Sorghum is one of the main staple foods in semi-arid tropics and ranks fifth among cereal crops in the world (Zohary and Hopf, 2000). Sorghum belongs to Gramineae family, subfamily Panicoideae and the tribe Andropogoneae. It is native to tropical and subtropical regions of Africa, Asia, and all continents in addition to the South West Pacific and Australia. The genetic variability of Sorghum is large, probably because the crop is grown under diverse agro climatic conditions which affect the grain composition (Burleson, et al., 1956; Waggle, et al., 1967; Deosthale, et al., 1972).

Sorghum have 30 different species, out of them Sorghum vulgare is main edible species. It is annual or short-term perennial grows up to 4 m or more height, panicle 8-40cm long, loose or contracted, sessile spikelets 4- 6 mm long. In India, Sorghum vulgare occupies an area of nearly 9.5 million hectares (25% of world sorghum area), 5.0 M ha in rainy season (Kharif) and 4.5 M ha in post rainy season (rabi) and the productivity average is 1000 kg/ha in the rainy season and 600 kg/ha in the post rainy season (Seetharama et al., 2006). In Rayalaseema the average yield of sorghum in last 3 years is 700 kg/ha (www.authorsteam.com).

In Andhra Pradesh the yield of sorghum was 1000 kg/hectare in 2003 (ICRISAT, 2004) and it was decreased to 470 kg/hectare in year 2010 (http://www.ibef.co.in/download/Andhra_Pradesh_060710.pdf). The area of sorghum crop also decreased to 9.5 M ha from 17.0 M ha in the last 30 years. Several factors were responsible for the declining trend in the production and crop area of sorghum. They are unpredictable and erratic distribution of rainfall
in Rayalaseema region, declining soil fertility due to nitrogen deficiency, some other biotic and abiotic stresses.

Sorghum is considered to be a “powerhouse of nutrition” (Schaffert and Gourley, 1982; Woods, 2000; Herbst, 2001; Hunsigi, 2007) and it is the nutritious cereal which makes healthy diet. *Sorghum vulgare* (100g) shows nutrient values as Energy- 1,418kJ (339kcal), Carbohydrates- 74.63g, Dietary fiber- 6.3g, Fat- 3.30g and Protein- 11.30g (Sankara Rao and Deosthale, 1980). Sorghum also having P (352 mg), Mg (171 mg), Ca (15 mg), Fe (4.2 mg), Zn (2.5 mg), Cu (0.44 mg), Mn (1.15 mg), Mo (0.06 mg) and Cr (0.017 mg). Sorghum is rich in sources of B-complex vitamins. Some yellow-endosperm varieties of sorghum contain β-carotene which can be converted to vitamin A by the human body. Detectable amounts of other fat-soluble vitamins, namely D, E and K, have also been found in sorghum grain (Taur et al., 1984).

Now-a-days Sorghum importance in dietary requirement and production of biofuels assumed significance. In drought areas, like Rayalaseema most of the people use *Sorghum vulgare* as food like Jonna rotte, Sangati, Soups, Cakes, Bread, starch. It also used as sorghum syrup or sorghum molasses (Ghanekar et al., 1992; Schaffert, 1992). Other than these uses, sorghum used for the production of Ethanol, Starch, adhesives and paper (Dayakar Rao et al., 2004; Huligo et al., 2004; Ratnavathi et al., 2004; Shukla et al., 2006; ICRISAT, 2006; Rao et al., 2006a; 2006b). *Sorghum vulgare* is heat tolerant or drought area crop, and are especially important in arid regions. Economic well
being of Chittoor District especially Tirupati surrounding rural people are very much dependent on Sorghum crop.

Sorghum also used as fodder for millions of the cattle. The FAO (2005) reported that 440,000 square kilometres were devoted worldwide to sorghum production in 2004. Sorghum grain is used primarily as a maize substitute for livestock feed because their nutritional values are very similar. Some of the Sorghum hybrids are commonly grown for feed to deter birds. It contains a high concentration of tannins and phenolic compounds, which allows the grain to be digested by cattle.

The sorghum shows nutraceutical property by inhibiting the protein glycation (Farrar et al., 2008). Sorghum is used for the treatment of celiac disease and wheat allergies due to its antiradical and antioxidant activities. It keeps bones and teeth healthy giving energy to the body and also it controls arthritis and weight of the body. Sorghum is rich in phenolics and tannins which are proven anticancer and cardioprotective constituents. By this, the demand of Sorghum crude oil in international market was increased (Yakadri and Murali, 2009). *Sorghum vulgare*, as herbal remedy, is capable of boosting blood levels (cure anemia), get rid of inflammation, pain and repeal cell damage (antioxidant). It raises cellular immunity in those who are gripped by Acquired Immune Deficiency Syndrome (AIDS) in human.

*Sorghum vulgare* is an exhaustive crop and needs more nutrients especially nitrogen. Nitrogen available in environment in many forms like
nitrogen gas (N\textsubscript{2}), ammonia (NH\textsubscript{3}) and nitrate (NO\textsubscript{3}⁻). However, nitrogen availability is limited in many soils, and although the earth’s atmosphere consists of 78% nitrogen gas (Howard and Rees, 1996), plants are unable to use this form of nitrogen. Nitrogen is a component of many biological macromolecules including nucleic acids and proteins.

The productivity will be decreased due to the deficiency of essential nutrients like nitrogen. *Sorghum* plants manifest the nutritional deficiency of nitrogen is noticeable by stunted growth and yellowing leaves starting at the bottom of the plant and moving gradually upward as nitrogen deficiency persists (http://www.thebestgardening.com/2010/01/plant-nutrition-needs-and-efficiencies.html).

To overcome the disadvantages by nitrogen fertilizers and availability of nitrogen to improve the yield of the crop, the farmers use different methods like chemical fertilization and Intercropping with legumes etc. The Intercropping system also has its own disadvantage like, reduction of nodulation and N\textsubscript{2} fixation in Groundnut (Nambiar et al., 1983). Nitrogen fertilizers keep nutrient levels at an optimum level, protect against disease and control weeds, resulting in healthier crops and consistent quality and quantity of yields. Other than the advantages of these nitrogen fertilizers they also show some disadvantages like, excessive application of commercial fertilizers can leads to excessive nitrate levels in forages, causing nitrate toxicity in the cattle (Halsey, 1998). Plants can not absorb excess nitrogen it shown to leaching into the groundwater nearby rivers and ultimately the ocean. High levels of nitrogen in the water can create
algal blooms, large growths of algae that imbalance the delicate ecosystem to the detriment of other aquatic species. The fertilizers became expensive day by day and its usage leads to increase in the cost of cultivation which cannot effort by the Rayalaseema poor formers, leads in the decreased productivity of Sorghum.

So, The Biological Nitrogen Fixation (BNF) is most suitable strategy, which accounts for up to 65% of the biosphere’s total available nitrogen. Majorly BNF is contributed by symbiotic interaction of bacteria of the genus *Rhizobium* with leguminous plant (Newton, 2000; Gage, 2004).

BNF having many advantages over nitrogen fertilizers by reducing costs of production. Field trials have been shown that the N captured by crops due to the use of *Rhizobia* inoculants costing $ 3.00/ha is equal to fertilizer N costing $ 87.00. Contamination of water resources from leaching and runoff excess fertilizer was decreased by the use of BNF. It improves the quality of dietary protein of legume seed (Silva and Uchida, 2000). The BNF efficiencies are at least twice that of nitrogen fertilizers, which are used to non-leguminous plants like *Sorghum vulgare*. 4.20 million tones of nitrogen is fixed in India through BNF per year where farmers expenses about Rs. 4410 crores every year for urea (http://www.iiss.nic.in/Technology9.doc). So, the economic, environmental and agronomic advantages of BNF make it a cornerstone of sustainable agricultural systems.
Some plant species formed mutualistic symbioses with nitrogen fixing prokaryotes and some eukaryotes. The organisms that can directly utilize atmospheric nitrogen as a nitrogen source are called diazotrophs. They are able to live independently in soil. These are belongs to the kingdoms Eubacteria and Archaebacteria (Young, 1992). Genera like *Rhizobium, Bradyrhizobium* and *Azorhizobium* are symbiotic with legumes where as the genus *Frankia* with some non-legumes plants. They fix nitrogen with the help of the enzyme nitrogenase. These organisms account for about 60% of the total annual fixed nitrogen (Kim and Rees, 1994).

Biological Nitrogen Fixation generally occurs under anaerobic or micro-aerophilic conditions, which converts atmospheric N$_2$ into ammonia (Arp, 2000). The legume-*Rhizobium* symbiotic system is the most efficient system which contributes more nitrogen to the ecosystem and to food production.

A number of non-leguminous plants are recognized to have the ability to fix nitrogen either through exogenous or endogenous symbiosis with N$_2$-fixing microorganisms. This can be a potential source of nitrogen for agriculture and can be of greater economic importance. Some cereal crops of commercial importance like rice, wheat, maize and millets are found to have association with microorganisms that are capable of assimilating atmospheric nitrogen. There is only one non-legume association reported, that of *Trema aspera* with a 'cowpea-type' strain of *Rhizobium* (Trinick, 1973). Researchers have to make various attempts to extend the host range of *Rhizobium* from legumes to non-legumes through genetic manipulation.
The efforts to make cereal crops especially Wheat, Rice and Sorghum relatively self reliant in nitrogen demands are desirable because of i) Heavy requirements for nitrogenous fertilizers which are manufactured from non-renewable energy resources, ii) economic reasons, especially in developing countries, iii) Low efficiency of nitrogen fertilizer use by plants and iv) Environmental pollution due to potential nitrogenous fertilizers (Azam, 2002).

In the present study, we selected *Sorghum vulgare* as experimental plant to provide associative nitrogen fixation to decrease the usage of nitrogen fertilizers and increase the yield of the crop for rainfed cultivated farmers. The transfer of *nod* genes from *Rhizobium* species along with inducers for nodulation was considered to be the most suitable strategy to achieve symbiotic nitrogen fixation in non-legumes. Peoples and Crasswell, 1992; Wani and Lee, 1992, screened and selected for efficient Rhizobial species from different legume plants and obtained *Rhizobium meliloti* from Alfalfa plant, which fixes high nitrogen content i.e., 100-300 Kg/ha. We also followed the same procedure for screening and selection of efficient Rhizobial strain for induction of nodules and nitrogen fixation in *S.vulgare*.

In recent years molecular genetic analysis of nodulation (*nod*) genes have been in the focus of various laboratories studying *Rhizobium*-legume interactions. In numerous species and strains of the three genera, *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, *nod* genes have been identified and sequenced (These studies have led to the assignment of over thirty nodulation genes). Nodulation genes are named as *nod* or *nol*.
In *Rhizobium meliloti* the essential *nod* genes were localized on indigenous plasmids (also called symbiotic or *sym* plasmids) in two clusters, located about 25 and 13 kb down-stream from the *nifHDK* operon on an 8.5 kb and on a 6.8 kb ECORI fragment, respectively (Kondorosi *et al.*, 1984). By Site directed Tn 5 mutagenesis, a *nod* gene cluster of about 2.5 – 3.0 kb was found within the 8.5 kb region. These results suggested that the 8.5 kb fragment contains *nod* genes determining functions necessary for nodulation of a wide range of legume hosts. The other, 6.8 kb region contains two *nod* gene regions which probably determine host specificity of nodulation (*hsn* genes) (Kondorosi *et al.*, 1984). *Nod* and *hsn* genes of *Rhizobium*, named as *nodA, nodB, nodC, nodD, nodE, nodF, nodG, nodH, nodI, nodJ, nodK, nodL, nodM, nodN, nodO, nodP, nodQ, nodS, nodSU, nodT, nodU, nodV, nodW, nodX, nodY, nodZ, nolA, nolE, nolFGHI, nolP.*

The host range of the plant–bacterial interaction is governed by the nodulation (*nod*) genes. Some of these *nod* genes, *hsn* genes affect host specificity. The other genes (common *nod* genes include *nodABC* and *nodD*) perform general functions which are necessary for nodulation of any host (non-specific) (Long, 1989a). *hsn* genes are generally differs from common *nod* genes and they are not found similar in all strains and species of *Rhizobia* (Kondorosi *et al.*, 1984). *nodD* is a member of *LysR* family transcriptional regulator (Henikoff *et al.*, 1988), positively regulates the transcription of approximately 25 *nod* genes required for the production and export of Nod factor, in response to flavonoids (Schell, 1993; Gage, 2004). The cellular
location of \textit{nodD} is cytoplasm and cytoplasmic membrane. (Mulligan and Long, 1985; Rossen \textit{et al.}, 1985; Gottfert \textit{et al.}, 1986; Horvath \textit{et al.}, 1987). \textit{nodD} also plays a crucial role in the early recognition events between \textit{Rhizobia} and legumes (Gyorgypal \textit{et al.}, 1991; Sclaman \textit{et al.}, 1992).

The induction of \textit{nodABC} and \textit{hsn} genes requires the regulatory gene \textit{nodD} in conjunction with a plant signal, flavonoid (Firmin \textit{et al.}, 1986; Peters \textit{et al.}, 1986; Redmond \textit{et al.}, 1986; Zaat \textit{et al.}, 1987; Gyorgypal \textit{et al.}, 1991; Demont \textit{et al.}, 1994). \textit{nodD} binds to a 50-bp conserved DNA region, called the nod box (Rostas \textit{et al.}, 1986), upstream of the inducible \textit{nod} genes. NodD is DNA binding protein, which is encoded by \textit{nodD} gene acts as a positive transcription activator (Hong \textit{et al.}, 1987; Fischer \textit{et al.}, 1988). It is the medium sized protein with 32-36 KDa molecular weight. NodD protein contains a common motif in their DNA target sites, designated the LysR motif (Goethals \textit{et al.}, 1992).

The regulation of nodulation gene expression by NodD in \textit{Rhizobia} was reviewed by Schlaman \textit{et al.}, (1992). NodD shows certain flavonoid specificity that restricts \textit{nod} gene induction in plants and takes part in determining host specificity (Horvath \textit{et al.}, 1987; Spaink \textit{et al.}, 1987). Under normal laboratory growth conditions, \textit{nodD} is expressed constitutively (Rossen \textit{et al.}, 1985) but the other \textit{nod} gene operons are not induced.

\textit{pGEMT} easy vectors are linearized vectors with a single 3\textsuperscript{1}- terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve
the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases (Mezei and Storts, 1994; Robles and Doers, 1994). These are high copy number vectors containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows identification of recombinants by blue/white screening on indicator plates. By these advantageous properties we used pGEMT vector (Promega) in the present study to ligate amplified gene.

Subsequently pET-24a was used as binary vector. The pET-24a vector carry an N-terminal T7 tag sequence plus an optional C-terminal His tag sequence. This vector differs from pET21a only by their selectable marker (Kanamycin vs. ampicillin resistance). Unique sites are shown on the circle map (Fig 3.3). The sequence is numbered by the pBR322 conversion, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown in Fig 3.3. The f1 region is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer. pET-24a is a 5310 bp plasmid. It is linearized vector with a single 3'-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR
products generated by certain thermostable polymerases (Mezei and Storts, 1994; Robles and Doers, 1994).

An *Escherichia coli* strains like BL21, BL21DE3, BL21DE3 strain is general protein expression strain that lack both the lon protease, which can degrade proteins. The genotype of this strain is *E.coli* BF-dcm ampT hsnS (rB-mB-) gal λ(DE3) these competent cells that provide varying levels of expression control with T7 promoter-driven vectors, such as the pCAL vectors and the pET vectors.

The induction method for BL21(DE3) competent cells is Isopropyl-1-thiol-β-D-galactopyranoside (IPTG) induction of T7 polymerase from lac UV5 promoter. The BL21(DE3) competent cells are an all-purpose strain for high-level protein expression and easy induction.

The root exudates of leguminous plants contains compounds such as flavonoids and betaines, produced via the isopropanoid pathway, which induces *nod* genes to synthesize Nod factor (Broughton *et al.*, 2000; Perret *et al.*, 2000). Flavonoids are a class of natural AEIs (Auxin Effluent Inhibitors), some of which can regulate PIN (pin-shaped inflorescence) activity and localization (Peer and Murphy 2007). Flavonoids are synthesized by all plants. They have diverse structures and many functions, eg: they can act as antioxidants, enzyme regulators, and molecular signals for rhizobial *nod* gene expression, flower pigments, UV protectants and antimicrobials (Winkel-Shirley, 2001). Flavonoids interact specifically with the protein product of the
nod gene and the active form of NodD is believed to activate transcription through promoters of nod operons. This response is even clearer in these rhizobia for which nod itself was activated by the NodD-flavonoid complex (Burn et al., 1987). The product of nodD gene in Rhizobium interacts with the flavonoid compounds secreted by the roots and this follows the activation of other nod genes in a host dependent manner (Sanjuan et al., 1994).

Flavonoids are group of ubiquitous and diverse molecules produced via the phenylpropanoid pathway in higher plants. They have diversified in form and function with the evolution of land plants. Flavonoid biosynthesis occurs in most plant parts, flavonoids play variety roles in plants including protection against UV damage and pathogenic microbes, acting as pigments or co-pigments in influencing flower color, modulating auxin distribution, and as signal molecules to symbiotic microbes (Taylor and Grotewold, 2005). The role of flavonoids in nodulation is intriguing because it is possible that these compounds might play multiple roles during the process. Legume-rhizobial nodulation begins with signal exchange between the symbiotic partners. This is initiated by flavonoid and isoflavonoid compounds exuded through the plant roots (Peters et al., 1986). The rhizobial symbionts recognize specific flavonoid signals produced by compatible host legumes and respond by producing a novel, lipo-chitooligosaccharide signal (Nod signal). The type of flavonoid molecules secreted by the plant and the ability of the rhizobial species to recognize these molecules, and then induce Nod signal biosynthesis is the earliest step in determining host-specificity (Fisher and Long, 1992). On the
roots of a compatible host plant, these bacterial Nod signals induce a series of physiological and developmental responses leading to the formation of a functional nodule. One of these responses is cell division in cortex cells to initiate the nodule primordium. Thus, flavonoids might play a crucial role as auxin transport regulators during nodulation (Mathesius et al., 1998). The third role that flavonoids play during nodulation is to act as Nod signal inducers inside the plant roots. This secondary induction of Nod signals is thought to be responsible for an additional level of host specificity (Madsen et al., 2003; Parniske and Downie, 2003).

For several decades, flavonoids were largely presumed to function solely as inducers of rhizobial nod gene expression (nod genes are associated with Nod signal biosynthesis in Rhizobia) and as chemo-attractants to concentrate the compatible Rhizobium at the root surface (Rolfe, 1988; Stougaard, 2000). However these roles were based largely on in vitro studies. For example, exogenous addition of isoflavones was shown to induce the expression of nod-lacZ fusions in Bradyrhizobium japonicum (Banfalvi et al., 1988). Such studies strongly suggested that the flavonoids has essential role in the nodulation process. But no genetic evidence to suggest a definitive role of flavonoids in nodulation, because of the altering flavonoid profiles of a legume species.

Apart from other compounds in root exudates, flavonoids are known as key signaling compounds in a number of plant-microbe interactions. In roots of legume nod factors induce the accumulation of flavonoids resulting in the
secretion of more flavonoids by the root, which further stimulate the production of nod factors by the bacteria (Recourt et al., 1992, Dakora et al., 1993, Schmidt et al., 1994, Bolanos-Vasquez and Werner 1997). Apart from their function in the Rhizobium-legume interaction, flavonoids also act as signaling compounds in the arbuscular micorhizal symbiosis and in different plant-soil pathogenic interactions (Recourt et al., 1992).

There are several reports on induction of nodule-like structures termed as para-nodules on cereal roots using different plant hormones like 2,4-D, NAA, BAP and Zeatin. Among different plant hormones, 2,4-D is a synthetic auxin, was found to be the best in inducing nodular outgrowth in cereals (Ridge, 1992). The term para-nodules were introduced by Tchan and Kennedy (1989) to describe the chemically induced nodules, since they differ from the naturally occurring legume nodule. These induced nodule-like outgrowths are modified lateral roots with carbon reserves (as starch in amyloplasts) similar to those found in the cortex of roots, and microorganisms are able to modulate or interfere with the development of these outgrowths (Ridge, 1992).

It was suggested more than 50 years ago that hormones were involved in nodule induction on plant roots and this concept was demonstrated by the use of cytokinin which could induce psuedonodules on tobacco roots (Arora et al., 1959) by cortical cell division. One of the early steps in nodule induction, could be induced by auxins and cytokinins excreted by the infecting *Rhizobia*. In Alfalfa roots, localized trans-Zeatin produced by *R.meliloti* was found to induce the formation of nodule-like structures (Cooper and Long, 1994). The
use of 2,4-D and polyethylene glycol is loosening the cell wall of roots which helps the entry of *Rhizobia* into the roots and form nodule like structures (Cocking *et al.*, 1995; Christiansen-Wengier, 1996).

A number of laboratories have pursued plant transformation methods to avoid tissue culture or regeneration. In many cases these methods have targeted meristems or other tissues that ultimately give rise to gametes (Chee and Slighton, 1995; Birch, 1997). Successful *Agrobacterium*-mediated transformation is routinely used for the transformation of many monocots by tissue culture methods. It has been reported in Rice (Hiei *et al.*, 1994; Aldemita and Hodges, 1996; Rashid *et al.*, 1996; Hiei *et al.*, 1997; Toki, 1997), Maize (Ishida *et al.*, 1996), Barley (Tingay *et al.*, 1997), Wheat (Cheng *et al.*, 1997) and recently, Sorghum (Zhao *et al.*, 2000; Henrique *et al.*, 2004). The following multiplicity of factors that influence transformation is probably the reason for *Agrobacterium*-mediated transformation in monocotyledonous plant species has been difficult to achieve (Ishida *et al.*, 1996; Hiei *et al.*, 1997). Several factors are important in transformation, the type and developmental stage of the infected plant tissues, the concentration of *Agrobacterium tumefaciens*, the composition of the media used for co-cultivation and tissue culture, the selectable marker genes used, the type of vector and the plant genotype being among the factors that influence transformation (Hiei *et al.*, 1997). A critical point in developing an efficient transformation protocol is to find the right combination of the many factors that act together during transformation.
Scope of the study:

Development of any technologies for increased nitrogen use efficiency or decreased usage of nitrogenous fertilizers is research priority, especially in crops like *Sorghum vulgare*, which is mostly grown by rainfed resource farmers. New agriculturally significant properties may not necessarily have to arise by selection from natural populations, but through genetic manipulations can yield plants with new qualities.

If a BNF system could be assembled in the non-legume plants, it could increase the potential for nitrogen supply because fixed nitrogen would be available to the plants directly, with little or no loss. Such a system could also enhance resource conservation and environmental security, besides saving farmers from the economic burden of purchasing fertilizer nitrogen for crop production. Thus, a significant reduction in the relative use of fertilizer N can be achieved if atmospheric N is made available to non-legumes directly through an effective associative system with some of the characteristics of legume symbiosis. Recently, several approaches were developed in the area of recombinant DNA technology to succeed this objective.

Our main focus was on establishing efficient colonization of *Rhizobia* for significant Biological Nitrogen Fixation in non-leguminous Sorghum plant and also observing changes in root morphology.

In the present study, we studied the induction of the *para*-nodules by cloning process for introducing of *Rhizobium nodD* gene (which is regulatory
and transcribes the other *nod* genes) into *Sorghum vulgare* by *Agrobacterium*-mediated *in planta* technique. Simultaneously, *Sorghum vulgare* seedlings and plants were treated with 2,4-D and flavonoid Naringenin.

**Objectives of the study:**

The main objective of the present study is to produce the Nodulation in non-leguminous plant *Sorghum vulgare* by *Agrobacterium*-mediated transformation of *nodD* gene and treating the plantlets with 2,4-D and flavonoid Naringenin.

Another objective is to develop an easy, efficient and rapid protocol for the genetic transformation of the *nodD* gene into *Sorghum vulgare* without involving tissue culture.