6. DISCUSSION

Mulberry belongs to the genus *Morus* which was positioned under the family Moraceae as placed by Bentham and Hooker. Worldwide the genus *Morus* has more than 150 different species (Srivastava et al. 2006), among them in India reported wild genus were *M. alba* L., *M. indica* L., *M. serrata* Roxb. and *M. laevigata* Wall. *ex* Brandis. Among different reported species of *Morus*, *Morus alba* (white mulberry) gains special attention as it was solely used for feeding silkworm larvae. Besides being used as fodder, leaves of white mulberry holds multi-dimensional uses. Kumar and Chauhan (2008) reported that the use of different parts of white mulberry *viz.* fruit, leaf, bark, stem and root has been well documented in Chinese medicine. Łochyńska and Oleszak (2011) observed that leaves of mulberry act as an effective phyto-medicine to fight against Type-II Diabetes, due to the presence of quercetin and 1-deoxynojirimycin. Different flavonoids based compounds like rutin, isoquercitrin, albanol B, astragalin, quercetin, and morusin present within the leaves of mulberry helps to reduce generated oxidative stress inside tissue system (Yen et al. 1996). Zafar et al. (2013) reported that leaf extract of white mulberry was highly effective against hypertensive, hyperglycaemic and hyperlipidemic activities. In some part of the world, leaves of mulberry were also reported to be used in the preparation of tea and juice (Wilson and Islam 2015).

In all the above stated multipurpose use of white mulberry, leaves can be stored for long time after drying to prevent any contamination mediated by pathogens, except for feeding silkworm larvae which requires freshly collected healthy leaves (Dong et al. 2017). The nutritional quality of the mulberry leaves greatly determines the quality and quantity of silk produced by the larvae (Manjula and Kumari 2015). Miyashita (1986) stated that leaf quality of white mulberry act as an independent factor contributing more than 30% cocoon production success rate. Depending on this property in the last three decades huge emphasis has been given for developing mulberry genotype bearing high yielding and high nutritional quality. AR11, AR12, BC259, C776, G2, G4, K2, RC1, RC2, RF5135, RF5175, S1, S13, S34, S36, S1635, S146, Sahana, Tr4, Tr10 and V1 are the high yielding cultivars of white mulberry that are mostly used for propagation and silkworm feeding.

Preservation of these high quality leaves of white mulberry becomes necessary during rainy season, as supplementation with wet leaves was not productive as it increases mortality rate of silkworm. Conventionally the traditional practisers during rainy season preserve the collected leaves by rapping in a moist cotton cloth. Preservation by this traditional mode results in decrease of moisture and nutrient content of leaves, leading to the production of inferior quality cocoons. Dong et al. (2017) reported that mulberry leaves maintain the water and nutritional balance inside the body of silkworm. So drop off in nutritional quality of leaves during preservation will hold back normal growth and development rate of larvae. An alternative method for improving productivity during rainy season was a pressing demand to overcome the above stated problem. Preservation of mulberry leaves by the application of efficient preservative solutions may assist to retain the nutritional quality of leaves at post-harvest stage.

The major obstacle that arises during preservation of mulberry leaves using preservative solution was proliferation of microbes within preservative solution that enters into the lumen of xylem through conducting pathway. Increase in count of bacteria inside xylem vessels causes blockage, besides these polymeric compounds like lignin, suberin and many others also gets accumulated inside lumen and there by blocking the normal conducting pathway (Jedrzejuk et al. 2012), accelerating the process of senescence and thereby inhibiting the process of post-harvest shelf life extension.

Present study deals with an attempt to identify an effective preservative solution bearing efficient antimicrobial property that will prevent occlusion of xylem vessel at post-harvest stage of preservation. The preservative solution must be biologically active which will directly or indirectly accelerate enzymatic function that will participate in ROS mitigation, retention of primary metabolites and thereby involving in postharvest shelf-life extension of mulberry leaves. The entire background process from selection of suitable cultivar of mulberry to the selection of suitable preservative solution was described underneath.

6.1. Screening of mulberry cultivars

Locally and commercially available six cultivars of white mulberry were evaluated in terms of macro- and micro-morphological attributes for predicting suitable

cultivar for carrying out the post-harvest preservation experiments. Significant difference was noted among the six cultivars on comparing macro-morphological parameters. Among all the cultivars maximum and minimum lamina length and breadth was recorded in Guangdong (195.04 and 177.80 mm) and TR10 (138 and 88.40 mm) respectively. On comparing petiole length, it was observed that Guangdong displayed smallest petiole length (32.10 mm), while S1635 showed highest length of 47.70 mm. Thus, Guangdong represents advance morphological form as it bears large lamina with small petiole size. The decreasing order of leaf surface area among the cultivars can be represented as Guangdong > S1635 > BC259 > TR10 > V1 > S1. Menon and Srivastava (1984) stated that assimilation of carbon dioxide by plant through the mechanism of photosynthesis and gain in biomass of lamina are directly associated. As Guangdong cultivar of white mulberry bears large surface area, so it is more probable to gain greater biomass through photosynthesis which might favour its yield.

For proper adaptation of different cultivars at different geographic location aperture of stroma, size dimension of stomata and its adjoining cells plays important role. Susheelamma and Datta (1993) reported that those cultivars of mulberry that are having small opening and size dimension of stomata could retain moisture content of leaves more efficiently than the cultivars having large stomatal openings. Beside this it has been reported that photosynthetic efficiency depends to some extent over stomatal frequency and dimension of stomata present on surface of the lamina (Maghsoudi and Moud 2008). Among the six cultivars under present study, it was observed that S1 bears smallest average stroma length (9.48 μm) and breadth (1.40 μm). On comparing the cumulative length and breadth of stroma and guard cell it was observed that S1 lowers the ranking. Beside this S1 also displayed lowest stomatal frequency. Thus, it may be stated that as S1 represents least stomatal dimension along with minimum stomatal frequency, so it bears greater possibility to retain the internal moisture content in a better way as compared to other cultivars helping and thus showcasing greater adaptability in tropical and sub-tropical areas. Present observation was strongly supported by PCA analysis which reveals that length and breadth of lamina positively correlates while stomatal parameters *viz*. stoma length and breadth, frequency and index both individually or in association with guard cell and subsidiary cell. This finding points to the fact that with increase

in lamina dimension, stomatal attributes will simultaneously increase. From this it might be concluded that mulberry cultivar with smaller leaves is more preferable at post-harvest stage because with increase in lamina size, frequency of stomata also increases which might play negative role by reducing the moisture level of leaves, thereby decreasing leaf quality.

Present study also reported extensive variation in length and breadth of trichome in all the studied cultivars. Presence of both glandular and non-glandular trichomes was reported from all cultivars. BC259 had the shortest trichomes (89.40 µm) while S1 showed longest trichomes (253 µm) in terms of length. Trichome exhibits mechanical barrier against the activity of herbivory caused by insects (Baur et al. 1991). On comparing idioblast and trichome density it was observed that the Guangdong and BC259 cultivar had the maximum number of idioblast (45.29) and trichome (55.08) per millimetre square of microscopic field respectively, while TR10 and S1 displayed the least number of idioblast (18.55) and trichomes (28.36) density respectively. The type and density trichomes present puts significant impact on the overall acceptability of leaves by the larvae. Kesavacharyulu et al. (2004) reported that with increase in trichome density, acceptability of leaves by larvae decreases considerably. In sericulture industry acceptability of particular cultivar of mulberry by silkworm larvae as supplement was considered as most important as any other parameters (Singhal et al. 2010). As S1 cultivar displayed least trichome density, it may be predicted that it will be more acceptable by silkworm larvae and thus by silk industry than any other cultivars of white mulberry.

Thus, from the above outcome and from the study of correlation matrix it may be stated that most significant attributes were positively correlated among each other. Lamina length showed direct correlation with lamina breadth; similarly attributes of stomata and trichome were also positively correlated among each other. The positive correlation between lengths of internode and petiole is very significant which proposes that developing leaves obtain sufficient space for proper growth; besides this the upper whorled leaves were not overlapping the lower leaves due to which efficiency of photosynthesis might get enhanced leading to accumulation of more photo-assimilates. Vein islet functions in proper and uniform distribution of photosynthetic products and it showed positive correlation with length and breadth of lamina, which reveals that greater the size of lamina more is the number of vein

islet and thereby greater the distribution of photo-assimilates leads to the development of healthy leaves on maturity which may accomplish the industrial requirement. Thus, present findings were highly significant as it may help to categorize suitable cultivar for proper purpose.

6.2. Selection of suitable preservative solution for post-harvest shelf life extension

Screening of ideal preservative solution was the basic pre-requisite for post-harvest shelf life extension. Different class of chemicals were used during screening, out of which one class comprises group of hormones *viz.* indole acetic acid, kinetin, benzyl adenine and gibberellic acid. Yu et al. (2009) while working on blue mold rot of apple stated that IAA greatly enhances natural resistance power of plants during post-harvest stage which extends shelf life by inhibiting proliferation of microbes. Cytokinins like kinetin besides maintaining normal growth process of plants maintains vigor and delays the process of leaf yellowing (Seema et al. 2011), while benzyl adenine delays senescence by slowing the process of chlorophyll degradation, thereby maintaining chloroplast activity, thus promoting expression of chloroplast genome maintaining photosynthetic process (Kumari and Solankey 2018). Further it was reported that degradation of chlorophyll and photosynthetic apparatus that take place during senescence were accelerated by the action of abscisic acid and ethylene which gets inhibited or delayed by the exogenous application of cytokinins (Richmond and Lang 1957) and in some systems by the application of auxins and gibberellins (Smart 1994). Jordi et al. (1995) proposed that bioactive gibberellins delays chlorophyll loss thereby slows down the process of senescence. Current study displayed inefficiency of hormonal solutions in extending post-harvest shelf life of mulberry leaves. Senescence of leaf at post-harvest stage involves degradation of macromolecules like chlorophyll, protein, nucleic acid and components of plasma membrane, and thereby subsequent mobilization of a number of degradation products to other parts of the plant. Breakdown of chlorophyll molecule causes yellowing of the leaves, the most noticeable visible symptom of senescence (Fang et al. 1998). Beside appearance of yellow patches over leaves, leaves preserved in higher concentration of indole acetic acid, kinetin and benzyl adenine displayed tissue browning indicating loss of internal moisture content with increase in storage time. Cellular loss of water causing internal dehydration was the

major cause of tissue browning (Landrigan et al. 1996). Cellular dehydration causes loss of turgor pressure (Alzamora et al. 2000) which causes drastic change in qualitative attributes such as colour, texture and many more that ultimately leads to post-harvest enzymatic browning of tissues (Agüero et al. 2008).

Beside hormones, phenolic phyto-hormone *viz*. salicylic acid an endogenous regulator of physiological processes that mostly involves in defence mechanism of disease resistance (Luo et al. 2011) and polyamine *viz*. putrescine that protects plant cells against various environmental stress (Davarynejad et al. 2015), also fails to extend the shelf life of mulberry at post-harvest stage. Sodium nitroprusside, a nitric oxide generator, that was reported to extend vase life by inhibiting ethylene production (Naing et al. 2017) and by enhancing or maintaining antioxidant enzymatic and non-enzymatic activity (Shi et al. 2016) when used to prolong shelf life of mulberry leaves failed to produce effective result.

The major challenges faced during preservation of leaves are wilting and yellowing of leaves, as well as xylem blockage due to microbial proliferation. Silver nitrate and silver thiosulphate are the most commonly used silver ion as preservatives acting as an ethylene blocker, thus delaying senescence (Subhashini et al. 2011). By acting as ethylene blocker both silver nitrate and silver thiosulphate delays the process of senescence in flowering plants and thus preventing abscission (Kofranek 1985; Knee 1992). Salts of silver ions help in extending the shelf life by maintaining the continuity of water column, as it prevents colonization of microbial organisms within the xylem lumen (Ohkawa et al. 1999). Butt (2005) reported that silver nitrate solution at 150 ppm concentration extends the vase life of *Rosa hybrida* by 4 days. Similarly, application of silver thiosulphate prolongs the vase life of cut rose flower stem up to 10 days (Reid et al. 1980). Ten minute pulsing of silver thiosulphate increases the post-harvest vase life of cut carnation flowers (Dimalla and Staden 1980). Liao (2000) observed that ethylene production within cut rose decreases by the application of silver thiosulphate at post-harvest stage. Although plenty of work has already been done on evaluating the effect of silver nitrate and silver thiosulphate as ACC synthase blocker, but most of the work remains confined in preservation of economically important flowering plants/ twigs and almost no work was carried out for evaluating the effect of silver ions in preservation of economically important leaves at post-harvest stage along with predicting the

mechanism of senescence retardation. Present study showed that among the used two salts of silver, silver nitrate at low concentration showed potentiality to extend the shelf life of mulberry leaves at post-harvest stage which the other silver salts *viz*. silver thiosulphate fails to extend. Silver nitrate solution (more precisely at 10 ppm concentration) showed potentiality to retain their physical and chemical property of preserved mulberry leaves as that of fresh leaves for a period of at least 7 days, signifying that as an anti-senescence mediator, silver nitrate acts as a effective preservative for mulberry leaves.

On preserving mulberry leaves with silver nitrate solution, characteristic colour transformation from transparent to brown that was recorded with time indicated the formation of nanosilver solution, as the solution displayed SPR peak of ~459 nm. Literature survey proposes that appearance of SPR peak in the wavelength range of 400 – 500 nm indicates nanosilver formation (Sastry et al. 1997). Thus, it was actually the nanosilver which was formed during the course of preservation of mulberry leaves helping in extending post-harvest shelf life by 7 days and thereby maintaining fresh texture of leaves as that of fresh leaves. Obtaining such striking observation, present work was directed in the track of determining ideal nanosilver solution suitable for extending shelf life of mulberry leaves at post-harvest stage and the entire results were compared with respect to silver nitrate, performing as positive control and distilled water acting as negative control.

6.3. Phytosynthesis and characterization of silver nanoparticles

Biogenic synthesis of silver nanoparticles was initially confirmed by colour transformation of silver nitrate solution. Annamalai et al. (2014) stated that the organic constituents and the dielectric medium of the bio-extract was mainly responsible for colour transformation during nano formation. Further validation was carried out using more specific technique, UV-Vis spectrophotometer. Sastry et al. (1997) reported that appearance of spectral peak in the wavelength range of 400 – 500 nm confirms nanosilver formation. In the present study the characteristic UV-Visible spectra displayed SPR band at ~441 nm conforming phytosynthesis of silver nanoparticles.

FTIR spectral peak of phytosynthesized nanosilver and plant extract appeared almost at the same position with minor deviation due to reduction process (Bhakya

et al. 2016). FTIR spectra strongly support the participation of different components of plant extract in bioreduction process of metallic salts into silver nanoparticles. Ganesh Babu and Gunasekaran (2009) stated that during nanoparticle formation interaction between metal salts and biomolecules takes place through the association of functional groups. Bands shifting from 3675.839 cm^{-1} of plant extract to 3633.411 cm^{-1} in nanosilver was due to –OH stretching vibration, while positional shifting from 3425.126 cm^{-1} to 3430.912 cm^{-1} corresponds to N–H mode of vibration which overlapped with –OH vibrational stretching of phenolic and alcoholic compounds (Loo et al. 2012; Ahmed et al. 2016). The peaks at 2919.843 cm^{-1} and 2854.272 cm^{-1} corresponds functional vibrations of $–CH_2$ and $–CH_3$ groups. These peaks were not detected in plant extract possibly due to nosiness of –OH vibration stretching. Strong intense peak at 1627.706 cm^{-1} corresponds vibration of 1˚ and 2˚ amines (Khalil et al. 2014; Sivakumar et al. 2012) and 1386.636 cm^{-1} represents C–N vibration stretch, most likely representing amide I protein bands of leaf extract (Gurunathan et al. 2015). The band at 1066.495, 1020.209 cm^{-1} of leaf extract and 1033.709 cm^{-1} of nanosilver depicts strong C–O– and C–OH vibrational stretching of alcohol, carboxylic acid, ester and ether bond of proteins and carbohydrates present in the isolated leaf extract (Yuen et al. 2005; Shankar et al. 2004). Obtained peak at 761.782 cm^{-1} of extract showed positional displacement in the direction of higher wave number at 796.4961 cm^{-1} in phytosynthesized nanosilver indicates N–H vibration of 1˚ aliphatic amines. Absorption band at 572.7829 and 518.7832 cm^{-1} correspond vibration of C–C skeleton of branch alkanes. Thus, FTIR spectra, clearly indicates the presence of carbohydrate, proteins and probably different group of secondary metabolites in the mulberry leaf extract that are involved in the bio-reduction of silver ion and also acting as bio-stabilizing agents.

SEM micrograph displayed spherical nanoparticles that were clumped at some regions. These clumping of particles were probably due to cross linking (Shankar et al. 2017) or may be due to solvent evaporation during preparation of sample (Jagtap and Bapat 2013). Elemental profiling through EDX analysis displayed most intensified peak at 3 KeV representing abundant presence of silver. Due to SPR, silver present in nanoparticles showed powerful peak at 3 KeV confirming the formation of silver nanoparticles (Magudapathy et al. 2001; Das et al. 2013). EDX spectra also showed the presence of carbon and oxygen indicating the presence of

alkyl chain as stabilizing agent during the phytoreduction of metallic silver (Puchalski et al. 2007), supporting the observation of FTIR analysis. TEM and HR-TEM analysis showed synthesized nanoparticles have size dimension <10 nm and >40 nm. The variation in particle size distribution was primarily due to clustering of particles at some places (Jagtap and Bapat 2013). TEM micrograph displayed that the periphery of the synthesized nanoparticles were thinner than centre, indicating the participation of protein molecules as capping agent (Ahmad et al. 2010). The polydispersity was obtained to be 11.35% indicating that most of the nanoparticles remained in monodispersed phase; similar observation was also been reported by Ibharim (2015) and Banala et al. (2015) while synthesizing nanosilver using banana peel and papaya leaf extract respectively. Bio-application of nanoparticles largely depends on shape, size and polydispersity index of the synthesized particles (Bansal et al. 2010). Agnihotri et al. (2014) reported that bioactivity of nanosilver mostly antimicrobial activity was inversely related to the size of the nanoparticles. Current study showed synthesis of smaller size nanoparticles, supporting their bioactive nature.

XRD pattern of dried nanosilver generated four prominent diffraction peaks at 38.14˚, 44.26˚, 64.46˚, 77.41˚ corresponding to (hkl) values of (111), (200), (220), (311) Bragg's reflections plane that matches with JCPDS library file no: 04-0783 confirming the formation of face centered cubic silver. Premasudha et al. (2015) and Awwad et al. (2013) while synthesizing nanosilver using extract of *Eclipta* and Carob leaf respectively reported almost similar orientation pattern of XRD spectra. Besides normal peaks of silver stated above, three extra peaks at 27.86˚, 32.26˚ and 46.11˚ were noted and these peaks probably represents the organic constituents of extracts that were responsible for bioreduction of silver ion (Roopan et al. 2013). SAED pattern showed four prominent circles of bright diffraction spots and these spots were corresponds to (111), (200), (220) and (311) Bragg reflection planes (Amin et al. 2012). Beside these the lattice constant value of most intense peak (111) which was obtained to be 4.084 Å matches exactly with the reported standard value of silver in JCPDS file no: 04-0783. Similar observation on biogenic silver nanoparticles was also proposed by Anandalakshmi et al. (2016) and Mehta et al. (2017).

The size of the particles obtained from DLS analysis *viz*. 29.68 nm is greater than that determined through TEM and XRD analysis, this may be due to the fact that DLS takes into consideration the associated capping and reducing agents during size measurement (Singhal et al. 2011). Besides size dimension for biological activity of nanoparticles, stability plays a significant role (Aiad et al. 2013). Nano stability was measured in terms of zeta potential by measuring the surface charge of the synthesized nanoparticles. Nanoparticles displaying greater positive or negative value of zeta potential will repulse each other, which will prevent nanoparticles for agglomeration and there by increases particle stability (Haider and Mehdi 2014). Nanoparticles having zeta potential greater than +30 mV or less than -30 mV are considered to remain stable without agglomeration for longer period (Zhang et al. 2008). Current study reports zeta potential of +37.4 mV, indicating highly stable nanoparticles synthesized using mulberry leaf extract. Positive value of zeta potential was probably due to development of attraction between positively charged capping agents by electrostatic force with the synthesized nanoparticles (Hedberg et al. 2012). Thus, above study clearly indicates that mulberry leaf extract bears complete potentiality to synthesize stable and bioactive silver nanoparticles that can be used to extend the shelf life of mulberry leaves at post-harvest stage.

6.4. Bioactivity assessment of phytosynthesized silver nanoparticles

Biosynthesized silver nanoparticles exhibit significant scavenging activity against oxidative molecules. The functional groups present in leaf extract which are mostly involved in reduction of silver ion are principally responsible for antioxidant activity (Patil et al. 2015). The lipophilic radical DPPH was usually used as model test for determining the scavenging activity of free radicals against synthesized nanoparticles and natural compounds due to its high stability (Bhakya et al. 2015). DPPH in its radical state displayed maximum absorbance at 517 nm, which steadily decreases by reduction caused by antioxidants (Blois 1958). In present study scavenging activity of DPPH increases with rise in concentration of synthesized nanoparticles. DPPH scavenging activity was also displayed by plant extract but extend of activity was considerably less than synthesized nanosilver. Findings of the current study was supported by earlier workers who also reported more or less similar outcome while working with silver nanoparticles synthesized using extract of *Iresine herbstii* (Dipankar and Murugan 2012) and *Alpinia katsumadai* (He et al.

2017). Potassium persulfate oxidizes ABTS to generate $ABTS^{+}$, a blue chromophore causing oxidative damage (Moteriya et al. 2017). Assessment of $ABTS^{+}$ scavenging activity was mostly conducted to access the potentiality of hydrogen donors and antioxidant agents that remained in the biological samples (Ajayi and Afolayan 2017). Silver nanoparticles synthesized with mulberry leaf extract displayed strong $ABTS^{+}$ activity. Strong $ABTS^{+}$ scavenging activity of phytosynthesized nanosilver was also reported by Shanmugam et al. (2016). Silver nanoparticles also displayed considerably strong nitric oxide scavenging activity and this potentiality was generated due to the presence of electronegative property of nitric oxide radical which silver nanoparticles utilizes for reduction by donating electron (Rodriguez-Gattorno et al. 2002). Patil et al. (2015) proposed that nitric oxide plays vital role in different processes of bio-regulation, but its excessive production or accumulation may cause several disorders. Superoxide, a weak anionic radical bear the potentiality to generate two harmful radicals *viz.* hydroxyl radical and singlet oxygen that generates oxidative stress (Elmastas et al. 2006). Reddy et al. (2014) stated that inside living system, accumulated superoxide directly reacts and damages nucleic acids and proteins, besides this superoxide mediated generated hydroxyl radical causes cellular damage by reacting with fatty acid in polyunsaturated state that were associated with phospholipids (Halliwell and Gutteridge 1990). In present study phytosynthesized silver nanoparticles displayed elevated superoxide scavenging activity which was supported by the work of Reddy at al. (2014) that showed 60% superoxide scavenging potentiality of nanosilver synthesized using fruit extract of *Piper longum*. Metal chelating activity is the ability of the antioxidant molecules to destabilize the formation of Ferrozine-Fe^{2+} complex (Inbathamizh et al. 2013). Current study indicated high metal chelating potentiality of phytosynthesized silver nanoparticles with respect to standard.

Biosynthesized silver nanoparticles also displayed significant antimicrobial activity against both gram positive and gram negative bacteria. It was observed that for inhibiting the growth of gram-positive bacteria, higher concentration of nanosilver was required while efficiency against gram negative bacteria was determined to be effective at comparatively lower concentrations of nanosilver.

Such striking observation was possibly due to the fact that metallic ions at higher concentration can smoothly bind to the surface of gram positive bacteria (Beveridge

and Fyfe 1985), while at lower concentration the rigid composition of cell wall of gram positive bacteria creates difficulty for nanosilver to penetrate the bacterial cell wall (Ibrahim 2015). Earlier workers reported the phytosynthesis silver nanoparticles by using extracts of *Artocarpus heterophyllus* (Jagtap and Bapat 2013), *Lycopersicon esculentum* (Maiti 2014), *Capparis spinosa* (Benakashani et al. 2016), *Sida acuta* (Nisha et al. 2017), *Aloe vera* (Abalkhila et al. 2017) and *Eriobotrya japonica* (Rao and Tang 2017) depicting strong bactericidal activity against different strains of microorganisms.

Silver nanoparticles showed minimum inhibitory concentration against gram negative bacteria. Low MIC against gram negative bacteria was probably due to the presence of thin peptidoglycan layer in the cell wall that can be easily penetrated by nanoparticles (Shrivastava et al. 2007). Silver nanoparticles inhibit bacterial growth by releasing silver cations that internally damages cellular organization (Paszek et al. 2012). Stoimenov et al. (2002) reported that the driving force that causes attraction between negatively charged microbial membranes with positively charged nanoparticles leading to bactericidal activity was electrostatic force. Silver nanoparticles inhibit microbial growth by interacting with negatively charged sulphur and phosphorous containing cellular constituents like nucleic acids (DNA/ RNA) and proteins (Nel et al. 2009; Jung et al. 2008). Although in recent years different workers have proposed different mode of action of nanosilver against microorganisms but exact mode of action requires to be analysed further.

6.5. Time Kinetics, Process variation, stability and bioactivity assessment of phytosynthesized silver nanoparticles

Hamouda et al. (2019) stated that phytosynthesis of nanosilver was a time dependent process. Current study reports requirement of more than 10 hrs for the entire bioreduction process to complete. Kumar and Yadav (2008) reported that prolong is the reaction time greater is the probability for production of large size dimension of biosynthesized silver nanoparticles with greater degree of polydispersity. Current study reports completion of mainstream reaction within first hour of initiation. Kumari et al. (2016) reported that appearance of single spectral peak was the indicator of formation of spherical shaped metallic nanoparticles, while appearance of two or more SPR peaks indicated anisotropic particles. Present study also reports appearance of single SPR peak, indicating generation of spherical shaped particles.

During the first hour of reaction, SPR peak appeared at 429 nm which gradually got positional displacement to 432 nm and finally got stabilized at and around 441 nm indicating red shift with time. Red shift of spectral peak indicates large size nanoparticles (Prathnaa et al. 2011). Lina et al. (2010) stated that positional shifting of spectral peak with time depends on number of factors, including size and surface morphology of the synthesized nanoparticles, its dielectric medium, coupling of colloids and absorbed solutes. Hasan et al. (2018) reported that prolong reaction time significantly effects distribution of nanoparticles along with development of large size particles. Large size particles are biologically less dynamic (Kaya et al. 2016), thus execution of process variation will assist to demarcate ideal condition for the phytosynthesis of silver nanoparticles using extract of mulberry leaf that will produce biologically more efficient nanoparticles.

On synthesizing silver nanoparticles with silver nitrate at five different concentrations, it was noted that 10^{-4} and 10^{-5} M concentrations of silver nitrate solution remained transparent at the end of the bioreduction process signifying the inability of such low concentrations to produce bioactive nanoparticles. UV-Vis spectra also displayed no absorption peak for silver nanoparticles phytosynthesized with 10^{-4} and 10^{-5} M silver nitrate solution. Nanoparticles formed with 10^{-1} M silver nitrate solution showed aggregation and precipitation within 24 hour of phytosynthesis, displaying nanoparticles instability. Similar experimental outcome was reported by Mohapatra et al. (2015) while synthesizing nanosilver using lemon extract. Instability of synthesized nanoparticles with 10^{-1} M silver nitrate was supported by zeta potential which displayed no zeta value on analysis representing highly unstable nature. Synthesized 10^{-2} M nanosilver also showed zeta instability value of -2.11 mV, as zeta potential showing more positive than +30 mV or more negative than -30 mV are accounted to remain in stable condition for longer duration without coalescence (Kadu et al. 2011). Further indication of coalescence nature of 10^{-2} M nanosilver was evident from long term validity assessment which showed steady increase in absorbance value with increase in preservation period. Izak-Nau et al. (2015) reported that during storage, agglomeration of nanosilver was the probable reason for increase in spectral absorbance value. Among the five concentrations of silver nitrate used for silver nanoparticle biosynthesis, 10^{-3} M concentration was identified to be the most suitable concentration having zeta

stability of +74.7 mV and its SPR peak pattern remained almost uniform during storage period, indicating long term stability. On assessing bioactivity by preserving S1 genotype of mulberry leaves with synthesized silver nanoparticles for 7 days, it was observed that silver nanoparticles biosynthesized with 10^{-1} M and 10^{-2} M silver nitrate solution was not suitable for preservation, as yellowing of the leaves and thereby consequent drop off in chlorophyll content was noted from 4^{th} day onward of preservation. While 10^{-3} M silver nanoparticles due to its stable nature were capable to keep holding of its bioactive form during post-harvest preservation and thereby maintaining the fresh texture and shelf life till the last day of preservation. Biogenic silver nanoparticles synthesized using solution silver nitrate at a concentration of 10^{-3} M was also reported to extend the post-harvest shelf life of horticultural crops including *Chrysanthemum* (Carrillo-López et al. 2016), *Gerbera* (Mohammadiju et al. 2014), Carnation flower (Koohkan et al. 2014) and Tuberose (Bahrehmand et al. 2014).

On monitoring nanoparticle synthesis at different volume (2.5, 5, 10, 15, 20 ml) of mulberry leaf extract maintaining the concentrations of silver nitrate constant at10^{-3} M, it was noted that with increase in volume of extract decrease in SPR spectra (blue shift) was taken place. Shankar et al. (2017) reported that red or blue shift of SPR spectra depends on shape, size and nature of organic constituents present in contiguous medium. Bar et al. (2009) stated that presence of excess biomolecules beyond a certain limit ceases nanoparticle formation and was the probable reason for decrease in SPR spectra at high concentration of mulberry leaf extract. Thus, proportion analysis between leaf extract to silver nitrate clearly indicated that 1:10 ratio was the ideal combination for nanosilver phytosynthesis. Similar proportion was reported by Yu et al. (2019) while studying nanosilver green synthesis using leaf extract of *Eriobotrya japonica*. TEM mediated size distributional analysis showed direct relationship with extract volume. It was observed that nanosilver synthesized with 5 ml mulberry leaf extract amalgamated with 45 ml 10^{-3} M silver nitrate produced small size particles with polydispersity of 10.14%. It was proposed that biological activity of silver nanoparticles was dependent on number of factors including size, shape, surface chemistry, type of reducing agents and coating/capping agents, reactivity of particles in solution, agglomeration, and dissolution rate (Carlson et al. 2008). Among them size and morphological

uniformity are the key requisite for bio-functionality (Zhang et al. 2016). The kinetics of nanoparticle uptake by cellular system indicates that size of nanoparticle puts impact over bio-energetic properties *viz.* enthalpy and entropy that administer the communication between nanoparticles and living system (Li et al. 2010). Maximum cellular uptake and cellular functionality was performed by nanoparticles bearing smaller dimension (Hoshyar et al. 2016). In the current study small dimension and uniform particle size distribution was provided by nanoparticles synthesized with 5 ml plant extract, beside this it also displayed long term storage stability. Thus, nanosilver synthesized with 5 ml leaf extract was the most suitable extract volume for nanosilver biosynthesis.

In sericulture for improving yield of rearing, many cultivars of mulberry have been developed overtime. The phytochemical constituent and nutritional value of one cultivar differs from the other. So, to forecast which cultivar is most appropriate for nanosilver synthesis, UV-visible spectral reading of synthesized nanosilver with all the cultivars under study was taken and the obtained spectral reading suggested S1 and S1635 as suitable cultivar for silver nanoparticle biosynthesis, as they portrayed SPR peak in the region of low wavelength than others signifying small size particles. Shaik et al. (2018) proposed that the appearance of spectral peaks at lower wavelength was an indicative signal of small sized nanoparticles. Kaya et al. (2016) observed that smaller the size of synthesized nanoparticles superior was the bioactivity, indicating superior effectiveness of nanosilver synthesized from extracts of S1 and S1635. Further it was noted that peak intensity and sharpness of S1 was more acute and prominent than S1635 where the nature of the SPR peak was slightly blunt. Velgosová and Mražíková (2017) stated that more the peak broadening greater was the possibility of forming nanoparticles with different size and shape, thus rejecting nanosilver formed with leaf extract of S1635 as it bore likelihood of forming nanoparticles of uneven size and shape. Considering the entire observable facts, it might be stated that S1 was the most appropriate cultivar for nanosilver formation.

On analysing impact of light over nanosilver formation it was observed that rate of nanosilver formation under sunlight was enhanced by more than 10 times in comparison with diffuse light, while it took nearly 3 days under dark for colour transformation to take place. In the report of Srikar et al. (2016), where light

mediated green synthesis of nanosilver was performed using aqueous extract of *Prunus amygdalus*, it was stated that no nanoparticle formation was taken place under dark condition, while formation rate of nanoparticles got enhanced by 60 times under direct sunlight than normal dispersed light. Madhu et al. (2019) on synthesizing honey-mediated nanosilver observed that under sun light rate of nano formation got induced in contrast to dark and dispersed light. The driving force that leads to rapid bioreduction of silver nitrate under direct sunlight is the presence of greater number of photons leading to the biosynthesis of silver nanoparticles (Srikar et al. 2016). UV-Vis spectral analysis showed SPR at 459 nm, 441 nm and 447 nm respectively for nanosilver produced under sunlight, diffuse light and dark. Appearance of SPR peak at diverse wavelengths was a general sign of dissimilarity in particle size, indicative of large size particles with progressive shifting in UV-Vis peak towards right (Siriwardane and Cook 1985). Appearance of high wavelength plasmon peak in nanosilver synthesized under dark was probably because it has taken long time period for bioreduction to take place, as distribution of particle size was inversely proportional to time of reduction (Hasan et al. 2018). Z-average nanoparticles size determined through DLS analysis showed more than 2.5 fold particle size of nanosilver under sunlight than diffuse light. Mankad et al. (2020) reported that it was nature of light exposure during nanoparticle biosynthesis upon which DLS mediated particle size was largely dependent on. Obtained numerical value of zeta potential in the present study clearly indicates that nanosilver biosynthesized under diffuse light displayed greater stability than the remaining conditions. The stability of the silver nanoparticle synthesized under diffuse light was probably due to the presence of large quantity of positive charge particles in the outer surface which repulsed each other, preventing particle agglomeration (Mankad et al. 2020). The trend of particle size distribution observed from TEM analysis appeared similar to DLS analysis showing large size particle under the condition of sunlight followed by diffuse light and dark. The only variation appeared in the size dimension *i.e.*, average particle size obtained from TEM analysis was much less in dimension than that obtained from DLS analysis, this was probably because of DLS while analysing particle size, not actually taking consideration of only metallic core but also measured the dimension of the biomolecules present on the surface as capping or stabilizing agent (Aziz et al. 2014). Present study showed nanosilver biosynthesized under diffuse light depicted minimum particle size dimension with

greater monodispersed nature which was in agreement with the report of Bansal et al. (2010) which proposed that biological activity of the nanoparticles was mostly dependent on particle size and monodispersed nature.

On analyzing pH adjusted nanosilver bio-formation it was noted that at acidic pH, nanosilver formation was ceased while with increase in alkalinity range, rate of biosynthesis was enhanced. Literature study reported that addition of bases like NaOH enhanced the rate of bio-reduction thereby promoting synthesis of silver nanoparticles (Wang et al. 2005), supporting the above observations. SPR analysis displayed no spectral peak at lower pH while SPR peak amplitude increased with raise in alkaline range. At acidic pH SPR peak does not appear due to repression of nano formation (Samari et al. 2018), while intense SPR peak at neutral and alkaline pH was probably due to ionization of phenolic group present in the leaf extract (Gavade et al. 2015). Similar observation has been reported when silver nanoparticles was synthesized using cyanobacterium *Oscillatoria limnetica*, displaying transparent solution without any SPR peak at low pH and generated gradient of brown colouration with acute peak with increasing pH level (Hamouda et al. 2019). Birla et al. (2013) reported that at acidic pH, due to aggregation of nanoparticles, plasmon peak became broader presenting low value absorbance. Anigol et al. (2017) reported that alkaline pH was most appropriate for nanoparticle formation with finest plasmon peak formed at pH 9; current reporting was in agreement with the present spectral observation which also displayed least wavenumber of 420 nm at pH 9. Stability analysis through zeta potential in alkaline range displayed multiple peaks revealing polydispersive and instable nature, not fruitful for bioactivity. Lack of stability of nanoparticles under alkaline range was probably due to lack of stabilizing agent consequence to agglomeration (Vanaja et al. 2013). Stable nano formation occurred in the neutral zone around pH 7 was producing zeta potential of +36.4 mV. Silver nanoparticle synthesized with extract of *Aegle marmelos* and *Psidium guajava* at pH 7 showed optimum particle size with maximum bioactivity (Christopher et al. 2015; Sarsar et al. 2014), supporting current observation. Similarly, nanosilver biosynthesised with leaf extract of mango displayed optimum pH of 7 for biosynthesis (Samari et al. 2018). Maximum stability of nanoparticles at and around pH 7 was probably due to the prevailing surface charge on the particles which maximized the repulsive force among particles

enhancing nano stability (Elemike et al. 2017). TEM mediated size distributional analysis displayed elevation in particle size dimension with increase in alkalinity, with smallest particle size in neutral pH. Hasan et al. (2018) stated that the size and shape of the synthesizing nanoparticles was greatly influenced by the pH of the reaction medium as it alterd the nature of photochemical present in the prepared plant extract, and thereby affecting the binding potential with the metallic ions.

Firdhouse and Lalitha (2013) while performing green synthesis of nanosilver using extract of *Amaranthus polygonoides* observed that with increase in temperature rapid phytosynthesis takes place. This observation was in supporting with the current experimental outcome which showed accelerated rate of nano formation with increase in reaction temperature. Absorbance of SPR increased with increase in temperature and was probably due to the fact that the rate of synthesis of nanoparticles got elevated with increase in reaction temperature (Tahir et al. 2015). Current study reports constant value of λ_{max} at all temperature range, indicating uniformity in particle size distribution as positioning of plasmon band largely depends on size and shape of synthesized nanoparticles (Roy et al. 2017). DLS mediated hydrodynamic diameter analysis of synthesized nanoparticles reveals that at high temperature, particle size increases, similar observation was reported from TEM analysis. This is because particles that are in Brownian motion, their degree of momentum increases with increase in temperature resulting in higher degree of collisions between adjoining particles causing aggregation, thus forming larger size dimension (Chiad et al. 2013). Beside this, increase in Van der Waals force of attraction between nanoparticles in the solution was the probable reason behind raise in particle size (Banerjee and Nath 2015). On analyzing zeta potential mediated electrostatic stabilization, it was noted that except nanosilver formed at 25˚C all other failed to attain stable range. Particles having zeta potential outside range of stability fails to generate necessary repulsive force resulting in aggregation forming large size particles, explaining the possible cause for increase in nanoparticle size at low and high temperature (Ahmad et al. 2018). Thus, nanosilver biosynthesized under optimum condition of 25°C carries an additional advantage over high temperature ranges, as it will prevent denaturation of protein molecules present in the mulberry leaf extract (acting as capping and reducing agent) (Cicek et al. 2015).

Singh et al. (2018) proposed UV-Vis spectroscopy was an ideal tool for studying formation and stability of synthesized metallic nanoparticles. On assessing long term stability through UV-Visible spectral reading for nanoparticles synthesized at different pH and temperature range it was observed that bioreduction that took place at pH 7 under 25˚C produced most stable nanoparticle that could be stored for longer period. With increase in storage duration, gradual red shift in SPR peak was observed for nanosilver biosynthesized at alkaline pH indicating aggregation of nanoparticles resulting in large size with highly instable nature, as red shift in the wavenumber of spectral peak indicates instability (Tripathi et al. 2009). Nanosilver synthesized by the process of chemical reduction displayed no characteristics change in the plasmon peak position in the reaction mixture till the period 30 day (Udapudi et al. 2012). Present study reports better performance of nanosilver synthesized by green technology presenting additional stability under defined physical condition of pH 7 and 50˚C. Long term storage stability of biogenic colloidal nanosilver has also been reported by Velgosová and Mražíková (2017). Chartarrayawadee et al. (2020) proposed that for long durability and stability of biogenic silver nanoparticles, nature of associated stabilizing and capping agents are of key importance. Thus it may be stated that due to the presence of sufficient potentiality in phyto-constituents of mulberry leaves extract, long time stability of synthesized nanosilver at pH 7 and 25˚C was ensured.

Application of mulberry leaves was well documented in sericulture industry, as they are used as feeding material for lepidopteron insect *Bombyx mori* (Prabu et al. 2012). During rainy season silk farmers store these leaves at post-harvest stage, as feeding wet leaves increase larval mortality rate. During storage, normal conducting pathway gets blocked by microbial proliferation resulting in early senescence. Use of preservative solution having potent antimicrobial property like nanosilver may solve the sticky situation. On preserving mulberry leaves at post-harvest stage for 7 days with nanosilver solution biosynthesized at different temperature range with leaf extract of mulberry adjusted to different pH it was observed that best quality of leaf preservation was attained on preserving leaves to nanosilver solution prepared at room temperature adjusted to neutral pH. Thus, nanosilver biosynthesized under room temperature and at neutral pH range was the most bioactive. Bioactive nanosilver was reported to bear significant antimicrobial property as they have the

potentiality to alter structure of microbial membrane, as well as causing damage to DNA backbone and inter cellular proteins content (Nabikhan et al. 2010).

Post-harvest leaf quality was also assessed through chlorophyll content, as alteration in chlorophyll content serve as potential marker for detecting postharvest stress level that causes changes in composition of cellular membrane (DeEll et al. 1999). According to Toivonen (1992), chloroplasts and mitochondria are considered as one of the most sensitive membrane systems in postharvest physiology. On analyzing retention pattern of chlorophyll in the present study, it is clear that silver nanoparticles phytosynthesized with mulberry leaf extract maintaining pH 7 and 25˚C produces best bioactive nanoparticles effective in post-harvest shelf life extension of mulberry leaves. Decrease in chlorophyll content during vase life, as revealed by preserving mulberry leaves in other condition of process variation was mainly due to accelerated senescence of harvested leaves resulting in loss of metabolic protein and chlorophyll content and there by accelerating the process of ageing (Perera et al. 2009).

6.6. Determination of least effective concentration of screened nanosilver solution

Estimation of least effective concentration of phytosynthesized silver nanoparticles will be helpful in applied field as literature reports proposes that at significantly higher concentration there was even likelihood of generation of abiotic stress hampering normal cellular physiology, leading to the stimulation of oxidative stress (Courtois et al. 2019; Jaskulak et al. 2019). Thus, preservation of mulberry leaves using diluted solution of bioactive silver nanoparticles was more cost-effective and less toxic than directly using raw solution. On preserving leaves of mulberry with bioactive nanosilver (10^{-3} M) at three different dilutions *viz*. 10x, 20x and 40x, it was noted that efficient preservation was obtained till the last day by the application of nanosilver up to 20x dilution as apparent by greenish physical texture of the leaves and withholding of uniform content of chlorophyll. Dilution beyond 20x was found to be unproductive as yellowing of leaves was noted along the mid vein. Thus 10^{-3} M nanosilver, diluted 20 times was determined to be the least effective concentration at which preservation was achieved, and its concentration was estimated to be 6 ppm. Thus, in the downstream experiments attempts were made to

evaluate the effect of preserving mulberry leaves with 6 ppm nanosilver solution, by comparing with 6 ppm silver nitrate that served as positive control and distilled water was serving as negative control.

6.7. Assessment of preservative potential of phytosynthesized silver nanoparticles at least effective concentration

Major hurdle which was faced during preserving mulberry leaves was the gradual manifestation of senescence with raise in days of preservation. Senescence is primarily categorized by breakdown of photosynthetic pigments consequence of which is gradual decrease in photosynthetic rate and photosynthesis to respiration ratio (Souri et al. 2018). Beside this differences that were noted on supplementing senescence leaves to silkworm larvae includes poor growth index, decline in cocoon and shell weight, and enhanced larval mortality rate. At the onset of stress plant or plant part like leaves adopts several senescence retardation cellular mechanisms and its up- and down-regulation in presence of preservative solution *viz.* nanosilver and silver nitrate was predicted for determining their efficacy. In presence of nanosilver and silver nitrate solution as preservative, enhanced activity of different enzymatic and non-enzymatic antioxidant activity and declining ROS content was noted which ultimately resulted in prolonging of post-harvest shelf life of mulberry leaves.

Result of present study indicated significant reduction of total chlorophyll content in leaves preserved in distilled water with increase in days of preservation. Decrease in content of chlorophyll with onset of stress is directly associated with the activation of enzyme chlorophyllase leading to the degradation of chlorophyll (Santos 2004). Current study displayed significant negative correlation ($p \leq 0.01$) between generation of ROS and chlorophyll degradation. With rise in days of preservation, excessive accumulation of $O_2^{\bullet-}$ and H_2O_2 inside cellular system leads to the generation of oxidative stress. Ishida et al. (2014) stated that the major site for ROS production inside leaves was chloroplast lumen. With the onset of senescence, photosynthetic competence decreases, as a consequence CO_2 fixation reduces and generation of ROS increases by using the unutilized energy of the chloroplast (Xie et al. 2015). It was reported that senescence causes isolation of chloroplast and generation of $O_2^{\bullet-}$ through reduction of cellular O_2 by means of photosynthetic electron transport chain (Allen and Hall 1973). The generated $O_2^{\bullet-}$ by the action of the enzyme superoxide

dismutase gets transformed into H_2O_2 (Asada 2006). Generated ROS is proficient in causing oxidative injury to carbohydrates, lipids, proteins, and nucleic acids, while excessive generation and accumulation of ROS leads to programmed cell death (Ismail et al. 2015; Anjum et al. 2015). Generated $O_2^{\bullet-}$, H_2O_2 in the present study showed direct correlation with the increasing content of MDA. MDA is considered as stress indicator associated with cell and organelle damage (Wang et al. 2010). Multiple regression analysis of positively and negatively correlated parameters associated with chlorophyll content indicated major involvement of MDA during stress in degradation of leaf chlorophyll content (Table 69).

Table 69: Statistics summary of multiple regression analysis keeping chlorophyll constant

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	0.988[a]	0.976	0.974	0.032820	
2	0.996[b]	0.991	0.989	0.021129	
3	0.999[c]	0.998	0.997	0.011702	2.524

[a]Predictors: (Constant), **MDA**

[b]Predictors: (Constant), MDA, SOD

[c]Predictors: (Constant), MDA, SOD, FLAVANOL

[d]Dependent Variable: **TOTAL CHLOROPHYLL**

The outcome of the present observation was supported by the earlier works, indicating that the enhancement in lipid peroxidation causes degradation of chlorophyll (Orthoefer and Dugan 1973; Imamura and Shimizu 1974). Rajinder et al. (1980) reported that it was lipid peroxidation of chloroplast membrane that causes reduction in chlorophyll content during leaf senescence. Pearson correlation study stated that leaves preserved in solution of nanosilver showed significant ($p \leq 0.05$) enduring of chlorophyll content than dH_2O set. Present result suggests that nanosilver and silver nitrate both acts as an effective preservative as they help to prevent ROS generation and lipid peroxidation which causes retention of chlorophyll content. In comparison with distilled water, silver nitrate displayed high efficacy in nullifying generated H_2O_2 ($p \leq 0.05$), while nanosilver proved to establish itself as improved preservative solution as it holds the potentiality to check

generation of $O_2^{\bullet-}$ and H_2O_2 and preventing lipid peroxidation ($p \leq 0.05$). Thus, prevention of excessive ROS generation and its subsequent cellular accumulation helps in averting peroxidation of plastid and other internal sub-cellular membranes and thus maintaining chlorophyll content, extending post-harvest shelf life (Fig. 189).

By enhancing antioxidant enzymological activity leaves preserved in nanosilver and silver nitrate solution displayed appropriate stress management policy. SOD transforms $O_2^{\bullet-}$ into H_2O_2 (Ighodaro and Akinloye 2018), current study demonstrates gradual uplifting of SOD content in leaves preserved with nanosilver solution, while reversed trend was noted for distilled water set. Apel and Hirt (2004) reported that enzymes like CAT, APX and GPOX are principally synthesized by the plant for scavenging excess generated H_2O_2 produced during induction of stress by the activity of SOD. Present study displayed a mounting trend of CAT, APX and GPOX activity with increase in days of preservation in nanosilver and silver nitrate sets. While CAT activity increases in leaves preserved in nanosilver and silver nitrate solution up to 6 days, in distilled water set CAT activity fails to boost during preservation, representing its incapability to nullify the toxic effect of H_2O_2. Moreover, CAT activity displayed direct correlation with content of chlorophyll ($p \leq 0.05$), demonstrating H_2O_2 detoxification by CAT helps in retention of chlorophyll with increase in days of preservation. APX and GPOX activity also showed growing trend till the last day of preservation in leaves preserved with nanosilver and silver nitrate solution. In negative control (distilled water set), activity of ascorbate peroxidase increased up to 4 days after which rapid decline in activity was recorded, while GPOX mediated defensive activity was to a great extent higher in dH_2O set than leaves preserved in nanosilver and silver nitrate solution till the final day of preservation. Apel and Hirt (2004) reported that if during severe stress CAT alone fails to nullify generated H_2O_2, as a compensatory mechanism, activity of APX and GPOX increases. In the present study as distilled water set failed to raise CAT activity during preservation, so probably by increasing APX and GPOX activity, leaves tried to detoxify generated and accumulated H_2O_2. Obtained result showed that in distilled water set APX failed to detoxify generated H_2O_2 from four days onwards after which the role was played by GPOX as single protecting agent. A rapid rise in GSR and GST activity till 4 days was occurred in distilled

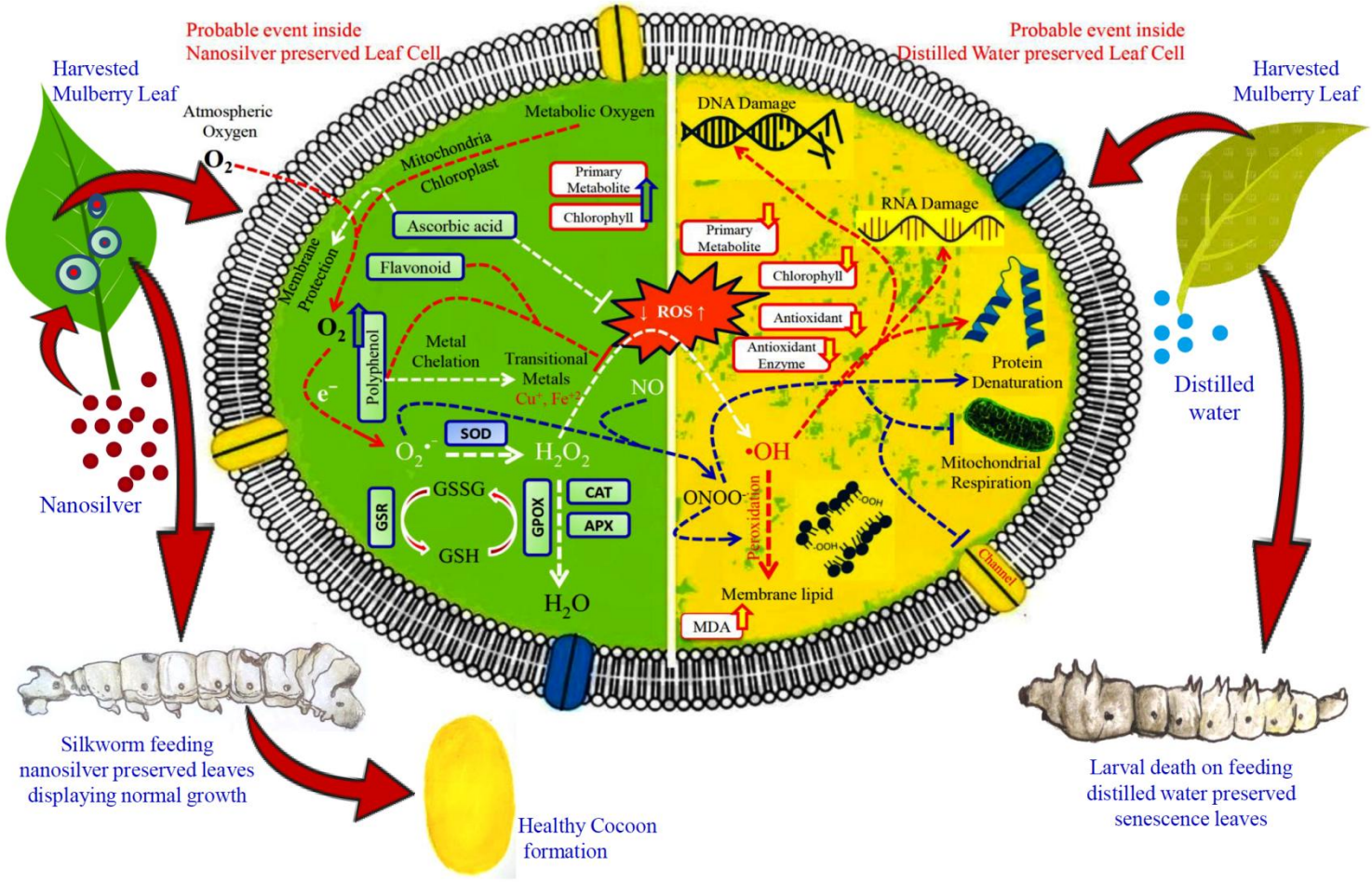

Fig. 189: Probable mechanism of action leading to post-harvest shelf life extension in nanosilver preserved mulberry leaves

water set failed to raise CAT activity during preservation, so probably by increasing APX and GPOX activity, leaves tried to detoxify generated and accumulated H_2O_2. Obtained result showed that in distilled water set APX failed to detoxify generated H_2O_2 from four days onwards after which the role was played by GPOX as single protecting agent. A rapid rise in GSR and GST activity till 4 days was occurred in distilled water set, signifying high stress accumulation in which the biological tissues tried to nullify by activating their protective measures. GST was reported to neutralize different abiotic damage caused during stress enforcement (Ding et al. 2017) and also protects tissues against oxygen toxicity (Mannervik and Danielson 1988). On the contrary GSR is a flavo-protein oxidoreductase playing vital role in plummeting biotic stress by maintaining pool of ascorbate and glutathione in reduced state inside the chloroplast (Edwards et al. 1990). Gradual increase in GSR and GST activity was noted in leaves preserved with nanosilver solution till the last day indicating the potentiality of nanosilver solution in strengthening the biological tissue system for tolerating both abiotic and biotic stresses.

A lofty increase in total glutathione concentration was observed in leaves preserved with nanosilver solution from 4 to 6 days after which drop off in concentration was noted while no such sharp increase was taken place for leaves preserved in silver nitrate solution, but a gradual escalating trend was maintained till last day, reflecting an attempt to minimize oxidative stress. Leaves in distilled water set failed to increase the threshold content of glutathione and thus led to early senescence due to build-up of oxidative stress. According to Noctor et al. (2012) glutathione is a low molecular weight thiol which is non-protein in nature that performs multiple functions in biosynthetic pathways, including antioxidant activities and detoxification of toxins which the other thiols cannot perform. The non-enzymatic antioxidant activity of glutathione lies in its capability to generate another non-enzymatic antioxidant, ascorbic acid by taking part in ascorbate-glutathione cycle (Blokhina et al. 2003). Ascorbic acid, one of the most important non-enzymatic antioxidants was reported by Sminnoff (2000) to subsist mostly in reduced from inside chloroplast lumen and can directly takes part in scavenging $O_2^{\bullet-}$ and H_2O_2 via APX activity (Noctor and Foyer 1998). Current study reported increased content of ascorbic acid in leaves preserved in nanosilver and silver nitrate solution indicating their ability to detoxify generated free radicals by involving APX, reported earlier

and thereby prolonging postharvest shelf life. In addition to ascorbate and glutathione pool, leaves in nanosilver solution also maintained more or less steady pool of carotenoids which bears significant dissimilarity with diminishing pool of distilled water set ($p \leq 0.05$). Carotenoids belong to the class of lipophilic antioxidants, synthesize inside plastids, and thus probably play role in preventing accumulation of ROS inside chloroplast (Rachhi 2013).

Besides glutathione, ascorbic acid and carotenoids, phenolics (total phenol, orthodihydric phenol and flavonoid) is another cluster of non-enzymatic antioxidant actively taking part in stress defending mechanism. The most important defensive function of polyphenols resides in their ability to perform scavenging of lipid free radicals (Blokhina et al. 2003). According to Rice-Evans et al. (1997) free radical scavenging activity of phenolic compounds lies in their capability to donate electron and in chelating metal ions in their transitional state. Polyphenols by acting as chelator for metal ions block metal ion mediated conversion of $O_2^{\bullet-}$ and H_2O_2 in to highly reactive hydroxyl radical (•OH) and thereby preventing nucleic acid damage (Perron and Brumaghin 2009) and thus providing path for continuous synthesis of defensive compounds through enzymatic mechanism, involving in enhancing shelf life. Present study reflects almost similar trend as mentioned above, with increase in days of preservation content of phenol and orthodihydric phenol increases in leaves preserved in preservative solution displaying activation of their potentiality to overcome generated stress. Result of distilled water set also presents an increasing trend but the extent of boost in comparison to nanosilver and silver nitrate was much less. Flavonoid content does not display any significant change with increase in preservation period, however in distilled water set from 4 days onward a decreasing drift was noted signifying inability to overcome generated stress.

Scavenging activity of free radicals and metal chelating activity was measured for evaluating the potentiality of secondary metabolites in preventing stress mediated by free radicals and metal ions, mentioned above. It was noted that with increase in preservation period $ABTS^+$, DPPH, SO and NO scavenging activity increased in mulberry leaves preserved with nanosilver solution, in contradictory, in silver nitrate and distilled water set $ABTS^+$, DPPH and NO activity decreased while SO activity remained almost stationary. Literature survey also reports significant free radicals scavenging activity (Sundararajan et al. 2016; Phull et al. 2016) and metal ions

chelating activity (Madhanraj et al. 2017) of phytosynthesized nanosilver. It revealed that the scavenging potential of 6 ppm nanosilver solution protects cellular and sub-cellular tissue system from the toxic effect of free radicals.

During the entire course of preservation significant ($p \leq 0.05$) retention of cellular protein content was recorded for leaves preserved in nanosilver and silver nitrate solution. Retention of total and reducing sugar in nanosilver set was also observed to be significant ($p \leq 0.05$). In extending post-harvest longevity, water relationship and balance plays a key role (Jowkar et al. 2013). Due to the microbial proliferation, water relation gets interrupted, blocking the cut end and thereby preventing water conducting pathways (van Doorn 1997; Liu et al. 2009). Mazumdar (2014) reported that solution of nanosilver by lowering microbial load can prolong post-harvest vase life of commercially important flowering twigs at low concentrations. It has also been reported that as an preservative both nanosilver and silver nitrate solution was effective, but the rate of water conduction was much higher when nanosilver was used as preservative (Hatami et al. 2013), thereby helping in prolonging shelf life by retaining the content of primary metabolite to optimum level, allowing the cellular synthesis of defensive enzymes needed to overcome generated stress. Current study reports elevated trend of proline content in all the preservative sets signifying activation of ROS scavenging mechanism (Hayat et al. 2012) and in doing so stabilizing cellular proteins and membrane integrity (Matysik et al. 2002). Thus, through biochemical analysis it might be stated that biogenic silver nanoparticles at 6 ppm concentration bears the potentiality to extent the shelf life of mulberry leaves.

6.8. Protein and isozyme profiling of preserved mulberry leaves through gel electrophoresis and prediction of differential expression through OHR-LCMS analysis

Maintenance of proper balance between water uptake and rate of transpiration plays key role in prolonging post-harvest shelf life (Jowkar et al. 2013). Interruption in water uptake pathway at post-harvest takes place primarily due to microbial proliferation and cut end blockage (Liu et al. 2009; Van Doorn 1997). Microbial blockage enhances ROS production and accumulation resulting in inter- and intra-cellular damages leading to acceleration in senescence process (Das and Roychoudhury 2014; Merzlyak and Hendry 1994). Byczyńska (2017) reported that

nanosilver solution can extend the post-harvest vase life of many flowering twigs by plummeting microbial count. Hatami et al. (2013) reported that both nanosilver and silver nitrate solution carried the potentiality of extending shelf life at post-harvest stage through activation of defensive enzyme and by maintaining the content of primary metabolites, but the water conduction capacity was found to be comparatively more when nanosilver was used as preservative. Current study reflects retention of post-harvest shelf life of mulberry leaves by 7 and 5 days respectively when nanosilver and silver nitrate was used as preservatives as manifested from gel banding pattern. Uniformity in the nature of gel banding pattern of leaf proteins preserved in nanosilver solution almost till the final day of preservation clearly indicates the existence of strong antimicrobial activity in biogenic silver nanoparticles which by dipping microbial load maintains the continuity of xylem column. Maintenance of xylem integrity prevents ROS accumulation through consistent distribution of antioxidants and defensive enzymes and thus preventing degradation of macromolecules like pigments, proteins, and nucleic acids delaying senescence.

The nutritional quality of mulberry leaves significantly influenced growth and development of larvae and subsequent formation of cocoon (Krishnaswami 1978). The principal components of mulberry leaves are water, carbohydrate, protein, minerals and crude fibre, disproportion of which causes alteration in metabolic activity inside larval body, hampering growth and other metabolic activities (Srivastava and Elangovan 2011). Sengupta et al. (1972) proposed that silkworm larvae for their normal growth and development requires amino acids, proteins, sugars, and vitamins. Leaf protein of mulberry was the primary requisite during all stages of larval development, particularly during fifth instar, causing development of silk gland that produces silk cocoon during spinning (Sekar et al. 2016). Current study displayed significant potentiality of silver nanoparticles as preservative in prolonging post-harvest shelf life of mulberry leaves, as evident from gel banding of SDS-PAGE displaying retention of uniform protein content till the last day of preservation.

NADPH oxidase is one of the specialized enzyme complexes responsible for the generation of ROS inside tissue system (Marschall et al. 2016). Across membrane, NOX complex from NADPH donates electron to molecular oxygen leading to

generation of ROS (Lambeth 2004). Superoxide was the most frequent and common ROS, produced by NADPH oxidase on transferring electron to molecular oxygen (Bedard and Krause 2007). Produced superoxide further reacts to form other ROS *viz.* hydrogen peroxide, peroxide anion and hydroxyl radical (Soneja et al. 2005). In present study two isoforms of NOX enzyme were observed in preserved and fresh leaves and the relative density of NOX in distilled water preserved leaves appeared greater in amplitude than leaves preserved in other preservative solutions. Beckman and Ames (1998) stated that during the process of ageing, the major contributor causing cellular damage was ROS generation and accumulation. The obtained gel pattern clearly indicates that leaves preserved in distilled water showed greater accumulation of ROS, leading to cellular damage, thus promoting senescence. The stress which was generated during leaf preservation results in the generation of excessive volume of superoxide (O_2^-) either by the activation of NOX enzyme or through electron transport chain (Alscher 2002). To overcome the damage caused by accumulated superoxide, plant system produces an intercellular enzyme namely superoxide dismutase (SOD) which converts generated O_2^- to H_2O_2 and O_2 (Fukai and Ushio-Fukai 2011). SOD banding pattern displayed two isoforms in the current study. The relative density of isoform 1 in nanosilver and silver nitrate appeared equivalent to fresh leaf while isoform 2 showed maximum intensity in nanosilver set, indicating the greater potentiality of nanosilver set to overcome generated stress. The hydrogen peroxide which gets accumulated inside tissue system by the action of the enzyme SOD is toxic to living tissue system, as it is considered as an active reactive oxygen species. To overcome the accumulated ROS i.e., H_2O_2, an antioxidant enzyme namely catalase (CAT) acts which nullifies H_2O_2 in two steps leading to the production of one molecule of O_2 and two molecule of H_2O (Nandi et al. 2019). On-gel analysis revealed that CAT activity was higher in nanosilver and silver nitrate sets, while it failed to uplift in distilled water set. Probably during post-harvest preservation, the stress which was accumulated in the form of H_2O_2 to nullify that leaves preserved in preservative solution gains potentiality to synthesize adequate CAT enzyme which the leaves preserved in distilled water fails to do, resulting to ROS mediated tissue damage. Another vital antioxidant enzyme, protecting cellular damage along with catalase is peroxidase (POD). Defensive activity of POD lies in its ability to convert produced H_2O_2 and ROOH into H_2O and R-OH (Liu et al. 2010). POD banding pattern in the current study displayed the

presence of one isoform which was elevated in preserved leaves in comparison to fresh leaves. Among preservative solutions, nanosilver manifested better POD activity, indicating its defensive potentiality to nullify stress and there by retaining healthy texture of leaves which when supplemented to larvae might produce good cocoon quality.

The differentially expressed SDS band on analysing through OHR-LCMS identified 34 protein types which were categorized into seven KEGG pathways as revealed through STRING analysis. Five identified proteins support photosynthesis among them PSAB, PSAA and PSAJ are the component of PS I while PSBB and PSBC are the component of PSII. PSBB and PSBC are 47 and 44 KDa protein respectively of PS II and remains associated with 15 chlorophyll a and 2 – 3 β-carotenes molecules. Beside this, they showed remarkable structural homology with six N-terminal helices of PSAA and PSAB of PS I, as they crossed the membrane six times. Both PSBB and PSBC cultivate photons and thereby generate electrons required to run the process of photosynthesis, as they participate in dual function of light harvesting and water oxidation process (Barber et al. 2000). PSAA, PSAB and PSAJ are the three out of fifteen core subunits associated with Photosystem I (PS I). Jensen et al. (2007) stated that PSAA (82.5 KDa) and PSAB (83.2 KDa) participates directly with electron transport cofactors *viz*. P700, A_0, A_1, Fx, FA, FB and 4Fe–4S. PSAJ is a protein of molecular mass 5 KDa, having 2 molecules of Chl a as cofactor principally involved in stabilization of core antenna molecules (Fromme et al. 2001). Lu and Zhang (1998) stated that decrease in activity of PS I and II takes place during loss of function of chloroplast, and among the two Photosystems, PS II was more affected than PS I. Kotakis et. al. (2014) stated that during chlorophyll degradation, gradual decrease in PS II activity takes place leads to senescence. Detection of five differentially expressed photosynthetic proteins clearly indicates the ongoing process of photosynthesis inside tissue system of postharvest mulberry leaves preserved in nanosilver solution, thereby representing healthy and working status of different chlorophyll molecules that eventually delays the process senescence. Identified CAT 1 participates in MAPK signalling pathway, encodes catalase enzyme; participates in mitigation of oxidative stress by nullifying generated H_2O_2 to H_2O and O_2 (Nandi et al. 2019). Differential expression of CAT enzyme clearly indicates the activation of stress management activity inside mulberry leaves during

preservation. CAT on gel assay for nanosilver preserved leaves also detected up-regulated catalase activity, was in strong agreement with the present observation. CAM 5, another MAPK signalling protein, participates in the activation of serine threonine protein kinase that helps to maintain homeostasis of ROS during stress (Virdi et al. 2015). Thus, expression of CAM 5 in elevated quantity inside tissue system prevents accretion of excessive reactive oxygen species, and prolonging shelf life. Among proteins involved in oxidative phosphorylation, current study identified subunit C of V-type ATPase which in plant are primary-active proton pump that works at sub-cellular level functioning as housekeeping enzyme during management of stress (Ratajczak 2000). Subunit α of F-type ATPase is a regulatory subunit, belonging to component F1 which uses electrochemical proton gradient for synthesis of energy rich ATP from ADP and Pi. The generated energy rich phosphate bonds help to operate different metabolic processes prolonging shelf life during preservation (Okamoto and Futai 2018). Okumuraa et al. (2016) proposed that H^+ ATPase plays diversified role and maintains the processes like membrane potential, secondary transport, stomatal opening, phloem loading and nutrient transport through root. Besides these, H^+ ATPase also participates in maintaining alkaline condition of stroma during daytime, thereby activating dark reaction of photosynthesis i.e., Calvin cycle (Peters and Berkowitz 1998). Thus, identified ATPase on one hand maintains light and dark processes of photosynthesis and on other hand promoting cellular processes, indirectly delaying the upcoming of senescence during preservation. Identified NAD(P)H-quinone oxidoreductase protects cellular membrane against oxidative damage by activating superoxide scavenging process at tissue level (Atia et al. 2014). This property of NAD(P)H-quinone oxidoreductase was noteworthy as it will ensure nullification of generated oxidative stress during preservation. Identified methylerythritol-4-phosphate pathway provides precursor molecules for biosynthesis of chlorophylls, carotenoids and gibberellins (Yang et al. 2012). Thus, enzymes identified through terpenoid backbone biosynthesis pathway indirectly promote biosynthesis of chlorophyll, maintaining greenish texture of leaves at post-harvest stage in nanosilver solution. The proteins detected under metabolic pathway *viz*. carbon fixation and carbon metabolism pathway comprises all the above identified proteins. Thus, metabolic pathway was a broad summery of all the pathways identified through KEGG.

6.9. Evaluation of preservative potential of preservative solutions by assessing microbial count

Potentiality of nanosilver solution as strong antimicrobial agent can be established from the observation of the present study where no CFU count was recorded after incubation of nanosilver solution used as preservative. Antibacterial activity of biogenic nanosilver solution was also reported when bioreduction took place using extract of *Pelargonium sidoides* (Kgatshe et al. 2019), *Tectona grandis* (Rautela et al. 2019) and *Melissa officinalis* (Ruíz-Baltazar et al. 2017). Silver nitrate also displayed preservative potentiality, but it showed abortive response to prolong shelf life of mulberry leaves to the level that was exhibited by nanosilver solution; this is most likely due to the fact that silver nanoparticles showed superior antimicrobial property than silver nitrate (Salman 2017). Yin et al. (2020) reported that wide spectrum of antibacterial activity of nanosilver was probably due to its ability to penetrate bacterial cell wall, causing alteration in structure of cell membrane and eventually causing cell death. The antimicrobial property of nanosilver was due to high surface-area-to-volume ratio (Loo et al. 2018), and with decrease in particles size, this ratio increases. Synthesized silver nanoparticle in the size range of 10 – 100 nm was reported to exhibit bactericidal activity against both Gram positive and negative bacteria (Morones et al. 2005). Silver nanoparticles with small dimension can effortlessly adhere to the bacterial cell wall and penetrate membrane easily causing more toxicity to the bacteria resulting in quick bactericidal consequence, stopping microbial proliferation (Zhang et al. 2016). Present study reports average nanoparticle size dimension ~14 nm (estimated through TEM particle size distribution) which was in good agreement with above explanation, indicating strong antimicrobial property of phytosynthesized silver nanoparticle, there by inhibiting microbial mediated xylem blockage.

6.10. Determination of preservative aspect of nanosilver solution against different cultivars of mulberry

Among four out of five studied cultivars of mulberry selected in the present study *viz.* S1, S1635, TR10 and BC259 were recommended for cultivation in hilly and foot hill regions of North and North-East India (Sastry 1984; Ravindran et al. 1997). The geographic location of present study area urged the requirement of evaluating the

effect of post-harvest preservation over these cultivars. Foliage of mulberry serve as only food source of silkworm (Vanitha and Narayanaswamy 2019) and thus the nutritional quality of mulberry leaves has a direct bearing on cocoon harvest (Abedian et al. 2020). Amino acids and protein content of mulberry leaves influence the post cocoon parameters (Machii and Katagiri 1991). Physical texture and chemical attributes of mulberry leaves directly influences the leaf consumption and digestive property of silkworm larvae (Das and Vijayaraghavan 1990). Thus, assessment of leaf quality of different cultivars of mulberry post preservation becomes essential to evaluate consequence of preservation over physical and chemical properties of leaves. Among the studied cultivars S1 displayed maximum response to preservative solutions, thus presented its physical and chemical properties almost equivalent to that of fresh leaves, till the last day of preservation. Superiority of S1 cultivar of mulberry over other was also reported by Kumar et al. (2013) and Ghosh et al. (2000) while studying effect of mulberry varieties over post rearing attributes. Among the preservative solution, 6 ppm nanosilver showed maximum potentiality to extend post-harvest shelf life of all the studied cultivars of mulberry with best performance was displayed for S1 cultivar. Thus, present study clearly demonstrates that S1 cultivar of mulberry was the superior cultivar in terms of extending post-harvest shelf life by the application of nanosilver as preservative solution.

6.11. Histochemical detection of stress and xylem blockage

Microscopic observation of transverse section of fresh petioles showed traces of vascular blockage in all the five cultivars under study indicating presence of natural blockage. Leaves preserved in distilled water when examined at the end of preservation period, showed large number of partially and completely blocked lumens, consequence to leaf senescence. Hassan (2005) and Ieperen et al. (2001) reported that during extending post-harvest shelf life, major obstacle that occurs was xylem blockage inhibiting normal conducting pathway, thereby promoting tissue damage in early stage of preservation. One of the most important causes behind tissue damage was the accumulation of hydrogen peroxide at the wounded site. Current study reports accumulation of H_2O_2 and thereby disintegration of plasma membrane in leaves preserved in distilled water for 7 days. Sharma and Dubey (2005) reported that disintegration of plasma membrane was indicated by dark

deposition of stain caused due to the presence free radicals. Jedrzejuk and Zakrzewski (2009) reported that proliferation of microbial organisms, accumulation of toxic materials inside xylem vessels and creation of air bubble inside vascular tissue are the foremost reasons preventing post-harvest shelf life. Meeteren et al. (2006) stated that post-harvest tissue longevity depends on size of the xylem vessels, as large diametric vessels can transport more sap and are not completely clogged-up under stress. Present microscopic observation displayed similar anatomical architecture of leaf petioles in all the five cultivars under study. The only variation lied in the size and number of xylem vessels, which played significant role in prolonging post-harvest shelf-life by maintaining uninterrupted water column from bottom (cut end) to the top (leaf apex). Present study displayed no significant correlation between diameter of xylem vessels with proportion of partial and complete blocked vessels. Among the five cultivars under study, Guangdung and S1 displayed highest and lowest diameter of xylem lumen respectively, but maximum blocked vessels were observed for BC259, which ranks after Guangdung in terms of lumen diameter. Jedrzejuk at al. (2012) while studying post-harvest shelf life of cut *Clematis* stem reported similar observation.

Nanosilver and silver nitrate solution extends postharvest shelf life as they prevent formation of xylem obstacles and thus maintaining continuous water column. Microbial proliferation was another major cause of vascular blockage that inhibits normal conducting pathway (Durkin and Kuc 1966). Beside microbes, pith pores might get blocked by the macromolecules secreted by bacteria, putting a cutback in normal conducting rate (De Stigter and Broekhuysen 1986). Silver nanoparticles acts as an effective antimicrobial agent in inhibiting bacterial proliferation as revealed by no CFU count in the preservative solutions. Marousky (1976) reported that presence of bactericidal agent in the preservative solution reduces the bacterial count and thereby prolongs shelf life. Nabikhan et al. (2010) reported that microbial inhibition property of nanosilver was due to their capability to cause structural change within microbial membrane, penetrating the cellular membrane and interacting with DNA through phosphate backbone and protein through sulphur containing amino acids and there by inhibiting growth and proliferation of microbes. Solgi (2014) stated that due to large surface area, nanosilver showed better antibacterial activity in comparison to other silver salts. Present study reported

similar observations, where both silver nanoparticles and silver nitrate displayed potentiality to extend postharvest shelf life of mulberry leaves, but the extent of prolongation was more in nanosilver than silver nitrate. Nanosilver solution also displayed better bactericidal activity than silver nitrate as revealed from CFU count. Due to the proven incidence of active potentiality of silver nitrate and nanosilver as preservative, mulberry leaves preserved in them represents better retention of plasma membrane integrity and less accumulation of H_2O_2 at cut end. Current observation was also supported by principal component analysis, which presented more proximity of silver nanoparticle preserved leaves towards freshly collected leaves, than leaves preserved in solution of silver nitrate. The clustering of principal compounds in the direction of negative axis of PC1 displayed the aggregation of the principal compounds (nanosilver and silver nitrate) with freshly cultivated leaves. Such aggregation was due to the fact that blockage number of preserved leaves appeared almost similar to the number of blockages naturally observed under microscope in fresh leaves. The physical positional distribution displayed that leaves preserved in nanosilver and silver nitrate were positioned 2.4146 and 4.0519 units apart from fresh leaves which clearly showed more proximity of leaves preserved in nanosilver solution with the blockage number of fresh leaves than silver nitrate preserved leaves.

Neumann et al. (2010) stated that in addition microbial mediated blockage of vascular transport, occlusion of xylem lumen can occur by the deposition of different types of polymeric colloidal compounds that are commonly present in xylem sap of plants. Current study reports protein mediated vascular lumen blockage by Bradford staining in foliage during postharvest preservation with maximum number of blocked lumens were recorded in distilled water preserved leaves. Proteomic records of xylem sap revealed the presence of different types of portable proteins including glycine-rich proteins, proteases, protease inhibitors, chitinase like proteins, lipid transfer proteins, peroxidase like proteins, wall metabolic enzymes and many others (Bhutz et al. 2004). The transport of these protein molecules along with xylem sap mostly depends on rate of vascular transport, which when gets reduced causes aggregation and agglutination, thereby clumping of xylem lumen takes place (Neumann et al. 2010). Van Doorn (1997) reported that during post-harvest stage, blockage developed at the cut end or at the wound site was the major

cause behind developing imbalance between uptake of water and rate of transpiration, thereby decreasing the pulling efficiency of water, hampering normal flow rate. Phytosynthesized nanosilver probably acts by preventing the formation of plugging at the cut end, maintaining normal rate of conduction and thus prolongs the post-harvest shelf life by seven days. Silver nanoparticles inhibiting microbial aggregation was also reported by Biswas et al. (2020) and Tavakoli et al. (2017) while studying extension shelf life of coconut endosperm and nuts respectively.

Another reason of vascular lumen blockage was unwarranted deposition of impermeable layer like lignin and suberin. Rhodes and Wooltorton (1978) reported that rate of bio-production of lignin and suberin are usually gets enhanced at cut end, while their polymerization from monomer to polymer leads to the creation of occlusion that takes place by the action of different oxidative enzymes. Present study depicts the presence of lignin and suberin as blockage material inside xylem lumen inhibiting the normal conducting pathways. Among the studied cultivars, BC259 and TR10 represents highest number of lignin deposition, while BC259 and Guangdung represents highest number of suberin deposition, signifying greater activation of polymerizing enzymes in these cultivars leading to development of big sized depository plugs blocking vessels. Presence of suberin plug at cut end was also reported by Cline and Neely (1983) while studying the process of wound healing in *Geranium* stems. Among the used preservative solutions, it was observed that mulberry leaves preserved in distilled water showed maximum deposition of lignin and suberin inside xylem lumen causing obstruction in normal conduction pathway and thereby promoting the rate of senescence as revealed by yellow texture of the leaves. Lignin and suberin mediated occlusion of xylem lumen was also reported in leaves preserved in silver nitrate and nanosilver solution but the count of blockage was negligible in comparison to the blockage number showed by leaves preserved in distilled water. Agglomerative hierarchical clustering also presented similar interpretation as mentioned above. Dendrogram have placed leaves preserved in distilled water as an out-group due to its dissimilarity with remaining preservative solutions in terms of blockage number. Whereas leaves preserved in silver nitrate and nanosilver solution forms a solitary cluster along with fresh leaves demonstrating their resemblance with one another in terms of blockage number. The preservative potentiality of nanosilver solution was further evidenced from

SEM imaging, which displayed the presence of adequate number of complete and partial blockage inside xylem lumen of leaves preserved in distilled water set for seven days while leaves preserved in nanosilver solution displayed almost negligible number of vascular blockages. The current interpretation clearly indicates the potentiality of silver nanoparticles in inhibiting microbial proliferation inside xylem vessels and thus maintaining intact conducting pathway by preventing aggregation of biomolecules. Heat map colour matrix demonstrates that along the horizontal axis, in right part, the colour matrix appeared mostly dark, while the opposite side appeared lighter, indicating formation a greater number of partial blockages than complete blockage. In the vertical axis, yellow coloured matrix that appeared in the petiole section of freshly collected leaves displayed insignificant number of blocked vessels, as yellow colour represents lower value. The colour matrix of silver nitrate and nanosilver preserved leaves appeared in the intermediate position i.e. in the yellowish green zone, however the intensity of green colour is more towards silver nitrate preserved leaves than nanosilver preserved leaves representing more number of vascular blockage in silver nitrate set. While leaves preserved in distilled water displayed reddish green colour matrix indicating large number blockages, with intensity of red colour being more towards right side signifying a greater number of partial blockage than complete blockage. Heat map examination clearly suggests that in comparison to distilled water and silver nitrate, nanosilver acts as an efficient preservative for preserving leaves at post-harvest stage.

Thus, it may be stated that during preservation at post-harvest stage, clustering of macromolecules inside xylem lumen and microbial proliferation causes obstacle in usual conducting pathway (Fig.190), leading to excessive accumulation of ROS, causing membrane breakdown resulting in senescence, as apparent from yellowing of the leaves that were preserved with distilled water. Nanosilver showed effectiveness in preventing microbial propagation and ROS generation, thus prolonging shelf life. Further it was observed that 6 ppm silver nitrate solution showed more effectiveness than equivalent concentration of silver nitrate as indicated by a smaller amount number of partial and complete blockage and more prolonged shelf life. Thus, at post-harvest stage preserving mulberry leaves, with 6 ppm nanosilver solution will assist to overcome the problem of supplementing

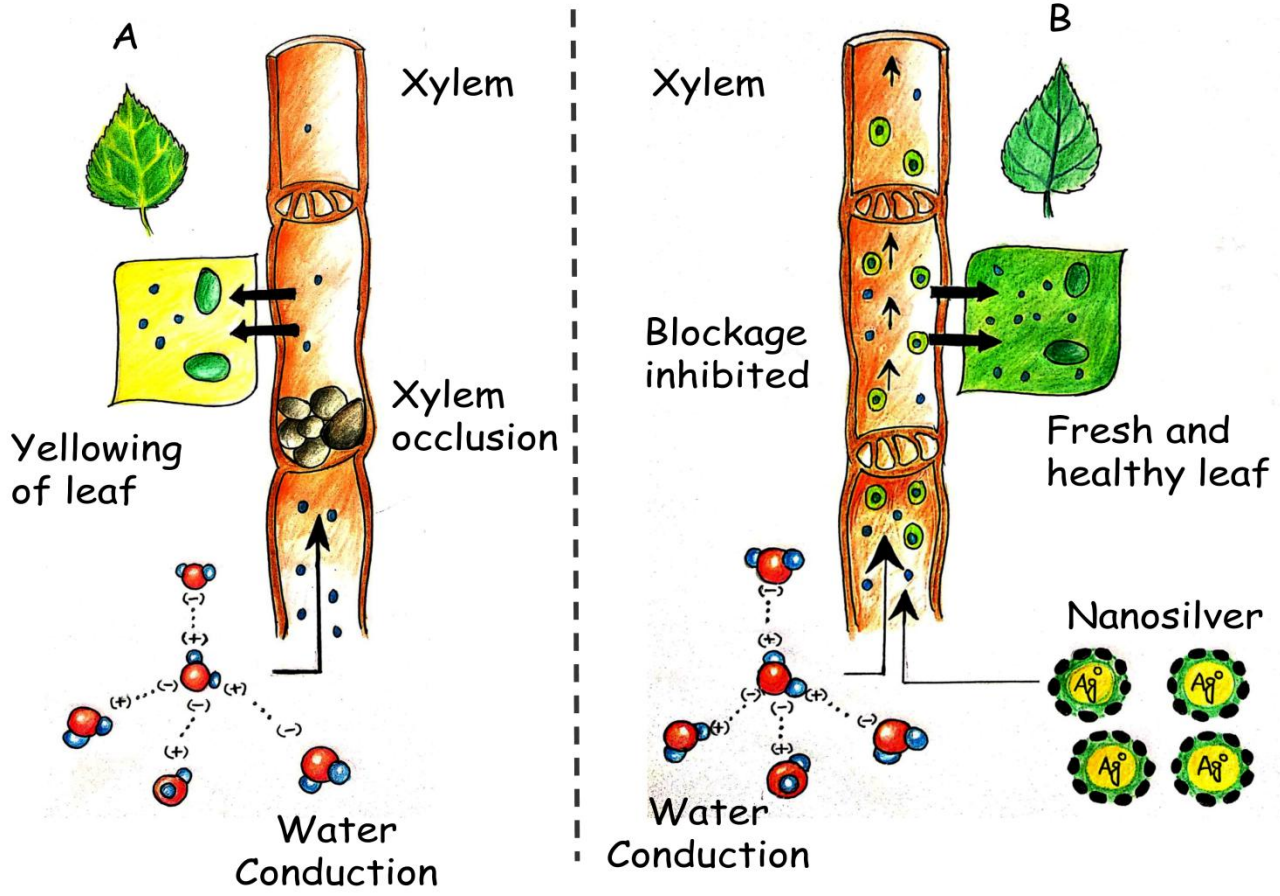

Fig. 190: Diagrammatic model of post-harvest vascular blockage and its inhibition through nanosilver application

larvae during rainy season, as the above mentioned module will help to maintain fresh texture of leaves.

6.12. Transcriptome profiling of preserved mulberry leaves using Illumina platform

Effective preservation of mulberry leaves at post-harvest stage will put significant impact in the global growth of silk industry in two aspects; firstly, it will promote the engagement of landless farmers and secondly it will increase the rearing productivity during rainy season. Above described study in this aspect identified phytosynthesized silver nanoparticle at 6 ppm concentration significantly retains physical texture of leaves till day 7 post-harvest. Earlier observation signifies that nanosilver delays the process of senescence by preventing accumulation of molecules capable of causing oxidative damage through upregulation of defensive processes and thereby preventing deterioration of primary metabolites. In the current study attempt was made for identifying novel genes responsible for extending post-harvest shelf life of mulberry leaves upon nanosilver application with respect to leaves preserved in distilled water for same duration.

Transcriptome analysis using Illumima platform serves as a handy and economical tool for generating information on gene and associated sequences. Illumina based transcriptome assembly although based on shorter read archive technology but generates a wide range of transcriptome coverage helpful in identification of genes involved in different processes (Xu et al. 2013). Current study using Illumina platform (Hiseq 4000) generated ~191 million raw reads among the studies treatments of mulberry (NS7 and CO7), out of which ~71% high quality reads (base score ≥30) were used for downstream analysis. The quantity of obtained high quality reads remained within the bracket area of reads required for analysing differential expression (Liu et al. 2014). Obtained N50 value of the assembled transcripts (>2000) with median contig length >500 indicates good QC report of the sequencing data for downstream analysis (Karako-Lampert et al. 2014). N50 value reported in the current study was almost similar with the N50 value reported for *Morus laevigata* (2,086) and *Morus serrata* (2,140) (Saeed et al. 2016). On vetting out transcripts ≤200 bp, Trinity identified 1, 57,982 assembled transcripts and 81,952 unigenes, which was in good agreement with earlier studied mulberry transcripts

(Wang et al. 2018; Du et al. 2016). The assembled transcripts showed ~55% BLAST hit with plant NR database, which was significantly greater with previous transcript studies on mulberry (*Morus alba*) that showed 41.08% hit (Guan et al. 2018). NR database hit was supported by PMN hit which detected ~56% unigenes. Previously studied other *Morus* spp. showed 52% (*Morus laevigata*), 53% (*Morus serrata*) and 59% (*Morus indica*) and 67% (*Morus atropurpurea*) NR BLAST hit (Saeed et al. 2016; Rukmangada et al. 2019; Dai et al. 2015). Transcriptome analysis within the members of the family Moraceae showed 76% (*Artocarpus heterophyllus*), 74% (*Ficus carica*), 47% (*Ficus hirta*), NR BLAST hit (Hu et al. 2016; Li et al. 2020; Yu et al. 2015). Members of the order Rosales depict 71% (*Pyrus communis*, Rosaceae), 76% (*Humulus lupulus*, Cannabaceae), 54% (*Ziziphus jujube*, Rhamnaceae), 46% (*Boehmeria nivea*, Urticaceae), and 41% (*Rubus idaeus*, Rosaceae) NR BLAST hit (Ou et al. 2015; Mishra et. al. 2018; Li et al. 2014; Liu et al. 2014; Travisanya et al. 2019). Analysis of distributional similarity of the assembled transcripts to other plant species with respect to Nr database showed maximum homology with *Morus notabilis* (~62%), indicating mapping of most of the transcript with *Morus notabilis* draft genome. Comparable observation was reported by Wang et al. (2018) and Dai et al. (2015) while studying De novo assembly in *Morus alba* and *Morus atropurpurea* respectively. Besides *Morus notabilis* which belongs to the family Moraceae, *Morus alba* transcripts in the current study also showed sequence similarity with *Quercus suber* (Fagaceae), *Vitis vinifera* (Vitaceae), *Trema orientalis* (Cannabaceae). Moraceae and Cannabinaceae come under the order Rosales where Fagaceae and Vitaceae come under the order Fagales, and Vitales respectively. Evolutionary the order Rosales, Fagales, and Vitales are considered as sister clade (Ravi et al. 2006; Zeng et al. 2017), thus transcriptome data correlates significantly with evolutionary relationship among the sequence identical species, with *Morus notabilis* being the closest relative of *Morus alba*. Sequence similarity index indicates ~60% of the assembled unigenes mapped more than 80% of their length, ~31% mapped within the range of 80 – 50% of their length, while remaining ~8% had mapped similarity less than 50%. Similar observation was prescribed by Zhou et al. (2019) while studying De novo assembly of *Taxus* sp.

Present study also deals with the identification of SSR markers that are most frequently utilized for the development of cost-effective marker system helpful for

studying genetic diversity, genetic structuring, demography, relatedness, and development of linkage maps. The number of SSR obtained in the current study was much higher than that was reported earlier for *Morus alba* (31,799) (Du et al. 2016). SSR count was also found to be on greater scale than other species of the family Moraceae *viz.*, *Morus multicaulis* (33,285), *Morus laevigata* (12,206), *Morus serrata* (11,843) (Wanga et al. 2014; Saeed et al. 2016). Presence of at least 1 SSR was documented in most of the studied transcripts. Most abundantly found transcript types were tri-nucleotide followed by di-nucleotide which was in accordance with the observation of Victoria et al. (2011), stated that tri-nucleotide repeats were most frequent in higher plant groups (angiosperms) whereas in lower group of plants di-nucleotide repeats were predominant. The high percentage of mono-nucleotide repeats was probably due to the mechanical errors in NGS sequencing technology (Sarika et al. 2013). SSRs with A/T motif (~98%) were the most abundant mono-nucleotide repeat, while in di-nucleotide AG/CT (~31%) leads the ranking, similar to the report of Park et al. (2019) on *Lychnis kiusiana*. AAAT/ATTT (~25%) and AAAAT/ATTTT (~12%) was the most frequent tetra- and penta- nucleotide repeat, similar observation was displayed by Tulsani et al. (2019) and Li et al. (2014) while performing SSR analysis of *Coriandrum sativum* and *Ziziphus jujuba*. SSRs with long repeats are considered to be highly polymorphic (Tóth et al., 2000), present study showed SSRs with length range between 11 – 20, 21 – 30, and 31 – 40 bp chronologically displayed greater number of SSRs. The variation in length among the repetitive units was probably because different repetitive sequences may have appeared in different generation during the course of evolution (Gao et. al. 2013). The frequency of occurrence of repetitive units decreased as the number of nucleotide increased. Cho et al. (2000) stated that appearance of repetitive motifs within plant gnome and its length distribution was the consequence of applied selection pressure during the course of evolution.

Preservative potentiality of effective preservative solution depends on its ability to retain primary metabolites and to prevent accumulation of compounds responsible for elevating the process of senescence. Among the primary metabolites, chlorophyll and photosynthetic apparatus acts as an effective marker system for assisting effectiveness of preservative solution. Change in chlorophyll content below optimum level was an indicating symbol of plant stress (Lichtenhaler 1996). Leaf

chlorophyll content was a monitoring marker of overall plant health, particularly in applied field where plant health puts direct impact over productivity (Shah et al. 2017). Leaf quality was affected by number of factors including chlorophyll distribution (its decomposition and synthesis), nutrient content (its distribution), and genetic expression (Pavlović et al. 2014). The content (Chl a + Chl b) and ratio of chlorophyll (Chl a: Chl b) varies from species to species, but within a species it should be specifically maintained for proper growth and long-time survival (Li et al. 2018). Maintenance of nutrient level was essential for proper functioning of photosynthetic machinery (Schertz 1928). Any deficiency in pigment level will put significant impact over plant growth resulting in decrease in net primary productivity (Hosseinzadeh et al. 2016). Earlier stated observations showed post-harvest retention of chlorophyll content in NS7 in comparison to FR0 (fresh harvested leaves), while CO7 fails to do so. Quantitative data was supported by qualitative observation (morphological) showing presence of yellowish patches in leaves preserved in distilled water, indicating senescence. Leaves preserved in nanosilver solution morphologically displayed retention of greenish texture almost similar to that of fresh leaves. Qualitative and quantitative observation was supported by expression analysis using NGS technology showing proteins assembled under photosynthetic processes (Light reaction: Photosystem I and Photosystem II; Dark reaction: Calvin cycle), chloroplast and chlorophyll development and assembly are mostly up-regulated in NS7. Less expression of the photosynthetic and chloroplast associated proteins in CO7 was the foremost cause for the activation of senescence related processes. Decrease in water transport significantly promotes decomposition of chlorophyll, accelerating leaf yellowing (Li et al. 2018). In the study prescribed above it was reported that during post-harvest preservation, blockage of xylem lumen by microbial proliferation and by accumulation of secondary polymerized molecules interferes with water conducting pathway in CO7, promoting senescence and wilting. Down-regulation of photosynthetic associated processes in CO7 was probably due to alterations in the structural and functional capability of the chloroplasts and associated proteins. Senescence mediated cutback in photosynthesis was mainly due to breakdown of protein functioning, pigment content and membrane lipids degradation (Panda et al. 2013). STRING round 2 qualified, Cytoscape topological score based selected major chloroplast associated up-regulated (NS7 vs CO7) genes were AOR, PnsB3,

PSBO2, CYP38, GLU2, EGY1, PSAA, PSAB, PSBA, PSBB, PSBD, and Lhca6. Of them PSAA, PSAB, PSBA, PSBB, PSBC, and PSBD was identified by KEGG to be directly associated with photosynthetic process. PSAA and PSAB which showed >20 fold expression level was encoded by chloroplast genome and shows greater level of sequence homology (Rochaix et al. 2000). PS I mediated electron transport system comprises PSAA and PSAB, remains associated with P_{700} in heterodimeric forms and with cascade components A_0 (CHLa), A_1 (phylloquinone) and Fx (4Fe-4S protein) (Hoj et al. 1987). It was reported that genomic expression of PSAA depends upon expression, synthesis, and thylakoid membrane integration of PSAB (Vallon et al. 1993). Current study showed greater expression profile of PSAB than PSAA, supporting the above observation. PSBD participates in PS II as reaction centre D2 protein which promotes assembly of PSBA, reaction centre subunit D1. PSBB and PSBC encodes core antenna subunits CP47 and CP43 respectively binding Chl a, and thereby promotes binding of upstream oxygen evolving enhancer (OEE) proteins (Minai et al. 2006 and Barber et al. 2000). It was noted that synthesis of one chloroplast-encoded protein associated with photosynthesis process was promoted by the expression of another protein from the same protein complex. Current observation was in accordance with the earlier reports of Choquet and Vallon (2000) and Choquet and Wollman (2002). Reduction in expression of PSBD reduces the expression of both PSBA and PSBB (Bennoun et al. 1986), similarly expression of PSBA and PSBB are also directly correlated (de Vitry et al. 1989). As expression profile of most of photosynthetic genes are interrelated thus obtained differentially expressed among treatments (NS7 vs CO7). MCODE mediated sub-networking analysis also supports the above observation showing two independent strong network co-ordinations of chloroplast and photosynthetic proteins, and photosynthetic light reaction proteins. OHR-LCMS mediated analysis of differentially expressed electrophoresis generated gel protein band of mulberry leaves preserved in nanosilver solution in earlier reported was in good agreement with the transcriptome profile, as both the expression profile identified up-regulation of some common photosynthesis associated proteins (gene product). Carotenoids which remain associated as light harvesting molecule with PSBB and PSBC (Barber et al. 2000) was found to retain its content in NS7 till the last day of preservation. Carotenoids participate in non-photochemical quenching, thus act as a vital antioxidant for eliminating generated singlet oxygen (McElroy and Kopsell 2009).

Another important photosynthetic protein differentially expressed in NS7 was PSBO2 (an extrinsic subunit of PS II associated with water oxidizing complex) and it has been reported to play key role in stabilizing catalytic manganese cluster (Mn_4Ca-cluster) (Enami et al. 2008) and in electron transport of PSII (Takahashi et al. 2017). BiNGO identified involvement of PSBO2 and Lhca6 in response to light stimulus (abiotic stress management). The defensive role of PSBO2 resides in their ability to dephosphorylate damaged D1 protein for degradation and thereby maintaining its turnover number (Lundin et al. 2007). Plant nanobionics has identified integration of biogenic nanoparticles into the chloroplast of the plant cell thereby playing potential role in extending shelf life of crop plants (Sridhar et al. 2020). Maintenance of photosynthetic and chloroplast processes was also carried out by an oxidoreductase, AOR representative of zinc binding dehydrogenase protein family by detoxifying reactive carbonyls species formed during lipid peroxidation (Takagi et al. 2016).

Maintenance of cellular redox homeostasis was foremost pre-requisite for extension of shelf life at postharvest stage. In postharvest stage generation of oxidative stress can rarely be avoided, thus extension of shelf life can only be done through mitigation of generated ROS. Increase in concentration of cellular oxygen molecule beyond optimum level results in the generation of ROS either in the form of $^1O_2$ (singlet oxygen) or in the form of O_2^- (superoxide), H_2O_2 (hydrogen peroxide), and •OH (hydroxyl radical) by accepting electron (Adiletta et al. 2018). Nucleus, chloroplasts, mitochondria, glyoxysomes, and peroxisomes are the key ROS generating sites, damaging various cellular components (Hodges et al. 2004). Carbohydrates, proteins, lipids, nucleic acids along with other cellular macro and micro molecules are the target site of ROS action (Roy et al. 2017). Current study showed decrease in protein, and carbohydrate (total and reducing sugar) content in CO7 by more than 60% with respect to FR0, while NS7 able to maintain the primary metabolite content almost similar to that of FR0. Whereas the content of stress markers *viz.* hydrogen peroxide, MDA, superoxide, and proline were found to be significantly enhanced in CO7 with respect to FR0. The content of stress markers also increases in NS7, but the extent of enhancement was insignificant with respect to FR0. For nullifying cellular and sub-cellular damage caused by imbalance of ROS, tissue system requires efficient antioxidative (enzymatic and non-enzymatic)

repair system that can detoxify generated ROS (Toivonen et al. 2003). Transcriptome analysis has identified significant number of proteins involved in scavenging activity in NS7 including CSD1, LOX1, LOX6 and DOX1. CSD1 (superoxide dismutase) catalyses the dismutation of superoxide generated by photosynthetic electron transport reactions to hydrogen peroxide (Asada et al. 1987). KEGG identified participation of LOX1, and LOX6 in linoleic acid metabolism, which thereby participates in jasmonic acid biosynthesis and metabolic process as identified by BiNGO. LOX1, and LOX6 (lipoxygenase), and DOX1 (α-dioxygenases) through the production of oxylipins, involves in stress (biotic and abiotic) responses (Bell and Mullet 1993) by delaying senescence through controlled chloroplast destruction (Springer et al. 2016). It has been reported that LOX1 mutant causes enhanced hydrogen peroxide and MDA accumulation and thereby promoting cellular damage causing senescence (López et al. 2011). It has been stated that the activity of DOX1 increases in tomato and in *Arabidopsis* leaves under oxidative stress generated due to bacterial proliferation (Bannenberg et al. 2009). In plants DOX1 was reported to perform tissue-protective role and the degree of tissue damage was inversely proportional to the cellular level of α-dioxygenases (Ponce de León et al. 2002). Silver nanoparticle was reported to promote plant growth by preventing microbial and other pathogenic proliferation (Ndlovu et al. 2020). Implementation of nanosilver has identified more than 20 disease resistance protein types among them significantly expressed defensive proteins were RPP8, PBS1, PAD4 and WAK1 in NS7. BiNGO identified involvement of RPP8, PBS1, and PAD4 in innate immune response, which restricts pathogen growth through hypersensitive response (Parker et al. 1997). PBS1 comes under R-gene family and participating in effector-triggered immunity response (Ade et al. 2007). WAK1 was reported to be a transmembrane serine/threonine-protein kinase performing diverse role in cell expansion, morphogenesis and development (Saintenac et al. 2018). WAK protein through cell wall integrity-sensing mechanism provides defensive response against wounding, pathogen attack and oxidative stress (Kohorn and Kohorn 2012). WAK1, and PAD4 was found to be involved in MCODE generated sub-network clustering involving in stress associated defence response along with WRKY53, CRK10, CRK6, and AT3G28580. Another MCODE generated defensive line that were identified were NAD-ME1, ALDH2B4, ALDH10A8, NADP-ME3, and PHS2. KEGG analysis recognized the involvement of NAD-ME1, and NADP-

ME3 in carbon fixation process of photosynthetic organisms as they participate in oxidation of malic acid to pyruvic acid and CO_2 (Tronconi et al. 2008). Besides performing metabolic role in photosynthetic process NAD-ME1, and NADP-ME3 are also reported to provide defensive role by participating in lignin biosynthesis process and by maintaining cytoplasmic pH level (Martinoia et al. 1994). ALDH2B4, and ALDH10A8 (aldehyde dehydrogenase) maintains aldehyde homeostasis within the cell and provides defence by scavenging toxic aldehydes (Rodriguesn et al. 2006). In support with present study, it was reported that ALDH genes over expression provides defence against abiotic stress (Kotchoni et al. 2006). ATR1 another defensive protein identified by MCODE and BiNGO involves in stress management by inducing hypersensitive response (HR) there by providing resistance to bacterial pathogens (Sohn et al. 2007).

Dismantled chloroplast was associated with degradation of internal macromolecules causing lack of nutrient accumulation, promoting senescence. Degradation of polymeric-molecules to monomeric form causes their transport through phloem, altering sink-source relationship (Buchanan-Wollaston et al. 2003). In post-harvest stage, these molecules may get released through the cut end, accelerating senescence as in CO7. One such molecule was sucrose whose internal concentration decreases upon senescence activation by the action of the enzyme invertase. Matsushita and Uritani (1974) reported an increased level of acid invertase in sweet potato tuber upon ageing. Current study identified BFRUCT4 (acid beta-fructofuranosidase 4), an acid invertase differentially expressed in CO7 (>50 fold) probably causing continued mobilization of sucrose products through cut end, decreasing internal sugar concentration, decreasing shelf life. BFRUCT4 involves in sucrose catabolism and transport was also reported by Aluru et al. (2009), play vital role in sink metabolism, in support with present observation.

Leaf senescence is the summative response of multiple factorial operations working together in a step wise manner. Senescence is a process of cell degeneration and death operated in a systematic manner under tight genetic control with an attempt to enhance the survival of the plant. The regulation and execution of senescence at cellular and sub-cellular level was controlled by mode of action of different hormones, receptors, and transcription factors (Ahmad and Guo 2019). Most of the phyto-hormones *viz.* ethylene, abscisic acid, cytokinin, auxin, gibberellic acid,

brassinosteroids, jasmonic acid, and salicylic acid regulate both stress induced and maturity dependent leaf senescence (Jibran et al. 2013). Ethylene besides promoting senescence, involves in stress related gene regulation involve in plant survival and growth response (Achard et al. 2006). Cao et al. (2007) reported that CTR1 (constitutive triple response 1) mutant plants displays higher longevity than wild type plants due to continuous ethylene signalling. Current study also reports differentially expressed CTR1, down regulated in NS7, indicating the ability of nanosilver solution to extend shelf life at post-harvest stage. In contrary, plants bearing over expressed form of EIN4 become less tolerant to stress thereby promoting senescence as observed for CO7 (Shi et al. 2012; Jibran et al. 2013). MCODE identified strong network interaction between RAN1, CTR1, and HMA5 involving in copper mediated ethylene response. RAN1 and HMA5 was reported to work upstream of ETR1 (ethylene receptor 1), delivering copper to the receptor, promoting ethylene binding (Binder et al. 2010). In ETR1 mutant binding of copper ion was inhibited and thus binding of ethylene was also ceased (Rodriguez et al. 1999). Similarly, in NS7 down regulation of ETR1 was due to down regulation of differential expression of RAN1 and HMA5, inhibiting the process of senescence. ARF1 (auxin response factor 1) regulates senescence by regulating the expression of some senescence-associated genes (Noh et al. 1999). It was reported that in ARF1 mutant plant expression of SAG12 (senescence associated gene 12) gets suppressed, consequently senescence gets suspended (Kong et al. 2013). Down regulation of ARF1 in NS7 was the probable reason for long post-harvest shelf life.

Among different cellular and sub-cellular transporters, the expression profile of ABC class transporters gets uplifted during stress as they plays key role in detoxification of xenobiotics and transport toxic substances (Lee et al. 2005). Literature survey reveals that ABC transporters participate in abiotic stress response by excluding cellular harmful compounds including heavy metals (Martin et al. 2000). Current study identified 3 differentially expressed ABC transporters in CO7 *viz.* ABCC8, ABCG15, and ABCG33 forming MCODE generated separate sub-network. Class G-ABC transporters are mainly involved in regulating plant-pathogen defence by excluding microbial toxins out of plant body (Hwang et al. 2016). Probably accumulation of excessive toxic compounds inside CO7 causes enhanced expression of ABC class transporters with an attempt to mitigate

generated stress. It was reported that expression ABCC8 gets highly enhanced during fruit ripening, and the process was related with chlorophyll degradation and anthocyanin accumulation in the fruit (Shi et al. 2020). Similarly, enhanced expression of ABCC8 in CO7 was probably associated with the process of senescence mediated leaf yellowing. Another transporter differentially expressed in CO7 was CNGC4 (cyclic nucleotide-gated ion channel 4), non-selective cation channel involves in defensive responses (Moedera et al. 2011). CNGC4 through enhancing cellular Ca^{+} level participates in expression of pathogenesis related genes (PR-gene) and in hypersensitive response (Dietrich et al. 2020).

Phyto-immune system gets hyperactivated during stress and senescence management which leads to the identification of another MCODE generated defensive line in CO7 *viz*. APS2, APS3, NIR1, WIN1, and PPC1. KEGG identified involvement of APS2 (ATP-sulfurylase 2), and APS3 (ATP-sulfurylase 3) in metabolic pathways including biosynthesis of amino acids and 2-oxocarboxylic acid metabolism. ATP-sulfurylase catalysed the synthesis of adenosine-5-phosphosulfate which gets incorporated in to cysteine. Cysteine through redox-reactions participates in various abiotic stress managements through the synthesis of antioxidant molecules *viz*. glutathione, homo-glutathione, and phytochelatins (Anjum et al. 2015). Akbudak et al. (2019) stated that ATP sulfurylase activity was up-regulated under stress probably for maintaining the pool of sulphur containing amino acids *viz.* methionine and cysteine which will counterbalance generated ROS through the synthesis of antioxidant molecules. Probably for mitigating generated toxic substances, CO7 has up-regulated the expression of APS2/3 inside tissue system for protecting rapid degradation of cell and cellular components. Similarly, NIR1 encodes nitrite reductase 1, one of the two enzymes along with glutamine-dependent asparagine synthase 1 involves in nitrogen assimilation during stress management (Egea et al. 2018). PPC1 (Phosphoenolpyruvate carboxylase 1) was an indicator of dehydration stress, and in leaves its upregulation indicates excessive water loss due to interruption in stomatal closure process (Taybi et al. 1999). In leaves PPC1 generally participates in carbon and nitrogen metabolism (Shi et al. 2015) but over expression was an indicator of osmotic stress condition (Zhao et al. 2019). Over expression of PPC1 in CO7 might be due to internal water deficit which may occur due to the generated inability to conduct water as a consequence of xylem blockage

as reported earlier.

Thus, transcriptome analysis identified significant retention of photosynthetic and chloroplast proteins in NS7 responsible for green texture and maintaining shelf life through proper regulation of photosynthetic machineries. Shelf life extension in NS7 was achieved probably through enhanced expression of proteins involved in providing defence by scavenging toxic substances including ROS. Probably blockage of xylem vessels inhibiting conducting pathways and leakage of storage carbohydrates through cut end caused rapid wilting associated senescence through accumulation of ROS in CO7. However enhanced expression of different transporters and immune-modulators were observed in CO7 but their collective summation failed to prolong the shelf life by more than 3 days.

6.13. Performance assessment of silkworm larvae on supplementing with preserved mulberry leaves in terms of feeding, rearing and protein profiling

Bothikar et al. (2014) reported the significance of leaf quality on larval rearing and formation of cocoon. Deviation from leaf nutritional content puts noteworthy impact over cocoon production (Gawade and Medhe 2010; Quader et al. 1992). On rearing bi-voltine silkworm larvae with preserved mulberry leaves it was observed that larvae supplemented with nanosilver preserved leaves showed equivalent rearing performance and cocoon parameters as that of larvae supplemented with fresh leaves. The obtained result clearly indicates sufficient retention of essential metabolite inside nanosilver preserved mulberry leaves at post preservation stage. Nguku et al. (2007) reported that supplement of high protein diet efficiently increases the quality of cocoon shell. Current study also reflects alike observation, as maximum drop off in leaf quality was observed for distilled water preserved leaves, so supplementing these leaves resulted in decreased of both cocoon quality and quantity.

The two most vital parameters influencing cocoon-related parameters were rearing seasons and mulberry leaf quality (Rahmathulla 2012). Mulberry leaves serve as exclusive nutrition source viz. carbohydrates, protein, vitamins and minerals depending on which good quality cocoon crops are produced by larvae (Tang et al. 2005; Ravikumar 1988). The silk gland of Silkworm larvae bears a unique ability for transforming ingested leaf protein into fibre of silk protein. It was studied that the

amino acids obtained from metabolism of mulberry leaf protein, are utilized by the larvae for proper growth and development and cocoon formation (Borah and Boro 2020). Silkworm feeding nutrient deficient leaves will puts significant impact on growth and cocoon production both qualitatively and quantitatively (Cristina and Marghitas 2011). Carbohydrate and protein are the two most essential constituents of mulberry leaves playing decisive role in silkworm rearing (Ito and Mukaiyama 1970). While leaf protein is crucial for growth of larvae, development of silk gland, silk production and cocoon characters, carbohydrate provides major energy source during accretion and secretion of silk fibre from silk gland (Sajgotra et al. 2017). Rearing records of multi-voltine silkworm larvae suggests that GI of larvae supplemented with leaves preserved in nanosilver and silver nitrate solution remains almost same as that of larvae supplemented with freshly cultivated leaves. At the same time significant ($p \leq 0.05$) drop off in GI was noted when fed with leaves of distilled water set. Larval development depends mainly on leaf protein content, and retention of GI signifies retention of protein in leaves preserved in preservative solution. Multiple regression analysis (Table 70) of significantly correlated parameters related with protein content in preserved leaves indicates that larger the superoxide scavenging potentiality in preserved leaves less is the ROS mediated oxidation of proteins and thus more is the native protein content of the leaves.

Table 70: Statistics summary of multiple regression analysis keeping protein constant

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	0.995[a]	0.991	0.990	1.212853	
2	0.997[b]	0.994	0.993	1.020261	1.843

[a] Predictors: (Constant), **SUPEROXIDE**

[b]Predictors: (Constant), SUPEROXIDE, MDA

[c]Dependent Variable: **TOTAL PROTEIN**

Impact of feeding on larval productivity can be estimated in terms of effective rate of rearing (Giridhar and Sivarami Reddy 1991). Current studies displayed best ERR in larvae fed with nanosilver preserved leaves. Low effective rate of rearing in

larvae fed with distilled water preserved leaves reflects the inability of larvae to produce quality cocoon, as the mortality rate of the larvae is high. Productivity in sericulture can be described in terms of three most significant commercial parameters namely cocoon and shell weight and shell ratio (Sajgotra et al. 2017). Larvae supplemented with nanosilver preserved leaves displayed best cocoon and shell weight and shell ratio than larvae supplemented with leaves preserved in silver nitrate and distilled water. Penkov et al. (1988) and Periaswamy and Radhakrishnan (1985) reported that cocoon yield and thereby cocoon and shell ratio depends on the nutritional content of the leaves. Rorie et al. (1983) proposed that protein content of leaves directly influences rearing performance in terms of effective rate of rearing, cocoon and shell weight and shell ratio. Thus probably due to high retention of nutrient content, nanosilver preserved leaves displayed cocoon parameters sericulture can be described in terms of three most significant commercial parameters namely cocoon and shell weight and shell ratio (Sajgotra et al. 2017). Larvae supplemented with nanosilver preserved leaves displayed best cocoon and shell weight and shell ratio than larvae supplemented with leaves preserved in silver nitrate and distilled water. Penkov et al. (1988) and Periaswamy and Radhakrishnan (1985) reported that cocoon yield and thereby cocoon and shell ratio depends on the nutritional content of the leaves. Rorie et al. (1983) proposed that protein content of leaves directly influences rearing performance in terms of effective rate of rearing, cocoon and shell weight and shell ratio. Thus probably due to high retention of nutrient content, nanosilver preserved leaves displayed cocoon parameters equivalent to that of fresh leaves. It has also been reported that physical and internal biochemical property of mulberry leaves directly influences larval consumption and digestion efficiency (Zannoon et al. 2012; Das and Vijayaraghavan 1990). Thus probably due to more ingestion, digestion and conversion ratio of larvae supplemented with nanosilver preserved leaves displayed better rearing performance than others, demonstrating the ability of green synthesized silver nanoparticles as an effective preservative.

The post cocoon characters i.e., quality of silk filament obtained, mostly depends on quality of supplement provided during rearing (Hassanein and EL Shaarawy 1962). It has been reported that greater the length of silk filament and lesser the length of non-breakable filament, more superior is the obtained silk (Kamimura and Kiuchi

1998). Estimated average filament length and non-broken filament length was more in nanosilver set than silver nitrate set, in comparison to silk produced by larvae supplemented with fresh leaves, indicating better preservative potentiality of green synthesized nanosilver solution. Silk obtained from nanosilver set displayed greater value of rendita (quantity of green cocoons required to fabricate 1 kg raw silk) than silk produced from larvae fed with fresh leaves, indicating high effectiveness of nanosilver solution over cocoon parameters. Kumararaj (1972) stated that greater the occurrence of filament break, inferior will be the reelability of fiber causing increase in rendita value. Thus, probably due to greater frequency of filament break in silver nitrate set displayed less rendita value than others, indicating superiority of nanosilver solution to produce better quality of silk.

In the current era of plant and animal science, activity-based protein profiling is an important and powerful functional proteomics tool used to identify the actual involvement of proteins or pathways in performing a particular function (Morimoto and van der Hoorn 2016). The electrophoresis-based banding pattern of silk gland proteins displayed sixteen distinct bands. Raman et al. (2007) reported the presence of 10 – 15 gel bands in 5^{th} instar male larvae of silkworm supplemented with hydrolysed soy protein *viz.* P-soyatase, supporting current observation. Relative density based graphical representation displayed band density of nanosilver and silver nitrate lane almost similar to that of fresh lane, supporting the shelf life prolonging capacity of preservative solutions. The protein content was the most significant nutritive component of mulberry leaves, serving in the growth form of silkworm larvae, as well as in the maturity of silk gland (Mahmoud 2017). Decline in band intensity of distilled water set evidently indicates depletion in protein content in silk gland, which may be due to the drop off in leaf quality with increase in days of preservation as apparent from physical state of leaves. The obtained result of distilled water set was furthermore supported by the result of SSW and SCW which also displayed declining trend in comparison to other treatments. Hou et al. (2010) mentioned that haemolymph surrounds the tissue system of insects and acts as essential depository for energy and nutrition. Electrophoresis of proteins present in haemolymph showed the incidence of eight visible bands and the banding pattern among treatments appeared almost like that obtained for silk proteins. Haemolymph present in the open circulatory system of insects helps in delivering nutrient and

energy content throughout the body of silkworm (Arrese and Soulages 2010). Haemolymph also plays vital role in innate immune response by protecting the body of silkworm against fungal and bacterial attack (Mullen and Goldsworthy 2003). Banding pattern of haemolymph protein in the current study clearly indicates that the energy content and nutrient efficiency was lesser in the larvae supplemented with distilled water preserved leaves. This decline in energy content may be the primary reason for larvae in distilled water set, failing to spin during cocoon formation and rise in their mortality rate. Thus, it might be concluded that larvae reared in fresh leaves and leaves of nanosilver and silver nitrate set showed better immune response than the larvae of distilled water set. The guts of the larvae hold diverse types of digestive enzymes that bear the efficiency to convert the organic molecules obtained from leaves ingestion into useful bio-products (Lokesh et al. 2012). It was reported that in larvae the mid-gut protein plays role in growth and metamorphosis (Zhang et al. 2011). Result obtained from electrophoresis of larval stomach proteins clearly indicated that larvae supplemented with nanosilver treated leaves produces high amount of digestive enzymes for leaf digestion leading to maintenance of nutrient efficiency. On analysing the electrophoresis pattern of fat body associated protein, eight protein bands were identified. Through comparative analysis it was found that the larvae supplemented with nanosilver and silver nitrate preserved leaves displayed high level of fat protein expression than the larvae supplemented with fresh leaves. Meng et al. (2017) stated that the function of fat body in insects is equivalent to that of mammalian liver. The fat body plays significant role as nutrient luggage compartment, beside this it also acts in immune system, homeostasis, and metabolic detoxification (Arrese and Soulages 2010). High level of expressed protein in nanosilver set signifies elevated nutrient contents and better homeostatic regulation.

Further evaluation of effect of feeding preserved leaves was conducted through isozyme profiling. NOX is a stress indicator as it is responsible for generating superoxide molecules by transforming metabolic electron from NADPH to molecular oxygen (Shen et al. 2019). In silk gland protein, elevated NOX activity was noted for larvae fed with distilled water preserved leaves, indicating generation of excessive ROS molecules inside silk gland, inhibiting proper development of the gland. The obtained result further indicated increase in mortality rate in larvae fed

with distilled water preserved leaves, which also showed formation of low quality cocoons. One of the most important defensive enzymes present in the body of silkworm throughout their life cycle is superoxide dismutase (Kobayashi et al. 2019). Superoxide dismutase plays important roles in immune responses inside silk gland by inhibiting any generated superoxide during spinning (Guo et al. 2016). Segal et al. (2000) also reported the occurrence of different antioxidant defensive enzymes like thioredoxin, peroxidase and superoxide dismutase in the silk protein that give defensive response against generated ROS during spinning. SOD activity in larvae fed with nanosilver preserved leaves appeared almost comparable to that of larvae fed with fresh leaves, reflecting the existence of equivalent quantity of defensive activity even after 7 days of preservation. Besides superoxide, another pro-oxidant, capable of causing oxidative damage to important biomolecules on crossing physiological limit was hydrogen peroxide (Sahoo et al. 2015). Hydrogen peroxide is capable of causing lipid peroxidation within the cell of insects thereby inhibiting lipid mediated developmental activities (Downer 1985). Antioxidant enzyme catalase acts to overcome the stress caused by hydrogen peroxide (Keilin and Hartree 1943). CAT destroys cellular hydrogen peroxide to produce water and oxygen (Nandi et al. 2019). The activity of CAT isozyme was found more in the larvae supplemented with preserved leaves than larvae fed with fresh leaves. The elevated catalase activity was probably due to greater production of H_2O_2 on feeding preserved leaves inside silk gland which was nullified by the detected two CAT isozymes. Thus, on an overall aspect it can be stated that among the supplemented preserved leaves, leaves preserved in nanosilver solution displayed better antioxidant activity thus helping silkworm larvae to develop proper silk gland, thereby producing economically effective cocoons.

OHR-LCMS analysis of differentially expressed silk gland proteins identified different functionally active cellular and metabolic proteins. Among the identified proteins, actin-related protein 2/3 complex subunit involved in the development of branched actin networks. Couble et al. (1984) reported that the posterior cells of silk gland contain abundant microfilament of 50 – 70 Å diameter situated towards cellular apex. Besides actin, tubulin alpha chain was also identified and was also reported to be concentrated in the cells of posterior silk gland, which was also identified to be the key site for production of fibroin protein (Tashiro et al. 1968). It

was reported that inside silk gland of fifth instar larvae microtubule and microfilament plays dynamic role in protein secretion leading to the development of cocoon (Sasaki and Tashiro 1976). Identified mediator (RNA polymerase II subunit 7) was involved in regulation of transcriptional system (Blazek et al. 2005). Identified mediator molecule remains associated with RNA polymerase II and directly take part in the regulation of RNA polymerase II specific genes, which might be helpful in the development of silk gland of larvae. Identified Glycine-tRNA ligase promotes the process of translation by catalysing the attachment of glycine to glycine specific tRNA. Transferrin protein which was identified binds and transfer iron at cellular and sub-cellular level in ferric state. Geiser and Winzerling, (2012) reported that alongside transporting iron, transferrin plays active role in towards developmental process, reproduction and antioxidant defence response. Thus, through activation of antioxidant defence mechanism, transferrin protects metallic compounds mediated cellular damage, promoting growth. Another important identified protein was pyridoxal phosphate homeostasis protein, an active form of vitamin B6. Chen et al. (2019) reported that pyridoxal phosphate act as cofactor for more than hundred types of vitamin B6 dependent enzymes that take part in various metabolic activities *viz.* glucose metabolism, amino acid metabolism and in lipid syntheses. Thus, presence of pyridoxal phosphate in activated form inside silk gland protein leads to the genomic expression of large number of proteins which might play active role in development of silk gland. Identified protein, phenoloxidase (subunit-1) was reported to be responsible for the intercellular production of a variety of polyphenolic compounds that directly or in-directly participate in variety of defensive responses (Ankola and Puttaraju 2017). It was also reported that phenoloxidase also protects larvae from infection against various microorganisms (Eleftherianos and Revenis 2011). Beside these, two sex specific larval storage protein namely Sp1 and Sp2 were identified which were probably synthesized inside larval fat body and their synthesis relates directly to the nutritional condition inside larval body (Sumio Tojo and Kobayashi 1980). Detection of sex specific larval storage protein indicates that leaves preserved in nanosilver solution contains sufficient retention of nutritional content that might be useful for larval development and moulting.