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Analysis of n-alkanes in leaf epicuticular wax of three cultivars of winged bean [*Psophocarpus tetragonolobus* (Stickm.) DC.]

Sunanda Ray[1], Jagatpati Tah[1] and Amalendu Sinhababu[2]*

[1]Cyto-genetics and Molecular Biology, Centre for Advanced Studies in Botany, Department of Botany, The University of Burdwan, Burdwan-713104, W. B., India
[2]Natural Product Laboratory, Department of Chemistry, The University of Burdwan, Burdwan-713104, W. B., India

Abstract : The plant cuticle, the protective waxy covering over the aerial parts of the higher plants represents specific pattern. Leaf epicuticular waxes of fresh and matured leaves of three cultivars of winged bean [*Psophocarpus tetragonolobus* (Stickm.) DC.] were extracted in n-hexane and have been analysed for Thin Layer Chromatography (TLC), Infrared Spectroscopy (IR), Gas Chromatography (GC) and Scanning Electron Microscopy (SEM). The study of epicuticular wax in those cultivars showed remarkable variations. Leaf cuticular wax mainly consists of straight chain aliphatic hydrocarbons with a variety of substituted groups. Scanning Electron Microscopic studies of the leaf cuticular layers and the extracted hydrocarbons revealed specific patterns in three cultivars studied. Among the three cultivars, amount of n-alkane is comparatively high in EC38821B, but low in EC27886. Nineteen n-alkanes from n-C_{16} to n-C_{35} (except n-C_{21}) have been found varying in composition in the mature leaves of three cultivars studied. The predominant n-alkanes were C_{29} and C_{31} while C_{20} and C_{26} were only present in minor amounts. The study may serve as a tool in chemo taxonomical work, along with other characteristics may be useful to identify the cultivars.
Keywords : Epicuticular wax, GC, n-Alkanes, Winged bean, SEM.

Introduction

Winged bean [*Psophocarpus tetragonolobus* (Stickm.) DC.] is significantly important as a high protein multipurpose legume. Plant is taller and larger than common bean. The tuberous rooted twining annual vine bears clusters of purplish flowers and pods with four jagged wings. Leaves are trifoliate. The versatile, but neglected winged bean is a highly nutritious, easy to grow legume with edible flowers, pods and leaves. It has similar nutritional profile to soybeans, but contains more calcium[1, 2]. The aerial parts of the terrestrial plants are covered by cuticle. It is primarily composed of cutin, a polyester matrix of hydroxyl and hydroxyl epoxy C_{16} and C_{18} fatty acids[3] which is overlaid and embedded with long chain hydrocarbon waxes e.g. primary and secondary alcohol, aldehydes, alkanes, ketones, and fatty acids[4]. Epicuticular wax prevents uncontrolled water loss or uptake through the epidermis[5-7]; protects the plant parts against UV radiation[8]; defends against attack by pathogens[9-11] and insects[12]. Cuticular wax also controls surface wettability by reducing water retention on the plant surfaces[13-15].

The amount of epicuticular wax or bloom[16-17] varies from species to species, cultivar to cultivar[18] parts of a particular plant and also it differs from one season to another. Deposition of wax on the plant surface creates specific ornamentation pattern which might be useful in chemotaxonomy[19-21].

The aim of the present study was to characterize n-alkane profile of the leaf epicuticular waxes and also to establish the variation among the cultivars by TLC, IR, GC and SEM techniques. The study was the continuation of the work done in seven cultivars of winged bean[18].

Materials and methods

General Procedure

Seeds of three cultivars of winged bean [*Psophocarpus tetragonolobus* (Stickm.) DC.] were collected from the Regional Station of Plant Genetic Resources (NBPGR) at Akola (Maharashtra, India). Those were EC38821B (H), EC27886 (I), and IC95227 (J). Healthy seeds of those three accessions were sown at the experimental field of Crop Research Farm (23°53'N latitude and 83°25'E longitude), Department of Botany, The University of Burdwan, West Bengal, India. Fresh and mature leaves (100 g) of each cultivar were collected during the winter season (December, 2011). Leaves were rinsed with distilled water and dried by paper towel. Dried leaves were then dipped in 2L of n-hexane (AR Grade, SRL, India) for 45 min at room temperature. The crude extracts were then filtered through Whatman (Maidstone, UK) filter paper No. 41 and the solvent was removed under reduced pressure. The total extract in each case was weighed after complete removal of the solvent (Table 1). The extract was then fractionated through preparative thin layer chromatography (TLC) using carbon tetrachloride (AR Grade, Merck, India) as the mobile phase. The TLC plates (0.5 mm thick) were prepared with silica gel G (Merck, India) using Unoplan coating apparatus (Shandon, London). The hydrocarbon band (R_f =0.9) was identified through co-TLC studies with standard hydrocarbon samples (Sigma, USA). The single hydrocarbon band was eluted from the layer with chloroform (AR Grade, SRL, India) and it showed no absorption for any detectable functional group in the infrared region (IR).

The purified hydrocarbon fraction was analysed directly by GC on a Hewlett Packard (HP; Palo Atto, CA, USA) Agilent 6890 Plus instrument fitted with a HP-5 capillary column (30 m × 0.32 mm i. d.; film thickness 0.25 µm) and a flame ionization detector (FID). The temperatures of the injection and detector ports were set at 300°C. The oven temperature program was initially 170°C for 1 min, then rose to 5°C/min to 280°C, and finally held for 15 min. The carrier gas was nitrogen at a flow rate of 1.5 mL/min. Components were characterized by co-GLC run with n-alkane standards obtained from Sigma (USA). Standard error was calculated following Singh and Chaudhary[22].

Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy of surface waxes of fresh mature leaves of *P. tetragonolobus* (Stickm.) DC. and hydrocarbons separated from total wax have carried out in the month of December, 2011. Small portion of the leaf samples and hydrocarbon samples were mounted on aluminium stubs with double sticky tape and coated with 20 nm of gold using IB2 ion coater. Samples were examined and photographed in a Hitachi Scanning Electron Microscope (Model: Hitachi S-530 Scanning Electron Microscope, Hitachi Ltd., Tokyo, Japan) at an accelerating potential of 20-25 KV.

Results

The study shows variation of n-alkane profile in leaf epicuticular wax of three cultivars of winged bean [*P. tetragonolobus* (Stickm.) DC.]. Variation of the amount of surface wax from the mature leaves of three cultivars of winged bean and surface n-alkane in fruiting stage (December) is exhibited in Table 1. Nineteen n-alkanes from n-C_{16} to n-C_{35} (except n-C_{21}) have been found varying in composition in the mature leaves of three cultivars studied (Table 2). The ratio of odd to even number of total alkanes, amount of odd and even number of n-alkanes, branched chain n-alkanes of all three cultivars are presented (Table 2). GC-FID chromatograms of the purified hydrocarbon fractions are exhibited in figures 1-3. Scanning Electron Microscopic pictures of epicuticular wax of winged bean reveal different patterns of wax deposition on the leaves of the three cultivars studied. SEM pictures from the upper surfaces of the leaves and of hydrocarbons isolated from total wax of three cultivars are presented in Figure 4.

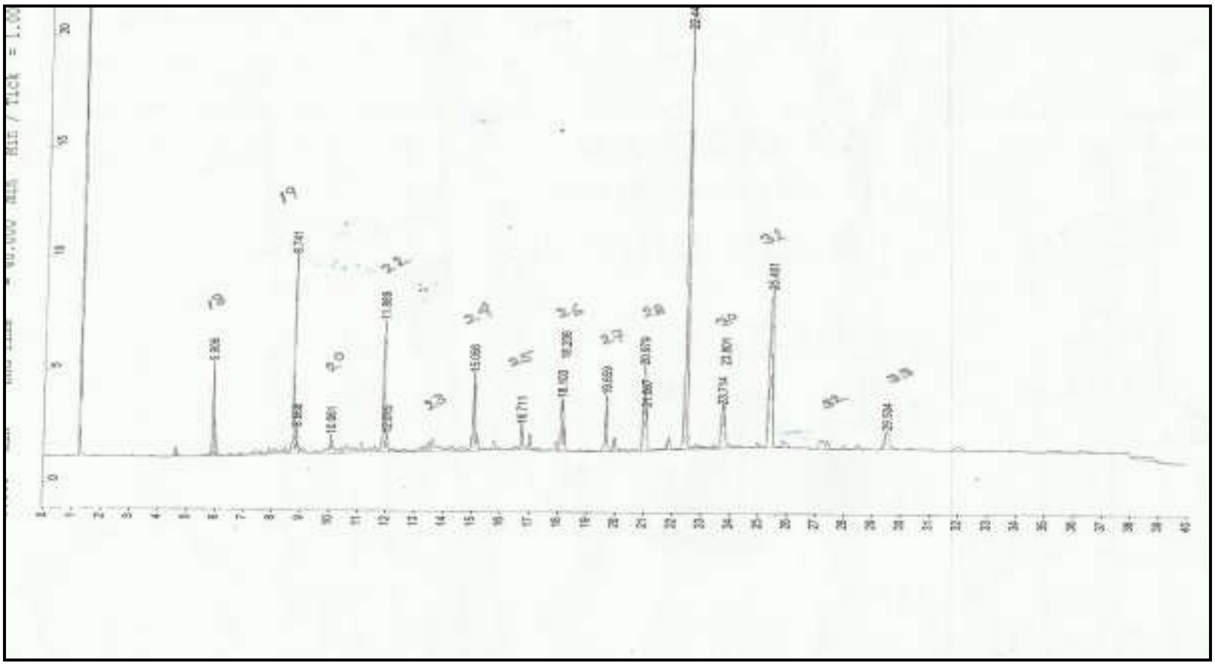


Figure 1 GC-FID chromatograms of the purified hydrocarbon fractions of the cultivar H

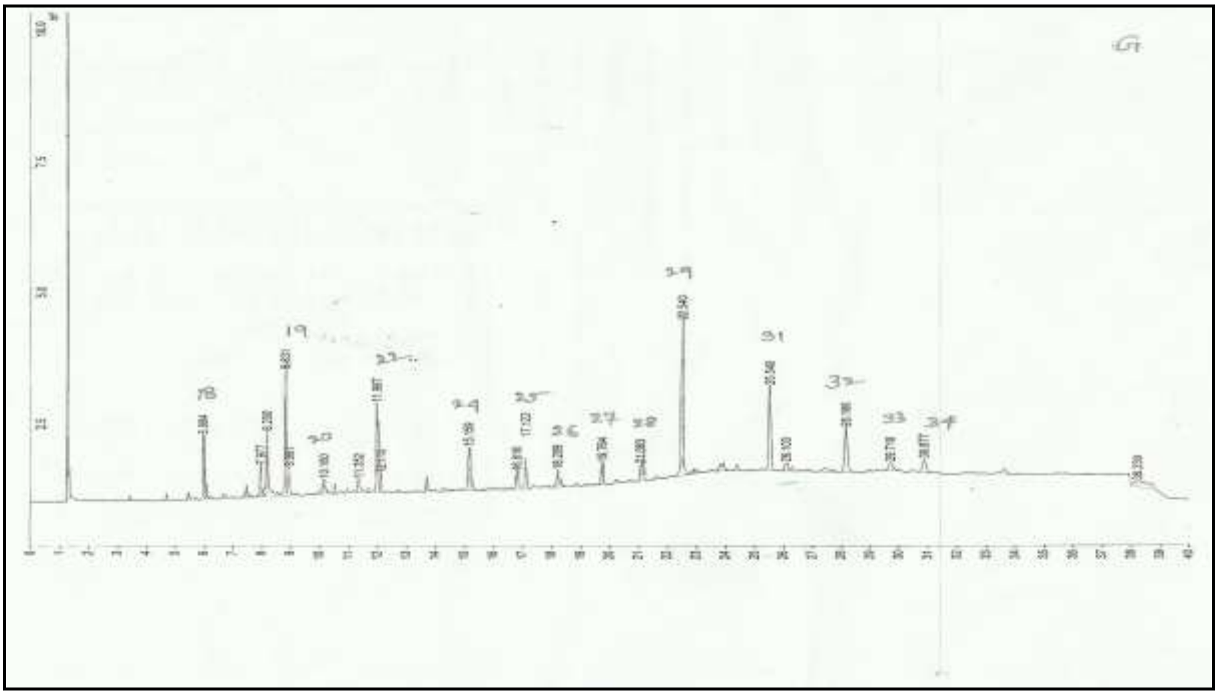


Figure 2 GC-FID chromatograms of the purified hydrocarbon fractions of the cultivar I

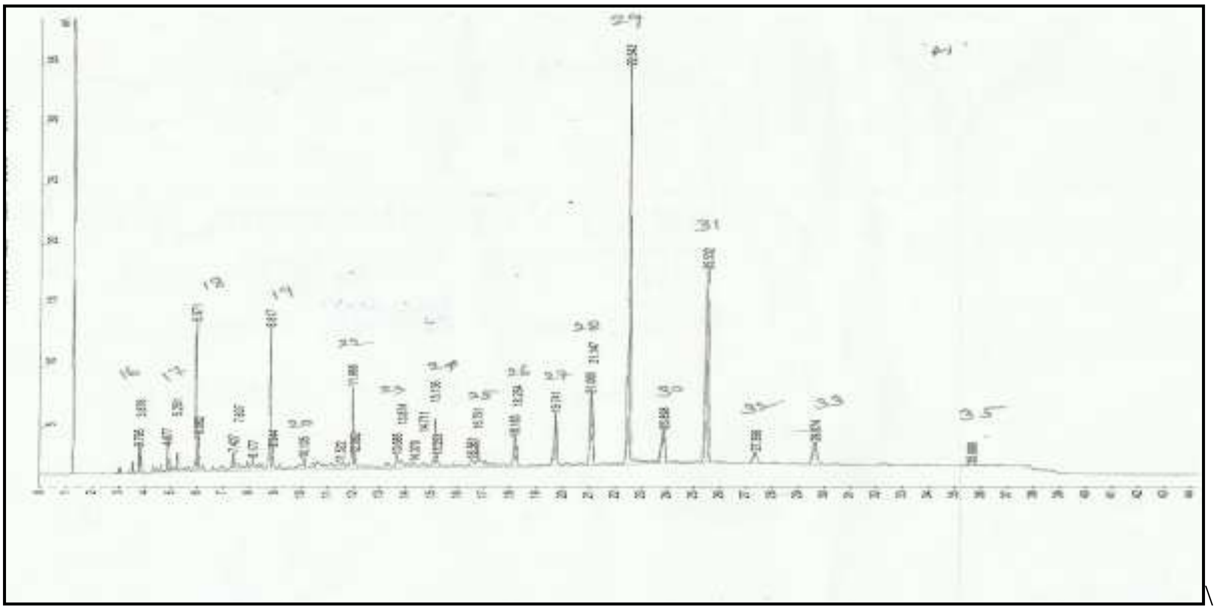
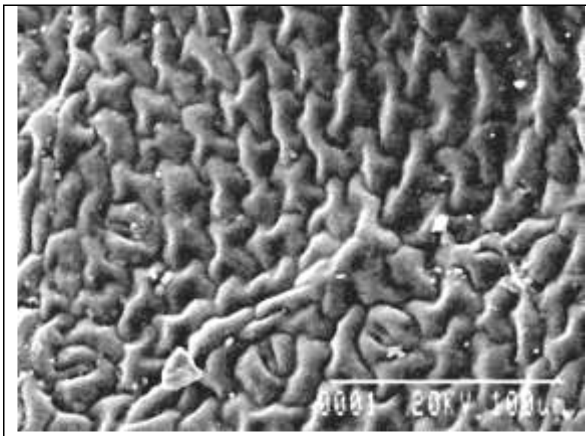
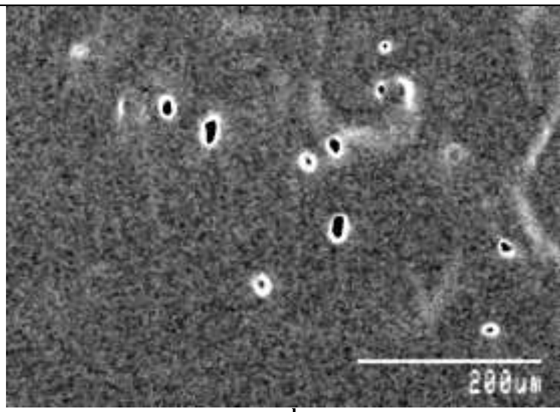


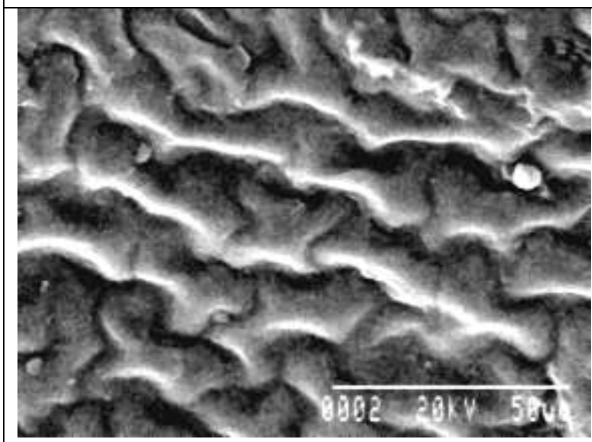
Figure 3 GC-FID chromatograms of the purified hydrocarbon fractions of the cultivar J



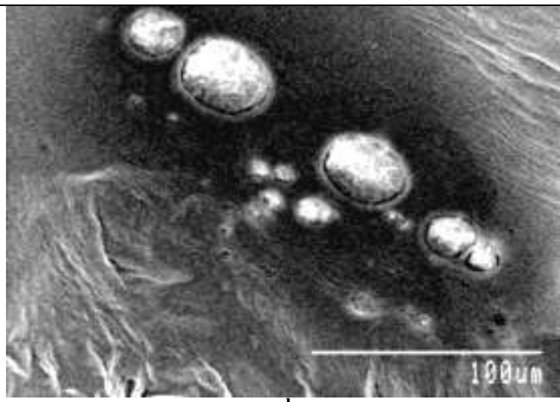
H[a]



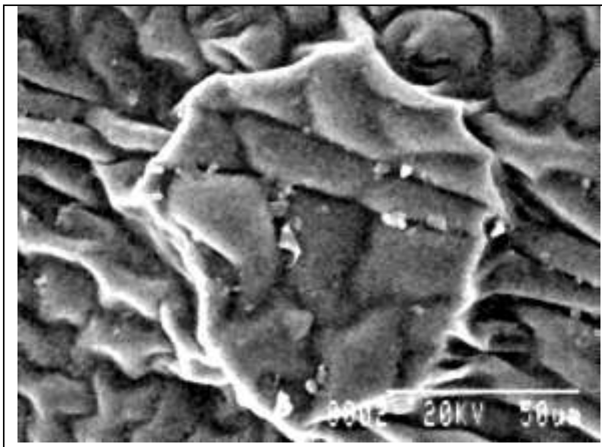
H[b]



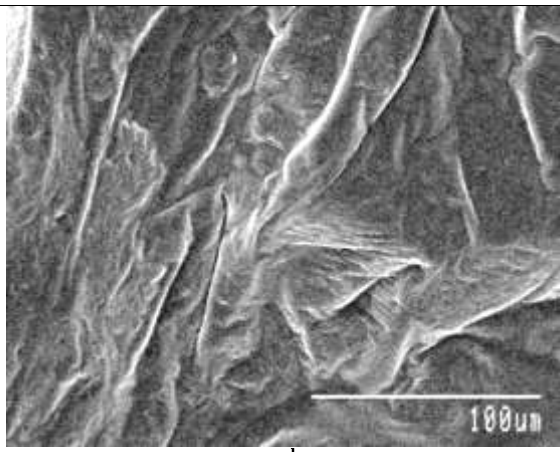
I[a]



I[b]



J [a]



J [b]

Figure 4 SEM pictures of different cultivars (H to J) of *P. tetragonolobus* (Stickm.) DC. [a]epicuticular wax of upper surface of leaf [b]hydrocarbon isolated from total epicuticular wax collected from leaves

Table 1 Variation of amount of extractable surface wax from the fresh leaves (100 g) of *P. tetragonolobus* (Stickm.)DC. and surface n-alkanes present there in the cultivars studied

Name of materials	**EC38821B (H)**	**EC27886 (I)**	**IC95227 (J)**
Amount of surface wax extracted (mg)	84.59±0.66	78.36±0.63	79.50±0.64
Hydrocarbons (n-alkanes) in total (%)	35.47±0.42	29.88±0.39	32.95±0.41
Other constituents in extractable leaf surface wax (%)	64.53±0.57	70.12±0.60	67.05±0.59

n= 3, ± SE

Table 2 Varietal differences of hydrocarbon constituents (mol %) of surface wax of leaves of winged bean [*P. tetragonolobus* (Stickm.) DC.]

n-alkanes (carbon number)	**EC38821B (H)**	**EC27886 (I)**	**IC95227 (J)**
C14			
C15			
C16			0 82±0.06
C17			0.77±0.06
C18	3.81±0.14	4.27±0.14	5.55±0.17
C19	10.01±0.22	10.93±0.23	6.31±0.18
C20	1.64±0.09	1.84±0.09	0.96±0.07
C21			
C22	7.64±0.19	7.66±0.19	4.38±0.15
C23			0.63±0.05
C24	4.91±0.15	4.07±0.14	2.61±0.11
C25	1.63±0.09	3.04±0.12	1.84±0.09
C26	1.17±0.07	1.65±0.09	1.08±0.07
C27	3.73±0.13	2.29±0.10	3.42±0.13
C28	5.07±0.16	1.73±0.09	2.55±0.11
C29	28.74±0.38	17.31±0.30	28.27±0.38

C30	2.30±0.10		2.26±0.10
C31	14.82±0.27	11.32±0.24	15.21±0.28
C32		7.95±0.20	1.59±0.09
C33	3.14±0.12	2.01±0.10	3.37±0.13
C34		2.74±0.11	
C35			0.63±0.05
C36			
C37			
C38			
Total n-alkanes	88.61±0.67	78.83±0.64	82.26±0.65
Branched chain alkanes	11.38±0.24	21.17±0.33	17.74±0.30
Composition ratio of odd numbers to total alkanes	62.07±0.56	46.90±0.49	60.45±0.56
Ratio of odd and even numbered alkanes	2.33:1	1.47:1	2.77:1
Odd	62.07	46.9	60.45
Even	26.54	31.91	21.8

n= 3, ±SE

Discussion

In the present study nonacosane (n-C_{29}) and hentriacontane (n-C_{31}) are the predominant alkanes in the three cultivars studied. This data is in accordance with that obtained in seven cultivars of winged bean studied[18]. The amount of nonacosane in cultivar EC27886 is 17.31% whereas in the cultivars EC38821B and IC95227 it is almost similar i.e. 28.74% and 28.27% respectively. Next predominant alkane present in leaf epicuticular wax is hentriacontane, the amount of which varies from 11.32% in EC27886, 14.82% in EC38821B, and 15.21% in IC95227. It is quite interesting to note that nonadecane (n-C_{19}) is present in these three cultivars, though absent in other seven cultivars studied earlier (Ray et al. 2014). The amount of nonadecane is also quite high in these three cultivars i.e. 6.31% in IC95227, 10.01% in EC38821B, and 10.93% in EC27886.

In cultivars EC38821B and EC27886 n-C_{23} is absent, but in cultivar IC95227 it is present in very small amount (0.63%). Pentriacontane (n-C_{35}) is absent in cultivars EC38821B and EC27886, but present in very small amount in cultivar IC95227 (0.63%). Tetratriacontane (n-C_{34}) is absent in cultivars EC38821B and IC95227; but present in cultivar EC27886 (2.74%). Dotriacontane (n-C_{32}) is absent in EC38821B, but present in EC27886 (7.95%) and IC95227 (1.59%). Hexadecane (n-C_{16}) and heptadecane (n-C_{17}) are absent in EC38821B and EC27886, but present in IC95227. n-alkanes from C_{16} to C_{18} are liquids, whereas C_{19} is semi solid and higher alkanes (C_{20}, etc.) are solids. The greater the number of carbons in the alkane, the more is known about its solid architecture. The solid long chain alkanes protect the plants against water loss; prevent leaching of important minerals by rain. Cuticular wax provides protective coatings on leaves and stems that have important physiological and ecological functions in plants[21, 23]. Plants are capable of adapting to changing environmental conditions by producing different relative amounts of these compounds which has been recognised as biomarkers of climatic change[23]. Studies on n-alkanes of leaves have explored the compounds as a taxonomic marker, mainly due to the high species-specific coherence in n-alkane distribution pattern[24]. SEM pictures of epicuticular wax on the upper surface of leaves (Figure 4 H^a, I^a and J^a) of winged bean cultivars shows deposition of wax in specific pattern. In cultivar H, wax deposition in the form of finely reticulate pattern is found. Wax deposition in broadly reticulate pattern is seen in cultivar I. In cultivar J, wax deposition in broadly reticulate with uneven deposition of wax at places is observed. Neither trichome nor stoma is found on the upper surface of the cultivars studied. SEM images of pure hydrocarbons (Figure 4 H^b, I^b and J^b) isolated from the crude extract of leaves shows smeared waxy substances along with droplets like structures arranged in scattered manner. The present observation is very well supported by the earlier findings of Prasad and Inamdar[25]. In case of cultivar H, smear is almost uniform and particulate type; in cultivar I, large droplets of various sizes are noticed along with fine particles[21] but wax deposition in the form of folded plate like sheets is found in cultivar J.

From Table 2 it has been concluded that odd numbered n-alkanes are greater in relative amount than even numbered n-alkanes. Ratio of odd and even number of alkanes is quite similar in EC38821B (2.33:1) and IC95227 (2.77:1) but different in EC27886 (1.47:1). The predominant character of C_{29} and C_{31} indicates that the plant belongs to higher plant group. SEM pictures differentiate upper surfaces of the leaf and nature of alkanes in the suface wax. The total work again reveals that the purpose of this experimental works will be useful as a taxonomic marker of the plant and also for the study of genotypic variation in all the cultivars of *Psophocarpus tetragonolobus* (Stickm.) DC.

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