Nitrogen Fixation in Seabuckthorn (*Hippophae rhamnoides* L.)

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ABSTRACT

Seabuckthorn (*H. rhamnoides* L.) is an ecologically important pioneering plant, having enormous agricultural and forestry interests. On the basis of seabuckthorn root nodule ultra-structure, Frankia endophyte has been included in the ascomycetales. One Frankia strain belongs to aerobic Frankia group, while other strain belongs to microaerophiles. On the other hand, some Frankia strains are non-promiscuous and fall in to 4 major host-specificity groups. Although Frankia strains infecting Rhamnales are the member of cross-inoculation group, still in some cases, Frankia isolate has been found to be genera specific in its nodulation capacity. Endophytic vesicles arise from the hyphal terminal swelling and the endophytic vesicle structures are thought to play an important role in the metabolism of symbiotic process. The solitary hyphal actinomycete develops into highly complex system of hyphae and multi vesicles in course of endophytic development, which are more efficient in N_2-fixing capability as compared to single-lobed nodules. Nodular endophyte has been shown to acquire 7 different shapes in different parts of nodules, during 4 different seasons of the year. During spring and summer, endophyte alternation of generation occurs through infection mycelium, spring sporangium, spring spores and vesicles, while in autumn and winter, it occurs through declined vegetative mycelium, decaying vesicles, winter sporangium, winter spores, and bacteroid like cells.

There is a close correlation between nodule numbers and the root age, with young roots having the highest numbers of nodules. A correlation has been established between the nodular nitrogen-fixing activity and the vesicles population per unit of cell area. AIL along the vegetative period, the vesicle population varies according to the cultivation conditions. Vesicle clusters in the nodules, are supposed to be the sites of endophytic nitrogenase activity. Nitrogenase activity from all the Frankia sources has been found to be thermo sensitive. Root nodules of seabuckthorn have been found to fix 180 kg of nitrogen per ha per year. Nitrogen fixation capacity in seabuckthorn nodules has been found to vary with some of the biological parameters, such as stage of plant growth, plant variety and age of the plant and physical parameters, including temperature, sun-shine, on seasonal and daily basis. Wide variation has been observed in the number of nodules per unit area, with maximum number of nodules lying at the depth of 10 and 60 cm below the soil surface, whereas there are very few nodules below 60 cm depth or in the surface layer.

Key words: H. rhamnoides, root nodules, nodulation, ultra-structure, Frankia, host-specificity, symbiotic genes and nitrogen fixation.

INTRODUCTION

Owing to immense global interest aroused, of lately, in seabuckthorn, due to its very important medicinal and nutritionally rich repertoire of diverse biologically active biomolecules, its systematic and scientific cultivation is gaining momentum in the form of well established commercial orchards in many countries of the world. The curative, health and nutritional potential of this wonder plant are well recognized with great enthusiasm and interest throughout the world to reap and realize its unmatched and hitherto unknown benefits. Seabuckthorn not only provides these direct benefits, but also help in bringing out the innumerable indirect benefits including denuded marginal land reclamation of the fragile mountainous systems, stabilization and protection of slopes, prevention of spreading of blow out lands, phyto-remadiation and greening of industrial waste lands, prevention of floods, conservation of watersheds besides soil fertility and physicochemical properties of soil, soil and water conservation, and last but not the least improving the lively hood of less privileged people of the mountainous regions by way of providing fuel, fodder and fertilizer as well as generating employment avenues and by improving their socio-economic status (Lu Rongsen, 1992).

The well-developed root system of this plant not only improve soil fertility through its root nodular N_2-fixation with the help of Frankia-symbiotic association (Jike and Xiaoming, 1992), but also add to soil organic matter through litter and root and litter decomposition. Coupled with dense canopy, seabuckthorn plant help in the rainwater interception, which ultimately reduce raindrop splash induced soil surface scouring and top soil erosion on the floor of seabuckthorn stand (Quanzhong et al1989). Due to its N_2-fixation capability and further soil fertility improvement, this plant has got very high ecological significance, being important pioneer plants, in the rehabilitation of other plant species, as it helps in the better growth and survival of other wild plants, including poplar, willow etc. growing in or near seabuckthorn stand and having very varied agricultural and forestry interest (Quanzhong et al, 1989). The present paper discusses the development and ultrastructure of nodular endophyte in relation to N0-fixation, culture, growth, classification and taxonomy of endophyte, nodular nitrogen fixation, biochemistry and metabolism of nodules, effect of physical, chemical and biological parameters on N_2-fixation, infectivity potential, cross inoculation groups and relation between soil factors, endophyte inoculum, nodulation and N_2-fixation efficiency etc.

ECOLOGICAL SIGNIFICANCE

Seabuckthorn and other non-leguminous N-fixing trees and shrubs have been reported to be ecologically important pioneer plants in an Iberian Peninsular region and their agricultural and forestry interests were enumerated by Bermudez (1977). Root nodules of seabuckthorn have been found to fix 180 kg of nitrogen per ha per year, which greatly improves the fertility status of the soil (Jike and Xiaoming, 1992).

NODULAR ENDOPHYTE DEVELOPMENTAL ULTRA STRUCTURE AND ANATOMY

The nodular endophytic ultrastructure in seabuckthorn was studied and it was observed that the endophyte had different shapes of hypha, vesicles and bacteroid cells (Catner et al., 1970). Electron microscopic studies of the developmental ultrastructure of nodular endophyte in seabuckthorn showed that endophytic vesicles arise from the hyphal terminal swelling and proposed that endophytic vesicle structure might be playing an important role in the metabolism of symbiotic process (Cardner and Catner, 1973). Maistrenko (1974) also arrived at the same conclusion that seabuckthorn endophyte has diversified forms. Root nodules actinomycete was isolated from H. rhamnoides and Baumeister and Kausch (1974) demonstrated its further reinfection in the in vitro grown plants. They observed that endophyte and periendophytic space was separated from the seabuckthorn root nodule cells' cytoplasm through a membranous envelope, similar to the one found in Legume-Rhizobium symbiotic association. They also found that actinomycete infected cells had increased level of auto-and heterolytic capacity.

Through transmission electron microscopic observations, Cardner (1976) observed winter sporangia and winter spores in seabuckthorn root nodules. Root nodule endophytic ultra structure of up to four and a half months old seabuckthorn sand grown seedlings was studied and it was concluded that there was maximum N_2 fixation after 60 days onward, which was comparable with soyabean (Andreeva et al., 1979). Beri et al. (1986) discovered that infection hypha intrude through protoplasm bridge, beside discovering spring sporangia and spring spores for the first time. From nodules of different seasons, they found 4 different kinds of mycelia with different forms and functions, including infections, reproducible, vegetative and bacteroid like mycelia.

In yet another exhaustive study, Andreeva et al. (1990) investigated the ultrastructure and composition of the cell wall and capsule of the actinomycete in root nodules of seabuckthorn, Elaeagnus argentea, Shepherdia argentea, Alnus crispa, A. tenuifolia and A. rubra, through electron microscopy and electron-microscopic cytochemistry. They found that the actinomycete vesicle cell wall consisted of electron-dense inner and outer layers containing lamellae and a central electron-dense inner and halo whose volume increased with vesicle maturity. They proposed that the complicated cell wall structure provide protection to nitrogenase from the damaging effect of oxygen. The cell wall was made up of layers of glycolipid and polysaccharide.

Zhang et al. (1995) intensively studied the detailed ultra structure of root nodule endophyte of seabuckthorn during different seasons of the year. They found that nodular endophyte acquire 7 different shapes in different parts of the nodules during 4 different seasons of the year. They concluded that the endophytic alternation of generation during spring and summer seasons occur through infection mycelium, spring sporangium and spring spores and vesicles. On the other hand, during autumn and winter seasons, alternation of generation takes place by means of declined vegetative mycelium, decaying vesicles, winter sporangium and winter spores, and bacteroid like cells. They showed that endophyte perrinate through winter spores, and bacteroid like cells. They also found that reinfection process of Frankia endophyte in seabuckthorn was very similar to Alnus cremastogyme root hair Frankia infection, which is in agreement with the previous report by Beri et al. (1986)

RELATIONSHIP BETWEEN NODULAR ULTRA STRUCTURE AND NITROGEN FIXATION

A very elegant relationship was established between the nitrogen fixing capability and endophyte infected root nodule cells ultra structure in seabuckthorn during early to multilobbed root nodule ontogenic development (Andreeva et al., 1980). They found that the solitary hyphal actinomycete ultra structurally turn into highly complex system of hyphae and multi vesicles in course of endophytic development, that were more efficient in

N_2fixing capability, as compared to single- lobed nodules. In a yet another study, the anatomical ultra structure of root nodules of Russian seabuckthorn for 6 months from May to October, was investigated, which directly correlated the number of nodule cell vesicles with nitrogen-fixing capability, that was found to be maximum in late June (Andreeva et al., 1982). They reported that during winter, all vesicles got degraded, however a few endophytic hyphae of Frankia survived the winter in each nodule and started the fresh infection cycle by infecting new cells, formed in the growing season.

In one study, the ultrastructure of the endophytic actinomycete in infected cells of seabuckthorn nodules was investigated, to analyze the relation between middle lobbed nodular cells vesicles number and their nitrogen fixing capability under varied conditions of seabuckthorn cultivation (Andreeva et ai, 1982). They demonstrated that all along the vegetative period, the vesicle population varied according to the cultivation conditions. The most significant changes in the vesicles population were observed during nodulation in the seedlings, while the changes in 3-years-old plants were non-significant. They found that molybdenum deficiency in the nutrient medium drastically reduced vesicle formation in the nodular cells, with a consequential decrease in the nitrogen-fixing capacity. They established a correlation between the nodular nitrogen-fixing activity and the vesicles population per unit of cell area.

Andreeva et al. (1982) also studied the relationship between endophytic vesicle formation in cells of seabuckthorn nodules and their nitrogen-fixing activity. They found that the population of endophytic vesicles per unit cell area, increased with age in young seedlings and varied with mean air temperature in adult plants. They concluded that nitrogen-fixing activity was directly proportional to the number of endophytic vesicles per unit cell area and the vesicle formation was severely affected by molybdenum deficiency, which also reduced the N-fixing activity.

ENDOPHYTE CULTURE AND GROWTH

The role of molybdenum in formation and functioning of the symbiotic nitrogen-fixing system in seabuckthorn nodules in 1-year-old transplanted sand cultured green house grown variety of 'Dar Katuni' seedlings was studied (Zhiznevskaya et al., 1983). They concluded that ammonium molybdate at the rate of 30 mg/kg sand decreased both the root nodule nitrogen fixing activity and the population of endophytic vesicles in the nodular cells, whereas ammonium molybdate at the rate of 330 mg/kg sand nodules fixed maximum nitrogen and produced maximum nodular biomass. Zhang and Torrey (1985) also concluded that optimal pH for nodulation was 7.0 and both NH_4^+ and NO_3^- were found to be the inhibitor of root nodulation, with NH_4^+ resulting in severe inhibition. While studying the growth of 3 Frankia isolates from Alnus rubra, H. rhamnoides and Shepherdia argentae under varying culture conditions, it was concluded that pH of the nutrient medium ranged from 6.0-7.1 for their optimum growth (Zhitskaya et ai, 1987). They correlated the Frankia culture productivity in an inoculum dose and aeration dependent manner.

The nodulated and non-nodulated plants of Alnus glutinosa and H. rhamnoides ssp. rhamnoides were studied by growing them with different sources of N_2, with and without acidity control in solution. It was concluded that under controlled pH, NO_3^- supplementation alone drastically reduced dry matter yield. On the contrary NH4 supplementation either alone or along with NO_3^- under controlled pH, led to increased dry matter yield, but the final dry matter yield of N_2 fixing seabuckthorn was low, whereas presence of NH_4^+, without acidity control greatly reduced dry matter yields. They concluded that roots nitrate reductase activity in A. glutinosa was more than H. rhamnoides (Troelstra and Blacquiere, 1986).

When the growth of 8 weeks old non-nodulated seabuckthorn seedlings supplemented with combined nitrogen, against nodulated seedlings without any nitrogen supplementation, was compared, it was found that the growth was much better in a supplementation mixture of NH_4^+ and NO_3^- in terms of maximum fruit yield, but root nitrate reductase activity was greatly repressed. In case of plants, supplemented with NO_3^- alone, entirely root based in vivo nitrate reductase activity was demonstrated. It was suggested that in the nodulated plants, on the contrary, least nodule population drastically reduced the plant growth and it was found that seabuckthorn used NH_4^+ compounds, preferentially as a major source of nitrogen compared to NO_3^- compounds amounting to only 1/5th of the total nitrogen assimilation, when used in combination. The highest rates of nitrogen assimilation and ion uptake were observed in plants supplemented jointly with NO_3^-+ NH_4^+ compounds. It was observed that in the presence of NH_4^+, in the absence of any pH control, specific ion uptake and carboxylate generation rates were maximally reduced and growth was suppressed by 1/3rd (Troelstra et al., 1987).

CLASSIFICATION AND CHEMO TAXONOMY

Maistrenko et al. (1974) characterized the seabuckthorn root nodule endophyte ultra-structure and included it into the ascomycetales. Interestingly enough, Zhitskaya et al. (1987) in yet another study found that ANP 140105 Frankia strain belonged to group of aerobic Frankia, while ANP 190105 Frankia strain belonged to microaerophiles. They also proposed optimized culture conditions for each Frankia strain to increase the biomass concentration per unit of nutrient medium.

The nodulation capability of pure in-vitro cultured 50 strains of Frankia in actinorhizal plant species was tested and actinorhizal host inoculation groups were defined. It was concluded that Frankia strains were non-promiscuous and all the Frankia test strains were classified into 4 major host-specificity groups (Baker, 1987). Nazaret et al. (1989) tested the nodulation capability of various Frankia isolates in C. equisetifolia, C. glauca and H. rhamnoides and concluded that isolates could be classified on the basis of their infection potential into 2 groups. Some isolates that did not reinfect the original host plant, instead nodulated H. rhamnoides, whereas other isolates effectively infected the original host plants.
The role of glycosides in the taxonomy of Frankia by analyzing the relative sugar contents of 79 Frankia strains, isolated from Alnus spp., Myrica spp., Comptonia sp., Elaeagnus spp., Shepherdia spp. and Hippophae root nodules, was confirmed by Laurent et al. (1987). They found that 2-O-methyl-D-mannose, a sugar abundantly present throughout the genus Frankia, was the most discriminant sugar, concentration variation of which showed a significant differentiation between both host specificity groups Alnus and Elaeagnus with a classification success rate of 97% at 2 weeks growth against 78% after 8 weeks growth. Frankia isolates from Myrica gale nodulated plants from both host specificity groups had intermediate sugar content, between the Alnus and Elaeagnus host specificity groups.

NODULE BIOCHEMISTRY AND METABOLISM

Huss-Danell et al. (1982) studied the occurrence and localization of enzymes, involved in glycolysis, the tricarboxylic acid cycle and the glyoxylate cycle in root nodules of Alnus glutinosa and H. rhamnoides ssp. rhamnoides. They concluded that endophytic Frankia have incomplete glycolytic pathway, because the reversible reactions of the glycolytic pathway were occurring, both in the endophyte Frankia and the root nodule cytosol of Alnus and Hippophae. On the other hand, the irreversible reactions of glycolytic pathway were occurring only in the root nodule cytosol. They also proposed that the glyoxylate cycle enzymes are repressed in the root-nodule symbiosis.

Frankia Avcll cultivated in-vitro, were found to consume tween detergents and fatty acids as sole source of carbon, whereas various amino acids and N_2, NH4, N03 compounds were shown to be utilized, as main source of nitrogen from the synthetic media (Akkermans et al., 1983). They also described the nature of carbon compounds utilized by nodular Frankia during symbiosis in Alnus, Hippophae and Datisca. In another study, Chaudhary and Sajjad (1987) isolated and characterized Frankia strains form actinorhizal nodules of seabuckthorn and other non-leguminous N_2 fixers, for their capability to grow on various carbon and nitrogen sources, their in-vitro nitrogen-fixing ability and potential to nodulate original host plant seedlings.

NODULATION GENES

While studying the nodulation capability of various Frankia isolates in *C. equisetifolia*, *C. glauca* and *H. rhamnoides*, a definite relationship was established between nodulation characteristics and symbiotic genes among different isolates (Nazaret et al., 1989). A genomic DNA library of Frankia strains isolated from root nodule endophytes of Alnus, Casuarina and Hippophae was constructed and screened, using nodABC gene from R. meliloti as heterologous probe, to identify clones homologous to common nodulation genes of Rhizobium. Thirteen putative genomic clones were identified and recombinant plasmids from those clones were isolated, which had the sequence homology with Rhizobium nod genes, after confirmation by spot hybridization (Cui et al., 1990).

NODULAR NITROGEN FIXATION

An acetylene reduction assay was performed for comparative estimation of nitrogen fixation in the soil cores and detached nodules of different legumes and non-legume plants including seabuckthorn, in the sand dunes. It was estimated that the Lotus corniculatus was the main contributor of nitrogen fixation in the sand dunes (Waughman, 1972). In a very interesting investigation, McNiel and Carpenter (1974) studied the nitrogen fixation in root nodules of different woody plants through acetylene reduction activity and showed that seabuckthorn root nodules also fix nitrogen even after excising them from the plant roots. While in some other woody plants, no active nodules were found after excision.

EFFECT OF PHYSICAL, CHEMICAL AND BIOLOGICAL PARAMETERS ON NODULAR N_2 FIXATION

The effect of varying oxygen tension, temperature and sample size on acetylene reduction in the nodules of Alnus and H. rhamnoides was studied. Nodular acetylene reduction rate was correlated with a temperature range of 4°C to 20°C, and an oxygen tension range of 1-15%, without any further increment of nitrogen fixation rate in the nodules of either species, when the oxygen tension was increased even up to 20% (Waughman, 1972). In another study, Bound and Mackintosh (1975) compared the effect of nitrate-nitrogen on the nodule symbioses of nodulated Coriaria arborea and H. rhamnoides, using labeled N15.They concluded that, whereas the applied nitrogen promoted plant growth in seabuckthorn, nodule growth and rate of nitrogen fixation per plant was depressed. In another study, the effect of temperature on nitrogenase activity in the detached nodules of non-leguminous, leguminous and blue green algae was investigated, using acetylene reduction assay. It was concluded that nitrogenase activity from all the sources was thermo sensitive, and there was reduction in the blue-green algae thermo sensitivity of nitrogenase activity by low photo intensities (Waughman, 1977)

The nitrogenase activity through acetylene reduction assay in the excised and nodule homogenates of Alnus glutinosa, H. rhamnoides, Shepherdia canadensis and Myrica gale was studied and it was found that ATP in small concentration elicited maximum nitrogenase activity, whereas ATP-generating system inhibited nitrogenase acivity (Straten et al., 1977). They also found that there are vesicle clusters in the nodules and proposed that these are the sites of endophytic nitrogenase activity.

In another study, it was found, the nitrogen fixation capacity in H. rhamnoides nodules was comparable to that of Lupin and concluded that it varied with some of the physical parameters, such as stage of plant growth, plant variety, and age of the plant. It was also reported that haemoglobin and haemoglobin like compounds were not present in the H. rhamnoides nodules, and the endophytic Rhizobium was absent in these nodules, although anatomically, the seabuckthorn nodules were similar to legume nodules (Simonov et al., 1978).

In another investigation, the effect of temperature on the N_2-fixation capabilities in the excised root nodules of non-legumes and legume woody trees and shrubs including H. rhamnoides and Robinia pseudocacia, was studied, which showed maximum nitrogenase activity at 20°C and up to 30°C, no significant change was found in the activity and at 40°C, there was high temperature induced irreversible nitrogenase injury. At temperatures near 0°C, the nitrogenase activity was found to be very less (McNeil and Carpenter, 1980). The N_2-fixation in the seabuckthorn root nodules was studied and it was found that root nodular N_2-fixation capacity fluctuated with plant age, environmental factors, seasonally as well as daily between morning and evening (Zhao et al., 1985).

In a study, it was found that seabuckthorn root nodule nitrogen fixation enzyme activity was significantly influenced, by seasonal factors viz temperature and sunshine. The effect of slope direction on nitrogen fixation was also studied and found that it was independent of slope direction. It was shown that nitrogenase enzyme activity was affected by plant age in the shady slopes, while plant sex did not influence the nitrogenase enzyme activity (Wang et al, 1999).

INFECTIVITY POTENTIAL AND NODULATION CAPABILITIES

The infective potential of nodule homogenates from field-grown H. rhamnoides spp. rhamnoides was studied. It was concluded that prior to the appearance of H. rhamnoides in the dune forming sites, because of low inoculum of infective endophyte in these soils, their infective potential was very low. During the H. rhamnoides succession, due to the increase in the endophytic inoculum in the soil, their infective potential increased, which was gradually controlled by the environmental factors (Dremus, 1980). Two endophytic strains of actinomycete were isolated from C. equisetifolia nodules, which were capable of inducing nodulation in H. rhamnoides. The N_2- fixation activity of these strains in H. rhamnoides nodules was same as that of another endophytic strain isolated from H. rhamnoides. It was confirmed that two endophytic actinomycete were Frankia, having effective infectious and capability in H. rhamnoides (Gauthier et al., 1981).

Zhang et al. (1984) also isolated 3 new Frankia strains from aeroponically grown root nodules of C. cunninghamiana. Two of the typical red-pigmented Frankia strains, formed sporangia in vitro without combined nitrogen, produced vesicles and reduced acetylene. They induced nodulation in Elaeagnus and Hippophae, but not in Casuarina. They found that third typical Frankia strain was non-pigmented, formed sporangia in vitro, formed vesicles and reduced acetylene. It was able to induce nodulation in both C. cunninghamiana and C. equisetifolia, while it did not produce nodules in case of Elaeagnus. They also suggested about the possibilities of seedlings inoculation with available infective, effective strains for forestry and field application.

In a unique commercial venture, more than 7 million green house grown seedlings of actinorhizal plants for industrial plantation were inoculated with crushed-nodule homogenates and Frankia pure culture formulations, with a purpose of land reclamation and re-vegetation programme in northern Quebec and city of Montreal, Canada. It was found that inoculations with soil injection and spraying with greenhouse watering devices were equally effective in efficient nodulation of A. crispa, A. glutinosa, A. rugosa, Elaeagnus angustifolia, E. commutata, H. rhamnoides, Myrica gale and Shepherdia argentea (Perinet et al., 1985).

SYNERGISTIC AND ANTAGONISTIC EFFECTS

Frankia isolates from root nodules of C. equisetifolia collected from different locations in Senegal, Guadeloupe and Philippines were characterized. Two of these isolates induced root nodulation in polybag raised seabuckthorn seedlings. It was also observed that inoculation with the crushed nodules and the endomycorrhizal fungus Glomus mosseae combination in the sterile soil grown C. equisetifolia seedlings, increased the nodulation and N content to more than double compared to Frankia inoculation alone (Gauthier et al., 1983).

In another interesting study, Kobets and Andreeva (1983) found, that the roots and root nodule crude extract of non-leguminous nitrogen-fixers Alnus spp. and seabuckthorn drastically inhibited the growth of fungus H. annosum. On the contrary, the roots and root nodule crude extract from Elaeagnus, and leaf extract from Alnus and seabuckthorn weakly inhibited H. annosum growth. They suggested the use of root nodule mixture of Alnus spp. and H. rhamnoides with scots pine, on the sites susceptible to H. annosum.

CROSS-INOCULATION GROUPS, GENERA AND HOST SPECIFICITY

Infective and effective in vitro nitrogen fixing symbiotic endophyte, Frankia was isolated and was shown to nodulate Rhamnales, H. rhamnoides and Elaeagnus angustifolia, but failed to infect non-Rhamnales. It was suggested that theses Frankia strains infecting Rhamnales are the member of cross-inoculation group (Gauthier et al., 1984). In-vitro cultured Elaeagnus angustifolia L. plantlets, growing on nitrogen-free artificial soil substrate were inoculated with different pure cultured Frankia strains, isolated from Elaeagnus, Shepherdia and Hippophae host plants. It was shown that in-vitro cultured Elaeagnus angustifolia L. plants do not loose their capability of nodule formation and readily fix nitrogen (Bertrand and Lalonde, 1985).

Zhang and Torrey (1985) cultured the Frankia isolate from C. cunninghamiana nodules. When the seedlings of different Casuarina spp., Allocasuarina lehmanniana and plants of a variety of other species were inoculated with 15-30 days old Frankia pure cultures, they found that Frankia isolate was genera specific in its nodulation capacity, since it nodulated all Casuarina spp., but on the contrary there was no nodulation in other genera including H. rhamnoides.

Thirty strains of Frankia were isolated, from 6 provenances of M. gale, most of which were fastidious. Upon inoculation, it was concluded that of the easy growing isolates, 12 effectively infected both M. gale and Alnus glutinosa, while 5 did not show nitrogenase activity in Alnus glutinosa, while effective nitrogenase activity was found in Elaeagnus angustifolia and H. rhamnoides (Laurent and Lalonde, 1987).

Dobritsa et al. (1990) studied the nodulation ability and infectivity of 97 Frankia strain isolates from 14 actinorhizal plant species in Alnus glutinosa, H. rhamnoides, Elaeagnus angustifolia and Elaeagnus argentea. They found that of the 39 infectious strains, 26 isolated from Alnus spp. and Comptonia peregrina, could not nodulate Comptonia peregrina, while 12 strains form Elaeagnus spp., H. rhamnoides, Shepherdia argentea, and Colletia cruciata lost the capacity to infect Shepherdia argentea, and Colletia cruciata. From the cross-infection of the genus Alnus and of the family Elaeagnacease with Frankia isolates of E. argentea, they concluded that these Frankia strains represent a new host specificity group. They also proposed possible mechanisms of cross infectivity for widening the host plant range of Frankia.

CROSS INOCULATION POTENTIAL

The cross inoculation potential of nodular Frankia spp from H. rhamnoides subsp. sinensis, and many other non-leguminous N_2 fixers was tested and it was found that all Frankia strains could infect all the test host species. A conclusion was drawn that both the infection potential and nitrogenase activity of different Frankia strains was less in the new host background, although the more efficient strains in the original host retained their efficiency and capability to fix more N_2, even in the new host backgrounds. It was also found that in the new host background, spores were morphologically different than their native host (Du et al., 1988).

A Frankia strain from the root nodules of Atriplex cordobensis was isolated, which could re-nodulate its host and had the capacity to fix N_2. Two Frankia isolates from Colletia spinosissima and Ceanothus americanus infected Atriplex cordobensis, one Frankia isolate from H. rhamnoides and one atypical isolate from Casuarina equisetifolia, which did not nodulate its host of origin but nodulated Elaeagnus angustifolia, also infected Atriplex cordobensis. Of all 4 isolates, the least effective was the atypical strain from Casuarina equisetifolia. One isolate from Ceanothus americanus efficiently fixed N_2, and significantly increased the dry weight of the host plant, but without any change in the N concentration of the plant. It was concluded that the Frankia isolate from Atriplex cordobensis was a member of the Elaeagnaceae-Rhamnaceae cross-inoculation group (Caucas and Abril, 1996).

ENDOPHYTIC NODULE DISTRIBUTION

The numbers, weights, and ages of root nodules in a homogenous 6 to 7-year-old stand of seabuckthorn in a coastal sand-dune area were studied. Wide variation in the number of nodules per unit area were found, with maximum number of nodules lying in the depth of 10 and 60 cm below the soil surface, whereas there were very few nodules below 60 cm depth or in the surface layer. A close correlation between nodules numbers and the root age, with young roots having the highest numbers of nodules was found. It was concluded that nodule decay plays a highly significant role, both in the endophytic distribution in the soil and the process of nodule formation (Oremus, 1979). Chaudhary et al. (1981) surveyed the non-leguminous nitrogen fixing angiosperms from Pakistan, and reported actinorhizal nitrogen fixing root nodules in all the species of non-leguminous angiosperms including Hippophae except Rubus ellipticus, which did not have nodules.

In a study on the extent of endophytic root nodule in seabuckthorn and inoculant effectiveness in Shanxi province soils, it was found that soils prior to seabuckthorn growth had very small population of nodular endophyte and the maximum quantity of root nodule endophyte was in the top 10-30 cm soil layer. It was concluded that inoculation was more effective in yellow loam type of soil, and increased yield (Yuan, 1989).

RELATION BETWEEN SOIL FACTOR, ENDOPHYTE INOCULUM, NODULATION AND NITROGEN FIXATION EFFICIENCY

The effect of additional inoculation of Frankia spp. nodule homogenate on actinorhizal plants grown in non-sterile soil was studied and concluded that after 2 years of inoculation, nodule size and stem length increased significantly, compared to non inoculated plants. Even during the first growing season after seabuckthorn nodule homogenates inoculation, and specifically with Frankia culture, the root nodule population and their size significantly increased, as compared to control plants (Stupar et al., 1989). The level of root nodulation and N_2 fixation in sand, alluvial meadow soil, calcareous chernozem, leached chernozem soil types in pot grown Robinia pseudoacacia, Alnus glutinosa and H. rhamnoides plants were studied. It was claimed that N_2 fixation was maximum in R. pseudoacacia raised on sand, and with A. glutinosa and H. rhamnoides on alluvial meadow soil. It was also found that R. pseudoacacia adapted better to the soil substrate compared to other two species, and was the most efficient N_2 fixer (Aleksandrova et al, 1994).

FACTORS AFFECTING NODULATION GROWTH

In Netherlands, the factors affecting nodulation growth of H. rhamnoides L. ssp. rhamnoides in the soils from early and post optimum successional stages of dune formation were investigated. It was concluded that the heavy nematode infestation of the post optimum-soil was main factor responsible for root deformation, necrotic nodules, darker roots, less root hairs, more short lateral roots, higher percentage of dead roots and dry matter content and subsequent reduced nodulation and poor phosphate uptake in Hippophae (Oremus and Otten, 1981).

CONCLUSIONS AND FUTURE STRATEGIES

The studies of developmental ultra-structure and anatomy of nodular endophytes in various non- leguminous nitrogen fixers has provided a great insight in to the mode of infection, the development of root nodules, besides the functional relationship of nodules and different structures of endophyte with special reference to N_2- fixation, apart from the effect of different mineral nutrients, pH condition, age of the plant, seasons, soil factors including other biological, physical and chemical parameters on the nitrogenase activity and overall N_2 - fixation efficiency. In addition the studies also provided a clear picture about various metabolic events and biochemical intractions occurring between the root nodules and endophyte, in addition to the establishment of nodulation characteristics and symbiotic genes among different endophytes, apart from showing genetic sequence homology of Frankia genomic clones with Rhizobium nod genes. Classification and chemotaxonomic studies has helped immensely to discern the different Frankia strains in to discrete and distinctive host specific groups and cross inoculation groups among different genera, on the basis of their infection capacities, infectivity, cross inoculation potential and nodulation capabilities. No doubt that all those studies has helped us to under stand the very basics of nitrogen fixation intricacies, still we need to know and understand the many hitherto unknown physiological and biochemical aspects of symbiotic association between Frankia-seabuckthorn in particular and symbiotic associations between other nitrogen fixers with other leguminous as well as non- leguminous plants, in general, if we have to reap the real benefits of symbiotic nitrogen fixation. This knowledge will greatly help us not only in bringing many non- nitrogen fixer monocots as well as dicots, annuals, biennials and perennials plants of agri-horticultural and silvicultural importance under the category of symbiotic nitrogen fixers, but also to reduce our dependence on nitrogenous fertilizers to great extent, beside protecting our environment from further degradation.

Apart from that, there is an urgent need of hour to search and domesticate more efficient strains of N_2-fixers, with higher nodulation capacity, broad host range, easy adaptability to tolerate extremes of physical, chemical and biological environments, soils, and climatic factors, which can directly improve soil fertility and its physicochemical properties, besides improving overall biomass and yield of the plant. In addition, we also need to search, isolate and improve the native microflora from the rhizospheric zones of leguminous and non-leguminous plants, for noble symbiotic organisms, other than N_2-fixers, such as PGPR's, siderophores, phosphate, potassium and iron solubilizers to harness their natural capabilities to support the healthy plant growth without further polluting and degrading our environment, by reducing our dependence on the costly, synthetic fertilizers and other toxic agrochemicals.

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