Chapter 5

Discussion

The results (Figures 4.2 - 4.5, Tables 4.2 - 4.5) indicated that soybean *Glycine max* cv. CM 60 was suitable for growing in Leonard jars under the experimental conditions when both nitrogen-free media pH 5.0 and 6.8 were used. It is noteworthy that *Glycine max* cv. CM 60 (as well as SJ 4 and SJ 5) have been selected for use by Agricultural Research Stations in several parts of Thailand (Table 1.1). Judging from the results of the average plant dry weight, *B. japonicum* S78 was found to have higher nitrogen-fixing potential than *B. japonicum* S76 and S162 at pH 5.0. *B. japonicum* S162 was found to have higher nitrogen-fixing potential at pH 6.8 than the other two strains under the experimental conditions.

One remarkable finding was growth of *B. japonicum* S76, S78 and S162 was found in unbuffered pHs possibly due to slight alkalinization when grown in the acidic pH range and due to slight acidification when grown in the alkali pH range (Table 4.7, Figure 4.6). Table 4.7 indicated that the acidification of culture medium when the initial medium pH was in the alkali range was more marked than the slight alkalinization observed when the initial medium pH was in the acidic range. Suwat Saengkerdsub (1999) also found that when YMB medium was not buffered, the final pHs were changed to more suitable pHs for growth of *B. japonicum*. Glenn & Dilworth (1994) hypothesized that when the initial pHs were in the acidic range, alkali products might be secreted so the final pHs were increased. Similarly, when the initial pHs of the medium were in the alkali range, acidic products might be secreted by the cells resulting in less alkali final pHs. Previous results also indicated similar acidification and alkalinization of the unbuffered culture medium (Chansa-ngavej & Singhaboonpong, 1996).

As pH of the medium decreased, the number of *B. japonicum* cells were found to decrease. This result is in accordance with previously published results on the reduction of rhizobial number in acidic soils (Richardson & Simpson, 1988).

The determination of qualitative and especially the quantitative differences in polypeptide bands as shown in Figures 4.10-4.19 was difficult. Protein extraction and SDS-PAGE separation of soluble intracellular proteins had been performed at least twice

for each experiment. Although care was taken to determine the protein concentration carefully, it was found out that slight overloading or underloading of samples could lead to darker or fainter bands. All the quantitative differences in intracellular proteins reported in the experiments had been determined after careful comparisons of results of repeats of the experiments.

Intracellular protein profiles of mid-log phase *B. japonicum* S76, S78 and S162 cells grown in unbuffered media (Figures 4.7) did not reveal any qualitative or quantitative changes in intracellular proteins. It is interesting to note that in the central part of Thailand (Lop Buri, Sukhothai) soybean growers do not lime the soils before planting soybean seeds (Information from personal communication with officers of Lop Buri and Sukhothai Field Crops Research Stations). The results might preliminarily indicate that the use of SDS-PAGE did not detect marked qualitative and/or quantitative differences in proteins which might have taken place in response to growth in unbuffered media. The findings that there was neither qualitative nor quantitative change in intracellular protein profiles of *B. japonicum* S76, S78, S162 when cultured in buffered yeast extract mannitol medium at pH 6.0 to 9.0 might indicate that the three strains of *B. japonicum* which were isolated from acid soil, pH 5.25, could grow at a wide pH range.

After at least two extractions and SDS-PAGE separation of intracellular proteins of the three *B. japonicum* strains upon medium pH changes from pH 5.5 to pH 6.0, 6.5, 7.0 and careful comparisons of the protein profiles, it was determined that there were no changes in the intracellular protein profiles after the pH shifts as shown in the representatives of protein profiles in Figures 4.10 to 4.19 except some occasional disappearance or decrease in quantity of the 53 kDa polypeptide within the first 24h of the pH shifts. The findings that no other polypeptide was found to change in quantity when the 53 kDa was found to change in quantity might indicate the labile nature of the polypeptide. The labile nature of the 53 kDa polypeptide is interesting and is being under investigation. Preliminary results on SDS-PAGE separate of intracellular soluble proteins extracted from *B. japonicum* S76 after pH shifts from 5.5 to 6.0 for 45 minutes at 5 minute intervals did not reveal the disappearance of the 53 kDa polypeptide (Appendix E).

The experimental results revealed no major changes in intracellular protein profiles of *B. japonicum* S76, S78 and S162 except that a 53 kDa polypeptide was found

to be a labile protein which might disappear or decrease in quantity upon cell manipulation and pH shifts under aerobic conditions. It cannot at present be ruled out that expression of some genes may operate under anaerobic conditions. Considering that *B. japonicum* might thrive in a partially anaerobic condition underground where it is attracted to the soybean root rhizosphere for the initiation of the nodulation processes, one might be able to detect changes in gene expression governing the nodulation processes under (partially) anaerobic conditions. Changes of gene expression under (partially) anaerobic conditions in *B. japonicum* have never been explored.

Aliabadi et al (1988) reported that in *Salmonella typhimurium* the expression of the anaerobiosis – inducible gene, *aniG*, was controlled by changes in external pH under aerobic and anaerobic conditions. Maximum expression was observed under anaerobic conditions at an external pH of 6.0. Significant transcriptional activity was also observed under aerobic conditions at an external pH of 6.0. This was in contrast to *hyd* gene encoding hydrogenase in *S. typhimurium* whose expression was dependent upon anaerobiosis and varied with external pH. The pH dependence of *hyd* gene expression disappeared under fully aerobic conditions (Aliabadi et al, 1988).

Although the design of the experiments was in such a way that simulated the changes of pH upon liming of acid soils, it was discovered that the real life situation concerning the effects of changes of pH upon liming should include (partially) anaerobic conditions which are operating in the case of the presence of *B. japonicum* underground and moving towards germinating soybean seeds which are planted underground. It is expected that further experiments on SDS-PAGE separations of intracellular proteins from *B. japonicum* S76, S78 and S162 under (partially) anaerobic conditions might reveal changes in the protein profiles upon pH shifts. Partial anaerobiosis could be achieved by overlaying 250 ml flasks containing culture medium with sterilized paraffin oil.

Further Recommendations for Future Research

The research conducted for this thesis is only the tip of the iceberg. More comprehensive field research needs to be conducted on a large scale. For example, analyses of soils from sampling sites for pH, Al, available P, K^+, NH_4^+ and NO_3^-, before

and after lime application need to be carried out in order to assess the effects of liming on factors that may influence nitrogen fixing ability. Unkovich et al (1996) reported that in an acid soil pasteur of clover (*Trifolium subterraneum* L.) in southern Australia, liming increased the nitrate but not ammonia contents of the soils possibly due to beneficial effects of increased pH on nitrification. In fact, the influences of the biochemical reactions in the Nitrogen Cycle on nitrogen fixation and soybean productivity are largely unknown at present.

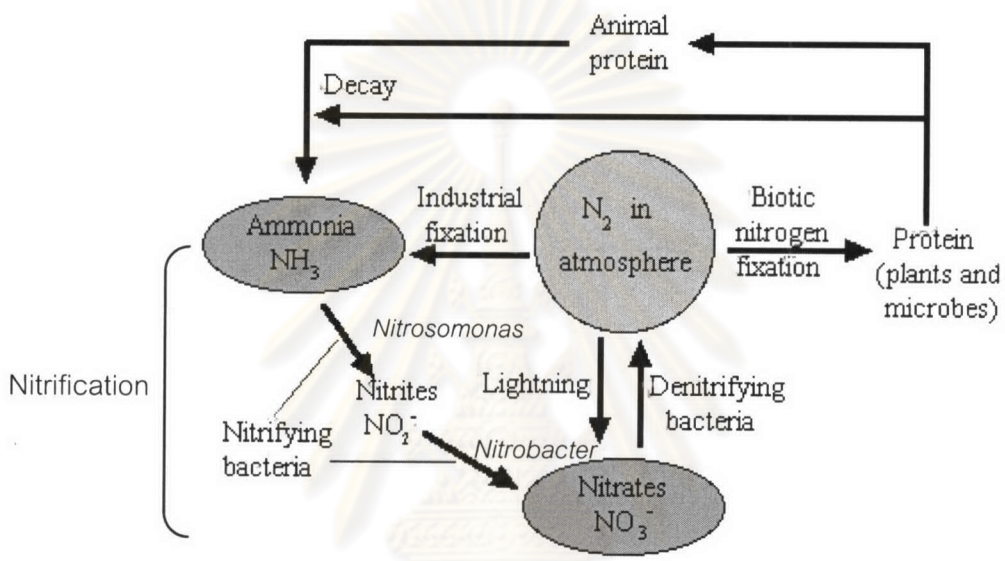

Figure 5.1 The Nitrogen Cycle (http://www.users.rcn.com)

Since nitrate concentrations are known to inhibit nitrogen fixation (Luciñski et al, 2002), Unkovich et al (1996) stated that in the case of clover (*Trifolium subterraneum* L.) and *Rhizobium trifolii* relationship while soil liming might reduce concentrations of toxic aluminium, the counter-active effect of increases in mineral N concentrations needed to be considered in order to improve soil environments to be conducive to sustained maximal symbiosis establishment and function.

2. In the experiments reported in this thesis, the three strains of *B. japonicum* cells had been acclimatized to pH 5.5 before the pH shifts to pHs in the acidic range (pH 6.0, pH 6.5) and the neutral pH. Therefore polypeptides which are responsible for acid tolerance should either disappear or reduce in quantities when cells were transferred to pH 7.0. In order to confirm the presence of proteins for acid tolerance in the three strains

of *B. japonicum*, mid-log phase cells grown in yeast extract mannitol broth (YMB) pH 7.0 could be transferred to YMB medium with acidic pHs. SDS-PAGE profiles of proteins extracted from cells grown in acidic pHs should reveal the appearance of the proteins for acid tolerance. Another set of interesting experiments to be conducted is the transfer of *B. japonicum* cells acclimatized to growth either at pH 5.5 or pH 7.0 to low buffered pH medium (pH 4.0, 5.0, to see if the phenomenon of Acid Tolerance Response (ATR) as reported by O' Hara & Glenn (1994) occurs.

3. In order to fine-tune the detection of differential gene expression in *B. japonicum* S76, S78 and S162 in response to pH shifts, intracellular soluble protein profiles could be obtained for cells grown anaerobically 1, 2, 3, 4, 5 and 6 hours after the pH shifts.

4. The nature of the acidic or the alkali products which *B. japonicum* secrete in response to growth in unbuffered yeast mannitol media pH 4.0-9.0 should be determined.