
Synopsis of the Thesis entitled

Evolutionary Patterns in the Secondary Growth of Tropical Lianas

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Introduction

Lianas are one of the fascinating groups of plants that intelligently evolved and adapted and modified themselves from self-supporting to the climbing habit. They are non-self-supporting members of unrelated lineages (Putz & Mooney, 1991; Cabanillas & Hurrell, 2012; Sperotto *et al.*, 2020; Souza-Baena *et al.*, 2021; Leme *et al.*, 2020). For the extension growth, they are dependent on an object or host that supports to climb on the external support to find mechanical assistance for vertical development towards the light. One of the categories of climbing plants is lianas (woody climbers) that are perennial, woody non-self-supporting members dependent on other plants for their support to climb (Putz, 1984; Putz & Holbrook, 1991; Liang *et al.*, 2017). Climbing plants are characterised by the presence of narrow stem and they increase their stem thickness by various means by developing the cambial variant (Carlquist 1988, 2001). Unlike most of the eudicots, they increase in stem diameter by various growth trajectories like formation of successive cambia phloem wedges, inter-and intraxylary phloem, compound or polystelic condition or fissured stems (Metcalfe & Chalk 1950, 1983; Obaton, 1960; Putz & Mooney, 1991; Isnard & Silk, 2009; Angyalossy *et al.,* 2015; Schnitzer *et al.,* 2015, Tamaio & Somner, 2010; Tamaio *et al.*, 2011). Most of the non-self-supporting plants have slender and narrow stems (Field *et al.,* 2012) but occasionally they may develop various stem conformations (Carlquist 1988, 2001).

Throughout the process of evolution from self-supporting habit towards non-self-supporting growth forms, these plants have not only transformed their internal structure of conducting elements but also revised the external morphology that helps them to climb or entwine around the supporting object or host (Rowe *et al.*, 2004; Isnard & Silk, 2009). Darwin (1865) for the first time categorized climbing plants into five categories based on their mode of climbing mechanism and modification of aerial organs which work as the specialized structures playing a strategic role in their climbing behaviour as twiner (e.g., *Ipomoea*, *Merremia*, *Convolvulus*, *Cocculus hirsutus*); leaf climbers (*Clematis gauriana*, *Gloriosa superba*, *Smilax zeylanica*);

tendrilar climbers (*Passiflora, Cucurbits, Serjania*); root climbers (*Vitis, Epipremnum aureum* [money plant], *Ficus pumila*) and hook climbers (*Artabotrys odoratism, Ancistrocladus benomensis, Calamus*) (*cf.* Isnard & Silk, 2009).

The climbing habit evolved by achieving stem flexibility and several structural modifications in the mechanical tissue (Rowe & Speck, 1996, 1998, 2005; Rowe *et al.*, 2004; Angyalossy *et al.*, 2015; Lekhak *et al.*, 2018). Stem flexibility is accomplished by changing growth patterns and comparatively profusion of parenchyma cells and reduction in the mechanical tissue, wide rays, forming phloem wedges and by developing various kinds of cambial variants (Obaton, 1960; Carlquist, 1988, 2001; Pace *et al.*, 2009, 2018; Gerolamo *et al.*, 2020). Among these alterations, modification in the secondary growth is one of the ways to advance hydraulic competence and increase stem flexibility (Carlquist, 1988; Ewers & Fisher, 1991; Rowe *et al.*, 2004; Isnard & Silk, 2009; Schnitzer & Bongers, 2011; Rajput *et al.*, 2012, 2013; Lekhak *et al.*, 2018) for climbing or twining on the host that protects the conducting tissues from internal injury during stem torsion (Carlquist, 1988). The narrow stem diameter and altered mechanical tissue of the stems in climbing plants act like a cable and help them to protect the vessel from cavitation while an abundance of axial parenchyma helps them during external and internal injuries (Carlquist, 2001; Rowe *et al.*, 2004; Rowe & Speck, 2005; Isnard & Silk, 2009; Schnitzer & Bongers, 2011; Rajput *et al.*, 2012b, 2013; Lekhak *et al.*, 2018). Alterations in the pattern of the secondary thickening in lianas may vary from species to species or may be unique and characteristic to certain tribes and families or may be shared by allied genera (Rajput *et al.*, 2021). Alterations in the structure and composition of the stems of non-self-supporting plants are usually known to occur only after the initiation of the climbing habit (Caballe, 1998; Carlquist, 2001; Rowe *et al.*, 2004; Rowe & Speck, 2005; Isnard & Silk, 2009; Rajput *et al.*, 2012b, 2013; Angyalossy *et al.*, 2015; Lekhak *et al.*, 2018; Chery *et al.*, 2020; Tamaio *et al.*, 2021). During the early stages of the secondary growth (i.e., before the development climbing habit), young plants or their branches show typical secondary growth which is characteristic of self-supporting habit (Rowe & Speck, 1996, 2005). Most of the non-self-supporting members show development of various types of cambial variants that are hypothesized to result in response to climbing habit (Carlquist, 1988, 2001; Isnard & Silk, 2009; Angyalossy *et al.*, 2015; Lekhak *et al.*, 2018; Rajput *et al.*, 2021), including the formation of successive cambia. All these cambial variants have been categorized variously by earlier researchers (Schenck, 1895; De Bary, 1984; Pfeiffer, 1926; Metcalfe & Chalk, 1950, 1983; Boureau, 1957; Obaton, 1960; Philipson & Ward, 1965; Carlquist, 1988, 2001). These classifications of variant secondary growth include extensive monographs (Pfeiffer, 1926), diagrammatic illustrations (Schenck 1893; Chalk & Chattaway, 1937) or cellular details and outline diagrams of the monograph (Schenck 1893; Obaton, 1960). Anatomically, these classifications also include, bicollateral vascular bundles, medullary bundles, cortical bundles, intraxylary (internal) phloem, interxylary (included) phloem, concentric rings of xylem alternating with phloem, stem become elliptical, flattened, lobbed, fissured xylem or furrowed in cross-section, stems lobed. Among them, the family Sapindaceae is of special interest and treated separately due to a unique type of cambial variant (referred to as compound xylem i.e., polystelic xylem) (Tamaio *et al.*, 2011, 2021). With this brief background, the present study attempts to understand the evolution of structural alterations in the growth patterns and structure of the secondary xylem by studying their developmental stages in selected tropical lianas with the following objectives.

Objectives of the proposed study:

1. To study the origin and development of procambium and vascular cambium.
2. To study the structure and development of primary and secondary vascular tissue before the climbing habit.
3. To study the behaviour of meristem (pattern secondary growth) that is responsible for secondary growth after adapting the climbing habit.
4. To study the structural alterations induced in the secondary xylem and phloem in response to the shift from self-supporting to climbing habit.
5. To correlate these alterations in the secondary tissues with ecological wood anatomy.

Materials and Methods

Collection of samples and Fixation**:** Stem samples of various thicknesses (shoot tip to maximum possible thickness available) were collected from the five individuals each of the species growing naturally or under-cultivation. Stem samples of a total of 52 different species belonging to 10 angiosperm families and 2 Gymnosperm families were collected from various bio-geographical locations throughout the country (including some samples from Durban, South Africa and Osa Peninsula, Costa Rica). To study various developmental stages; stems with different diameters starting from shoot tip to mature stems were harvested 10 cm above the ground level and subsequently, samples were collected from every 50 cm interval towards up to the apex. Stem pieces measuring 40-60 mm in length were cut and fixed immediately in FAA after the collection (Berlyn & Miksche, 1976). After 48-72 hours of fixation, samples were transferred to 70 % ethanol for further processing and storage.

Sample preparations, sectioning and photography**:** For the macro-morphological study, thick stem samples of suitable sizes were trimmed using a sharp blade and allowed to dry for a few minutes and photographed with Canon (DSLR – EOS 1500D) or by using the stereo zoom microscope (Leica S6D & Leica 205C fitted with Leica IC90E camera) and sometimes using a mobile camera (Nokia 7.2). For histological preparations, young and soft stems were processed through a Tertiary Butyl Alcohol (TBA) series, embedded in paraffin wax after infiltration (Berlyn & Miksche, 1976) and sectioned using a Leica RM 2035 rotary microtome. Thick stem samples were sectioned directly on Leica SM 2010R sliding microtome. Transverse, radial and tangential longitudinal sections of 15-20 µm thickness obtained from both, soft and hard samples were dehydrated through an ethanol-xylene series and mounted in DPX after staining with the Safranin-Astra blue combination (Srebotnik & Messner, 1994). Microphotographs of important results were taken using a Leica DME 2000 trinocular photomicroscope with Leica DFC 295 firewire digital camera.

Maceration: Dimensional details of the secondary xylem derivatives were studied by maceration of the outermost secondary xylem adjacent to the vascular cambium. Small portions of the secondary xylem were sliced into small pieces with a razor blade and treated with Jeffrey’s fluid (Johansen, 1940). These xylem slices were incubated at 55-60 ^{o}C for 24-36 hours washed thoroughly with distilled water and stained with 0.5% aqueous safranin. Lengths of the vessel elements and fibres were measured from the macerated material while dimensional details of sieve tube elements, ray height and width (of both xylem and phloem) were measured from the tangential longitudinal sections while vessel diameter (tangential) was measured from the transverse sections as recommended by IAWA committee (1989). Thirty measurements for each cell type were taken randomly to get the mean and standard deviation. Mean and SD was calculated using Microsoft Excel 2007.

Results

Throughout angiosperm evolution particularly in lianescent lineage, several plant families independently developed cambial variants, with certain variants being so distinctive and characteristic to each group and may be solely recognized by their stems topographies (Caballe, 1993; Angyalossy *et al*. 2015). Climbing or non-self-supporting plants are characterized by the presence of cambial variants like successive cambia, functionally inverse cambia, inter-and intraxylary phloem, internal/intraxylary phloem cambium, compound/polystelic stems, phloem wedges and furrowed xylem. As shown in Table 1, several climbing members of various families that are dominated by climbing habits were investigated histologically in the present study. All the members were characterized by one or other type of variant secondary growth (including *Gnetum ula* from the gymnosperms) except *Ampelocissus latifolia*, *Cayratia auriculata*, *Cissus quadrangularis*, *C. repanda*, C. *rotundifolia*, *Dalbergia volubilis*, *Cissampelos pareira*, *Coscinium fenestratum*, *Stephania japonica*, *Tinosporia cordifolia*, *T. sinensis*, *Vitis vinifera* and from gymnosperms *Ephedra foliata* and *E. karumanchiana* showed a regular way of secondary growth with a single ring of the vascular cambium like other eudicots.

During the primary growth, most of the eudicots (including species with cambial variants) show a regular pattern of growth i.e., procambium initiated as a small sector at several places and its bifacial activity led to the development of vascular bundles that were arranged in a ring. Subsequently, these vascular bundles were interconnected interfascicular cambium and formed a complete ring of the vascular cambium. However, in the present study, members of families like Apocynaceae, Convolvulaceae, Cucurbitaceae and Loganiaceae also showed presence of primary variant (intraxylary protoxylem) along with regular primary growth. In all the investigated species, after the initiation of the secondary growth, the vascular cambium differentiated xylem centripetally and phloem centrifugally. In the initial stage of the secondary growth, in some species showed characteristics of the self-supporting plants (elaborated in thesis) but as the secondary growth progressed, composition and structure of the secondary xylem altered suddenly and showed a structure that is typical to the non-self-supporting or climbing habit.

Stem conformation (outline) in transverse view: In the transverse view, a structural outline is one of the important features to study the mechanical architecture, ecology, and adaptation towards the climbing habit. It also indicates the strategy of secondary growth by which they reach the canopy of the supporting trees/objects. As compared to self-supporting plants, non-self-supporting plants like vines, herbaceous climbers, and woody liana possess narrower stems. Due to their narrow stems and reduced mechanical tissue, they use various other strategies so that they can facilitate climbing on the supporting objects/hosts by promoting extension growth. Initially, when the individual plants are young, all the species studied in the present investigation showed circular stem conformation in transverse view. However, with the increase in age and the stem diameter, stem outline in transverse view in some members changed into various shapes other than circular (Table 1).

***Structure of young stem and initiation of primary growth*:** In all the speciesinvestigated, the young stems were circular in outline and they were composed of single-layered, oval to circular or barrel-shaped thin-walled parenchymatous epidermis. The epidermis was followed by one to a few cell-wide hypodermis. The hypodermal cells were oval to polygonal and thin-walled cells. Unlike other species, in *Cayratia auriculata* the hypodermis was distinct and composed of radially elongated cells. The hypodermis was followed by several cells wide cortex that was composed of compactly arranged oval to polygonal, isodiametric, thin-walled parenchyma cells with or without intercellular spaces. In young stems, the cortex and central stele were separated by continuous ring pericyclic fibres. However, with the increase in age, the ring of fibres was broken due to tangentially stretching in response to increase stem diameter that resulted in splitting of complete ring into small segments of pericyclic fibres. Isolated or groups of randomly distributed sclereids (*Campsis radicans*, *Strychnos bredemeyeri*. *S. andamanensis*) or laticifers were common in the members of the Convolvulaceae, Apocynaceae and Asclepiadaceae. An endodermis consisted of the thin-walled isodiametric or tangentially elongated barrel, shaped parenchymatous cells while some species like *Turbina corymbosa*, *Valaris solanacea, Strychnos bredemeyeri, Beaumontia jerdoniana, Campsis radicans* endodermis was indistinct. Though the endodermis and pericycle appeared indistinct, they can be discerned by the presence of pericyclic fibres. A central portion of the young stem was occupied by the parenchymatous pith. Depending on the species, a cluster of the external protophloem and protoxylem began to differentiate as vascular bundles in the 4-7th visible internodes. Several vascular bundles that were composed of one to two protoxylem elements and a cluster of three to four protophloem elements on either side of the procambium initiated in the subsequent internodes. These vascular elements become distinctly visible with the enlargement of metaxylem elements in the 7-9th internodes. Subsequently, adjacent vascular bundles were interconnected by forming interfascicular cambium to form a complete ring of the vascular cambium. This newly initiated vascular cambium was functionally bidirectional to deposit the secondary xylem centripetally and phloem centrifugally. The centrally located pith consists of thin-walled parenchyma cells that may be isodiametric or irregular in shape and size. Species like *Beaumontia jerdomiana* (Apocynaceae), *Dicranostyles ampla*, *Hewittia malabarica* (Convolvulaceae), *Leptadenia reticulata*; *L. pyrotechnica* (Asclepiadaceae, Apocynaceae), *Maripa nicaraguensis Turbina corymbosa* (Convolvulaceae), *Vallaris solanacea* and *Wattakaka volubilis* (Apocynaceae) also showed the presence of interxylary phloem at the pith margin.

***Xylem structure from the transition from self-supporting to climbing habit*:** During the initial period of growth, the young shoots of some members like *Arrabidaea candicans* (Bignoniaceae), *Beaumontia jerdoniana*, *Dicranostyles ampla*, *Entada rheedii*, *Maripa nicaraguensis*, *Serjania mexicana*, *Strychnos bredemeyeri*, S. *andamanensis* and *Valleris solanacea* showed typical secondary xylem of self-supporting habit before the initiation of climbing habit. Such a xylem was relatively hard and stiff and possessed relatively narrow vessels, higher number of xylem fibres and parenchyma cells were either absent or minimal.

***Structure of the secondary xylem after climbing*:** After the initiation of the climbing habit, considerable alterations were observed in the structure of the secondary xylem. Unexpectedly, the production of parenchyma, vessel frequency and vessel diameter were increased with the shift from the self-supporting to the climbing stage. Subsequently, ray width also increased in most of the members while species like *Arrabidaea candicans, Beaumontia jerdoniana* and *Valleris solanacea* retained uni-biseriate rays while multiseriate rays developed rarely.

***Formation of the secondary xylem in plates*:** In the members of Menispermaceae like *Anamirta cocculus, Cissampelos pareira, Cocculus hirsutus, C. pendulus, Coscinium fenestratum*, *Diploclausia glauscence*, *Pachygon ovata*, *Stephania japonica*, *Tinosporia cordifolia*, *T. sinensis*, and *Tiliacora racemosa* (including trees like *Cocculus laurifolius*), *Cissus quadrangularis, C. repanda*, *Vitis vinifera* of Vitaceae and gymnosperm like *Ephedra foliata* and *E. karumanchiana* showed the formation of xylem in radial plates. Formation of such a xylem resulted in response to relatively wide rays and narrow sector of the fascicular sector of the cambium that produces axial elements. Development of xylem in plates was observed in species with regular secondary growth and species with successive cambia.

***The abundance of parenchyma* (*both axial and ray*)**: The abundance of unlignified parenchyma in the wood of scandent plants is attributed as a feature that provides flexibility to the stem and protects large and vulnerable vessels against stem torsion and damage. The secondary xylem of the plants of the family Fabaceae (*viz. Canavalia gladiata, C. virosa, Entada rheedii, Phaseolus lunatus, Pueraria tuberosa*) and Vitaceae (viz. *Ampelocissus latifolia*, *Cayratia auriculata*, *Cayratia trifolia, Cissus quadrangularis*, *Cissus repanda* and *Tetrastigma bracteolatum*) possesses abundant unlignified axial parenchyma whereas libriform fibres were restricted around the vessels or distributed as small islands within the parenchymatous background. Fibres form a sheath around vessels, which is also known as "cable construction"

Formation of successive cambia: Present study revealed the development of successive cambia from various cell types like pericycle parenchyma, cortical parenchyma cells, from the dilating phloem parenchyma (both ray and axial) or both ray and axial parenchyma cells of the xylem. Based on their origin, formation of successive cambia may be categorised as:

i). *Initiation of successive cambia from pericyclic parenchyma or derivatives*: In *Antigonon leptopus, Cyratia trifoliata, Dicranostyles ampla, Maripa nicaraguensis, Moutabea gentry, Tetrastigma bracteolatum* and *Turbina corymbosa* the increase in stem thickness was achieved by forming successive rings of cambia. These cambia were initiated from the pericyclic parenchyma cells. After a short period of secondary growth, parenchymatous cells (likely pericycle) located outside the protophloem acquired meristematic activity and gave rise to the first ring of successive cambium.

ii). *Dilatation phloem ray parenchyma and initiation of neo-formed vascular cylinders*: Dilatation of phloem ray parenchyma is a common feature in majority of the eudicots. The degree of dilatation is highly variable from species to species and is correlated with the adjustment to the increase in diameter in response to the secondary growth of the stem. In this process, ray cells may either extend tangentially or increase tangentially by radial divisions. The extent of radial divisions in rays was observed maximum in some of the investigated species which consequently led to the development meristem that was structurally and functionally similar to the vascular cambium. In species like *Hewittia malabarica*, *Phaseolus lunatus* and *Turbina corymbosa* the development of neo-formed vascular cylinders was observed from such a dilatation meristem. Most species in the family Convolvulaceae are characterized by the presence of successive cambia from the pericycle parenchyma while some members complete their lifecycles with a single vascular cambium. Unlike other members of the Convolvulaceae, the additional cambia in *Hewittia malabarica* and *Turbina corymbosa* do not connect to form a complete ring of the successive cambium and form individual vascular cylinders. Similar vascular cylinders were also observed in *Phaseolus lunatus*, which is reported for the first time in the family Fabaceae.

iii) *Initiation of successive cambia from cortical parenchyma*: Among the species investigated, the development of successive cambia from the cortical parenchyma cells was observed in the members of the Menispermaceae, (like *Anamirta cocculus*, *Cocculus hirsutus, C. laurifolius, C. pendulus, Cyclea peltata, Diploclisia glaucescens, Pachygone ovata* and *Tiliacora racemosa*) and *Rhynchosia pyramidalys*

(Fabaceae). There is a difference of opinion regarding the initiation of the successive cambia in Menispermaceae which is discussed in detail in the thesis.

iv) *Development of functionally inverse cambia*: Development of inverse cambia is a rare feature and is reported in a few members. In the present study formation of such functionally inverse cambia is reported herewith in *Argyreia elliptica*, *Canavalia gladiata*, *C. virosa*, *Entada rheedii*, *Dicranostylis ampla*, *Phaseolus lunatus*, *Turbina corymbosa* and *Hewittia malabarica*. During the initiation of successive cambia, the parenchyma cells formed before the cambium initiation, cells located on the inner margin of the meristematic zone differentiate into conjunctive tissue and serve as a site for the initiation of inverse cambia in future, whereas cells on its outer margin become the source for the further successive cambia.

***The proliferation of xylem rays and formation of ray cambium*:** In *Canavalia gladiata*, *C. virosa*, *Hewittia malabarica*, *Phaseolus lunatus*, *Pueraria tuberosa* and *Turbina corymbosa* the marginal ray cells of the multiseriate rays acquired meristematic activity and initially formed only phloem elements. However, as time passed it also showed differentiation of the secondary xylem. Initiation of the ray cambium and differentiation of the xylem and phloem from the ray cambium coincide with the reproductive phase of samples collected in the present study. The development of additional conducting elements (both secondary xylem and phloem) from these ray cambia and the proliferation of ray cells may be playing a crucial role in the rapid translocation of minerals and nutrients, and reserved food materials respectively.

Development of interxylary phloem: Interxylary phloem are strands of sieve tubes and associated parenchyma cells embedded within the secondary xylem of the stems and roots. In the present study, the development of such phloem was observed in *Canavalia virosa*, *C. gladiata*, *Entada rheedii*, *Hewittia malabarica*, *Leptadenia reticulata*, *L. pyrotechnica*, *Phaseolus lunatus*, *Pueraria tuberosa*, *Strychnos bredemeyeri*, *S. andamanesis* and *Turbina corymbosa*. However, this definition broadly includes the phloem formed by successive cambia as well as by a single ring of cambium.

Development of intraxylary phloem and intraxylary phloem cambium: Internal or intraxylary phloem are strands of phloem differentiating at the pith margin. The occurrence of intraxylary phloem is documented in 30 families of eudicots including families like Apocynaceae, Convolvulaceae and Loganiaceae. In the present study development of such phloem is documented in *Beaumontia jerdomiana, Campsis radicans, Dicranostyles ampla, Hewittia malabarica, Jacquemontia pentantha, Leptadenia reticulata, L. pyrotechnica, Maripa nicaraguensis, Strychnos bredemeyeri, Turbina corymbosa, Vallaris solanacea*, and *Wattakaka volubilis*. In all the investigated species formation of intraxylary phloem was observed from the initiation of primary growth except in *Campsis radicans*. Development of such a phloem in this species was observed only after the initiation of secondary growth.

Development of phloem wedges* (*furrowed xylem*) *and lobbed stems: Phloem wedges are the discrete variant portions of the secondary xylem that derive from the small segments of the vascular cambium due to shift from the regular pattern and deposit less xylem and more phloem. The evolution of the climbing habit induced several alterations in the mechanical architecture of the stem to increase stem flexibility and growth patterns. Formation of such phloem wedges is one of the important strategies of the climbing plants to enhance the stem flexibility against the stem twisting and internal injury due to swinging of the stems in response to strong wind currents or movement of arboreal animals.

Some of the species investigated in the present study also showed a special case of growth pattern which is not been reported so far from the Indian species. In the present two such taxa, (*Serjania circumvallate* and *S. mexicana* of the family Sapindaceae) collected from the Osa Peninsula of Costa Rica are investigated as a special case of growth pattern for the climbing habit.

***A special case of variant growth*:** As compared to other members investigated, a unique pattern of primary and secondary growth was observed in *S. circumvallata*. Among the three species (*S. circumvallata*, *S. mexicana* and *Cardiospermum helicacabum*) of Sapindaceae investigated here, all three represent different growth trajectories. *C. helicacabum* showed regular secondary growth like most eudicots while *S. circumvallata* and *S. mexicana* showed primary and secondary variant growth respectively. In the former case (i.e., in *S. circumvallata*), the development of polystelic condition was observed from the primary growth. After the establishment of the vascular cambium, some of the cells located within the pith region began to form xylem and phloem and subsequently developed 3-5 vascular cylinders giving an appearance like 4-6 vascular cylinders were clubbed together. In contrast, later species (*S. mexicana*) showed phloem wedges like the members of the tribe Bignonieae but the only difference was these wedges were uneven in number followed by it also showed the development of neo-formed cylinders external to the phloem wedges.

The obtained results are discussed with the available literature to correlate the evolution/origin variant structures with climbing habits.

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