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An Approach to Improve the Post-harvest Leaf Quality in Indian Mulberry (*Morus alba* L.)

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AUTHORS CONTRIBUTION

HARI SINGH MEENA : Performed the experiments and drafted the manuscript;
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ABSTRACT

Mulberry (*Morus* species) leaves are the sole source of food for the silkworm (*Bombyx mori* L.) hence, high-quality foliage is the key to the productivity of the silk industry. Rapid water loss from the harvested leaves is one of the major reasons for quality loss affecting silkworm feeding behaviour. Previous research in our laboratory indicated that epicuticular wax is essential in maintaining leaf moisture in harvested leaves of mulberry. In the present study, an agronomic approach was designed and tested to increase the cuticular resistance and enhance moisture retention capacity in the harvested leaves in two genotypes of mulberry, Dudia white (DW) and Matigara white (MW). Controlled irrigation and mild drought stress increased leaf epicuticular wax and drought-stressed plants had a significantly higher wax load (465.31 and 556.29 µg dm^{-2} in Dudia white and Matigara white, respectively) at 40 per cent soil field capacity (FC) when compared to the plants grown at 100 per cent FC. The harvested leaves showed higher moisture retention capacity (MRC) of 92 and 84.74 per cent at 40 per cent FC compared to 62.55 and 57.10 per cent at 100 per cent FC in Dudia white and Matigara white, respectively. Leaves from water-limited plants had significantly lower water loss of 3.4 and 5.9 per cent under 40 per cent FC compared to control plants (6.6 and 9.1%). The technique used in this study increased MRC and helped in preserving leaf quality. The approach developed is cost-effective and can be easily adopted in farmer's fields.

Keywords : Epicuticular wax, Post-harvest water content, Water stress, MRC, FC, Mulberry

MULBERRY (*Morus alba* L.), a member of the *Moraceae* family, is commercially grown for its leaves, which are the sole food source for the monophagous silkworm (*Bombyx mori* L.). Hence, the availability of quality foliage and its yield is vital for the success of the sericulture industry (Thomas and Nataraja, 2022 and Thinnaluri *et al.,* 2015). India is the second largest producer of raw silk after China (Anonymous, 2022). The leaf is harvested and fed to the silkworm, and the time lag between foliage harvest and silkworm feeding causes post-harvest water loss, reduces feed quality and hence affecting silkworm growth and cocoon yield (Mamrutha *et al.*, 2010). Any interventions that sustain leaf moisture status, especially during post-harvest handling of the foliage, would benefit the sericulture industry.

Post-harvest water loss occurs mainly through the cuticular pore rather than the stomata since stomata close spontaneously (Brodribb *et al.,* 2014: Duursma *et al.,* 2019). Maintaining post-harvest water content by reducing water loss will improve the leaf quality and silk yield. A desirable trait that aids in the preservation of post-harvest leaf quality from water loss is the higher amount of wax on the leaf surface (Rahmathulla *et al.,* 2006; Mamrutha *et al.,* 2010). In mulberry trees, the importance of leaf surface wax in regulating leaf moisture loss has been demonstrated, with a positive correlation between surface wax load and moisture retention capacity (Mamrutha *et al.,* 2010). Leaf surface waxes are a complex mixture of very long-chain fatty acids and their derivatives that are produced through a series of biochemical pathways

(Samuels *et al.,* 2008; Kunst and Samuels, 2009). Chemical characteristics of the epicuticular wax load and wax crystal morphology are the primary determinants of the permeability of the plant cuticle (Mamrutha *et al.,* 2010, 2017). Many regulatory proteins of wax biosynthesis have been identified, including SHINE1/Wax Inducer1 (SHN1/WIN1) in *Arabidopsis thaliana* (Broun *et al.,* 2004). Previously, our group had attempted to improve mulberry leaf wax content through a transgenic approach, and the leaves of *AtSHN1* expressed mulberry plants had significantly higher wax and reduced post-harvest water loss (Sajeevan *et al.,* 2017). Agronomical ways to improve epicuticular wax and increase cuticular resistance will be one of the viable strategies to reduce post-harvest water loss. In the present study, an attempt has been made to understand the influence of drought stress on epicuticular wax load and the post-harvest water loss in Indian mulberry.

MATERIAL AND METHODS

Plant Material and Imposition of Limited Water Condition Treatments ?

Pencil-sized mulberry cuttings of Dudia white and Matigara white with 2-3 buds were raised in a pot with a capacity of 25 kg, containing garden soil, farm yard manure (FYM) and sand mixed at 2:1:1 ratio. After five months, plants with uniformly established foliage were used for the experiment. The plants were subjected to water-limited conditions of 60 and 40 per cent field capacities (FC), gravimetrically (Karaba *et al.,* 2007). Plants maintained at 100 per cent FC served as the control. After two weeks of stress imposition, stress intensity was confirmed by measuring soil moisture content using a soil moisture probe.

Determination of Relative Water Content (RWC), Moisture Retention Capacity (MRC) and Rate of Water Loss from Leaves

The relative water content in both genotypes was measured 14 days after the imposition of a limited water condition. The leaf disc ware made from well-developed fifth leaf from the apical node. Uniform four plants were selected and three leaves from each were used. The relative water content was calculated using the formula below (Pieczynski *et al.,* 2013).

$$\text{RWC (\%)} = \{(\text{FW}-\text{DW}) / (\text{TW}-\text{DW})\} \times 100$$

where,

FW = fresh weight

DW = dry weight

TW = turgid weight.

Moisture retention capacity (MRC) was estimated as described by Mamrutha *et al.* (2010). Leaves were harvested early in the morning, and the fresh weight was immediately recorded. Leaf weight was recorded at hourly intervals up to 5 hours for estimating the MRC.

For determining water loss, healthy leaves were collected in the early morning, and leaf discs were prepared using a single-hole punch with a diameter of 1 cm. The fresh weight of leaf discs were recorded immediately after collection, and the weight loss was recorded at hourly intervals. The water loss was calculated using the following formula.

$$\text{Water loss} = (\text{FW}_0 - \text{FW}_1)$$

where,

FW_0 is the initials fresh weight

FW_1 is the weight after subsequent hour

SPAD Chlorophyll Meter Reading (SCMR)

The SPAD metre is a portable device that gives the indirect measurement of leaf chlorophyll content. SPAD chlorophyll meter reading (SCMR) value is a non-destructive indicator of plant health. The SPAD-502 (Minolta, Japan) meter was used to record SCMR values for both control and limited water-treated plants after 14 days of the imposition of limited water conditions.

Measurement of Gas Exchange Parameters

The LI-6800XT portable photosynthesis measurement system (Model: LI-COR, Lincoln, Nebraska, USA) was used to measure the leaf gas exchange parameters of mulberry plants under both fully saturated and limited water conditions. The photosynthetic rate (µmol m^{-2} s^{-1}), stomatal conductance (mol m^{-2} s^{-1}), intercellular CO_2 (µmol mol^{-1}) and transpiration rate (mol m^{-2} s^{-1}) were recorded from the fifth fully developed leaves from the apical node. Gas exchange parameters were recorded between 9:00 to 11:00 a.m. on clear sunny days. Chamber CO_2 was maintained at 400 ppm, PAR 1200 µmol m^2 s^{-1}, RH 65-70 per cent and chamber temperature 30°C (Nataraja and Jacob, 1999).

Analysis of Epicuticular Wax Content

Cuticular wax content was determined using a colourimetric method based on the colour change by the reaction of wax with acidic potassium dichromate, ($K_2Cr_2O_7$), as described by Mamrutha *et al.* (2010). The fresh leaf sample was immersed in chloroform (10 mL) for 15 seconds to extract the leaf surface wax. Total wax content was estimated by recording the absorbance of the reaction mixture at 590 nm. Carnauba wax (Sigma, St. Louis, Missouri, USA) was used for developing the standard curve (Samdur *et al.,* 2003) and the total cuticular wax was expressed as µg dm^{-2}.

Statistical Analysis

The experiment was carried out using a completely randomized design (CRD) (Gomez and Gomez, 1984), and GraphPad Prism was used to analyze the data. The data's statistical significance was determined using one-way ANOVA.

RESULTS AND DISCUSSION

Stress is Conferred by Assessing RWC, SCMR and Gas Exchange Parameters

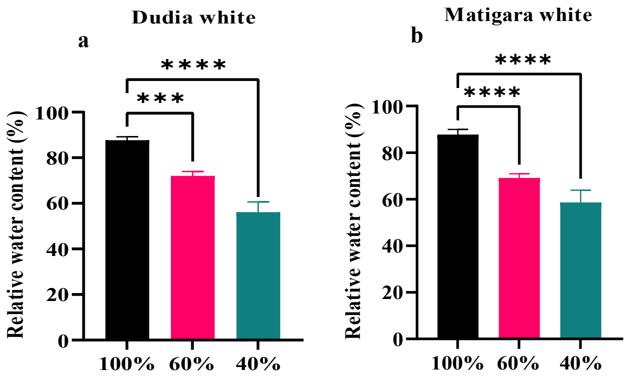

Fig. 1: Relative water content determined from the leaves of five month old mulberry plants grown under fully irrigated (100% FC) and water-limited conditions (60% and 40% FC). The error bars represent ± SE, and statistical significance was indicated by an asterisk (***$P < 0.001$, and ****$P < 0.0001$)

The relative water content (RWC) gives the approximate estimation of leaf water status in terms of cellular hydration. A significant reduction in RWC was observed in plants grown under limited water conditions when compared with fully saturated plants. About 17 and 36 per cent reduction has been observed in Dudia white in 60 and 40 per cent FC respectively (Fig. 1a). Similarly, in Matigara white 21.17 and 33.02 per cent reduction has been observed under 60 and 40 per cent FC, respectively (Fig. 1b). Plants exposed to limited water conditions have also showed lower SCMR values when compared to fully saturated plants. Under 60 per cent FC, the SCMR value was reduced by 0.09 per cent in the Dudia white, while a significant reduction (11.52%) was observed in the Matigara white genotype. Under 40 per cent FC, the SCMR value was 9.18 per cent, in the Dudia white (Fig. 2a) and 18.02 per cent reduction was observed in Matigara white genotypes (Fig. 2b). This indicates the reduction in chlorophyll content due to moisture limited conditions. Under limited water conditions (60 and 40% FC) had lower CO_2 assimilation rates (12.02 and 8.01 µmol m^{-2} s^{-1}), intercellular CO_2 (202.15 and

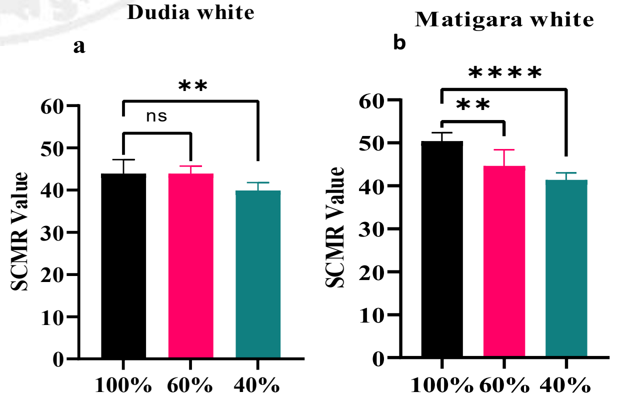

Fig. 2. SCMR value recorded from the leaves of five -month-old mulberry plants grown under fully irrigated (100% FC) and water limited conditions (60% and 40% FC). The error bars represent ± SE, and statistical significance was indicated by an asterisk (**$P < 0.01$, ****$P < 0.0001$ and ns non significant)

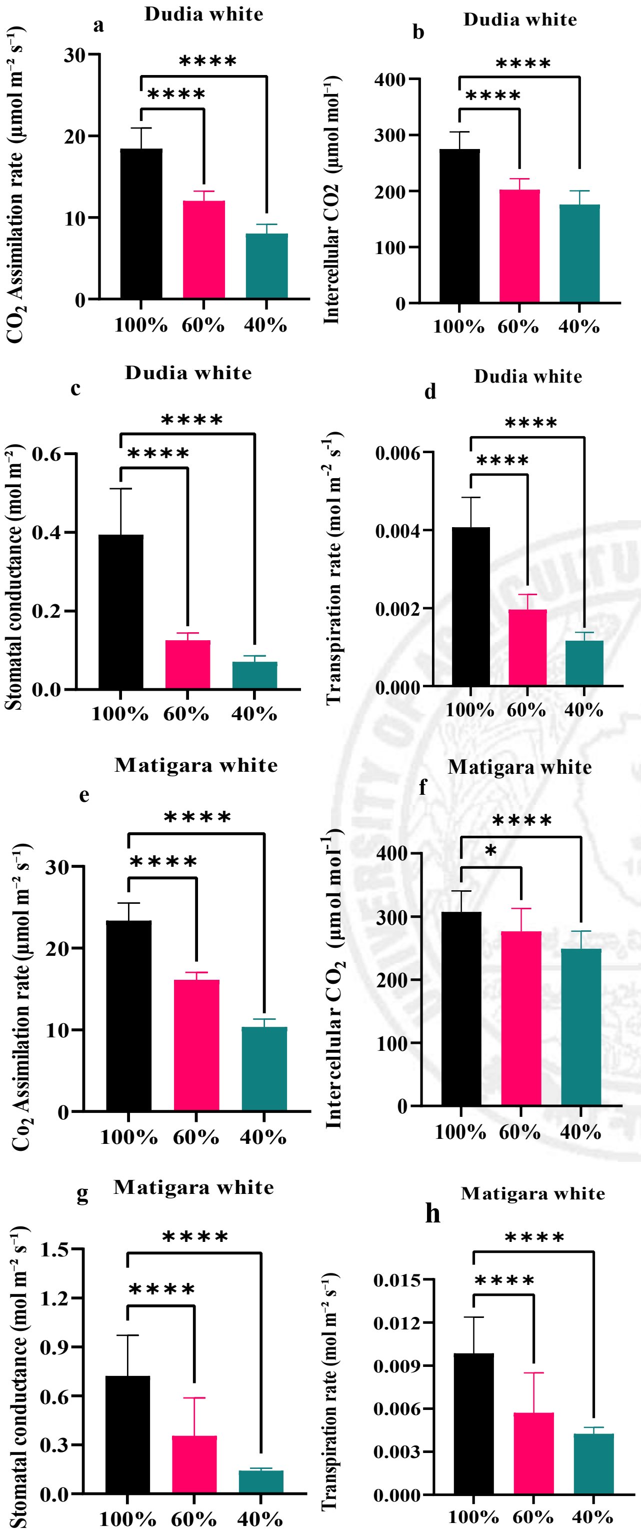

Fig. 3: Gas exchange parameters recorded from the leaves of mulberry plants grown under fully irrigated (100% FC) and water-limited conditions (60% and 40% FC). The error bars represent ± SE, and statistical significance was indicated by an asterisk (*$P < 0.05$ and ****$P < 0.0001$)

175.49 µmol mol^{-1}), stomatal conductance (0.12 and 0.06 mol m^{-2} s^{-1}) and transpiration rate (0.0019 and 0.0011 mol m^{-2} s^{-1}) in Dudia white (Fig. 3a-d), while CO$_2$ assimilation rates (16.08 and 10.33 µmol m^{-2} s^{-}), intercellular CO$_2$ (276.70 and 249.16% µmol mol^{-1}), stomatal conductance (0.35 and 0.14 mol m^{-2} s^{-1}), and transpiration rate (0.0056 and 0.0042 mol m^{-2} s^{-1}) respectively in Matigara white genotypes (Fig. 3e-h). This reduction in leaf gas exchange parameters indicates the disturbances in photosynthesis due to the stress imposed. An immediate response of plants to water limitation is a reduction in stomatal conductance and this stomatal limitation is the main cause for the reduction in transpiration and assimilation rate. (Tezara *et al.,* 1999; DeglInnocenti *et al.,* 2009 and Misson *et al.,* 2010).

Limited Water Condition Enhanced the Epicuticular Wax Load and Improved the Post-harvest Leaf Quality

Leaf hydration is the essential aspect of the harvested mulberry leaf, which determines the silkworm growth and cocoon yield. Leaf moisture content is the imperative parameter for determining the quality of harvested leaves (Khan *et al.,* 2007 and Rahmathulla *et al.,* 2006). Moisture retention capacity is a key factor in determining the post-harvest water content (Mamrutha *et al.,* 2010). Plants grown in limited water conditions showed higher MRC after 5 hours of

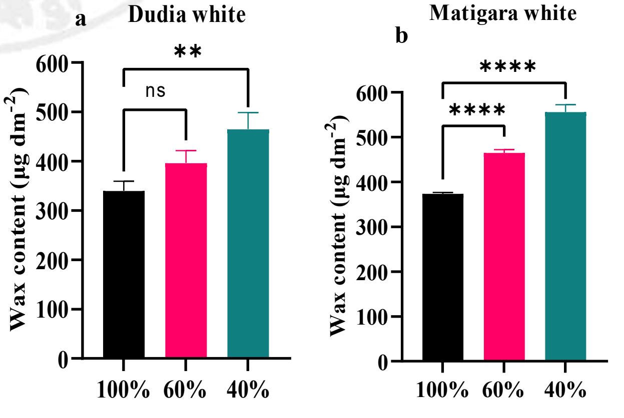

Fig. 4: Total surface wax load in the leaves collected from the leaves of five month old mulberry plants grown under fully irrigated (100% FC) and water-limited conditions (60% and 40% FC). The error bars represent ± SE, and statistical significance was indicated by an asterisk (**$P < 0.01$, ****$P < 0.0001$, and ns non-significant)

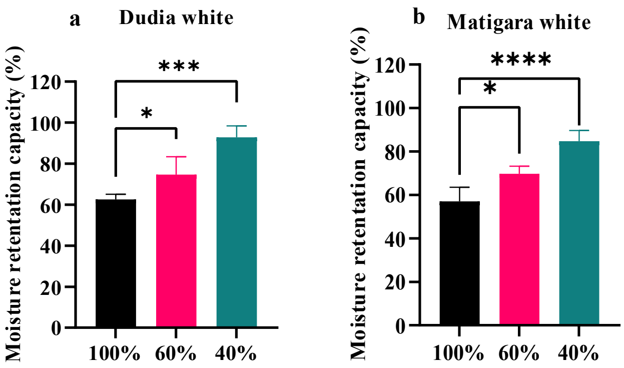

Fig. 5: Moisture retention capacity in the leaves of five months old mulberry plant grown under fully irrigated (100% FC) and water-limited conditions (60% and 40% FC). The error bars represent ± SE, and statistical significance was indicated by an asterisk (*$P < 0.05$, ***$P < 0.05$ and ****$P < 0.0001$)

harvesting. Under limited water conditions plant showed 19.37 and 48.39 per cent MRC in 60 and 40 per cent FC in Dudia white (Fig. 5a). Similar results have been observed in Matigara white genotype. The MRC values were 22.11 and 48.40 per cent under 60 and 40 per cent FC, respectively, which were significantly more than fully saturated plants (Fig. 5b). These results indicate that limited water stress can improve the MRC of the harvested leaf. One of the major components that influence the MRC in harvested leaves is leaf cuticular wax. The epicuticular wax load was found to be significantly higher in plants exposed to limited water conditions as compared to fully irrigated plants. Under 60 and 40 per cent FC, the epicuticular wax load increased by 14.24 and 36.90 per cent in the Dudia white (Fig. 4a), and 24.38 and 48.79 per cent, respectively, in the Matigara white compared to fully irrigated control plants (Fig. 4b). Earlier studies have proved that mild drought stress increases the cuticular wax biosynthesis and composition (Kosma *et al.,* 2009), to prevent the nonstomatal water loss from leaf (Sajeevan *et al.,* 2017). Positive correlations have been reported between wax content and drought condition in various crops such as sorghum (Jordan *et al.,* 1984), barley (Febrero *et al.,* 1998), rice (Zhu and Xiong, 2013), and wheat (Richards *et al.,* 1986). Mild drought stress can trigger the cascade of signalling events that upregulate genes that involve wax biosynthesis (Sajeevan *et al.,* 2017). Over expression of SHN1/ WIN1 resulted in increased wax deposition and improved water relations in plants (Sajeevan *et al.,* 2017). CER1, CER3, CYTB5, MAH1, KCS2, ECR, and WSD1 genes are important in terrestrial plants for cuticular wax biosynthesis and reduce cuticular wax water loss, and protect underlying tissues from drying out (Kosma *et al.,* 2007). This increase in MRC under limited water conditions can be accounted by increased wax load in plant leaves. A positive correlation between the MRC and the epicuticular wax load was observed in mulberry leaves in Dudia white (Fig. 7a) and Matigara white (Fig.7b). Similar results of a strong positive relationship between surface wax load and MRC of harvested mulberry leaves were recorded by Mamrutha *et al.* (2010). Water loss after leaf harvesting was significantly lower under limited water conditions when compared to fully irrigated plants. Water loss after the 5th hour was 6.6, 5.6 and 3.4 g in 100, 60 and 40 per cent FC, respectively, in

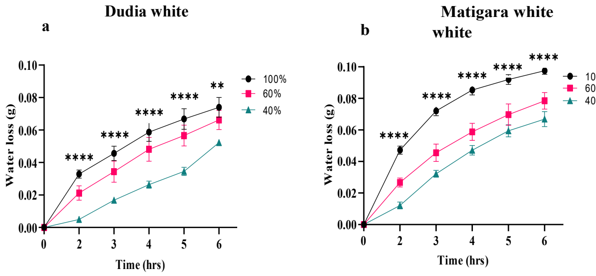

Fig. 6: Water loss from the harvested leaves of mulberry plants grown under fully irrigated (100% FC) and water-limited conditions (60% and 40% FC). The error bars represent ± SE, and statistical significance was indicated by an asterisk (**$P < 0.01$ and ****$P < 0.0001$)

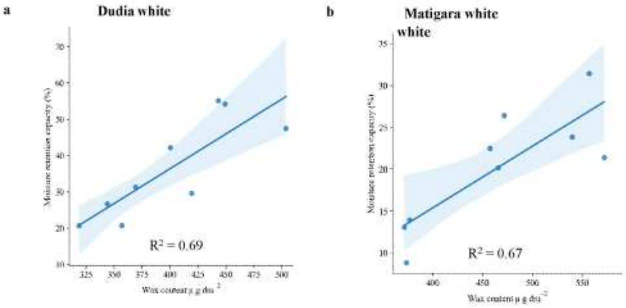

Fig. 7: Correlation between MRC (%) and leaf surface wax content (μg dm^{-2}) in mulberry leaves collected from five months old mulberry plants grown under fully irrigated (100% FC) and water-limited conditions (60% and 40% FC)

Dudia white (Fig. 6a), while the values were 9.1, 6.9 and 5.9g in 100, 60 and 40 per cent FC respectively, in Matigara white (Fig. 6b). Leaves had significantly more water content in 60 and 40 per cent FC when compared to fully irrigated plants after 5 hours. Both the mulberry genotypes retained more water after harvest under water-limited conditions (Fig.8a and 8b). These results indicate that mild drought stress can prevent rapid dehydration in harvested leaves. In many plant species, drought-induced wax production has been shown to enhance water relations by reducing water loss through the cuticle (Cameron *et al.,* 2006; Jenks *et al.,* 2001; Kim *et al.,* 2007a; Kim *et al.,* 2007b; Rahmathulla *et al.,* 2006).

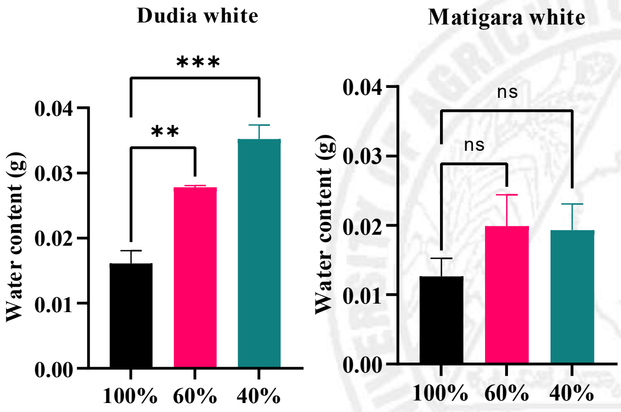

Fig. 8: Water content in harvested leaves of mulberry plants grown under fully irrigated (100% FC) and water limited conditions (60% and 40% FC). The error bars represent ± SE, and statistical significance was indicated by an asterisk (**$P < 0.01$, ***$P < 0.001$and ns is non-significant).

Our study showed that limited water condition enhances the epicuticular wax content in mulberry leaves, which is an important barrier to post-harvest water loss. Leaves from plants exposed to mild water stress conditions had higher wax and better moisture retention capacity and reduced post-harvest water loss. Higher moisture retention capacity will have a positive impact on silkworm feeding. Mild water stress conditions can easily be implemented under field conditions.

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