Chapter 4
Results & Discussion

4.1.0 SOME SURVEY FACTS

The study site with total area of 23427.62 ha is popularly known as Lachen Valley and is surrounded by sacred Pangal Reserve Forest. The middle and southern ridges (Lachen and Chaten) are moderately populated Zema IA, Zema IB, Zema II, Zema III, Puchi and Chako have very less density of population and rest of the area has temporary huts constructed for seasonal agriculture. Due to severe winter and heavy monsoon, the area sometimes remains cut off from the rest of the world as there is only one road considered to be the lifeline connecting Lachen from rest of the world. No information on authentic published climatic and topographical data of this area was available, but during my regular visits for three years it was revealed that the temperature in the study area ranges from minimum of -5°C at upper ridges of Zema III to 21°C in the lower ridges of Chaten.

All the maps used for ecological studies at fig. 4.1 (A, B, C and D) of the study area were prepared using 1:50000 scale, geo-coded India Remote Sensing (IRS) 1D LISS III False Colour Composite (FCC), Survey of India (SOI) 1: 50000 scale maps (78A/5, 78A/6, 78A/9 and 78A/10) and GIS using ERDAS ver. 9.0 and Arc map 8.3 software, along with ground truthing, data collection and extensive surveys as elucidated in 3.2.0 of chapter III.

4.1.1 Land use and land cover

Lachen valley has a very interesting admixture of land use and land cover covering 7605.19 ha, including part of Pangal Reserve Forest. Here, *Hippophae salicifolia* D. Don (sea buckthorn) plant is found growing naturally in different areas in pure or

Table 4.1: Vegetation classification with area revealed from the land use and land cover map of the study area using GIS ERDAS.

Vegetation Class	Area (ha)
Dense Evergreen Forest	7605.19
Open Evergreen Forest	6610.25
Scrub Forest	4466.69
Forest Blank	2183.19
Barren Rocky/ Stony area	728.94
River-water Channel area	509.88
Lakes	3.56
Grassland/Grazing land	323.50
Snow cover-Perennial	762.00
Hippophae-bamboo	19.83
Hippophae-broadleaf	60.90
Hippophae area	89.60
Hippophae-conifer	59.51
Hippophae-Alnus	4.59
Total	23427.62

mixed clusters. Table 4.1 and fig. 4.1A reveals different vegetation class and their areas in hectare and fig. 4.2A illustrates the percentage of different vegetation and land cover of the study area. It is seen that major part of the study area is occupied by dense evergreen forest (33%) which is due to the part of Pangal Reserve Forest. The pie chart presents total area covered in percentage by different land use and land cover parameters through legends shown in the map. In areas like forest blank, barren rocky/stony areas, river-water channel areas, lake, grass land and snow cover (perennial), presence of *H. salicifolia* was not observed. Beside these areas, *H. salicifolia* stands could be observed in different vegetation combinations like *Hippophae*-conifer, *Hippophae*-broad leaf, *Hippophae-Alnus*, *Hippophae*-bamboo, etc. During digitization and assignment of training sites to the IRS satellite data through GIS (as stated above) for creating map, only the areas which covered more than 1 pixel in the satellite data were accepted by the system. This was due to very small patches of *Hippophae* growing areas on the ground, which became 1pixel or less on the FCC data. In this work *Hippophae* area was taken up manually through intensive survey, ground truthing and data interpretation, to authenticate the ground data, which could not be verified only through image data. This was later on assigned

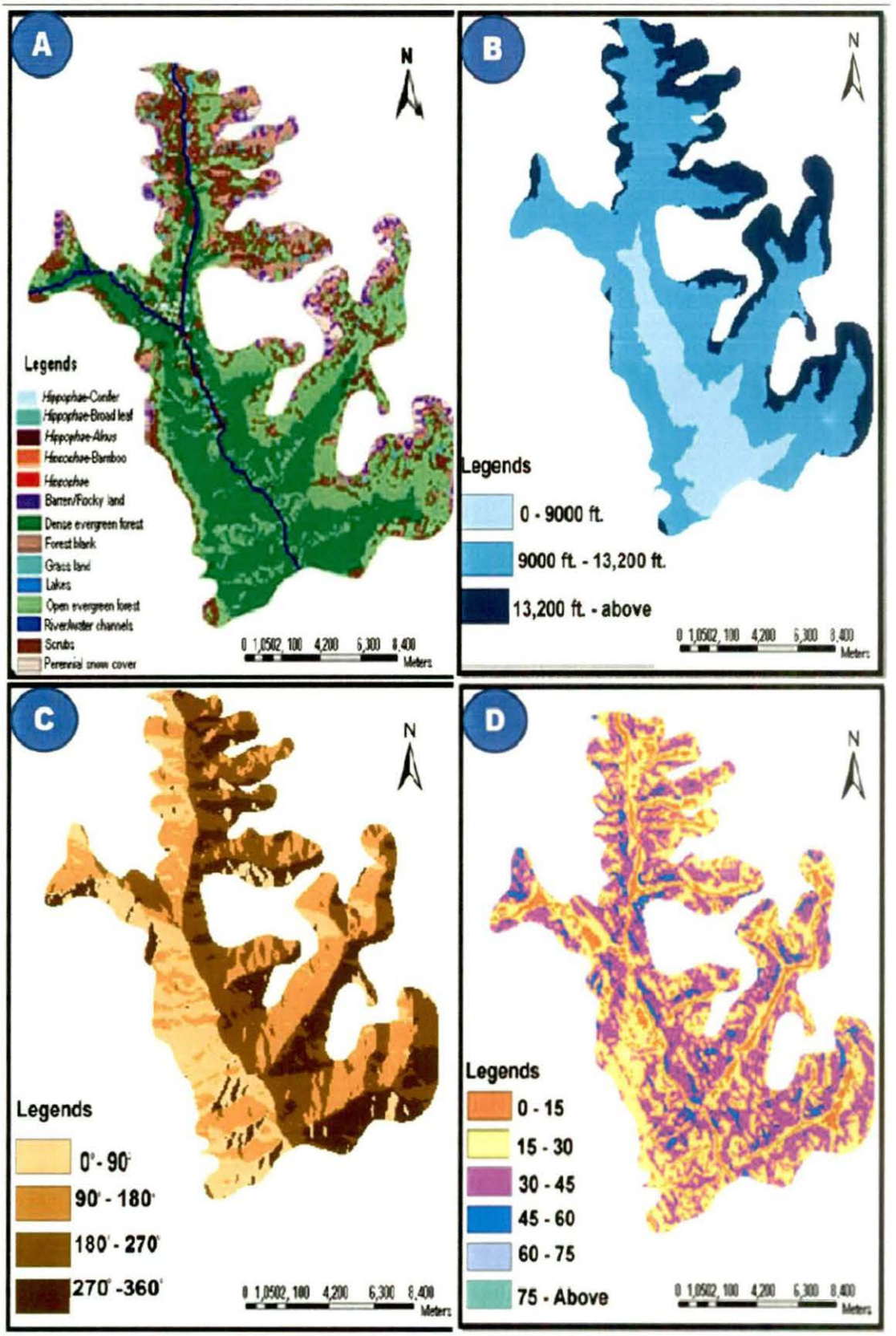

Figure 4.1: Ecological maps of the study area using IRS 1D LISS III FCC, SOI topo -sheets (78A/5, 78A/6, 78A/9 and 78A/10) and GIS ERDAS ver. 9.0 and Arc map ver. 8.3 software. A: Land use and land cover map, B**:** Elevation map. C: Aspect map and D: Slope map

Table 4.2: Area covered in ha by different elevation range revealed through GIS.

Elevation range (ft.)	Area (ha)
0-9000	4093.69
9000-13200	13274.90
13200-above	6058.99
Total	23427.58

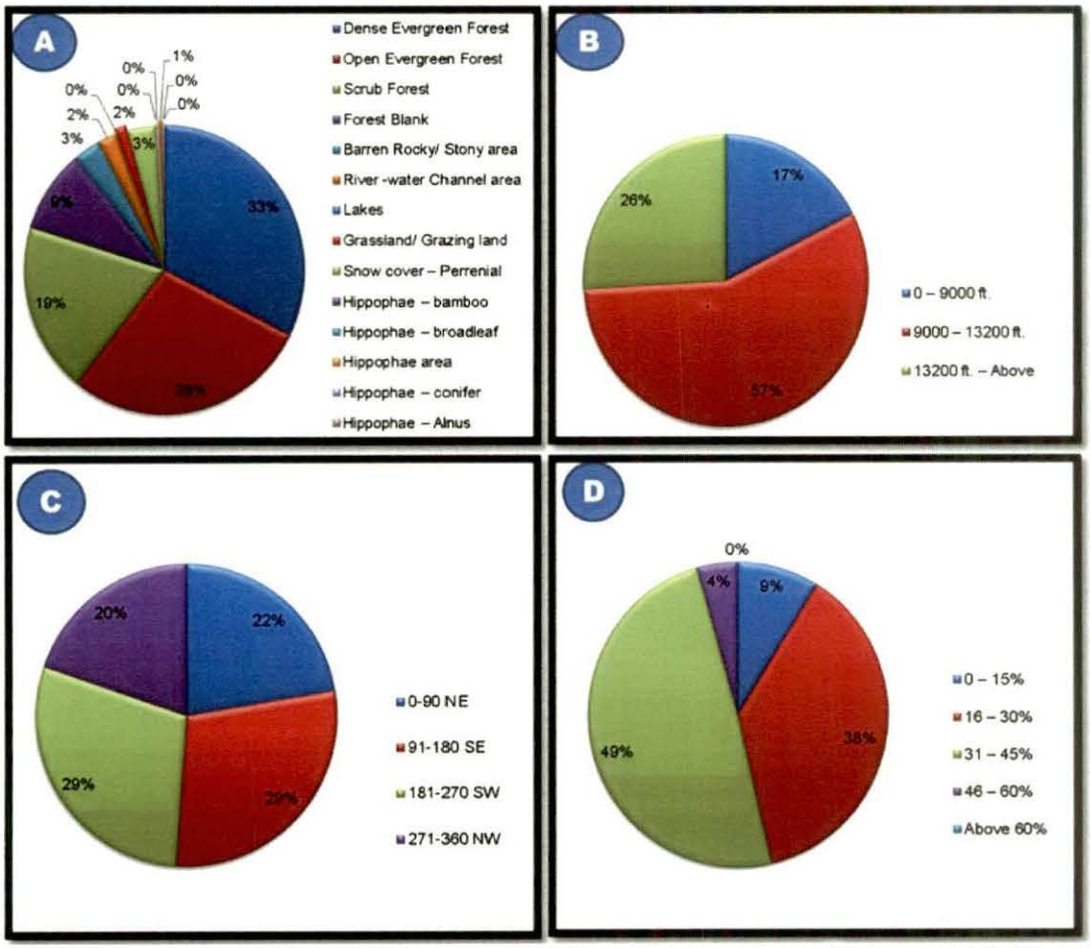

Figure 4.2: Pie chart showing percentage of area covered by A: Different land use and land cover; B: Range of elevations; C: Different aspects and D: Range of slopes

to GIS to produce the land use and land cover map.

Such kind of predictive land use and land cover maps covering vegetation have been used in remote sensing applications to improve land cover classifications based on digital satellite data by Strahler (1981); Cibula and Niquist (1987). In these studies, various models were created from ground samples using satellite data (FCC) followed by GIS to extrapolate across unsampled areas. Similar work had been carried out by Roy *et al.* (2001) on *H. rhamnoides* L. of Lahul and Spiti in Himachal Pradesh (India), using IRS IC LISS III FCC and GIS Arc-view software. Here, GIS was

Table 4.3: Area covered in ha by different aspects revealed through GIS

Aspect range	Aspect	Area (ha.)
$0\text{-}90^0$	NE	5241.0273
$91^0\text{-}180^0$	SE	6737.7199
$181^0\text{-}270^0$	SW	6825.3649
$271^0\text{-}360^0$	NW	4623.6112
Total		23427.723

limited to digitization for classifying the satellite data. However, I have created GIS map with ecological information of even inaccessible areas through intensive surveys and collection of field data using high resolution satellite imageries and Survey of India topo sheets, as stated above.

Such inventory map (Figure 4.1A) providing ecological information proves to be a boon for the researchers, planners, foresters and conservationists towards identification and mapping of important floral species and simultaneously applying noble conservation practices.

4.1.2 Elevation

The map at fig. 4.1B shows different height ranges of the study area separated by colours. From the map supported by ground data, it was found that elevation of the study area ranged up to 13,200ft. The GPS aided ground data in the *H. salicifolia* growing in different range of elevations are illustrated at table 4.2 compliments the data at table 4.5. Further, Pie chart at fig 4.2B and table 4.2 of the study area, elaborates that maximum area covering 13274.90 ha of land falls at an elevation range of 9000ft.-13200ft. covering 57% of the total area. 17% total area falls under altitudinal range up to 9000ft. covering 4093.69 ha. and the remaining 26% of the area falls under 13200ft. and above, covering 6058.99 ha land. Detailed observations of data in pie chart at fig. 4.2B and altitudinal data in table 4.5 shows that most of the *H. salicifolia* plants grow at

Table 4.4: Area covered in ha by different slopes revealed through GIS

Slope Range (%)	Area (ha.)
0-15	2073.61
16-30	8808.19
31-45	11513.96
46-60	1028.37
Above 60	3.73
Total area	23427.86

an elevation ranging from 9000-13200ft. compared to 0-9000ft. Ground reality also confirms that there was no *H. salicifolia* growing above 13200ft. covering 26% of my study area because the area was mostly covered by snow, grass lands and barren lands. This may be due to the geological makeup of the Sikkim mountains because reports by Rongsen, (1992), Rajchal (2009) and Singh *et al.*, (1995) suggests that *H. salicifolia* grows beyond this altitudinal region.

4.1.3 Aspect

The cardinal or aspect in the map at fig. 4.1C, of the study area, are based on angle measured clockwise due north called azimuth (measured between 0-360°). Table 4.3 and fig. 4.2C elucidates all the inter-cardinal directions (aspects) and their area covered, viz., north east (NE) covering 22%, south east (SE) and south west (SW) covering 29%, and north west (NW) covering 20% of the area. SE and SW aspects cover equal area in the study site

Table 4.3 also reveals the individual area coverage in hectares, for each aspect. From the ground survey it was observed that NW aspects received less sunlight than SE aspect and was mostly covered with snow or were barren lands, grass lands or scrubby vegetation. SW aspect, which is opposite to NE aspect, received more sunlight and vegetation also flourished better in these area.

Observation during survey and data collected by GPS navigation at table 4.5 also reveals that SE aspect had healthy and taller tree stands of *H. salicifolia* compared to other aspects and tree stands at gully and drainage areas receiving less sunlight. Out of 20 quadrates, 9 slopes faced SE, 5 faced SW, 4 faced NE and 1 each slope faced north and east. This clearly indicates that *H. salicifolia* is a sun loving species, which prefers growing mostly in sun facing SE aspects. My results corresponds to the reporting made by Rajchal, (2009); Rongsen, (1992) and Singh and Awasthi (1995), where these authors have also advocated on growth preference of sea buckthorn in open sun facing aspects. Since such kind of study is the first of its kind on *H. salicifolia* of Sikkim.

4.1.4 Slope

Slopes are important ecological component in identifying constraints and evaluating potential environmental impacts related to landform alteration along with vegetation successions. Fig. 4.1D, table 4.4 and fig. 4.2D reflects

the fact that the study area consists of hilly slopes with virtually no plain land. The table and the figures illustrates the slopes in the study area ranged from gentle (0-15%) to very steep slopes (above 60%), individual classification of slope area in ha. and percentage of slope area covered. It was also observed that moderate slope ranging from 16-30% to 31-45% occupies maximum area of 11513.96 and 8808.19 ha. covering 49% and 38% of the geographic location, respectively. Above this percentage of slopes, the terrain started becoming steeper and there was decrease in vegetation.

H. salicifolia growing area covered slopes ranging from 16-30% and 31-45% slope landscape mostly with SE aspects. This was similar to the reports of Rongsen (1992) and Singh (1998) that *Hippophae* prefers growing in sun facing aspect i.e., SE aspect. The slope map gives an idea about the terrain condition and also helps in future planning and management of the area by different section of researchers.

Geo-physico-chemical studies of all the quadrates carried out during survey reflected interesting ecological facts of the field (table 4.5). It was observed that *H. salicifolia* was growing naturally and was mostly concentrated in the river banks or riverine areas (RA) of Lachen River and torrential drainages connecting it. The plant was also found to be growing in non-river side or non-riverine (NR), torrential areas, slopes, village road side, grazing forest, torrential banks and landslide areas. They were growing either in small patches of pure populations or mixed with other vegetation (fig. 4.1A). It was also found that about 4-5 year old plant was found growing in the middle of the high current Lachen River forming a small island, which indicated the strength of its root and its ability to hold the fragile slopes of sea buckthorn growing area.

A very interesting admixture of two actinorhizal plant species was observed at an altitude of 7845ft. in quadrate (Q) 15. Here *Alnus nepalensis* population ended from the south and *H. salicifolia* population started towards north facing SE aspect. Majority of *H. salicifolia* growing areas were fragile having new soils with great risk of mass movement and flash floods.

Some old *H. salicifolia* forests with tall trees were observed in Zema II+ (Q2) and below Lachen (Q17, Q18 and Q19 as referred in table 4.5). Other locations like Dozam, Zema IIB and

Zema IID also had old stands, but fewer in populations due to human interferences for fuel and fodder, slowly being replaced by naturally regenerated plants. This forest clusters showed association with *Arundinaria* sp. Platt and Brantley (1997) suggested that *Arundinaria gigantea* basically a species of river flood plains of the south, is fostered by burning regimes. However, bamboo (*Arundinaria* sp.) and *Hippophae* can be observed in many patches of the study area also supports the idea of forest fires in the past. Results also indicated that *H. salicifolia* is well associated with *Daphniphyllum* sp. *Rhododendron* sp. *Tsuga* sp. *Populus* sp., etc. (table 4.6), which makes this species a major player in the ecological balance of this fragile area.

The study area consisted of various admixtures of soil combinations (loamy, sandy, clayish, etc.), colours (brown to dark brown) and pH ranging from 4.5 to 6.9. The site cover of natural *H. salicifolia* orchard ranged from 20% to 61-80% (table 4.5). Furthermore, any area with geographical boundaries of 27°42′758″ to 27°46′839″N and 88°31′041″ to 88° 33′639″ E at an altitude ranging from 7845 to 10206ft. with pH and aspect stated above was found suitable habitat for *H. salicifolia* in North Sikkim.

From the socioeconomic interaction it was understood that the sea buckthorn was used in fencing of apple orchards, fodder for cattle, firewood and the fruit pulp was used in treating common problems like stomach ache, common cold and fever. Ripe fruit juice of sea buckthorn was used as natural dyes for colouring endemically woven clothes and blankets. Need for fuel wood and construction has led people to cut down forest trees including sea buckthorn plants. The plant also serves a good dietary supplement for the birds and other animals (Fig 1.5).

4.1.5 Vegetation analysis

Understanding the forest structure is important to describe different ecological processes and also to model the functioning and dynamics of forest (Elouard *et al.*, 1997; Sukumar *et al.*, 1992).

In table 4.6 properties of vegetation with reference to vegetation composition and functional attributes are expressed on species basis. The density (D) measurement (number of individual species present), relative dominance (Rdom) (largest species in terms of its presence), Frequency (F)

Table 4.5: Field data compilation of the study area showing different geo-physico-chemical parameters.

	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10
Location	Zema II	Zema II +	Zema II A	Zema II B	Below Dozam	Dozam +	Dozam	Zema II D	Zema II C	Zema I +
Date	3.12.2008	3.12.2008	3.12.2008	3.12.2008	4.12.2008	4.12.2008	4.12.2008	4.12.2008	4.12.2008	5.12.2008
Time	1140 hrs	1350 hrs	1445 hrs	1620 hrs	1030 hrs	1200 hrs	1430 hrs	1515 hrs	1645 hrs	0745 hrs
Altitude (ft)	10206	9990	9340	8958	9728	9960	9370	9060	9015	8985
Latitude	27°46'83 8'N	27°46'32 6'N	27°45'56 4'N	27°45'42 0'N	27°46'83 9'N	27°46'25 4'N	27°45'70 7'N	27°45'50 1'N	27°45'37 4'N	27°45'41 4'N
Longitude	88°32'79 0'E	88°32'66 9'E	88°32'07 6'E	88°32'33 9'E	88°32'79 0'E	88°31'04 1'E	88°31'72 9'E	88°32'17 1'E	88°32'50 1'E	88°32'26 1'E
Soil colour	Brown	Brown	Brown	Dark brown	Brown	Brown	Dark brown	Dark brown	Brown	Brown
Soil temp (°C)	3 80	10.0	10.0	5.0	10.0	18 0	6.0	5.0	5.0	2.0
Soil moisture (%)	42	25	10	40	30	20	15	16	15	80
Soil pH	6 6	6.8	6.6	6.6	6.6	6.7	6.6	4.5	6.25	6.4
Air temp (°C)	15	17	6	4	18	24	7	8	4	2
Humidty (%)	5	5	5	58	7	5	12	12	22	30
Aspect	SE	SE	SE	SE	SE	SE	SW	SE	SE	SE
Topography	Vertical hill	Hilly	Hilly	Hilly river side	Hilly	Hilly	Hilly river side	Hilly	Hilly	Hilly
Site cover (%)	21 – 40	21 – 40	41 60	21 – 40	21 – 40	21 – 40	61 – 80	20	20	21 – 40
Vegetation type	Forest	Forest	Forest	Forest	Forest	Forest	Village road side	Grazing Forest	Forest	Well drained forest
Management	Natural	Natural	Natural	Natural	Natural	Natural	Natural	Natural	Natural	Natural
Nodules location	Tap root	Tap root	Tap root / lateral roots	Tap root / lateral roots	Tap root	Tap roots / lateral roots	Tap root	Tap root	Tap root	Tap root
Nodules Growth form	Clumped	Clumped & scattered	Clumped	Clumped	Clumped	Scattered	Clumped & scattered	Clumped	Clumped	Scattered

Table 4.5: continued.

	Q11	Q12	Q13	Q14	Q15	Q16	Q17	Q18	Q19	Q20
Location	Zema I (A)	Zema I (B)	Puchi (Zema I)	Chako River	Below Chaten	Chaten	Above Chaten	Below Lachen	Below Lachen	Chako River
Date	5.12.2008	5.12.2006	5.12.2006	5.12.2006	5.12.2006	5.12.2006	5.12.2006	6.12.2006	6.12.2006	6.12.2006
Time	0900 hrs	0945 hrs	1100 hrs	1215 hrs	1410 hrs	1545 hrs	1630 hrs	0800 hrs	0850 hrs	1030 hrs
Altitude (ft)	8948	9185	9017	8814	7845	8525	8854	8450	8299	8337
Latitude	27°45'272 "N	27°44'87 7'N	27°44'40 7'N	27°43'62 1'N	27°42'75 8'N	27°42'83 6'N	27°43'36 5'N	27°44'00 2'N	27°43'48 5'N	27°43'54 4'N
Longitude	88°32'540 "E	88°32'58 4'E	88°32'80 2'E	88°33'02 8'E	88°33'63 9'E	88°33'37 9'E	88°33'11 5'E	88°33'21 6'E	88°33'48 5'E	88°32'82 7'E
Soil colour	Brown	Brown	Brown	Dark brown	Brown	Brown	Brown	Dark brown	Black	Brown
Soil temp (°C)	4.0	6.0	4.0	10.0	8.0	8 6	4.5	6 2	4.5	4 5
Soil moisture (%)	18	30	65	30	55	10	35	90	20	60
Soil pH	6.4	6 6	6.5	6 8	6.5	6 9	6.7	6 3	6.8	6 4
Air temp (°C)	6.0	13.0	8.0	13.0	10.0	10.0	6.0	6 0	4.0	11.0
Humidity (%)	30	24	20	12	22	27	30	31	33	35
Aspect	East	North East	South East	South West	North	North East	South East	South East	North east	North East
Topography	Hilly	Hilly near dry torrent	Hilly	Well drained hill	Hilly	Hilly road side	Hilly river side	River valley	Plain	Hilly
Site cover (%)[all plants]	21 – 40	61 – 80	21 – 40	21 – 40	21 – 40	21 – 40	21 – 40	41 – 60	21 – 40	21 – 40
Vegetation type	Forest	Forest	Forest	Forest	Forest	Forest	Forest	River side forest	Old Forest	Forest
Management	Natural	Natural	Natural	Natural	Natural	Natural	Natural	Natural	Natural	Natural
Nodules location	Tap root	Tap roots / lateral roots	Lateral roots	Lateral roots	Lateral roots	Tap roots / lateral roots	Tap roots / lateral roots	Crown area / tap roots	Lateral roots	Lateral roots
Nodules Growth form	Scattered	Clumped	Clumped	Clumped	Scattered	Scattered	Scattered	Clumped	Scattered	Scattered

Table 4.6: Phyto-sociological characterization of vegetation in Lachen Valley with reference to *H. salicifolia*

Name of Species	A	D	F	BA	Rdom	RD	RF	IVI
Hippophae salicifolia D. Don	189	94.50	100.0	31.51	72.07	45.99	24.4	**142.5**
Daphni cannabiana Wall.	37	18.50	40.00	1.20	2.74	9.00	9.76	21.50
Alnus nepalensis D. Don	30	15.00	15.00	1.37	3.14	7.30	3.66	14.10
Rhododendron sp.	28	14.00	30.00	0.86	1.98	6.81	7.32	16.11
Tsuga dumosa (D. Don) Eichler	26	13.00	40.00	5.07	11.59	6.33	9.76	27.67
Populus deltoids Bartr.	24	12.00	25.00	1.10	2.51	5.84	6.10	14.45
Chenga sp.	13	6.50	10.00	0.26	0.58	3.16	2.44	6.19
Leucosceptrum cannum Smith	13	6.50	15.00	0.26	0.58	3.16	3.66	7.41
Betula utilis D. Don	9	4.50	25.00	0.33	0.76	2.19	6.10	9.05
Lonicera glabrata Wall.	8	4.00	10.00	0.31	0.72	1.95	2.44	5.10
Taxus baccata L.	8	4.00	15.00	0.47	1.08	1.95	3.66	6.68
Pteris villosa Windham	7	3.50	20.00	0.14	0.31	1.70	4.88	6.90
Rubus kumaonensis Balakrishnan	7	3.50	15.00	0.29	0.67	1.70	3.66	6.04
Auracaria sp.	3	1.50	15.00	0.22	0.49	0.73	3.66	4.88
Elsholtzia flava (Benth.) Benth.	2	1.00	5.00	0.04	0.09	0.49	1.22	1.80
Acer cappadocicum Gled.	2	1.00	5.00	0.04	0.09	0.49	1.22	1.80
Elaeagnus Elaeagnus L.	1	0.50	5.00	0.02	0.04	0.24	1.22	1.51
Juniperus recurva Buch.-Ham. ex D. Don	1	0.50	5.00	0.02	0.04	0.24	1.22	1.51
Populus gamblei Dode	1	0.50	5.00	0.18	0.40	0.24	1.22	1.87
Piptanthus nepalansis (Hook.) D. Don	1	0.50	5.00	0.02	0.04	0.24	1.22	1.51
Betula alnoides D. Don	1	0.50	5.00	0.02	0.04	0.24	1.22	1.51

(wide distribution of species among the same plots) and Importance Value Index or IVI, which is the expression of reasonable measure to assess the overall significance of species by taking different properties of species in vegetation (Phillips, 1959), were the important attributes considered along with other parameters like Abundance (A), Basal area of each tree species per hectare (BA), Relative density (RD) and relative frequency (RF). IVI among all was the important parameter to assess floral diversity and species association with *Hippophae salicifolia* in all the quadrates referred above and expressed at fig. 4.3. IVI also reflected the overall importance of each species in the community structure of the study area.

In table 4.6 we can see common plant associates of *H. salicifolia* and also the pattern of change in IVI of twenty one tree species of the study site. Of all the tree species *Hippophae salicifolia* recorded the highest IVI value (142.45)

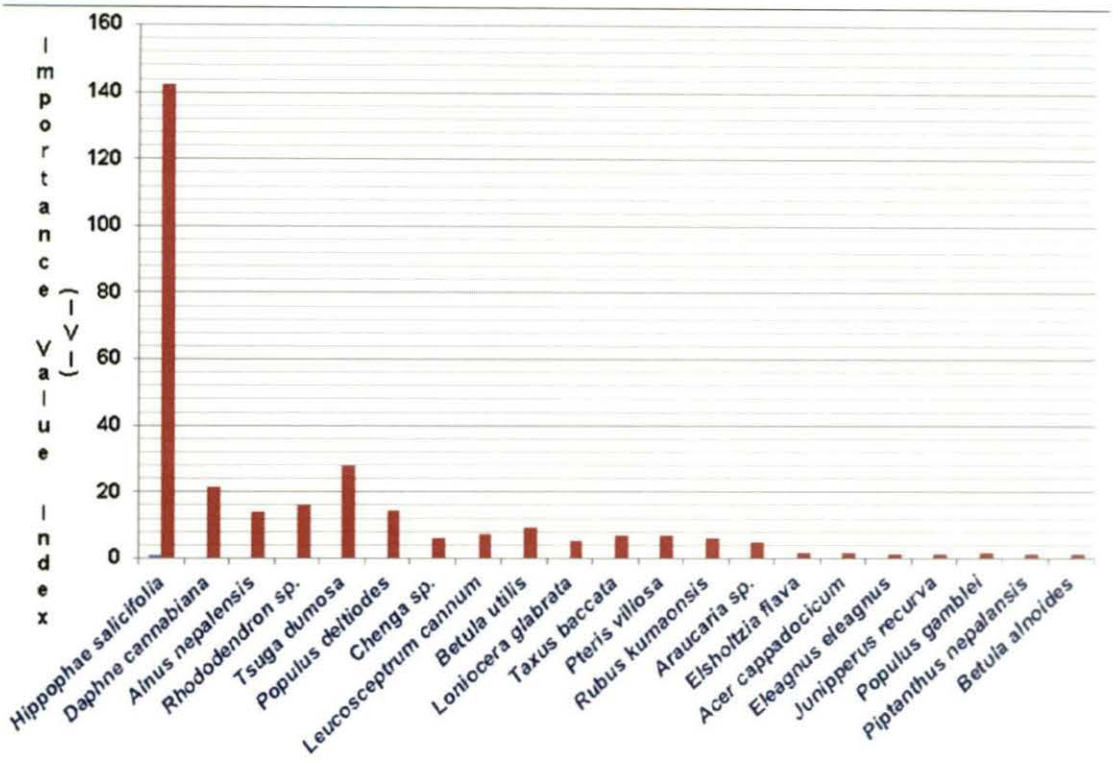

Figure 4.3: Graph representing IVI sequence of vegetation of the study area

Table 4.7: Results of soil analysis

UNIVERSITY OF NORTH BENGAL

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Dist.: Darjeeling, West Bengal, India
Phone: 0353-2699415 (0)

(Tea Board, Government of India, Approved Soil Testing Laboratory)
SOIL TESTING REPORT

Reference No: PGDTM / 30 / 08 / 377
Name of the Garden / Party: Mr B. C. Basistha Date: 22.04.2008

Lab#	*Sample code*	*Electrical Conductivity m.mho/cm*	*Organic carbon %*	*Organic matter %*	*Nitrogen %*	*Available K_2O ppm*	*Available P_2O_5 ppm*	*Available sulphurp pm*	*Chloride mg/g*	*Silt %*	*clay %*	*Sand %*
17277-16296	Q 1	0.26	2.19	3.78	0.30	**38.43**	21.76	**11.78**	**0.002**	4.00	34.00	62.00
	Q 2	0.16	0.57	0.98	0.08	76.25	26.11	22.59	0.005	3.00	28.00	69.00
	Q 3	0.23	2.08	3.59	0.29	46.97	21.68	23.57	0.004	5.00	30.00	65.00
	Q 4	0.11	0.78	1.34	0.11	80.53	**15.23**	27.50	0.007	**2.00**	**20.00**	**78.00**
	Q 5	0.26	0.97	1.67	0.13	78.08	29.38	25.35	0.006	2.00	27.00	71.00
	Q 6	0.19	0.49	0.84	0.07	84.79	43.52	26.51	0.005	4.00	26.00	70.00
	Q 7	0.14	0.64	1.10	0.09	93.94	26.67	25.53	0.008	3.00	22.00	75.00
	Q 8	0.28	1.03	1.78	0.14	42.70	65.29	24.12	0.005	2.00	**35.00**	63.00
	Q 9	0.14	1.08	1.86	0.15	64.05	27.21	29.46	0.004	2.00	26.00	72.00
	Q 10	0.31	0.99	1.71	0.14	67.10	26.17	33.38	0.007	4.00	30.00	66.00
	Q 11	**0.06**	0.86	1.48	0.12	71.98	**76.17**	39.28	0.008	4.00	34.00	62.00
	Q 12	0.16	0.97	1.67	0.13	81.13	29.66	**41.24**	0.006	2.00	34.00	64.00
	Q 13	0.23	0.86	1.48	0.12	78.08	19.58	26.51	0.005	4.00	31.00	65.00
	Q 14	0.24	0.95	1.64	0.13	87.23	36.99	35.35	0.004	**6.00**	33.00	**61.00**
	Q 15	0.34	0.41	0.71	0.06	**146.40**	26.88	27.50	0.006	5.00	31.00	64.00
	Q 16	0.29	3.24	5.59	0.45	91.53	26.87	21.60	0.007	2.00	28.00	70.00
	Q 17	0.24	**0.35**	**0.60**	**0.05**	109.80	38.09	33.38	0.005	4.00	30.00	66.00
	Q 18	0.23	1.24	2.14	0.17	104.92	28.29	26.51	**0.009**	2.00	30.00	68.00
	Q 19	**0.62**	**3.56**	**6.14**	**0.49**	84.79	24.65	26.66	0.008	3.00	30.00	67.00
	Q 20	0.35	2.70	4.65	0.37	109.19	30.46	35.36	0.005	4.00	26.00	70.00

with Rdom=72.07, RD=45.99 and RF contributing 24.39. This species had homogeneous patches in the study site, which can be matched with the tabular data showing very good presence i.e., 100% frequency. Out of all the tree species, *Tsuga dumosa* D. Don Eichler (IVI=27.67), *Daphniphyllum cannabiana* Wall (IVI=21.50) and *Rhododendron* sp. (IVI=16.11) were found to be very closely associated with *Hippophae salicifolia* (IVI=142.45). *Juniperus recurva* Buch. -Ham, ex D. Don (IVI=1.51), *Elsholtzia flava* (Benth.) Benth. (IVI=1.80), *Piptanthus nepalensis* (Hook.) D. Don (IVI=1.51) and *Betula alnoides* D. Don (IVI=1.51) showed greater disturbance and much lower IVI in stands of the sample plot. Thus this gives information about the most important species in the sample systems.

Table 4.8: Biophysical data of *H. salicifolia* related to nodule quantification*

Location	Age of plant (yrs)	Plant height (ft.)	Plant canopy (ft.)	Tillering	Soil pH	No. of Nodules per plant	Weight of nodules/plant (gm)
Riverine	4.69±1.18	16.45±3.85	11.45±4.26	1.35±0.59	6.63±0.21	56.0±21.93	125.7±102.76
Non riverine	4.25±1.12	14.65±4.57	10.67±3.69	2.15±1.50	6.68±0.15	22.2±9.51	33.45±19.65

**Values are averages of 20 independent determinations ± standard deviation*

4.1.6 Soil analysis:

The result of soil analysis is shown at table 4.7. Soil moisture and pH can be referred from the table 4.5. Organic matter and nitrogen percentages ranged from 0.60-6.14% and 0.05-0.49% in Q 17 and Q 19, respectively. Soil moisture ranged from 10% at Q 3 to 90% Q18 below Chaten with gorgeous shady terrain. Statistically there was not much variation in soil pH of different Quadrates in all the forest types. The percentage of organic carbon (OC) ranged from 0.35% at Q17 to 3.56% at Q19. Similarly, the percentage of silt, clay and sand ranged from 2-6%, 20-35% and 61-78%, respectively. Available potassium,

phosphorous and sulphur ranged from 38.43ppm (Q1) to 146.40ppm (Q, 15), 15.23ppm (Q4) to 76.17ppm (Q12) and 11.78ppm (Q1) to 41.24ppm (Q12), respectively. Chloride ranged from 0.002 (Q, 1) to 0.009mg/g (Q, 18). The electrical conductivity (EC) which plays a vital role in germination of seeds in the soil, ranged from 0.06 (Q11)-0.62m.mho/cm. EC less than 1.0m.mho/cm is regarded to be normal and suitable for germination, whereas 1 -2 and 2-3m.mho/cm and above are critical for germination and critical for salt sensitive crops. Above 3m mho/cm is injurious to all crops (Jackson, 1979).

However, from the analysis it is observed that there was not much leaching of nitrogen in the soil. Though the soil conditions of the study site were stressed with fewer amounts of minerals available in the soil, it was adequate for other species to succeed and regenerate due to the presence of this actinorhizal species. Among all the quadrates, Q19, the area below Lachen with dense *H. salicifolia* old forest was observed to be more adequate for regeneration and succession of other plant species along with *H. salicifolia*. Overall, the soil content of the study area is suitable for natural regeneration *of H. salicifolia.*

There are reports that major proportions of phosphorous in the soil are stored in the forms, which are not available to the plants (Murphy, 1958). This phenomenon compliments the explanation of low phosphorous content (15.23-76.17ppm) in different soil samples collected from the study area.

According to Banerjee and Chand (1981), low percentage of organic carbon in the soil is an indicative of forest fire in the past. The amount of organic carbon revealed from the soil test report of my study area suggests that there may have been a forest fire in the past. I have found higher amount of organic carbon percentage in the riverine area (table 4.5) compared to non-riverine area.

4.1.7 The plant morphology:

Sea buckthorn (*Hippophae salicifolia*) plants are erect, bushy, shrubby tree (3ft.-4ft.) or trees up to 35ft. (fig 1.2A), with average standard deviation (SD) height of 16.45±4.62 in riverine and 10.67±3.69 in the non-riverine areas. The age of plant was estimated to be 4.69±1.18 in riverine and 4.25±1.12 in the non-riverine areas (table 4.8). The trunk was brown in colour with its

circumference ranging from 10 cm to 3.5ft. at chest breast height (CBH). However, unlike in my study area, *Hippophae salicifolia* tree with a height of 56ft. tall has been reported from Mustang by Rajchal, (1998).

The young branches that looked reddish brown to green in some cases and was profuse, alternate, unevenly distant and sometimes whorled. Tillering was seen in many trees, shrubby and bushy plants. New plant growth was observed mainly in bushy plants from a rhizomatic stolon like connectivity resulting to a growth of a new plant. This was rarely seen in higher plants. The canopy size varied from 11.45±4.26 to 10.67±3.69, irrespective of height and development of the plant.

From the table 4.9a and table 4.9b, it was interesting to observe that average leaf length (up to 14.4cm) and average inter-nodular distance (up to 14.7cm) were more in plants growing in non-riverine areas compared to riverine plants. Similarly, average leaf width (up to 2.35 cm), average pedicel length (up to 0.48 cm) and average inter-branch distance (up to 82.80 inches), were observed to be more in plants growing in and around riverine areas. Holland and Steyn (1975) are of the opinion that morphological changes may be related to soil moisture and distribution, and thus such minor changes might have also been observed in plants located at different ecotypes of my study area. Such minute detail analysis during ecological studies makes the study more interesting.

Repeated visits to the study area in different seasons revealed that during spring and summer seasons, the numbers of thorns increased, looked greenish and were spiny. The same had reduced in number with their maturity due to branching (with leaves) or breakage in the autumn and winter seasons. The reason behind increase in number of thorns during early season may be due to the emergence of young flushes and for protection of the younger fruits before they were matured. It was also observed that the trees species were less thorny than the shrubby or bushy species. In some area of Zema II and its adjoining river bank, *Hippophae salicifolia* had sought branches and a big foliage canopy. In Lachen town, near the monastery male and female trees measuring 35ft. and 25ft. with trunk diameter of 4.2ft. and 3.5ft., respectively, has been standing (fig. 1.2A).

Table 4.9a: Biophysical parameters of randomly selected *Hippophae salicifolia* from riverine areas of the study area

Plant location	Average leaf length (cm)	Average leaf width (cm)	Average pedicel length (cm)	Average Inter Branch distance (Inch)	Average internodular distance (cm)
Riverine	9.43	**0.97**	0.36	29.85	6.7
Riverine	**5.75**	1.27	0.38	27.43	8.2
Riverine	6.47	1.41	0.34	24.26	3.9
Riverine	9.41	**2.35**	0.47	22.40	4.5
Riverine	8.50	1.41	0.36	20.32	4.5
Riverine	**10.30**	1.63	0.45	28.15	3.2
Riverine	5.80	1.42	0.42	**82.80**	6.5
Riverine	8.67	1.40	0.39	12.90	**13.2**
Riverine	10.11	1.45	0.41	22.00	4.1
Riverine	7.00	1.10	0.29	14.00	**2.3**
Riverine	9.33	1.22	**0.25**	28.89	3.4
Riverine	9.23.	1.34.	0.32	16.43	4.5
Riverine	8.75	1.79	0.33	27.35	5.7
Riverine	10.25	1.87	0.38	24.25	5.2
Riverine	8.76	1.29	0.42	**12.80**	4.3
Riverine	9.15	0.91	0.35	21.45	3.4
Riverine	8.97	1.37	**0.48**	25.66	3.2
Riverine	9.88	1.42	0.34	31.02	4.2
Riverine	8.79	1.56	0.37	26.64	3.8
Riverine	7.54	1.45	0.33	23.79	3.6

Table 4.9b: Biophysical parameters from non-riverine areas of the study area

Plant location	Average leaf length (cm)	Average leaf width (cm)	Average pedicel length (cm)	Average inter branch distance (Inch)	Average inter nodular distance (cm)
Non Riverine	7.52	1.36	0.43	24.26	14.7
Non Riverine	7.25	1.38	0.46	13.10	5.6
Non Riverine	14.41	1.16	0.44	20.68	5.4
Non Riverine	7.33	1.00	0.27	35.24	3.2
Non Riverine	8.76	1.13	0.41	36.49	2.9
Non Riverine	8.02	1.37	0.39	16.71	2.8
Non Riverine	9.53	1.32	0.39	13.72	11.3
Non Riverine	6.41	1.56	0.33	26.04	5.6
Non Riverine	6.37	1.36	0.40	17.72	6.0
Non Riverine	7.36	1.46	0.38	18.75	3.6
Non Riverine	12.45	1.35	0.42	17.41	11.2
Non Riverine	7.37	1.32	0.38	26.11	7.9
Non Riverine	8.12	1.17	0.43	18.56	8.5.
Non Riverine	10.12	1.25	0.41	32.13	5.2
Non Riverine	8.97	1.31	0.29	12.21	4.3
Non Riverine	9.45	1.28	0.38	34.26	2.8
Non Riverine	8.96	1.26	0.42	18.23.	4.4
Non Riverine	8.65	1.37	0.39	19.86	3.9
Non Riverine	6.74	1.31	0.34	18.26	6.1
Non Riverine	7.66	1.27	0.36	25.32	3.3

4.1.7.1 Leaf:

Leaves of *Hippophae salicifolia* were small pedicillate, entire, linear, lanceolate, acute, alternate, dorsiventral with ventral side covered with silvery stellate scales and pubescent hairs

giving willow like shiny feature. These stellate scales help in reducing moisture (Rongsen, 1992). Leaf size varied in different growing conditions and even in a single plant. Leaves of plant growing in and around riverine areas were bigger in size than plants growing in slightly stressed conditions, which may be due to better moisture conditions. Mean leaf length calculated from 100 leaves per individual plant of 20 different plants each, from riverine and non-riverine locations revealed that leaf measured 5.75 to 14.41cm in length and 0.97 to 2.35cm in breadth. Yang *et al* (1999) have carried out study in the size of leaves in higher and lower altitudes, which states that leaf size of higher altitudes were bigger in size compared to lower altitudes. Average pedicel length ranged from 0.25 cm to 0.48 cm (table 4.9a and table 4.9b). In the report of Rajchal (1998) on *Hippophae salicifolia* of Mustang district of Nepal, leaves were usually 3-8 cm long and 0.4 cm wide.

4.1.7.2 Flower

The male and female flowers were small in size and markedly different in their appearance. Male floral buds consisted of 4 to 6 flowers in cluster, while the female flowers possess only one flower and rarely two in the current season growth and in the older branches. Both male and female flowers had no petals and possess four stamens in male flowers and one pistil with conspicuous single ovary in female flowers. Often, floral buds were found mixed with the vegetative buds and were rarely in pure. The male floral buds appeared slightly bigger than the female floral buds, which was similar to the report of Li. and Mc Loughlin (1997).

With the passage of time, repeated plant tagging and observation of morphological characters, growth pattern, with respect to seasons was done for three consecutive years. From this, it was found that the species was a regular fruit bearing plant with no cases of alternate year fruit bearing character, but there was plant-to-plant and annual variations in total fruit production. The species was a distinct dioecious with male and female flowers in different plants. The male to female plant ratio in the natural habitat of Lachen was observed to be about 1:20.

4.1.7.3 Fruits and seeds

Sea buckthorn fruits are unique to other fruits or berries. The juicy calyx tube or the pulp and the seeds are of economic importance. It was observed

that the fruits took 11-14 months to ripe from flowering. The ripe fruits were orange or reddish yellow in colour and were measured 4 to 6mm in length and 3 to 6mm in width with test weight of 0.97 gm. A small black pustule was observed in mature fruit, with a notch. Yadav *et al.* (2006) have reported that fruit of *H. salicifolia* of Garhwal Hills, India were ovate shaped with its length ranging from 5.78 mm to 7.92 mm and width from 5.51 to 7.24 mm in all the locations. This indicates variation of the species in different ecological conditions, because geographically, Sikkim falls east to the Garhwal Hills.

The fruit of *Hippophae salicifolia* bore a dark brown, shiny, globose, single seeds measuring 3.5-5 mm in length and 2-4 mm in breadth with 1-2.5 mm thickness. The seeds look shiny due to the content oil in it (Fig 1.3E). The weight of 100 seeds of this plant makes 0.970 gm. (Basistha and Adhikari, 2003).

Hippophae salicifolia fruits were persistent in some of the branches for many months, even till next season fruit matured. Viviparous germination from some of these shrinked, dark yellow-brown fruits has also been observed and reported (Basistha, 2001).

4.1.7.4 Root:

For the plants having height of 3ft.-6.5ft., roots were seen mostly confined around 3ft. to5ft. in the soil. In non-riverine areas, a long taproot was found in most cases penetrating down the surface up to 1.5ft. to 3.5ft. Approximately two years old plant was measured with root length of 3.2ft. at Zema III at an altitude of 10206ft. Roots were found restricted to comparatively less depth in the soil with maximum percentage of humus and black soil compared to sandy and rough soils. Both main root and tap roots were observed in the plant species.

A case of aerial nodulation was also observed in *Hippophae salicifolia* (unpublished). Similar type of aerial nodulation too has been reported in *Casuarina equisitifolia* by Prin and Rougier (1987). In general, the root nodules (Fig 1.4) varied in size from 1 cm to 10 cm in diameter. The root nodules were milky white during the monsoon and late monsoon but observations showed that with gradual lowering of temperature followed by dry season, the colour of nodules changed to yellowish to brown due to secretion of phenolic compounds.

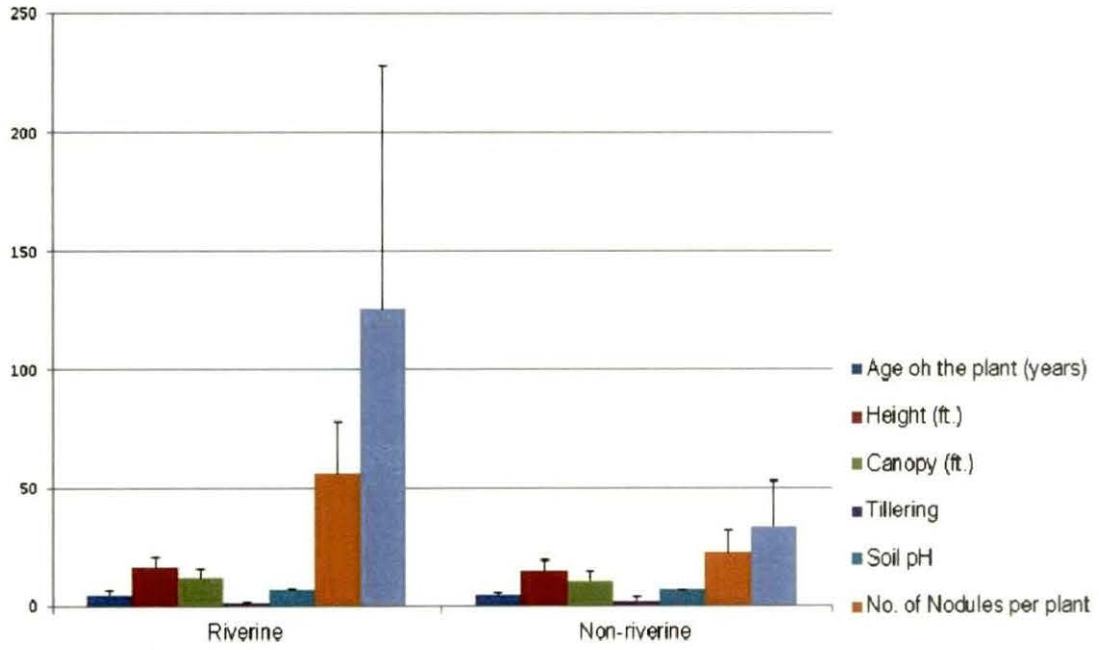


Figure 4.4: Standard Deviation (SD) graph showing quantification of nodules of *H. salicifolia* in relation with various parameters from riverine and non-riverine areas of

4.1.8 Root nodule quantification

Study on quantitative analysis of nodulation from 40 different individual *H. salicifolia* plants (20 plants each from riverine and non-riverine areas), selected randomly were carried out taking biophysical parameters to see if any of these parameters affected nodulation of the plant. The species growing in high biomass possessed lesser number of nodules compared to plants in the stressed conditions with less biomass but the nodulation percentage was 100%.

Table 4.8 illustrates the standard deviation (SD) of age of *H. salicifolia* trees in years, their height in feet, canopy in feet, soil pH, number of nodules per individual plant and total weight of nodules per plant in grams, for forest stands in riverine and non-riverine areas.The plants formed a dense scrub reaching heights of up to 16.45±3.85ft. in riverine and 14.65±4.57ft. in the non-riverine areas. The age of the plants appeared to be 4.69±1.18 years in riverine areas and 4.25±1.12 years old in non-riverine areas. Root nodules were located in the soil at depths ranging from 3 cm to 120 cm, which were mainly located in the tap roots and lateral roots. Numbers of nodules per plant in stands of *H. salicifolia* were counted as 56.0±21.93 in riverine area and 22.2±9.51 in non-riverine areas and total nodule weight per plant was recorded as

Table 4.10: Germination performance of *Hippophae salicifolia* seeds in different

Soil type	Days to initial Germination	Days to 50% germination	Total germin.(%)	Growth/month (Avg.) cm
Black soil	18	26	40	1.9
Brown soil	17	**21**	80-85	**0.7**
Perlite	17	25	**85-90**	1.9
Sandy soil	**13**	22	80-85	**1.8**

125.7±102.76 gm. in riverine and 33.45±19.65 gm. in non-riverine areas. pH in this experimental finding was recorded between 6.63±0.21 in riverine and 6.68±0.15 in non-riverine areas.

Figure 4.4 and table 4.8 explain number and weight of nodules per plant were more in taller and older *Hippophae salicifolia* plants growing in riverine areas compared to non-riverine areas. It was interesting to note that nodules were quantified more in riverine areas than the non-riverine areas. The other factors like canopy size, tillering, pH did not show any remarkable effect on nodular quantity. In this quantification experiment, being the first initiative of its kind for *Hippophae salicifolia* of Sikkim, both living and necrotic nodules were considered with an objective to see the quantity of nodules per plant. The graph also shows the age of plant with a remarkable standard deviation compared to other parameters in the graph.

Despite of ecological significance of *Hippophae* L in temperate areas, there are very less reports on nodulation works from the fields. In the report on *H. rhamnoides* L. growing in coastal areas of Lincolnshire (England) by Stewart and Pearson (1967) it is also suggested that number as well as weight of nodules increased with age of plants up to 13 years. Oremus (1979) has also illustrated Akkermans's report stating 1-2 years plants growing along the Dutch coast were richly nodulated compared to older plants. In the course of this study, it was also observed that young roots of younger plants growing in riverine areas did not have necrotic nodules and also had more number of nodules compared to roots of older plants and older roots of younger plants irrespective of their height. Quantification of nodules of *Hippophae salicifolia* in the riverine areas of Lachen valley and the results reported by Stewart and Pearson (1967) and Oremus (1979) have shown

Table 4.11: Effect of GA_3 on root and shoot growth of sea buckthorn seedlings

Treatment (ppm)	Root growth (cm)	Shoot growth (cm)
Control	2.02	2.0
600	2.80	5.75
700	**6.30**	**8.07**
750	4.85	5.00
800	4.25	4.47
CD at P 0.05	8.67	4.95
CD at P 0.01	11.9	Non-significant

some similarities irrespective of different parameters taken for each experiment.

The reason behind higher number of nodulation and thus increase in weight of nodules per plant in the riverine areas may be due to more moisture contents in sandy soils of river banks. It was seen during the monsoon that constant rain and flash floods washed away the biomass of riverine areas, thereby leaving the soil with little nutrients compared to the soils of non-riverine areas, which did not face constant flash floods and had less soil erosions. Such natural activities made the soil stressed left with little nutrition for the plant compelling it to nodulate more. Reports by Schwencke and Carua (2001); Dawson (1990); Sen (1996) also suggests that actinorhizal plants can grow well in stressed soil conditions and also nodulate more in these conditions. I did not go through any such report on comparison of nodular richness in *Hippophae* L or other actinorhizal plants growing in riverine and non-riverine areas, but reports on leguminous plants suggested that flash floods characterizes loss of nitrogen from the soil by leaching and de-nitrification which is not common in plants growing in waterlogged or non-riverine areas (Barrios and Herrera 1994, Moreira and Franco 1994, Saur, *et al.* 1998). Nitrogen being same in all cases, may have been leaching in riverine sandy soils of Lachen valley also, thereby forcing *H. salicifolia* to nodulate more compared to non-riverine plants. Further moisture too played a great role in gaining the weight of the nodules in riverine areas.

4.2.0 GROWTH OF HOST PLANTS

Table 4.12: Response of chemicals in germination of sea buckthorn seeds

Treatment(s)	Days for initial germination	Days to 50% germination
H_2SO_4 (1.5N)	11	16
GA_3 (700ppm)	**08**	**10**
IBA (1ppm)	09	11
NAA (5ppm)	09	11
Control (H_2O)	17	20

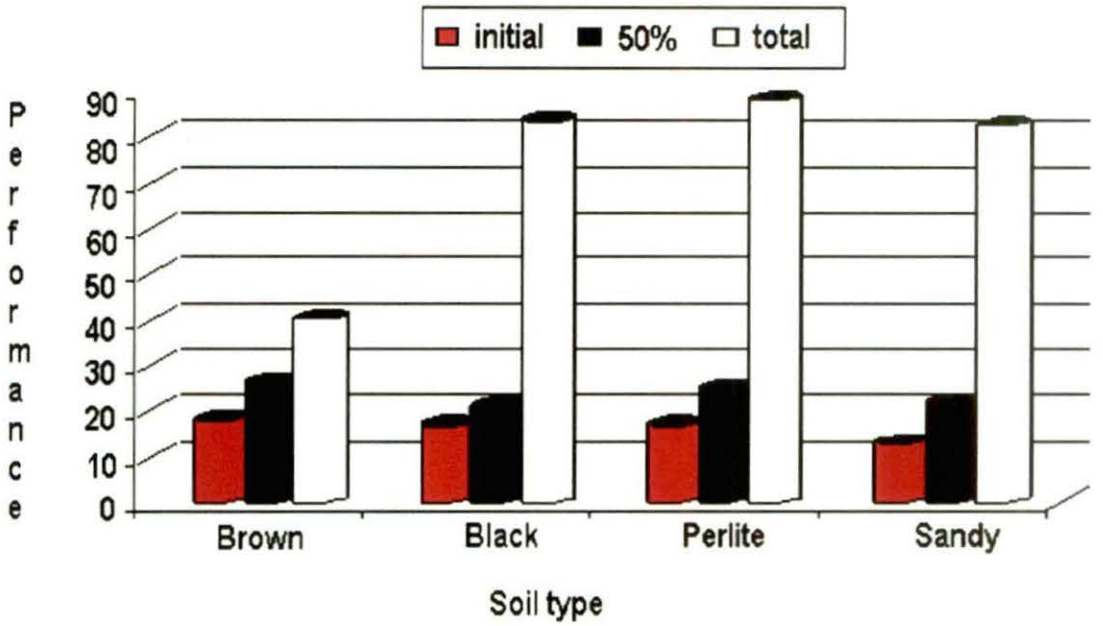


Figure 4.5: Germination of *Hippophae salicifolia* seeds in different soil based media

4.2.1 Seed germination

Propagation of seeds of *Hippophae salicifolia* was studied in different soil combinations. Table 4.10 presents the details on germination and growth pattern of seedlings in different soil based media in the field during the month of May-June at Gangtok (4,700ft.) with temperature ranging from 19°C-27°C.Rongsen (1992) reported that only 13.2% sea buckthorn seeds germinate at 12°C over a period of 47 days but if the temperature is between 24 to 26°C, 95% of the seeds germinate within six days.

Result of germination performance from the table 4.10 shows that germination in artificial media (Kel-perlite) and black soil took 17 and 18 days, respectively compared to faster germination in sandy soil, which took only13 days. This may be due to better soil aeration and temperature absorption by sands compared to the other two soils tried. Better 50% germination of the seeds was seen in brown soil, which just took 21 days compared to other soil combinations. Germination of 85-90% was seen in perlite soil with better average growth (1.9 cm per month), but perlite being an artificial media may not be cost effective and readily available every time for rural purpose. The table 4.10 and fig. 4.5 suggests brown soil to be better for germination (80-85%) and growth of *Hippophae salicifolia* seeds in the present condition. Sandy soils may be better for initial growth but may not fulfill the nutritional

Table 4.13: Response of growth hormones in successful formation of callus and rooting after 90 days of plantation in sea buckthorn cuttings

Treatments (ppm)	Total rooting		Total callus formation		Root length (cm)	
	(Nos.)	(%)	(Nos.)	(%)	Total	Mean
IBA 50	7	35	8	40	**43**	4.8
IBA 55	8	40	9	45	14.6	1.8
IBA 60	**9**	**45**	9	45	31.5	3.93
NAA 300	3	15	7	35	7.5	2.5
NAA 350	0	04	20	-	-	-
NAA 400	0	0	4	20	-	-
IAA 50	2	10	10	50	2.2	1.1
IAA100	5	25	**12**	**60**	4.7	**9.4**
IAA 200	3	15	9	45	25.5	8.5
Control (H_2O)	5	25	1	5	19.5	3.9

Figure 4.6: Different stages of growth of *Hippophae salicifolia* raised from treated seeds

requirement of the seedlings for later growth and development. Black soil, perlite and the sandy soils gave better result during the initial germination period but brown soil that may not be responsive for seed germination during the initial stage produced 50% of germination faster than perlite, black and sandy soil. Hence, table and figure stated above suggests that seeds

germination performance is better in brown soil.

All the seeds after attaining the growth of 2-3 inches were transferred to normal local soil mixed with some organic manure, showed good growth performance in the hardening shed (fig. 4.6).

4.2.2 Germination performance using active substances:

Study of germination performance was carried out in two different ways as a result two types of data have been obtained as an outcome of these two experiments on seed germination and growth of seedlings. The results are illustrated in table 4.11 and table 4.12.

The findings at table 4.11 showed that there was increased growth of roots and shoots of the sea buckthorn seeds when treated with at 700ppm GA_3, compared to other concentrations (600, 750 and 800ppm GA_3).The root growth was found significant both at 0.05 and 0.01 critical difference (CD), while shoot growth was found significant only at 0.05 CD.

The results in table 4.12 suggests that seeds treated with GA_3 (700ppm) attended 50% germination by 10 days from the date of sowing while seeds in control took 20 days' time for the same. The seeds treated with NAA (5ppm), IBA (1ppm), H_2SO_4 (1.5N) germinated in13, 11 and 16 days, respectively. The roots developed after treatment method also successfully produced nodules in a similar manner to those at natural habitat in their later stages, when they were transplanted to sandy soil-based media in10 inches poly-bag.

The findings at table 4.12 also states that when *H. salicifolia* seed were subjected to different physiologically active substances to break dormancy, the response of GA_3 (700ppm) was positive in reducing the time span of germination of seeds, which took only 8 days compared to more days by other treatments like H_2SO_4 1.5N, IBA 1ppm, NAA 5ppm and control (H_2O). Subedi and Adhikari (2001) reported that soaking seeds for 48 hrs. followed by hot water treatment gave better germination results. Koller *et al.* (1962) reported that seedling grows best at that temperature where the seed germinates, growth of roots and shoots significantly increased when seeds were soaked in plain water for 24 hrs. at 22-24°C (room temperature) and then treated with GA_3 at 700ppm. In this case, seeds gave better performance when soaked in different

concentration of chemicals stated above, just for 12 hrs. at room temperature (20-25°C).

It is understood from the present study that the difficulty in growing plantlets from seeds may be reduced using growth regulators like GA_3 and IBA at a specified concentration. This method proved better germination and growth than the naturally regenerating seeds.GA_3 is well known germination stimulator (Vijaya *et al.,* 1996; Soyler and Khawar, 2007). As a result of GA_3 treatment, there is a mobilization of stored reserves inside seeds (Soyler and Khawar, 2007) and it also promotes fast disappearance of large quantity of abscisic acid (ABA)-regulated polypeptides, in dormant seeds (Nicolas *et al.,* 1997). This experiment may be useful for faster plant regeneration related to eco-conservation works. *Hippophae salicifolia* can be planted in other similar areas of altitude and habitat in the dry temperate region to gain its multifarious economic values. Nursery raised seedlings of this species, through pretreated seeds are advantageous and cost effective for mass regeneration, plantation and greenery development programmes at temperate mountain deserts.

4.2.3 Propagation by cuttings:

Different parameters considered for the results of this experiment (fig. 4.7) are as under:

- Time of plantation=August-September.
- Average length of cuttings=25 cm.
- Average weight of cutting=75gm.
- Average buds per cutting=20 nos.
- Average number of damaged buds per cuttings=10.
- Average number of nodes on the cutting of 50cm=40.
- Cutting position while planting=vertical.
- Crop geometry=2 x 2m.
- Spacing (row to row) on planting=200cm.
- Cutting for replacement of damage/ dead cuttings=15%.
- Type of set/cutting to be used for plantation=Hard wood.
- Number of cuttings used=2500
- No. of cuttings required for replacement=20% of 2500=500.
- Total cuttings required=2500+500=3000.

From the results recorded after 30 days (one month) of cuttings treated with

Figure 4.7: Cuttings raised in different soil compositions after treatments. D represents the growth of *Hippophae salicifolia* in polybag in the hardening shed after treated with IBA 60ppm

different concentrations of IBA, NAA, IAA and control (H_2O), it was revealed that cuttings treated with IBA 60ppm showed 45% total rooting and 45% of total callus formation with a significant root length of 31.5% and showed better results among all the treatments used for propagation of cuttings. The callus formation (60%) and mean root length (9.4cm) was however, observed to be higher in IAA at 100ppm treated cuttings.

Among all the three concentrations of IBA (50ppm, 55ppm and 60ppm) used for treatment, the cuttings responded well to IBA 60ppm and responded least to IBA 50ppm. Similarly for the cuttings treated with different concentrations of IAA (50ppm, 100ppm and 200ppm), IAA 100ppm showed better performance compared to IAA 50ppm and IAA 200ppm,

which gave mixed results (table 4.13). NAA 300ppm showed 35% success in callus formation and 15% success in root formation with mean root length of 2.5cm was among the least preferred concentration for the treatment of cuttings. Optical observation during this experiment also revealed that though both hard wood and soft wood equally gave response to the growth regulators, but the survival rate of hard wood was observed to be more to that of soft wood.

The findings at table 4.13 are related with the findings of Donald (1987) who reported that treatment of cuttings of *Pinus ellitti* with 1% IBA was successful for root initiation. Similar type of results was also reported by Tsar-kova-TF (1988) and Rongsen (1992) at 50ppm IBA in the cuttings of *Hippophae rhamnoides* L.

My result provides considerable guidance towards successful propagation protocol for large scale cultivation of this species for orchard development under horticultural model, afforestation, rehabilitation and conservation of degraded lands in the high altitudes of Sikkim. This technique is also inexpensive, easier to perform and less labor intensive than micro-propagation (Soni, 2010), which also reduces the fruiting duration of the plants compared to the plants raised from seeds. Apart from this technique of propagation, it shall also give the true to type of the cultivar for economic usages, if propagated after careful selection of accession with desired characters.

4.2.4 Layering

The observations of layering showed that 13 out of total 20 layerages (65%) showed successful rooting and foliage growth. It was also observed that some of the rooted branches were already nodulated with 2-6 lobed nodules, somewhat whitish in colour and intact with the root mass. This experiment was done in the field during the month of May. The layerages were collected by cutting from the mother plant in the field during the month of September, and transported to the trial plot at Gangtok (4,700ft. amsl) for propagation experiments. Plantation was done in sandy soil bed of 1ft. height at 0.5 x 0.5ft. plant spacing. The layerages had good success rate in the experimental plot without any treatments. Similar layering experiments have been reported by Papp (1982).

Agro-techniques study on *H. salicifolia* revealed some interesting facts. In the

trial plot it was interesting to note that the seedlings of even smaller size produced sufficient root mass and formation of nodules were even high as compared to the plant from natural habitat. Nodulation was even better in the sandy soil with sufficient gravel and organic matters added with cow dung manure. The average number of nodules from 100 seedlings (5-15cm) was recorded at 12 per plant. The root mass was observed to be poor in case of water logged soil.

The present study on agro-techniques also revealed that the plant can be artificially explored to several other dry temperate areas of Sikkim with similar climatic conditions. Cultivation of this economic and eco-friendly plant with scientific approaches may fulfill the requirements of high altitude inhabitants. Germination of seeds using perlite type soil or brown soil media, use of growth regulator treated cuttings for mass propagation programmes and thus development of forest vegetation along with soil binding and biomass production at difficult areas, can be taken as viable package of practice for sea buckthorn cultivation in Sikkim and thus be addition of economy to the marginalized farmers of the remote terrain.

4.3.0 ANTIOXIDANT:

4.3.1 Plant Yield

The yield of the different parts of plant and different extracts has been shown in table 4.14. This table shows the percentage of yield from male leaf, male bark, female leaf and female bark extracted in water (W), Acetone (A) and Methanol (M).

4.3.2 Total phenolic and flavonoid content

Phenolic compounds can equally contribute to the antioxidative action. The amount of total phenol and flavonoids in leaf and bark of male and female of *H. salicifolia*, separately, are explained in table 4.15. The flavonoid content was measured as Quercetin equivalents (QE) and phenol concentrations were measured against Gallic acid equivalent (GAE). These extracts had phenolic levels ranging from 99 to 1459 mg/g GAE. Among all the extracts, water had the highest content of total phenolic and the lowest content was observed in acetone extract. Total phenolic content of methanol was intermediate between water and acetone extracts. Various flavonoids in these extracts ranged from 135 to 707 mg/g of QE, which also followed similar pattern as

Table 4.14: Plant yield v/s plant part

Plant Part	Yield Percentage*		
	Water (W)	**Acetone (A)**	**Methanol (M)**
Male leaf	8.5	4	12.5
Female leaf	10.3	2.5	11.7
Male bark	11.73	3.3	12
Female bark	9.8	1.5	10.45

**Values are in triplicates*

phenolic content, in terms of extract. Highest flavonoid content was present in the aqueous extract of male bark (707±62 mg/g QE) followed by male leaf (419±32 mg/g QE) in comparison to female plant parts. Similarly, phenol contents also followed the same pattern, where aqueous extract of male leaf male leaf (1454±62 mg/g GAE), female leaf (1459±53 mg/g GAE), male bark (1453±82 mg/g GAE) and female bark (1377±62 mg/g GAE) were recorded.

4.3.3 Antioxidant activities

Evaluation of antioxidant activities of the different type of fractions of *Hippophae* were carried out using three methods, viz, 2,2-diphenyl-1-picryl-hydrazyl(DPPH) radical scavenging activity, ferric reducing/antioxidant power (FRAP) and hydrogen peroxide (H_2O_2) scavenging assays.

4.3.4 Free radical scavenging activity (DPPH method)

Goyal, *et al.*, 2010 has reported that DPPH is a stable free radical with a characteristic absorption at 517 nm and this is widely used to study the radical-scavenging activity of natural antioxidants.

It was found that simplest method to interpret data of DPPH was by plotting absorbance against substrate concentration, extending the concentration range beyond the end-point to define the subsequent section

Table 4.15: Concentration of phenols and flavonoids in different extracts of *Hippophae salicifolia*

	Phenols (µg/g GAE)*			***Flavonoids (µg/g QE)****		
	Water	Acetone	Methanol	Water	Acetone	Methanol
Male Leaf	1454±62	137±42	**558±11**	419±52	163±32	315±32
Female Leaf	**1459±53**	**157±62**	329±32	351±22	**203±52**	255±12
Male Bark	1453±82	99±32	276±53	**707±62**	135±12	267±42
Female Bark	1377±62	107±3	447±63	415±19	139±14	**347±12**

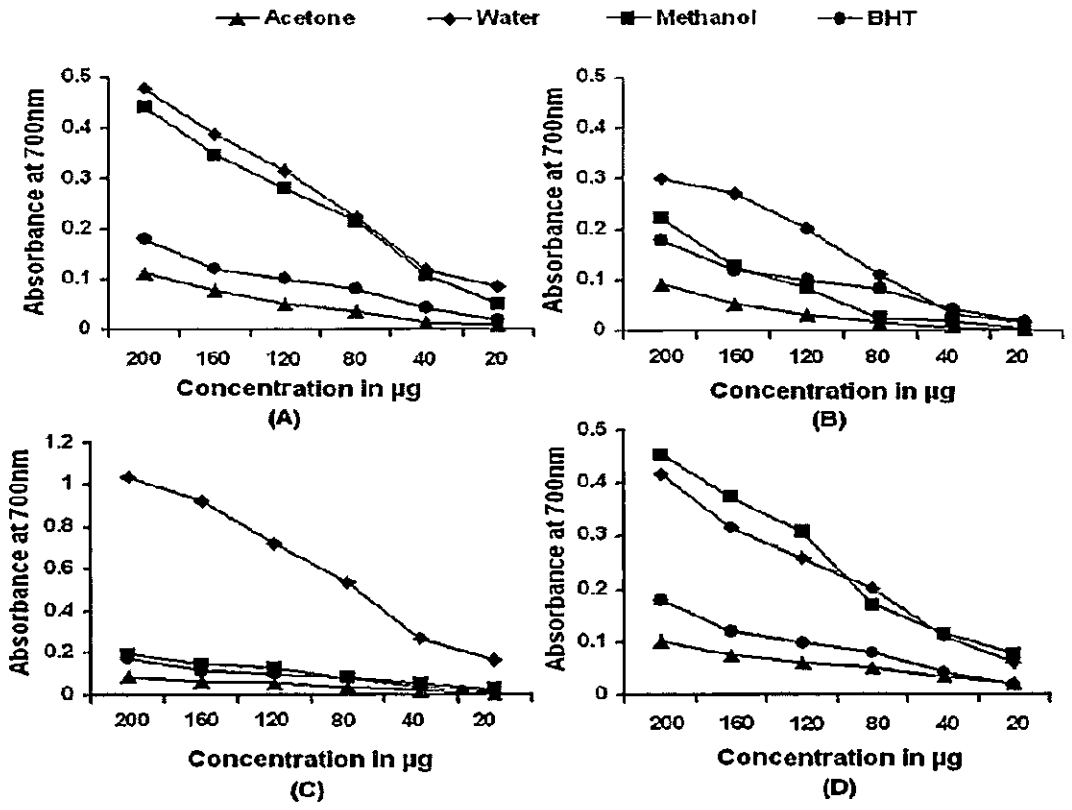


Figure 4.8: Reducing power assay of (A) Male leaf, (B) Female leaf, (C) Male bark and (D) Female bark compared with standard (BHT) at 700nm. Values are in triplicates±standard deviation

of the plot to enable more accuracy in defining the intersection point. This allowed any residual colour from the reduced DPPH and also any inherent absorbance from the substrate itself at a working wavelength. For definiteness, the substrate concentration used had to be in the reaction cuvette in absence of any DPPH and alternatively the amount in moles of substrate added to the reaction vessel can be used.

Table 4.16, 4.17, 4.18, and 4.19 illustrates the result of DPPH assay. The plants showed high antioxidant capacity, quenching ability ranged from 5%-90%. Male leaf (86%) showed highest scavenging in water extract followed by female leaf (82%), male bark (84%) and female bark (83%) at 0.6, 0.6, 0.4 and 0.8 mg/ml, respectively. At the same time, acetone extract of *H. salicifolia* plant parts could not reach 50% level. 0.8 mg/ml concentration of methanol extracts containing male leaf and female bark also showed 90% quenching ability. Male bark (1.0 mg/ml) and female leaf (1.0 mg/ml) expressed 88% and 83%. It was found that these methanolic extracts of *H. salicifolia* showed excellent activities with the concentrations tested (Ara and Nur 2009).

4.3.5 Reducing Power Assay

Another significant indicator of antioxidant, i.e. the reducing capacity of the extract, was also observed at an appreciable level. Fe3+/Fe2+ transformation (by an electron donation) activity was investigated in the samples for measurement of reductive ability. The amount of Fe2+ complex can be examined by measuring the formation of Perl's blue at 700 nm (Ebrahimzadeh *et al.*, 2010). Increase in absorbance was an indicative of increase in reductive activity. Figure 4.8 showed the reducing power of different parts of *H. salicifolia* ranging from 0.021 to 1.041 Abs at concentrations ranging from 20-200 µg/ml of the extract. The reducing capacity of extracts in different plant parts of *H. salicifolia* were as follows: water (W) > methanol (M) > acetone (A). Water extract of male bark (1.04) showed the highest reducing activity. In different plant parts, reducing power from highest to lowest range were as follows: Male bark (W) > Female bark (M)>Male leaf (M)>Male leaf(W) >Female bark(W)> Female leaf(W) >Male leaf(A)>Female bark(A) >Female leaf(A)>Male bark(A).

Report by Okuda *et al.* (1983) states that reducing power of tannin inhibits the formation of lipid peroxides, thereby preventing liver injuries. In another report by Goyal *et al.* (2010) it is mentioned that the reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. This is an indicative of medicinal property of sea buckthorn.

4.3.6 H_2O_2 radical scavenging activity

Tables 4.16-4.19 illustrates that the ability of quenching H_2O_2 in methanol extract of male leaf (100%), female leaf (99%), male bark (100%), female bark (98%) was higher compared to acetone and water extract. Table 4.20 showed the scavenging of hydrogen peroxide gradually increased with the increase in concentration of the extract as well as the standard (BHT).

4.3.7 Correlation study

Observations made in the past clearly indicates that there is a close association between overall phenolic content and antioxidant activity, like reducing power and radical scavenging effect on DPPH radicals (Li *et al.*, 2003). Similarly, indications that correlation coefficient can be used as an indicator of antioxidant capacity were established after linear

relationships between these parameters had been found for *L. cuneata* extract (Kim and Kim, 2007). From our experimental observations it was found that there were high correlations between the phenolic levels of *H. salicifolia* extract and antioxidant activities, which suggested that phenolic compounds acted as antioxidants in this plant species.

To determine relationship between antioxidant activity and phenols of *H. salicifolia* extract and fractions, a positive linear correlation was established between phenol content and DPPH. (Linear correlation coefficient of Male Leaf R^2 (W)=0.7273, R^2 (M) =0.8451, R^2 (A)=0.6741, Female Bark R^2 (W)=0.8845, R^2 (M)=0.5409, R^2(A) =0.7024, Male Bark R^2(W)=0.4623, R^2 (M)=0.9144, R^2(A)=0.4462, Female Leaf R^2(W)=0.8386, R^2(M)=0.9256, R^2 (A)=0.9354 (figure 4.9).

To evaluate antioxidant activity of total phenolics and flavonoides in this study, three different kinds of extracts

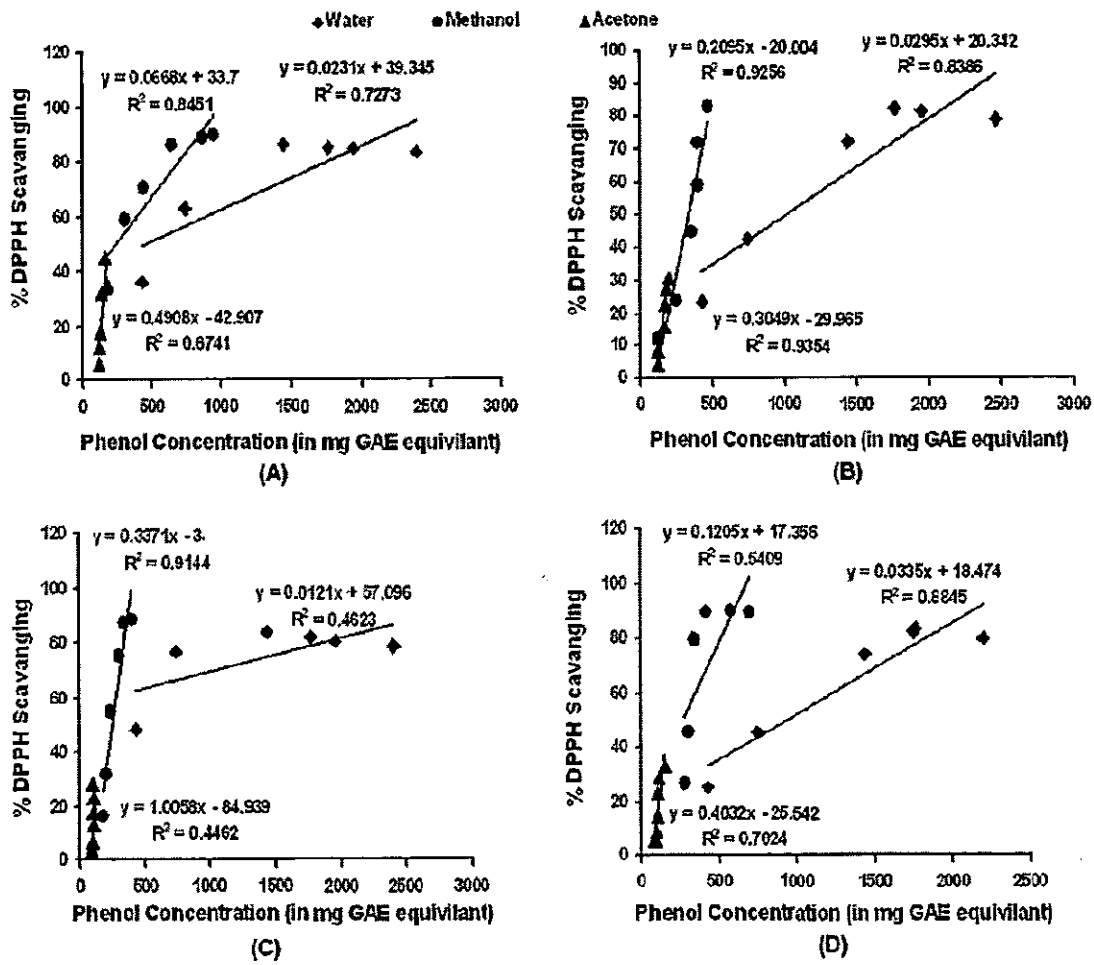


Figure 4.9: Correlation between DPPH and total phenol content in water, methanolic and acetone extract of (A) Male leaf, (B) Female leaf, (C) Male bark and (D) Female bark. Values are in triplicates±standard deviation

Table 4.16: Percentage inhibition* (%I) of radical scavenging of male leaf of *Hippophae salicifolia*

Extract concentration (mg/ml)	Male Leaf					
	DPPH			H_2O_2		
	Water	Acetone	Methanol	Water	Acetone	Methanol
0.1	36±21	6±67	33±21	5±53	57±28	92±23
0.2	63±55	12±51	59±11	51±23	65 ±71	94±30
0.4	**86±89**	18±71	71±26	67±51	75±50	96 ±71
0.6	85±12	32±12	86±30	78±55	92±20	97±24
0.8	84±10	35±76	**90±66**	89±21	83±40	99±53
1.0	83±12	**45±26**	89±21	**95±10**	**96±11**	**100±33**

Table 4.17: Percentage inhibition* (%I) of radical scavenging of female leaf of *Hippophae salicifolia*

Extract concentration (mg/ml)	Female Leaf					
	DPPH			H_2O_2		
	Water	Acetone	Methanol	Water	Acetone	Methanol
0.1	23±61	4±41	12±66	54±11	80±28	93±54
0.2	43±10	8±91	23±21	64±91	85±26	95±11
0.4	72±12	16±80	45±90	73±32	89±18	96±33
0.6	**82±31**	22±97	59±25	82±44	92±60	97±43
0.8	79±61	27±88	72±33	90±30	96±20	98±21
1.0	82±21	**30±12**	**83±22**	**95±13**	**98±34**	**99±81**

Table4.18: Percentage inhibition* (%I) of radical scavenging of male bark of *Hippophae salicifolia*

Extract concentration (mg/ml)	Male Bark					
	DPPH			H_2O_2		
	Water	Acetone	Methanol	Water	Acetone	Methanol
0.1	48±61	4±20	16±16	5±7	57±65	92±32
0.2	77±81	7±62	31±40	51±10	65±98	94±40
0.4	**84±67**	13±61	55±10	67±17	75±91	96±89
0.6	82±77	18±56	75±20	78±66	83±17	97±21
0.8	80±17	23±69	87±56	89±99	92±18	99±42
1.0	78±18	**28±61**	**88±78**	**95±53**	**96±12**	**100±64**

Table 4.19: Percentage inhibition* (%I) of radical scavenging of female bark of *Hippophae salicifolia*

Extract concentration (mg/ml)	Female Bark					
	DPPH			H_2O_2		
	Water	Acetone	Methanol	Water	Acetone	Methanol
0.1	23±61	5±11	26±11	54±34	93±76	80±55
0.2	46±16	8±17	46±41	64±16	95±16	85±68
0.4	74±61	14±78	79±99	73±64	**96±10**	89±65
0.6	**83±56**	23±67	89±53	82±83	95±62	92±44
0.8	82±67	28±22	**90±89**	90±77	94±53	96±65
1.0	80±31	**33±91**	89±16	**95±7**	94±10	**98±47**

**Values are in triplicates±standard deviation* (for all the tables of this page)

Table 4.20: Radical scavenging activities of BHT as standard at different concentrations

Standard concentration (mg/ml)	DPPH	H_2O_2
0.1	33±11	34±4
0.2	46±6	43±6
0.4	58±8	57±16
0.6	73±15	62±13
0.8	82±36	70±8
1.0	**90±10**	**75±16**

**Values are in triplicates±standard deviation*

(aqueous, methanolic and acetone) were used from four different parts of *H. salicifolia*. Similar study of antioxidant evaluation was also carried out by Negi, *et al.* (2005) in *Hippophae rhamnoides*. It is for the first time that this experiment has contributed to a better understanding of antioxidant properties of different parts in *H. salicifolia* of Sikkim.

In my study the presence of phenolic and flavonoid compounds of all the parts were more in aqueous extracts compared to methanol and acetone extracts, which means these compounds are hydrophilic and thus seeps into water during extraction process (Ayansor, *et al.*, 2010) or the phenolic and flavnoid contents were more in aqueous extract may be due to the difference in polarity of different solvents under study (Kostic *et al.*, 2010).

Li *et al.* (2003) reported that flavonoids affect membrane permeability by inhibiting membrane bound enzymes like ATPase and phospholipase A2, which may explain the antioxidative action of *H. salicifolia*. Ferguson (2001) suggested that flavonoides contain anion radicals and hence serves as health promoting compounds, which supports the ethno-traditional use of this plant in treating stress related problems and skin ailments like treating burns, scabis, cracking of skin, impetigo, keratosis, cures xeroderma and prickly heat (Negi *et al.*, 2005).

After incubation for 20 minutes, the DPPH scavenging activity of aqueous and methanol extracts were higher compared to acetone extracts. As the concentration of the extract ranging from 0.1 to 1.0 mg/ml was gradually increased, the percentage inhibition of DPPH too gradually increased and similar activity was also exhibited by the standard (BHT). This may be due to elevated levels of the active compounds along with the polyphenols in both aqueous and the methanolic

extracts, which have higher hydrogen donating ability to scavenge DPPH radicals attributing their higher antioxidant activity (Chueng *et al.*, 2003)

Hydrogen peroxides are highly diffusible and an important Reactive Oxygen Species (ROS) because of its ability to penetrate biological membranes and most importantly it causes activation of nuclear translocation of transcription factors NF-$_K$B, which subsequently allows the transcription of genes and leads to the inflammation and Syndrome X (Goyal *et al.*, 2010; Ghaisas *et al.*, 2008), though, it may be toxic if converted to hydroxyl radical in the cell (Wang *et al.*, 1998). Scavenging of H_2O_2 by the plant extracts may be ascribed to their phenolics, which donate electron to H_2O_2, thus reducing it to water.

This is the first study of its kind showing male bark had high level of reducing capacity, may be because of more phenolic compounds, which are potent antioxidants. The outcome also showed that there was increase in reducing power of the plant extract along with increase in extract concentration (Akinpelu *et al.*, 2010).

Studies carried out by Kim and Kim (2007); Haraguchi *et al.* (1992) found that there was a good linear correlation between total phenolic compound content and the antioxidant activity in different plants. Nagai *et al.* (2004) reported that there was also no significant relationships between the antioxidant activities (determined using three different methods namely FRAP, DPPH and carotene bleaching assays) and total content of phenolic compounds shown by some other researcher. Therefore, this positive linear correlation showed that the sample with highest total phenol content likewise had the highest DPPH values, whereas the sample with lowest total phenol content produces the lowest reducing power values. Ailments such as diabetes mellitus, cancer, liver diseases, renal failure and degenerative diseases are as a result of deficient natural antioxidant defence mechanism. Such diseases can be cured by polyphenols, which are the principal plant compounds that have antioxidant activity (Middha *et al.*, 2009). Table 4.14 exhibits aqueous and methanolic fractions containing the highest amounts of phenolic compounds and both fractions exhibited higher antioxidant activities. The antioxidant activities of phenolic compounds were primarily due to their redox properties,

Table 4.21: Quantification of DNA isolated from the root nodules of different samples after purification and the quantity taken for PCR

Sample code No	A_{260} nm	A_{280} nm	A_{260}/A_{280}	Amount of DNA (ng/µl)	Amount of DNA for PCR (ng/µl)	PCR Result
SIHF01	0.075	0.043	1.74	375	46.87	Band visible
SIHF02	0.064	0.036	1.77	320	45.71	-do-
SIHF03	0.155	0.085	**1.82**	775	45.58	-do-
SIHF04	0.093	0.054	1.72	465	46.50	-do-
SIHF05	0.159	0.089	1.78	795	**46.76**	-do-
SIHF06	0.080	0.44	1.81	400	44.44	-do-
SIHF07	0.029	0.016	1.81	145	**36.25**	-do-
SIHF08	0.044	0.025	1.76	220	40.00	-do-
SIHF09	0.038	0,022	**1.72**	190	38.00	-do-
SIHF10	0.213	0.120	1.77	1065	46.30	-do-
SIHF11	0.028	0,016	1.75	140	46.60	-do-

which allowed them to function as reducing agents, hydrogen donors and singlet-oxygen quenchers (Goyal *et al.*, 2010). Taken together, the high correlation confirmed that the polyphenol constituents of *H. salicifolia* may be responsible for its high antioxidant activity during the DPPH assay.

4.4.0 ISOLATION OF *FRANKIA*

4.4.1 Surface sterilization

There are different types of microorganisms present on the surface of the root nodule, which act as contaminants and hinder the growth of *Frankia* in pure culture It was mandatory to remove these contaminants in order to get *Frankia* clusters in pure culture. Of all the techniques applied, the technique of serial no D at chapter III, (3.10.2 D) gave positive result. Success of this technique may be due to the efficient elimination of microbial contaminants associated with the nodule surface. H_2O_2 are potential oxidizing chemical agents that are efficient in eliminating contaminants from the surface of the nodule without causing harmful effect to the cortical cells where *Frankia* were confined.

Frankia is a very slow growing organism. Each *Frankia* colony originates from a single unit called positive cells and were termed as units able to form *Frankia* (UFF) (Diem and Dommergues, 1982). Only the positive cells that came in contact with the nutrient media formed a colony. The UFF broadly referred either to specific structures or hyphae or cluster of hyphae, which was able to grow from the nodule. Berry and Torrey (1979) had already reported that *Frankia* originates from clusters of hyphae, which may be suggestive, that hypahal

clusters originating from the sliced nodule may be more suitable for initiating colonies than crushed nodules (Sen, 1996).

4.4.2 Culture media for isolation

The culture medium formulation or used in isolation of new *Frankia* strains is critical for success, still till date no universal or *Frankia* strain specific medium has been formulated. Different kinds of media have been described by Lechevalier and Lechevalier (1990) and Myrold (1994) (see review section for details). Many *Frankia* isolation media are supplemented with various chemicals and yeast extract believed to promote *Frankia* growth.

Frankia has been found to grow well on a minimal medium containing buffered salt solutions with one carbon source like succinate, pyruvate propionate, etc., which can be changed depending on media (Myrold, 1994). From different types of isolation media (refer Appendix I).

Of all the media opted for the work, DPM (Baker and O Keef, 1984) with sodium propionate as carbon source and Q_{mod} (Lalonde and Calvert, 1979) were best suited for *Frankia* isolation from *Hippophae salicifolia* root nodules. Sodium propionate is the preferred carbon source compared to carbohydrates (Myrold, 1994). For further sub-cultures, Q_{mod} medium supplemented with Lecithin also gave positive results, but contaminations in this medium were of great concern.

4.4.3 Nodulation test

The nodulation test conducted in *H. salicifolia* seedlings inoculated with 45 days old pure culture grown in sterile Hoagland's nitrogen free solution showed 40-50% of the plants formed 1-2 sometimes 3 nodules in 30-40 days. In a similar type of study Diem *et al.* (1983) reported 80% nodulation in 14-20 days. *H. salicifolia* being the plant of cold temperate region perhaps showed some hesitance to nodulate freely in the controlled conditions of Gangtok. It was also observed that plants without root hairs hardly nodulated, which indicated and supported the idea that root hairs were the prerequisite for nodulation. Apart from this it was also seen that out of 2-3 pre nodules per plant, only 1-2 developed into creamy or milky white nodules with 1-1.5mm diameter later became brownish in colour.

4.4.4 *Frankia* under light microscope

Sections from the root nodules of *H. salicifolia* were subjected to light

microscopy, which revealed that the section contained clusters of thin hypha in the cortical region. The nodule lobe section had outer epidermis covering parenchyma cells and cortical region. The vascular bundles were surrounded by a thin layer of endodermis. The parenchymatous cells too had infections along with cortical region. All the cortical cells showed infection with dense staining. Distinct cell walls were visible, within which vesicles were present in a mucilaginous material. Only the vesicles and hyphae in the outer periphery could be stained and were viewed. The average colony measured about 300µm (fig. 4.10).

Light microscopy was the preliminary means to identify *Frankia* based on morphological characters. Many reports have suggested that *Frankia* are filamentous, branched bearing vesicles (the site of nitrogenase activity) and presence or absence of sporangia and spores (Benson and Silvester, 1993; Pradhan *et al.*, 1999). The *Frankia* isolates from pure culture of *H. salicifolia* also showed mat of septate filaments or hyphae ranging from 0.6-1mm bearing vesicles measuring between 0.3-0.7mm in diameter (fig. 4.11). Pradhan *et al.* (1999) have reported that DPM was most effective in inducing vesicles and sporangia in most of the isolates of *Alnus nepalensis* compared to Q_{mod} medium. In this case also the isolates revealed sporangia in terminal or intercalary positions. Three months colonies produced in DPM did not exceed more than 1mm in diameter. Some pinkish to off white pigmentation were also observed in the colonies developed in the pure culture. After going through the review of literatures by Normand and Lalonde (1986) and Mullin and An (1990), it was understood that still limited information have been generated on *Frankia* compared to *Rhizobium*. One reason behind this may be due to limited number of isolation and identification of *Frankia* in pure culture. Since the first isolation in 1978, problem of obtaining pure culture isolates and their growth requirements are still restricting further studies on genetics, ecology and physiology of *Frankia* symbiosis. Even after formulation and improvement of different culture media, *Frankia* still remains a slow growing organism requiring longer period to get ready for experiments (Simonet *et al.*, 1991). Even after many attempts there are reports of very few isolates of *H. salicifolia*, which is yet to be described

in details.

4.4.5 Plant infectivity test

Infection tests of *Hippophae salicifolia* seedlings grown in earthen pots, showed 40-50% plants formed 4-8 nodules in 4-6 weeks. Similar works by using the actinorhizal plant as a trap plants to assay plant nodulation using pot system with solid media has been carried out with *Alnus, Casuarina*, and *Myrica* (Cranell *et al.*, 1994; Zimpfer *et al.*, 1997) and *Ceanothus* sp. (Jeong and Myrold, 2003). Dobritsa and Novik (1992) reported that sea buckthorn has an active, systemic mechanism for feedback control of nodulation that suppresses further nodule formation and prevents excessive nodulation. *Hippophae salicifolia* is a plant growing naturally in tropical cold desert and predominant in sandy soils at the river banks (Singh *et al.*, 1995; Rajchal, 2009). This may be the probable reason for not getting clusters of nodulation as natural habitat in *Hippophae salicifolia* when grown in controlled environmental conditions. High NaCl concentration, low phosphorous supply and high combined N_2 supply reduces nodulation in *H. rhamnoides* (Jian, 1989). So making the soil more stressed by reducing the amount of manures and composts, more roots curling were observed in young and developing roots. But care had to be taken during watering where little more watering than the optimum quantity led to damping off and death of the plants. When plants of 1-2 years, grown in controlled environment, were transferred to the experimental site (different from its natural environment), the plants did not show progressive growth in the first year with drastic reduction on nodulation rate to 1-2 nodules per plant. But after subsequent year there were some growth in plants as well as nodulation also increased to an average of 3-4 nodules per plant. Though survivals of the plants were 70-80%, the nodulation was not to the mark as it was expected. The environmental factor of the soil might have reduced nodulation in the plant at new environment. However, it is understood that *Hippophae salicifolia* can be infected by artificial *Frankia* inoculants.

4.5.0 ISOLATION OF DNA OF *FRANKIA* FROM ROOT NODULES

The difficulty in isolating pure culture of *Frankia* and its sluggish growth created major hurdles in studying the genetic diversity of *Frankia*. However, with the advent of better primer

designing ability it is now possible to develop PCR primers, which will amplify a section of *Frankia* genome from mixed cultures or from the DNA samples directly collected and isolated from the environment. The amplified products are then subjected to restriction digestion to get PCR-RFLP profile of wild *Frankia* strains.

As mentioned in materials and methods, DNA was isolated directly from the root nodules collected from 11 different places of Lachen Valley, Sikkim. Various methods were followed but method of Bajwa *et al.* (2005) with minor modifications was found to be the most effective one. Genomic DNA of *Frankia* can be isolated directly from the root nodules (Simonet *et al.,* 1991). Extraction of DNA directly from nodules and amplification by PCR have also been previously reported for studying phylogenetic relationships between *Frankia* reference strains and un-isolated *Frankia* strains in nodules of *Alnus*, *Coriaria*, and *Dryas* species (Rouvier *et al.,* 1996). The method I followed was useful to examine the possible genetic diversity occurring among *Frankia* strains of *H. salicifolia* in the tropical forests of Lachen, North Sikkim. Though the quantity of DNA yields in this method was less, it produced whitish precipitate of nucleic acid, which after further purification was subjected to PCR amplification. Bright bands could be seen on 1.5% agarose gel with 7µl of ethidium bromide. This approach avoided the bias-inducing process of isolation of *Frankia* strains from field collected nodules.

4.5.1 *Frankia* DNA purification:

The root nodules contained large amounts of phenolics, RNA, proteins and polysaccharides. These compounds hinder in DNA isolation and isolated crude DNA may not be suitable for PCR amplification. Addition of CTAB in DNA extraction in the first instance helped in elimination of large amount of polysaccharides from DNA preparation. During the later stages of extraction, RNA could also be removed by treating the sample with 10µl RNase (10mg/ml) followed by equal volume of Phenol: Chloroform: *Iso*-amyl alcohol (25:24:1) and simultaneously by Chloroform: *Iso*-amyl alcohol (24:1) to the samples dissolved in 500 µl 1X TE buffer, at least twice.

4.5.2 DNA quantification

Frankia DNA was quantified using

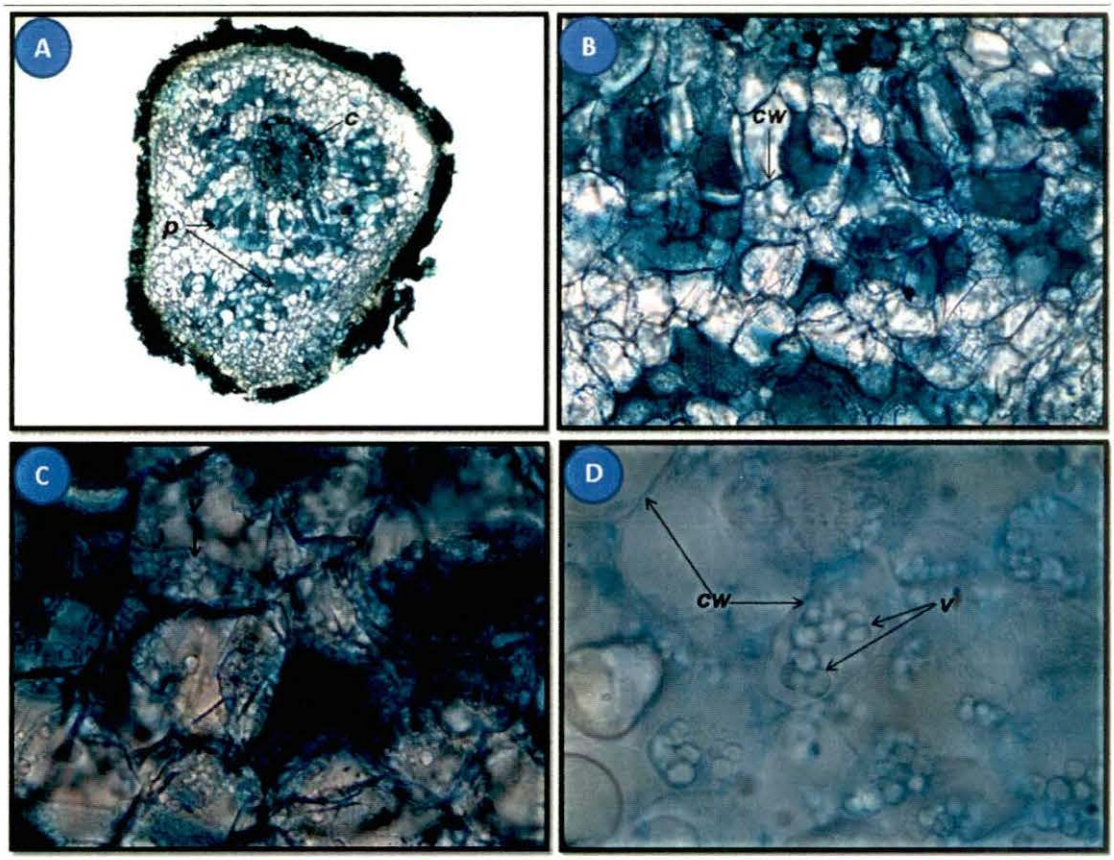


Figure 4.10: Transverse section of root nodule of *Hippophae salicifolia* under light microscope showing *Frankia* infection. A: T. S. under 10X magnification cortical (*c*) and parenchymatous (*p*) regions are infected with *Frankia*; B: T. S. under 40X magnification. Infection can be seen partly in cortical cells and parenchyma region, the cell wall (*cw*) and infected regions are prominent; C: TS closer views of infected cells with 100X magnification; vesicles are prominent inside the cell wall of each cell. D: it's the closer view (lens zoomed); prominent *Frankia* vesicles with bi-lobed and tri-lobed structures are visible in the infected parenchymatous cells

spectrophotometric analysis showing A_{260}/A_{280} ratio around 1.8. This ratio was chosen for further PCR based methods which was having less impurity and gave a bright visible band on subjecting the PCR product to electrophoresis.

Frankia DNA extraction, purification and quantification steps allowed optimum DNA from the root nodules of *Hippophae salicifolia*. However, modifications made during the process by RNase treatment allowed me to obtain substantial amount of pure DNA for PCR amplification. Table 4.21 illustrates the details of amount of root nodules taken and the total DNA isolated after purification and quantification. From the table it also clear that DNA to be taken for PCR amplification after quantification should be in the range of 30-50ng/µl. Though, theoretically ratio of (A_{260}/A_{280}) 1.8 is the ideal value of ratio, in my study PCR amplification was obtained at A_{260}/A_{280} ratio values

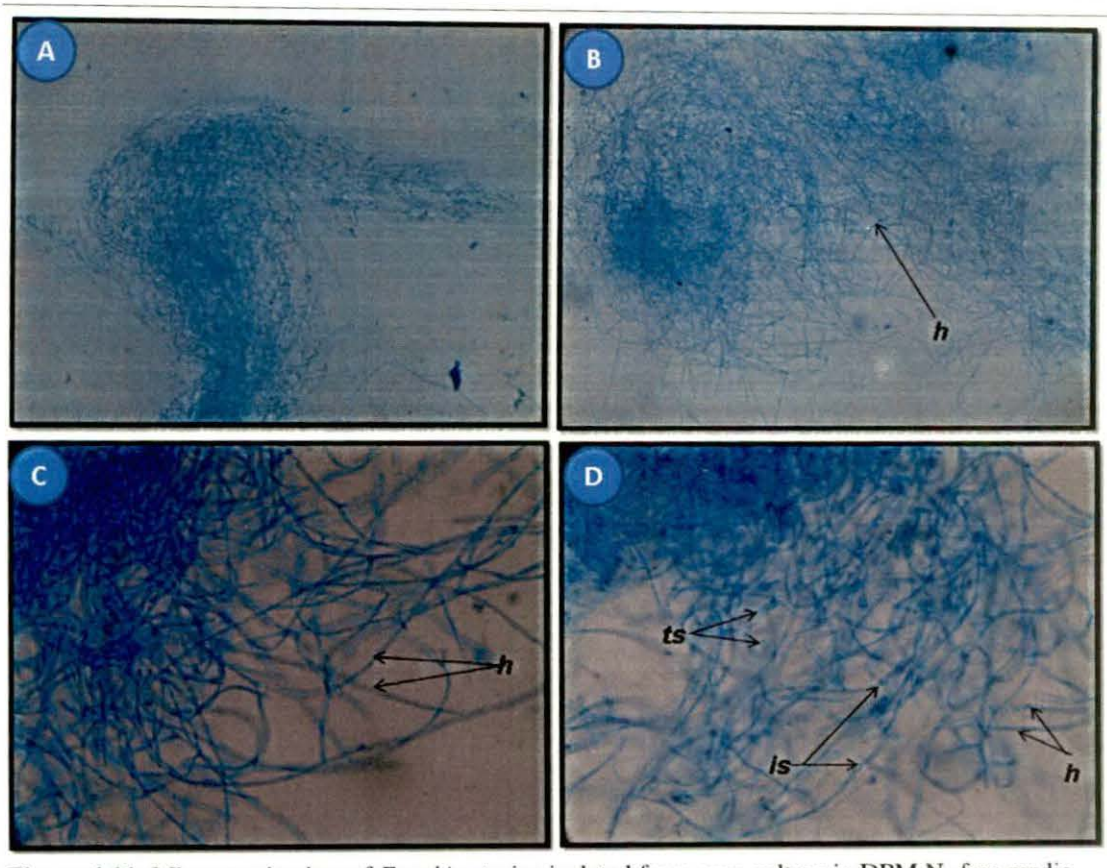


Figure 4.11: Microscopic view of *Frankia* strains isolated from pure culture in DPM N_2 free media (Baker and O'Keef, 1984). A & B shows the dense mat of hyphae under 40X optical lens; C & D reveals distinct septate hypha (*h*), terminal sporangia (*ts*) and intercalary sporangia (*is*) with 100X magnification (oil immersion)

ranging from 1.72 to 1.82.

4.6.0 POLYMERASE CHAIN REACTION

DNA obtained from the root nodules were composed of mixture of DNA from the host plant, the *Frankia* and possibly contaminating bacteria, which may not have been eliminated during the surface sterilization and peeling of nodules. Hence, *Frankia* specific primers were required for obtaining exclusive *Frankia* genome bands. Forward and reverse primer (16 mer) compatible to *Elaeagnus* specific *Frankia* (5' GGGGTCCKTARGGGYY 3') was designed in our laboratory (refer chapter III) and reverse primer FGPL 2054, (5' CCGGGTTTCCCCATTCGG 3') (Simonet *et al.*, 1991), was used to obtain specific amplification of *Frankia* DNA from *Hippophae salicifolia* root nodule isolates (fig. 4.12).

DNA isolated from different sets of nodules collected from different parts of the study site yielded ~1000 bp band on amplification with a set of forward and reverse primers, as stated above.

Distinct band in agarose gel was visible when the annealing temperature was set at 54^{0}C for 1 minute. Even 1^{0}C increase or decrease in annealing temperature effected the band formation. 36.25-46.76ng/µl concentration of DNA template in 1µl was used for PCR amplification, which gave distinct bands. Out of all the DNA isolates subjected to PCR amplification, 11 isolates (SIHF01 to SIHF11) formed distinct bands although few gave low signals, presumably because of lesser amount of DNA in some samples. The results of some amplified samples in presence of DNA-molecular weight markers have been presented in the fig. 4.12.

4.7.0 PHYLOGENY AND GENETIC DIVERSITY:

In the present study BLAST programme was used to compare the 22 sequences namely SIHF01f to SIHF11f and SIHF01r to SIHF11r (Accession No. FR773720 to FR773741) for homology with sequences available in the GenBank. All the sequences showed highest identity with 16S Ribosomal RNA gene of an uncultured *Frankia* sp. isolate (fig. 4.13). The sequences, which were used for preparing phylogenetic tree, along with their accession number and percentage of identity obtained in BLAST programme are presented in the table 4.22.

Phylogenetic analysis from the figure 4.14 revealed that the entire 16S rRNA gene sequences of *H. salicifolia-Frankia* isolate (inside the red box) clusters reside close other *Frankia* strains (inside the blue box) but forms a cluster of its own. Amongst the *Frankia* strains, newly sequenced isolates of *H. salicifolia* showed close proximity with that of *Frankia* EAN1pec and *Frankia* EuI1c. Both these strains infect the plant *Elaeagnus,* which belong to the family Elaeagnaceae. *Hippophae salicifolia* is also the member of the same family. Of the newly sequenced isolates, FR773729 formed an out group in comparison to the ten other isolates with boot strap value of 95%. The sequences used in phylogenetic analysis their names, abbreviations, genes and GenBank accession numbers are given in table 4.23.

The phylogenetic tree in the fig 4.14 also reflects a broad genetic diversity among the *Frankia* strains of the *Elaeagnus*-infective group. Reports on close phylogenetic affinity (up to 99%) among the *Frankia* of *Hippophae* and

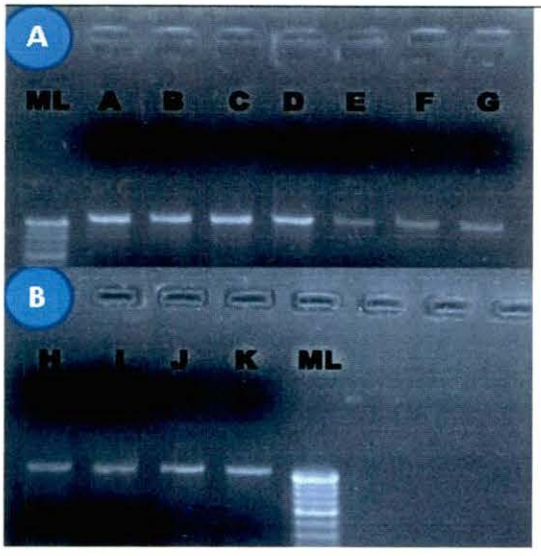
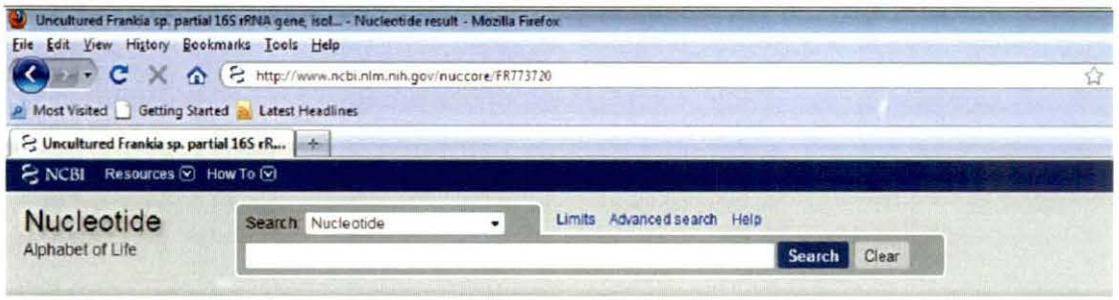


Figure 4.12: Agarose gel (1.5%) electrophoresis of PCR amplified partial 16S rRNA of DNA isolates from root nodules of *Hippophae salicifolia* collected from different locations of the study area. ML=100 bp Marker (1x100bp); lanes A to K represents PCR bands of SIHF01-SIHF11 *Frankia* isolates

Elaeagnus infective group, irrespective of their different geographical locations, have been reported by Chen *et al.* (2008). From the fig. it is also observed that *Frankia* strains of different host specificity (in red and blue boxes) have the tendency to form close clusters with other organisms of close lineages. The sequences of *Frankia* strains present in *Gymnostoma* and *Ceanothus* nodules are phylogenetically close to *Elaeagnus*-infective strains (Navarro *et al.*, 1997; Murry *et al.*, 1997). However, in the process of diversity study of *H. salicifolia-Frankia* strains, it can also be concluded that *Elaeagnus, Gymnostoma, Hippophae, Ceanothus* and all *Elaeagnus*-specific *Frankia* strains have evolved from a common ancestor. Further the close proximity of the *Hippophae salicifolia-Frankia* can also be related and with the altitude of the study area (may refer table 4.5 table 4.22 for quadrates and accession numbers, respectively). The isolates have formed close clusters with almost similar types of altitude and climatic conditions. In a similar type of study, it has been revealed that altitudinal variations affect *Frankia* distribution (Jeong and Myrold, 1999; Oakley *et al.*, 2004)

4.8.0 PCR-RFLP DATA ANALYSIS USING POPGENE SOFTWARE:

From all the samples a total of 18 scorable fragments were generated (table 4.24), out of which 11 were polymorphic. The primer was tried thrice and was reproducible. The number of polymorphic bands per enzyme ranged from one in *Msp*I to five in *Hpa*II with an average of 2.75 (say 3) polymorphic bands per endonuclease (enzyme). These clearly visible, repeatable and polymorphic bands were scored and used for further analysis (fig.4.15). Due to high specificity of the recognition sites, closer cleavage pattern in agarose gel are seen when two sequences have higher similarity (Nei and Li, 1979)



Uncultured Frankia sp. partial 16S rRNA gene, isolate SIHF01f

GenBank FR773720.1

FASTA Graphics

Go to:

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LOCUS       FR773720                770 bp    DNA     linear   ENV 14-JAN-2011
DEFINITION  Uncultured Frankia sp. partial 16S rRNA gene, isolate SIHF01f.
ACCESSION   FR773720
VERSION     FR773720.1  GI:318608262
KEYWORDS    ENV.
SOURCE      uncultured Frankia sp.
  ORGANISM  uncultured Frankia sp.
            Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
            Frankineae; Frankiaceae; Frankia; environmental samples.
REFERENCE   1
  AUTHORS   Basistha,B.C., Thakur,S., Pradhan,S. and Sen,A.
  TITLE     Molecular diversity of Hippophae associated Frankia found in Lachen
            valley of Sikkim, India
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 770)
  AUTHORS   Sen,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-JAN-2011) Sen A., Molecular Genetics Laboratory,
            Department of Botany, University of North Bengal, Rajarammohunpur,
            Siliguri, 73013, INDIA
FEATURES             Location/Qualifiers
     source          1..770
                     /organism="uncultured Frankia sp."
                     /mol_type="genomic DNA"
                     /isolate="SIHF01f"
                     /isolation_source="host root nodules"
                     /host="Hippophae salicifolia"
                     /db_xref="taxon:181582"
                     /environmental_sample
                     /country="India:Lachen Valley, Sikkim"
     gene            <1..>770
                     /gene="16S rRNA"
     rRNA            <1..>770
                     /gene="16S rRNA"
                     /product="16S ribosomal RNA"
ORIGIN
        1 gctcgtgtcg tgagatgttg ggttaagtcc cgcaacgagc gcaacccttg tcctatgttg
       61 ccagcgcgtc atggcgggga ctcataggag actgccgggg tcaactcgga ggaaggtggg
      121 gatgacgtca agtcatcatg gcccttacgt cttgggctgc acacatgcta caatggcggg
      181 tacaaagggc tgcgataccg caaggtggag cgaatcccaa aaagccggtc tcagttcgga
      241 tcgggggtctg caactgcgcc ccgtgaagtc ggagtcgcta gtaatcgcag atcagcattg
      301 ctgcggtgaa tacgttcccg ggccttgtac acaccgcccg tcacgtcacg aaagtcggta
      361 acacccgaag ccggtggccc aaccttgtgg gggggagccg tcgaaggtgg gaccggcgat
      421 tgggacgaag tcgtaacaag gtagccgtac cggaaggtgc gggtggatca cctccttttt
      481 aaggagcttt ctggctgggt gccttctgta gtggaggggtg tctggtccag agcgaagacc
      541 agctggatgg ttggtctggg tgctcatggg tgtggaacgc tgactatgtt tctgtctggt
      601 ctggtctgtt gtccgggggt cctgtgtgtt ctctgggtgg tgggttgtct gggtggatcg
      661 gtacgctgtt gggttgtgag ggagtgagtt gccctcgtgt acgatcctgc ctttctgctg
      721 gcccgttggg gctggtggga ggcaggtttg cgggctggtt cgtcgttggg
//
```

Figure 4.13: Partial 16S rRNA sequence of uncultured *Frankia* sp. from *Hippophae salicifolia* root nodule named as SIHF01f and its accession number provided by the GenBank

Table 4.24 also reveals polymorphism ranged from 50-71.4%. Restriction digestion enzyme *Msp*I and *Hae*III showed 50% polymorphism while *Hpa*II revealed 71.4% polymorphism. An intermediate polymorphism in between the range was shown by *Alu*I (66.6%). The overall polymorphism of 61.11% was observed.

Quantitative measure of genetic divergence or genetic distance calculated from the Nei's original

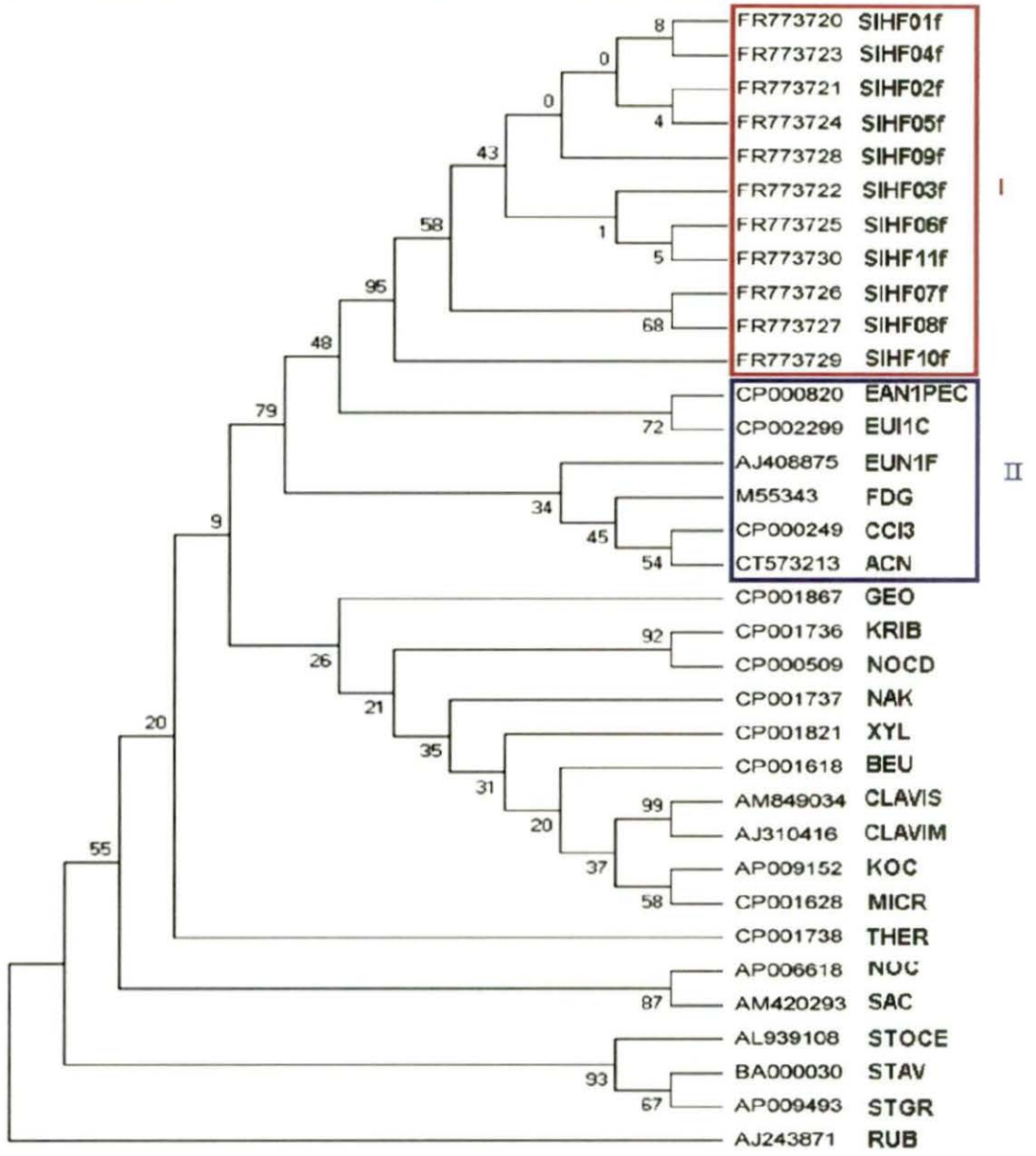

Figure 4.14: Most parsimonious tree (neighbour joining) showing the relationship of 16S rRNA of *Hippophae salicifolia-Frankia* isolates FR773720-FR773730 with other GenBank-published sequences. The red box indicates *Hippophae salicifolia-Frankia* isolates and the blue box indicated other *Frankia* isolates. Numbers at nodes indicate the bootstrap percentage scores out of 1000 replicates

measure of genetic distance (Nei, 1972) averaged 0.36 and varied from 0, lowest between *Frankia* strains HISF03 & HISF02, HISF05 & HISF02, HISF05 & HISF03, HISF06 & HISF02, HISF06 & HISF03 and HISF06 & HISF05 to maximum genetic distance of 0.6931 between HISF04 and HISF01 strains (table 4.25).

The dendrogram constructed on the basis of Nei's genetic distance utilizing the UPGMA method (fig. 4.16) showed two main clusters. These

Table 4.22: Diversity analysis of the partial 16S rRNA sequences of the eleven different *Hippophae salicifolia* root nodule DNA samples of the present study area along with their GenBank accession numbers

Isolates (Location)	Sample code No	Length of the sequence (nt)	Maximum similarity (%) with other sequences in GenBank	Accession No.
Zema III (Q1*)	SIHF01	770	96% with Uncultured Frankia sp. isolate H1S2Hp 16S ribosomal RNA	FR773720
Chaten (Q16)	SIHF02	687	96% with Uncultured Frankia sp. isolate H1S2Hp 16S ribosomal RNA gene, partial (DQ988981.1	FR773721
Chako River (Q14)	SIHF03	702	96% with Uncultured Frankia sp. isolate H1S2Hp 16S ribosomal RNA gene, partial (DQ988981.1	FR773722
Dozam (Q6)	SIHF04	723	95% with Uncultured Frankia sp. isolate H1S2Hp 16S ribosomal RNA gene, partial (DQ988981.1	FR773723
Below Lachen (Q19)	SIHF05	732	95% with Uncultured Frankia sp. isolate H1S2Hp 16S ribosomal RNA gene, partial (DQ988981.1	FR773724
Zema II B (Q4)	SIHF06	798	96% with Uncultured Frankia sp. isolate H1S2Hp 16S ribosomal RNA gene, partial (DQ988981.1	FR773725
Puchi (Q13)	SIHF07	539	99% with Uncultured Frankia sp. isolate H1S2Hp 16S ribosomal RNA gene, partial (DQ988981.1	FR773726
Zema II D (Q8)	SIHF08	780	99% with Uncultured Frankia sp. isolate H1S2Hp 16S ribosomal RNA gene, partial (DQ988981.1	FR773727
Below Lachen (Q 18)	SIHF09	730	95% with Uncultured Frankia sp. isolate H1S2Hp 16S ribosomal RNA gene, partial (DQ988981.1	FR773728
Zema I+ (Q10)	SIHF10	797	95% with Uncultured Frankia sp. isolate H1S2Hp 16S ribosomal RNA gene, partial (DQ988981.1	FR773729
Chako River (Q20)	SIHF11	640	96% with Uncultured Frankia sp. isolate H1S2Hp 16S ribosomal RNA	FR773730

*Q represents quadrates of table 4.5.

clusters revealed the relatedness between the 11 *H. salicifolia-Frankia* studied. Cluster I consists of 7 isolates, which is the larger cluster compared to the other one, which consists of 4 isolates. Cluster I can be divided into two main groups: the first one comprising HISF02, HISF03, HISF05 and HISF06 with genetic proximity or similarity measures of 0.0000. Similarly the second group comprises HISF04 and HISF09 with genetic proximity of 2.85792. Cluster II comprises HISF07, HISF11, HISF08 and HISF10 with their genetic proximity given in fig. 4.16.

Careful observation of the PCR-RFLP dendrogram and its correlation with the table 4.5 (for quadrates, geographical conditions and the altitude from where

the samples were collected) and table 4.22, it is clearly understood that the *Frankia* isolates belonging to similar or near to similar altitudes have formed a close proximity. The altitude of group one of cluster I ranges between 8299-8814ft. Similarly the altitude of cluster II isolates ranges from 8937-9960ft. Similarly in the dendrogram it can also be seen that HISF01 showed a genetic proximity of 24.87490 and is placed far away from other isolates of close proximity. This sample was collected at an altitude of 10206ft. Except for the altitudinal differences in the second group of cluster I [HISF04 (9960ft) and HISF09 (8450ft)], almost all have shown close proximity based on close altitudinal ranges. The second group of cluster I indicates that apart from altitude, some other factor may also affect the genetic diversity of *Frankia*. PCR based 16S rDNA-RFLP studies have been used in eco-biotechnological

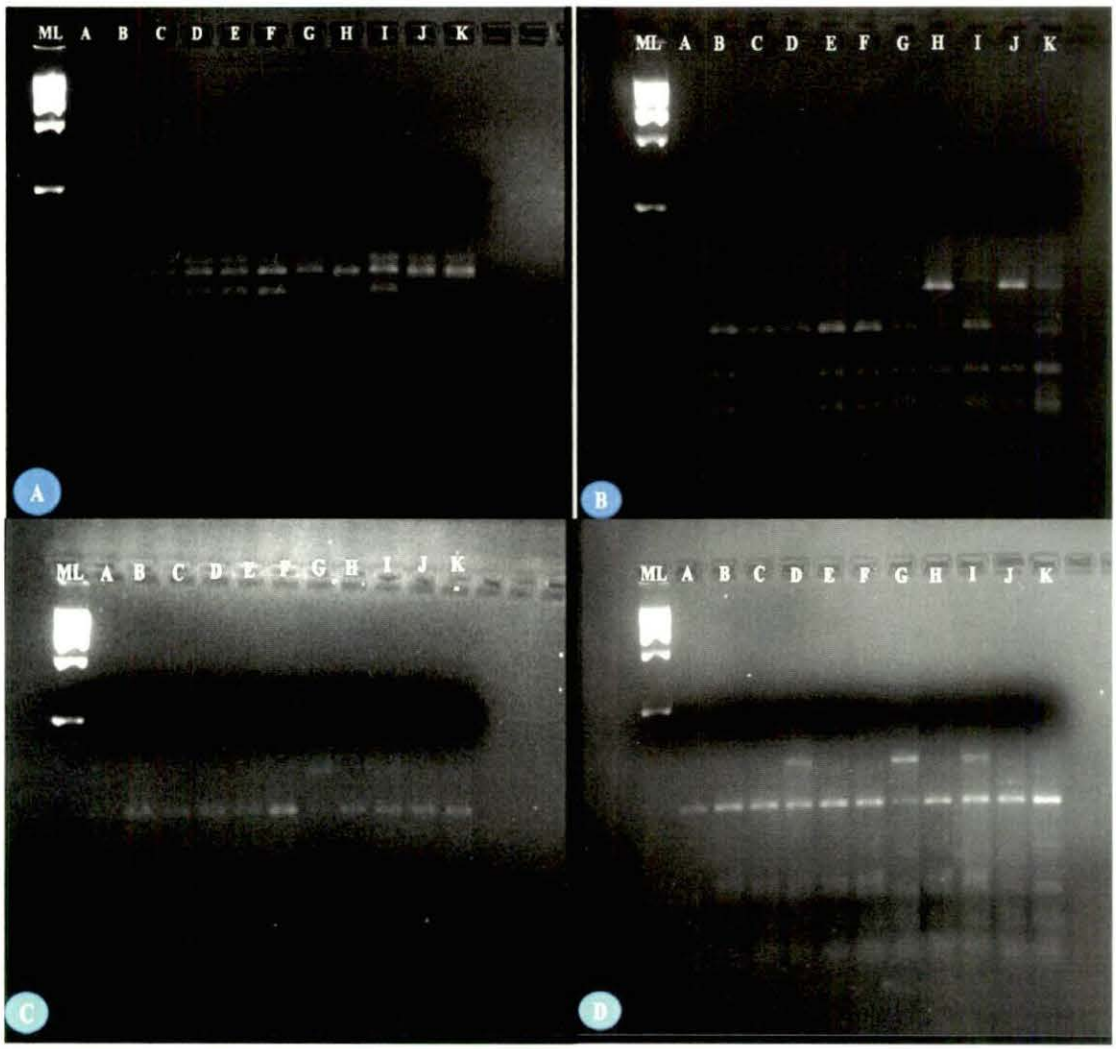

Figure 4.15: Restriction digestion gels of PCR amplified 16S rRNA *Frankia* DNA of *H. salicifolia* using different enzymes. Lane ML=1kb molecular ladder (λDNA), Lane A-K=SIHF01-SIHF11 (11 isolates). A=Restriction digestion using *Alu*I, B=Restriction digestion using *Hae*III, C=Restriction digestion using *Msp*I, and D=Restriction digestion using *Hpa*II

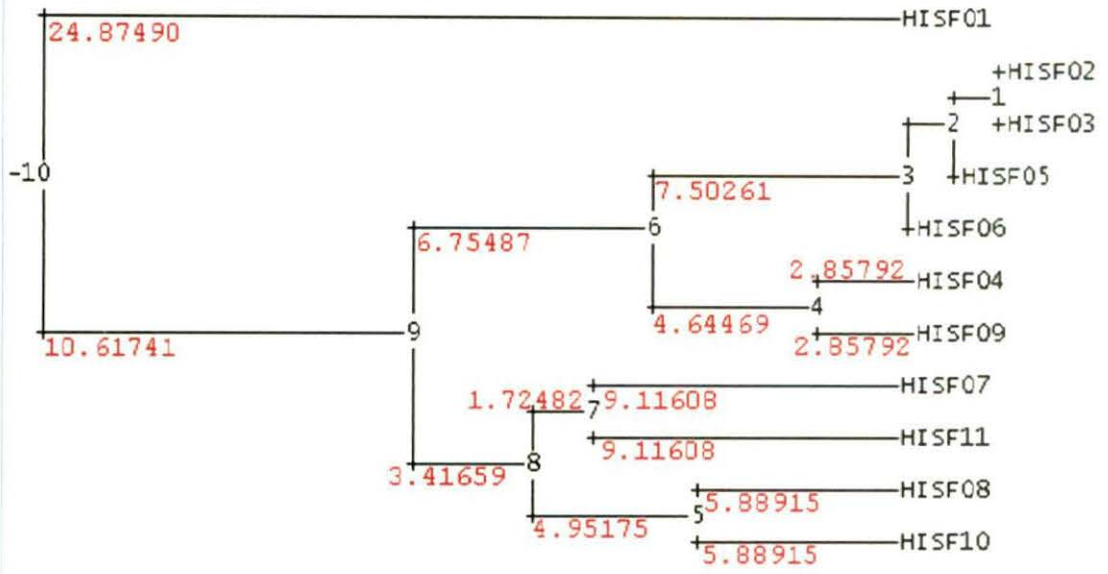

Figure 4.16: Dendrogram based on Nei's (1972) Genetic distance: Method=UPGMA modified from NEIGHBOR procedure of PHYLIP Version 3.5

studies as a procedure for studying the diversity of strains isolated from nature (Schraft *et al.*, 1996; Urakawa *et al.*, 1997; Vallaeys *et al.*, 1997).

In this study it has been found that, apart from various other factors discussed above and in the foregoing chapters, environmental conditions (elevation, aspect, slope), soil composition, geographic and microclimatic conditions of Lachen Valley of North Sikkim have been found to play a major role genetic diversity of *Frankia* species found in the root nodules of *H. salicifolia*.

2004; Elith *et al.*, 2006). The use of such kind of database in environmental niche modelling (Soberon and Peterson, 2005) with relation to the species is proving to be a powerful means in understanding how abiotic factors like temperature, precipitation, seasonality (Graham *et al.*, 2004; Wiens and Graham, 2005), aspect, elevation, slope, etc. influences the geographic limits of lineages and species. To address the idea I have made an approach to use remote sensing satellite FCC data along with Survey of India maps (both 1:50000 scale) in generating an environmental map (land use & land cover, slope, elevation and aspect) to study the environmental factors of *H. salicifolia* growing area. Land use and land cover data proves especially valuable for predicting the distribution of both individual species (Jennings, 2000) and assembly of different species (Kerr *et al.*, 2001) across broad areas that could not otherwise be surveyed because of

the tough and some inaccessible terrains of the study area.

Variations in environmental factors like nutrient concentration, nutrient availability, temperature, pH, etc. have been found to affect the kinetics of soil microbes directly. The environmental factors act as a gene controlling enzyme system (McArthur *et al.*, 1988). Therefore, there must be a relationship between genetic diversity of *Frankia* populations with their

Table 4.23: Sequences used in phylogenetic analysis with their sequence abbreviations, description and GenBank accession numbers

Sequence code (GenBank)	Description	GenBank ac. No.
SIHF01f	Uncultured Frankia sp. partial 16S rRNA gene from Hippophae salicifolia root nodule isolate	FR773720
SIHF06f	-do-	FR773725
SIHF04f	-do-	FR773723
SIHF03f	-do-	FR773722
SIHF02f	-do-	FR773721
SIHF09f	-do-	FR773728
SIHF05f	-do-	FR773724
SIHF11f	-do-	FR773730
SIHF07f	-do-	FR773726
SIHF08f	-do-	FR773727
SIHF10f	-do-	FR773729
EAN1PEC	Frankia sp., complete genome	CP000820
EUI1C	Frankia sp., complete genome	CP002299
EUN1F	Frankia sp. partial 16S rRNA gene, Sn5-8 isolate	AJ408875
FDG	Frankia sp. 5S, 16S, and 23S ribosomal RNA genes, complete sequences.	M55343
CCI3	Frankia sp. Complete genome.	CP000249
ACN	Frankia alni str. ACN14A chromosome, complete sequence.	CT573213
NOC	Nocardia farcinica IFM 10152 DNA, complete genome.	AP006618
SAC	Saccharopolyspora erythraea NRRL2338 complete genome	AM420293
THER	Thermomonospora curvataDSM 43183, complete genome.	CP001738
XYL	Xylanimonas cellulosilyticaDSM 15894, complete genome.	CP001821
GEO	Geodermatophilus obscurusDSM 43160, complete genome.	CP001867
KRIB	Kribbella flavida DSM 17836, complete genome.	CP001736
NOCD	Nocardioidessp.JS614, complete genome	CP000509
NAK	Nakamurella multipartite DSM 44233, complete genome.	CP001737
KOC	KocuriarhizophilaDC2201 DNA, complete genome.	AP009152
MICR	Micrococcus luteusNCTC 2665, complete genome.	CP001628
BEU	Beutenbergia cavernae DSM 12333, complete genome.	CP001618
CLAVIS	Clavibacter michiganensis subsp. Sepedonicus, complete genome.	AM849034
CLAVIM	Clavibacter michiganensis, partial 16S rRNA gene, strain P 250/01.	AJ310416
STCOE	Streptomyces coelicolo rA3(2) complete genome; segment 5/29.	AL939108
STAV	Streptomyces avermitilisMA-4680 DNA, complete genome	BA000030
STGR	Streptomyces griseus subsp. Griseus NBRC 13350 DNA, complete genome.	AP009493
RUB	Rubrobacter xylanophilus partial 16S rRNA gene.	AJ243871

Table 4.24: Genetic polymorphism of *Frankia* detected by RFLP marker by various enzymes

Enzymes used	Bands generated	Polymorphic bands	%age of polymorphism
AluI	3	2	66.6
HaeIII	6	3	50
HpaII	7	5	71.42
MspI	2	1	50
Total	**18**	**11**	**61.11**

Table 4.25: Nei's measure of genetic distance (RFLP) for pop ID codes (SIHF01-SIHF11)

Pop ID	SIHF01	SIHF02	SIHF03	SIHF04	SIHF05	SIHF06	SIHF07	SIHF08	SIHF09	SIHF10	SIHF11
SIHF01	0										
SIHF02	0.4925	0									
SIHF03	0.4925	0	0								
SIHF04	0.6931	0.1178	0.1178	0							
SIHF05	0.4925	0	0	0.1178	0						
SIHF06	0.4925	0	0	0.1178	0	0					
SIHF07	0.5878	0.3254	0.3254	0.1823	0.3254	0.3254	0				
SIHF08	0.4055	0.1823	0.1823	0.3254	0.1823	0.1823	0.2513	0			
SIHF09	0.5878	0.1823	0.1823	0.0572	0.1823	0.1823	0.1178	0.2513	0		
SIHF10	0.4055	0.3254	0.3254	0.4952	0.3254	0.3254	0.2513	0.1178	0.4055	0	
SIHF11	0.3254	0.2513	0.2513	0.4055	0.2513	0.2513	0.1823	0.1823	0.3254	0.1823	0

environmental conditions. Studies have also been conducted on microbial populations to show close relation between the genetic diversity and environmental changes of a particular ecosystem (Nevo *et al.*, 1984) and some evidences have proved positive relation between genetic polymorphism and environmental heterogenecity (Hedrick *et al.*, 1976). The *Frankia* population diversity has been shown to be affected by ecological factors determined by elevation, like temperature, precipitation, etc. and soil composition parameters like pH, OC, moisture, type, etc. (Chen *et al.*, 2008).

As has already been discussed in previous chapters, *H. salicifolia* are good sources of dietary supplement, natural antioxidants including carotenoids, vitamins, phenols, flavonoids, and a high level of antioxidative property against free radical species. The total antioxidant activity can vary from plants growing in different environmental conditions, and these may affect overall protective benefits of human health, which needs

to be further investigated. Antioxidant study from the leaf and bark of male and female plants of *H. salicifolia* is the first report from Sikkim Himalayas but there may not be any report on antioxidant (phenolics, flavonoids, etc.) of the host plant affecting genetic diversity of *Frankia* in *Hippophae.*

It has been understood that during biotic interactions, plant secondary metabolites and phenolics give positive signals, during plant-environment interactions. One such interaction is during the early stage of nodule formation and establishment of symbiotic relationship, phenolic based reorganisation mechanism between the host and bacterial symbiont is involved (Gould and Lister, 2006). These signal interaction mechanism is well studied in mycorrhiza (Steinkellner *et al.*, 2007) and legumes (Cooper, 2004) but very little is known about the role of phenolics in the symbiosis between actinorhizal plants and *Frankia,* and no attempts have even been made to study this type of signal interactions in *Hippophae salicifolia.* There has been a report on the responsiveness of *Frankia* strains to *Myrica gale* fruit exudates phenolics with their symbiotic compatibility by Popovici *et al.* (2010). However, my study apart from providing the antioxidative property of *H. salicifolia* of Sikkim has opened up a new scope for using these compounds to correlate the symbiotic interaction of host-microorganism and genetic diversity of *Frankia* to get a better cultivar of this ecologically and economically important plant species.

Quantification of root nodules from *H. salicifolia* plants growing in different locations of the study area can be attributed to assessing the population size of *Frankia* in the study area. Jeong and Myrold (2003) have also stated that counting of nodule from different actinorhizal plants and can be the indirect means of measurement of population size of host infective *Frankia* population in the soil.

Vegetative propagation of *H. salicifolia* using different means like cuttings using different combinations and proportions of growth regulators and layering in different soil combinations was studied. Further propagation through seeds in combination with different chemicals to study breaking of dormancy was carried out in this study. The species showed its survival and adaptability in a different condition from its natural habitat. The outcome of this study may not be directly involved in the study of

genetic diversity but the outcome of this can be useful in planning, getting desired selected plant accession and faster propagation compared to natural condition. The root nodules out of these propagated plants could have given an interesting result, if *Frankia* could have been isolated from them in pure culture. Insufficient nodules per plant for genomic DNA isolation hindered study of genetic diversity due to environmental and time factor to complete this research work, which will be taken up in future.

During the study of genetic diversity of *Frankia* associated with *Hippophae* of Lachen, I took up the environmental parameters of the study area, which can also reflect the plant-environment relationship on the genetic diversity of *Frankia* through the host. Intense studies have revealed that host plant controls the root nodulation and nitrogen fixation processes in actinorhizal plants (Dobritsa and Novik, 1992; Wall and Huss-Danell, 1997; Valverde and Wall, 1999). Environmental factors like light, water, soil nutrients availability, soil pH, etc. and bacterial factors such as physiologic state, population and ability to fix nitrogen are also thought to effect nodule development, growth and function in actinorhizal plants (Huss-Danell 1997). In my study also it was revealed that the roots of *H. salicifolia* growing in the riverine areas possessed more nodulations compared to non riverine areas. However, the difference in diversity observed could be understood by the characteristics of the niches occupied by the plant, which in turn controls its microsymbiont in the root. Therefore we can conclude that environmental factors control the genetic diversity of *Frankia* in the root nodules of the actinorhizal plant and *H. salicifolia* cannot be an exception.

Earnest beginning in the study of microbial aspect of *Frankia* species started only 15 years ago when first strain was announced. It was also found that *Frankia* were slow growing organisms and much of intervening time has been spent on establishment of different techniques of growing *Frankia* and pursuing hypothesis related to the nature of organism, its vesicle, spores and physiology evolved from the past studies. If we see from the global perspective, fair amount is known about *Frankia* strains on phylogenetic position, structure and function of spores and vesicles, physiology of carbon and nitrogen metabolism, ecology in the soil and

nodule. Recently some details have started pouring in about the genetics of this unique microorganism. In my study, I also isolated *Frankia* strains of *H. salicifolia* in pure culture, which may also be the first isolation from the Hills of Sikkim. The same has been reflected in the results and discussion chapter (chapter IV). This has led to the understanding of the structure of this isolated strain and also helped in conducting plant infectivity test. The amount of *Frankia* colonies could not suffice the isolation of genomic DNA from pure culture, hence genomic DNA for its genetic diversity was isolated from the root nodules directly, as has been done in many cases of such studies in various actinorhizal plants.

During the infectivity test *in vitro* and *in situ* the *Hippophae* plants showed positive results by getting infected with the *Frankia* inoculums. This has not only showed that *Hippophae-Frankia* strains are spore+ but has also opened up an area to carry out more work to study communication mechanism between the actinomycetes and the host actinorhizal plants in the rhizosphere.

Phylogenetic comparisons of *Frankia* and its close lineages are promising approach to understand the evolution of the organisms. Out of 21 actinorhizal genera described (Benson and Silvester, 1993), 11 genera fulfilling Koch's postulate could have their 16S rDNA sequence determined and compared for its diversity study (Normand *et al.*, 1996).

For the presence or absence of actinorhizal plants, climatic and soil conditions are the factor that always influences *Frankia* (Dawson *et al.*, 1989; Huguet *et al.*, 2004; Oakley *et al.*, 2004). This study focuses on single *Frankia*-nodulating species growing in variable microclimatic region at different elevations, aspects and slopes of Lachen valley. Total of 11 DNA isolates gave positive results when subjected to PCR. Study of genetic diversity using *H. salicifolia-Frankia* genome and comparing it with other genome sequences having 16S rRNA, and also PCR-RFLP study, provided some interesting facts related to genetic diversity of *Frankia* strains of *H. salicifolia* occupying this niche with physically untraceable climatic conditions. It is understood that distribution of this *Frankia* strain is also basically affected by altitudes or the host plant growing habitat. Reports by Dai *et al.* (2004); Igual *et al.* (2006); Jeong and Myrold (1999); Oakley *et al.* (2004) and few others supports this

findings that topography and altitude plays a vital role in composition of *Frankia* community. Soil in the study area was almost the same throughout, so not much difference was observed due to soil conditions or their compositions. Although some authors like Burleigh and Dawson (1994); Crannell *et al.* (1994) have co-related that soil pH, organic matters, etc. may influence *Frankia* genotypes but from various studies and results expressed in chapter IV, especially with reference to *Frankia,* even soil pH or organic matters, had no major role to play in *Frankia* population and distribution in Lachen valley. Some ecological factors like altitude, aspect, slope and microclimatic conditions like temperature and moisture in the *H. salicifolia* growing area have shown the genetic diversity among its *Frankia* strains, perhaps through host or direct impact, and also may be the major determinants of *Frankia* population in the study area. Similar work based on *Frankia* population diversity on *H. rhamnoides* taking few ecological parameters focussing on elevation and plant cover by Chen *et al.* (2008) supports my findings.

With the advent of many innovative and informative researches throughout the world on evolution and genetic diversity of *Frankia* (in the soil or in different actinorhizal hosts or in different ecological conditions involving different ecological and environmental parameters), many opportunities to study this organism are coming up. It is felt that there are still more potential working areas in the days to come, which include studies on genetics and molecular genetics of the host plant infection process, symbiotic interaction, the phylogeny and taxonomy of those strains that have yet to yield infective strains and physiological studies in relation to symbiosis. Above all, there is a great need to understand integrated phylogenetic and taxonomic studies of *Frankia* strains to understand its actual phylogeny and taxonomy, which will lead to universal identity of this microorganism. Presently, out of many phylogenetic studies carried out by researchers globally, the outcome is based either on a particular host, region or environmental factor. Very few comparisons have been found, which is not sufficient to present the actual status of *Frankia* strains on a global basis. The work is difficult and time consuming too, but such genetic studies would help to lay a base with a

unifying hypothesis to answer questions like why actinorhizal nodules form on certain distantly related plants but not on other plants of the same family. This may share co-evolution theory of both the symbionts and their diversity.

Broadly, actinorhizal plants play an important role in different environmental conditions. *Hippophae salicifolia* growing in the Lachen valley of Sikkim too has been found to occupy an important and influential position in the existing ecosystem, especially in the context of climatic changes, which are proposed to occur in the next few decades. Since these species can survive and ameliorate the effect of vegetation losses because of nutritional deficiency in the soil, they can be a pioneering species to keep the vegetation integrity of the area but its practical issue of actinorhizal symbiosis needs to be addressed along with genetic diversity, which will not only explain its phylogeny and evolution but shall also help in understanding better variant of *Frankia* strain for future planning of better eco-friendly cultivar.