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Comparative foliar anatomical study of six different varieties of *Piper betle* L.

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ABSTRACT

The present investigation on a few selected cultivars of *Piper betle* L. was undertaken mainly to understand the anatomy and tissue components of the stem, leaves and petiole. This was accomplished by using basic anatomy techniques such as free hand and microtome sectioning. The varieties selected were: HY1, HY2, JB, KB, LV and SC. Major aspects of the present inquiry were - macro morphology, anatomy and histochemistry. The main focus of the study was on the internal structure of leaves and petioles to understand the role of specialised cells like enlarged hypodermal cells and mucilage cavities in retaining moisture and thereby preserving the shelf life of harvested leaves.

KEYWORDS: Anatomical study, *Piper betle*, Hypodermis, Secretary cell, Mucilage cavity

INTRODUCTION

Piper betle Linn. (Piperaceae), a dioecious, annual creeper, climbing by many small adventitious rootless, grows to a height of about one metre, generally grown in hotter and damper parts of the country. It is extensively found in damp forests and is propagated in India and other countries in South-East Asia, such as Vietnam and China. In India it is found in Uttar Pradesh, Bihar, Bengal, Orissa, Tamilnadu, Andhra Pradesh and Karnataka. In Tamilnadu, three varieties of *P. betle* leaves are accessible mostly Sirugamani, Karpoori and Vellaikodi. It is used in a variety of decoctions, in curing wounds, burns, impetigo, furunculosis, eczema, and lymphangitis and juice is beneficial stomatic. Kammaru (a variety of *P. betle*) leaf has a good level of juice that heals pharyngitis, abdominal pain and swelling. Generally betle leaf cures urticaria and as per ayurvedic medicine, it recovers the loss of equilibrium between the three 'humours', namely, Vatha, Pitha and Kapha. The roots and fruits are well-known for the treatment of malaria, and asthma (Bhattacharya *et al.*, 2007).

Chemical Constituents

The *P. betle* leaf has been described to have Piperol-A, Piperol-B, and methyl piper betlol and they also have been isolated (Chopra *et al.*, 1956). The betle leaves have starch, sugars, diastases and an essential oil composed of terpinen-4-ol, safrole, allyl pyrocatechol monoacetate, eugenol, eugenyl acetate, hydroxyl chavicol,

eugenol, piper betol and the betle oil contains cadinene carvacrol, allyl catechol, chavicol, p-cymene, caryophyllene, chavibetol, cineole, estragol, etc. as the key components. Phytochemical analysis on leaves revealed the presence of Alkaloids, Tannins, Carbohydrate, Amino acids and Steroidal components. The chief component of the leaves is a volatile oil in the leaves from different countries, called Betle oil which contains 2 phenols, and betle phenol (Chavibetol and Chavicol). Codinene has also been found.

Traditional Uses

The paste of *P. betle* leaves assorted with salt and hot water able to be administered for filariasis. For curing obesity, one *P. betle* leaf mix with *Piper nigrum* is prescribed for two months.

Juice of *P. betle* with honey is accommodating to treat coughs, dyspnoea, and indigestion, among children. Leaves of *P. betle* smeared with oil are useful on the breasts of lactating women; it is supposed to promote milk secretion. A local application is recommended for inflammatory swelling such as orchitis, arthritis and mastitis. For childhood and old people, leaves are mixed with mustard oil, warmed and applied to the chest for treatment to reduce cough and dyspnoea. Recovers bad breath, and body odor and prevents tooth decay. Prevents and treats vaginal ejection, and reduce itching of the vagina. Stop bleeding in the nose. It contains vitamins such as thiamine, niacin, riboflavin and carotene. In India, leaves are used for curing

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eczema, lymphangitis, asthma and rheumatism. A paste of leaves is applied on cuts and wounds. Roots with black pepper used to generate sterility in women.

The oil is used for irritation in the throat, larynx, and bronchi, gargling and inhalation in diphtheria. Juice of leaves is used as stomachic and febrifuge (Vandana & Shalini, 2014).

MATERIAL AND METHODS

Source of Plant Material

The present investigation was carried out in six different varieties of *Piper betle* viz., Hybrid I (HY1), Hybrid 2 (HY2), Jaipur Bangla (JB), Karpoori (KAR), Tenkasi Variety (TV) and Sirugamani (SG). Plant cuttings were collected from Sugarcane Research Station, Sirugamani, Tamil Nadu, India.

Cuttings were raised in the department nursery without the addition of organic or inorganic fertilizers under irrigated condition. The seventh internode (middle of the plant) of each variety was collected, cut into small segments of uniform size and used for anatomical investigations.

Anatomical Investigation

Fresh free-hand transverse sections of leaf, stem and petiole were taken and observed with the help of a compound light microscope (Nikon Alphaphot). Specific stains and reagents were employed to detect the presence of starch and oil following the standard procedures described by Krishnamurthy (1988), Harris and Oparka (1994) and Dayanandan *et al.* (1996).

Microtome sections were taken following the standard paraffin embedding technique described by Johansen (1940). Six samples of stem, leaf and petiole from six different varieties were employed in the study. Fresh segments of the stem, leaf and petiole were cut into small pieces (5 x 5 x 10 mm) and fixed in formalin acetic acid-alcohol (FAA) for 48 hrs. Then the materials were dehydrated with alcohol series viz., 25%, 50%, 75% and 100%. Then the materials were cleared by xylol series of 25%, 50%, 75% & 100% and kept for 4 - 6 hrs. Materials were in 100% xylol and wax infiltration was commenced. The specimens were infiltrated with paraffin wax. The paper boats were used for casting the blocks and they were stored in the refrigerator prior to microtome sectioning.

Transverse sections were taken at a thickness of 18 μm and mounted onto slides with Haupt's adhesive, using 4% formalin as a flooding solution. Sections were stained with Toluidine blue 'O' (Feder & O' Brien, 1968). After staining the slides were air dried, dewaxed by passing through two changes of pure xylene and mounted using DPX and the slides were made permanent.

RESULTS

The present study on the anatomy of a few selected cultivars was mainly aimed to widen the basic anatomical details of *P. betle*,

besides understanding the role of various tissue components of the betle leaf (Table 1). Varieties selected for these investigations include: HY1 (Hybrid Variety 1 - male), HY2 (Hybrid Variety 2 - female), Jaipur Bangla (JB), Karpoori (KP), Tenkasi Variety (TV) and Sirugamani (SG) (Figures 1a - 1f).

Leaf Anatomy

In all the varieties studied the upper epidermis is single-layered, thin-walled and bound by a thin layer of the cuticle (Figure 2a). The epidermal cells are isodiametric in shape but the size varies in the different varieties studied (Figures 2b & 2c). Trichomes are present just above the epidermis, they are uniseriate and unicellular. The trichome has a basal stalk cell and by itself appears conical or oval in shape. However, in varieties HY2 and Sirugamani the trichomes are three-celled. The hypodermal cells are larger than the epidermal cells and form two layers. They appear cubical in shape with slight folding and measure about 38 μm length in all the varieties and width varies from 52 - 78 μm . Large sized hypodermal cells were observed in Karpoori, followed by HY2, JB and Sirugamani (Figures 2b & 2c). The cells are closely packed with no intercellular spaces. The Hypodermis also shows secretory cells and mucilage canals (Figure 2d). Stomata are completely absent on the upper epidermis. Like the upper epidermis, the lower epidermis is also single-layered and is about the same length as the upper epidermal cells but the breadth is slightly reduced, so the cells appear oblong in shape (Figure 2c). Stomata are uniformly distributed on the lower surface. Stomata is of anomocytic type. The stomatal frequency varies in different cultivars as follows: HY1 128/mm², HY2 138/mm², JB 116/mm², Karpoori 124/mm², LV 128/mm², Sirugamani 137/mm². The lower hypodermal cells are only one layered except in the regions between two vascular bundles where it forms a double layer (Figure 2a). In all the varieties studied two different types of trichomes could be distinguished- unicellular and uniseriate multicellular.

The mesophyll consists of palisade cells and spongy parenchyma, thus making it bifacial (Figure 2a). Palisade appears to be in a single stratum throughout the greater length of the leaf. Secretory cells also occur in between the palisade cells. The spongy tissue is made of irregularly shaped cells. The mesophyll tissue is also characterised by the presence of calcium oxalate and fine sand crystals. These crystals could be clearly observed under polarized microscope (Figure 2e). The presence of granular crystals in leaf lamina especially in spongy parenchyma was earlier reported in *P. betle*. In the midrib region there is a single, large bowl-shaped vascular bundle (Figure 2a). The hypodermis in this region is substituted by a group of collenchyma cells. Above the vascular bundle there is a single large mucilage canal measuring about 100-150/ μm (Figure 2e). Thick-walled xylem elements are present and phloem occurs beneath the xylem.

The most distinguishing feature of betle leaves as understood by Chibber (1913), Metcalfe & Chalk (1950) and Raman *et al.* (2012) is the hypodermal layer of the leaf with lateral wall folding (Figure 2e). This indicates a provision for reducing the

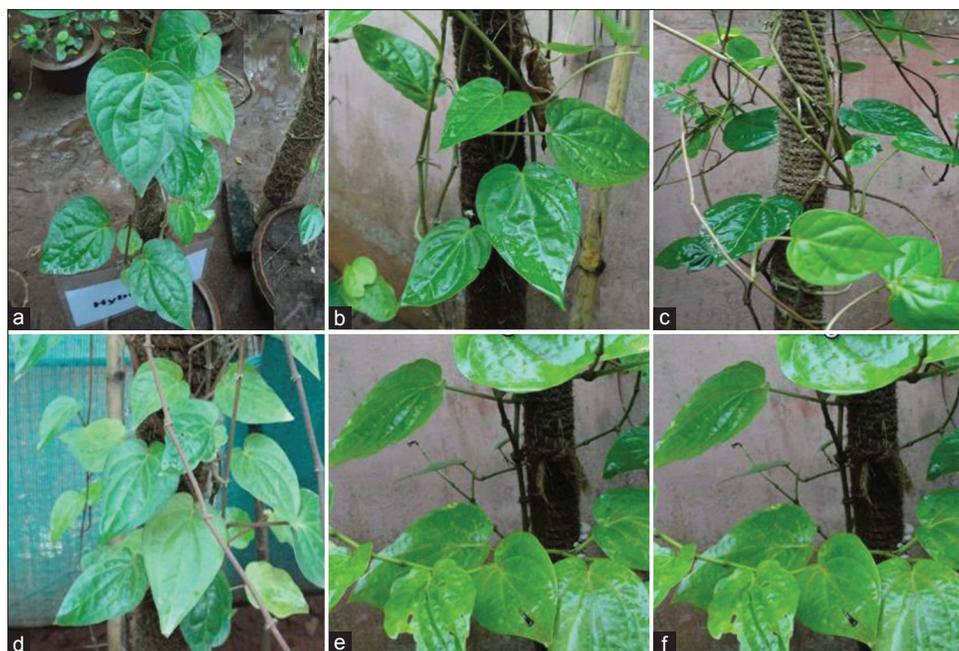


Figure 1: Habit of *P. betle* cultivar a) Hybrid I (HY1), b) Hybrid II (HY2), c) Jaipur Bangla (JB), d) Karpuri (KAR), e) Tenkasi variety (TV) and f) Sirugamani (SG)

size of the cell lumina of the hypodermal aqueous cells when necessary. Fully turgid these cells measure about three times more than the size of epidermal cells (Metcalf & Chalk, 1950). In the selected varieties also similar features were observed in the hypodermal cells on both upper and lower surfaces. Chibber (1913) has reported that the hypodermal cells are highly silicified and this intends to prevent the collapse of the aqueous hypodermis. Besides, the unicellular trichomes are similar to that of hydathodes in structure and absorb water from outside when necessary and available (Raman *et al.*, 2012). The basal cell of trichome facilitates entry of water into leaves.

The ten cultivars of *P. betle* have shown some structural similarities. The four-layered upper and two-layered lower epidermis was observed in all the varieties of *P. betle* studied. Crystals and oil reserves were found in the epidermal cells. Some varieties were found to have more stomata and trichome frequency. Multicellular tector trichomes were seen on the abaxial surface of the midrib. Similar observations were made in the present study also where varieties HY2 and Sirugamani have 1 - 3 celled trichomes. The development of tracheoids and idioblasts from the vessel elements has been noticed. These tracheoids are suggested to have water-holding capacity. They are connected to the vein endings in the leaves. Such tracheoids were also observed in the present study in the HY2 variety (Figure 2c).

The glossy nature and presence of trichomes on the leaf surface are intended to reflect strong light falling on it. The aqueous hypodermal layer helps to store water and it is significant that this layer is thick (two-layered) on the upper surface compared to the lower surface (single-layered). Of the contents present in leaves mucilage and oil-secreting cells are abundant thus making *P. betle* plant to reduce heat and severity of water loss.

Petiole

Invariably in all the varieties the outline of the petiole in cross-sectional view is convex on the lower side and concave on the upper. The petiole has a single layer of the epidermis, the cells of which are slightly papillose. Trichomes are found just above the epidermis, which is uniseriate and 1 - 4 celled. But JB and KAR have 5 - 6 cells (Figure 2g). Just below the epidermis there is a discontinuous layer or girdle of collenchyma which is about 7 - 8 layers except in Sirugamani which has 5 - 6 layers only. The number of girdles observed were 10 - 11 as against 8 reported by Chibber (1913). The centre of the petiole is occupied by a large mucilage canal (Figure 2f). Secretory cells and calcium oxalate crystals (sand and rod-shaped crystals) are found scattered in the parenchyma region (Figures 2h-2j). The vascular bundles of the petiole are schizostelic and arranged in a ring (Figure 2f). There are seven large alternating bundles with four small bundles except in LV where there are five large bundles alternating with five small bundles, in addition to the two or three-minute stipular ones (Figure 2k). A single large mucilage canal is present in the center of the petiole; two small mucilage canals are present on both sides. Among these HY2 contains several mucilage canals maximum of 9, one opposite to each vascular bundle and arranged in 'U' shape in addition to the large central canal (Figure 2i). Calcium oxalate and sand crystals were also observed in the ground parenchyma cells. Similar observations were made by Murthy (1973) and Raman *et al.* (2012).

Piper betle and *Piper sarmentosum*. HY1 variety contain only three mucilage canals- one large central and two small one on either side of the larger canal (Figures 2e & 2f). In varieties JB, Karpuri and LV only one single central large mucilage canal is present, however smaller mucilage canals are absent (Figure 2l).

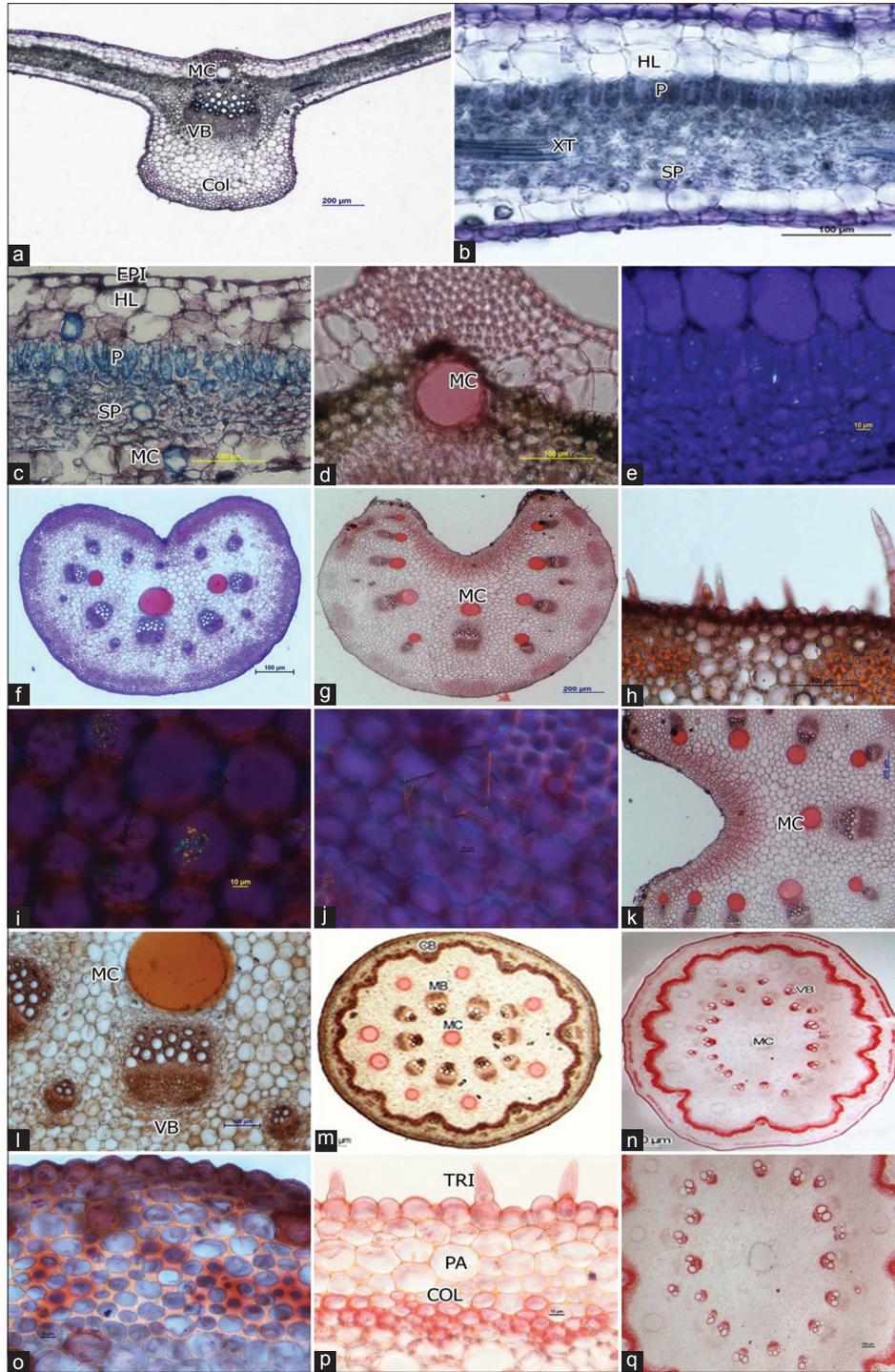


Figure 2: a) Leaf lamina section across the mid rib region stained with TBO shows epidermis (EPI), collenchyma (COL), mucilage cavity (MC), few vascular bundles (VB) and trichomes (TRI), b) Enlarged view of leaf lamina shows two layers of palisade cells, c) Enlarged view of leaf lamina shows two layers of palisade cells, d) Leaf midrib region shows large mucilage canal, e) Leaf section viewed under in polarized light show the presence of crystals in mesophyll layers (CRY), f) Section of entire petiole HY1 stained with TBO A single mucilage canal is present in the center of the petiole, two small canals present both sides F, g) Section of entire petiole HY2 stained with safranin several mucilage canals, one opposite to each vascular bundle, arranged in U shape, central one larger than the other (9) h) Petiole section shows 1 - 6 celled trichomes in Karpuri, i) Petiole section viewed under in polarized light show the presence of sand shaped crystals, j) Petiole section viewed under in polarized light show the presence of rod shaped crystals (CRY), k) Enlarged view of HY2 mucilage canal, l) Petiole cross section shows only one mucilage canal is present in the center of the petiole in JB, LV and Kar, m) Ground plan of male stem, n) Ground plan of female stem, o) Cross section of HY2 stem stained with Safranin presenting the prominent arrangement of collenchymas 5 - 6 layer stand, p) Cross section of HY2 stem stained with Safranin presenting the prominent arrangement of epidermal cells and trichomes and q) HY2 enlarged view of a section showing distinct sclerenchyma strands (SCL) sunken large vascular bundles (10 - 13)

Table 1: The distinguishing anatomical features of *Piper betle* leaf, stem and petiole

Plant part	Features	HY1	HY2	Jaipur Bangla	Karpoori	Tenkasi Variety	Sirugamani
Leaf	Non glandular trichomes	1 - 2 celled	1 - 3 celled	1 - 2 celled	1 - 2 celled	1 - 2 celled	1 - 3 celled
	Upper epidermis	Stomata absent, Hypodermal layers: 2 layers in upper epidermis and single layer in lower epidermis	Stomata absent, Hypodermal layers: 2 layers in upper epidermis and single layer in lower epidermis	Stomata absent, Hypodermal layers: 2 layers in upper epidermis and single layer in lower epidermis	Stomata absent, Hypodermal layers: 2 layers in upper epidermis and single layer in lower epidermis	Stomata absent, Hypodermal layers: 2 layers in upper epidermis and single layer in lower epidermis	Stomata absent, Hypodermal layers: 2 layers in upper epidermis and single layer in lower epidermis
	Stomata on lower epidermis	Anomocytic (Frequency 128/mm ²)	Anomocytic (Frequency 138/mm ²)	Anomocytic (Frequency 116/mm ²)	Anomocytic (Frequency 124/mm ²)	Anomocytic (Frequency 128/mm ²)	Anomocytic (Frequency 137/mm ²)
	Palisade cells	Single layer					
	Secretory cells in mesophyll	Oil cells, mucilage cavity	Oil cells, mucilage cavity	Oil cells, mucilage cavity	Oil cells, mucilage cavity and anthocyanin pigments	Oil cells, mucilage cavity	Oil cells, mucilage cavity
	Silica crystals	Ground tissue and leaf lamina					
	Non glandular trichomes	1 - 4 celled	1 - 4 celled	1 - 5 celled	1 - 6 celled	1 - 3 celled	1 - 4 celled
Petiole	Collenchyma	Present in patches forming (7 - 8 layers) slightly lignified cells	Present in patches (7 - 8 layers) slightly lignified cells	Present in patches (7 - 8 layers) slightly lignified cells	Present in patches (7 - 8 layers) slightly lignified cells	Present in patches (7 - 8 layers) slightly lignified cells	Present in patches (5 - 6 layers) slightly lignified cells
	Calcium oxalate crystals in cortical parenchyma	Sand crystals and rod shaped crystals					
	Vascular bundles	7 large alternating with 4 small bundles and 3 stipulodes	7 large alternating with 3 small bundles and 2 stipulodes	7 large alternating with 4 small bundles 2 stipulodes	7 large alternating with 4 small bundles and 2 stipulodes	5 large alternating with 5 small bundles stipulodes absent	7 large alternating with 7 small bundles stipulodes absent
	Mucilage canal	A single mucilage canal is present in the center of the petiole, two small canals present both sides	Several mucilage canals, one opposite to each vascular bundle, arranged in U shape, central one larger than the other (9)	Only one mucilage canal is present in the center of the petiole	Only one mucilage canal is present in the center of the petiole	Only one mucilage canal is present in the center of the petiole	A single mucilage canal is present in the center of the petiole, two small canals present one side and single canal is present the other side
	Non glandular trichomes	1 - 2 celled	Rare, 1 celled	1 - 2 celled	1 - 4 celled	1 - 2 celled	1 - 2 celled
Stem	Cortex	Collenchyma 2 - 3 layered interrupted, cells with slightly thickened walls, and fibers with thinly lignified walls present in the collenchymatous outer cortex	Collenchyma 5 - 6 layered interrupted, cells with slightly thickened walls, and fibers with thinly lignified walls present in the collenchymatous outer cortex	Collenchyma 3 - 4 layered interrupted, cells with slightly thickened walls, and fibers with thinly lignified walls present in the collenchymatous outer cortex	Collenchyma 2 - 3 layered interrupted, cells with slightly thickened walls, and fibers with thinly lignified walls present in the collenchymatous outer cortex	Collenchyma 2 - 3 layered interrupted, cells with slightly thickened walls, and fibers with thinly lignified walls present in the collenchymatous outer cortex	Collenchyma 3 - 4 layered interrupted, cells with slightly thickened walls, and fibers with thinly lignified walls present in the collenchymatous outer cortex
	Mucilage canal	Central one large mucilage canal, a rings of smaller canals seen between two rings of vascular bundles (7 - 8)	Central one large mucilage canal, a rings of smaller canals seen between two rings of vascular bundles (10 - 13)	Central one large mucilage canal, a rings of smaller canals seen between two rings of vascular bundles (7 - 8)	Central one large mucilage canal, a rings of smaller canals seen between two rings of vascular bundles (7 - 8)	Central one large mucilage canal, a rings of smaller canals seen between two rings of vascular bundles (7 - 8)	Central one large mucilage canal, a rings of smaller canals seen between two rings of vascular bundles (6 - 7)
	Vascular bundles	Two concentric Rings					

In the variety Sirugumani there are three small mucilage canals in addition to the large central one

Stem

In the transectional view, the stem shows an outer rind region composed of an epidermis made of thick-walled cells and externally covered by a thick cuticle (Figures 2m & 2n). The epidermal cells are papillose (Murthy, 1973). Unicellular or bicellular trichomes are found on the surface except for Karpoori which has 4-celled trichome. Inner to the epidermis, the cortex is present, composed of three distinct regions. The outermost region of two to three layers of parenchyma cells (Figures 2o & 2p). The middle region of 3 - 4 layers of collenchyma cells. The presence of a collenchymatous layer about 8 - 15 cells wide was observed and reported by Raman et al. (2012). Variety HY2 contains 5 - 6 layers and SG contains 3 - 4 layers. An innermost region of 2 - 3 layers of chlorenchyma cells and secretory cells (oil cells) are also observed in the cortex. No definite pericycle and endodermis could be seen (Chibber, 1913). There is a very conspicuous mucilage canal in the center of the stem with 10 - 15 µm. In addition to this a ring of 7 - 8 smaller canals is also found separating the two rings of vascular bundles. The numerous vascular bundles which are collateral and open are arranged in two concentric rings (Figures 2m & 2n). The bundles of the outermost ring are always greater in number than those of the inner, but they are not uniform in their size. The ring has about 8 - 9 large bundles 16 - 18 smaller bundles. There is a prominent sclerenchymatous strand (Figure 2q). The inner ring has 7 - 8 bundles except for HY2 which has 10 - 13 bundles (Figure 2n). Spaces between the vascular bundles are occupied by parenchyma cells which are comparatively smaller than the pith cells.

The anatomical characteristics of the leaf, petiole and stem in all the varieties studied are typically xeromorphic which could help in preserving the aroma and shelf-life longevity of *P. betle* leaves.

Morphoanatomical key to the varieties studied

1. Stem ellipsoidal, rough, trichomes absent; cuticle; mucilage cavity; collenchyma 5 - 6 layers; vascular bundle 10 - 13.....HY2
2. Stem oval, rough, trichomes 2 - 4; collenchyma 2 - 3; vascular bundle 6 - 8; mucilage cavity 6 - 7
 - 2.1 Stems red
 - 2.1.1 Ridged.....Sirugamani
 - 2.1.2 Non-ridged.....Karpoori
 - 2.2 Stems green
 - 2.2.1 Ridged.....Jaipur Bangla
 - 2.2.2 Non-ridged
 - 2.2.2.1 Mucilage cavity 7 - 8.....Tenkasi variety
 - 2.2.2.2 Mucilage cavity 9 - 10.....HY1

CONCLUSION

The internal structure of leaves, stem and petiole, though the gross internal structure was similar, however minor variations were observed. All the vegetative organs are bound by a thin

layer of cuticle and in all the varieties studied two different types of trichomes could be distinguished- unicellular and uniseriate multicellular. In the leaves the hypodermis is single-layered. The hypodermis also shows secretory cells and mucilage canals. Stomata are completely absent on the upper epidermis. Like the upper epidermis the lower epidermis is also single-layered and is about the same length as the upper epidermal cells but the breadth is slightly reduced, so the cells appear oblong in shape. Stomata are uniformly distributed on the lower surface. Stomata are of anomocytic type. The mesophyll tissue is also characterised by the presence of calcium oxalate and fine sand crystals.

Invariably, in all the varieties the outline of the petiole is convex on the lower side and concave on the upper. Secretory cells and calcium oxalate crystals (sand crystals) are found scattered in the parenchyma region. The unique feature of the petiole is the presence of collenchymatous girdles below the epidermis; these girdles are variable in the number of layers which is from 5 - 8, in the varieties studied.

In a transectional view, the stem shows an outer rind region composed of an epidermis made of thick-walled cells and externally covered by a thick cuticle. The presence of a collenchymatous layer about 2 - 8 cells wide and oil cells are also observed in the cortex. There is a very conspicuous mucilage canal in the center of the stem 10 - 15 µm. In addition to this a ring of 7 - 8 smaller canals is also found separating the two rings of vascular bundles.

Regarding the abundant presence of mucilage canals in the stem and petiole, it is indicative of reducing the rate of transpiration. Further mucilage is hydrophilic and absorbs moisture content from the atmosphere and completely becomes dissolved. The presence of discontinuous collenchyma strands in stem and petiole explains the supportive nature of this tissue towards climbing and twining nature. The anatomical characteristics of the leaf, petiole and stem in all the varieties studied are typically xeromorphic which could help in preserving the aroma and shelf-life longevity of *P. betle* leaves. Morpho-anatomical key has been framed for the varieties studied.

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