Helicoidal cell wall architecture of *Margaritaria nobilis* fruits

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The bright and intense blue-green coloration of the fruits of *Margaritaria nobilis* (Phyllanthaceae) was investigated using polarisation-resolved spectroscopy and transmission electron microscopy. Optical measurements on freshly collected fruits revealed a strong circularly polarised reflection of the fruit that originates from a cellulose helicoidal cell wall structure in the pericarp cells. Hyperspectral microscopy was used to capture the iridescent effect at the single-cell level.

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Keywords: structural colour; helicoidal structures; circular dichroism; cellulose

1 INTRODUCTION

In some plants, the cell walls of selected tissues exhibit helicoidal architecture, in which multiple adjacent wall layers are composed of aligned cellulose fibrils that rotate along a helical screw [1]. Despite this regular construction, considerable flexibility exists in the dimensions and geometry of the multilayered structure [2]. In the special case when the dimension of the helicoid, defined by the distance between two planes with closely similar fibril orientation (pitch p), is comparable with the light wavelength and is constant within the cell wall, these structures are capable of selectively reflecting light of specific coloration and polarisation. In particular, they reflect circularly polarised light at a wavelength defined by $\lambda = 2np$, (where n is the refractive index of the fibrils) and with handedness that depends on the handedness of the helicoid [3].

Helicoidal cell-wall architecture has been reported in a broad range of land plants, including mosses, ferns, gymnosperms and angiosperms [2, 4]. This apparently complex cell-wall structure occurs in tissues that include thick-walled cells [5], including epidermis, sclerenchyma and xylem, and in many different plant organs, including leaves, stems and fruits [1, 6–10]. For example, structural colour obtained from helicoidal architecture has been reported in leaves of plants from a range of different habitats, [11–14]. However, with a few exceptions (e.g. hazelnut [15], Pollia [10, 16]), this structure has rarely been studied in fruits and seeds, which often possess thick-walled tissues that are resistant to desiccation. Most fruit colour is produced by pigmentation [17], but a few plant species produce highly metallic and intensely coloured fruits by means of a nano-structured multilayered cell wall, including the commelinid monocot *Pollia condensata* [10, 16] and the rosid eudicot *Margaritaria nobilis* [18, 19].

In this paper, we use both polarisation-resolved spectroscopy and electron microscopy to present a detailed optical analysis of fresh fruits of *Margaritaria nobilis* (Phyllanthaceae), a forest tree from tropical Central and South America. In this species, the fruits possess a green exocarp, which splits after they become detached and fall to the forest floor [18, 19]. The remaining exposed in-

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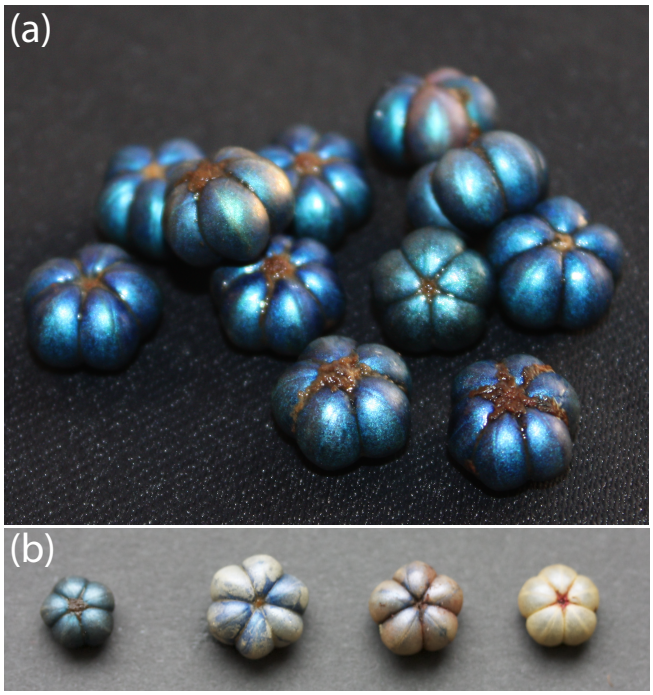

Figure 1: *(a)* Fresh fruits of *Margaritaria nobilis*. The intense metallic coloration of the fruits is the result of selective reflection from a helicoidal cellulose structure in the cell walls of the endocarp. *(b)* Fruits at successive stages of desiccation, from left to right: fully hydrated to dry.

ner part of the fruit wall exhibits a metallic greenish-blue colour, particularly in humid environments, that is attractive to birds such as jays and doves [18]. These birds consume the fruits and hence act as dispersal agents. This striking optical response has inspired material scientists to fabricate bio-inspired photonic fibres [19]. The results obtained here demonstrate that, similarly to the case of *Pollia condensata* [10], the strong intense coloration of *Margaritaria nobilis* fruits is due to a helicoidal cellulose structure in the endocarp cell walls. The optical measurements are confirmed by high resolution electron microscopy of the tissue showing Bouligand pattern typical of helicoidal architectures [20].

2 MATERIALS AND METHODS

2.1 *Plant Material*

For optical and microscopic analysis, fresh fruits were collected in Panama under permit $SEX/P - 59 - 13$ to Dr Edmund Tanner (issued 23/10/2013 by the Direccion de Areas Protegidas y Vida Silvestre). Fruits were refrigerated and then sent directly to Cambridge, UK. For examination using light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM), fruits were also obtained from the Royal Botanic Gardens, Kew, either from alcohol-preserved specimens (collected from Brazil by Milliken in 2011) or dried herbarium specimens from two separate collections, the first collected by Spruce in 1855, and the second collected by Belem and Mendes in 1964.

2.2 *Microscopy*

Optical imaging was performed using a customised Zeiss optical microscope equipped with epi-illumination and a 10× objective. Unpolarised light from a halogen lamp served as illumination for imaging. A polariser and a quarter-waveplate mounted onto independent motorised rotation stages were selectively inserted into the optical path to perform polarisation-resolved imaging.

For SEM imaging, dried fruit material was fractured, mounted on an aluminium stub, coated with platinum using a sputter coater (Emitech K550), and examined using a Hitachi S-4700 SEM at 2 kV.

For TEM imaging, fruits were cut into small fragments and fixed in 3% phosphate-buffered glutaraldehyde followed by immersion in 1% osmium tetroxide. Fixed samples were taken through a graded ethanol and LR white resin series prior to embedding in an epoxy resin. Ultrathin sections (50–100 nm) were cut using an ultramicrotome (Reichert-Jung Ultracut) and collected on formvar-coated copper slot grids. Initial results using post-staining with uranyl acetate and lead citrate (as used for fruits of *Pollia condensata*, [10]) failed to reveal a helicoidal ultrastructure, which could only be resolved when these stages were omitted, see Supplementary Figure 1. Samples were imaged in a Hitachi H-7650 TEM equipped with an AMT XR41 digital camera.

2.3 *Spectroscopic Characterisation*

Reflectance spectra of the fruit surface were measured on a microscopic scale (spot size: ≈ 10 µm) which allowed the collection of optical signals from individual cells. The halogen lamp of the microscope served as light source in bright-field configuration. Light reflected from the sample passed back into the objective and was coupled in confocal configuration with a 100 µm core optical fibre connected to a spectrometer (QE65000, Ocean Optics, 200 – 880 nm). The reflection spectra were normalised with respect to a Silver mirror (Thorlabs). Spectra and images were collected using unpolarised illumination and a circularly polarised filter for right-handed (RH) and left-handed (LH) light detection. The Hyperspectral images were collected in the same configuration using an additional liquid crystal filter (CRI, Varispec) that was inserted in front of the CCD imaging chip.

3 RESULTS

3.1 *Fruit Anatomy*

Each fruit of *Margaritaria nobilis* consists of several (4?6) segments, each containing a single seed, (Figures 1 and 2). The entire structure is enclosed in a pericarp that consists of two layers: an outer papery exocarp that dehisces at fruit maturity and an endocarp

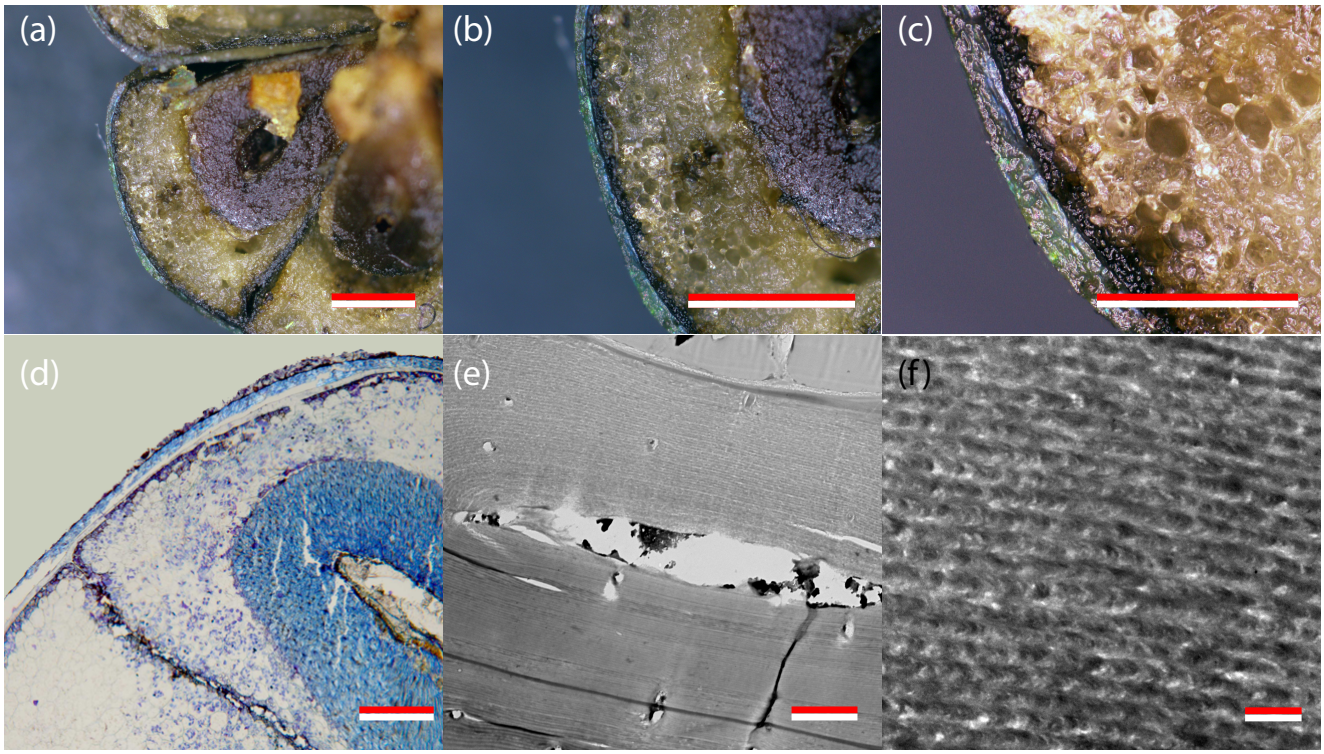

Figure 2: Anatomy of *Margaritaria nobilis* fruit. *(a,b,c)* Transverse section of a fresh fruit shown at different magnifications. *(d)*ransverse section of fruit stained with toluidine blue. The pericarp is the light blue outermost layer. *(e,f)* EM transverse sections of wall of a single pericarp cell, with multilayered structure visible in *(e)*, and Bouligand arch pattern, the fingerprint of helicoidal cell-wall architecture, visible at higher magnification in *(f)*.Scale bars: 1mm in *(a)* and *(b)*, 0.5 mm in *(c)*, 200μm in *(d)*, and 4μm in *(e)* and 0.5μm in *(f)*.

consisting of c. 4?5 layers of thick-walled cells (Figure 2; see also figure 1 of [18]). The endocarp is about 1mm thick, and the average thickness of the cell wall is about $10 - 15\mu$m. When the fruit is fresh or well hydrated the colour of the remaining fruit is metallic blue or green. Fruits have a more pearlescent white appearance when they are completely dry (Figure 1(b)). Transverse sections of fresh fruits (Figure 2(a,b,c)) show that the blue-green coloration of the fruits comes from the endocarp, which consists of thick-walled cells (Figure 2, (d),(e),(f)). When the fruit is fresh, the seeds are hydrated and adhere perfectly to the endocarp. In the dry state, the seeds shrink, and the endocarp is separated from the seeds by an air layer that prevents light absorption and therefore decreases the contrast and the hue of the structural coloration [21]. TEM cross-sectional imaging shows the multilayered helicoidal architecture of an endocarp cell wall (Figure 2). At low magnification, the structure appears as a simple multilayer (Figure 2(e)). However, with higher magnification and resolution (Figure 2(f)), the Bouligand arch pattern is visible and the twisting of the individual cellulose microfibrils organised in a helicoidal structure is clearly recognisable. The fact that some preserved or fresh material failed to show the helicoidal structure, which is also much less obvious than in *Pollia condensata* [10], could be partly due to relatively loose packing of the microfibrils and/or to a different mixture of secondary polysaccharides in the cell wall.

3.2 *Optical Characterisation*

Figure 3 shows the optical response of a fresh fruit illuminated at different polarisation configurations. In particular, *(a)* shows an optical micrograph of the fruit without any polarisation filter either in collection or illumination. In this image, the colour reflected from the cell wall and an additional reflection that originates from the air-fruit interface can be observed. In the cross-polarisation configuration (illuminating polarising light and collecting with linear polarisation perpendicular to the illumination), the colours are still visible and their contours became sharper because we collect only the signal that comes from the multilayered structure. This result already indicates the presence of circular dichroism, and excludes the possibility that the fruit structure behaves as a standard multilayer as previously hypothesized [19]. The helicoidal nature of the optical response became clear when using circularly polarised filters. In this configuration, the colour is observed only in the left-handed (LH) circular polarisation channel, and only a small amount of light is observed in the right-handed (RH) channel, probably scattered from inner cells tilted with respect to the surface of the fruit. It is interesting to note that here all cells reflect only left-handed circularly polarised light, in contrast with cells of *Pollia con-*

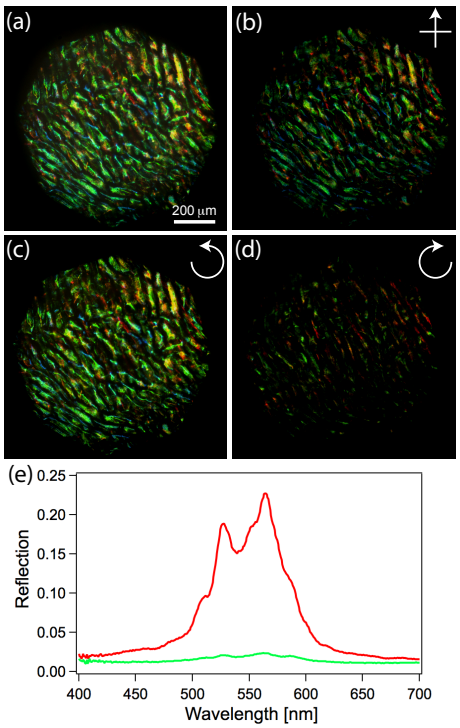

Figure 3: Optical response of *Margaritaria nobilis* fruit. *(a)* Micrograph picture obtained using a 10× magnification objective in epi-illumination without any polarisation filter, the same area is also imaged in cross polarisation channel *(b)*, and in left *(c)* and right *(d)* circular polarisation configuration cahnnels. The two spectra in figure *(e)* are collected from the same area in left (red line) and right (green line) circular polarisation channels.

densata, in which both handednesses were observed [10]. Bright field spectra taken at the single-cell level using a 20X magnification objective are reported in Fig.3*(e)* and*(e)*, respectively. In the left polarisation channel (red line) different intense peaks are visible in the spectral region between 500 and 550 nm. In the opposite channel, only a wavelength-independent response of about 2% is recorded. This difference is the result of the specular reflection from the interface between air and the outer layer of the endocarp, suggesting that its refractive index is about 1.5.

In order to capture iridescence at the single cell level we investigated the fruit using hyperspectral-microscopy. The shape of the epidermal cells of *Margaritaria nobilis* can be approximated as cylinders. As observed by [19], when illuminating the cells with an objective with Numerical Aperture NA=0.45, the light reflected from different cells has different colours, due to the geometry of the cells. This effect, typical of every multilayer structure with spherical or cylindrical geometry, reveals the iridescent nature of the colour, as reported in Fig. 4. However, the effect is averaged out when the fruit is illuminated with diffuse light, and the iridescence disappears, leaving only an intense 'metallic' coloration. See also Supplementary Figure 2.

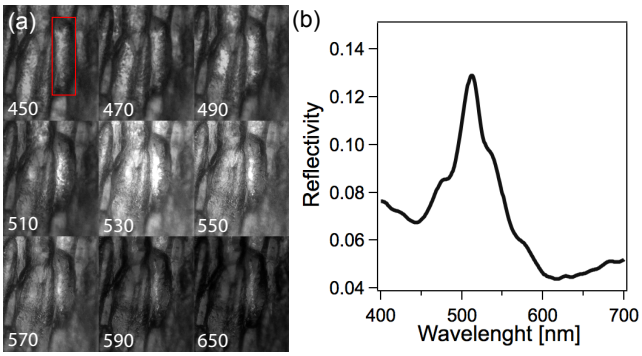

Figure 4: Iridescence at the single cell level in *Margaritaria nobilis* fruit measured using hyper-spectral microscopy. *(a)* Image sequence (from top to bottom and right to left) is obtained in epi-illumination using a 20× magnification objective (NA=0.45) without any polarisation filter in illumination and with a coloured liquid crystal filter before the camera. The same area is imaged changing the transmission wavelength of the liquid crystal filter (450-470-490-510-530-550-570-590 and 650 nm) with intervals of 10 nm. *(b)* Spectrum measured from cell highlighted in *(a)*. All the images in Figure *(a)* are normalised with respect to the spectrum reported in *(b)*.

4 DISCUSSION

Our results demonstrate that the intense blue-green coloration of the fruits of *Margaritaria nobilis* is a structural effect resulting from a helicoidal cellulose structure in the multi-layered cell walls of the pericarp. The results of our optical measurements are confirmed by high resolution electron microscopy of the tissue showing Bouligand pattern typical of helicoidal architectures [20]. The chiral nature of the optical response of the fruit of *Margaritaria nobilis* resembles that of the fruit of *Pollia condensata*, except that in *Margaritaria nobilis* only left-handed polarisation is reflected, while both LH and RH circular polarisation can be measured in *Pollia condensata* [10].

These two species are relatively distantly related among flowering plants: textitPollia condensata is a commelinid monocot and *Margaritaria nobilis* is a rosid eudicot. Therefore, the helicoidal structural response in the fruits of these two species is clearly a example of convergent evolution of metallic fruit colour. Both species produce fruits lacking soft tissues, and therefore offer little nutritional reward to potential seed-dispersers (Cazetta:2008co, 2012PNAS..10915712V). Although the diversity and evolution of fruit colour remains imperfectly understood [17], some studies suggest that brightly coloured non-nutritious fruits are likely to be mimetic, where the plant deceives potential dispersers such as birds by mimicking the colour of other species with fleshy nutritious fruits that grow in the same habitat [18]. This form of mimicry may allow efficient seed dispersal without the energetic cost of providing a food reward to the disperser.

Interestingly, a related example of helicoidal archi-

tecture facilitating seed dispersal occurs in some plant species with mucilagenous seed coats that adhere to passing animals. For example, in the seed coat of quince, the outer cell layers possess helicoidal thickenings that produce a slime consisting of scattered microfibrils that result from unravelling helicoidal arrays[22]. Future studies on the internal geometry of cell walls in a diverse range of plant tissues could provide further clues concerning the construction and properties of this highly ordered and multifunctional cell-wall architecture.

5 CONCLUSIONS

In conclusion, our study provides a detailed correlation between the anatomy of the fruit of *Margaritaria nobilis* and its optical response. Our results demonstrate that, as in the case of *Pollia condensata* [10], the intense blue-green coloration of this fruit is a structural effect resulting from a helicoidal cellulose structure in the multi-layered cell walls of the pericarp. This helicoidal architecture is common in plant tissues [7], and interestingly, a related example of helicoidal architecture facilitating seed dispersal occurs in some plant species with mucilagenous seed coats that adhere to passing animals. Future studies on the internal geometry of cell walls in a diverse range of plant tissues could provide further clues concerning the construction and properties of this highly ordered and multifunctional cell-wall architecture. However, even though the development of such structures in nature has not yet been undestood, material scientists have been inspired by such bright coloration and bio-inspired photonic fibres [19] and films [23] have been produced using different strategies.

Acknowledgments

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Supplementary Material

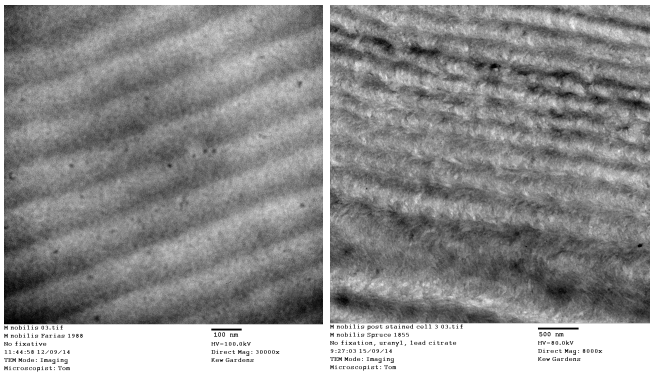

Figure S 1: TEM cross sectional images of *Margaritaria nobilis* fruit: (a) post stained cell wall of fresh *Margaritaria nobilis* specimen from herbarium compared with (b) specimen collected by Spruce in 1855.

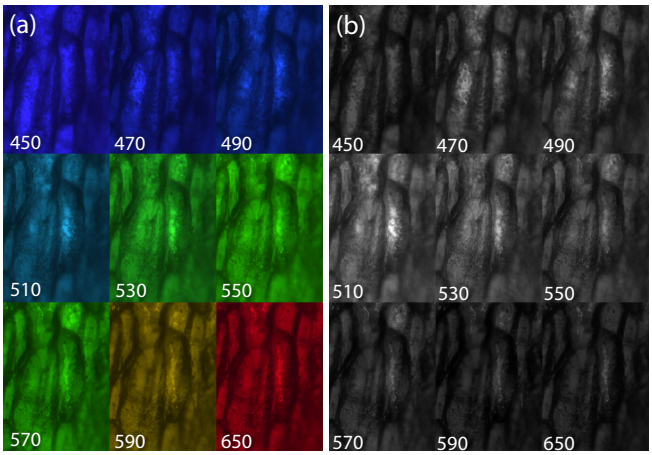

Figure S 2: Raw data on the iridescence on the single cell level of *Margaritaria nobilis* fruit obtained with hyperspectral microscopy as shown in Figure 4 *(a)*. Set of micrograph pictures obtained with a 20× magnification objective (NA = 0.45) in epi-illumination with the liquid crystal filter in real colour. Processed image as reported in Figure 4*(a)* where the averaged intensity is normalised with respect the spectrum reported Figure 4*(b)*.