CHAPTER - III

3.1). *Selection of plant species*:

Non-self-supporting plants are unique groups of vascular plants and they have narrow stems versus large photosynthetic areas (i.e., crown). They are adapted to climb over the host to cope with competition for above and below-ground resources. Based on their climbing strategy for harvesting the above-ground resources, they may be classified as vines, climbers, twiners, lianas and stranglers (Darwin, 1865; Gentry, 1985; Hegarty, 1992; Rowe *et al.*, 2004; Isnard & Silk, 2009; Bohn *et al.*, 2015). Depending on the strategy for climbing on the host, their stem anatomy also varies accordingly. In the present study, non-self-supporting plants from different categories like vines, climbers, twiners, lianas and stranglers are selected from the families that are dominated by climbing habit to understand the evolution of adaptive anatomical characteristics.

3.2). *Field visits and collection of samples*:

Based on the above-mentioned criteria, species belonging to different families were selected and are mentioned in Table 1. Since all selected species were not available in Gujarat, stem samples of these species were collected from various locations throughout the country. In the present study, some of the samples were received from other countries under collaborative research work (Table 1). The reason for including foreign samples is just to compare the behaviour of species growing in another continent that has similar behaviour to Indian samples. To trace the ontogeny of structural alteration in the growth pattern, sample of both primary and secondary growth stages was collected for the investigation. Samples were collected from five individuals of each species in the primary and secondary growth phases. For primary growth, samples were collected in the vegetative, reproductive phase and thick (20-30 mm, for perennial) and fully grown (for annuals at fruit dispersal stage) stems. For primary growth samples were collected from the shoot tip up to 25 visible nodes while for secondary growth, samples were collected from the main stem. To trace various

developmental stages, samples of various stem thicknesses were collected from five individuals for each species. For secondary growth, samples were collected 10-12 mm above the ground and continued to collect after every 50 cm interval of the stem till it reached five mm thickness from the base towards the apex. A list of the species selected in the present investigation is provided in table 1, along with the place of collection and maximum thickness of the stem sample available for the study.

3.3). *Processing of the Samples in the Field and Laboratory:*

3.3.1). *Killing and fixation*:

Besides collection and proper identification, appropriate fixation of the collected samples is a crucial feature for better sectioning and good-quality slide preparation. Therefore, after collection, it is very much essential and much important to fix it as early as possible. In the present study, samples were fixed in Formaldehyde-Acetic Acid-70% Alcohol (FAA i.e., 10:5:85 respectively) solution as described by Berlyn and Miksche (1976). Stem samples were cut into proper sizes depending on the thickness and type of material collected. As mentioned above, primary growth samples starting from the shoot tip up to 25 nodes were collected, coiled in the ring form and fixed as it is while thick samples were cut into 50-60 mm long pieces and fixed immediately. Some of the samples were also collected in polyethene zip lock bags containing cotton buds with a fixative solution to avoid drying and dehydration of the tissue. It also helps to reduce air embolism and is one way of immediate fixation in field conditions. After reaching the laboratory, these samples were subjected to a low vacuum to remove the air bubbles and proper fixation of the collected samples. Depending on the material thickness (stem diameter) and size of the samples, were transferred to 70 % alcohol. After 48-72 hours of fixation, samples were transferred to 70% of alcohol for further processing and preservation.

3.3.2). *Microscopic preparations*:

After killing and fixation, samples were sectioned using either by rotary microtome or with the help of a sliding microtome (Leica SM2010R). The selection of

microtome methods for sectioning was decided based on the tissue hardness, stem thickness and type of material to be sectioned. Hard and thick stems with secondary xylem containing lignified tissue were sectioned directly by using the sliding microtome while soft and delicate samples like apical shoot, primary tissue and xylem having abundant unlignified parenchyma cells were processed through paraffin embedding and sectioned using the rotary microtome.

***A). Rotary microtome sectioning*:**

Soft and young tissues with primary growth are difficult to hold in specimen holder of sliding microtome; therefore, such tissues were infiltrated with paraffin and embedded in paraffin (Berlyn & Miksche, 1976) and sectioning was done using a rotary microtome (Leica RM2035) to obtain 15-20 µm thick sections. The steps followed in paraffin processing are described as follows:

a) *Dehydration*: Fixed samples of soft material were washed thoroughly in running tap water followed by rinsing in distilled water to remove even a very minute trace of fixative from the material. After washing, these samples were trimmed into small pieces using a sterile surgical blade and aspirated using a vacuum pump at low pressure to remove the air bubbles to avoid floating of samples during the dehydration process. Trimmed samples were passed through the graded series of Tertiary Butyl Alcohol (TBA) for dehydration. For dehydration, these samples were kept in small glass vials containing various concentrations of TBA (*viz*. 15%, 35%, 50%, 70%, 85%, 95% and 100%). The incubation time for each sample was decided based on the sample size and hardness. On an average, for each concentration, samples were kept about 6-8 hrs. or sometimes up to 12 hrs. if the sample is hard. After reaching 100% TBA, three changes of pure TBA were given as described by Johansen (1940) and Berlyn and Miksche (1976).

b) *Infiltration*: Dehydrated samples were infiltrated using paraffin wax (Qualigens Paraffin wax with ceresin, M.P.: 55 – 60 ^{o}C). After 3rd change of pure TBA, a small amount of Para-TBA was added gradually every 3-4 hrs. till the TBA in

the vial reached saturation. Subsequently, vials containing samples were shifted to the oven at 45-50 ^{o}C temperature and the lids of the vials were kept open to evaporate the TBA. As paraffin melts, more wax was added periodically to compensate for evaporating TBA so that samples remain completely inside the liquid paraffin. The melting point of paraffin wax and temperature of oven were selected based on the type of tissue under processing for sectioning.

c) *Embedding*: Samples infiltrated with paraffin were given three changes of pure paraffin to remove the traces of TBA. Subsequently, these samples were transferred into paper boats containing freshly melted wax for moulding purposes. Immediately after pouring into moulding paper boat, (i.e., when the paraffin wax was melted and in a liquid state) samples were arranged properly in the orientation of interest (i.e., in the transverse, tangential and radial longitudinal plane) for sectioning. After proper arrangement and orientation of processed samples/tissue, paper boat was allowed to cool down for proper hardening of wax within the moulding boat.

d) *Sectioning and mounting*: The paper of moulding boat was removed after complete cooling of wax and the embedded samples were trimmed to remove the extra wax. They were properly trimmed to get a perfect cubic-shaped paraffin block; subsequently, the block was mounted on wooded blocks for sectioning. After mounting on wooden block once again fine trimming was done to get the perfect paraffin ribbon containing sections embedded within it. Sections of 12-15 µm of thickness were taken in transverse, radial and tangential longitudinal planes using rotary microtome. Paraffin ribbons obtained after sectioning were cut into 4-5 cm long strips and 1-3 strips (depending on sample thickness) were mounted on the glass slide coated with Haupt's adhesive. Before mounting sections, one drop of Haupt's adhesive (Johansen 1940) was applied on the slide and rubbed till it completely dries. These dried slides were used to arrange the paraffin ribbons containing sections. The slides were flooded with 2 % formalin and gently heated on a slide warmer for the proper spreading of sections. Warming the slides on a slide warmer helps in fast evaporation due to heating and gelatin used in Haupt's adhesive getting coagulated properly which helps to adhere sections on the slide. Thereafter, slides were kept for

drying for 2–3 days or more to achieve complete drying. Completely dried slides were passed through the downgraded series of Xylene – Ethanol to remove the paraffin wax and sections were brought to aqueous (rehydration) condition (Berlyn & Miksche, 1976) for staining purposes.

***B). Sliding microtome sectioning*:**

Thick stem samples that has hard and lignified xylem tissue were sectioned directly with the help of a sliding microtome (Leica SM2010R). Before sectioning, thick stem samples were washed thoroughly in running water for 10 min and trimmed into proper size to fit properly into the sample holder of the microtome. Sections of 15-20 µm thickness were obtained in various planes i.e., in a transverse, tangential and radial longitudinal orientation. From the several sections obtained, thin and intact sections were chosen and tied on a slide using the cotton thread.

3.3.3). *Staining and mounting*:

Sections obtained with the help of both, rotary and sliding microtome were further processed for the staining, dehydration and mounting. Before staining, it is necessary to ensure that the embedded sections were free from paraffin wax and rehydrated properly. Slides mounted with sections when ready for staining were treated with Safranin and fast green (Johansen, 1940) or Safranin - Astra blue (Srebotnik & Messner, 1994; Vasquez-Cooz & Mayer, 2002) and duration for the staining was standardized as per requirement. Depending on the sample (i.e., species), staining time varies from species to species. Sections obtained using both the types of microtomes (i.e., rotary and sliding) were stained as described above.

Rehydrated sections were obtained using the paraffin embedding procedure, and sliding microtome sections were stained in 0.5% aqueous solution of safranin ‘O’ for 1-2 hrs. (or depending on the type, amount of secondary tissue and the thickness of section). Stained sections were washed thoroughly and carefully in running tap water until the sections stop diffusing the stain. Finally, sections were rinsed twice with distilled water and subsequently transferred to 0.5% Astra blue

(aqueous) solution as described by Srebotnik and Messner (1994) or Vasquez-Cooz and Mayer (2002) for a few minutes and processed through upgraded series of ethanol-xylene, and mounted in Dibutyl Phthalate Xylene (DPX). In the case of fast green staining, thoroughly washed sections after safranin was passed through the upgraded series and brought to 95 % ethanol. Thereafter, sections were stained with 0.25 % fast green FCF (Johansen, 1940) that was prepared in 95% ethanol. After staining with a fast green, samples were washed with absolute alcohol and processed further for mounting by using ethanol-xylene series. To study the callose deposition on the sieve plates, some of the sections were also stained using the Tannic Acid-Ferric Chloride-Lacmoid combination method (Cheadle *et al.*, 1953).

3.3.4). *Maceration, measurements and analysis*:

To study the general morphology of the xylem elements and to obtain quantitative details (like length, the diameter of the vessel elements, fibriform vessels, tracheids, fibre tracheids and libriform fibres) maceration of secondary xylem was carried out. A small portion of the secondary xylem adjacent to the cambium (and the xylem formed from the outermost successive cambium [if successive cambia develop]) was sliced with the help of a sharp razor blade and macerated with Jeffrey's solution (Berlyn & Miksche, 1976) at 56 ºC – 60 ºC for 24-48 hrs. Treatment time for these slices may vary from species to species and depending on the composition of lignified tissue. After maceration, samples were washed thoroughly with water and stained with 0.5% aqueous Safranin before mounting them in glycerine. Temporarily mounted slides were used for recording the dimensional measurement of various elements of the secondary xylem. Measurements were carried out by conventional method i.e., using the ocular micrometry technique.

The length of wide vessel elements and the length and width of narrow vessels (fibriform vessels, if present), libriform fibres and fibre tracheids were measured from the macerated material while the tangential diameter of wide vessels was measured from transverse sections as per the recommendation of the IAWA committee (IAWA 1989). Length and width of sieve tube elements, xylem ray height

and width were taken using a tangential longitudinal section. For every individual element, thirty measurements were taken randomly and subjected to statistical analysis like mean, standard deviation and standard error. Term internal cambium and functionally inverse cambium were adopted from Rajput *et al.* (2008) while term fibriform vessels were adopted from Carlquist and Hanson (1991) and Carlquist (1988, 2001). Wood characteristic, description and terminology is followed as per IAWA Committee (1989) and Carlquist (2001). For cambial variants, the terminology is followed as suggested by Angyalossy *et al.* (2015).

3.3.5). *Macro and Micro photographs*:

Macrophotography of complete stem was done after sectioning of the thick stem. Wood blocks of the complete stems were allowed to dry for some time. Subsequently, these wood blocks were used to take photographs of the complete stem by using a Canon camera (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). Some of the wood blocks of thick stems were finely trimmed and polished using various grades of sandpapers for macrophotography of complete stems. Micro-photographs of important results were taken using Leica DMC 2900 fire wire digital fluorescent sensitive camera mounted on the above said microscope.

Note: In place of their newly approved names of certain plants, which are shown in brackets in Table 1, we have used synonyms in present investigation. The use of synonyms is necessary since names of such plants have been change may be after collections or publishing of the plant anatomical findings.

Table 1: List of species, place of collection, stem outline, maximum stem thickness available for the present study and growth pattern (*Legends*: F = Furrowed xylem, IP = interxylary phloem, IPX = intraxylary phloem cambium, INP = intraxylary phloem and SC = successive cambia, XP = Xylem in plates, PX = Parenchymatous xylem, NX = Normal secondary xylem, CP = Compound xylem).

Sr. No.	Name of the taxon	Family	Place of collection	Stem outline	Stem thickness	Growth pattern
1	*Beaumontia jerdoniana* Wight	Apocynaceae	Amboli forest (Western Ghats, Maharashtra state; Botanical Garden, The M. S. Uni. Baroda, Vadodara, Gujarat state	Circular	2 - 25 mm	IP, IPX
2	*Leptadenia reticulata* (Retz.) Wight & Arn.		Botanical Garden, The M. S. Uni. Baroda, Vadodara, Gujarat state	Circular	3 – 15 mm	IP, IPX, INP
3	*Leptadenia pyrotechnica* (Forssk.) Decne.		Bet Dwarka, Gujarat, Navinal (Kachchh region), Gujarat state	Circular	5 - 15 mm	IP, IPX, INP
4	*Vallaris solanacea* Kuntze		Botanical Garden, The M. S. Uni. Baroda, Vadodara, Gujarat state	Circular	2 - 25 mm	IP, IPX
5	*Wattakaka volubilis* (L.f.) Stapf.		Kaparada, Dangs, Gujarat state	Circular	3 – 30 mm	IP, IPX
6	*Arrabidaea candicans* (L. C. Rich.) DC.	Bignoniaceae	Forest of Banejos de Sierpe,	Circular	5 – 15 mm	F

			Peninsula de Osa, Costa Rica.			
7	*Campsis radicans* (L.) Seem.		Home garden at Vadodara & Anand, Gujarat	Circular	3 - 25 mm	IP, IPX, INP
8	*Cardiospermum helicacabum* L.		Road side area in Central Maharashtra state	Circular	3 – 30 mm	NX
9	*Dicranostyles ampla* Ducke	Convolvulaceae	Forest of Banejos de Sierpe, Peninsula de Osa, Costa Rica.	Circular to triangular	5 - 20 mm	IP, IPX, INP, SC
10	*Hewittia malabarica* (L.) Suresh		University of KwaZulu-Natal, Durban (South Africa); Campus of The M. S. Uni. Baroda, Vadodara (Gujarat, India)	Circular-elliptic; variously lobbed	5 – 30 mm	F, IP, IPX, INP, SC
11	*Jacquemontia paniculata* Hallier f.		Home garden at Vadodara, Gujarat	Circular-elliptic	3 – 10 mm	F, IP, INP, IPX
12	*Maripa nicaraguensis* Hemsl		Forest of Banejos de Sierpe, Peninsula de Osa, Costa Rica	Circular to triangular	5 - 20 mm	IP, IPX, INP, SC
13	*Turbina corymbosa* (L.) Raf. *(Ipomoea corymbosa* (L.) Roth)		Botanical garden of the Shivaji University, Kolhapur, Maharashtra state	Flat, highly irregular, and variously lobbed	3 – 40 mm	F, IP, IPX, INP, SC
14	*Zanonia indica* L.	Cucurbitaceae	Road side area near	Circular	2 – 35 mm	XP, PX

			Udupi, Karnataka state			
15	*Canavalia gladiata* (Jacq.) DC.	Fabaceae	Panas, Dangs, Gujarat state	Circular	3 – 25 mm	INP, PX
16	*Canavalia virosa* (Roxb.) Wight & Arn.		Timba-Tuwa, Panchmahal, Gujarat state	Circular	3 – 20 mm	INP, PX
17	*Dalbergia volubilis* Roxb.		Kaparada, Dangs, Gujarat state	Circular	2 – 25 mm	NX
18	*Entada gigas* (L.) Fawc. & Rendle		Banejos de Sierpe, Península de Osa, Costa Rica	Circular to variously lobbed		INP, PX
19	*Entada rheedii* Spreng.		Kaparada, Dangs, Gujarat state	Circular to variously lobbed	5 – 30 mm	INP, PX
20	*Phaseolus lunatus* L.		CTC Campus, Costa Rica	Circular, irregularly lobbed		INP, PX
21	*Pueraria tuberosa* (Roxb. ex Willd.) DC.		Kaparada, Dangs, Gujarat state & Junagadh Hills, Gujarat state	Circular, irregularly lobbed	20 – 55 mm	INP, PX
22	*Rhynchosia pyramidalis* (Lam.) Urb.		Monte Verde, Costa Rica	Stem flat, ribbon like	3 – 15 mm	SC
23	*Strychnos bredemeyeri* (Schult.) Sprague & Sandwith	Loganiaceae	Banejos de Sierpe, Península de Osa, Costa Rica	Circular	5 – 20 mm	IP, IPX, INP
24	*Strychnos andamanensis* A.W.Hill		TBGRI, Trivendrum, Kerala state	Circular	2 – 10 mm	IP, IPX, INP
25	*Anamirta cocculus* (L.) Wight & Arn.	Menispermaceae	Waghai Botanical Garden, Gujarat state	Circular	2 – 20 mm	SC, XP
26	*Cissampelos pareira* L.		Areas near Vadodara, Gujarat	Circular	2 – 8 mm	XP

			state			
27	*Cocculus hirsutus* (L.) W.Theob.		Vadodara, Gujarat state; Bhorkheda Maharashtra state	Circular	5 – 70 mm	SC
28	*Cocculus laurifolius* DC.		TBGRI, Trivendrum, Kerala state	Circular to elliptic	10 – 90 mm	SC
29	*Cocculus pendulus* (J.R.Forst. & G.Forst.) Diels		Zariya Mahadev, Chotila, Gujarat state	Circular to elliptic	5 – 50 mm	SC, XP
30	*Coscinium fenestratum* (Gaertn.) Colebr.		TBGRI, Trivendrum, Kerala state	Circular	5 – 15 mm	XP
31	*Cyclea peltata* (Lam.) Hook.f. & Thomson	Menispermaceae	Radhanagari Forest, Maharashtra state	Circular	3 – 10 mm	SC
32	*Diploclisia glaucescens* (Blume) Diels		Radhanagari Forest, Maharashtra state	Circular	5 – 15 mm	SC
33	*Pachygone ovata* (Poir.) Diels		Radhanagari Forest, Maharashtra state	Circular	3 – 8 mm	SC
34	*Stephania japonica* (Thunb.) Miers		Panhala Ghat, Maharashtra state	Circular	2 – 8 mm	XP
35	*Tiliacora racemosa* Colebr. (*Tiliacora acuminata* (Lam.) Miers)		Aurangabad, Maharashtra state	Circular	3 – 20 mm	SC, XP
36	*Tinosporia cordifolia* (Willd.) Hook.f. & Thomson		Vadodara, Gujarat state	Circular	2 – 60 mm	XP
37	*Tinospora sinensis* (Lour.) Merr		Road side areas Arunachal Pradesh state	Circular	5 – 15 mm	XP
38	*Moutabea gentry* T. Wendt	Polygalaceae	Forest of Banejos de Sierpe, Peninsula de Osa, Costa Rica.	Circular to elliptic	20 – 80 mm	SC
39	*Antigonon leptopus* Hook. &	Polygonaceae	Botanical Garden,	Circular,	5 – 30 mm	SC

	Arn.		The M. S. Uni. Baroda, Vadodara, Gujarat state	irregularly lobbed		
40	*Serjania circumvallata* Radlk.	Sapindaceae	Banejos de Sierpe, Peninsula de Osa, Costa Rica	Tri-lobed, Compound xylem	3 – 25 mm	CP
41	*Serjania mexicana* Willd.		Banejos de Sierpe, Peninsula de Osa, Costa Rica	Circular	5 – 20 mm	FX
42	*Ampelocissus latifolia* (Roxb.) Planch.	Vitaceae	Ahwa-Mahal, Dangs, Gujarat state	Circular	5 – 25 mm	PX
43	*Cayratia auriculata* Gamble (*Cyphostemma auriculatum* (Roxb.) P.Singh & B.V.Shetty)		Junagadh Hills, Gujarat state	Circular	2 – 15 mm	PX
44	*Cayratia trifolia* Domin *(Causonis trifolia* (L.) Mabb. & J.Wen*)*		Timba-Tuwa, Panchmahal, Gujarat state	Circular to elliptic	5 – 25 mm	SC, PX
45	*Cissus quadrangularis* L.		Botanical Garden, The M. S. Uni. Baroda, Vadodara, Gujarat state	Square to quadrangular	2 – 25 mm	PX, XP
46	*Cissus repanda* Vahl		Kaparada, Dangs & North Gujarat, Gujarat state	Circular	5 – 50 mm	PX, XP
47	*Cissus rotundifolia* Vahl		Ornamental, Vadodara city, Gujarat state	Circular	3 – 10 mm	NX
48	*Tetrastigma bracteolatum* (Wall.) Planch.		Dangs, Gujarat state	Flat, ribbon like	5 – 25 mm	SC, PX, XP
49	*Vitis vinifera* L.		Nashik, Maharashtra state	Circular	3 – 20 mm	NX, XP

Gymnosperms

50	*Gnetum ula* Brongn. *(Gnetum edule* (Willd.) Blume*)*	Gnetaceae	Udavi, Radhanagari, Maharashtra state	Circular to elliptic	5 – 30 mm	SC, XP
51	*Ephedra karumanchiana* SK Patel, SM Patil, RS Patel, RN Kachhiyapatel & KS Rajput	Ephedraceae	North Gujarat, Gujarat state	Circular	5 – 25 mm	NX
52	*Ephedra foliata* Boiss. ex C.A.Mey.		Navinal, Kachchh, Gujarat state	Circular	3 – 20 mm	NX