Differential occurrence of epicuticular wax and its role in leaf tissues of three edible aroids hails from north eastern hill region of India

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Localization of epicuticular wax (EW) content in leaf tissues and its interaction on leaf protective mechanisms of three edible aroids, *Alocasia*, *Colocasia* and *Xanthosoma* were assessed. Scanning electron microscopy depicted the occurrence of EW in leaf tissues which was higher in *Colocasia* (10.61 mg dm^{-2}) and *Xanthosoma* (11.36 mg dm^{-2}) than in *Alocasia* (1.36 mg dm^{-2}). The result highlighted the interface of EW between the leaves and its internal and external environments. EW acted as a protecting barrier against deleterious solar radiation in term of sun protecting factor (*SPF*). Occurrence of EW also effectively managed leaf pigmentation, moisture retention, cellular membrane integrity against the invaders. *Colocasia* exhibited superhydrophobic properties with higher static contact angle (*CA*) >150^{o} than hydrophobic *Xanthosoma* and *Alocasia* with CA ranged between 99.0^{o} to 128.7^{o}. *Colocasia* EW highly influenced the qualitative and protective mechanisms of leaf. Aroids are the cheapest sources of edible EW among the terrestrial plants could be used in food, agricultural and industrial applications.

Keywords: epicuticular wax, aroid leaves, *Colocasia*, *Alocasia*, *Xanthosoma*

1. Introduction

Aroids are important minor food crops, belong to the Araceae family, cultivated widely in the tropics of the world[1,2]. Edible aroids are one of the cheapest sources of carbohydrates and dietary energy, thus, have social and economic significance on daily nutrition intake for about 400 millions of people around the world[3]. In spite of low share among the tuber crops, production of aroids exceeded 10.13 million tonnes worldwide[4].

Alocasia, *Colocasia*, *Xanthosoma*, among the edible aroids, are the one of the most preferred vegetables in Southeast Asian countries. Leaves, pseudo-stems and corms are consumed as vegetables and traditional medicines by the tribal communities of north eastern hill region of India[5,6]. These aroids were being used as folk medicines in the ancient world[7] due to high antioxidant, anti-inflammatory, anti-nociceptive and anti-carcinogenic properties[8,9].

Apart from the food and medicines, Aroids have a lot of possible applications as animal feed, carbohydrates, energy and waxes for various industrial uses[10]. Aroid starch could be a substitute for 40% of the biodegradable plastics[11]. Aroids also contain higher epicuticular wax in the leaf tissues among the terrestrial plants. Edible and biodegradable waxes from the aroids are still need to be explored.

Plant derived hydrophobicity and non-toxicity edible waxes have ample scope in food industries especially for food production and protection[12]. Wide use of carnauba, beeswax or petroleum based waxes in food industries warrants the consumer's preference and health hazards[13]. Aroids can be cultivated under harsh environments[3] and exploration of edible wax from natural resources would add momentum to the food and post-harvest industries[14].

Epicuticular wax in leaf tissues act as a protective barrier against several biotic and abiotic factors[15]. In spite of being an important biological constituent, studies on role of epicuticular wax in leaf tissues of aroids are limited. Assessment of cuticular wax needs more attention to understand its involvement in leaf physiological processes. The present study was focused on the role of epicuticular wax in leaf tissues of three different aroid species cultivated in north-eastern hill region of India.

2. Material and methods

2.1. Collection of leaf samples and wax extraction

Fresh leaves of Alocasia (A), Colocasia (C) and Xanthosoma (X) were collected from ICAR Research Complex for North Eastern Hill Region (ICAR RC NEHR), Imphal valley, Manipur, India located at 24°50'N latitude, 93°55'E longitude and altitude of 860 m above mean sea level. Each leaf was processed for wax estimation and analyses on leaf characters were performed immediately. The experiments were carried according the regulations, with the approval of the competent authority.

2.2. Scanning electron microscopy (SEM) of aroid leaves

Scanning Electron Microscopy (SEM) was used for the observation of the microstructure of fully opened leaves of *Alocasia*, *Colocasia* and *Xanthosoma*. Samples were cross sectioned using a scalpel; the cut was always performed in the same direction. Samples were mounted on holders and coated with gold as described by Pieniazek and Messina (2017)[16]. Microscopic evaluation was performed using a scanning electron microscope (JOEL-JSM 6390LV, Japan). Observations of the samples at magnification of at 500X, 1000X, 1500X and 2000X were obtained for image analysis. Brightness and contrast are the most important variables that must be controlled during the acquisition of images; therefore, the values of these parameters were kept constant for each magnification during the process of image acquisition[17].

2.3. Extraction and estimation of epicuticular wax

The extraction of the epicuticular wax was performed using chloroform as solvent[15]. Extraction process was performed at 15, 30, 45, 60, 90, 120 and 180 seconds in order to optimise the extraction process. Eight different leaves (n=8) of each plant were taken for wax estimation. The experiment was repeated thrice.

The surface area was measured by digital image analysis using Image J software, and the amount of wax was obtained by extraction dipping the leaves in chloroform during different times[18], then followed by the evaporation of chloroform[15]. The results were calculated using the following equation. Samples were analysed in triplicate.

$$\text{Wax content} = \frac{Ww}{AL}$$

Where, W_w is the weight of the wax in mg, and AL is the area of the leaf in cm^2.

2.4. Sun protector factor (SPF)

The wax extracted from the three plants were dissolved in methanol at different concentrations (4 mg ml^{-1}, 2 mg ml^{-1}, 1 mg ml^{-1} and 0.5 mg ml^{-1}). Samples were analysed using a absorbance scan (Eppendorf, Germany) measuring every 5 nm from 290 to 320 nm in a UV-Vis spectrophotomete[19]. *SPF* was calculated using the following equation,

$$SPF = \text{CF x } \sum_{290}^{320} \text{EE}(\lambda) \text{ x I}(\lambda) \text{ x Abs}(\lambda)$$

Where, Abs is the absorbance of the sample, CF is a correction factor (=10), and $\text{EE}(\lambda)$ x $\text{I}(\lambda)$ is the product of erithermal efficiency spectrum and the solar simulator intensity spectrum, which was tabulated following the methodology of Sayre et al. (1979)[20].

2.5. Contact angle and wettability

Leaves with and without waxes were (n=8 each) fastened to a flat surface with tape in front of a white background. A drop of water (0.01ml) was placed on the surface of the leaves with and without wax. A digital camera with macro lens placed perpendicularly to the sample was used to capture an image. Contact angle value was determined by Image J software[20]. The experiment was repeated thrice with three replications.

In order to observe the wettability, the extracted wax was dissolved in chloroform at different concentrations (100 mg ml^{-1}, 75 mg ml^{-1}, 50 mg ml^{-1}, 25 mg ml^{-1} and 0 mg ml^{-1}). 0.25ml of each solution was poured in 3x3 cm^2 filter paper. Once the chloroform was completely evaporated, 0.01ml droplet was placed on top of each sample and the time until its completely absorbed was measured.

2.6. Chlorophyll stability index (CSI)

Chlorophyll content (Ch) of the treated samples (ChT) and chlorophyll content of control samples (ChC) were analised using a SPAD-502 portable leaf greenness meter (Minolta Corp, Romsey, NJ). Samples were exposed to 56°C for 30min in a water bath to determine the pigment stability. CSI was calculated following the equation as derived by Mohan et al. (2000) [22].

$$\text{CSI} = \frac{\text{ChT}}{\text{ChC}} \text{x } 100$$

2.7. Colour parameters

Samples of the three plants, waxed and dewaxed, were illuminated using a lamp (TL-D Deluxe, 169 Natural Daylight, 18W/965, Philips, NY, USA) with a colour temperature of 6500 K 170 (D65, standard light source) and a colour-rendering index (Ra) close to 90%[23].

Eighteen images from one side of each sample and eight regions of interest of each image were taken on the matte black background using the following camera settings: 174 manual mode with the lens aperture at f of 4.5 and speed 1/125, no zoom, no flash, 175, 3088 × 2056 pixels resolution and stored in JPEG format.

The algorithms for pre-processing of full images, image segmentation and colour quantification were processed by Adobe Photoshop CS6 (v18.0 Adobe Systems Incorporated, 2012, USA). *L*, *a* and *b* values were transformed to CIE *L**, *a** and *b**.

2.8. Relative water content (RWC) and leaf moisture loss

RWC and leaf moisture loss was determined following the methods of Perez-Perez *et al*. (2007)[24] and Bueno *et al*. (2020)[25], respectively. Eight leaves were cut into squares (5x5 cm^2) using a scapel and weighted in order to obtain the fresh weight (FW).

Leaves were dipped in distilled water at 22°C during 4 h to obtain the turgid weight (TW) and the samples were dried in a hot air oven (REMI, India] at 70°C for four days. RWC was calculated using the following equation,

$$\mathrm{RWC}(\%) = \frac{\mathrm{FW}-\mathrm{FD}}{\mathrm{TW}-\mathrm{DW}} \mathrm{x}\ 100$$

Leaf moisture loss was analysed at 15s intervals using an electronic balance (Shimadzu Analytical, India).

2.9. Cell membrane Injury (CMI)

CMI was determined by comparing the electric conductivity (EC) of waxed and dewaxed leaves submerged in water for 22h and after 2h of heat stress treatment at 70°C. The electrolytic leakage related with the cell injuries was estimated with the variation on the conductivity[26]as follows:

$$\%\mathrm{Injury} = 1 - \frac{1-(\mathrm{T1}/\mathrm{T2})}{1-(\mathrm{C1}/\mathrm{C2})}\ \mathrm{X}\ 100$$

Where, C_1 and C_2 are the EC of the water before and after submersion of leaves for 22h, respectively. T_1 and T_2 are the EC of the water before and after submersion of leaves for 22h with heat treatment for 2h, respectively.

2.10. In vitro Phytophthora colocasiae infectivity assay

Fungal infection in the leaf tissues was detected by staining with trypan blue as stated by Fernandez-Baustia *et al*. (2016)[27]. Fresh leaves per plant were collected and inoculated with 10µL of *Phytophthora colocasiae (Pc)* spore suspension (15000 ml^{-1} spores) on the dorsal surface of the leaf. The leaves were placed in petriplates at room temperature (25±2ºC) and *Pc* infectivity was observed at different time points at 2, 4 and 6h.

Infected leaves were boiled in 1.5 ml of trypan blue solution for 1 min. The leaves were decolourized in 1ml of bleaching solution by boiling at 60° C for 1 h and bleaching solution was discarded. Each leaf was mounted on glass slide with the help of glycerol and viewed under a light microscope (Magnus Opto Systems, New Delhi, India).

2.11. Statistical analysis

All the data were analyzed by analysis of variance (ANOVA) using XLSTAT statistical software (XLSTAT Premium 2020.2.1, Adinsoft, NY). Differences among the mean values were compared using *Tukey's* test[28] and were considered statistically significant when $P<0.05$.

3. Results and discussion

3.1. Surface properties, extraction process and estimation of epicuticular wax

Leaf surface properties were visualized with SEM prior to wax extraction (Fig. 1A). Micrographs showed the localization, distribution and abundance of epicuticular wax (EW) in leaf cuticles of the three tested aroids. Upon extraction, EW concentration varied significantly among *Alocasia* (1.36 mg dm^{-2}), *Colocasia* (10.61 mg dm^{-2}) and *Xanthosoma* (11.36 mg dm^{-2}) leaf samples (Fig. 1B). *Alocasia* leaves exhibited 10-fold lower EW as compared to *Colocasia* and *Xanthosoma*.

In the present study, we have optimized the wax extraction process for the three aroid species by dipping the leaf pieces in chloroform for 1 min to obtain pure white wax crystals (Fig. 1C). The time point beyond 1 min resulted in green colouration of the solvent and wax which indicated the removal of leaf chlorophyll. The amount of

wax content in leaf epidermis, its chemical composition and crystallization pattern increased the protecting capacity of the leaves[29]. EW plays an important role in maintaining the leaf and plant quality[30].

3.2. Sun protection factor (SPF)

SPF increased significantly ($P<0.05$) with increasing concentration of wax in three aroid leaves (Fig. 2). *Alocasia* registered higher mean *SPF* (2.02) when compared to *Xanthosoma* (1.35) and *Colocasia* (0.24). Sun protection activity depends on the ability to prevent the plants from deleterious UV radiation led mutagenesis[31]. Higher *SPF* was positively correlated with the protective mechanisms and negatively correlated with adverse effect of ultraviolet (UV) radiations[32].

Our results revealed that, *Alocasia* leaves showed 10-fold higher *SPF* than *Colocasia* and 2-fold higher *SPF* than *Xanthosoma*, which could be explored as a potential natural sun protector.

3.3. Contact angle (CA)

Fig. 3 showed significant differences ($p<0.05$) in *CA* of three aroid leaves. *CA* of the leaves decreased significantly while de-waxed in comparison to the leaves with wax. *Colocasia* leaves exhibited superhydrophobicity with higher CA (153.1^o) followed by *Xanthosoma* (128.7^o) and *Alocasia* (105.7^o). The static *CA* in de-waxed leaves of *Colocasia*, *Xanthosoma* and *Alocasia* were observed to be 132.0^o, 102.9^o and 99.7^o, respectively.

Static CA >90^o and <150^o was considered as hydrophobic[33]. Surface with static *CA* more than 150^o is regarded as superhydrophobic[34] which probably is due to micro and nano scale hierarchial topography in the leaves.

According to the classification[34], *Colocasia* leaves represented superhydrophobicity similar to the 'Lotus' hydrophobic state which is a special state of Cassies's superhydrophobic state. Similar results were reported by Kumar and Bhardwaj (2020)[35]. *Xanthosoma* exhibited a transitional hydrophobic state between Wenzel's and Cassie's state; However, *Alocasia* showed Wenzel's state with lowest static *CA* and poor hydrophobic capacity due to lower wax content. Results showed that the hydrophobic properties diminished once the wax was removed from the leaves due to the role of epicuticular wax in static *CA* and hydrophobicity. The hydrophobic capacity maintained in *Colocasia* even without wax may be related with the surface structure of the leaf (Fig. 1A).

3.4. Wettability

Wettability test showed the capacity of epicuticular wax to repel environmental water and protect the leaf surface. In our study, the sample filter paper piece coated with aroid wax persisted the water resistance significantly (**https://drive.google.com/file/d/1SlAchDLY1aveMY2A0PSjhvHoXyLSKHIB/view?usp=sharing**). Results showed that filter paper without wax coating instantly absorbed the water droplet when compared to the filter paper with wax. The resistivity varied significantly ($p<0.05$) among the three types of aroid wax coating.

Wettability showed a linear tendency of higher wax concentration correlated with higher water resistance and hydrophobicity. As shown in the above video link, *Colocasia* wax coating persisted longer resistance to the water droplet which justified its superhydrophobicity. Oner and McCarthy (2000)[36] reported that there was a correlation in wettability of various synthetic compounds with hydrophobicity and surface topography. Leaf epicuticular wax film was successfully examined as a model hydrophobic system[37]. On the other hand, *Alocasia* and *Xanthosoma* wax showed poor hydrophobicity, lower resistivity when compared to *Colocasia* wax.

3.5. Chlorophyll content and chlorophyll stability index (CSI)

Statistical differences ($p<0.05$) in chlorophyll content and stability index were observed among the *Colocasia, Xanthosoma and Alocasia* leaves. Higher SPAD values for chlorophyll content (55.9) were obtained for *Colocasia* followed by *Xanthosoma* (34.4) and *Alocasia* (12.6) (Fig. 4A). In de-waxed leaves, SPAD values decreased significantly ($p<0.05$) in *Xanthosoma* (27.3), *Colocasia* (25.8) and *Alocasia* (9.2). *Colocasia* exhibited higher CSI followed by *Xanthosoma* and *Alocasia* (Fig. 4B). However, chlorophyll content degraded faster in *Colocasia* upon removal of EW which signified the role of EW in maintaining the leaf chlorophyll content. Medeiros et al. (2017)[38] reported that removal of leaf EW lowered leaf chlorophyll content which could be related to reduction of cuticular layer thickness[39] and dismantling of thylakoid membrane[40].

3.7. Colour Parameters

Colour parameters (l^*, a^* and b^* values) had significant differences ($p<0.05$) among the tested aroid leaves with and without wax (Fig. 5). Leaf brightness (l^*) decreased when time was increased. The greenish leaf colour is related to a^* values which also decreased when time was increased. Decreases in a^* value is probably due to the chlorophyll degradation. During leaf pigment degradation, increases in yellow colour (b^*) also played an important role to manipulate leaf greenness.

Leaf discoloration in *Colocasia* under de-waxed conditions was higher when compared to leaves with wax. *Xanthosoma* showed similar values of l^*, a^*, b^* when compared to wax and de-waxed leaves. Epicuticular wax exhibited more predominant role in *Colocasia* leaf protection than in *Alocasia* and *Xanthosoma*. Similar results on leaf colour pigmentation using quantifiable RGB model was reported by Chen et al. (2020)[41]. The colour variation is related to the chlorophyll degradation and also with other biological, chemical and gas exchange processes occurring during photorespiration[42].

3.7. Relative water content (RWC) and leaf moisture loss

As shown in Fig. 6, *RWC* varied significantly ($p<0.05$) in the range of 76.1-94.7% in waxy leaves and 73.1-85.6% in de-waxed aroid leaves. *Alocasia* recorded higher *RWC* followed by *Xanthosoma* and *Colocasia* both under wax and de-waxed conditions. Significant differences ($p<0.05$) were observed for in *RWC* in de-waxed *Alocasia* leaves, showed highly decrease in *RWC* when compared to in *Colocasia* and *Xanthosoma*. Lower wax content in *Alocasia can be* attributed to higher reduction in *RWC.* In *Colocasia* and *Xanthosoma* the samples showed less reduction in RWC, may be due to theirs higher wax content.

EW played an important role in preventing the leaf moisture loss in waxy leaves. Upon wax removal, *Xanthosoma* exhibited rapid moisture loss when compared to *Colocasia* and *Alocasia*. The rapid moisture loss occurred either due to lack of wax content or formation of cuticular cracks upon wax removal[43]. On the other hand, *Colocasia* leaves showed a high dehydration rate in wax and de-waxed conditions, which can be related with the thinner leaf structure. Rapid moisture loss is one of the major factors that affect the leaf quality and EW evidently helped leaf moisture retention in the tested aroids.

3.8. Cell membrane injury (CMI) and in vitro Phytophthora colocasea infectivity

EW helps to membrane stability and acts as a protecting barrier against several environmental factors and invaders[43]. In our study, *Alocasia* showed significantly higher *CMI* as compared *Colocasia* and *Alocasia* under both wax and de-waxed conditions (Fig. 7A). Higher *CMI* attributed by higher electrolytic leakage which was evidently

related to the lower wax content in the leaf tissues of *Alocasia. Xanthosoma* and *colocasia* exhibited lower *CMI* proportionate to their higher EW.

Leaves of Aroids family, *Colocasia*, in particular, usually experienced leaf blight disease caused by the fungal pathogen *Phytophthora colocasea* Racib (*Pc*). Fig. 7B shows the intensity of *in vitro Pc* infestation assayed using Evan's blue staining. The blue coloration showed the damage caused by *Pc*. *Xanthosoma* leaves showed less cellular disruption compared to *Alocasia* and *Colocasia*. Higher cellular damage was observed in *Colocasia* due to several cell wall constituents such as pectine, cellulose and hemicellulose.

On the other hand, the wax solubility might be another reason of rapid cellular depletion. However, the de-waxed leaves showed higher incidence when compared to waxed leaves which could be used to predict the role of EW on *Pc* prevention. Evidence of natural wax preventing disease incidence was reported by several authors[44,45]. Results showed that the presence of EW in leaf tissues sustainably inhibit electrolytic leakage which in turns defends the cellular damage caused by *Pc*.

4. Conclusions

Differences among epicuticular wax and its interaction between the qualitative and protective mechanisms in leaf tissues of three edible aroids, *Alocasia*, *Colocasia* and *Xanthosoma* were observed. *Colocasia* and *Xanthosoma* exhibited higher EW similar to lotus leaves which can be considered as the most pronounced edible wax rich terrestrial plants. Interestingly, *Colocasia* leaves showed superhydrophobic surface with higher contact angle and better wetting properties. Lower values of occurrence of EW showed negative impact on *SPF*, leaf chlorophyll content, moisture retention ability, prevention of electrolytic leakage and cellular disruption caused by invaders. In summary, the results of the study revealed that the leaf epicuticular wax coverage in aroids strengthens leaf epidermis and improve the physiological processes. The evidence provides further exploration of the wax structure and composition from the edible underutilized aroids to better understand its food, agricultural and industrial applications.

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Author contributions

FP participated in conducting the experiments, data analysis, interpretation and drafting the manuscript. MD participated in experimentation, data analysis, interpretation, reviewing the manuscript. VM validated the data and reviewed the manuscript. MRS conceived and designed the experiment, validated and interpreted the data, revised and reviewed the manuscript. All authors have viewed and approved the present form of the manuscript.

Competing interests

The authors have no competing interests.

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Legends to Figures

Figure 1A–C. Leaf ultrastructure of the aroid leaves (*Alocasia*, *Colocasia* and *Xanthosoma*) before extraction of epicuticular wax (A); Epicuticular wax content of the three aroids (B); Wax structure of the aroids (C).

Figure 2. Sun protection factor (*SPF*) of *Alocasia*, *Colocasia* and *Xanthosoma* at different wax concentrations.

Figure 3. Contact angle (*CA*) of *Alocasia*, *Colocasia* and *Xanthosoma* leaves under wax and dewax conditions.

Figure 4A–B. Chlorophyll content (SPAD value) [A] and chlorophyll stability index (*CSI*) [B] of *Alocasia*, *Colocasia* and *Xanthosoma* leaves under wax and dewax conditions.

Figure 5. Colour scheme (l^*, a^*, b^* values) of *Alocasia*, *Colocasia* and *Xanthosoma* leaves under wax and dewax conditions.

Figure 6. Relative water content (*RWC*) and moisture loss of *Alocasia*, *Colocasia* and *Xanthosoma* leaves under wax and dewax conditions.

Figure 7A–B. Cell membrane injury (*CMI*, Electrolytic leakage) [A] and *Phytophthora colocasiae* infectivity assay [B] of *Alocasia*, *Colocasia* and *Xanthosoma* leaves under wax and dewax conditions.

Video link: https://drive.google.com/file/d/1SlAchDLY1aveMY2A0PSjhvHoXyLSKHIB/view?usp=sharing