Chapter 1

INTRODUCTION

Hevea brasiliensis, the Para rubber tree, often simply called *rubber tree*, is a perennial tropical plant, belonging to the family Euphorbiaceae (Archer & Audley, 1987) and is one of the most economically important members of the genus *Hevea*. It is of major economic importance because of its sap-like extract (known as latex) which is the primary source of natural rubber. Of the ten latex bearing species reported (Wycherley, 1992), *Hevea brasiliensis,* accounts for 99% of world's natural rubber production (George and Panikkar, 2000). The rubber tree is a renewable, sustainable, non- polluting and environment friendly source of elastomer in sharp contrast to synthetic rubber manufactured from petroleum base (Jacob, 2000).

The rubber tree initially grew only in the Amazon Rainforest. The discovery of the vulcanization procedure in 1839 and the demand for natural rubber led to the rubber boom in that region. However, Brazil was not the site of successful commercialization of rubber. Instead, rubber cultivation got established in the Southeast Asian regions. Since it was abundant and cheap, rubber became a major industrial raw material. Now, rubber is harvested in Africa, Central and South America and in Asia. Hence rubber, a rain-forest species of the Amazon Basin has become an Asian crop, with Asia producing 92% of the world's natural rubber in Malaysia, Thailand, India, Sri Lanka, China and South Vietnam.

In India, its cultivation was started in 1870's by the British and by the first half of the 20^{th} century many plantations were successfully established in the country. The growth attained by the Indian rubber plantation industry since its commercial beginning in 1902 has no parallel in the agricultural scenario of the country. The Indian rubber plantation industry is ahead of all the other major natural rubber producing countries in the world in terms of growth, productivity, production and the extent of price realization at the farm gate.

Equatorial monsoon climate prevailing in the tropics in between l0°S and 8°N latitudes is the best suited for rubber cultivation. The climatic conditions in this region include plentiful rainfall (about 2000 mm or more per year and equally distributed round the year), warm and humid conditions, absence of prolonged drought and extremes of temperature. Also rubber thrives best in deep well-drained loamy soil, covered by natural undergrowth or leguminous cover-crop and protected from erosion (Reed, 1976). It tolerates but does not flourish in areas with pronounced dry seasons and temperature fluctuations. It also tolerates some water logging and a wide range of pH (4–8).

The traditional rubber growing region in India stretches from Kanyakumari district of Tamil Nadu in the South to the South Canara district of Karnataka state in the North. This is a narrow stretch of about 700 km long along the foot hills of the Western Ghats up to an altitude of about 450 metres above MSL. Six agro-climatic sub-regions can be identified in the traditional rubber growing belt like Kanyakumari**,** South, Central and North Kerala, South West Karnataka**,** and tropical high altitude. Although agro-climatic conditions are generally suited for rubber cultivation in the traditional belt, regional variations exist in the amount and distribution of annual rainfall, duration of rain free summer, temperature fluctuations, etc. Well distributed rainfall in the Kanyakumari region is one of the most important climatic factors that is responsible for making plantations in this region the most productive.

The growing demand for natural rubber all over the world has emphasized the need for substantial increase in the total production of natural rubber. Productivity enhancement alone cannot bridge the gap between production and consumption and the only alternative is to extend the area of cultivation. Scope for expansion of the crop in the traditional belt is little due to non-availability of cultivable land. The only practical solution therefore is to extend rubber cultivation to non-traditional regions where the climatic conditions are not optimum. Rubber is now being cultivated in non-traditional regions like Tripura, Assam, Meghalaya, Nagaland, etc. in the north eastern regions, Maharashtra and Goa in the North Konkan region and West Bengal, Orissa, Andhra Pradesh and Madhya Pradesh in the eastern region. Though warm humid equable climate and a well distributed annual rainfall of 2000 mm are ideal for optimum growth and yield of *Hevea*, the weather parameters in such non-traditional areas are not quite favourable as these regions experience various abiotic stresses like either extreme drought or low temperature.

A number of abnormal environmental parameters such as drought, cold, high temperature, high light intensity, nutrient imbalances, etc. either alone or together affect the growth and yield of *Hevea* in both traditional and non- traditional regions. Abiotic stresses lead to dehydration or osmotic stress through reduced availability of water for vital cellular functions and maintenance of turgor pressure. Stomata closure, reduced supply of CO_2 and slower rate of biochemical reactions during prolonged periods of dehydration, high light intensity and high and low temperature conditions lead to increased production of Reactive Oxygen Intermediates (ROI) in the chloroplasts ultimately ending up with irreversible cellular damage and photo inhibition.

Apart from these abiotic stress factors, *Hevea* also undergoes other stress factors such as tapping induced wounding, stimulation of yield by ethephon (which increase the internal ethylene level), wintering related senescence and various diseases. Ethephon stimulation which is practised in *Hevea* to increase rubber yield leads to long term damages to the bark. Such high intensity of latex harvest leads to a phenomenon called Tapping Panel Dryness (TPD) commonly known as brown bast, during which the latex vessels in the tapping panel exhibit intermittent dry regions that do not ooze latex thus eventually resulting in cessation of latex flow and drying of entire panel. In most cases, dryness spreads to the adjacent virgin panel, affecting the entire circumference of the trunk. TPD is a major concern as it renders the tree non-productive thus leading to loss of revenue.

Apart from these factors, there are other biotic factors that affect the productivity of *Hevea*. The major diseases affecting *Hevea* are pink disease, black stripe (black rot), patch canker, dry rot, mouldy rot, bark necrosis, etc. (Kothandaraman and Idicula, 2000). Brown root disease, white root disease, red root rot, dry root rot, stinking root rot, *Poria* root rot, black root disease, purple root disease are the major root diseases (Rajalakshmy and Jayarathnam, 2000). About 90 species of fungi are known to attack *Hevea* trees, the most prevalent ones being *Botryodiplodia elactica* and *B. theobromae, Colletotrichum heveae* (leaf spot), *Fomes lamaensis* (brown root rot), *Gloeosporium heveae* (die-back), *Oidium heveae* (powdery mildew), *Pellicularis salmonicolor* (pink disease), *Phytophthora* *palmivora* (causing fruit rot, leaf-fall, black thread, and die-back), *Polystichus occidentalis* and *P. personii* (white spongy rot), *Sphaerella heveae* (rim bright), *Sphaerostilbe repens* (red rot) and *Ustulina maxima* (charcoal rot) (Reed, 1976).

Most of the diseases and some of the pests that occur on rubber in the traditional belts are also seen in the non- traditional areas, with varying levels of intensity and damage. In all the states of the north eastern region, powdery mildew is the most severe one. Wide spread occurrence of *Gleosporium* leaf disease, abnormal leaf fall (caused by *Phytophthora*), *Corynespora* leaf disease, Pink disease, Brown root disease, etc. are widely seen in non-traditional areas.

The most important disease of rubber in India is abnormal leaf fall caused by *Phytophthora* for which rainfall is the most important predisposing factor influencing its initiation and spreading. *Corynespora* leaf fall caused by the fungus '*Corynespora cassiicola'* is currently the most destructive leaf disease affecting rubber in many Asian and African countries. The pathogen affects young and old leaves in both immature and mature rubber. It causes leaf spot and eventually leaf fall. High humidity and a temperature between 28 and 30 ºC, humid air and cloudy weather favour the spreading of disease in the entire plantation during refoliation period (Situmorang *et al*., 1996). Cassicolin, the phytotoxin produced by this fungus is responsible for the *Corynespora* leaf fall disease.

In India, clones found susceptible to *Corynespora* leaf disease include RRII 105, RRII 118, RRII 300, RRII 305, PR 107, PR 255, PR 261, RRIM 600, PB 86, PB 235, PB 255, PB 260, PB 311, Gl 1 and Tjir 1 of which the susceptibility of RRII 105 is of major concern due to the large extent of area it occupies (Jacob, 1997). The disease can be controlled by physical, chemical and biological control methods. In India, 1% Bordeaux mixture or 0.24 percent Zineb (Dithane Z78) was earlier recommended for control of *Corynespora* disease in nurseries (Ramakrishnan and Pillai, 1961b). Later 0.1% carbendazim (Bavistin) spray (Rajalakshmy *et al*., 1980) and 0.2% mancozeb (Dithane/Indofil M45) (Jacob, 1997) were also recommended.

Among various control measures, biological methods are preferred as these methods are eco- friendly and do not have any side effects. Biological control method has emerged as an effective and economic method for disease management (Jeyarajan *et al*., 1999). Biological control of plant pathogen naturally occurs at some level in all agricultural ecosystems. Now a days more importance is given to bio control method of disease control in order to minimise the use of hazardous chemicals. Bio control agents have the potential to supplement (or) replace chemical pesticides. They also offer additional advantage like improved plant growth which is not possible with the latter. For e.g. Bio protectants in the rhizosphere can protect the plant from soil-borne plant pathogens throughout the crop growth periods. The need for such alternative disease control measures to supplement or replace chemical fungicides was realized as a result of higher expenditure involved, apart from its threat to the ecosystem and the possible development of resistance in pathogens.

In the case of fungal infection, biological control is accomplished by degradation of chitin in the cell wall of the fungus. Chitin is an insoluble, linear polymer of β-1, 4 linked NAG (N-acetyl glucosamine) residues found in fungal cell wall as well as in exoskeleton of insects and nematodes, which are major pathogens and pests of crop plants. It is the most abundant nitrogen bearing organic compound in nature. Chitinases are ubiquitous enzymes of bacteria, fungi, animals and plants which can hydrolyze the β-1, 4-linkage between N-acetyl glucosamine residues of chitin. The development of research in plant defence mechanisms has led to special attention on chitinases, since they were the first pathogen-induced proteins whose function was identified. Though in *Hevea*, chitinase is expressed as a pathogenesis related (PR) protein (Neuhaus, 1999), its levels are insufficient to prevent the development and spread of the fungal disease. If the levels of chitinase could be raised by some means, the spreading of the disease could be prevented.

Plants harbour a heterogeneous population of both pathogenic and nonpathogenic endogenous microorganisms that include fungi, bacteria, actinomycetes, etc. Their association has substantial impact on plant health and fitness while they can be detrimental, neutral or beneficial. Detrimental microbes include plant pathogens, while beneficial microbes (bacteria form a major portion of total endophytes) play an important role in the sustainability of agro ecosystems.

Beneficial microbes are generally used to promote plant growth and to control various diseases (Manjula *et al*., 2002).

Endophytes can also protect host plants from insect herbivory (Clay, 1988; Clark *et al.,* 1989) and other fungal pathogens (Carroll, 1988). They are also being used as bio regulators to induce resistance against diseases, as biological control agents against certain pathogens (Bissegger and Sieber, 1994) and also in the biological control of undesirable weeds (Dorworth and Callan., 1996). Endophytes could be isolated from surface of disinfected plant tissue or extracted from the plant, if they are found not visibly harming the plant, e.g. *Bacillus, Agrobacterium, Enterobacter, Pseudomonas*, etc. They have been found to occur in seeds, ovules, stems, leaves, fruits and tubers. Endophytic bacteria are also reported to inhibit growth of pathogens by production of antimicrobial compounds like antibiotics (Leyns *et al.,* 1990) and siderophores (Schroth and Hancock, 1981). Some endophytic bacteria are having antagonistic action against some of the fungal pathogens also. Several mechanisms are known to be involved in antagonistic interaction. Antibiosis is one of the major mechanisms involved in antagonism.

Recent studies revealed the presence of endosymbionts such as *Bacillus subtilis* in the intercellular regions of almost all parts such as leaf, stem, bark, etc. of natural rubber trees (Philip *et al*., 2005). As endosymbionts are generally known to play a major role in rendering resistance against fungal pathogens and in growth regulation, presence of endosymbionts in *Hevea* could be made advantageous in so many ways. If they can be genetically manipulated to deliver specific gene products into the host system with chitin degrading genes like chitinase, spread of fungal diseases can be prevented. This approach also has an edge over the conventional genetic transformation of the host system (the rubber plant) with genes imparting various stress tolerance. The transformed *Bacillus* cells can be specifically applied just before the onset of the diseases on the vulnerable parts of the plant thus avoiding the whole crop transformation.

Bacillus belongs to the phylum firmicutes, the low G+C gram-positive bacteria. The genus *Bacillus* is the largest in the order Bacillales. The genus contains gram positive endospore forming, chemo heterotrophic rods that are usually motile and peritrichously flagellated. It is aerobic, or sometimes facultative, and catalase positive. *B. subtilis* is a ubiquitous bacterium commonly recovered from water, soil, air and decomposing plant residue. The bacterium produces an endospore that allows it to endure extreme conditions of heat and dessication in the environment.

As the genomic DNA sequencing had been completed in various strains of *B. subtilis*, it is of major importance in scientific and industrial field. *B. subtilis* is totally harmless to man and has infact rendered him invaluable services. Its ability to grow *in vitro* and to produce abundant quantities of enzymatic substances is widely used by the food industries, detergent manufacturers and some textile industries. It is an important source of production of enzymes such as amylase, protease, etc.

B. subtilis is being used in commercial production of extra cellular enzymes, for e.g. alpha-amylase of *B. amyloliquefaciens.* Some strains produce insect toxins, peptides, antibiotics and antifungal agents. Some are being used in agricultural crop protection purposes. *B. subtilis* is also being used as fungicide on flowers and ornamental seeds and on agricultural seeds including cotton, vegetables, peanuts, soybeans etc. This is also being used as a soil inoculant in horticulture and agriculture. This organism competes with certain disease causing fungus by colonizing the newly developing root system of the plant. *B. subtilis* is also being used as a model organism, since it is highly amenable to genetic manipulation.

All the scientific and industrial applications of *B. subtilis* have previously been on trial and error basis of various experimentations. The availability of its complete genomic information makes it possible to identify and understand the action of about 4,000 coding genes that control its function, its regulation. It is also possible to choose the sites of foreign gene integration to improve efficiency of the cloned gene and its expression.

B. subtilis has been considered as an attractive host because of several advantages such as:

- it is non pathogenic and is considered as a Generally Regarded As Safe (GRAS) organism.

- it has no significant bias in its codon usage
- it is capable of secreting functional proteins directly into the culture medium (at present about 60% of the commercially available enzymes are produced by *Bacillus* species) and
- a large body of information concerning transcription, translation, protein folding, secretion mechanisms, genetic manipulation and large scale fermentation have been already acquired (Harwood, 1992; Meima *et al.,* 1995; Westers *et al.,* 2004; Wong, 1995).

Expression of recombinant genes in host cells are largely obtained with the help of expression vectors which are often derivatives of plasmid pBR322 containing the necessary transcription and translation start signals. They also have useful restriction sites next to these sequences so that foreign DNA can be inserted with relative ease. Some expression vectors contain portions of the *lac* operon and can effectively regulate the expression of the cloned gene.

Employing the genetically modified endosymbionts to save the plants from biotic and abiotic stress situations is now considered a better alternative to the conventional methods or the transgenic plants. The transformed endosymbionts if applied to the vulnerable parts of the plant before the onset of the stress or disease can save the plants from various stresses by producing the appropriate protein. Also if the transformed cells can colonise the intercellular regions of a developing embryo, they can confer disease or stress resistance to the plant. On these bases, this research work was conceived and executed with the following objectives:

- To standardize an efficient transformation protocol for endosymbiotic *B. subtilis* cells.
- To genetically alter a recombinant vector for the constitutive expression and secretion of antifungal protein/chitinase in endosymbiotic *B. subtilis* cells.
- To validate the expression of chitinase in transformed *B. subtilis* cells.
- And to explore if this could be employed as an efficient system to prevent the spread of fungal disease (*Corynespora* leaf disease) in *Hevea*.

The basic concept of this study is to employ the native endosymbionts of *Hevea* which can survive in the intercellular regions of various tissues of *Hevea* to express gene of interest. This system of transformation does not affect the plants' genetic set up as integration of foreign gene in its (*Hevea*) genome is not envisaged. This system of over-expression can be utilized only when it is required i.e. just prior to the onset of the *Corynespora* leaf fall incidence. So, the advantageous factors such as the relative easiness with which the cells can be transformed and multiplied, higher levels of expression of desired protein and the possibility of choosing the right time of application (i.e. just prior to the onset of disease/stress situations) makes this system of gene expression a more advantageous one.

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