Chapter 2
Review of Literature

2.1.0 HISTORY OF *HIPPOPHAE* L. (SEA BUCKTHORN) RESEARCH

Hippophae L. commonly known as sea buckthorn has been in the use of mankind since ages. Tibetan medical 'Tantras' called *Sibuyidian* mentions that the Tibetan medical classic *rGyud-bzhi* (Four books of pharmacopoeia), written by Yu Tuo Yuan Dan Kong Bu that was completed during the Chinese Tang Dynasty (618 to 907 A.D.), contains 84 different set of prescription prepared from sea buckthorn (Rongsen, 1992). The original version of *rGyud-bzhi* is thought to have been written in Sanskrit around the 4th Century A.D., later translated into Tibetan language and submitted to the royal court (Tsarong *et al.,* 1981). History of sea buckthorn, botanically, can also be traced back to the age of Vedas. Its utilities were also mentioned in ancient Greek philosophy such as Dioscorides and Theophrastus. In ancient Greece, sea buckthorn was used as a dietary supplement for horses. Feeding on leaves and young branches of sea buckthorn resulted in rapid weight gain and a shiny coat in horse (Rongsen, 1990).

Sea buckthorn was ethnically used by the Mongolians from 13th Century onwards. In 'A Selection of Mongolian Medicine', a 120 chapter book written by Losan Quepie, during the Quing Dynasty (1821-1850), mentioned 13 chapters were solely devoted to sea buckthorn and its clinical effects (Rongsen, 1990).

Siberians started cultivating sea buckthorn (*H. mongolica)* in 1930s (Kalinina and Panteleyeva, 1987). Apart from developing new varieties of the plants, Russians' also developed various medicinal preparations and health products for the astronauts of Sputnik satellite. Since then the genera was soon introduced to other parts of Russia (former Soviet Union), and neighboring countries (Trajkovski and Jeppsson, 1999).

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plant breeding, Sweden perhaps is the world's most diverse gene bank for *Hippophae*, which consists almost all the wild, cultivars and selections from different breeding programme available in the world (Bartish *et al.*, 2000).

Chinese and Russian researchers engaged in sea buckthorn research have made considerable contributions. The success story in sea buckthorn research and development programme from Tibet, Mongolia and Russia, etc. encouraged many Asian countries like Nepal, Bhutan, India and Pakistan to start their own sea buckthorn development programmes.

In India, research on sea buckthorn is being carried out in states like Himachal Pradesh, Jammu and Kashmir (Lahul, Spiti, and Ladhak) and Sikkim (Singh 1998; Basistha and Adhikari, 2003).

2.1.1 Legends associated with sea buckthorn

Genghis Khan, the Mongol conqueror, in the12th century ordered his armies to eat sea buckthorn berries, to improve stamina and prevent altitude sickness.

The Tibetan doctors first understood the value of sea buckthorn and used it in the 8th century. The Tibetans and the Mongolians mastered the economic importance of this genus for centuries.

It is believed that the deserted war horses of the Greek army, in the 12 B.C., survived and became even stronger and shiny after wandering for long time in sea buckthorn forests. It is also believed that sea buckthorn was used as a diet for race horses by the Greeks.

In another legend, sea buckthorn leaves were preferred diet of a Pegasus, the winged horse.

In some ancient kingdom, execution of convicts by dropping them in a boiling barrel of sea buckthorn oil, gave chance for the convicts to survive.

Cosmonauts of Mir spaceship of former USSR used sea buckthorn creams to protect themselves from cosmic radiations and as a supplement of oxygen.

2.2.0 ANTIOXIDANT PROPERTIES AND CHEMISTRY

In the process of economic development, with the increase in income, human society tends to care more about their health. Therefore, demand for healthy herbal organic foods developed from various plants has also increased. Production of more efficient and productive food items by

the researchers are on demand. One such plant with multiple qualities is *Hippophae* L. (sea buckthorn).

Hippophae L. has been used as food, beverages and medicine by the humans since decades, especially in Russia and Tibet (Yang and Kallio, 2001) Sea buckthorn has attracted attention world over due to its nutritional and medicinal values (Xu *et al.*, 1994; Beveridge *et al.,* 1999). The diversity in biochemical and nutritional expressions shown by this plant are due to its distribution, origin, climate and methods of extraction (Wang, 1990; Zeb and Malook, 2009).

Antioxidants can react with free radicals during the oxidation process by acting as a reactive species scavenger and liberating catalysts, so antioxidants can be used to reduce the oxidative process (Gulcin *et al.*, 2005). Bioactive compounds like ascorbic acid, carotenoids, tocochromanols and phenols are antioxidants. Sea buckthorn, apart from being a repository of vitamins, minerals and essential bioactive substances (Xurong *et al.,* 2001), also has potential antioxidant activity (Velioglu *et al.*, 1998; Halvorsen *et al.*, 2002; Nilsson *et al.*, 2005) attributed to its flavonoids, oils, vitamin C (Li and Schroeder, 1996) and some essential fatty acids with strong antioxidant activities (Yan and Liu, 2000). These compounds have the ability to inhibit cancer, atherosclerosis (a common form of arteriosclerosis in which fatty substances form a deposit of plaque on the inner lining of arterial walls) (Visonneau, 1997) built up immune system and decrease obesity (Letchamo and Lobatcheva, 1997; Houseknecht, 1998) treating pulmonary, hepatic, gastrointestinal and articulate diseases (Olziykhutag, 1969). Oils extracted from the seeds can be successfully used for treating various kinds of irradiation, mucosa, ulcers and burns. It can also be used against stomach cancer at its initial stage (Yang *et al.*, 1999; Yang and Kallio, 2003; Rongsen, 1992). It is estimated that more than 100 kinds of bioactive and antioxidant elements have been identified in leaves, fruits, bark and seeds (Rongsen, 1992; Rongsen, 1993; Singh and Awasthi, 1995; Singh, 1998; Singh *et al.*, 2001) and thus, has high values in development of medicine, herbal and health care products (Lu and Ma, 2001). The polyphenols in sea buckthorn fruit have antioxidant properties and thus can be used for repairing damages caused by free

radicals (Yao and Tigerstedt, 1992; Yang and Kallio, 2001)

The leaves possess anti-inflammatory properties (Ganju *et al.,* 2005; Padwad *et al.,* 2006). Leaf extract is also used in ointments for treating burns, skin cracks, scabies, impetigo, keratosis and cures xeroderma (Rongsen, 1992). The young leaves contain high nutrient, carotenes and flavonoids. Generally, vitamin C in leaves is higher than the fruit. It is also used as one of the most important raw materials for the extraction of vitamins and flavonoids. Sea buckthorn leaves also controls the growth of cancer cells in liver (Zhao *et al.*, 1999).

Fruits of sea buckthorn are rich source of vitamin C (300-1600 mg/100gm), which is 04-100 times more than any vegetable fruit, high amount of organic acid (2-4%), especially mallic acid which is higher than *Citrus* sp. (1-2%) (Rongsen, 1992). Fatty acids, organic acid, tannic acid (Singh, 1998), Palmitic acid (about 34%), oleic acid (about 32%) and palmitoleic acid (about 26%) are the main constituents of pulp oil. Higher quantities of unsaturated fatty acids (around 86%) are found in seed oils. Seeds also contain vitamin C, large amount of carotenoids, vitamin E, flavonoids, kaempferol, fatty acids, triacylglycerol, phytosterols, sugar, organic acids, proanthocyanidins and phenolic compounds (Abid *et al.*, 2007; Fan *et al.*, 2007; Li *et al.*, 2007). Fruits contain globulins and albumin proteins, carotene, saturated and unsaturated fatty acids, free amino acids, flavonoids and vitamin E (Rongsen, 1993; Singh, 1998).

Many works on nutritional attributes of sea buckthorn has been carried out but review of literature did not provide any substantiation on antioxidant and nutritional properties of *Hippophae salicifolia* D. Don. There are few reports on antioxidants of leaves (Geetha *et al.,* 2003), seeds (Negi *et al.,* 2005) and fruits (Eccleston *et al.,* 2002) of *H. rhamnoides*. Research on detailed chemistry and antioxidant property of *H. salicifolia* is perhaps, yet to be done.

2.3.0 AGROTECHNIQUES OF SEABUCKTHORN

Hippophae L. (sea buckthorn), due to its beauty, was introduced as an ornamental plant in botanical gardens of Europe. Though sea buckthorn was well known to the human society since ages, plantation works through different means started only few decades ago. Its hydrophilous character

(Ansari, 2003) helps the plant to grow at places with 400-600 mm annual rainfall (Rongsen, 1992; Ansari, 2003; Dhakal, 2001). Besides its economic and ecological qualities, sea buckthorn is also regarded as the *"slave of degraded land"* (Constandache and Dinca, 2009).

Realizing the importance of sea buckthorn in different aspect of human life, including commercial interests, different agrotechniques and propagation techniques on this wonder plant has been carried throughout the world in recent years. *H. salicifolia* of Sikkim Himalayas is untouched of major researches till date apart from its illustration in the seven volumes of "Flora of British India" by Sir J. D. Hooker (1872-1897). Agrotechniques of sea buckthorn through different methods like cuttings, seed propagation (Auauzato and Megharini, 1986), layering, etc. have been carried out in the past, but the response of plants in different environmental conditions have been found to be different.

Naturally, propagation of sea buckthorn through seeds is the main way for its occupation into new habitat (Lisenkov *et al.,* 1969). This genus is also thermophilic, so the seeds can germinate at a temperature between 24°C-26°C (Ansari, 2003) with viability up to 3 years (Rajchal, 2009).

Various techniques like scarification, hot water and rooting hormone treatments have been employed for propagation of sea buckthorn seeds but research on use of growth regulators on germination response could not be reviewed in literature for seed propagation. Though seeds may have higher emergence rate (Rajchal, 2009), now a days, seed propagation is basically used for breeding and introduction works, forest land reclamation, protective and ornamental purposes and sometimes for root stocks (Rongsen, 1992).

Despite of significant biological variations in sea buckthorn seeds from varying ecological and geographical areas, each population have individuals with extended period of seeds germination (Bobodjanov and Kabulova, 2005). Many economic yields may be lost in the plant fruits grown from seeds due to higher level of heterozygosis. Therefore, cultivation of sea buckthorn for economic uses needs propagation techniques where genetic uniformity of the seedlings is ensured, keeping its integrity and similarity with the selected mother plant.

H. rhamnoides L. is propagated both sexually and by vegetative means. Through vegetative propagation, plants can be propagated on hill slopes and rivers banks (Rongsen, 1990) with proper spacing (1m within the row and 4m between rows) depending on terrain and male: female ratio between 1:6 and 1:8), adequate manure and water supply (Li and Schroeder, 1999). Propagation establishment of *H. rhamnoides*, through hard and soft wood cuttings, seed propagation (with or without treatment of different growth regulators) (Auauzato and Megharini, 1986; Ansari, 2003; Basistha and Adhikari, 2003; Basistha *et al.,* 2009b; Rongsen, 1992; Singh 1998) is well suited in well drained, deep, sandy loam soil with sufficient organic matters (Li and Schroeder 1999; Li and Oliver, 2001).Propagation through tissue culture (Liu *et. al.,* 2007; Vescan *et al.,* 2009) and layering (Basistha *et al.,* 2009b) has also been adopted in this genus.

Sea buckthorn can grow well in pH 6-7. For general growth and fruit production in future, soil acidity and alkalinity (except at extreme levels), may not be a limiting factor, but requires adequate soil nutrients with phosphorus for better fruit qualities and high yield (Li, 2002). Use of Nitrogen fertilizer to the plants inoculated with *Frankia,* not only delays nodulation but also has adverse effect in nodulation (Akkermans *et al.,* 1983; Montpetit and Lalonde, 1988; Bosco *et al.,* 1992). This was not the same with other actinorhizal plants. When studied in culture solutions, nodulation reduced at lower pH in some actinorhizal plants including *Alnus glutinosa.* High nodulation rate has been observed in soil pH between 5.5 and 7.2 In soil pH of 4.5, the viability of endophyte also decreased (Griffiths and. McCormick, 1984).

H. salicifolia, despite of having large population in Sikkim Himalayas, systematic and scientific studies are still immature for exploiting its true potential. It remains underutilized due to lack of further research and development.

2.4.0 DISTRIBUTION OF ACTINORHIZAL PLANTS

It was well established that genus *Frankia* was an actinomycetes and the plants bearing such associations with *Frankia* were called "non-leguminous plants" until the term 'actinorhizal' was coined in the First International Conference held at Harvard Forests in Petersham, Massachusetts, (USA) in

April 1978, replacing the term "non leguminous" (Tjepkema and Torrey, 1979; Newcomb and Wood, 1987), and published in the proceeding titled *Botanical Gazette*: 140 (suppl.) in 1979 (Huss-Danell, 1997). Researches have also led to the understanding that symbiotic relationship between the filamentous gram +ve bacteria of the order actinomycetales–*Frankia* and fine roots of certain angiosperms form the actinorhizal associations and the host plants involved in these kind of associations are known as Actinorhizal plants (Tjepkema and Torrey, 1979; Huss-Danell, 1997).

Frankia, unlike *Rhizobium*, are filamentous, branched, gram +ve actinomycetes, which have symbiotic association with large number of woody dicotyledons, whereas *Rhizobium* is a unicellular gram –ve bacteria that has symbiotic association with only legume family plants excepting *Parasponia* of Ulmaceae family (Obertello *et al.,* 2003).

Actinorhizal plants form a key component in natural ecosystem, agro-ecosystem and agro forestry by substantiating fixed nitrogen (1-150 kg N/ha/y) to these systems (Torrey, 1978; Dawson, 1983; Russo, 2005). Actinorhizal plants are the source of nitrogen rich organic matter mainly in the forest soil and play primary role in the dynamics and biodiversity of terrestrial ecosystems.

According to Baker and Schwintzer (1990), 288 species with 24 genera belonging to 8 families of actinorhizal plants have been reported so far. Similarly, Benson and Silvester (1993) have reported actinorhizal plants having 194 species with 24 genera, but without details. However some reviews state that there are more than 200 species of actinorhizal plants, which includes 25 angiosperm genera belonging to 8 families distributed among 4 orders *viz.* Fagales, Cucurbitales, Fabales and Rosales (Soltis *et al.,* 1995; Wall, 2000; Schwencke and Caru, 2001). Of the 25 genera of actinorhizal plants reflected in some literatures, the 25th genera might have been referred to *Rubus*, which as per Bond (1976) and Becking (1984) is an actinorhizal genus. But Stowers (1985) did not agree *Rubus* to be a truly actinorhizal plant. Even Jeong and Myrold (2003) have reported that actinorhizal plants consist of 24 genera, which is agreed by the majority of researchers in this field (Table 2.1).

Actinorhizal plants are woody dicots

except *Datisca* sp., which is herbaceous plant (Franche *et al.*, 1998) and are found throughout the world accept Antarctica (Table 2.1). *Alnus* and *Elaeagnus* species are found in the hilly region of the tropics (Myrold, 1994). These plants are pioneering species of the temperate regions (excepting *Casuarina* and *Myrica*), which prefer to grow in open areas having sun facing aspects during their early succession. Due to their ability to utilise atmospheric N_2 through *Frankia,* they show positive growth in sandy and swampy soils with marginal nitrogen level and range of environmental stress (Dawson, 1990; Tredici, 1995) (may also refer to Table 2.1).

2.5.0 TAXONOMY AND EVOLUTION OF ACTINORHIZAL PLANTS

It is understood by now that actinorhizal plants cover a wide range of dicotyledonous plants, with fewer lineages in their taxonomic relations. They have both ancient and advanced characters (Bousquet and Lalonde, 1990; Sen, 1996). Actinorhizal plants are included in different families of which, all the genera of a particular family may not be actinorhizal, except in Elaegnaceae where all plants are actinorhizal (Benson *et al.*, 2004).

Different authors have tried to classify actinorhizal plants with some differences. The classification put forward by Cronquist (1988) and reported by Benson *et al.* (2004), reveals some interesting features (Table. 2.2). In Cronquist's classification, actinorhizal plants are placed under Magnoliopsida, where Rosales are not ascribed with the sub class Hamamelidales. Elaegnaceae and Rhamnaceae have been placed in different order other than Rosales, Datiscaceae was assigned to Violales (Dilleniidae) and Hamamelidales, Casuarinales, Fagales and Myricales were combined together. In contrast to the above, Benson and his co-workers presented classification of actinorhizal plants in a modified way. Here, Elaegnaceae and Rhamnaceae have been placed in the order Rosales. Similarly Datiscaceae has been ascribed with order Cucurbitales in contrast to Violales (Dilleniidae). In both the classifications, the order Rosales are not ascribed with sub-class Hamamelidales but, other sub-class placements are similar.

Traditional classification of actinorhizal plants were done on the basis of morphological characters,

Table 2.1: Distribution (after Baker and Mullin, 1992)* and habitat of representative genera of actinorhizal plants

Family	Genus	Native from following regions	Habitat
Betulaceae	*Alnus*	North America, South America, Europe, Northern Asia, Southern Asia	bogs, riparian
Casuarinaceae	*Allocasuarina*	Australia*	sand dunes, saline, desert, coastal areas
	Casuarina	Australia	
	Gymnostoma	Australia	
Coriariaceae	*Coriaria*	Australia, North America, South America, Europe	gravel, poor soils
Datiscaceae	*Datisca*	North America, Southern Asia	gravel streams
Elaeagnaceae	*Elaeagnus*	Northern Asia, North America, Europe, Southern Asia	Poor and stressed soils, disturbed sites, hilly slopes
	Hippophae	Europe, Northern Asia, Indian sub-continent, Tibet.	River banks, torrential and land slide areas, fragile slopes, gullies, disturbed soil.
	Shepherdia	North America	
Myricaceae	*Myrica*	Southern Africa, North America, South America, Australia, Southern Asia northern Asia	bog, ocean dunes
	Comptonia	North America	chaparral, upland
Rhamnaceae	*Adorphia*[b]	North America	
	Ceanothus	North America	
	Colletia	South America	semiarid soils, sand
Rosaceae	*Cercocarpus*	North America	gravelly soil
	Dryas	North America	the same
	Purshia	North America	

Australia* = Australia and/or Oceania
[b]Cruz-Cisneros and Valdes (1990).
***Source:** Dommergues, Y. R, 1997 and Berry, M. A., 1994 with some personal modifications.*

which are distantly related and classified into four of the six major subclasses of angiosperms (Cronquist, 1981) (Table 2.2). As per this morphological classification, Mullin and An (1990) suggested that symbiotic association must have evolved several times during the course

Table 2.2: Classification of Actinorhizal plants [a].

Subclass [b]	Order [c]	Family	Nodulation ratio	Genus
Hamamelidae	Fagales	Betulaceae	1/6	*Alnus*
		*Casuarina*ceae	4/4	*Allocasuarina*
				Casuarina
				Ceuthostoma
		Myricaceae	2/3	*Gymnostoma*
				Comptonia
				Myrica
Rosidae	Rosales	Elaeagnaceae	3/3	*Elaeagnus*
				Hippophae
				Shepherdia
		Rhamnaceae[e]	7/55	*Ceanothus*
				Colletia
				Discaria
				Kentrothamnus
		Rosaceae	5/100	*Retanilla*
				Trevoa[f]
				Cercocarpus
				Chamaebatia
				Dryas
				Purshia[g]
Magnoliidae	Cucurbitales	Coriariaceae	1/1	*Coriaria*
Dilleniidae		Datiscaceae	1/1	*Datisca*

[a] Compiled after Baker and Schwintzer (1990), Swensen (1996), Benson and Clawson (2000), and Schwencke and Caru (2001).
[b] According to the classification of Cronquist (1988).
[c] According to the classification of the Angiosperm Phylogeny Group (1998); all of these orders fall in the 'Eurosid I' group of eudicots.
[d] Number of nodulated genera over the total number of described genera in the family
[e] *Adolphia* may be actinorhizal, but has not been confirmed (Cruz-Cisneros and Valdés, 1991).
[f] *Talguenea* should be combined under *Trevoa* (Tortosa, 1992).
[g] *Purshia* and *Cowania* have been combined under *Purshia* (Henrickson, 1986).

of angiosperm evolution. Taxonomy of actinorhizal plants, though still unclear, their respective symbiotic association could be homologous as they fall under the same subclass (Swensen, 1996).

Actinorhizal plants have originated in the late cretaceous period and have diversified to different ecological niche (Magallon *et al.*, 1999). From the reports on pollen records, members of Rosales, Protales and Rhamnales was relatively advance but Fagales and Myricales might have evolved earlier than other actinorhizal members (Sen, 1996). It is also theoretically estimated that present actinorhizal plants, before their divergence into families and their respective genera, might have come in contact with *Frankia* in a selective

ecological niche that might have favoured the evolution of symbiotic association.

Another theory of evolution presumes that in the early cretaceous period, due to scarcity of available nitrogen (Bond, 1983) in the atmosphere, some woody, wind pollinated dicotyledonous plants had to forcefully associate with *Frankia.* But after this association, during the course of evolution, there might have been increase in the atmospheric nitrogen and some changes on certain advantageous and favourable characteristics for symbiotic association. So, some plants lost the symbiotic ability with *Frankia* but their genetic makeup of their symbiotic association of the past existed. Today certain phylogenetic studies (Normand and Bousquet, 1989) and DNA hybridization studies carried out on host plant (Bousquet *et al.,* 1989) supports this theory.

It is understood in the recent times that actinorhizal plants have been found more closely related through molecular phylogenetic studies on large samplings of symbiotic N_2 fixing species. Molecular analyses of flowering plants based on rbcL gene have revealed distinctiveness and have placed both actinorhizal and *rhizobia,* including *Parasponia* sp. (rhizobially nodulated), together in a single clade called the "core rosid" (Chase *et al.,*1993; Mullin and Dobritsa, 1996; Dawson, 2008). Soltis *et al.* (1995) reported that of the four sub-clades that contain N_2 fixing symbiosis, actinorhizal plants were found in three. They also suggested that all N_2 fixing plants have originated simultaneously along with several other non N_2 fixing plants. Restriction in nodulation activity in core rosids emerged with a hypothesis that the predisposition to form nitrogen fixing root nodule symbioses emerged once during angiosperm evolution (Soltis *et al.,* 1995).

Now, it is also concluded that actinorhizal plants occur in 10 families and are distributed among 5 of the 8 main lineages of the 'core rosid' group (Magallon *et al.,* 1999). Swensen (1996) combined *rbcL* sequence analysis with morphological and anatomical characters and showed that this rosid clade of actinorhizal symbiosis originated at least four times (Bousquet and Lalonde, 1990; Sprent, 1994), which is separated from other lineages by related non actinorhizal plants. This combined characteristics of molecular (*rbc*L sequence),

anatomical and morphological data, after further investigation by different workers, led to the conclusion that N_2 fixing actinorhizal symbiosis should have originated at least 3-6 times, independently (Roy and Bousquet, 1996; Swensen, 1996; Swensen and Mullin, 1997; Jeong*et al.*, 1999).

From these facts, diversity of actinorhizal plants have expressed symbiotic predisposition in number of times during their evolution (Clawson *et al.*, 2004). This is an indication that these plants (*Frankia* and *Rhizobium* associated) had inherent capacity to adhere symbiotically with soil microbes (Swensen and Mullin, 1997; Jeong *et al.*, 1999).

Classification approaches using molecular techniques for phylogenetic studies have given new perspectives to actinorhizal plants, providing important evolutionary and ecological insights (Benson and Dawson, 2007).

2.6.0 HISTORY OF *FRANKIA* RESEARCH:

The study of root nodules of non-leguminous plants was shrouded in the mist of misconceptions for a long period. Meyen (1829) first described non-leguminous plant nodule after discovering it from the roots of *Alnus glutinosa*, and concluded it to be a parasites growing in the roots of plants. In 1866, Wornin conducted anatomical studies of root nodules where he observed hyphae passing through the intracellular walls. The round vesicular swellings at the tips of these passing hyphae were considered to be a fungal spore by him and named the organism as *Schinzia alni* due to its resemblance to parasitic fungus *Schinzia cellulicolia* (Sen, 1996).

Professor A. B Frank, a Swiss microbiologist, considered the root nodules of both leguminous and non-leguminous plants as protein bodies and rejected the idea of presence of microorganisms in it. The name *Frankia subtilis* was first proposed in 1886 by J. Brunchorst to honor his mentor A. B Frank, after studying the cytological differences of non-leguminous and leguminous roots. Both Brunchorst and Frank considered the microorganism *Frankia to* be a fungus. After further studies, *Frankia* was classified as actinomycetes by Krebber in 1932 (Quispel, 1990).

Two publications to their discovery by Hellriegel and Wilfarth during 1886-1888 proved the legume root nodules could fix atmospheric nitrogen with the help of bacteria residing in the cortical

cell and proposed difference between nitrogen users and nitrogen accumulators, where Alders were included as nitrogen accumulators, initially, brought an end to a 10-year-old controversy about sources of nitrogen for growth of plants and opened up new frontiers of plant microbial science (Bottomley, 1912; Quispel, 1988; Sen, 1996).

After a decade, experiment of Hellriegel and Wilfarth was further strengthened by Hiltner in 1896 by reporting the presence of some kind of Bacteria in *Elaeagnus* and *Alnus* sp., who also proved and demonstrated that young Alders without root nodules cannot survive in N_2-free soil but when inoculated with root nodule supplements, gave positive results. In the year 1907, Hiltner also reported nitrogen fixing organisms in the root nodules of *Cycas* (Bottomley, 1912). These experiments led to comparison of leguminous and actinorhizal root microsymbionts and concluded that actinorhiza were not fungus but bacteria.

In another study, Beyerinck (following the steps of Koch and Pasteur) isolated bacteria from legume root nodule but when he again tried to infect the root of non-leguminous plant, it failed. This result concluded that the two microsymbionts are two different microorganisms (Brewin, 2002). This observations along with cytological differences among the leguminous and non-leguminous microsymbiont, led to understand that these two microorganisms were different from each other (Pawlowski and Bisseling, 1996).

The identity of the non-leguminous microsymbiont was not established unless Krebber classified it as actinomycetes in 1932 (Quispel, 1990) and reported by Becking *et al.* (1964), after viewing under electron microscope. It was only after this; the presence of actinomycetes in the root nodule of non-leguminous plant was established.

Further detailed study could be carried out only after isolation of this microsymbiont in pure culture by Torrey and his co-workers (Callaham *et al.,* 1978). After successful pure culture isolation of *Frankia* was carried out, a classical reviews by Newcomb and Wood (1987) and Benson and Silvester (1993), illustrated vivid descriptions of structure and ultra-structure of *Frankia* cells through electron microscopy. But, Benson and Silvester (1993) have also discussed

some difficulties in studying *Frankia* with electron microscope because of the delicate nature of some cellular components.

After this important historical research on *Frankia,* further researches gradually took place and are still going on.

2.7.0 TAXONOMY OF *FRANKIA*

Classification of *Frankia* was initiated even earlier than pronouncing the term 'actinorhiza' in place of the term 'non-legume', for nodule bearing plants, in the first conference on *Frankia* and actinorhizal plants (Tjepkema and Torrey, 1979), mentioned earlier. Before successful isolation of the endophyte, Becking (1970), after redefining the genus, placed it as a member of the order Actinomycetales in a new family, Frankiaceae. Using crushed nodules as inocula and host specificity (Lechevalier, 1994), he further went ahead and put up a proposal of establishing 10 species of this genus (*Frankia alni, F. elaeagni, F. brunchorstii, F. discariae, F. casuarinae, F. ceanothi, F. coriariae, F. dryadis, F. purshiae, and F. cercocarpi*). Since isolation of organism had failed number of times, he named the members as "obligate symbiotic organisms."

On the basis of host specificity, most of the *Frankia* were divided into four groups: (1) *Alnus* and *Myrica,* (2) *Casuarina* and *Myrica,* (3) *Myrica* and *Elaeagnus*, and (4) members of *Elaegnaceae* (*Elaeagnus, Hippophae* and *Shepherdia*) (Baker, 1987). This classification was considered to be incomplete because it was not possible to isolate *Frankia* from all actinorhizal plants and some strains in pure culture showed non-infective character and could not re-nodulate their hosts. Further some strains also lacked morphological, cytochemical and physiological criteria assigned to *Frankia* (Hahn *et al.,* 1989; Lechevalier, 1994). However, based on phylogeny (Lechevalier and Lechevalier, 1989), DNA homology (An *et al.*, 1985) and serology (Baker *et al.*, 1981), *Frankia* were divided in two sub-groups –A and B.

Only after isolation of different strains of *Frankia* in pure culture, a new dimension on *Frankia* taxonomy started emerging, especially when it was found that individual *Frankia* strains could nodulate plants of different orders and were not so rigid in host specificity (Benson and Silvester, 1993), analogous to *Rhizobium.* Many *Frankia* strains have

been isolated and attempts have also been made to classify them.

It may be noted that only one genus and no species of *Frankia* had been recognized till 1988, this may probably be because of differences in morphological and physiological characters of the genus. In Bergey's Manual of Systematic Bacteriology, vol. 4 (1989), *Frankia* are included among "actinomycetes with multilocular sporangia" (Lechevalier and Lechevalier, 1989). *Frankia* cannot be classified on the basis morphological characters because they are too diverse to identify (Weber *et al.*, 1988).

Classification attempt at molecular level was initiated. It was widely believed that hypervariable regions of 16S rRNA may help in phylogenetic classification at genus or species level (Harry *et al.*, 1991). Hypervariable region of 16S rRNA genes of 1020 to 1042 (*E. coli* numbering) for *Frankia* taxonomy differed in comparison with rRNAs of effective (N_2 fixing) and infective *Frankia* strains (Hahn *et al.*, 1989). Probes containing 20-22 nucleotides targeting this region failed to interact with other actinomycetes put in experiment but both types of probes from effective and infective *Frankia* strains reacted with the rRNA of *Nacordioides albus*. However, this could not be considered.

In another experiment, when Hahn *et al.* (1990) tried to exploit sequence position 180 to 240 as a probe, it reacted with all the strains tested but at higher temperatures some strains failed to hybridize, which brought difference in the sequence of other strains. Out of 22 strains only two (*Actinomadura* and *Microbispora*) tested reactive. This too indicated lack of probe specificity.

In a new approach to characterize genus specific *Frankia,* Simonet and his co-workers in 1991, designed two sets of primers and tried to solve through Polymerase Chain Reaction (PCR). The first one was universal primer targeted to nitrogen fixing bacteria, which gave negative result for non-nitrogen fixing bacteria. The second set of primer was targeted to specific *nif*H-*nif*D region of *Frankia.* Using these primers when DNA was amplified, genus specific length fragments was achieved. These fragments thus generated were *Frankia* specific and also related to *Geodermatophilus* (closely allied genus with *Frankia).* To separate the two genera, again two primers were designed to amplify 16S and 23S

1990), host specificity (Bosco *et al.*, 1992), phylogenetic study of carbohydrate uptake (Ganesh *et al.*, 1994), etc. with mixed success. In mid 80s and 90s of last century, molecular approaches like DNA-DNA relatedness analysis, (An *et al.*, 1985), Low-Frequency Restriction Fragment Analysis (LFRFA) (Beyazova and Lechevalier, 1992), 16S rDNA and 16S rRNA sequences (Nazaret *et al.*, 1991; Normand *et al.*, 1996; Clawson *et al.*, 2004), arbitrary primers (Sellstedt *et al.*, 1992), nitrogen fixation (*nif*) genes (Jeong *et al.*, 1999) and glutamine synthetase (Clawson *et al.*, 2004), etc. have been approached with some interesting facts in the molecular level.

DNA-DNA hybridization study for *Frankia* classification taken up by An *et al.* (1985) was perhaps the first of its kind and found high relatedness among most of the *Frankia* strains isolated from *Alnus* sp., but could not relate isolates of other species like *Elaeagnus, Casuarina*, etc. Similar study on diversity among 43 isolates of the genus *Frankia* was also conducted by Fernandez *et al.* (1989). In his study, out of these isolates, at least nine compatible genomic species among the strains were classified as three genomic species as *Alnus* compatible, five genomic species as Elaeagnaceae compatible and one genomic species as *Casuarina* compatible. Fernandez *et al.* (1991), further reported on the study of hypervariable region E_2 of different isolates of *Alnus*, members of Elaeagnaceae and Casuarinaceae conducted by Normand and his co-workers, where he had confirmed classification of isolates from *Alnus*, into genomic species through DNA-DNA relatedness, but varied with ribosomal sequence of strains of Elaegnaceae genomic species. Later Nazaret *et al.* (1991) compared all 9 genomic sequences using PCR technique to amplify 268 bp DNA segment of 16S rRNA gene, where except one; all the strains from one genomic species had same sequences and were also different to other genomic species.

Similar studies on DNA-DNA hybridization or nucleic acid hybridization, using 16S rRNA-23S rRNA as molecular markers (Simonet *et al.*, 1994; Benson *et al.*, 1996; Normand *et al.*, 1996; Clawson *et al.*, 1999; Ritchie and Myrold, 1999), use of PCR amplified 16S rRNA partial sequences from pure culture or uncultured endophytes from the root nodules (Mirza *et al.*, 1994; Simonet *et*

al., 1991; Simonet *et al.*,1994) or probing PCR products with specific forward and reverse primers to identify specific *Frankia* populations, in dot blots (Mirza *et al.*, 1994b) and in PCR assay (Simonet *et al.*, 1994,) have been carried out in the past. Nazaret *et al.* (1991), also constructed a phylogenetic tree from the sequences described by Fernandez *et al.* (1989), to measure phylogenetic relations using 16S rDNA sequences, which revealed *Alnus* infective, *Elaeagnus* infective and *Casuarina* infective groups in respective clusters.

The analysis of DNA using PCR techniques allowed the scientists to study the microbes with or without culturing it. This proved to be one of the powerful tools in the study of ecology and classification of *Frankia.* Along with applications of PCR technique used in the study of *Frankia,* Restriction Fragment Length Polymorphism (RFLP) of *nif* complex (*nif*A-B, *nif*K and *nif*H) has added a new dimension in classification and phylogenetic study of the genus. Initially, Nazaret and his research team in 1989 reported to have got homologous results from infective *Casuarina* sp. regardless of restriction enzyme used. Subsequently other researchers took up the work with different procedures to attain homologous results.

Various molecular procedures like RFLP analysis (Akimov and Dobritsa, 1992) of PCR amplified 16 S rDNA (Huguet *et al.*, 2001), IGS of 16S-23S rRNA operon (Rouvier *et al.*, 1996; Ritchie and Myrold, 1999), IGS of *nif*H -D (nitrogenase) genes (Cournoyer and Normand, 1994) *nif*D-K genes (Jaman *et al.*, 1993; Nalin *et al.*, 1997) and *gln*II gene (Cournoyer and Normand, 1994) have been applied to classify *Frankia* from pure culture or nodules. Apart from these procedures, Beyazova and Lechevalier (1992) used pulse field electrophoresis to separate bigger molecular weight fragments spliced with restriction enzymes having less number of restriction sites and named it Low Frequency Restriction Fragment Analysis (LFRFA) and confirmed that *F. alni* sub-sp. *pommeru* clusters differently than other *Frankia* strains isolated from *Alnus* sp.

Use of arbitrary primers during PCR (Sellstedt *et al.*, 1992) and certain primers meant for consensus motifs of different repetitive elements (REP, ERIC, DR, BOX or rep-PCR) in gram +ve microbe genomes have also been opted for characterization of *Frankia*

(Murry *et al.*, 1997; Jeong, 2001). Characterization of *Frankia* has also been done using antibiotic resistant markers (Tisa *et al.*, 1999), pigment production and isozyme polymorphism (Igual *et al.*, 2001).

Some recent works on *Frankia* taxonomy and diversity generating BOX-PCR fingerprints targeting the conserved region of the 16S rRNA, using rep-PCR technique with BOX primer on six *Ceanothus* sp. have been carried out by Murry *et al.* (1997) to show *Ceanothus* nodules and *Elaeagnus* infective *Frankia* share common ancestors.

Jose *et al.* (2003) conducted PCR of 16 *Frankia* isolates using two different primers. (a) DNA fingerprints using DR1R primer through rep-PCR, which showed strain specific banding pattern and (b) RAPD fingerprints using 879F (16S rDNA sequence of *E. coli*). Here it showed similar banding patterns with strain Ccl3 and UGL 020603 (isolated strain of different *Casuarina* sp. of different geographical origin), indicating that it may help in identifying *Frankia* genomes at species or sub-species level.

According to the studies conducted, *Frankia* has been divided into three clusters with members of each clusters with distinct host range (Normand *et al.*, 2007) Cluster 1 also known as "*Alnus* strains", include strains that can nodulate plants of Fagales, Betulaceae and Myricaceae (Normand *et al.*,1996), and a subclade (within cluster I) of "*Casuarina* strains" with narrow host range that can nodulate *Casuarina* and *Allocasuarina,* (both species from Casuarinaceae), under natural conditions (Benson *et al.,* 2004). Cluster 2 comprises "Rosaceous strains", which are not isolated in culture and only infect five members of Fagales and Rosales comprising Coriariaceae, Datiscaceae, Rosaceae and *Ceanothus* of the Rhamnaceae (Benson *et al.,* 2004; Vanden *et al.,* 2004). Cluster 3 comprises "*Elaeagnus* strains". They form effective nodules on members of the five families in the Fagales and Rosales, which include Myricaceae, Rhamnaceae, Elaeagnaceae and *Gymnostoma* of the Casuarinaceae (Benson *et al.,* 2004).

Gtari *et al.* (2010) are of the opinion that *Frankia* strains based on 16S rRNA, *nif*H and *gln* genes (ITS region), cultivability, morphology and infectivity, has been placed in four clusters. The classification put forward here is almost similar to the classification mentioned above, except

Frankia placed in cluster II, which have not been isolated in pure culture, despite of many attempts and are considered as 'obligate symbionts. Such atypical *Frankia* strains in association with *Ceanothus, Coriaria, Datisca*, and *Purshia* are incorporated in Cluster 4.

All approaches by different workers have not been made with an objective to classify *Frankia* species. Some studies have been made to study the *Frankia* strains from limited geographic area or from single genus or species (Benson *et al.,* 1984; Bloom *et al.,* 1989; Gardes and Lalonde, 1987). Gtari *et al.* (2010) after carrying out 16S-23S rRNA ITS sequence from 53 *Frankia* strains is of the opinion that this procedure is not useful to assign *Frankia* strains to their respective cluster or host because of less variability of *Frankia* genes among the strains and phylogenetic relations are sometimes ambiguous with nearest matching strains.

Presently, there is no universal *Frankia* taxonomy or any compilation on this subject. Analysis of RNA nucleotide sequence 16S rRNA has been widely accepted in *Frankia* systematics.

2.9.0 *FRANKIA*–THE MICROSYMBIONT

Frankia are nitrogen fixing, filamentous, sporulating, heterotropic, gram positive bacteria of the order Actinomycetales. These microorganisms have symbiotic association with the roots of different dicotyledonous angiosperms and some are free living actinomycetes in the soil too (Wall, 2000). This ecological significance has made this microorganism one of the important subjects for the researchers.

Frankia inhabit in three different niches: the root nodule, the rhizosphere and the soil. Based on characters like (i) ability to nodulate, (b) ability to fix atmospheric nitrogen, (c) unique morphological properties, bearing sporangia and vesicles, (d) presence of 2-O-methyl-D-mannose sugar (Mort *et al.*, 1983), (e) presence of type III cell wall (*meso*-diaminopimelic acid, glutamic acid, alanine, glucosamine and muramic acid) (Lechevalier, 1994; Myrold, 1994) and (f) high G+C% (68-72%) (Lechevalier, 1994; Gtari *et al.*, 2010), *Frankia* has been uniquely placed in this genus.

2.9.1 Morphology and anatomy

Actinorhizal nodules may have discrete branched lobes or be compact or may form nodule roots as in the case of *Casuarina* and *Myrica* (Berry and

Sunell, 1990). It has been understood that the host (actinorhizal) plant plays a significant role in modification of *Frankia* morphology (Berg, 1999; Jeong and Myrold, 2003). Unlike *Rhizobium*, *Frankia* induced root nodules are perennial and have different morphology and anatomy. Anatomically legume root nodules are central and within endodermis with tightly packed cortical cells, whereas in actinorhizal root the vascular tissue is centrally located to the infected cells and the nodule meristem is located at the distal tip of the nodule (Benson and Silvester, 1993; Wall, 2000).

2.10.0 SYMBIOTIC INTERACTION- A PROCESS

The morphological steps of actinorhizal nodule development have been described in several excellent reviews (Newcomb and Wood, 1987; Berry and Sunnell, 1990; Wall, 2000). Process of symbiotic association among these two genera has also attracted interests among many workers. Depending upon the host plants, two modes of infection of actinorhizal plants, after local colonization by *Frankia* have been described: intercellular and intracellular.

Intracellular infection via deformed root hairs as in case of *Alnus, Casuarina, Comptonia, Myrica,* etc. (Callaham *et al.*, 1979; Berry and Torrey, 1983). Here, the bacterial hypha enters the root anatomy via root hairs (Torrey, 1976) and infection proceeds intracellularly in the root cortex where *Frankia* endosymbiont is formed with vesicles and hyphal tip (Akkermans *et al.*, 1989). Simultaneously, there are cell divisions in hypodermis and cortex producing wall like material composed of pectin and hemicellulose, which is mixed with intense branching of *Frankia* hyphae within the cell (Berg, 1990) forming a small protuberance called prenodule (Callaham *et al.*, 1979). Prenodules are infected with *Frankia.* Unlike *Rhizobium*, nodule primordium in actinorhizal plants is formed on pericycle as a result of mitotic division, and not in cortex. Therefore, prenodule in the cortex infects primordium cells in the pericycle to form a nodule.

Intercellular penetration by *Frankia* hyphae takes place bypassing the root hairs that reaches directly to the cortex through middle lamella between the epidermal cells, e.g. *Ceanothus, Elaeagnus, Hippophae, Shepherdia*, etc. (Miller and Baker, 1985; Liu and Berry, 1991; Huss-Danell, 1997). In

this case, there is no division in the cortical cell leading to prenodule formation, instead cortical cells secrete electron-dense material rich in pectin and proteins into the intercellular spaces, where the hyphae grow (Racette and Torrey, 1989). As a result of hyphal infection and cell division, nodule primordium is formed similarly as above in pericycle and plant cells gets infected by *Frankia* in the cortex, developing to a nodule for further infections (Miller and Baker, 1985).

Mature actinorhizal nodules have multiple lobes, which consists of *Frankia* in cortical cells and central vascular tissue. Interestingly, even in *Parasponia–Rhizobium* association prenodule formation takes place during infection procedure (Lancelle and Torrey, 1984) and the nodules formed are also modified lateral root (Obertello *et al.,* 2003) like actinorhizal root nodules (Laplaze *et al.*, 2000).

During the process of infection, *Frankia* sometimes takes the support of soil bacteria to deform host roots. This idea was supported by Berry and Torrey (1983) and also Knowlton and Dawson (1983) by mentioning that *Alnus rubra* roots deform quickly and increase the rate of nodulation with the support of *Pseudomonas cepacia*, whose presence may not be compulsory in the process. There are also reports of signal exchange between *Frankia* and the host plant before infection initiates (van *et al.,* 1997; Ceremonie *et al.,* 1999). However, no such active plant or the *Frankia* has been identified (Obertello *et al.,* 2003).

Apart from purification of hemoglobin (Fleming *et al.,* 1987) and isolation of nodule-specific cysteine proteinase cDNA (Goetting-Minseky and Mullin, 1994), very little is understood about the molecular aspect of symbiotic association between *Frankia* and the plant. Recently it has been found that a set of actinorhizal nodulin genes are activated during differentiation of actinorhizal root nodules (Mullin and Dobritsa, 1996). Obertello *et al.* (2003) defined two types of actinorhizal nodulin genes based on their expression and pattern, by homology to legume nodulins. They are early nodulin genes (involved in nodule organogenesis or plant infection) and late nodulin genes (involved in metabolism for nodules functioning).

The type of infection and the form of vesicles (spherical-*Alnus, Hippophae*, etc., mono-septate elliptical-

Cercocarpus, Dryas sp., etc. and club or pear shaped *Myrica, Casuarina, Comptonia*, etc.) are determined by the host plant, and not by the microsymbiont (Akkermans *et al.*, 1989; Newcomb and Wood, 1987).

2.11.0 ISOLATION AND STRUCTURE OF *FRANKIA* IN PURE CULTURE

Pommer in the year 1959 reported first *in vitro* isolation of *Frankia* from the roots of *Alnus,* which unfortunately got lost, but his descriptions on the isolate matched with the present day *Frankia* strain-HFPCpI1. However, almost after 19 years, first successful isolation of *Frankia* from the root nodules *Comptonia peregrine* was reported by Torrey and his collogues (Callaham *et al.,* 1978), which helped in understanding *Frankia* more closely than before (Quispel, 1990).

Stowers (1987) reviewed successful isolation techniques of *Frankia* in pure culture using different techniques, like serial dilution (Diem *et al.,* 1982), micro-dissection (Berry and Torrey, 1979), OsO_4 surface sterilisation (Normand and Lalonde, 1982), filter exclusion (Benson, 1982; Weber *et al.,* 1988), and sucrose density fractionation (Baker and O'Keefe, 1984; Baker and Torrey, 1979; Burggraaf *et al.,* 1981). During isolation various techniques were applied and it was found that *Frankia* are slow growing, heterotrophic, aerobic organisms (Benson and Silvester, 1993).

Improvements in the culture media for *in vitro* studies of *Frankia,* from the past yeast extract media to present improvised composition of nutrient media, have now made the task easier and endophytes also grow much faster (Lalonde and Calvert, 1979). Now successful isolation of *Frankia* from almost all actinorhizal plants, except *Ceuthostoma, Kentrothamnus, Chamaebatia, Dryas* (Benson and Silvester, 1993) and *Adolphia* (Huss-Danell, 1997) have been reported. Use of flavonoids (quercetin) in the culture media to control fungal contamination of *Casuarina* isolates have also been reported as a new measure of faster and contaminant free method of isolation of *Frankia* (Sayed and Wheeler, 1999).

In pure culture, *Frankia* behaves as microaerophilic and mesophilic organism and grows densely with branched and septate hyphae, multilocular sporangia developing terminally or at intercalary position of the hyphae and vesicles (Burggraff and Shipton, 1982; Newcomb and Wood,

1987).

2.11.1 The hyphae:

Microscopic observations reveal that *Frankia* hyphae, with varying size of 0.5 to 1.5 µm, are mass of anastomose (fuse) and branched mycelia to form a dense mat. The cell walls of these organisms have been found to be composed of electron-dense materials in two layers: the base layer and the outer layer (Baker *et al.*, 1980; Benson and Silvester, 1993).

Reports also suggest that small round to spherical or rosary shaped, probably glycogen and lipid granules, are present in the hyphal cells (Benson and Silvester, 1993). Ribosomes and polyribosomes approximately of 300 nm are present in the hyphal cells. Large number of circular shaped (in cross section) cytoplasmic tubules (average 45 nm in diameter), underlining the cell septum and outside of the cell wall are also visible in the periphery of hyphae cytoplasm (Lancelle *et al.*, 1985). Newcomb *et al.* (1979) first reported an extracellular multilayered hyphae in a free living *F. alni* (HFPCpI1), but this envelop, which is usually present in vesicles, needs to be further studied and is a subject of debate (Benson and Silvester, 1993).

2.11.2 The sporangia

Sporangia are multilocular structures produced by all *Frankia* strains (Simonet *et al.*, 1994).Sporangium consists of spores, which are the reproductive structures of *Frankia* (Krumholz *et al.*, 2003). Each sporangium contains several hundred refractile infective spores that are 1000 times effective in nodule production than equal volume of hyphae (Burleigh and Torrey, 1990). These spores are released from sporangia on maturity (Callaham *et al.*, 1978) and are presumed that they help in dispersal and survival of *Frankia* but not much has been reported in this regard (Krumholz, *et al.*, 2003). Inclusion of 2µg/ml of ampicillin (antibiotic) in the culture medium increases sporulation (Ganesh, 1993).

Based on production of spores, two types of root nodule were distinguished -one as spore positive (Sp+), comprising organisms that sporulate in the root nodule, and the other as spore negative (Sp-), that sporulates in pure culture but does not form spores inside the root nodule (van Dijk, 1978). All *Frankia* strains isolated *in vitro,* till date, have genetic capability to produce spores at different levels, even if they have been isolated from Sp- nodules.

But all the isolates when re-inoculated back to their hosts could not differentiate sporangia *in planta* (root nodules) and thus defined as Sp- strains (Torrey, 1987; Simonet *et al.*, 1994). A typical Sp+ strain has not been isolated successfully (Simonet *et al.*, 1994) but there are two reports on successful germination of spores of *Frankia* in the laboratory (Caru *et al.*, 1997).

Isolation of *Frankia* strains from Sp+ nodules was more difficult than Sp- nodules (Schwencke and Caru, 2001). In some cases, Sp+ nodules led to the isolation of *Frankia* strains but these putative strains failed to differentiate sporangia in the root nodules when re-inoculated in different host species. So they were classified as Sp- strains. Following this two hypothesis were proposed: (i) the phenotypic change of Sp- and Sp+ may have been caused due to mutation thereby inhibiting or stimulating sporulation in the root nodule, and (ii) Sp+ and Sp- strains co-exists in a single nodule and at the time of isolation, Sp- strains outnumber Sp+ strains as former grows better in pure culture (Schwencke and Caru, 2001).

Sporulation in *Frankia* is not determined by the host rather it is determined by the genotype of the endophyte (Schwintzer, 1990). Reports suggest that 9 out of 25 genera of actinorhizal plants comprising six families (*Betulaceae*, *Myricaceae*, *Rhamnaceae*, *Elaeagnaceae*, *Casuarinaceae* and *Rosaceae*) have Sp+ nodules. *Hippophae salicifolia* too is associated with Sp+ *Frankia* strains.

The morphological division of both the strains (Sp+ and Sp-) were further characterised at the molecular level (Simonet *et al.*, 1994; Wall, 2000). Simonet *et al.* (1994), based on 16S rDNA sequences, suggested that these two *Frankia* strains isolated from *Alnus* were genetically distinct from each other.

Sporangia are multilocular structures located terminally or at intercalary position of the hyphae (Newcomb *et al.*, 1979). Each locule or segment in the sporangia contains many spores, which can survive in the host as well as soil devoid of host (Weber, 1986).

2.11.3 The vesicles

In pure culture, vesicles are small spherical, thick walled structures, surrounded by multilayered lipid envelope that arises from hyphae on a small stalk (Harriot *et al.*, 1991; Gomaa *et al.*,2008), whereas in *planta* their shapes vary from spherical to elliptical to club shaped (Wall, 2000).

Vesicles are either produced when there is marginal or no concentration of nitrogen in the medium or nitrogen enriched medium where the nitrogen compound cannot be degraded to ammonia (Zhang and Benson, 1992). During the formation of vesicles, the hyphal tip or side branches swell up to form pre-vesicles (1.5-2.0 µm), which may be non-septate (Wall, 2000) or septate near the base and appears phase dark during examination with phase optics (Newcomb and Wood, 1987). Finally these pre-vesicles mature to vesicles (2.0-4.0 µm) and are phase bright. Birefringence was observed under Polaroid light (Torrey and Callaham, 1982). From the experiment conducted by them using elegant freeze fracture techniques, they concluded the presence of a highly structured laminated layer, to be lipid monolayer. These layers were totally lost during normal fixation procedures, leaving a space around the vesicles called "void area" (Benson and Silvester, 1993).

Reports also suggest that vesicles harbor nitrogenase in *Frankia* symbiosis. Studies have correlated onset of nitrogenase activity with vesicle development in young nodules (Mian and Bond, 1978) and its appearance along with seasonal changes (Schwintzer *et al.*, 1982; Wheeler *et al.*, 1983). During senescence of vesicles, septum becomes irregular thereby ceasing nitrogenase activity and disappearance of cytoplasm. It was also reported that some vesicles rejuvenate to produce hyphae *in vitro* (Schultz and Benson, 1989).

2.12.0 GENETIC DIVERSITY OF *FRANKIA*

As mentioned before, actinorhizal plants in symbiotic association with *Frankia* represent mostly perennial, dicotyledonous trees or shrubs, except *Datisca* (Jeong and Myrold, 2003) and are globally distributed, except Antarctica (Simonet *et al.* 1999). They are being studied since 1829 (Schwencke and Caru, 2001). Along with the diversity of the host, there exists the diversity of the microorganism.

In the present time, various reviews and reports makes us understand that there is a high degree of genetic diversity among *Frankia* strains isolated from different niches, plant species, of same host plant or from a single nodule of specific location or different geographical areas (Cournoyer *et al.*, 1993; Myrold, 1994; Normand *et al.*, 1996; Clawson *et al.*,

1997; Clawson *et al.*, 1999).

Classification (Becking, 1970) and phylogenetic studies (Lechevalier, 1994) also aided in analysing the diversity of *Frankia.* Global distribution and adaptation of actinorhizal plants in different environmental stress (Tang *et al.*, 2003), climatic and nutritional conditions have led to the diversity of organisms. First isolation of *Frankia* strain in 1978 opened up even more options for the study of diversity. Since then number of *Frankia* strains have been isolated from several actinorhizal plants from different geographical origins, which have helped in understanding its host specificity, metabolism, taxonomy and genetics (Benson and Silvester, 1993).

DNA isolation directly from the root nodule, amplification through PCR using appropriate primers and sequencing has made it possible to analyze uncultured *Frankia* strains. Construction of phylogenetic trees using related 16S-23S rRNA gene sequences from the GenBank and comparing with the isolated sequence using bioinformatics tools and appropriate software have been carried out in the past for studying diversity of different endosymbiont of actinorhizal plants (Hahn *et al.*,1989; Honerlage *et al.,* 1994; Benson *et al.*, 1996; Lumini and Bosco, 1996; Rouvier *et al.*, 1996; Normand *et al.,* 1996; Clawson and Benson, 1998; Benson and Dawson, 2007; Sen *et al.*, 2008). Some works on genetic diversity of *H. salicifolia* has been done in India; however, no detailed work has been taken up in this genus of Lachen Valley of Sikkim Himalayas.

2.13.0 PROBLEM IDENTIFICATION

To combat the harsh climatic conditions and extreme winters in Lachen, North Sikkim, most of the people residing there rely on forest products to meet up their daily consumptions in terms of fuel and fodder. Apart from private agricultural land, which may be insufficient for agriculture, forests are also used for intercropping of different agricultural and horticultural products like cardamom and potatoes. Therefore, with the increase in population these trends may not be economically and ecologically viable every year and in the same locality for the local people due to ecological degradation. Besides that, Sikkim is an organic state, so there is a prohibition in the use of chemical fertilizers. Therefore,

sustainable environmental and resource conservation planning along with utilization of nitrogen naturally has become one the major target as survival strategies for the inhabitants of these high altitude areas.

Nitrogen being one of the important components of high altitude ecosystem, choice of naturally growing actinorhizal plant species like *H. salicifolia* with proper management and further scientific study can satisfy the long-term conservation and sustainable needs of the local population of this arid desert. Efforts should be made on scientific study of nitrogen fixing symbiotic association of this plant. *H. salicifolia* has remained virgin in this regard. However, some research on sporulation, host specificity, biochemistry, physiology and phylogeny using molecular techniques may further bring light on the endophyte and the host, for its future use. Choice of strain with maximum nitrogenase activity shall help in developing a better symbiont for more nitrogen supply to the plant and generation of biomass.

From the silvicultural point of view also, genetic differentiation of sea buckthorn plant needs to be understood to protect its genetic diversity and restoration in the forests. Though there are some reports on genetic diversity and genetic differentiation of sea buckthorn (Yao and Tigerstedt, 1993; Bartish *et al.*, 1999; Sun *et al.*, 2006), many have contributed mainly on origin, evolution and phylogeny of the genera (Bartish *et al.*, 2000 and 2006; Sun *et al.*, 2002; Sheng *et al.*, 2006). However, till date no major record of genetic diversity of *H. salicifolia* and its microsymbiont *Frankia* have been reported. Therefore, knowledge on genetic diversity and population genetics of this genus along with symbiotic relationship and diversity of its endophyte-the *Frankia*, still further needs to be worked out especially in this region.

"If we consider Jawaharlal Nehru's idea, that actual world situation is the result of the European domination in ancient social economical and military development; maybe sea buckthorn could have had another position in the history of plant utilisation"

.....Angel Proorocu