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Waxes from the Mat Sedge-*Cyperus pangorei* Rottb.

[1]J.F. Benazir, [2]V. Manimekalai and [3]P. Ravichandran
[1]School of Biotechnology, Dr. G.R. Damodaran College of Science,
Civil Aerodrome Post Coimbatore-641014, India
[2]Department of Botany, Sri Parasakthi College for Women, Courtallam-627 802, India
[3]Laboratory of Developmental Biology and Plant Biotechnology,
SPK Centre for Excellence in Environmental Sciences,
Manonmaniam Sundaranar University, Alwarkurichi-627 412, India

Abstract: *Cyperus pangorei* is a sedge extensively and exclusively used in silk mat weaving. It is a plant that grows along river banks and canals in rice fields and is considered as a weed, expect for its use in silk mat weaving. The objective of the present study was to analyze the chemical composition and wax micro morphology on the culms (stems) of *Cyperus pangorei* to decipher its role in silky texture of the mats and to know the abundance of waxes that may find use in commercial applications. Scanning Electron Microscopic (SEM) and GC-MS studies revealed several new chemical entities and their accumulation as cuticular wax layers. The analysis of components and morphological features of epicuticular waxes have provided information on four different classes of waxes and one very long chain wax ester-nona-hexacontanoic acid ester (C_{69}). The epicuticular waxes determined in this sedge also form first of its kind in Cyperaceae. The wax morphologies observed over the culm do not vary among the different culm regions. Wax morphologies such as thick amorphous film, fissured layers, thick crusts, platelets with orthorhombic symmetry and granules were observed.

Key words: *Cyperus pangorei*, culm, epicuticular waxes, micro morphology

INTRODUCTION

Plant surfaces are impregnated with waxes that are made by the epidermal cells. Plant waxes act as a protective barrier against water, UV, pathogens and insects could be protecting plant from the environment (Bargel *et al*., 2006). In addition, they are valuable raw materials for a variety of industrial applications. Wax mixtures obtained from different plant sources have different chemical compositions that in turn determine their physical properties and therefore are of immense value in industries. Waxes are mixtures of lipophilic compounds that are solid at room temperature, transparent to opaque and are ductile and easy to polish. Due to their special properties, waxes find applications in industries such as lubricants, adhesives, coatings, sealants, impregnation materials, adjuvants in formulations of bioactive compounds, automobiles, textiles, paper and specialty inks, pesticides, biofuels, candles, plastics and wood plastic composites, furniture, shoe polishing, house hold cleaners, cosmetics, dental treat products, drugs and food (Illmann *et al*., 1983).

To meet the demand for such industrial applications, waxes are currently chemically synthesized or obtained from living organisms (Illmann *et al*., 1983). The most important plant sources for commercial production of waxes are carnauba (*Copernicia cerifera*), candelilla (*Euphorbia cerifera*), ouricuri (*Syagrus coronata*), sugarcane and jojoba (*Simmondsia chinensis*). All these waxes are relatively rich in aliphatic esters varying in chain lengths of both acyl and alkyl groups that contain mixtures of cinnamtes, hydroxy esters, steryl esters, estolides and alkanes (Basson and Reynhardt, 1988; Holloway, 1984; Illmann *et al*., 1983; Jetter and Kunst, 2008).

Cyperus is one of the largest genera in Cyperaceae with 650-700 species spread all over the world; of these eighty species occur in India (Simpson *et al*., 2003). *Cyperus pangorei* (mat sedge) is especially used in making of the World famous Pathamadai silk mats from India (Ravichandran *et al*., 2005). *Cyperus pangorei* Rottb. is distributed all over India, Ceylon, Nepal and Burma (Mathew, 1991; Mabberley, 2005). It is both pantropical and temperate in distribution (Haines and

Corresponding Author: J.F. Benazir, School of Biotechnology, Dr. G.R. Damodaran College of Science, Civil Aerodrome Post Coimbatore-641014, India

Lye, 1993; Tucker, 1983). *Cyperus pangorei* was previously known as *C. tegetum* Roxb., *C. dehiscens* Nees, *Papyrus pangorei* Rottb. and *Papyrus dehiscens* Nees. It is a perennial; rhizome decumbent, 3-7 mm thick sedge, clothed with brown scales; roots light brown, 0.5-0.7 mm thick. Culm laxly tufted or arranged in a row along the rhizome, subaphyllous, stiffly erect, 70 - 270 cm tall, 3-20 mm thick, acutely triquetrous with slightly concave sides, dark green and smooth throughout (Fig. 1). Leaves reduced to 4-6 subaphyllous sheaths surrounding culm (Dassanayake and Fosberg, 1985). However, until recently, *C. pangorei* as a commercial plant has remained barely studied. Nevertheless, the interest in this crop seems to be increasing worldwide for its importance in the making of Pathamadai Silk Mats (Amalraj, 1990; Benazir, 2000; Balaji, 2005; Venkatesan, 2005).

In this regard the distribution and types of cuticular waxes were studied in various regions of *C. pangorei* culm. The objective of the present investigation was thus to analyze the chemical composition, type and micro morphology of epicuticular waxes in sedge culms that could find an important place in industrial applications.

MATERIALS AND METHODS

C. pangorei plants were collected from the wild along rivers of Tirunelveli District., Tamil Nadu, India and maintained in the department nursery at near natural growth conditions. The waxes were extracted from the surface of the culms and three regions were selected for wax analysis based on light microscopic examination for deposition of wax, viz., the lower most 2 inches of the culm, the middle 2 inch and the topmost 2 inch of the culm.

Wax extraction and analysis: The cuticular components were extracted in chloroform as described by Rhee *et al.* (1998). The extracted wax samples were characterized by GC-MS (Gas Chromatography-Mass Spectrometry) analysis at SITRA (South India Textile Research Association) laboratories, Coimbatore. The lyophilized waxes were analysed using Fison 8000 series Gas chromatography with Fison MD 800 Mass Selective Detector. The column used was DBMS (30-meter length and 0.25 μm diameter). The carrier gas was Helium with a flow rate of 1.5 m min^{-1}. The injector temperature was 270°C with the transfer line at 175°C. Splitless mode was used with an oven program at 50°C, which was held for 2 min and steadily increased to 130°C at the rate of 9°C min^{-1} and then to 280°C with a steady increase of 4°C min^{-1}. This temperature was held for 5 min. Compounds were identified with mass spectra of NIST95 database. For MS the conditions were full scan mode with a mass range 50-500 m/z and EI positive, ionization mode was used (Rhee *et al.*, 1998). The several components obtained were grouped into major classes using their chemical formula and the percentage of each class was calculated by taking average and the results were tabulated.

Scanning electron microscopy: Scanning electron microscopic pictures were taken from all the three regions of the culms. The plant material was mounted on aluminium holders, sputter coated with gold-palladium (Bal-Tec SCD005 Sputter Coater; 25 mA, 300 sec; Balzers, Switzerland) and examined by SEM (Zeiss DSM 962, 15 kV; Zeiss, Oberkochen, Germany). The sputtering conditions, depositing approximately 20 nm of the alloy on the samples, were optimized for the acceleration voltage used in the SEM (Reidel *et al.*, 2003). Simple statistical analysis like Students't' test and ANOVA were performed to interpret the results and their significance.

RESULTS AND DISCUSSION

The chemical composition and physical properties of the cuticle and classification of epicuticular waxes has remained under investigation by several biologists and chemists that has resulted in fascinating significant findings. The present study on the chemical composition and morphology of epicuticular waxes of *Cyperus pangorei* are first of its kind in sedges and reveal several wax entities and micro morphologies when observed under SEM.

Chemical composition of epicuticular waxes: GC-MS analyses of cuticular waxes revealed that *C. pangorei* culms contain several aliphatic waxes and some particularly abundant major compounds given in Table 1. The kind and quantity of waxes differ from region to region. The major wax classes include alkyl esters, n-alkanes, fatty acids and primary alcohols, however, the most abundant compounds are heptacosanoic acid methyl ester, dodecatrien-1-ol and 1-dotriacontanol.

Wax extracts from lowermost surface region of the culms were dominated by alkanes of chain length between C_{18} to C_{44}. The n-alkanes represented 17.5% of total epicuticular waxes from lowermost region of which tritetriacontane was the most copius followed by octacosane. Although, both odd and even number alkanes were present, even number alkanes were most abundant (C_{18} to C_{44}) and contributed 57.2% and odd number alkanes (C_{15} to C_{43}) made up 42.8% of the 17.5% of

Table 1: Epicuticular wax compound classes of *Cyperus pangorei* culms and processed culm strands

Culm region	n-alkanes	Alkyl esters	Fatty acids	Alcohols	Others
Lower most	17.5%	73.75%	0	0	8.75%
	C_{18}-C_{44}	C_{13}-C_{43}			
Middle	15%	50%	0	15%	20%
	C_{12}-C_{43}	C_{15}-C_{69}	0	C_{15}-C_{50}	
Upper most	17.5%	51.25%	12.5%	7.5%	11.25%
	C_{12}-C_{44}	C_{14}-C_{69}	C_{10}-C_{22}	C_{16}-C_{50}	
Processed culm strands	20%	66.25%	1.25%	7.5%	5%
	C_{12}-C_{44}	C_{16}-C_{69}	C_8	C_{10}-C_{50}	

Statistical analysis-students t test

Region of culm	't' value	Significant difference
Lower and Middle	0.320	Yes
Lower and Top	0.318	Yes
Lower and Processed	0.499	Yes
Middle and Top	0.220	Yes
Middle and Processed	0.294	Yes
Top and Processed	0.5	Yes

Degree of freedom: 8, For degree of freedom 8 at 5% level of significance the't' value is 2.30, Values lower than this show statistically significant differences

the total alkanes. Alkyl wax esters contributed to major type of constituents of the total epicuticular waxes of the lower most culm regions i.e., about 73.75% of the total waxes. Alkyl esters were of the chain lengths between C_{13} and C_{43} and comprised of alkane esters, fatty acid esters and carboxylic acid esters. Fatty acid esters were up to 50% of the total wax esters. The most predominant fatty acid ester was heptadecanoic acid methyl ester (C_{19}). No fatty acids, alcohols and sterols were detected in this region.

The middle region of the culm comprised mostly of very long chain alkyl esters of chain lengths between C_{15} to C_{69}, followed by alkanes (15%) chain lengths C_{12} to C_{43} and fatty alcohols (C_{15} to C_{50}, 15%); both primary and secondary alcohols were detected. No fatty acids were present and other components like sterols, triterpenoids, alkenes, thiols, ethers and benzopyrones made up the remaining 20%. The most predominating component in middle region of the culm was secondary alcohol; Dodecatrien-1-ol followed by heptacosanoic acid methyl ester and 1-Dotriacontanol. Unlike the lowermost region, wax esters made up only about 50% and were also characterized by the presence of fatty alcohols and sterols. In addition a very long chain wax ester nona-hexacontanoic acid (C_{69}) was detected. The presence of such a very long chain component in plant waxes has not been reported hitherto. Among the alkanes, even chain alkanes were predominant (C_{12} to C_{32}) similar to that of the lower most region. Alcohols were more abundant in the middle region (15%), all being primary alcohols with only Dodecatrien-1-ol and cyclohexanol being secondary alcohols, the primary alcohols were 1-dotriacontanol, 1-triacontanol and 1-pentacontanol, respectively.

Cuticular waxes of the top region were more or less alike, predominated by alkyl esters (C_{14} to C_{69}, 51.25%) followed by alkanes (17.5%, C_{12} to C_{44}), fatty alcohols (2.5%, C_{16} to C_{50}) and fatty acids (12.5%, C_{10} to C_{22}) and other trace components like benzopyrones and alkenes as given in Table 1. Fatty acids have been observed only in the uppermost culm region. The most abundant components of total waxes were 1-Dotriacontanol, followed by pentadecanoic acid, heptacosanoic acid methyl ester and tridecanol. The ratio of even chain alkanes was high (71.4%) when compared to odd chain alkanes (28.6%). Fatty acids present include octadecanoic acid, tetradecanoic acid, tridecanoic acid, decanoic acid, eicosanoic acid, hexadecanoic acid, docosanoic acid, pentadecanoic acid and heneicosanoic acid. Primary alcohols were the most abundant of the alcohols and traces of the secondary alcohols such as cyclohexanol were present. No sterols were detected.

In general, the chain lengths remained consistent in all three regions of the culms: alkanes (C_{12} to C_{44}), wax esters (C_{14} to C_{69}; C_{69} absent in the lowermost) and alcohols (C_{15} to C_{50}) in middle and upper most regions. All three different culm regions were abundant in wax constituents with statistically significant differences in major compound classes (Table 1).

Although epicuticular wax analysis has been extensively studied in dicotyledonous plants (Jeffree, 1996; Dragota and Reiderer, 2007), there are only few studies on monocotyledonous plants such as Hordeum, wheat, sorghum (Tarumoto, 2005; Gorb *et al.*, 2005), *Allium, Gloriosa, Yucca* and *Strelitzia* (Barthlott *et al.*, 1998) and none on sedges.

Wax esters are the most abundant components in *Cyperus pangorei* and have also been reported to be prevalent in other monocotyledonous species such as *Musa paradisiaca* (Baker *et al.*, 1982), rice, barley (Richardson *et al.*, 2005), maize and sorghum (Gorb *et al.*, 2005). The lowermost region of the culm comprising young tissues is also abundant in waxes and rich in the chemical composition. This illustrates that cuticle formation parallels cell expansion as also in the case of grasses. However, the very long chain fatty acid ester nona- hexacontanoic acid (C_{69}), alcohols and sterols were present only in the mature (middle and upper) regions of the culm, which negates the previous hypothesis that onset of wax deposition occurs at a very early age and developmental stage, however the amount and composition of waxes differ from one plant organ to another with changes during development, season, location and other environmental factors (Jenks *et al.*, 1995; Shepherd and Griffiths, 2006).

Wax morphology: Light microscopic studies revealed that an exceptionally thick cuticle bounded the epidermis measuring c. 100 μm and c. 150 μm, respectively

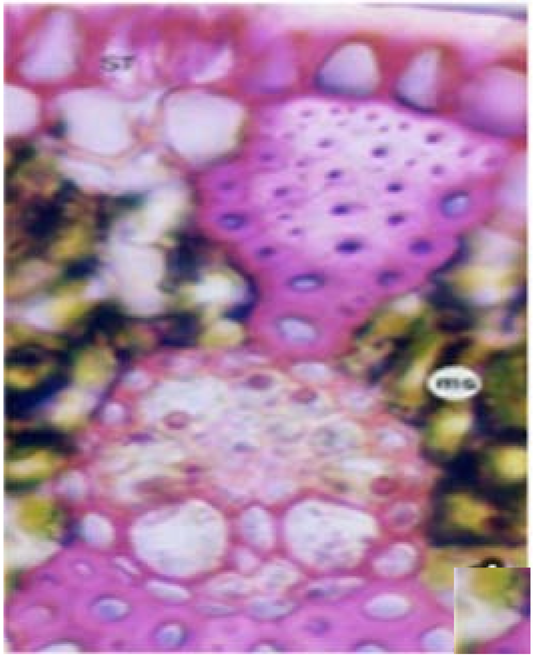

Fig. 1: A light microscopic view of the transverse section of the culm of *C. pangorei,* stained with safranin, showing a very thick cuticle with tooth shaped epidermal cells

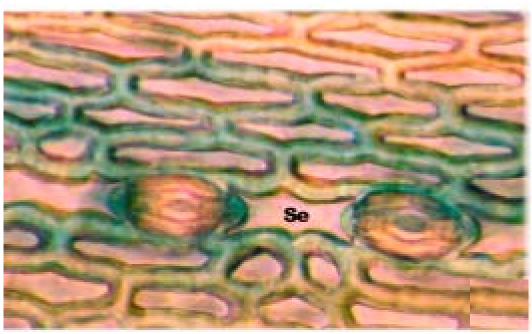

Fig. 2: The epidermal peel of the culm of *C. pangorei* showing a large amount of stomata with thick wax covering; Stain- Malachite green

(Fig. 1 and 2). SEM examination on culm surface exposed the presence of a thick wax layer (Fig. 3 to 8). Such wax covers the surface as an amorphous film, with irregularly shaped crystals, fissured layers (Fig. 3), granules (4), thick crusts (Fig. 5) and plates (Fig. 6). Abundant wax crystals were found near the stomata both on the dorsal and ventral sides (Fig. 7, 8).

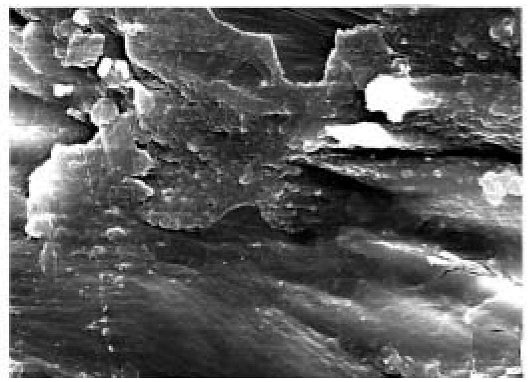

Fig. 3: An amorphous layer of wax on the surface of the culm with irregular crystals and fissured layers

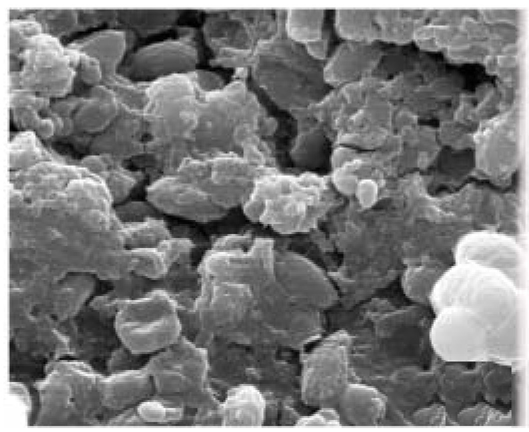

Fig. 4: Wax globular structures on the surface of *C. pangorei*

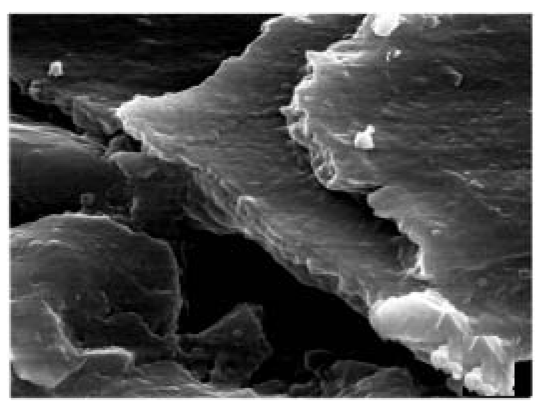

Fig. 5: Very thick wax crusts with characteristic morphology

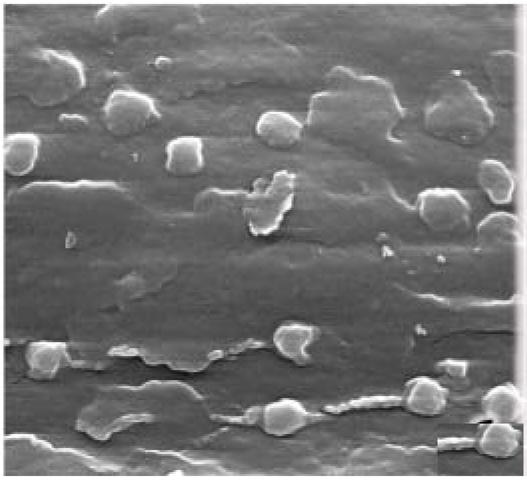

Fig. 6: Wax platelets with orthogonal symmetry

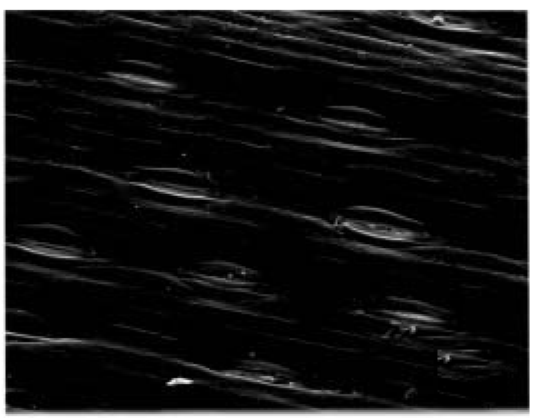

Fig. 7: Dense wax deposition and amorphous film on the stomata-on the surface

The epicuticular wax coverage of sedge culm is dense and non-uniform. It consisted of two superimposed layers of waxes, the upper that had crystalline structures and the lower, which was a thick amorphous film. The wax crystals varied greatly in size and form (Fig. 4). No projecting stalks were found. In the lower most region although the crystals were very densely arranged, they remain separated and do not form a particular network (Fig. 3). Dense aggregates and granules were abundant with a characteristic pattern (Fig. 4). These crystals were found attached to a thick amorphous film of wax and also around stomata with wax crystalline structures surrounding the stomatal pore. Very thick layers of crusts and fissured

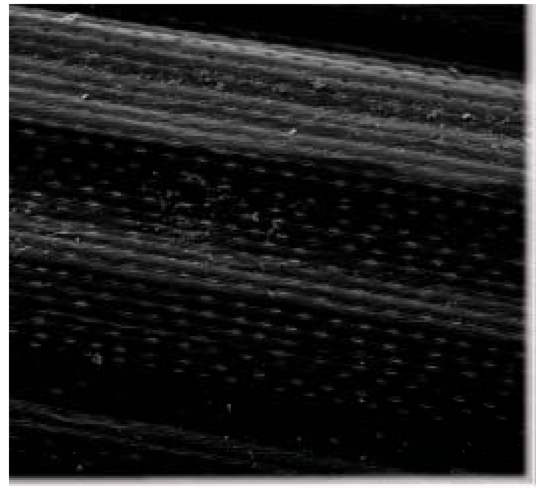

Fig. 8: Dense wax deposition and amorphous film on the stomata- inner side of epidermis

layers were not to be seen in the lower most region and the crystals appeared as if they were in the assembly process. The middle and upper region of culms consisted of very compact fissured layers and thick crusts with sculptured appearance (Fig. 5). Very thick waxy plates with orthorhombic symmetry and fissured layers were predominant (Fig. 6).

Assembly of epicuticular waxes exhibit great diversity and are determined by a principle component/dominant class or otherwise the total wax composition (Barthlott *et al.*, 1998; Meusel *et al.*, 2000; Ensikat *et al.*, 2006). The amorphous film of wax assemblage is attributed to the predominance of alkyl esters, alkanes and alcohols. Such films have also been reported from *Citrus* and *Pinus* (Jenks *et al.*, 1995). The alkanes form a plain layered structure whereas molecules with terminal polar groups like fatty acids and primary alcohols crystallize in a double layer structure (Koch and Ensikat, 2008). The wax films appear to be ubiquitous while thick layers and crusts are rare. Crusts have been reported from all major groups of plants including *Cynanchum sarcostemma, Copernicia cowellii and Buxbaumia viridis* (Barthlott *et al.*, 1998; Koch *et al.*, 2009). The formation of crusts over the amorphous film is mainly due to the presence of alkyl wax esters. The abundance of wax esters *Cyperus pangorei* culms may be contributing to the very thick crusts in the middle and upper regions.

Wax plates with orthorhombic symmetry exhibit one of the most common micro morphology in all type of plants and vary considerably in shape, chemical composition and spatial pattern (Barthlott *et al.*, 2003).

The orthorhombic symmetry observed in *C. pangorei* is similar to that of *Yucca* platelets (Koch *et al*., 2006). Even though the wax micro morphologies are implicated by the presence of several types of chemical constituents, the actual chemical composition of any individual crystal is not known.

The wax morphology is also influenced by environmental conditions during the crystallization process and also by the cuticle. Wax deposition is also further influenced by humidity, salinity, cold stress, mechanical stress, altitude and pollution (Shepherd and Griffiths, 2006).

Because of their unique composition, some of the wax esters from natural sources have proven to be important commercial commodities. All plant waxes are harvested more or less directly from plant surfaces, except for jojoba seeds. Plant cuticular waxes are therefore chemically much more diverse than all the other wax sources and this greater chemical diversity goes hand in hand with the variations in wax physical properties that are desirable for industrial applications.

CONCLUSION

The study reveals the rich diversity of waxes in the culms of the sedge *Cyperus pangorei*. The wax micro morphology demonstrates the fact that the chemical composition influences wax crystal patterns. The enormous amount of waxes as crystals on the surface of the culm may have a role in giving the silky texture to the mats. Further, the abundance and chemical diversity of waxes in *Cyperus pangorei* may be exploited as an attractive source for important commercial applications.

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