# CHAPTER I

## INTRODUCTION

Soybean rhizobia are nitrogen-fixing bacteria in root nodules of soybeans [*Glycine max* (L.) Merr]. At present there are three categories of soybean rhizobia : the fast-growers, the slow-growers, and the soybean rhizobia with variable generation times. Fast-growing soybean rhizobia were first isolated from root nodules of soybeans in the People's Republic of China (Keyser et al., 1982). In 1988, Chen et al. proposed the name *Sinorhizobium fredii* for fast - growing soybean rhizobia. Fast- growing soybean rhizobia with generation time between 1.2 to 4 hours consisted of two recognized species, *Sinorhizobium fredii* and *S. xinjiangense* (Chen et al., 1988, Peng et al., 2002). Slow-growing soybean rhizobia with generation time over 6 hours consisted of three recognized species (*Bradyrhizobium elkanii, B. japonicum,* and *B. liaoningense*) (Kuykendall et al. 1992 ; Jordan, 1982 ; Scholla and Elkan, 1984 ; Xu et al. 1995). *Mesorhizobium tianshanense* has variable generation times (Thomas - Oates et al., 2003).

Traditional authentication of fast- and slow–growing soybean rhizobia includes growth of isolates from soybean root nodules on yeast extract mannitol agar containing 25 $\mu g.ml^{-1}$ congo red. Slimy pinkish colonies were authenticated as soybean rhizobia by inoculation onto germination soybean seeds and detection of root nodules. These processes require approximately one month. The aim of this study is to develop multiplex PCR reactions to rapidly detect the presence of fast- and slow- growing soybean rhizobia.

According to Bergey's Manual of Systematic Bacteriology, Family Rhizobiaceae consists of four genera : *Rhizobium, Bradyrhizobium, Agrobacterium,* and *Phyllobacterium* (Jordan, 1984). Since multiplex PCR proposed to be developed in this research has to be specific for detection of fast- and slow- growing soybean rhizobia, the method must be tested against other plant – associated soil bacteria. Therefore, in

this research, developed multiplex PCR will be tested against DNA isolated from plant-associated *Agrobacterium tumefacians* TISTR 507 and *Xanthomonas campestris* TISTR 786 which causes bacterial spot disease on its host plants pepper and tomato. The bacteria enter the plant tissue via stomata or wounds and multiply in the intercellular spaces of the plant tissues (Stall, 1995). In addition, flagellated Gram - negative bacteria which produce extracellular polysaccharides such as *Proteus vulgaris* will also be tested for specificity of the developed multiplex PCR.

In order to design primers to differentiate between fast- and slow- growing soybean rhizobia in a multiplex PCR reaction, differences among the fast-and the slow-growing soybean rhizobia must be known. It is well established that fast- growing soybean rhizobia have no *nodY* while slow- growing soybean rhizobia contain *nodY* (Schlaman et al., 1998). Therefore, primers for the amplification of *nodY* are designed for use in the multiplex PCR which includes forward and reverse primers for *nodD1*. Since proteins NodD1 in fast- and slow- growers bind to different flavonoids in the perception process prior to nodulation, it was postulated that *nodD1* of fast- and slow-growing rhizobia may be different.

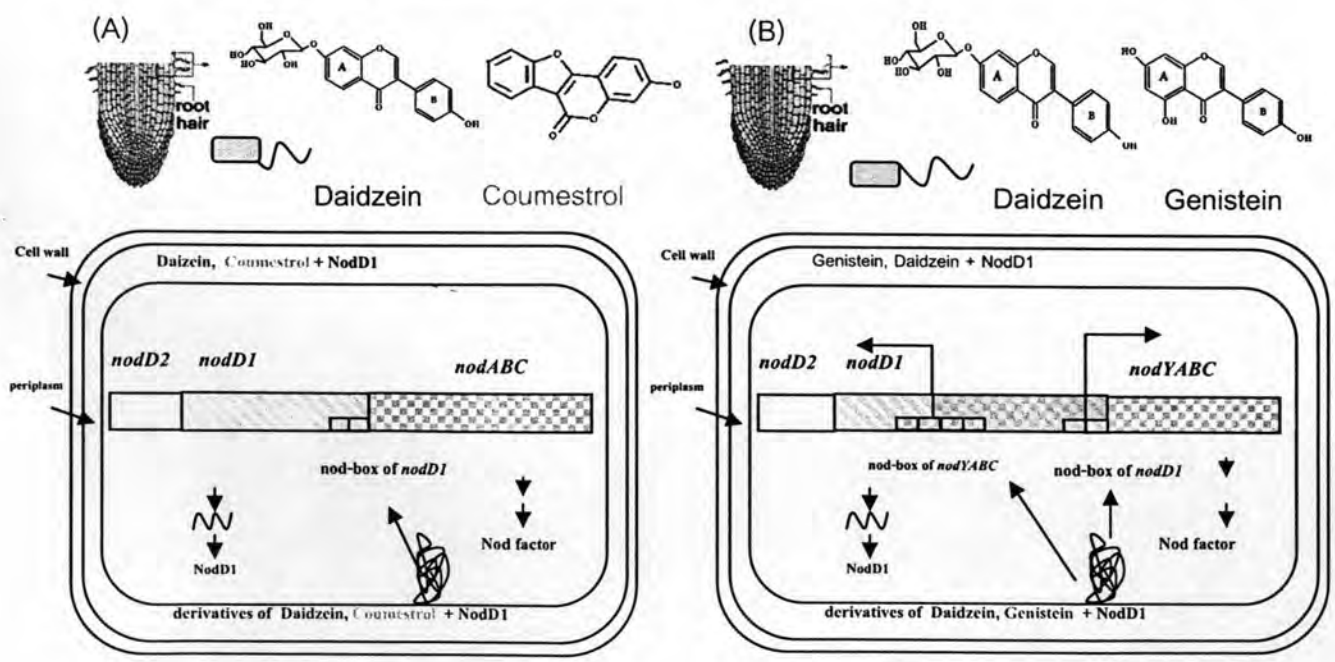


Figure 1.1 Diagramatic representations of early nodulation processes in (A) fast-growing soybean rhizobia (B) slow- growing soybean rhizobia.

Diagramatic representations in Figure 1.1 (A) and (B) show that roots of soybean cultivars such as cultivars McCall or Peking secrete flavonoids Daidzien or Coumestrol and fast- growing *S. fredii* or *S. Xinjiangense* move along the flavonoid gradients towards root hairs (Pueppke et al., 1998). On the other hand, roots of other soybean cultivars secrete flavonoids Genestein or Daidzein along whose gradients slow-growing soybean rhizobia move. Flavonoids diffuse into the periplasm of fast- growing or slow- growing respective soybean rhizobia where they bind to NodD1 proteins encoded by *nodD1* to form NodD1- flavonoid complexes which bind to promoter of *nodD1* called *nodD1* box to activate transcription of *nodD1*. Normally *nodD1* is constitutively transcribed. Upon binding of NodD1- flavonoid complex to *nodD1* box, the DNA is believed to bend to facilitate activation of transcription of *nodD1* (Fisher and Long, 1992). NodD1- flavonoid complex also binds to promoter of *nodABC* in fast-growing soybean rhizobia and to promoter of *nodYABC* in slow- growing soybean rhizobia to activate transcription of *nodABC* or *nodYABC* operons respectively. The structural genes *nodA, nodB,* and *nodC* encode enzymes NodA, NodB, and NodC which work in sequential order of NodC, NodB, and NodA. NodC is an N-acetylglucosaminyl transferase which catalyses the joining of N-acetylglucosaminyl units by ß - 1,4 linkages. NodB is an N- acetylase which catalyses the removal of an N- acetyl group from N- acetylglucosaminyl subunit at the non- reducing end. NodC is an N-acylase which catalyses the addition of an acyl group (C18:1) to the N-acetylglucosaminyl subunit at the non- reducing end. These three enzymes catalyse the synthesis of Nod- factors or lipo- chito- oligosaccharides. The role(s) of Nod- factors is still unknown (Stacey, 1995).

Once fast or slow- growing soybean rhizobia reach the root hairs, they attach to lectins which are carbohydrate- binding proteins specific for extrapolysaccharides produced by either fast- growing or slow- growing soybean rhizobia. At this stage, curling of root hairs is observed. Nod- factors may play a role(s) in the curling of root hair and the subsequent formation of an infection sac and infection thread which are invagination and elongation of cell membrane of root hair cells through which soybean rhizobia get into cortex cells of soybean roots to initiate cell division of cortex cells to form root nodules.

From the above- mentioned literature survey, it can be seen that fast- growing soybean rhizobia do not have *nodY* while slow- growing soybean rhizobia do have *nodY* . Also, NodD1 proteins produced by fast- growing and slow- growing soybean rhizobia bind to different soybean flavonoids. Therefore, it is likely that *nodD1* of fast- growing or slow- growing soybean rhizobia may be different. Hence, primers specific for amplification of *nodY* and *nodD1* are designed for use in multiplex PCR in this study.