Rubber Science, **33**(3): 262-274, 2020. RESEARCH ARTICLE

IDENTIFICATION OF RRII 400 SERIES CLONES OF *HEVEA BRASILIENSIS* USING ANATOMICAL TRAITS OF PETIOLE

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As a perennial tree crop, maintaining identity of different clones of *Hevea brasiliensis* is important in crop management system and research. Characterisation of RRII 400 series clones derived from same parental combinations for distinguishable phenotypic variations were very low and overlapping especially in the nursery stage. In this context, a set of distal pulvinus anatomical traits *viz.* shape of the vascular bundles in the abaxial and adaxial side, inter-vascular continuity, medullary bundles, intrusion of cortical cells into the pith, shape of pith *etc.* were used for identification of six *Hevea* clones *viz.* RRII 414, RRII 417, RRII 422, RRII 430, RRII 105 and RRIC 100. Among these, shape of vascular bundles, features of inter-vascular continuity and intrusion of cortical cells, proportion of vasculature and pith area in the stele are observed to be stable anatomical markers for identification of the above clones. RRII 414 showed a close affinity to the female parent RRII 105 and RRII 430 showed more resemblance to its male parent RRIC 100. The other two clones *viz.* RRII 417 and RRII 422 are intermediate with respect to these traits. Structural traits showed more resemblance between RRII 414 and RRII 422. Among the six clones studied, RRII 430 showed both morphological and anatomical identity with RRIC 100. It is obvious that anatomical characteristics represent the environmental adaptation of clones and hence could be used as reasonably reliable adjuncts for identification of *Hevea* clones.

Key words: Clone identification, *Hevea brasiliensis*, Natural rubber, Petiole anatomy

INTRODUCTION

A systematic approach on morphological and structural parameters of different plant organs *viz.* leaf, flower, secretory structures, seed topography, pericarp anatomy *etc.* have significant taxonomic importance (Gamble, 1935; Thomas, 1991, Thomas and Dave, 1994; Dave *et al.*, 1992; Kuriachen *et al.*, 1992). *Hevea brasiliensis*, the prime source of natural rubber, cultivated as plantation crop does not exhibit highly distinct clonal variations for morphological traits as it is evolved through genetic recombination from a narrow genetic base. During each stage of handling *viz.* genetic improvement of the crop through hybridisation programme, field evaluation of clones and various nursery level operations, utmost care should be taken to maintain identity of clones. Being a perennial crop with a gestation period of about seven years and an economic life span of more than 25 years, selection of genuine planting material should be judicious. In India,

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RRII 105 was the most popular high yielding clone till the recommendation and release of RRII 414 and RRII 430 in 2005 and RRII 417 and RRII 422 in 2009 (Licy *et al.*, 1992; Mydin *et al.*, 2011). Every year, large quantities of planting materials of different clones in the form of budded stumps, polybag plants and root trainer plants are required for plantation establishment. The Rubber Board owned nurseries cater to only 20 per cent of the requirement of planting material and hence majority of the growers depend on private nurseries for procuring the same. The multiple choices of high yielding clones have left the growers for assuring the authenticity of planting materials for field planting. There are instances where complaints of supply of spurious planting materials by commercial rubber nurseries have been brought to the notice of the Rubber Board either by the law enforcement agencies or farmers (Mercykutty *et al.*, 2002). Hence, a need based study on developing visual morphological descriptors of RRII 400 series clones was undertaken and the identification key was formulated based on distinct leaf morphological features for easy identification from the nurseries and field grown plants (Thomas *et al.*, 2006 a, b). The outcome of the study was disseminated to various stakeholders including farmers (Thomas, 2006). However in practice, it is observed that the influence of high genotype-environment interactions make the distinction of clones through visual morphological traits alone cumbersome. Leaves have a crucial role in plant systematics. A number of foliar anatomical studies in plant species have been carried out and used for taxonomic purpose (Noraini *et al.*, 2016; Olowokudejo, 2017). Hence, the present study using petiole anatomical features for distinguishing different clones with better accuracy was attempted.

MATERIALS AND METHODS

Six petiole samples each of the lowermost healthy leaf from the second matured leaf whorl of six clones *viz.* RRII 105, RRII 414, RRII 417, RRII 422, RRII 430 and RRIC 100 (Table 1) were collected during post-monsoon period from three year old plants in the experimental plots at the Rubber Research Institute of India. Samples were collected from six trees of each clone, and fixed in Formalin-Acetic acid-Ethyl alcohol mixture (Johansen, 1940). Thin hand sections in transverse plane from the distal end of the petiole (distal pulvinus; Figs. 1, 2) near petiole-petiolule juncture, proximal pulvinus, petiole, petiolule and midrib were taken and stained with Janus green B (Lazarow and Cooperstein, 1953), Methyl green (Baker and Williams, 1965), and Toluidine blue O (Johansen, 1940) for general histology, and Oil red O (Omman and Reghu, 2003) for latex vessels. Observations and photomicrographs were taken using Leitz Diaplan and Leica 2500 P polarisation microscope fitted with Leica Qwin image analyser system. Measurements on the extent of pith, vasculature, and length of vascular bundles, number of xylem units in the vertical array of each bundle and number of vessels in vertical file were recorded. Data analysis was carried out by one way ANOVA.

Table 1. **Clones and parentage**

Clone	Parentage	Country of origin
RRII 414	RRII 105 x RRIC 100	India
RRII 417	RRII 105 x RRIC 100	India
RRII 422	RRII 105 x RRIC 100	India
RRII 430	RRII 105 x RRIC 100	India
RRII 105	Tjir 1 x Gl 1	India
RRIC 100	RRIC 52 x PB 83	Sri Lanka

RESULTS AND DISCUSSION

The leaf of *Hevea brasiliensis* is constituted of long petiole, proximal and distal pulvini, and three leaflets attached to the petiole with distinct petiolule (Figs. 1, 2). Proximal and distal pulvini are characteristics of *Hevea* leaf, the size and orientation of which show clonal differences (Premakumari and Saraswathyamma, 2000). Petiole is large, horizontal with functionally active extra-floral nectary at the petiole-petiolule juncture.

Fig. 1. Trifoliate leaf of *Hevea brasiliensis*

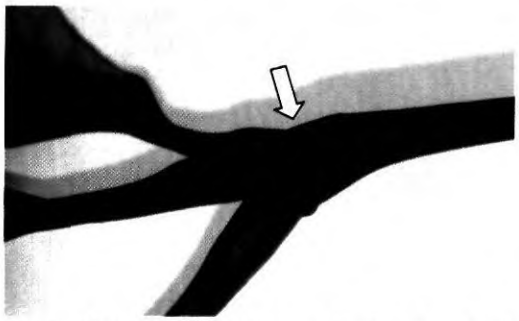

Fig. 2. Abaxial side of the petiole showing distal pulvinus (at arrow) near the petiole-petiolule juncture

Detailed leaf morphological characteristics of RRII 400 series clones of *Hevea* was reported by Thomas *et al.* (2006). Lower leaves from the second mature leaf storey are the best for recording petiole characters. Shape of the petiole falls into two categories *viz.* straight (RRII 417, RRII 422, RRII 430, RRII 105 and RRIC 100) or 'S' shaped (RRII 414). Thickness of the petiole showed wide variation among the clones: long and slender (RRII 414), not slender (RRII 422, RRII430, RRII 105, RRIC 100) or long and stout (RRII 417). Orientation of the petiole with respect to the stem varied from horizontal (RRII 417, RRII 430, RRIC 100), horizontal to downward (RRII 414), horizontal to upward (RRII 105) or downward (RRII 422).

For the present study, pulvinus (both proximal and distal), petiole, petiolule, midrib *etc.* were used for anatomical characterisation of clones, among which maximum diverse traits for clones were identified in the distal pulvinus of the petiole.

Cross sectional view of the distant pulvinus of the petiole has an inverted pentagonal outline (Fig. 3a). Epidermis is single layered isodiametric to radially elongated cells ensheathed by a thick cuticle. Followed by the epidermis is the cortical region constituted of 3-4 layers of collenchyma, sclerenchymatous patch and parenchymatous cells interspersed with latex vessels (Fig. 3b). The collenchymatous patch possesses chlorophyll. Sclerenchymatous patch have an internal lining of gelatinous layer (G-layer) as observed in the fibers of rubber wood (Reghu *et al.*, 1989). Druse type calcium oxalate crystals were found in the cortex, phloem and outer pith cells. Five collateral vascular bundles were present, of which two on either side at bottom are larger in size,

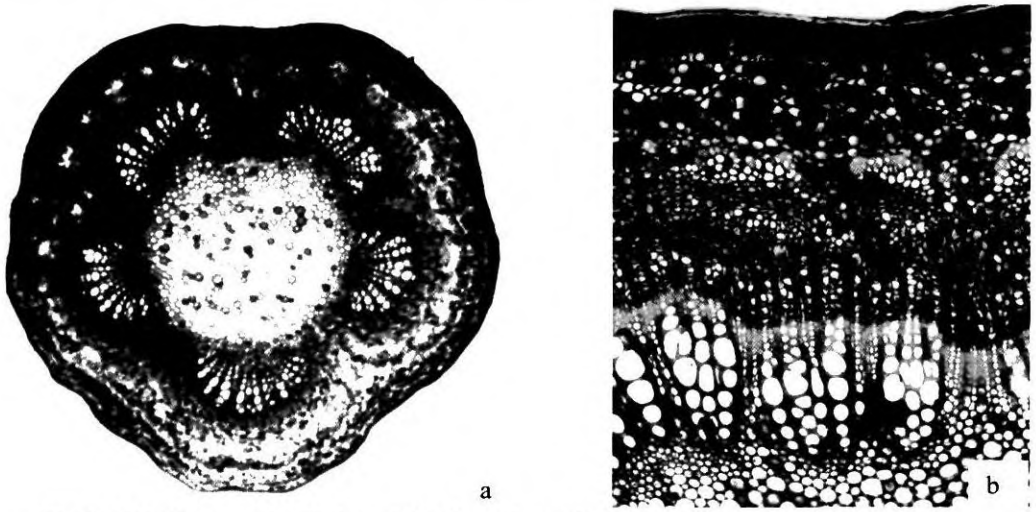

Fig. 3 a & b. Cross sectional view of distal pulvinus of petiole

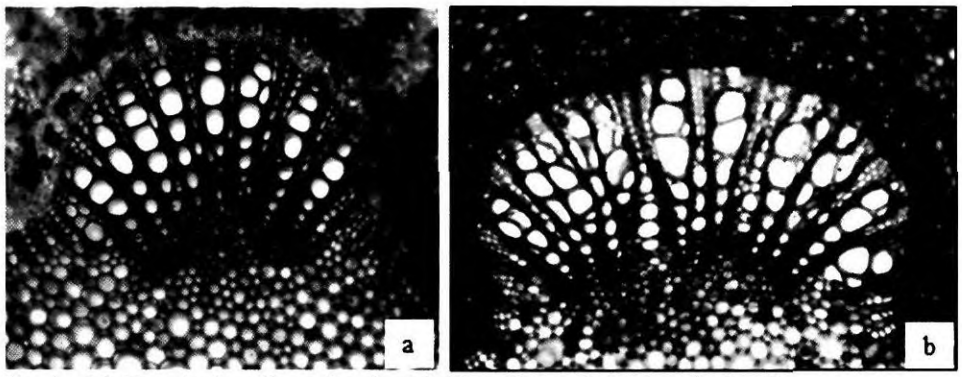

Fig. 4 a & b.Vascular bundle on the abaxial side with regular and necked outline

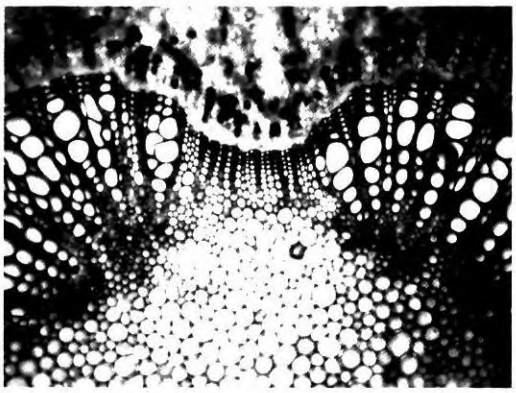

Fig.5. Inter-vascular continuity

third one is placed exactly perpendicular at the top and rest of two are laterally placed (Fig. 3). Bundles on the abaxial side have a regular or necked outline (Fig. 4a,b). Vascular bundles are distinct and arc shaped arranged around the pith. Each bundle maintains an inter-vascular continuity devoid of vessels (Fig. 5). This band with cell thickness ranges from 2-7 and is either continuous or incomplete at one loci between the adaxial bundles of the petiole (Fig. 6 a,b,c). Pith is either regular or irregular in shape due to the intrusion of cortical cell in the adaxial side of the petiole.

Table 2. **Anatomical diagnostic features of RRII 400 series clones**

Character	RRII 414	RRII 417	RRII 422	RRII 430	RRII 105	RRIC 100
Inter-vascular continuity	Discontinuous at one loci	Partially discontinuous at one to two loci	Discontinuous at one loci	Continuous	Discontinuous at one loci	Continuous
Thickness of inter-vascular continuity (no. of cells)	Thin (< 4)	Thin (< 4)	Thick (> 7)	Thick (> 7)	Thin (<4)	Thick (> 7)
Shape of abaxial bundle	Necked	Necked	Necked	Regular	Necked	Regular
Extent of adaxial bundles in the petiole	Half the total length of petiole	Uniform distribution	Intermediate	Uniform distribution	Uniform distribution	Uniform distribution
Intrusion of cortical cells into pith	Intruded	Not intruded	Intruded	Not intruded	Not intruded	Not intruded
Shape of adaxial bundle	C shaped	Regular	Horse shoe shaped	Regular	Regular	Regular
Medullary bundles	Present	Absent	Present	Absent	Absent	Absent
Size of pith	Large	Large	Small	Large	Large	Large

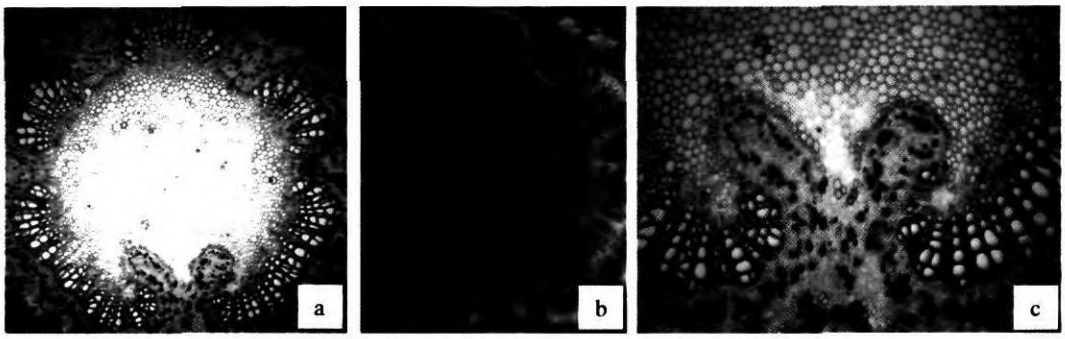

Fig. 6 a, b & c. Intrusion of cortical cells into the pith at the dorsal side of the petiole

Among the six clones, pith cells were comparatively small for RRII 414. Medullary bundles constituted of phloem encircled by xylem (Fig. 7) dispersed among the pith cells and were found in the adaxial side of petiole. This tissue mainly concentrated adjacent to the region where the inter-xylary discontinuity occurs and doesn't have any direct link with the main vascular cylinder of the petiole. Parenchyma cells interspersed with the protoxylem stained deep blue with Janus green that remains only for a short period of time after which discolouration occurs.

Table 2 depicts the predominant anatomical traits of five popular clones. A distinct intervascular continuity was characterised for RRII 430, whereas it was discontinuous at one loci in between the adaxial two vascular bundles of the petiole for RRII 105, RRII 414 and RRII 422, and intermediary with few scattered xylem tracheids in the discontinuous loci for RRII 417. This continuity was thick constituted with an average of seven layers of cells for RRII 430, RRII 417 amd RRIC 100 and for the rest of

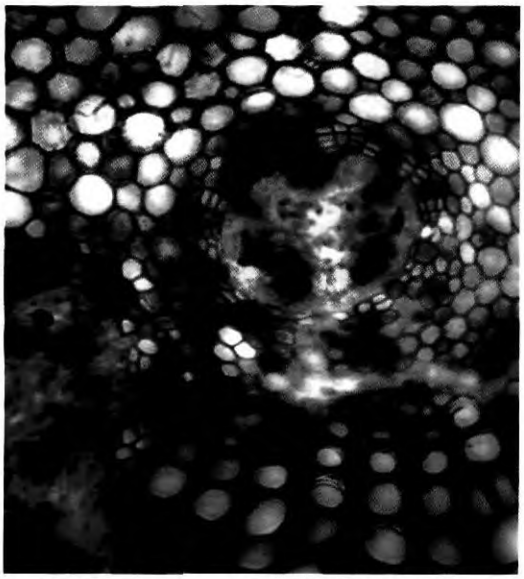

Fig. 7. Medullary bundles in the adaxial side of the pith

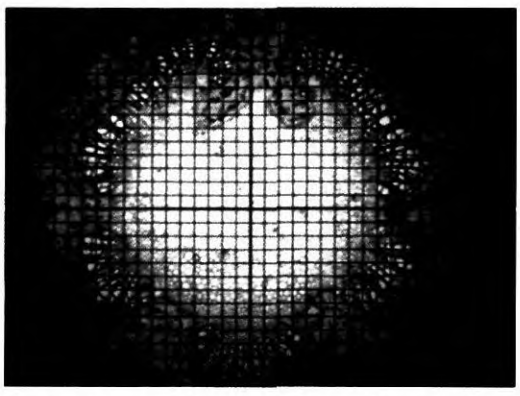

Fig. 8. Adaxial vascular bundles occupied half the total height of the petiole in cross sectional view

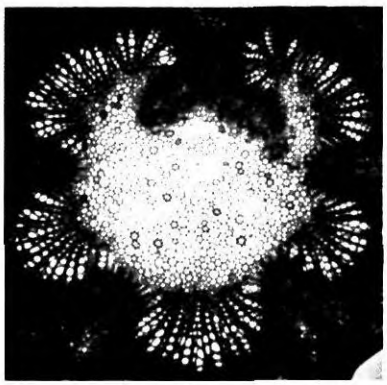

Fig. 9. C" shaped and 'horse shoe' shaped vascular bundle on the adaxial side of the petiole

the clones average number of cell layers is three. Vascular bundle in the abaxial side has a neck like outline except for RRII 430 where it appears to be a regular mount (Fig. 4). RRII 414 and RRII 422 have 1-3 rounded medullary bundles dispersed in the pith located adjacent to the inter-vascular continuity in the adaxial side. A patch of cortical cells bounded by an extension vascular tissue on the adaxial side intruded into the pith was characteristic for RRII 414 and RRII 422. Shape of the vascular bundle on the adaxial side is 'C' shaped for RRII 414 (Fig. 8), horse shoe shaped for RRII 422 (Fig. 9) and regular for rest of the clones. Both the bundles on the adaxial side are large and occupied half the total extent of the petiole thickness for RRII 414 (Fig. 8) and partly for RRII 422. Pith area was comparatively small with highly irregular outline due to the intrusion of cortical cells into the pith for RRII 422.

Number of xylem vessels in a vertical file in a bundle and total number of xylem vessel rows within a bundle showed significant clonal variation (Table 3). RRII 422 have the highest (8.00 – 8.33) number of xylem vessels in the individual vertical file with minimum variation for dorsal, ventral and lateral bundles. Number of xylem vessel rows in the bundles showed highest value for RRII 414 followed by RRII 422 and lowest value for RRIC 100. Length of vascular bundle in the dorsal side showed significant

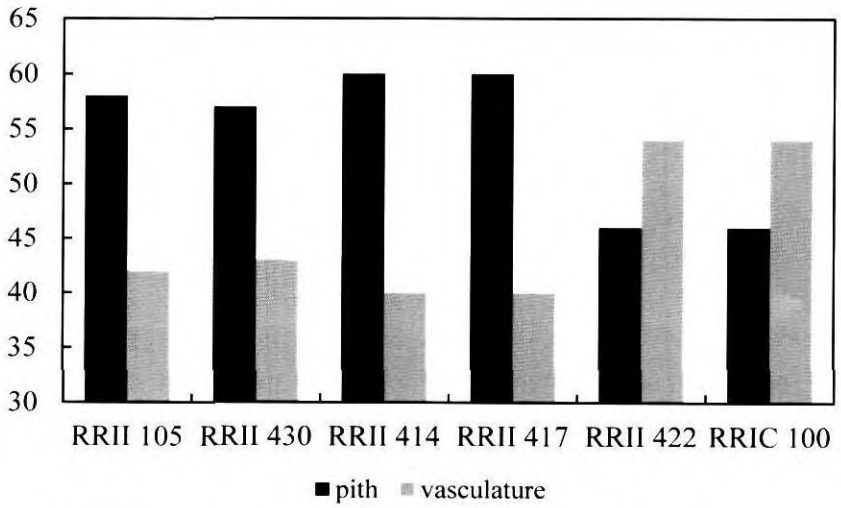

Fig. 10. Area of vasculature and pith in the stele of clones

clonal variation with higher values for RRII 414 (1149 µm) and RRII 430 (1102 µm). For all the clones length of lateral bundles have a lower value than dorsal and ventral bundles.

Proportionate area of pith and vascular tissue in the stele of the petiole showed drastic clonal difference (Figs.10, 11). Extent of pith recorded higher proportion for RRII 105, RRII 430, RRII 414 and RRII 417 while a reverse condition was observed for RRII 422 and RRIC 100. RRII 414 and RRII 417 recorded highest pith area compared to vascular tissue. Extent and shape of vascular tissue and pith is also evident. The strong intrusion of cortical cells into the pith and horse shoe shaped dorsal vascular bundles of RRII 422 has reduced the pith area substantially compared to other clones.

Taxonomic key based on anatomy of the petiole of *Hevea*

Anatomy of distal pulvinus of the petiole

1. Inter-vascular connection
   - 1.1. continuous– RRII 430, RRIC 100
   - 1.2. discontinuous at one loci- RRII 105, RRII 414, RRII 422
   - 1.3. partly-discontinuous at one loci – RRII 417
2. Thickness of inter- vascular connection
   - 2.1. Thick (average- 7) - RRII 430, RRII 422, RRIC 100
   - 2.2. Thin (average- 3)-RRII 105, RRII 414, RRII 417
3. Thickness of bundle sheath (no. of cells)
   - 3.1. 3-4 layers- RRII 105, RRII 430
   - 3.2. 5-7 layers- RRII 414, RRII 417, RRIC 100
4. Shape of vascular bundle (abaxial side)
   - 4.1. Regular mount- RRII 430, RRIC 100
   - 4.2. Necked- RRII 105, RRII, 414, RRII 417
   - 4.3. Intermediate - RRII 422
5. Medullary bundles in the pith
   - 5.1. Present - RRII 414, RRII 422
   - 5.2. Absent - RRII 105, RRII 417, RRII 430, RRIC 100
6. Position of the adaxial two vascular bundles
   - 6.1. Lower half of the petiole - RRII 414
   - 6.2. Equal distribution around the pith - RRII 105, RRII 417, RRII 430, RRIC 100
   - 6.3. Irregular - RRII 422
7. Cortical cell intruded into the pith
   - 7.1. Intruded - RRII 414, RRII 422
   - 7.2. Not intruded - RRII 105, RRII 417, RRII 430, RRIC 100
8. Shape of the adaxial vascular bundles
   - 8.1. Regular- RRII 105, RRII 417, RRII 430, RRIC 100
   - 8.2. "C" shaped - RRII 414
   - 8.3. Horse shoe shaped - RRII 422
9. Pith size
   - 9.1. Large - RRII 105, RRII 414, RRII 417, RRII 430, RRIC 100
   - 9.2. Small - RRII 422

Morphological classification for rubber based on a set of descriptors was first suggested by Dijkman (1951), followed by Jayasekara *et al.* (1984), Mercykutty *et al.* (1991) and Thomas *et al.* (2006), Sales *et al.* (2012). Considering the perennial nature of the crop with a long gestation period, utmost importance has been given for the identity of high yielding clones of *Hevea* in different rubber growing countries like Malaysia, Sri Lanka, Cote d' Ivoire and India since long (De Silva and Sachuthananthavale, 1961;

Table 3. **Characteristics of vascular bundle (VB) in different clones**

Clone	Characters								
	Length of VB (μm)			Length of VB (μm)			Length of VB (μm)		
	Dorsal	Ventral	Lateral	Dorsal	Ventral	Lateral	Dorsal	Ventral	Lateral
RRII 105	6.33	7.67	5.67	16.33	12.67	10.00	896.67	792.33	605.00
RRII 430	6.50	6.67	8.33	16.83	15.67	11.83	1102.50	965.83	671.66
RRII 417	6.17	6.17	7.17	16.67	14.50	11.83	975.00	817.50	632.33
RRII 414	6.67	7.67	7.17	20.50	12.67	13.67	1149.17	733.17	657.00
RRII 422	8.00	8.33	8.00	19.00	14.00	12.00	994.67	957.00	684.00
RRIC 100	6.17	6.67	6.00	10.50	11.00	8.67	692.00	808.00	689.00
CD	0.18	0.22	0.13	0.23	0.34	0.25	192.04	NS	NS
SE	0.06	0.08	0.05	0.08	0.12	0.09	66.50	65.55	52.57

Paardekooper, 1965; Jayasekara *et al.*, 1984; Mercykutty *et al.*, 1991, 2002; Penot and Rasidin, 1994; Delabarre and Binigno, 1994; Liyanage *et al.*, 2013). In the earlier reports including the studies carried out in India (Mercykutty *et al.*, 2002; Thomas and Saraswathyamma, 2004; Rao *et al.*, 2005; Thomas *et al.*, 2006) leaf morphological traits were used for taxonomic purpose. As part of multilateral clone exchange programme of the International Rubber Research and Development Board (IRRDB), also warrants the significance of identifying *Hevea* clones originated in different countries (Madhavan *et al.*, 2012, 2018; Mydin, 2018). Use of morphological markers is cost effective when compared to the use of biochemical and molecular markers for preliminary characterization of large number of accessions (Martinez *et al.*, 2003). Although many rubber clones do not exhibit highly distinct variations, most of them possess differences in certain minor, but more or less stable morphological features, which can be used for identification.

Taxonomic significance of leaf morphology, extra floral nectary on the petiole-petiolule juncture (Thankamma and George (1968); Antony and Madhavan (2015), intra-marginal venation pattern (Gomez and Hamzah, 1980; Martins and Zieri, 2003; Thomas and Saraswathyamma, 2004) and seed of *Hevea* (Saraswathyamma *et al.*, 2006) have been revealed earlier. Among the different plant organs used for identification, mature seed characteristics such as colour, shape and mottling can be used to identify clones with reasonable accuracy (Thomas *et al.*, 1996; Sebastian *et al.*, 2002; Rao *et al.*, 2005). Mercykutty *et al.* (2002) reported a comprehensive documentation of clone identification comprising of 24 primary and hybrid clones originated from various rubber growing countries. Detailed investigations to characterize the RRII 400 series clones based on yield and desirable secondary attributes have shown that distinguishable phenotypic and genotypic variations do exist among them, even though they are the progenies of the same parental combinations *i.e. RRII 105 x RRIC 100* (Saraswathyamma *et al.*, 2006). Thomas *et al.* (2006 a, b) developed a key of descriptors for the early identification of RRII 400 series clones including their parents (RRII 414, RRII 417, RRII 422, RRII 429, RRII 430, RRII 105 & RRIC 100) particularly based on the leaf characteristics.

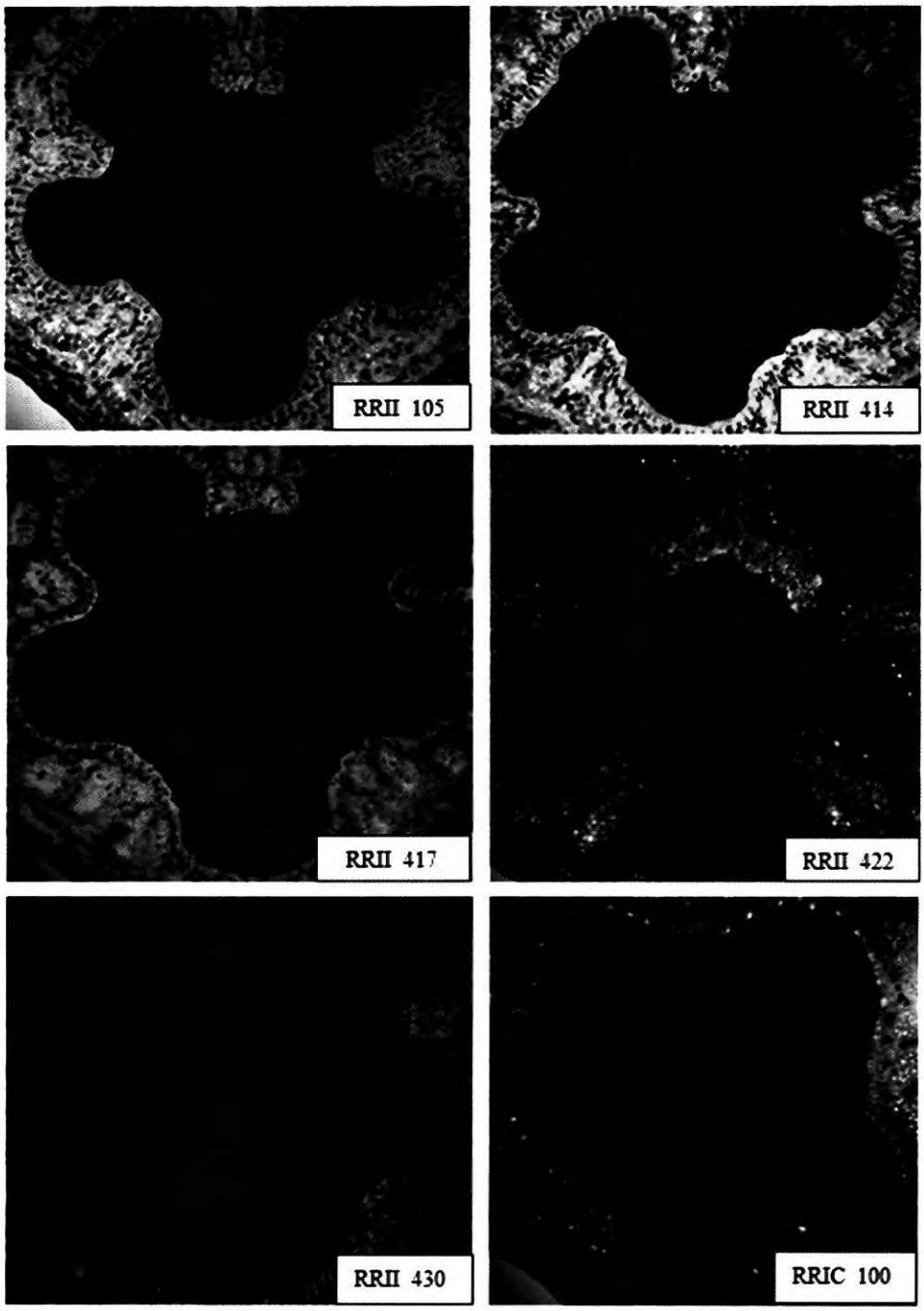

Fig. 11. Extent of vascular tissue and pith in the petiole of clones

Morphological distinction for some of the traits is relatively narrow or overlapping which can confound the identification (Thomas *et al.*, 2006 a, b). Moreover, quantitative traits like petiole length, size of leaves and height of the plants as well as certain other characters like colour and orientation are in general highly influenced by the environment and hence does not allow a clear separation among the clones. Hence, it is cautioned that a combination of as many traits as possible should be considered before drawing conclusive inferences (Thomas *et al.*, 2006 b). With respect to leaf morphological characteristics, RRII 414 shows a close affinity to the female parent RRII 105, and RRII 430 shows more resemblance to its male parent RRIC 100. The other two clones *viz.*, RRII 417 and RRII 422 are intermediate with respect to these traits. Among the six clones studied, RRII 430 showed both morphological and anatomical identity with RRIC 100. Structural traits showed more resemblance between RRII 414 and RRII 422.

It is obvious that compared to morphological traits, anatomical characteristics represent the environmental adaptation of clones and hence more reliable. A set of anatomical traits alone or in combination *viz.* inter-vascular continuity, occurrence of medullary bundles, shape and extent of adaxial and abaxial vasculature *etc.* are reliable features which can serve as an adjunct to the identification of the clones. The position of sectioning of the distal pulvinus appears to be crucial for revealing the distinct clonal variation. Many of the traits were observed to be prominent only in the distal pulvinus of the petiole, adjacent to the petiole- petiolule juncture.

The stability of these structural traits with respect to age in nursery plants, plants from bud wood nursery, young and mature plants, and agroclimatic variations needs to be addressed for considering them as a reliable anatomical marker for *Hevea* clone identification. Once stability of these traits is established irrespective of age and location, the descriptors can be extended to newer clones in the pipeline and the feasibility for use in clone registration in tune with DUS norms under PPV, FRA can be explored.

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