

CHAPTER II

LITERATURE REVIEW

2.1 Endophytic actinomycetes

According to Hallmann *et al.* (1997) and Azevedo *et al.* (2000), endophytic microbes are those which can be isolated from surface- disinfested plant tissue or extracted from inside the plant, and they do not visibly harm the plant. This definition includes internal colonist with apparently neutral behavior, as well as symbionts. Nevertheless, Matsukuma *et al.* (1994) and Okazaki *et al.* (1995) reported that the variety of actinomycetes inhabit a wide range of plants as either symbionts or parasites. Endophytic actinomycetes are attractive because their secondary metabolites might be promising sources of novel antibiotics and growth regulators of other organisms as suggested by Matsukuma *et al.* (1994) and Okazaki *et al.* (1995).

2.2 Beneficial effect of endophytic actinomycetes

According to Okazaki *et al.* (1995) the isolates of actinomycetes from leaves have different physiological characteristics from those existed in common soils although their appearance are obviously very similar. Sardi *et al.* (1992) suggested that a large number of *Streptomyces* strains isolated from healthy plants and the direct scanning electron microscopy investigation on internal tissues showed that there is a close relationship between the endophytic actinomycetes and roots, in which actinomycetes hyphal growth could have a favorable effect. The presence of *Streptomyces* inside the root tissues has an important role with regard to plant development and health. Their biological activity can interact with plant growth either

by nutrient assumption or by the in site production of secondary metabolites which stimulate or depress vegetative development. The antibiotic activities against *Staphylococcus aureus* and *Bacillus subtilis* were found in endophytic actinomycetes isolates from common bean, caupi bean and *Solanum lycocarpum* (Britto, 1998; Araujo *et al.*, 1999; Matsuura, 1988 and Maitan, 1993 cited by Azevedo *et al.*, 2000). The endophytic actinomycetes from rhododendron also had intense antagonistic activity against two major rhododendron pathogens, *Pestalotiopsis sydowiana* and *Phytophthora cinnamomi* (Shimizu *et al.*, 2000).

2.3 Usefulness of endophytic actinomycetes

Matsukuma *et al.* (1994) and Okazaki *et al.* (1995) reported that a variety of actinomycetes inhabit a wide range of plants as either symbionts or parasites. They also reported that several new or rare species of actinomycetes were discovered from plants and suggested that their secondary metabolites might be promising sources of novel antibiotics and growth regulators of other organisms. Actinomycetes, especially *Streptomyces spp.*, isolated from the rhizosphere of soil have proven to be excellent biocontrol agents of soil borne plant pathogens (Yuan and Crawford, 1995). Such an effective activity is largely dependent on secondary metabolites produced by these organisms.

Control of soil-borne diseases with endophytic actinomycetes biocontrol agents has elicited considerable interest. Increased concern about the environmental impacts agrochemicals in soil and ground water and the lack of effective chemical controls for many soil-borne diseases has stimulated this trend (Paulitz and Linderman, 1991).

Tokala et al. (2002) stated that endophytic actinomycetes (*Streptomyces sp.*) influenced pea root nodulation by increasing root nodulating frequency. When they colonized an increase in the average size of the nodules that form were increased. The vigor of bacteroids within the nodules was also improved because endophytic actinomycetes enhanced by nodular assimilation of iron and possibly other soil nutrients.

Thapanapongworakul (2003) isolated endophytic actinomycetes from sweet pea and one of the endophytic actinomycetes isolate P4 which showed effective antagonistic activities against various fungal disease belonged to genus *Streptomyces*. P4 could infect the other leguminous host plants such as navy bean, red kidney bean, adzuki bean, cowpea, soybean and Thai sweet pea. Under light room condition using nitrogen free medium for growing soybean, P4 inoculation had a trend to improve nitrogen uptake of the whole plant 83% over that of uninoculated control treatment. Dual inoculation of P4 and bradyrhizobial isolate improved nitrogen uptake (443%) as much as that of singly inoculation of *Bradyrhizobium* (438%).

At present, demand of agriculture products from organic farming has been increased world widely. The total value of organic food all over the world in 2000 was about 20,000 million US Dollars. The biggest market from organic food is EU, followed by USA and Japan (Import and Export Bank of Thailand, 2001).

The biotechnological potential of endophytic isolates assessed by their antagonistic activity and by *in vitro* production of enzymes, antibiotics, siderophores, and the plant growth hormone indole-1,3-acetic acid was generally high. (Sessitsch *et al.*, 2004.)

Endophytic actinomycetes are attractive because their secondary metabolites might be promising sources of novel antibiotics and growth regulators of other organisms as suggested by Matsukuma *et al.* (1994) and Okazaki *et al.* (1995). Quispel (1992) considered endophytic as those that established an endosymbiosis with the plants, whereby the plants receives an ecological benefit from the present of the symbiont such as increased stress tolerance or plant growth promotion.

The use of organic fertilizer or biofertilizer and biological control are the key factors for producing organic crops satisfactorily. Thus, nodule bacteria inoculation and the use of endophytic actinomycetes seems to be attractive means for good growth and satisfactory yield. It is very interesting to study the endophytic actinomycetes suitable for a biological control agent for soybean and its effects on root nodule bacteria.

2.4 Some beneficial soil microbes

In agriculture, the following microbes, N₂ fixing bacteria, cyanobacteria, phosphate and silicate solubilizing microorganisms, micorrhizal fungi and plant growth-promoting bacteria are considered as beneficial soil microbes. Inoculation of plants with beneficial bacteria can be traced back for centuries. By the end of 10th century, the practice of mixing naturally inoculated soil with seeds became a recommended method of legume inoculation in the first patent (“Nitrogen”) was registered for plant inoculation with *Rhizobium* sp. (Nobbe and Hilter, 1986, cited by Bashan, 1998). Eventually, the practice of legume inoculation with root nodule bacteria became common. Apart from legume inoculant, inoculation with nonsymbiotic, associative rhizosphere bacteria, like *Azotobacter*, was used on a large

scale in Russia in the 1930s and 1940s. The practice had inconclusive results and was later abandoned (Pubenchik, 1963, cited by Bashan, 1998).

2.5 Legume-Root Nodule Bacteria Symbiosis

The Leguminosae (Fabaceae) is an enormous plant family distributed worldwide, with 16,000 to 19,000 species in about 750 genera (Allen and Allen, 1981). Plants belonging to this family establish a symbiosis with bacteria from the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium* and *Allorhizobium* collectively known as rhizobia (Sawada *et al.*, 2003, Bottomley and Myrold, 2007). The result of this symbiosis is the fixation of atmospheric nitrogen in what is called biological nitrogen fixation (BNF). BNF is the enzymatic reduction of dinitrogen (N_2) from the atmosphere, to ammonia. The ability to fix nitrogen is only found in a variety of prokaryotic organisms, but not in eukaryotes. The nitrogen fixing bacteria have the enzyme system, nitrogenase, which provides the biochemical machinery for nitrogen fixation (Van Kammen, 1995).

Nitrogen fixation by legume- root nodule bacteria symbiosis plays a key role in world crop production. About 100 million tons N, valued at 50 billion US\$, is required annually for the production of the world's grain and oilseed crops. Of this amount, nitrogen fixation by the oilseed legumes, soybean and groundnut, and pulses supplies almost 20% (17 million ton N) (FAO, 1998). Legumes can only fix nitrogen if they are nodulated by effective, compatible root nodule bacteria. In many soils, populations of naturalized root nodule bacteria are presented insufficient number to nodulate the sown legumes. In other situations, there may be only low numbers of root nodule bacteria in the soil or they may be entirely absent. Under these conditions,

introduction of highly effective root nodule bacteria at the time of sowing of the legume will usually result in sufficient nitrogen being fixed by the crop to fulfill its requirement for growth (Herridge, 2002).

2.6 Characteristics of Root Nodule Bacteria

Root nodule bacteria are medium-sized rod-shaped cells, 0.5-0.9 μm in width and 1.2-3.0 μm in length. They do not form endospores, are Gram-negative and motile by a single polar flagellum or two to six peritrichous flagella (Somasegaran and Hoben, 1994). Root nodule bacteria are predominantly aerobic. They grow well in the presence of O_2 and utilize relatively simple carbohydrates and amino compounds. Optimal growth of most strains occurs at a temperature range of 25-30°C and a pH of 6.0-7.0. Generally most root nodule bacteria produce white colonies, but those that nodulate *Lotonosis bainesii* produce a characteristic red nonheme carotenoid pigment when cultured in yeast mannitol medium. Most root nodule bacteria only weakly absorb congo red dye, which is included in culture media for isolating root nodule bacteria. Other interesting and useful characteristics of root nodule bacteria are their growth reactions in the standard yeast mannitol medium containing bromothymol blue as the pH indicator. Fast growing root nodule bacteria produce an acid reaction in the yeast mannitol medium containing bromothymol blue (pH 6.8) while slow growers produce an alkaline reaction (Somasegaran and Hoben, 1994).

Root nodule bacteria are facultative microsymbionts that live as normal components of the soil microbial population when not living symbiotically in the root nodules of the host legume. Outside the root nodule, root nodule bacteria are mostly

found on the root surface (rhizoplane), soil around and close to the root surface (rhizosphere) and, to a lesser extent, non-rhizosphere soil. The increase in numbers of root nodule bacteria in the rhizosphere is a response to the excretion of nutrients by plant roots, especially the host legume (Somasegaran and Hoben, 1994).

The first edition of Burgey's Manual of Systematic Bacteriology (1984) adopted a classification divided the legume symbionts, previously allowed to the single genus *Rhizobium*, into two genera. Species comprising legume symbionts which grow fast and produce acid on a yeast mannitol agar medium (fast growers) were classified in the genus *Rhizobium*, and those which grow slowly and produce alkali were classified into the genus *Bradyrhizobium* (Jordan, 1984). Today, rhizobia fall into several genera and species within the Alphaproteo bacteria and are currently subdivided into six genera, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* (Bottomley and Myrold, 2007).

2.7 Root Nodule Bacteria as Symbiont

The free-living root nodule bacteria in the soil can enter the root hairs of the susceptible host legume by a complex series of interactions (Somasegaran and Hoben, 1994). The interaction of legumes and root nodule bacteria results in the formation of root nodules - new organs in which bacteria are able to fix atmospheric nitrogen into ammonia (Van Kammen, 1995). The development of nodule starts with the attachment of the root nodule bacteria to the root hairs of their host, which causes deformation and curling of root hairs followed by the formation of infection threads in the curled hairs. The root nodule bacteria enter the root through these threads. Concomitantly with the infection process, the bacteria induce cell divisions in the root

cortex, which leads to the primordium formation. The infection threads grow towards the centers of mitotic activity, and then enter primordium cells, and bacteria are released into the plant cells by an endocytotic process. Then the nodule primordium differentiates into a mature nodule. The final structure is a central core containing the root nodule bacteria and a cortical area that becomes occupied by the vascular system, which connects to the young root. The root nodule bacteria divide and differentiate into the form known as bacteroid. In these nodules, the root nodule bacteria are able to fix nitrogen and provide the host plant with ammonia for plant growth (Van Kammen, 1995).

2.8 Soybean-*Bradyrhizobium* Symbiosis

Soybean (*Glycine max* L. Merrill) is the most important grain legume crop in the world in terms of total production and international trade. Soybean seeds contain from 18% to 23% oil and from about 33% to 40% protein (Hymowitz *et al.*, 1998). It is grown on about 70 million ha world-wide and has been estimated to fix about 11 million ton N annually (FAO, 1998). It is likely that only 10-15 million ha (i.e. 14-21% of the total) are inoculated annually. However, virtually all of the 11 million ton N currently fixed by soybean results from either past and current inoculation. This is because soybean, for the most part, is grown on land that initially did not contain the soybean root nodule bacteria (Herridge, 2002). Estimated amount of nitrogen fixed by soybean- root nodule bacteria symbiosis under field conditions varied from 60-115 kg ha⁻¹ year⁻¹ (Evans and Barbar, 1977).

Bradyrhizobium was the first root nodule bacterial genus to be created in addition to *Rhizobium*. It was created to accommodate so-called "slow-growing

strains" of root nodule bacteria and for 10 years contained only one named species: the soybean-nodulating *B. japonicum* (Jordan, 1982). The existence of at least two genetically divergent types of *B. japonicum* was recognized and the group was subsequently split into two species with the creation of *B. elkanii* (Kuykendall *et al.*, 1992). A third genus of soybean-nodulating Bradyrhizobia, *B. liaoningense*, was created in 1995 to accommodate exceptionally slow-growing strains (Xu *et al.*, 1995).

2.9 Host-Strain Specificity

The legume- root nodule bacterial symbiosis exhibits widely differing degrees of specificity. In some instances, the symbiosis is highly specific in that a particular species or strain of root nodule bacteria can form an effective symbiosis association with only one particular legume species or variety (Somasegaran and Hoben, 1994). *Bradyrhizobium japonicum* strains differ in their ability to form nodules and to support nitrogen fixation and soybean yield (Bezdicsek *et al.*, 1978, Caldwell and Vest 1968, Rennie and Dubetz, 1984, Weaver and Frederick, 1974). Senaratne *et al.* (1987) reported that plant dry weight, nitrogen yield, percent nitrogen derived from the atmosphere and amount of nitrogen fixed were significantly influenced by specific combinations of host genotype and *Bradyrhizobium* strain.

A key feature of the symbiotic relationship between root nodule bacteria and legumes is the very high degree of specificity shown for effective nodulation of a particular host legume by a strain / species of root nodule bacteria. This specificity operates at both the nodulation and nitrogen fixation levels of symbiosis, and is a function of the exchange of specific chemical signals between the two partners (Denarie *et al.*, 1992 and Perret *et al.*, 2000). The specificity is controlled by the fact

that the chemicals can only be produced if the organisms contain the information for the synthesis of the chemicals on their genes. Thus the development and function of the symbiosis between legumes and their associated root nodule bacteria is controlled at a molecular level, and the development of effective nitrogen-fixing symbiosis is conditional on both partners containing appropriate sets of genes (Phillips *et al.*, 1997; Schlaman *et al.*, 1998; Perrret *et al.*, 2000).

2.10 Factors Affecting Legume- Root Nodule Bacteria Symbiosis

Since nitrogen fixation by legumes is a symbiotic process, environmental factors that affect the host legume and the root nodule bacteria must be optimal for establishing an effective nitrogen-fixing symbiosis.

Soil pH is an important environmental factor. Many of the soils of the world are affected by excess acidity, a problem caused by heavy fertilization with certain nutrients and by acid rain (Moawad, 2000). Such acid-soil conditions pose problems for the plant, the bacteria and the symbiosis (Giller and Wilson, 1993). The microbiont is usually more pH sensitive partner. Some root nodule bacterial species can tolerate acidity better than others, and tolerance may vary among strains within a species (Lowendorff, 1981, Vargas and Graham, 1988, Brockwell *et al.*, 1995, Hungria *et al.*, 1997). The optimum pH for root nodule bacterial growth is considered to be between 6.0 and 7.0 (Jordan, 1984) and relatively few root nodule bacteria grow well at pH less than 5.0 (Graham *et al.*, 1994).

Aluminium and Manganese toxicities are likely to be encountered in many tropical soils with soil pH 4.5 or less. Both plant roots and nodulation are adversely affected. Low soil pH is often associated with increased Al and Mn toxicity and

reduced Calcium supply. These additional stresses affect the growth of root nodule bacteria (Cooper *et al.*, 1983, Coventry and Evans, 1989, Campo, 1995), of the host legume (Andrews *et al.*, 1973; Kim *et al.*, 1985), and symbiosis (Murphy *et al.*, 1984, Brady *et al.*, 1990, Campo, 1995).

Nutritional factors in rhizosphere influence the growth of root nodule bacteria. Nitrogen limitation as the classic phenomenon is being investigated particularly with Bradyrhizobia association. Lopez-Garcia *et al.* (2001) demonstrated that root nodule bacterial nitrogen starvation has positive influence on the symbiosis of *Bradyrhizobium japonicum* with soybean plants. However, presence of some mineral nitrogen in the plant growth medium increased the number of nodules by as much as 50% (Allos and Bartholomew, 1959).

Starter doses of fertilizer nitrogen were recommended to improve nodulation and nitrogen fixation (Eaglasham *et al.*, 1983). Shortage of phosphorus will severely limit the formation of nodules and nitrogen-fixation. Molybdenum is an essential micronutrient to all plants and is required for the formation and function of the nitrogenase enzyme complex. Soils deficient in Mo produce poor and ineffectively nodulated legumes (Somasegaran and Hoben, 1994).

The size, effectiveness and competitiveness of the native or indigenous root nodule bacterial population are also important factors that influence the ability to achieve increased crop yield through inoculation. Soils often contain root nodule bacteria that are highly competitive against those applied in inoculants. Selected strains of peanut root nodule bacteria failed to increase yields in the presence of high populations of indigenous root nodule bacteria (Diatloff and Langford, 1975, Van der Merwe *et al.*, 1974).

While highly effective naturally occurring strains of *Bradyrhizobium japonicum* have been available for some time, the establishment of any new strains in the nodules of field-grown soybean is extremely difficult. Even at high inoculation rates, only a small portion of the nodules of field-grown soybean is formed by the inoculum strain (Caldwell and Vest, 1970; Ham *et al.*, 1971; Kuykendall and Weber, 1978).

2.11 Strain Selection

Selection of effective nitrogen-fixing strains of root nodule bacteria is a necessary first step in the production of high quality legume seed inoculants. Selected strains must have the capacity to nodulate the host legume and fix nitrogen under a wide range of field conditions. Thus, criteria for selecting strains may vary depending on local soil, environment and crop husbandry factors. Recognition of factors that restrict nodule formation and nitrogen-fixation is important since it is necessary to select strains to overcome these restrictions (Date, 1996).

The source of root nodule bacterial strains for a strain selection program can range from local isolates, to strains already tested in other parts of the region or country, to cultures from various overseas collections. The abundant diversity clearly present in soil populations of root nodule bacteria provides a large resource of natural germplasm to screen for desired characteristics (Sadowsky and Graham, 1998, Dilworth *et al.*, 2001). An essential desired characteristic for inoculum strains of root nodule bacteria is highly effective nitrogen-fixation with the intended host species, and in some instances there is a requirement for the strain to effectively nodulate a wide range of host legume species. Other beneficial characteristics include stress

tolerance, competitive ability against the indigenous strains, genetic stability and satisfactory growth and survival during procedures for manufacture of inoculum (Howieson *et al.*, 2000).

Preliminary screening is performed in the greenhouse, where numerous strains can be tested on several host varieties. Root nodule bacteria selected in greenhouse trials, where conditions are usually optimal, must then be evaluated in the field. Root nodule bacteria that adapt to the agronomic conditions under which the host legumes will be cultivated and that enhance crop production through nitrogen fixation can then be selected for inoculant production (Somasegaran and Hoben, 1994).

2.12 Nodule formation of legumes

According to Libbenza and Bogers (1974), nodulation of legumes needs the following steps: (1) accumulation of the root nodule bacteria in the rhizosphere (2) infection of the root hairs resulting in the information of infection threads, (3) growth of the infection threads into the cortex, and (4) induction of local meristematic growth in root cortex. Infection and nodulation are influenced by physical factors in the rhizosphere which affect both plant and root nodule bacteria (pH and calcium level, amount of combined nitrogen and other nutrients and temperature), by root nodule bacteria factors (numbers, strain competition, cross inoculation specificities and induced genetic change) and by plant genotype.

From the information reviewed by Dart (1974), some important points about the infection process are summarized. In the rhizosphere of legumes, there is a marked stimulation of root nodule bacterial numbers. Such stimulation causes by leguminous root exudates. Many strains of root nodule bacteria need biotin and some

thiamin for growth and these are found in root exudates. Different legumes produce different exudation of ninhydrin positive substances.

Some fungi, actinomycetes and bacteria can reduce nodulation perhaps by inhibiting root nodule bacteria, or root growth, or by altering the root metabolism so that it resist successful infection. Some fungi and actinomycetes occasionally stimulate the growth of root nodule bacteria, infection and nodulation of clover seedlings. Some root nodule bacteria are more sensitive than others are to actinomycetes and antibiotics.

Exposure of *R. trifolii* to some actinomycetes in culture reduced thus nodule forming and nitrogen fixing ability presumably because of some genetic change. Poor nodulation of clover in the field has been attributed to microbial antagonism in the rhizosphere preventing adequate colonization of the root by root nodule bacteria (Dart, 1974).

The bases of root nodule bacteria and legumes specificity are unknown. Anyhow the sugar containing compounds of both plant and root nodule bacteria may be involved in the recognition. It was postulated also that root nodule bacteria capable of infecting a legume release a specific polysaccharide, which induces more pectolytic activity by the root and that, this accounts for the cross-inoculation specificities. The pectolytic enzymes of some legumes are most active at pH 5 or 5.5 with little activity at more acid pH (Dart, 1974).

2.13 Number of Nodules

The number of nodules per plant was observed increasing from 30 to 72 DAS (day after sowing), but the nodule growth rate was markedly depressed by P

deficiency and acidity. A strong interaction effect phosphorus and lime on numbers of nodules were apparent at 50 and 70 DAS (day after sowing) (Nguyen, 1996).

2.14 Nodule dry weight

The effects of phosphorus and lime on nodule dry weight was similar with number of nodules, i.e., the weight of nodules were markedly depressed by phosphorus deficiency and acidity as well. Without lime or low lime rate the weight of nodules increased with phosphorus application. At high lime rates, there was no difference in the effects of phosphorus on nodule weight at all growth stages. Without lime, the nodule dry weight increased markedly with increasing rates of P. The nodule dry weight was increased when phosphorus was applied alone. Liming alone increased the nodule weight, but higher lime rate did not further increase the nodule dry weight in soybean. Phosphorus and lime had much higher effects on nodule dry weight when they were applied together (Nguyen, 1996).

2.15 Measuring Symbiotic Nitrogen Fixation

Evaluation of symbiosis is based on several measurable parameters. Short-term trials with Leonard's jars or sterile sand culture pots can provide an adequate basis for gross comparison of strains (Somasegaran and Hoben, 1994). Measurement have been based on pattern of nodulation, number of nodules, plant dry weight, plant color, and plant N content (Erdman and Means, 1952 and Halliday, 1984).

The shoot dry weight of plants harvested at floral initiation or after significant plant biomass accumulation is the generally accepted criterion for nitrogen-fixing

effectiveness, but nodule dry weight may also be employed. Nodule number is a less reliable indicator of strain effectiveness.

Effective nodules are generally large and are clustered on the primary and upper lateral roots. Maximum volume normally occurs in the late flowering stage. In contrast, ineffective nodules are small, numerous, and usually distributed throughout the root system. Total nodule mass formed by effective root nodule bacteria and the quantity of nitrogen fixed is linearly related (Zary *et al.*, 1978, Wadisirisuk and Weaver, 1985).

The measurement of activity in the nodules by the nitrogen-fixing enzymes, nitrogenase, may also be done. This is accomplished by means of the acetylene reduction assay, which is a measure of ethylene production and indicates nitrogenase activity. Total nitrogen accumulation in the shoot can be measured by the Kjeldahl method. Since total nitrogen content and nodule dry weight frequently correlate well with shoot dry weight, the latter parameter provides an acceptable basis for strain comparison. In recent years, the ureide technique has been developed for measuring nitrogen fixation. Ureides are a group of nitrogenous compounds including allantoin and allantonic acid. Some legumes produce large quantities of ureides when nitrogen is fixed symbiotically, but not when assimilated from soil mineral sources (Somasegaran and Hoben, 1994).

The research to determine whether N₂ fixation of four of the commonly-grown ureide-producing legumes, soybean (*Glycine max*), cowpea (*Vigna unguiculata*), mungbean (*V. radiata*) and black gram (*V. mungo*), could be quantified from a single sampling and N-solute analysis of xylem sap. They concluded that the seasonal %Ndfa can be accurately estimated using the xylem solute (ureide) method

from a single sampling of xylem sap during early pod-fill (R3.5) and that this simplification of the protocol of the technique may encourage expanded use. (Herridge and Peoples, 2002).

Isotopic techniques using N^{15} are also used for measuring nitrogen fixation (Douglas and Weaber, 1986), but the analysis is costly. The final proof of inoculation response must come from the field, when the seed and nitrogen yields at harvest are determined for grain legumes or from the dry matter production for forage legumes (Somasegaran and Hoben, 1994).

2.16 Dynamic of nitrogen fixation of soybean

Usually nodules are formed by 15-20 days after sowing (Tanner and Hume, 1978). In field condition, N_2 fixation process is started at 25-30 days after sowing; it will be increased at latter flowering stage and maintained until occurring of the first yellow leaf, at early pod-filling period (Bien et al., 1996). However, nitrogen fixation by field grow soybean may start as early as 12 days from planting (Mulonggoy and Gueye, 1990). Leaf color difference between nodulated and non-nodulated seedlings is frequently observed as 20 days after planting (15 days after emergence), N fixed from nodules is nearly half of the total N fixed if the plants nodulate rapidly (Wilson and Umbried, 1967).

Soybeans begin to fix significant quantities of N_2 from at 32 days (Ladha and Peoples, 1995). Total fixed N_2 doubles every six to ten days until the fixation rate peaks at late flowering or early seed development stages then the fixation rate tends to decline (Mulonggoy and Gueye, 1990). There is evidence indicated that the decline in the rate of fixation occurs because developing seeds compete so strongly for

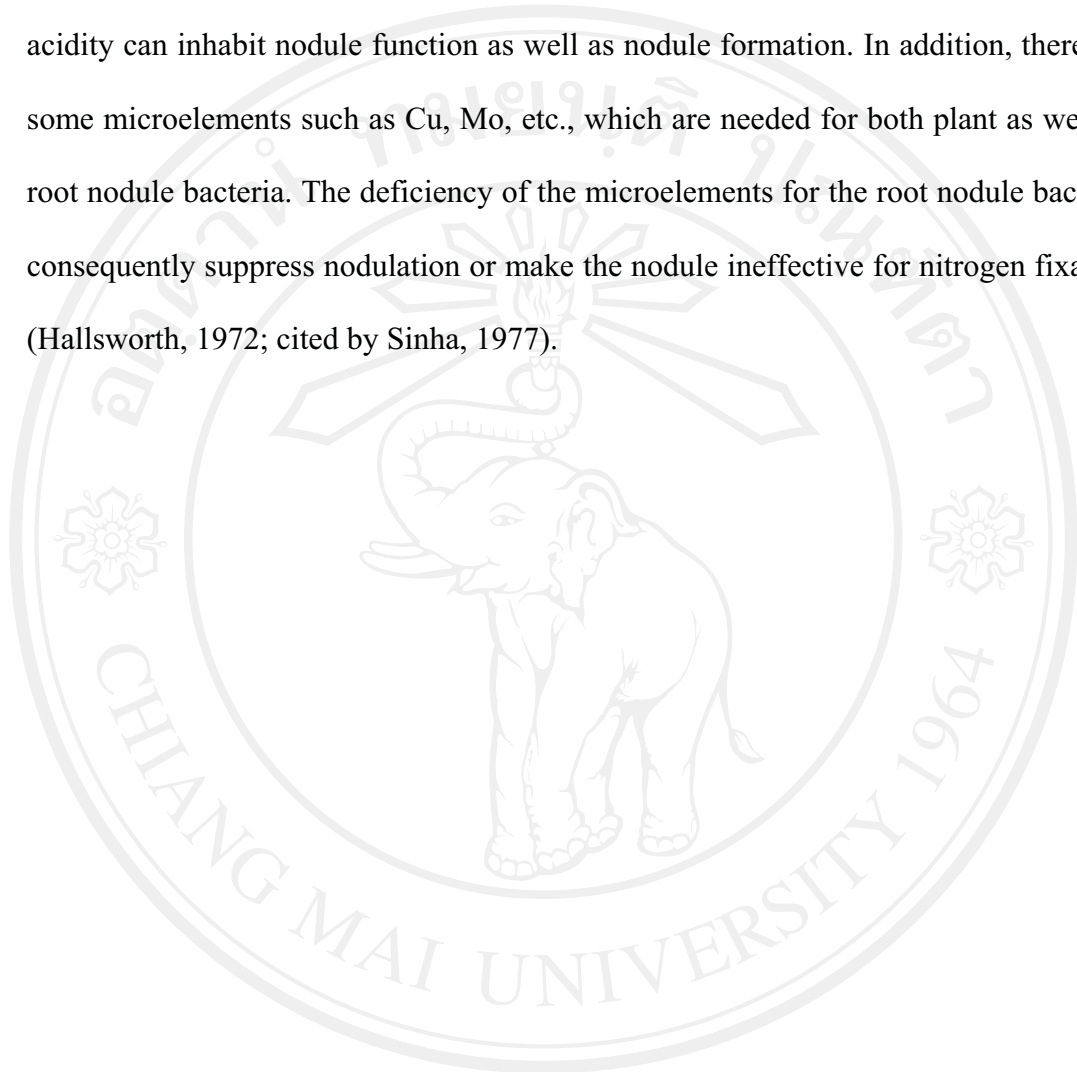
photosynthate that the nodules are not well enough nourished to continue rapid nitrogen fixation (Hinson and Hartwing, 1982). The maximum uptake of mineral nitrogen is reached at early flowering, followed by a rapid decline in absorption rate, while nitrogen fixation is maximized at early pod-filling and maintained at a high level until near physiological maturity (Cattelan and Hungria, 1994).

2.17 Factors effecting on N₂ fixation

Environmental factors affecting N₂ fixation include temperature, acidity, content and availability of nutrients such as nitrogen, phosphorus, calcium and molybdenum in the soil (FAO, 1984). It is often difficult to separate the effect of the above factors on inoculation success from their influence on symbiosis and nitrogen fixation. For example, acidity, as well as, calcium, aluminum and manganese concentration with interact and affect both bacterial proliferation, root-hair infection and plant growth (FAO, 1984). Temperature and moisture may also affect inoculation success with survival of root nodule bacteria affected by high temperature, although large differences intolerance to high temperature have been reported (ITTA, 1985).

The soil is not only a source of plant nutrients and water to the leguminous host plant but also is the medium from which nodules derive some of their nutrients because they seem to have this ability. Addition of organic matter in the soil enhances the number of nodules, the weight of nodules and the population of root nodule bacteria in the soil (Dart, 1974). A few definitive studies show that nodulation is more sensitive to NaCl and well symbiotic soybean is more sensitive than N-fertilized one (Bernstein and Ogata, 1966).

Low pH, low Ca, high Al and high Mn have received much attention as inhibitors of soybean nodulation (Muns, 1977). Calcium deficiency and perhaps acidity can inhibit nodule function as well as nodule formation. In addition, there are some microelements such as Cu, Mo, etc., which are needed for both plant as well as root nodule bacteria. The deficiency of the microelements for the root nodule bacteria consequently suppress nodulation or make the nodule ineffective for nitrogen fixation (Hallsworth, 1972; cited by Sinha, 1977).



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