowed to solidify. After 30 min, paper disks soaked in nanosilver solution of appropriate concentration was placed on the upper layer of the nutrient agar plate and was allowed to incubate inside an incubator maintaining at 30 – 37˚C. The zone of inhibition was calculated in millimetre scale after 24 hrs of incubation.

Antimicrobial activity of biogenic silver nanoparticles was also determined in terms of minimum inhibitory concentration (MIC). For estimation, 0.1 ml actively growing viable bacterial culture maintained at 10^6 CFU/ml was added to 50 ml freshly prepared sterilized nutrient broth and was allowed to incubate after mixing with different concentrations of silver nanoparticles. Growth of microbes was measured after overnight incubation at 37˚C and 120 rpm using UV-Visible spectrophotometer (SYSTRONICS – 2201) at 600 nm.

4.10. Process variation of nanosilver formation

For determining the ideal condition of nanosilver biosynthesis, effective in preserving mulberry leaves at post-harvest stage, following process variations were conducted.

4.10.1. Determination of effective concentration of silver nitrate for nanosilver formation

Nanosilver was synthesized with five different concentrations of silver nitrate *viz.* 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} M. Formed silver nanoparticles was evaluated in terms of colour, spectral peak, DLS, zeta potential and TEM for determining the most effective concentration suitable for nano formation.

4.10.2. Determination of appropriate volume of plant extract for nanosilver formation

Silver nanoparticle was synthesized with different volume of mulberry leaf extract *viz*. 2.5, 5, 10, 15, 20 ml keeping the volume and concentration of silver nitrate constant to 45 ml and 10^{-3} M. The purpose of the study was to identify appropriate volume of leaf extract suitable for nanosilver biosynthesis.

4.10.3. Determination of appropriate ratio of plant extract to silver nitrate for nanosilver formation

Nanosilver was synthesis at five different proportions of plant extract to silver nitrate *viz*. 1:10, 1:20, 1:50, 1:70 and 1:100 for determining the exact proportion suitable for nano formation by keeping the amount of plant extract constant to 1 ml and by varying the amount of silver nitrate.

4.10.4. Determination of suitable genotype of mulberry for nanosilver formation

In sericulture for improving yield, many genotypes of mulberry have been developed. The nutritional value of one genotype differs from other. So, to predict which genotype is most suitable for nano formation, extracts of five high yielding and commercially accepted genotypes of mulberry *viz*. S1635, TR10, S1, BC259 and Guangdung was selected for synthesizing silver nanoparticles using silver nitrate as base solution.

4.10.5. Determination of impact of light on nanosilver synthesis

For assessing impact of light on biosynthesis of silver nanoparticles, nanosilver was prepared at three different luminosity state of affairs. Biosynthesis was conducted under dark, diffuse light and direct sunlight having light intensity of 0 lux, 230 lux and 67000 lux (LX-101 Lux Meter) respectively. Formed silver nanoparticle was evaluated in terms of colour, SRP peak, DLS, zeta potential and TEM analysis.

4.10.6. Determination of impact of pH on nanosilver synthesis

Phytosynthesis of nanosilver was carried out by adjusting the pH of mulberry leaf extract with HCl (0.1 N) and NaOH (0.1 N). At first the actual pH of the leaf extract was measured using digital pH meter (Zesco make) and accordingly pH was adjusted by adding HCl or NaOH in drop-wise manner. The leaf extract was accustomed to five different pH *viz.* pH 2, 5, 7, 9 and 12, keeping one at neutral range (pH 7), two in acidic range (one high acidic pH 2 and one moderate acidic pH 5) and two in alkaline rage (one high basic pH 12 and one moderate basic pH 9). The adjusted pH of the extract was added to silver nitrate solution to prepare biogenic nanosilver in the ratio of 1:9. Effect of pH on nanosilver formation was comparatively analysed with respect to colour, SRP peak, DLS, zeta potential and TEM examination.

4.10.7. Determination of impact of temperature on nanosilver synthesis

Biosynthesis of nanosilver was carried out at different temperature range *viz.* 0˚C, 10˚C, 25˚C, 50˚C and 80˚C for evaluating the impact of temperature on nanosilver synthesis. For assessing the impact, temperature of silver nitrate solution was adjusted either by ice chilled water or by hot water and the temperature steadiness was maintained using thermometer. To the accustomed temperature of silver nitrate solution, plant extract was added leading to the production of silver nanoparticles. Impact of temperature variation on nanosilver biosynthesis was evaluated in terms of colour, UV-Vis spectra, DLS, zeta potential and TEM analysis.

4.11. Stability assessment of phytosynthesized silver nanoparticles

For evaluating long term stability, silver nanoparticles synthesized through different process variations was analysed periodically at an interval of 10 days for a duration

of 60 days using UV-Vis spectrophotometer (Systronics-2201). The SPR pattern generated graphically by plotting absorbance with respect to wavelength was comparatively analysed to predict the difference with respect to time of nanosilver storage.

4.12. Bio-potentiality assessment of silver nanoparticles prepared by process variation

Bio-potentiality assessment of silver nanoparticles prepared by different process variations was carried out by evaluating its preservative potentiality. Nanosilver prepared by varying different physical parameters was allowed to preserve mulberry leaves at post-harvest stage for a period of 7 days. During preservation periodic evaluation of physical texture and chlorophyll content was carried out for determining the ideal combination suitable for extending shelf life of mulberry leaves.

4.13. Screening of least effective concentration of silver nanoparticle

Nano silver was synthesized following the ideal combination as determined from process variation and was used to preserve S1 cultivar of mulberry leaves for a period of 7 days using three different dilutions *viz.* 10x, 20x and 40x for determining the least effective concentration suitable for extending post-harvest shelf life.

4.14. Evaluation of preservative potentiality of least effective concentration of phytosynthesized silver nanoparticles

The most commonly grown and used genotype of mulberry in this region, namely S1 genotype was used as experimental material. Juvenile, unhealthy, and diseased leaves were screened out for maintaining homogeneous experimental condition. Leaves were preserved up to 7 days in three different preservative solutions *viz.* phyto-synthesized silver nanoparticles (NS) having 6 ppm colloidal silver, silver nitrate (SN) solution containing 6 ppm silver ion concentration, serving as positive control and distilled water, acting as negative control. All the three preservative solutions were maintained at neutral pH (pH 7). The preserved leaves were evaluated through chemical profiling at regular interval of 1, 4, 6 and 7 days for determining the potentiality of preservative solutions in extending shelf life with respect to fresh leaves.

4.15. Quantitative chemical profiling of mulberry leaves at post preservation stage

At the end of preservation period mulberry leaves preserved in different preservative solution was evaluated in terms of quantitative chemical parameters for determining changes during the course of preservation.

4.15.1. Quantitative profiling of primary metabolite content

4.15.1.1. Determination of total chlorophyll content

Total chlorophyll content was determined according to the methods proposed by Arnon (1949). For extraction, 1gm preserved leaf was finely chopped and homogenized using a mortar and pestle with 20 ml 80% (v/v) acetone. The crushed leaf sample was centrifuged at 10,000 × g for 5 min at 25˚C. Obtained supernatant was re-extracted 2 – 3 times with 80% acetone for absolute extraction of pigments. The supernatants were collected and used as crude extract for determining total chlorophyll content at 663 and 645 nm using UV-Vis spectrophotometer (Systronics – 2201). The total chlorophyll content was calculated using the following benchmark formulation:

$$\text{Total chlorophyll } (\mu\text{g ml}^{-1}) = \{(20.20 \times \text{A645}) + (8.02 \times \text{A663})\}$$

Where A645 and A663 stands for absorbance value at 645 and 663 nm respectively

$$\text{Total chlorophyll content} (\text{mg g}^{-1}\text{FW}) = \{(\text{Total chlorophyll} \times \text{Final volume})/(\text{Initial weight of tissue} \times 1000)\}$$

4.15.1.2. Determination of total soluble protein content

Total protein content in preserved leaves was determined following the method prescribed by Lowry et al. (1951). For extraction, 0.5 gm cryo-frozen leaves were crushed using sodium phosphate buffer (pH 7.2) and centrifuged (REMI, C-24 BL) at 5,000 × g at -10˚C for 5 min. To the collected supernatant, 5 ml alkaline copper solution and 0.5 ml Folin-Ciocalteu reagent (Merck, AA3A620688, Batch# 8901668608596) was added and incubated for 30 min which leads to the development of blue coloured complex. The absorbance of the coloured complex was recorded at 660 nm and was standardized using bovine serum albumin (BSA)

(GeNei, 1650500501730, Batch# 5017) solution for determining the total protein content.

4.15.1.3. Determination of free proline content

Free proline content was determined according to the process described by Bates et al. (1973). For extraction, 1 gm leaf sample was crushed using mortar and pestle in 20 ml of 3% sulfosalicylic acid followed by UV-Vis spectral reading at 520 nm of the upper toluene layer collected from the reaction mixture containing 1000 µl extract, 2000 µl acid ninhydrin, 2000 µl glacial acetic acid and 4000 µl toluene.

4.15.1.4. Determination of total soluble sugar and reducing sugar content

Total soluble and reducing sugar content was estimated spectrophotometrically following the standard method that was prescribed by Sadasivam and Manickam (1996). Preserved leaf sample (0.1 gm) was extracted twice (5 ml each time) with 80% hot ethanol using mortar and pestle. The supernatant was collected through filtration and the ethanol was heat evaporated. On removal of ethanol, the final volume was brought up to 10 ml by adding double distilled water. Total soluble sugar content was estimated using Anthrone reagent (Himedia, GRM314-25G, Batch# 0000239756) at 620 nm by incubating the water extract in a boiling water bath for 8 min. Reducing sugar content was determined at 510 nm using Dinitrosalicylic acid and 40% Rochelle salt (sodium-potassium tartrate) solution.

4.15.2. Quantitative profiling of reactive oxygen species and lipid peroxidation

4.15.2.1. Determination of hydrogen peroxide (H_2O_2) content

Hydrogen peroxide accumulation was determined according to the protocol prescribed by Loreto and Velikova (2001). Preserved leaf sample (0.5 gm) was homogenized in 1% (w/v) tri-chloroacetic acid (TCA) (Himedia, GRM6274-100G, Batch# 0000243496) using mortar and pestle and centrifuged at 10,000 × g for 10 min at 4˚C. The supernatant collected was measured spectrophotometrically at 390 nm after reacting with 10 mM phosphate buffer (pH 7.0) and 1 M potassium iodide.

4.15.2.2. Determination of superoxide ($O_2^{\bullet -}$) content

Accumulation of superoxide ($O_2^{\bullet-}$) reactive oxygen species was evaluated following the method prescribed by and Elstner and Heupel (1976). For estimation, homogenization of 1gm leaf samples was done in 65 mM potassium phosphate buffer (pH 7.8) and was centrifuged at 5,000 × g for 10 min. The supernatant collected was allowed to react with 65 mM potassium phosphate buffer (pH 7.8) and 10 mM hydroxylamine hydrochloride. The resultant mixture was incubated for 15 min at 25˚C. Spectral absorbance was recorded at 530 nm after reacting with 17 mM sulphanilamide and 7 mM α-naphthylamine.

4.15.2.3. Determination of lipid peroxidation

Extend of lipid peroxidation was estimated by measuring malondialdehyde contents following the method prescribed by Davenport et al. (2003). For detection, 0.2 gm leaf sample was homogenized in 2 ml 0.5% (w/v) tri-chloroacetic acid and centrifuged at 10,000 × g for 10 min at 4˚C. From the collected supernatant, 2 ml was taken out and reacted with 2 ml 0.67% thiobarbituric acid (TBA) (Himedia, RM1594-25G, Batch# 0000177577). The reaction mixture was incubated for 30 min at 95˚C, followed by termination of reaction by ice water treatment. The spectral absorbance of the supernatant was recorded at 450, 532 and 600 nm. Measurement of MDA content was done using the following formula:

$$\text{MDA}\ (\mu\text{mol g}^{-1}) = [6.45(\text{A532} - \text{A600}) - 0.56\ \text{A450}]\ \times \text{Vt/W}$$

Where A600, A532 and A450 represents absorbance at 600, 532 and 450 nm and Vt = 0.0021 and W = 0.2 g.

4.15.3. Quantitative profiling of free radical scavenging activities and metal chelating activity

Free radical scavenging activity was estimated in terms of DPPH, ABTS, superoxide, nitric oxide scavenging activity and metal chelating activity following the method of Sidduraju et al. (2002), Li et al. (2009), Fu et al. (2010), Marcocci et al. (1994), and Dinis et al. (1994) respectively. For extraction, 1 gm preserved leaf sample was homogenized in 10 ml methanol using mortar and pestle and centrifuged at 10,000 × g for 10 min at 4˚C. The obtained supernatant was stored and was used as crude extract for the above cited assays.

DPPH scavenging activity was estimated at 517 nm by mixing 0.2 ml methanolic extract with 2 ml DPPH. ABTS activity was resolute by mixing 2 ml ABTS to 1 ml methanol extract and measuring the absorbance at 734 nm after incubating for 10 min. For predicting superoxide scavenging activity spectral absorbance was recorded at 560 nm after 30 min incubation under illuminated light (4000 flux) containing 1 ml NBT, 1 ml NADH and 10 µl PMS. For estimating nitric oxide scavenging activity 20 mM sodium nitroprusside and 3 ml Griess reagent was mixed with 0.5 ml phosphate buffer and to it 0.5 ml extract was added, and the absorbance value was recorded at 540 nm after 30 min incubation. Metal chelating activity was recorded at 562 nm by reacting 0.4 ml methanolic extract with 2 mM $FeCl_3$ and 5 mM Ferrozine. Scavenging activity was measured as percent inhibition and antioxidant activity was determined in terms of concentration where 50% reduction in free radical takes place denoted by IC_{50} value.

4.15.4. Determination of enzymatic antioxidant activity

For determining enzymatic antioxidant activity, the leaf samples (0.5 gm) were homogenized cryogenically in a pre-cool mortar and pestle. Crushed sample was immediately mixed with 0.1 M ice-cool potassium phosphate buffer (pH 7.5 for catalase, superoxide dismutase and glutathione disulphide reductase; pH 7.0 for glutathione peroxidase and glutathione S-transferase; pH 6.8 for ascorbate peroxidase) containing 0.5 mM EDTA. The crushed leaf buffer sample was centrifuged at 15000 × g for 15 min at -10˚C and the supernatant was used for enzymatic assay.

4.15.4.1. Detection of superoxide dismutase (SOD) activity

Superoxide dismutase (EC: 1.15.1.1) activity was predicted following the method of Esfandiari et al. (2007). For estimation, 50 µl enzyme extract was added to a reaction mixture containing 1500 µl 100 mM potassium phosphate buffer, 1000 µl distilled water, 100 µl 200 mM methionine, 10 µl 2.25 mM NBT and 100 µl 3 mM EDTA. Reaction was initiated by the addition of 60 µM riboflavin. Absorbance was recorded at 560 nm after incubated under light for 10 min. SOD activity was expressed as amount of enzyme reducing the absorbance of NBT by 50%.

4.15.4.2. Detection of catalase (CAT) activity

Detection method prescribed by Aebi (1984) was followed for estimating Catalase (EC: 1.11.1.6) activity with certain modifications. Enzyme activity was determined by adding enzyme extract to a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0) and 15 mM hydrogen peroxide. The reaction was monitored under UV-Vis spectrophotometer for 1 min at 240 nm and the resultant was used to predict enzyme activity expressed as unit (1unit = millimoles of H_2O_2 reduced min^{-1} mg $protein^{-1}$) by means of extinction coefficient of 39.4 M^{-1} cm^{-1}.

4.15.4.3. Detection of glutathione disulphide reductase (GSR) activity

GSR (EC: 1.6.4.2) activity was recorded under UV-Vis spectrophotometer for 1 min at 340 nm by mixing 1 mM glutathione disulphide to the reaction system containing 0.1 M potassium phosphate buffer (pH 7.8), 1 mM EDTA, 0.2 mM NADPH and 100 µl enzyme solution. The enzyme activity evaluated in terms of unit (1unit = µmol (NADPH) min^{-1} mg $protein^{-1}$) using extinction coefficient value of 6.2 mM^{-1} cm^{-1} (Hossain et al. 2010).

4.15.4.4. Detection of glutathione peroxidase (GPOX) activity

Glutathione peroxidase (EC: 1.11.1.9) activity was determined according to the method of Elia et al. (2003). For estimating GPOX activity, reaction mixture contains 100 mM sodium phosphate buffer (pH 7.5), 1 mM EDTA, 1mM sodium azide (NaN_3), 0.2 mM NADPH, 2 mM glutathione, 1 unit glutathione reductase and 0.6 mM hydrogen peroxide was reacted with 20 µl crude enzyme extract. Enzyme activity was recorded in terms of unit through oxidation of NADPH at 340 nm for 60 sec. Enzyme activity was expressed using extinction coefficient of 6.62 mM^{-1} cm^{-1} as µmol (NADPH) min^{-1} mg $protein^{-1}$.

4.15.4.5. Detection of glutathione S-transferase (GST) activity

Glutathione S-transferase (EC: 2.5.1.18) activity was estimated following the process of Hasanuzzaman et al. (2011). For estimating enzyme activity, reaction was made by adding 100 mM tris-HCl (pH 6.5), 1.5 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) with crude enzyme solution. Spectral reading was recorded for 60 sec at 340 nm and enzyme activity was expressed by using extinction coefficient of 9.6 mM^{-1} cm^{-1}.

4.15.4.6. Detection of ascorbate peroxidase (APX) activity

Estimation of ascorbate peroxidase (EC: 1.11.1.11) activity was determined according to the process of Chen and Asada (1989). For estimation, crude enzyme extract was added to 3 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate and 0.1 mM hydrogen peroxide. Spectral reading was recorded at 290 nm for 2 min. APX was expressed in terms of unit, where one unit was defined as quantity of enzyme required to put away 1 μm of ascorbate min^{-1} mg $protein^{-1}$.

4.15.5. Determination of non-enzymatic antioxidant activity

4.15.5.1. Determination of carotenoids content

Method prescribe by Lichtenthaler (1987) was followed for estimating the content of carotenoids present in preserved leaves. For estimation, 1 gm preserved leaf samples were homogenized in 80% acetone and supernatant was collected after centrifugation at 10,000 × g for 5 min at 25˚C. The spectral absorbance of the supernatant was determined at 470, 645, and 663 nm.

4.15.5.2. Determination of ascorbic acid content

Detection processes described by Omaye et al. (1979) was followed for estimating ascorbic acid content. Sample extraction was done by homogenizing 0.5 gm leaf samples in 10% (w/v) TCA, followed by centrifugation at 10,000 × g for 20 min at 25˚C to collect the supernatant. Detection was carried out by reacting 0.5 ml supernatant with 2% 2, 4-dinitrophenyl hydrazine in 0.5 N sulphuric acid and 10% thiourea. The absorbance of the reaction mixture was recorded at 520 nm after 3 hrs of incubation at 37˚C.

4.15.5.3. Determination of total glutathione content

Total glutathione content was detected according to the method prescribed by Griffith et al. (1979). For estimation, 0.1 ml extract was reacted with 0.3 mM NADPH in 20 mM potassium phosphate buffer (pH 7.5) and 6 mM 5'-dithio-bis (2-nitrobenzoic acid) and incubated for 3 min at 25˚C. After incubation 0.01 ml reduced glutathione was added and absorbance was recorded at 412 nm after the development of coloured complex.

4.15.6. Estimation of polyphenol content

Extraction of polyphenols present in preserved leaf sample was carried out by grinding 1gm leaf sample with 80% ethanol using mortar and pestle, followed by centrifugation at 10,000 × g for 20 min for collecting the supernatant. The supernatant collected was evaporated to dryness and the residue was re-extracted with double distilled water, using which estimation was carried out.

4.15.6.1. Detection of total phenol content

Total phenol content was calculated following the method of Malick and Singh (1980). To the 1000 µl water extracted sample 500 µl 50% Folin-ciocalteu reagent and 1000 µl 20% sodium carbonate was added and incubated for 1 hr. Proline content was estimated using gallic acid as standard after measuring the absorbance at 650 nm.

4.15.6.2. Detection of orthodihydric phenol content

Content of orthodihydric phenol was determined at 515 nm by adding 500 µl 0.05 (N) hydrochloric acid, 500 µl Arnow's reagent and 1000 µl 1 (N) sodium hydroxide to the aqueous extract following the method of Mahadevan and Sridhar (1986) using catechol as standard.

4.15.6.3. Detection of flavonoid content

Content of flavonoids was determined at 510 nm following the protocol of Atanassova et al. (2011) by adding 300 µl 5% $NaNO_2$, 300 µl 10% $AlCl_3$ and 2 ml 1 (M) NaOH to the aqueous extract and by using quercetin as standard.

4.15.7. Data analysis

Using General Linear Model, two-way analysis of variance (ANOVA) was performed to calculate the effect of preservative solutions on preservation of mulberry leaves using SPSS statistical package (IBM SPSS Advanced Statistics 20.0). Bivariate correlations study was performed using Pearson correlation coefficient to study inter-relationship among different metabolic parameters ($p \leq 0.01$ and 0.05) using SPSS. SPSS statistical tool was also used for performing multiple regression analysis (MRA). MRA was performed for determining the stress

parameters that are involved in degradation of essential primary metabolites. Graphs were generated using OriginPro 2018b software (b9.5.5.409).

4.16. Investigation of protein profile of preserved mulberry leaves

Mulberry leaves preserved in nanosilver, silver nitrate and distilled water was used for SDS gel electrophoresis and isozyme analysis for predicting the difference in banding pattern with respect to fresh leaves.

4.16.1. Sodium dodecyl (lauryl) sulphate (SDS) Gel Electrophoresis of leaf protein

4.16.1.1. Extraction of leaf protein

Preserved and fresh mulberry leaves were first treated with liquid nitrogen in a mortar and pestle, followed by addition of lysis buffer containing trichloroacetic acid, dithiotreitol and acetone and pasted thoroughly. The lysate was transferred to fresh eppendorf tube with a little more lysis buffer for accurate lysis of the cells, followed by vortexing for homogeneous mixing of the content. Followed vortexing, the tube was allowed to incubate at -20˚C for 40 min, for proper precipitation of protein in the buffer solution. At the end of incubation period, the tube was allowed to centrifuge at 14,000 × g for 30 min maintaining temperature consistency at 4˚C. The supernatant generated from centrifugation was cautiously discarded without disturbing the pellet. The pellet was mixed with 1000μl wash buffer containing dithiotreitol and acetone and incubated at -20˚C for 30 min. Post-incubation, centrifugation at 14,000 × g for 15 min was carried out and the supernatant was carefully discarded. The pellet was air dried and dissolved to rehydration buffer containing CHAPS and urea that would solubilise and denature the protein respectively.

4.16.1.2. Reagents and detection technique of Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS PAGE)

SDS PAGE mediated separation of protein was carried out in Bio Rad Mini PROTEAN Tetra Cell following the standard protocol as prescribed by He (2011).

4.16.1.2.1. Preparation of acrylamide and bisacrylamide solution

For conducting SDS gel electrophoresis, 29.2% acrylamide (w/v) (Himedia, GRM1110-500G, Batch# 0000279528) and 0.8% N'N'-bis-methylene-acrylamide (w/v) (Himedia, MB005-25G, Batch# 0000198755) was dissolved one after another in 100 ml double distilled water. Prepared stock solution was filtered by Whatman No. 1 filter paper and stored in dark glass bottle at 4˚C.

4.16.1.2.2. Preparation of sodium dodecyl sulphate (SDS) solution

For resolving gel and stacking gel 0.4% (w/v) and 0.8% (w/v) sodium dodecyl sulphate solution was prepared in 100 ml double distilled water respectively and stored at room temperature.

4.16.1.2.3. Preparation of Tris-HCl (pH 8.8) buffer for resolving gel

For resolving gel 1.5 M Tris-HCl (Himedia, MB029-100G, Batch# 0000420487) buffer solution was prepared by mixing 4.541 gm Tris free base with 15 ml of double distilled water. The strength of the solution was adjusted to pH 8.8 using 1(N) HCl. Final volume was made up to 25 ml with double distilled water and stored at 4˚C.

4.16.1.2.4. Preparation of Tris-HCl (pH 6.8) buffer for stacking gel

For stacking gel 1 M Tris-HCl (Himedia, MB030-100G, Batch# 0000089451) buffer solution was prepared by mixing 3.925 gm Tris hydrochloride with 15 ml of double distilled water. The strength of the solution was adjusted to pH 6.8 using 1(N) HCl. Final volume was made up to 25 ml with double distilled water and stored at 4 ˚C.

4.16.1.2.5. Preparation of sample loading buffer

Sample loading buffer was prepared by dissolving 24 mg Tris free base, 5 mg EDTA, 200 mg SDS and 1 gm sucrose into 19 ml of double distilled water and adjusted the solution strength to pH 8. Then 20 mg Bromophenol blue (Himedia, RM117-5G) was dissolved, and the solution was filtered using Whatman No.1 filter paper. At the end, 1 ml β-mercaptoethanol was added, bottled, and stored at 4˚C.

4.16.1.2.6. Preparation of electrophoresis running buffer (pH 8.3)

Running buffer was prepared by dissolving 3.03 gm Tris base, 14.4 gm glycine and 1 gm sodium dodecyl sulphate into 800 ml double distilled water. The strength of

the solution was adjusted to pH 8.3. Final volume made up to 1000 ml with double distilled water and stored at 4˚C.

4.16.1.2.7. Preparation of ammonium persulfate (APS) solution

10% ammonium persulphate (Himedia, GRM1095-500G, Batch# 0000142945) solution was freshly prepared prior to use.

4.16.1.2.8. Sample preparation for SDS gel electrophoresis

Protein sample was mixed with sample loading buffer in the ratio of 1:2, followed by heating at 95˚C for 60 sec and was gently loaded into the wells of stacking gel.

4.16.1.2.9. Gel formulations

Stacking and resolving gel were prepared by mixing double distilled water, 30% acrylamide-bisacrylamide, gel buffer and SDS in the proportion of 5% and 10% respectively.

4.16.1.2.10. Electrophoresis set up

At the onset of electrophoretic running, the electrode holding the gel was transferred onto the electrophoresis reservoir (tank) (Bio Rad, Mini PROTEAN Tetra Cell, Sl. No. 552BR028247) and into it, running buffer (pH 8.3) was added. Then the extracted protein samples were loaded to their respective lanes. Finally, the entire setup was eletrophoresed at 50-60 Volts using electrophoresis power stabilizer (GeNei, Model No. SLM-LVP-300, Sl. No. 20091011185).

4.16.1.2.11. Post electrophoresis gel staining

For staining the gel at the end of electrophoresis, solution mixture of methanol, acetic acid and double distilled water in the ratio of 5:1:4 was prepared and to it 0.25% Commassie brilliant blue R-250 (Himedia, RM-250, Batch# 0000157217) was added, dissolved and filtered with Whatman No.1 filter paper. The gel was immersed into the staining solution and was incubated for 6 hrs at room temperature.

4.16.1.2.12. De-staining of stained gel

The stained gel was transferred to the de-staining solution having mixture of methanol, acetic acid, and double distilled water in the ratio of 5:1:4 and washed gently 2 – 3 times, leading to the appearance of protein bands.

4.16.1.2.13. Gel scanning

For studying banding pattern and for identifying band intensity, the obtained gels were scanned under calibrated densitometer GS-800 (Model No. Powerlock 2100XL-USB) and the image obtained was analysed through image lab platform (version 5.1).

4.16.2. Orbitrap high resolution liquid chromatography mass spectrometry (OHR-LCMS)

The most prominent and differentially expressed SDS gel band of leaves preserved in solution of silver nanoparticles was analysed for liquid chromatography mass spectroscopy (Q-Exactive Plus Biopharma-High Resolution Orbitrap, Thermo Fischer Scientific Pte. Ltd.)

4.16.2.1. In-gel digestion of differentially expressed SDS gel band

The differentially expressed gel pieces were cut out and were rinsed with 0.1 ml 25mM ammonium bicarbonate (ABC). The gel pieces were then dehydrated by immersing in a mixed solution 0.1 ml of acetonitrile (ACN): 50mM ABC for 5 min in the ratio of 2:1 (Solution ‘A’). On removing supernatant, 0.1 ml 25mM ABC was again added and incubated for 5 min, followed by dehydration in solution ‘A’. This process was repeated 2 times before moving towards reduction steps for possible breakage of disulphide bonds. At the time of rehydration, the gel pieces were rehydrated in 50 µl of 10 mM dithiothreitol (DTT) and was allowed to incubate at 56˚C for 1 hr. After incubation DTT was poured off, and the gel segments were rinsed in 100 µl 25mM ABC. The gel pieces were incubated in dark for 30 min in 100mM IAA. After 30 min, IAA was discarded, and the gel cuts were rinsed with 1 ml ABC. After reduction the gel cuts were again dehydrated in the same manner as described above. Then the gel pieces were rehydrated with trypsin (Promega Sequencing Grade Trypsin) at 0˚C, mixed with 25 mM ABC and incubated for 30 min, until added trypsin gets absorbed. The gel slices were then transferred in a tube and incubated at 37˚C for 16 hrs with 25 mm ABC. At the end of incubation period,

extraction was carried out by pouring off supernatant and vortexing the gel slices with 0.1 ml extraction buffer having 50% ACN and 0.1% TFA for 10 min and was pooled into a single tube. After which again 100µl extraction solution was added containing 60% ACN and 0.1% TFA. The mixture was allowed to sonicate for 1 min (2 sec pulse, 1 sec gap, 25% amplitude), followed by 10 min vortexing and finally the supernatant was poured into the previously pooled sample. Extraction was again repeated with 80% ACN and 0.1% TFA and vortexed for 10 min, followed by collection of supernatants. Finally, the collected supernatant was vacuum concentrated to 20µl.

4.16.2.2. Zip Tip C18 for MS Analysis

Zip Tip analysis was carried out with the supernatant obtained from in-gel digestion. 10µl 100% HPLC-grade acetonitrile was sprayed into the tip to get rid of any waste, followed by tip washing with 0.1% TFA in MilliQ water (washing solution) to get rid of the waste. Bound peptides with Zip Tip were aspirated and were dispensed up to 10 cycles. After that 0.1% TFA in MilliQ water was added to get rid of the waste. The process was repeated five times. Finally, 3-5 µl elution solution (50% acetonitrile in 0.1% TFA) was poured into a clean sterile eppendorf tube. The eluent present in the Zip Tip was aspirated and dispensed 8 times in an airtight condition.

4.16.2.3. Column Details

4.16.2.3.1. Analytical Column

PepMap RSLC C_{18} 2 µm column, 100A × 50 cm was used for ultrahigh resolution separation. Pre-column: Acclaim PepMap 100, 100 µm × 2 cm nanoviper.

4.16.2.3.2. Mobile Phase

The mobile phases used in the column are as follows:

Solvent ‘A’: 0.1% Formic acid (FA) in MilliQ water

Solvent ‘B’: 80:20 (ACN: MilliQ water) + 0.1% FA

4.16.2.3.3. Run time of sample

The sample running was carried out for a period of 60 min.

4.16.2.3.4. Data analysis software

The obtained peptide sequences were analysed using Thermo Proteome Discoverer software (version 2.2).

4.16.2.3.5. Databases used

Morus alba database was used for identifying the leaf peptides sequences obtained from OHR-LCMS analysis.

4.16.2.4. Protein - protein networking analysis

The identified proteins were grouped, and their possible inter-relations were analysed using STRING under multiple protein interaction platforms to obtain the clustering network of protein-protein interaction.

4.16.3. Native PAGE detecting leaf isozymes

4.16.3.1. Preparation of plant extract for on-gel assay

For analysing on-gel isozyme patterning, leaves preserved in different preservative solution were first crushed in liquid nitrogen followed by mixing with phosphate buffer definite for different isozymes. After crushing, the buffer containing the sample was incubated for 30 min at -20˚C and then cold-centrifuged. After centrifugation the supernatant was collected and was stored at -20˚C for future use in reaction assay.

4.16.3.2. Electrophoresis set up

Isozyme on-gel patterning was conducted under freezer (≤ 4˚C) condition for preventing protein from denaturation following the standard protocol of Williams and Reisfeld (1964). Process of Native PAGE was more or less similar to that of SDS PAGE mentioned above with slight differences *viz*. no SDS solution was used, protein was not heat destabilized before sample loading and entire electrophoresis setup was maintained under cold condition (≤ 4˚C).

At the end of electrophoresis, the gel was collected out and was immediately stained with isozyme specific staining according to the nature of the isozymes to be detected.

4.16.3.3. Staining of isozymes

4.16.3.3.1. NADPH Oxidase (NOX)

Method prescribed by Frahry and Schopfer (2001) was followed for observing isozyme pattern of NADPH Oxidase. The gel obtained post electrophoresis was stained in a solution mixture of 10 mM Tris buffer (pH 7.4), 0.05 gm NBT (Himedia, RM578-1G, Batch# 0000246382) and 134 μM NADPH (Himedia, RM576-100MG, Batch# 00003277249). The gel was incubated in this staining solution under ice cold condition (inside freezer) until the visible band was developed.

The gels displaying isozyme banding pattern were scanned imaged under GS-800 calibrated densitometer (Model No. Powerlock 2100XL-USB). Band analysis from obtained gel image was done using image lab software (version 5.1).

4.16.3.3.2. Superoxide dismutase (SOD)

The isozyme pattern of superoxide dismutase activity was detected on gel according to protocol prescribed by Pereira et al. (2002). At the end of electrophoresis process, the gel was transferred on to the staining solution containing blend of 0.05 M potassium phosphate buffer (pH 7.0), 1 mM EDTA (Himedia, RM1370-100G), 0.05 mM riboflavin (Himedia, PCT0214-25G, Batch# 0000113139), 0.1 mM NBT and 300 μl TEMED (N, N, N, N-tetramethylenediamine) (Himedia, GRM1572-1ML, Batch# 0000214495) and was allowed to incubate under dark state at room temperature for 30 min. After incubation the gel was washed with double distilled water and kept into a light box until the isozyme banding pattern became visible.

4.16.3.3.3. Catalase (CAT)

On-gel detection of catalase isozyme was carried out by following the procedure of Woodbery et al. (1971). The gel obtained at post-electrophoresis stage was soaked in the solution of 5 mM H_2O_2 (Merck, CC2C620059) and incubated for 10 min. Post incubation the gel was rinse with double distilled water. The gel was then dark incubated in a solution containing equal proportion of 2% potassium ferricyanide (Himedia, GRM627-500G, Batch# 0000185664) (w/v) and 2% ferric chloride

(Himedia, GRM6353-500G, Batch# 0000340233) at 25˚C until bands were developed.

4.16.3.3.4. Peroxidase (POD)

Peroxidase activity was detected following the method given by Janda et al. (1999). At the end of electrophoresis, the gel was isolated from the gel plate and was soaked in a solution containing 5 mM benzidine dissolving in 0.05 M borate buffer. To the solution 5 mM H_2O_2 was added and was incubated in dark under ice-cold condition until visible bands were developed.

4.17. Determination of microbial load in preservative solution

For detecting microbial load (proliferation) in preservative solution pour plate method using nutrient agar media was followed as proposed by Sanders (2012). Preservative solutions (distilled water, nanosilver and silver nitrate) of different days (day 1 to day 7) were poured to sterilize Petri plates followed by addition of nutrient agar medium. On solidifying the plates were incubated for 24 hrs at 37˚C for detecting presence/ absence of microbial colonies.

4.18. Evaluation of preservative potentiality of phytosynthesized silver nanoparticles against five studied genotypes

Preservative potentiality of phytosynthesized silver nanoparticles was further evaluated against all the studied genotype with respect to physical and biochemical parameters. The objective of examination was to find out the response pattern of nanosilver against other genotypes.

4.19. Histochemical detection of preserved tissues

At the end of preservation period, transverse section of preserved leaf petioles was done using sharp blade and stained with different histochemical reagents for detecting the blockage and non-blockage scenery of xylem. Histochemical detection of xylem blockage was conducted with standard dyes *viz*. Bradford reagent, Azure B, Phloroglucinol-HCL and Sudan IV. A general account of the used stains, process of preparation and bio-component detected by the stain was enlisted below:

4.19.1. Light microscopic detection of xylem blockage

4.19.1.1. Preparation of Bradford reagent

For preparing Bradford reagent, 0.005 gm Coomassie brilliant blue (Himedia-RM344, Batch# 0000335258) was dissolved in 5 ml methanol. To this solution, 10 ml 8.5% (w/v) phosphoric acid and 85 ml double distilled water was added. The resultant solution was filtered to remove the precipitates (Bradford 1976). The section was stained for 1 – 2 min and observed under microscope (Olympus CX21iLED) for detecting protein accumulation.

4.19.1.2. Preparation of Azure B

Azure B stain was prepared by adding 0.15 gm Azure B (Sigma-Aldrich A4043-5G, Batch# 000348164) in 30 ml saturated alcoholic solution. To it, 100 ml double distilled water and 0.1 ml 10% potassium hydroxide was added. The solution was filtered and diluted to 1:20 ratio before use (Marshall and Lewis 1975). The section was stained for 3 – 4 min and observed under light microscope for detecting lignin deposition.

4.19.1.3. Preparation of Phloroglucinol-HCl

Phloroglucinol-HCl stain was prepared by dissolving 0.125 gm phloroglucinol (Himedia, RM834-25G, Batch# 0000052547) in 5 ml 20% ethanol and to it 1.25 ml HCl was added and mixed uniformly (Liljegren 2010). The section was stained for 2 min and observed under microscope for detecting deposition of lignin.

4.19.1.4. Preparation of Sudan IV

For preparing the stain solution, 0.005 gm Sudan IV (Sigma-Aldrich, 198102-25G) was dissolved in 10 ml 96% ethanol. The solution was filtered and to it 10 ml glycerol was added before use (Soukup 2013). The section was first rinsed in Sudan IV solution for 5 min followed by heating for few seconds. The solution was cooled at room temperature and the section was mounted with glycerol before observing under microscope for detecting suberin deposition.

4.19.2. Scanning electron microscopic detection of xylem blockage

Scanning electron microscopic analysis (Jeol JSM-IT 100) was conducted for detecting the post-harvest blockage pattern within petiole of all the five genotypes of *Morus alba* under study. The blockage nature was predicted after preservation in all

preservative solution viz. nanosilver, silver nitrate and distilled water and was compared with respect to fresh leaf petiole. For analysis, petiole section was dried on a carbon-coated copper grid and then micrographs were taken at different magnifications.

4.19.3. Histochemical detection of oxidative stress

At the end of preservation, the petioles of the leaves were cut into thin transverse section and stained with appropriate solution for studying the localization of hydrogen peroxide (H_2O_2) and detection of membrane integrity.

4.19.3.1. Detection of hydrogen peroxide localization

For detecting localization of hydrogen peroxide in the preserved leaves, the petiole sections were kept submerged for about 45 min in potassium iodide (Himedia, GRM10692-25G, Batch# 0000378816) solution (0.1M) and starch (4% w/v) reagent as proposed by Olson and Varner (1993). At the end of incubation period, the sections were examined under microscope.

4.19.3.2. Detection of plasma membrane integrity

The integrity of plasma membrane was detected according to the method prescribed by Yamamoto et al. (2001). Firstly, the transverse section of the petiole was stained using Evan's blue (Himedia, RM370-10G, Batch# 0000001622) solution (0.025% w/v in 100 mM $CaCl_2$) for 30 min and then was washed thrice with double distilled water for tissue clearing by removing the extra stain and observed under microscope.

4.19.4. Statistical analysis of anatomical data

For evaluating the relationship among used preservative solutions with respect to their potentiality to prevent vascular blockage by group clustering, Principal Component Analysis (PCA) was performed using XLSTAT 2017 statistical package. Agglomerative hierarchical clustering (AHC) was performed following Ward's method of dissimilarity matrix using XLSTAT 2017 to identify the inter-relationship among different preservative solutions suitable for enhancing shelf life at post-harvest stage. R software based heat map study (version 3.4.0) was conducted for predicting the nature of colour matrix between the cultivars under study with respect to staining pattern detecting vascular blockage.

4.20. Transcriptome analysis of preserved mulberry leaves using Illumina platform

4.20.1. RNA extraction and high-throughput Illumina sequencing

Standard Trizol RNA extraction protocol (1 ml Trizol reagent was mixed with 200 mg leaf tissue crushed in liquid nitrogen, vortex and incubated for 5 min at 25˚C; 0.2 ml chloroform/ml Trizol was added and vigorously shaking for 15 sec, incubated at 25˚C for 3 min and centrifuge at 14000 × g for 15 min at 4 ºC. To the upper aqueous phase isopropyl alcohol was added for precipitating RNA; incubated for 5 min followed by centrifugation at 12000 × g for 10 min at 4 ºC. The pellets were washed with 80% ethanol, by vortexing and centrifuged at 7000 × g for 5 min at 4ºC. The RNA pellets were dried out and dissolved in 40 – 80 µl RNAase-free water and stored at 80˚C) was used for total RNA extraction from nanosilver and distilled water preserved (7 days) mulberry leaves. RNAase free agarose gel electrophoresis was performed for measuring the integrity of isolated RNA (Laila et al. 2017) and the quantity was measured using Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA from both NS7 (7 days nanosilver preserved leaves) and CO7 (7 days distill water preserved leaves) was used for library preparation and sequencing. Library preparation was conducted following Illumina TrueSeq RNA library method as prescribed in TruSeq Stranded Total RNA Reference Guide (Illumina Technologies, San Diego, CA). Libraries of each sample (NS7 and CO7) were used for RNA sequencing using Illumina HiSeq 4000 platform (Illumina Inc., CA, USA), which were exposed to automated cycles of paired-end-sequencing (2 × 100 bp).

4.20.2. Pre-processing of RNA-Seq data set

Illumina raw reads were processed for removal of adapter sequences using AdapterRemoval (version 2.2.0). Reads having quality average score < 20 were filtered out from pair end and reads. Trimming of rRNA reads were achieved using Silva database (https://www.arb-silva.de/). The high quality reads with base quality score ≥ 30 were used for downstream analysis.

4.20.3. Prediction of simple sequence repeats (SSRs)

Mulberry transcriptome was analysed through MIcroSAtellite identification tool (MISA, https://.ipk-gatersleben.de/en/bioinformatics-tools/marker-data/misa/) for

detecting the presence of SSRs.

4.20.4. *de novo* transcriptome profile and differential gene expression analysis

Trinity (version 2.8.2) software package was used for *de novo* transcriptome analysis. Transcript ≤ 200 bp were screened out and the resultant high quality clean reads were assembled into contigs. Kallisto (version 0.44.0) was used for estimating transcript abundance, leading to the determination of cross-sample normalization TPM/TMM value, which was used to perform differential gene expression using EdgeR (version 3.6). Quantification of transcript expression level was determined in terms of fold change (FC). The expression profile of transcript that satisfied with $\log_2$FC ≥ 2 and ≤ -2 for up- and down-regulated genes respectively were considered as significantly differentially expressed transcript with P-value cut off ≤ 0.05 (Tafolla-Arellano et al. 2017). Significantly ($p<0.05$) differentially expressed up-regulated and down-regulated genes were expressed in terms of Violin and Volcano plot.

4.20.5. Functional annotation of differentially expressed genes

Generalized annotation and validation of differentially expressed genes was performed with NCBI plant NR database (http://www.ncbi.nlm.nih.gov/), and PMN (plant metabolic network) database (https://plantcyc.org/). While specific annotation with respect to *Arabidopsis* database was performed using Mercator (version 3.6) plaBi database (https://plabipd.de/portal/mercator-sequence-annotation) and revalidation was conducted with uniport database (https://www.uniprot.org/).

4.20.6. Functional enrichment analysis of differentially expressed genes

Functional enrichment through protein-protein enrichment analysis was studied using STRING (version 11.0; https://string-db.org/). STRING generated biological process (BP), cellular component (CC) and molecular function (MF) was aligned with respect to percentile ranking of the obtained GO (Gene ontology) score for obtaining top rank processes. STRING generated interaction data was passed through Cytoscape (version 3.7.1; https://cytoscape.org/) for obtaining the gene topological score. Sub-networking analysis of highly interconnected nodes was performed using MCODE (version 2.0.0) statistical package considering node density cut-off of 0.1 and node score cut-off of 0.2. Gene ontological study of

topologically selected differentially expressed genes having ≤50% average percentile rank was performed using BiNGO plug-in (version 3.0.4) at Cytoscape platform. BiNGO process was used to identify GO terms through the application of hypergeometric test, considering p-value cut off ≤ 0.05.

4.21. Validation of differentially expressed genes by quantitative real-time PCR

For validating differentially expressed up- and down-regulated genes quantitative real-time PCR (qPCR) analysis was carried out using gene specific primer sets. Sample preparation was done by vortex 0.1 gm of sample in a diethyl pyrocarbonate (DEPC) treated sterile 1.5 ml eppendorf tube containing 1 ml of Trisol (Aura Biotechnologies, India). The mixture was incubated at 25°C for 5 min. Following incubation 200 µl of chloroform (Himedia, India) was added and shaken vigorously for 30 sec, incubated for 5 min at 25˚C followed by centrifugation at 12,000 × g for 15 min maintaining at 4˚C. Post-centrifugation 1ml of 75% ethanol was added to the pellets and vortexed after discarding the supernatant, followed by re-centrifugation at 7,500 × g for 15 min maintaining 4˚C. The supernatant was removed, and the pellets were air dried and then mixed with 35 µl of DEPC treated water. The concentration and purity of extracted RNA was assessed using a UV-Vis spectrophotometer (Sartorius). Extracted RNA was treated with DNase to avoid the possibility of DNA contamination. 10 µl of DNase treated RNA was reacted with 5x RT buffer (4 µl), 25x dNTPS (0.8 µl), 10x random primer (2 µl), reverse transcriptase (1 µl) and DEPC water (2.2 µl) for cDNA synthesis at ice cold condition. The mixture was spun and was placed in the thermal cycler maintaining 25°C for 10 min, 42°C for 60 min, 85°C for 5mins, and then hold at 4°C for ∞. Synthesized cDNA was stored in -20°C for RT-PCR analysis after confirming by performing PCR with the house keeping gene Actin 3.

The Primers designed for the gene expression studies was carried out with Eurofin genomics PCR primer designing tool (https://eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design/). Real Time PCR relative quantification study was carried out in Applied Biosystems StepOne Real-Time PCR using the SYBR Green Chemistry (Sensifast SYBR HiRoxkit, Bioline,USA). Reaction mixture for qRT-PCR contains cDNA (0.5 µl), 2x SYBR Green Master Mix (5 µl), forward primer (10 µM, 0.5 µl), reverse primer (10 µM,

0.5 µl) and nuclease free water (3.5 µl). qRT-PCR programme was operated as follows: pre-denaturation at 95˚C followed by 40 cycles of 15 sec at 95˚C and 30 sec at 60˚C, followed by the steps of dissociation curve generation (20 sec at 95˚C, 60 sec at 60˚C, and 15 sec 95˚C). For accurate determination of gene expression values, raw fluorescence data (Ct values) generated by the real-time PCR instrument (Applied Biosystems) was exported to qBase plus software 13 which scales raw data to an endogenous control gene (Actin). Relative expression of a target gene (TG) was done using comparative Ct (ΔΔCt) method using StepOne Software (version 2.2.2) by applying following equation:

RQ $=2^{-\Delta\Delta CT} = 2^{\Delta CT\ (target)} \times 2^{\Delta CT\ (reference)}$.

Data was plotted graphically using OriginPro 2021 software (9.8.0.200).

4.22. Feeding experiment and rearing data collection

The overall rearing process was conducted under laboratory condition following the standard protocol of Krishnaswami et al. (1978).

4.22.1. Collection of silkworm larvae

For rearing purpose healthy and disease free 5th instar larvae were collected from Matigara Sericulture Complex and Naxalbari Sericulture Complex.

4.22.2. Experimental setup for rearing

The collected larvae were randomly selected and distributed uniformly in bamboo made trays in such a way that each tray was bearing 10 larvae. During rearing process larvae were supplemented with S1 genotype mulberry leaves. In one set freshly collected S1 leaves were given for feeding, while in remaining sets preserved leaves were given for feeding. At a regular interval of 4 – 5 hrs larvae were supplemented with preserved leaves. To avoid possibility of any contamination/ disease the rearing trays were cleaned twice a day.

4.22.3. Collection of cocoon parameters

During the entire rearing process, larval weight was recorded at regular interval. Besides this, mortality rate was also counted. When the larvae started spinning, they were transferred to spinning tray and left undisturbed. At the end of the trial growth index (GI), single cocoon weight (SCW), single shell weight (SSW), shell ratio (SR)

(%), effective rearing rate (ERR) (%) and mortality rate (MR) (%) were calculated by means of following formulas:

$$\text{GI} = \frac{\text{Final weight of larvae (gm)} - \text{Initial weight of larvae (gm)}}{\text{Initial weight of larvae (gm)}}$$

$$\text{WCS} = \frac{\text{Weight of 10 male cocoon (gm)} + \text{Weight of 10 female cocoon (gm)}}{\text{Total number of cocoon (20)}}$$

$$\text{SSW} = \frac{\text{Weight of 10 male shell (gm)} + \text{Weight of 10 female shell (gm)}}{\text{Total number of shell (20)}}$$

$$\text{SR (\%)} = \frac{\text{Single shell weight (gm)}}{\text{Single cocoon weight (gm)}} \times 100$$

$$\text{ERR (\%)} = \frac{\text{Total number of cocoon harvested}}{\text{Total number of larvae brushed}} \times 100$$

$$\text{MR (\%)} = \frac{\text{Number of death larvae}}{\text{Total number of larvae}} \times 100$$

4.22.4. Post cocoon analysis (cocoon quality assessment)

At the end of the rearing process obtained cocoons were analysed for cocoon characters (*viz*. average filament length, non-breakable filament length and average filament size) and reeling characters (*viz*. quantity of cocoon reeled, quantity of silk obtained, silk waste quantity, rendita (on good cocoons), percentage of raw silk recovery, percentage of raw silk and percent waste on silk weight) for understanding the effect of feeding preserved leaves over cocoon formation.

4.22.5. Data analysis of rearing parameters

General Linear Model of two-way analysis of variance (ANOVA) was used to calculate the effect of feeding preserved mulberry leaves on silkworm rearing system ($P \leq 0.05$) using SPSS statistical tool (IBM SPSS Advanced Statistics 20.0). Pearson correlation coefficient mediated bivariate correlations study was conducted using SPSS to study inter-relationship between leaf metabolic attributes with rearing parameters ($p \leq 0.01$ and 0.05). Rearing data were plotted using OriginPro 2021 software (9.8.0.200).

4.23. Investigation of protein profile of silkworm larvae fed with preserved mulberry leaves

Silkworm larvae fed with mulberry leaves preserved in nanosilver, silver nitrate and distilled water was used for electrophoretic analysis (SDS gel) and isozyme profiling for predicting difference in banding pattern with respect to larvae fed with fresh leaves.

4.23.1. Sodium dodecyl (lauryl) sulphate (SDS) Gel Electrophoresis of larval protein

4.23.1.1. Extraction of larval protein

Before the silkworm larvae starts spinning i.e., on the final day of 5th instar some larvae were selected from different preservation sets for dissection. Anatomical dissection of the larvae was conducted (Fig. 13) and different body parts *viz.* silk gland, stomach, haemolymph, and fat body (Fig. 14) were separately collected. Isolated body parts were crushed with phosphate buffer (pH 7.5), centrifuged and the supernatant obtained as source of proteins was stored at -20°C.

4.23.1.2. Reagents and detection technique of Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

Same as described under sub-sections of 4.16.1.2.

4.23.2. Orbitrap high resolution liquid chromatography mass spectrometry (OHR-LCMS) of differentially expressed larval proteins

Same procedure was followed as described under sub-sections of 4.16.2. The only difference was *Bombyx mori* database was used for identifying silk gland peptide sequences obtained from OHR-LCMS analysis.

4.23.3. Native PAGE detecting larval isozymes

Three isozymes were detected *viz.* SOD, Catalase and NADPH oxidase according to the protocol prescribed in 4.16.3.

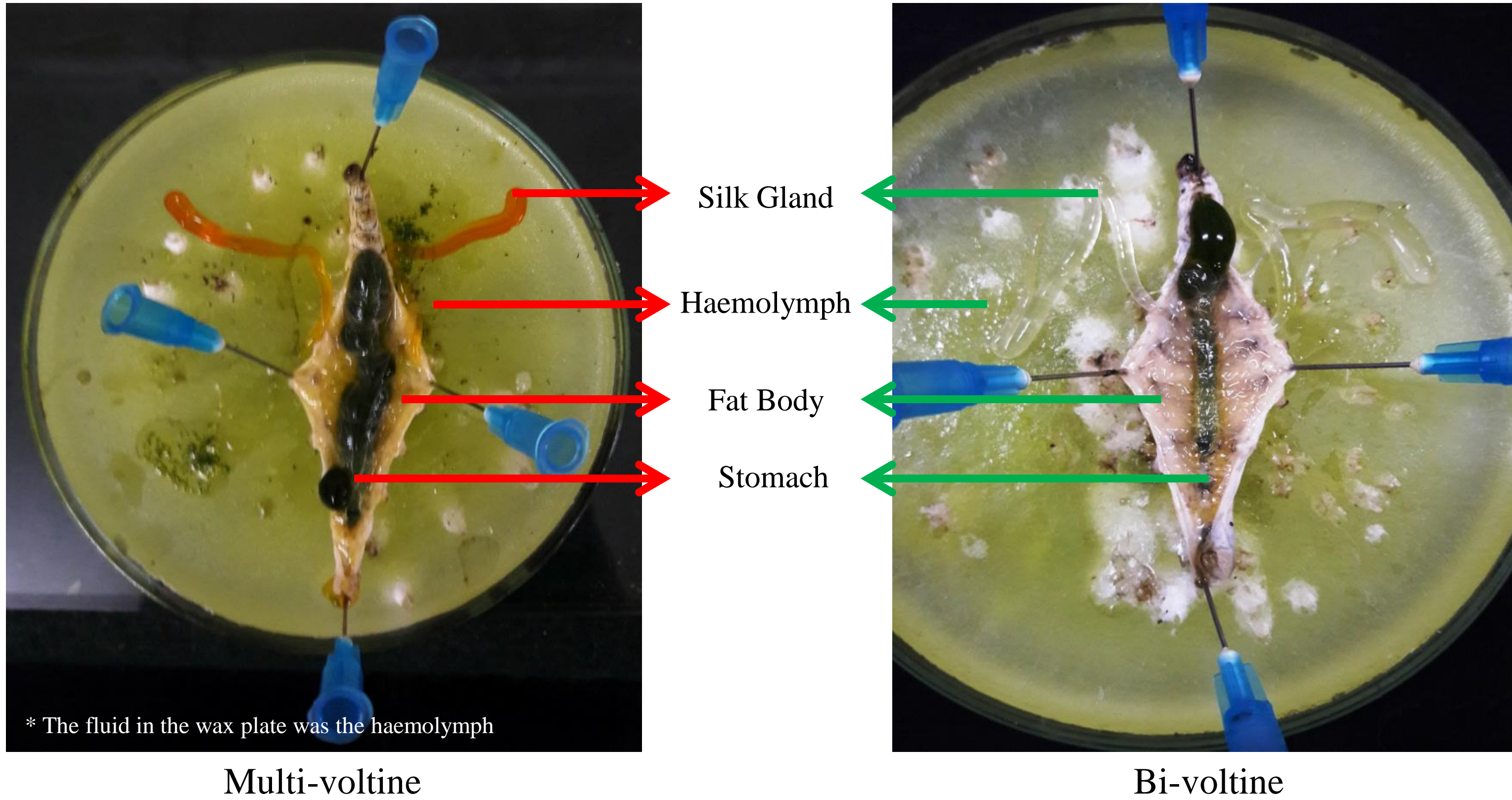
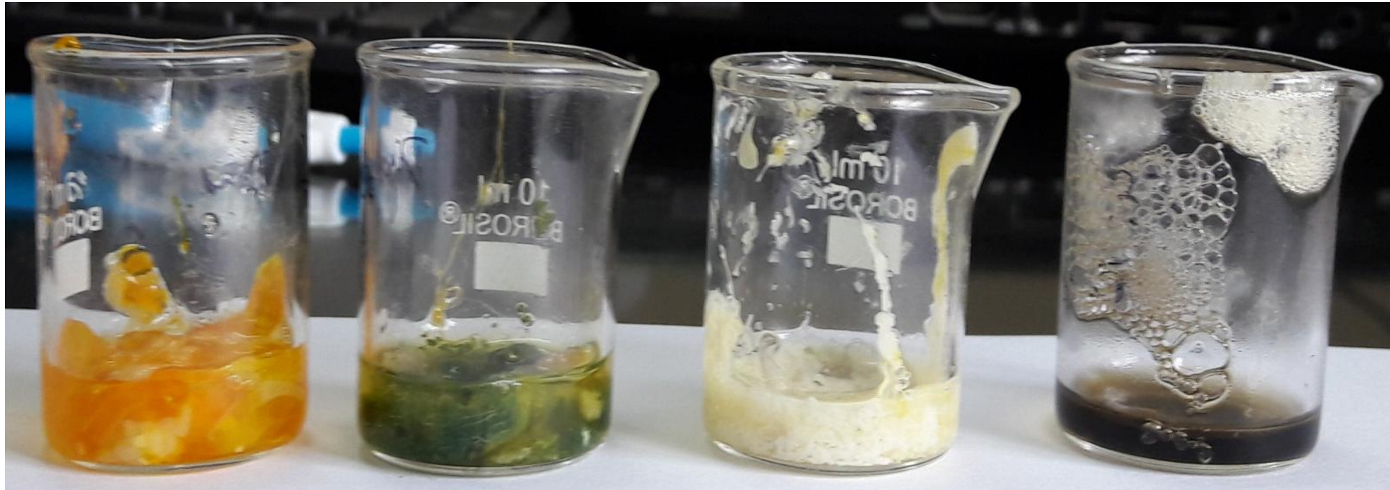


Fig. 13: Dissected silkworm larvae displaying internal body parts



Silk Gland Stomach Fat Body Haemolymph

Fig. 14: Isolated different body part of silkworm larvae obtained after dissection