SUPEROXIDE DISMUTASE GENE INTEGRATION AND EXPRESSION IN TRANSGENIC *HEVEA BRASILIENSIS*

THESIS

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May 16, 2005

Certificate

This is to certify that the thesis entitled "Superoxide dismutase gene integration and expression in transgenic *Hevea brasiliensis*" is an authentic record of original research work carried out by Mrs. Sobha S., at the Rubber Research Institute of India, Kottayam- 686 009 under my supervision for the award of the degree of Doctor of Philosophy in the Faculty of Science, Mahatma Gandhi University, Kottayam. It is also certified that the work presented in this thesis has not been submitted earlier for any other degree or diploma.

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DECLARATION

I hereby declare that this thesis entitled "**Superoxide dismutase** ase **gene integration and expression in transgenic *Hevea brasiliensis*"** is an authentic record of the research work carried out by me under the supervision of Dr. A. Thulaseedharan, at Rubber Research Institute of India, Kottayam. The work presented in this thesis has not been submitted earlier for any other degree or diploma at any university.

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CONTENTS

1

INTRODUCTION

In nature, plants are often exposed to a variety of abiotic stresses such as drought, temperature extremes, salinity, heavy metal toxicity and high CO_2 content. These environmental stresses are serious problems in agriculture as it adversely affects the growth and development of plants, causing significant yield reduction in most of the crops (Grover *et al.*, 2003*)*. An underlying mechanism for reduction in crop yield under stress conditions is excessive production of reactive oxygen species (ROS) that can damage lipids, nucleic acids, and proteins, leading to disruption of normal physiological processes. Green plant tissues may produce more ROS than animal tissues, because plants generate oxygen during photosynthesis and consume it during respiration. Further, because of their growth under high light intensities and a high cellular concentration of dioxygen, plants are subjected to oxidative stress. Increased antioxidant enzyme activities have been reported in response to heat and light conditions that cause sunscald in vegetables, fruits and flowers (Rabinowitch and Sklan, 1980). Therefore, there is a continuous need to scavenge these ROS to maintain the cell's integrity. Genetically modified plants which over-express antioxidant enzymes or some defense related proteins have better chance of survival under the stressed environmental conditions than non modified plants (McKersie *et al.*, 1999).

Natural Rubber (NR), has been used as an industrial raw material for the manufacture of about 35,000 products essential for mankind. NR has been reported to occur in more than 2000 plant species. Majority of them belong to the family *Moraceae, Euphorbiaceae* and *Appocynanaceae.* However, the rubber content in all these species is not adequate to cultivate them commercially as a source of natural rubber (George *et al.*, 1980). Occurrence of latex in the plant parts is a common feature to all these species. *Hevea brasiliensis* (para rubber tree) belonging to the family *Euphorbiaceae* is the major source of commercial natural rubber mainly because of its abundance in latex, quality and convenience of harvesting. In *Hevea brasiliensis*, natural rubber (cis-1,4-polyisoprene) is synthesized in specialized cells called laticifer cells (latex vessels) and stored in the form of latex.

Latex is a specialized form of cytoplasm present in almost all parts of the plant and is abundant in the laticiferous cells distributed in the bark of the main trunk. It is harvested from mature rubber trees through a process of controlled wounding of the bark, termed tapping (Fig. 1). The latex thus obtained by regular tapping of the mature trees contains about 30-45% rubber (Nair, 2000). In India rubber is cultivated in about 5.7 lakh hectares with an annual production of about 7.1 lakh tones (Rubber Statistical News, 2004). India ranks fourth among the major natural rubber producing countries in terms of total production and first in terms of productivity. The highest productivity could be attributed to the RRII 105 clone evolved by the Rubber Research Institute of India with an average yield of 2210 kg/ha/ yr (Mathew, 2005).

H. brasiliensis is an open pollinated, highly heterozygous perennial tree crop with a long breeding cycle. It is propagated using seeds or through vegetative propagation by bud grafting. Seeds were used as the planting material during the early periods of rubber cultivation. The major disadvantage of seed propagated trees is the lack of genetic uniformity, which results in wide variation in growth, yield, disease tolerance and other secondary characters. Later bud grafting became the accepted practice of propagation for commercial planting in all rubber producing countries. Bud grafting involves the grafting of suitable buds (scion) from high yielding trees on to assorted root stocks. The new clone thus formed is a two-part tree, comprising of a root system belonging to the stock part and a shoot system contributed by the bud of the donor. The advantage of bud-grafting is the uniformity exhibited by different individuals due to the uniform genetic constitution of the scion (Saraswathyamma *et al.*, 2000). However, a major disadvantage of this technique in rubber is that, the root stocks are derived from open pollinated highly heterozygous seedlings and hence lead to stock-scion interaction leading to intra-clonal variations in field performances (Combe, 1975; Seneviratne and Flegmann, 1996).

Increased crop productivity is the primary aim of any plant breeding programme. Hybridization and selection is the most important conventional method of *Hevea* breeding. This involves the process of repeated back-crossing and selection, which will take several generations before a set of desirable genes could be transferred to a cultivar. However, several factors restrict improvement of *Hevea* by conventional means. The perennial nature of the crop, the long juvenile period,

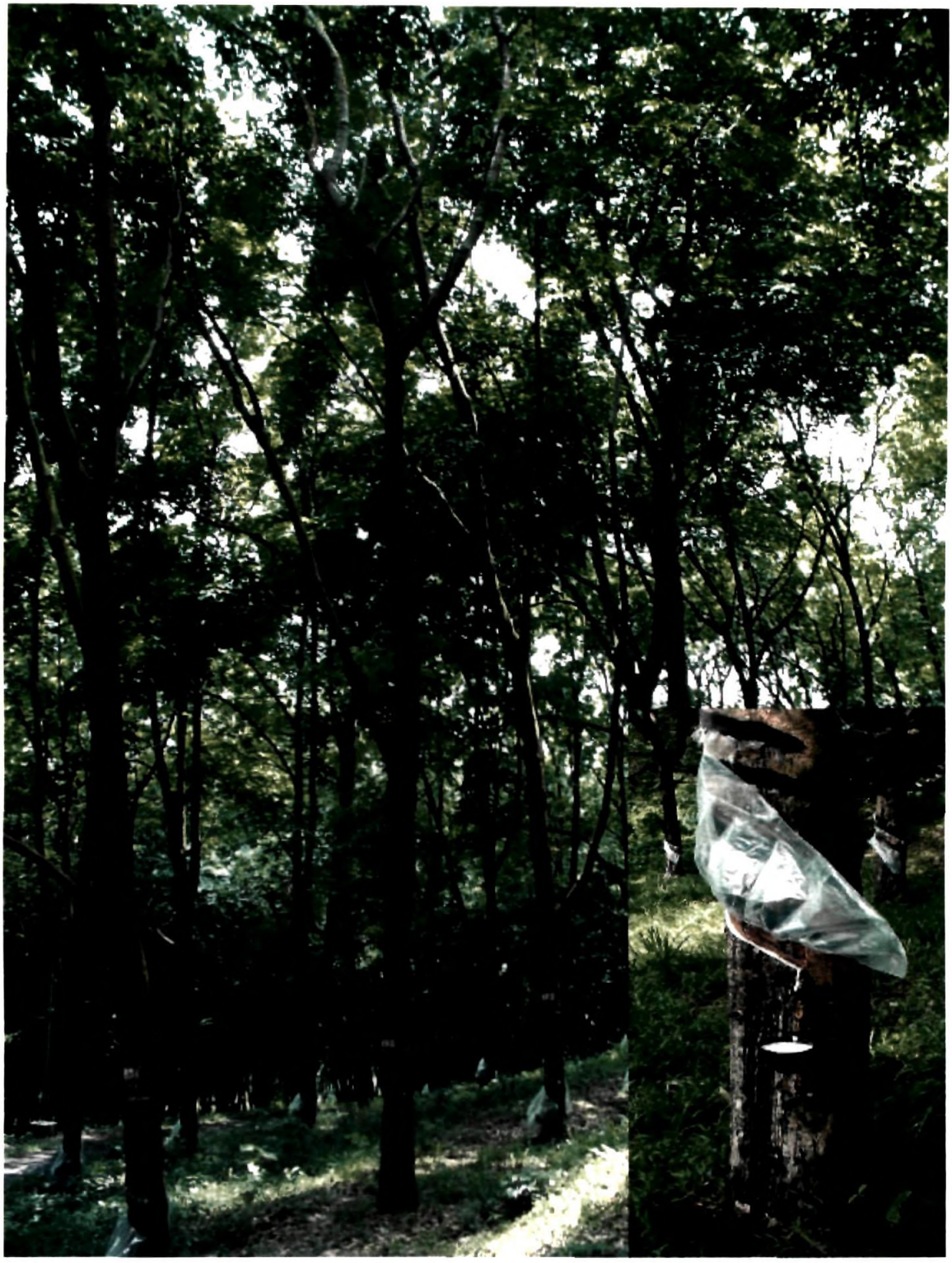


Fig. 1: A mature rubber plantation. Inset shows the trunk of a single tree under tapping

the heterozygous nature etc. are the main barriers for the slow progress in *Hevea* breeding. Further, attempts to generate hybrids are limited because of the seasonal and asynchronous flowering among different clones and poor seed set (below 5%) (Kavitha *et al.*, 1989). Transfer of useful traits from distantly related species which do not sexually cross with the crop plant is not possible through conventional breeding (Paroda, 1999).

With the ever increasing demand - supply gap in NR on the one hand and constraints in conventional breeding on the other, efforts on Research and Development, towards crop improvement are being extended to the generation of new clones through modern biotechnological approaches. Advances in molecular and cell biology over the past few decades have led to the development of a wide range of techniques for manipulating genomes. Genetic engineering is one approach that enables the transfer of genes of interest across sexual incompatibility barriers. Moreover, genetic transformation offers a novel approach to breeders by adding valuable genes for specific characters in a relatively short period of time. Regeneration of transgenic plants could result in accelerated crop improvement in both quality as well as quantity in a sustainable manner. Foreign gene integration by genetic engineering could be considered as analogous to plant breeding by hybridization, but it provides more precise and accurate adjustments to the genome (Sharma *et al.*, 2003a). The modification of plant genome through genetic engineering techniques have opened new vistas for the production of crop plants and forest trees with increased resistance to salinity stress, high temperature, disease, insect pest, drought and other environmental conditions.

Increasing demand for NR led to wide scale rubber cultivation in India not only in the traditional tracts of Kerala and Kanyakumari districts of Tamilnadu, but also to non-traditional/marginal areas of Karnataka, Maharashtra, Orissa, West Bengal and the North Eastern states which are exposed to a wide range of biotic and abiotic stresses. Unlike migratory birds and animals, plants cannot run away from adverse environmental conditions and they are often stuck where they germinate. They are exposed to variety of biotic and abiotic stresses in coordination with the changing environment. Plants respond to various abiotic stresses by altering their metabolic events, in order to adapt maximally to the changed environmental conditions. In plants, reactive oxygen species are naturally generated during plant metabolism and are enhanced as a consequence of various abiotic stresses like water

deficit, salinity, high light intensities (Scandalio, 1990; Gressel and Galum, 1994) and also, during infection by various plant pathogens. Several environmental stresses also impose their detrimental effects on plants via toxic oxygen species, which include air pollutions (ozone, sulphur dioxide), transient drought, cold and water logging (Bowler *et al.*, 1992; Gressel and Galum, 1994).

Oxidative stress is a constant burden of plants resulting from toxic oxygen species, such as superoxide anion, hydroxyl radicals and additional toxic oxygen species. An excess of ROS, causing oxidative stress, is harmful to cells as these molecules can damage nucleic acids, proteins and peroxidation of lipid membranes. The accumulation of ROS, particularly superoxide anion, causes a process of oxidative deterioration that ultimately leads to cell death (Thompson *et al.*, 1987). Therefore, protection of the cells from the damaging effect of free radicals is highly essential for their survival in aerobic environment. In *Hevea brasiliensis,* latex exploitation by the intensive tapping also induces wound related stress reactions in cells. Further, rubber yield in *H. brasiliensis* is closely related to resistance of laticiferous systems to oxidative stress.

In *Hevea brasiliensis*, tapping panel dryness (TPD), characterised by the browning and drying of the bark of the tapping panel, is a serious problem affecting mostly the high yielding clones as well as plants subjected to intensive tapping. TPD is generally considered as a physiological disorder. The first visible symptom is continued dripping of latex with low dry rubber content. Sometimes the initial symptom is developed as the partial dryness of tapping panel and the bark turns to light brown in colour. Significant yield loss occurred all over the rubber growing countries due to the incidence of TPD. Management of TPD is of potential economic interest as it renders the trees unproductive. Chrestin (1989) reported that TPD affected trees exhibited high levels of NAD(P)H oxidase activity, which leads to the formation of reactive oxygen species like superoxide dismutase (O_2^-) that damage lutoid membranes resulting in the cessation of latex production. In TPD affected bark, the free radical accumulation was found to be high with a reduced level of SOD activity (Das *et al.*, 1998).

Plants have evolved several defense systems against oxidative stress including the accumulation of low molecular weight compounds and enzymes involved in detoxification of reactive oxygen species (Scandalios, 1993).

Superoxide dismutase (SOD) [EC.1.15.1.1] is an essential and perhaps the most important component of the ROS scavenging mechanism, because it accelerates the spontaneous dismutation of superoxide radical to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Fridovich, 1986). Peroxidase and catalase enzymes eliminate the hydrogen peroxide. Thus the combined action of these enzymes convert superoxide radical and hydrogen peroxide to water and molecular oxygen, thereby abating the formation of the most toxic and highly reactive oxygen species.

Over-production of SOD to enhance the oxidative stress tolerance is envisaged ultimately, possibly resulting in increased crop yields under environmental stress conditions. Conventional method of *Hevea* breeding will take years to develop plants suited to such environmental conditions and tolerant to TPD. Moreover, *Hevea* being sensitive to *Agrobacterium,* availability of plant regeneration system through somatic embryogenesis and easy vegetative propagation that are rare in other woody species, make rubber plants amenable to genetic improvement through genetic transformation approach.

In view of the above facts the present study was under taken with the following **objectives**:

- Genetically transform *Hevea brasiliensis* callus with the cDNA sequence coding for Superoxide dismutase under the control of Figwort Mosaic Virus 34 S promoter.
- Optimize the conditions for plant regeneration from *Hevea* transgenic callus via somatic embryogenesis.
- Confirmation of the gene integration and the copy number through molecular approaches.
- Evaluate the over-expression of the integrated gene in response to abiotic stress at the mRNA and enzyme level.

2

REVIEW OF LITERATURE

Hevea brasiliensis is the major source of commercial natural rubber, which accounts for 99 percent of the world natural rubber production and 40 percent of the elastomer sale market. Over 90 percent of the natural rubber is produced in South East Asia. The traditional rubber cultivation area in India is Kerala and the adjoining districts of Tamilnadu and Karnataka states. Pressure on availability of land and socio-economic constraints necessitates the expansion of rubber cultivation to the non-traditional/marginal areas, which are exposed to extreme climatic conditions such as severe cold, elevated temperature and light intensities, leading to severe abiotic stresses. Tapping panel dryness (TPD), often associated with high yielding clones with extensive tapping, is another serious problem in rubber plantations all over the world resulting in significant yield loss. It has been reported that TPD is always associated with an accumulation of free radicals. Therefore, a high level of superoxide dismutase in the bark and latex could detoxify free radicals thus providing protection against TPD. Conventional methods of breeding would take several years to develop plants capable of withstanding such stress situations and for maximizing productivity in these areas. This necessitates the need for developing transgenic plants integrated with the gene coding for superoxide dismutase for tolerance to environmental stress as well as TPD.

The conventional methods of *Hevea* breeding and major constraints in crop improvement by conventional breeding are reviewed. Biotechnological approaches for crop improvement and the feasibility of developing transgenic *Hevea* plants integrated with the gene coding for superoxide dismutase, for enhanced tolerance to abiotic stresses and TPD is also reviewed. Several reports are available pertaining to model plants and other annual crop plants engineered for environmental stress tolerance. However, with respect to tree crops such information is limited.

2.1. Crop improvement in *H. brasiliensis*: Conventional methods, achievements and constraints

The original genetic material of the Para rubber tree, *Hevea brasiliensis* (Wild.ex Adr. De Juss.) Muell. Arg was introduced to South East Asia by Sir Henry Wickham in 1876 from the Amazon river basin in Brazil, the center of diversity of the genus (Annamma and Kavitha, 2000). During the early phase of breeding, latex yield per se was the main objective, and systematic breeding and selection resulted in a nearly ten fold increase in average yield i.e. 300 kg/ha/yr in the original material to an average yield of 2500 kg/ha/yr for the modern clones. Thus rubber breeding is indeed one of the outstanding success stories of this century (Simmonds, 1985; Annamma *et al.*, 1990). However, there are several problems associated with conventional breeding and selection that hamper quick release of clones.

H. brasiliensis is a predominantly cross pollinated, perennial tree crop. Crop improvement is mainly effected through hybridisation and selection. Artificial pollination between selected parental clones, evaluation of F_1 hybrids and selection of promising recombinants for commercial planting has been and still is the most important method of developing clones with desirable genetic constitution. The best genotypes in one generation (clones) are used as parents in the next cycle breeding from which improved genotypes are selected. This process was referred to as cyclical 'Generation-wise Assortative Mating' (Simmonds, 1985). Accordingly, primary clones developed from seedling trees served as parents in the first hybridisation series. The second and third level hybrids were evolved by crossing selected hybrid clones. These clones are evaluated in three consecutive phases viz., small scale, large scale and block trials. In India, breeding work was initiated since 1954, the programme is continuous and hand pollination is attempted every year. Clones designated as RRII 100, 200, 300 and 400 series were developed during the past few decades. Among these, RRII 105 is an earlier and most promising selection, resultant of a cross of two primary clones Tjir 1 and Gl 1 (Nair and George, 1969; Nazeer *et al.*, 1986; Annamma *et al.*, 1990).

The major difficulty in the aforementioned procedure is that this method is elaborate and time consuming, requiring about 30 years from nursery evaluation to the final release of a clone. There is no shortcut to systematic exploration of

large number of progenies at least during the initial stages of evaluation. Even though this concept of 'promotion plots' was introduced in Malaysia, by taking the promising seedlings from nursery directly in to a large-scale trial, it could not substitute the current procedure (Simmonds, 1985). Moreover, the nature of flowering and fruit set is yet another bottleneck in hybridisation programmes. In the traditional rubber-growing tract in India, flowering is restricted to only 1-2 months during February to March. This short period and also non–synchronization of flowering in certain potential clones, limits the production of sufficient legitimate families for making effective selection as well as the attempts for the desired cross-combinations. The average fruit set resultant of controlled pollination is generally less than 5%, even under best climatic conditions. The success rate varies widely with clones and seasons from less than 1 to 12% (Kavitha *et al.*, 1989; Chandrasekhar *et al.*, 2005).

Ortet selection or mother tree selection, the oldest breeding method involves systematic screening of seedling plantations for superior mother trees. Rapid progress with mother tree selection was achieved in Indonesia and Malaysia, which resulted in the release of a large number of early primary clones like Tjir 1, PR 107, GT 1, Gl 1 etc. Seedlings, though not comparable with high yielding clones in production potential, have special agricultural merits like resistance to diseases, drought etc. In India RRII 5, a promising selection for yield and RRII 33, a clone tolerant to abnormal leaf fall caused by *Phytophthora spp,* are resultant of ortet selection programme. The ortets also have to be evaluated in the same manner as that of hybrid clones. Nevertheless, now a days extensive seedling areas have been replaced by modern high yielding clones (Simmonds, 1985; Annamma and Kavitha, 2000). Apart from these, special techniques like polyploidy and mutation breeding have also been attempted on a limited scale, but the progress in these fields is rather slow (Markose *et al.*, 1981; Saraswathyamma *et al.*, 1980; 1988).

Lack of reliable early selection methods is yet another problem confronting the attempts to reduce the breeding cycle. Studies on the correlation between nursery level and mature phase yield have revealed only low to moderate correlation (Ong *et al.*, 1986) and selection based on nursery yield could only be a fair indicator of the mature yield, so that only mild selection should be adopted at

the immature phase to avoid the risk of loosing potential high yielders as well as useful recombinants for secondary characters (Simmonds, 1985).

At present, extensive areas are being cultivated with a handful of high yielding clones, which are more or less closely related. Genetic studies in general, have indicated inbreeding depression and unpredictable interactions when related parents are used for breeding (Gilbert *et al.*, 1973; Nga and Subramaniam, 1974). The performance of clones varies across locations because of genotype X environment interaction (Paardecooper, 1964; Jayasekera *et al.*, 1977). Efforts are taken to study the extent of interaction between genotype and environment so as to pick out the ideal clone for specific locations. RRII 105 clone performs well in the traditional tracts but it is susceptible to drought in terms of growth (Saraswathyamma *et al.*, 2000)

Traditionally the elite clones of *H. brasiliensis* plants are easily propagated through bud grafting which involves the grafting of selected clones on to seedling rootstocks. This ensures genetic homogeneity of the scion in this highly heterozygous species. Monoclonal seedlings are generally used as stocks which are not always vigorous, due to the possibility of inbreeding depression. As an alternative, polyclonal seed gardens with proven clones are to be raised for the production of good polycross root stocks, which again is a cumbersome procedure (Simmonds, 1985). In *Hevea*, the root stocks which are derived from cross pollinated seeds are highly heterozygous and hence lead to stock-scion interaction leading to intra-clonal variations in field performances (Combe, 1975; Seneviratne and Flegmann, 1996). In addition, the interaction of stock-scion on productivity and other secondary characters still remains an area less understood (Annamma and Kavitha, 2000). Propagation by root cuttings, though feasible in rubber, has the disadvantage that such plants lack tap roots and are wind susceptible (Simmonds, 1985). The micropropagation of elite clones with its own root system could reduce intra-clonal variation due to stock-scion interaction.

Despite the success of conventional crop improvement methods in evolving high yielding clones, rubber breeding has not yet gone far in breeding for specific character/situations. Essentially a small holder's crop, rubber is being pushed to poorer soils in drier, marginal areas experiencing various stress environments including drought, diseases, frost, wind etc. As such, specific priorities in breeding objectives vary with changing agroclimatic condition. Due

to the directional selection for yield, genetic improvement of many secondary characters influencing yield and overall performance has been ignored. Pressure on land availability and socio-economic constraints, further necessitates alternative approaches for developing clones capable of withstanding stress situations and hardy environments.

2. 2. Biotechnological approaches for crop improvement in *Hevea brasiliensis*

In recent years, the global consumption of natural rubber is steadily increasing and the production has also to be increased so as to meet the demand. Further, many of the recently developed high yielding *Hevea* clones are susceptible to one or more of the undesirable traits such as tapping panel dryness (TPD) - a physiological disorder, drought, leaf fall diseases caused by *Phytophthora* spp: and *Cornyespora* and, other environmental stresses like elevated temperature, high light intensity etc. The crop loss due to these traits is very heavy and there is an urgent need to introduce resistant genes into the high yielding clones. The narrow genetic base, highly heterozygous nature, the long breeding cycle, low seed set etc. are the major limitations of crop improvement by conventional breeding.

2.2.1. Marker assisted selection

Isozymes, microsatellites and restriction fragment length polymorphism (RFLP) have already been applied to rubber tree to investigate the polymorphisms between clones (Chevallier, 1988; Leconte *et al.*, 1994). Genetic diversity of the *Hevea* germplasm, including wild and cultivated materials, has been studied with isozyme and nuclear RFLPs (Chevallier, 1988; Besse *et al.*, 1994; Seguin *et al.*, 1996). Genetic mapping for rubber tree would be useful, in order to gain a better knowledge of the genome organization to enable comparison with other genera of the *Euphorbiaceae* family such as cassava to depict genetic basis of key quantitative traits such as resistance to South American Leaf Blight (SALB) and to develop marker-assisted selection for these traits (Fregene *et al.*, 1997). A saturated genetic linkage map of rubber tree (*Hevea* spp.) was developed based on RFLP, AFLP, microsatellite and isozyme markers by Lespinasse *et al.* (2000). Molecular techniques like RAPD, microsatellite, AFLP etc. could be effectively used for the development of molecular markers and genetic analysis in *Hevea*

(Venkatachalam *et al.*, 2004; Bindu Roy *et al.*, 2004). Molecular marker-assisted selection will help the plant breeder to identify and select the desired plants at an early stage and hasten the transfer of desirable genes in to cultivars (Mohan *et al.*, 1997).

2. 2.2. Development of *in vitro* plant regeneration system

Although, elite clones of rubber plants can be propagated easily through bud grafting, the interaction of uniform scions with the unselected seed derived stocks (stock-scion interaction) is a major problem. Development of protocols for the *in vitro* propagation of selected genotypes is highly desirable for mass production of true-to-type individuals (Thulaseedharan *et al.*, 2000). Even though, several reports on *Hevea* micropropagation using different explants, mostly derived from seedlings are available, an efficient protocol for large-scale micropropagation of elite *Hevea* clones is yet to be evolved.

Tissue culture in *Hevea brasiliensis* was initiated by Bouychou in 1953 with the objective of producing callus for the study of laticiferous system (Bouychou, 1953). This line of research was taken up by Chua in 1966 at the Rubber Research Institute of Malaysia (Chua, 1966). He observed callus induction from epicotyl and green stems of young *Hevea* seedlings on MS medium provided with a high concentration of sucrose (100 g/l). Later, Wilson and Street (1975) reported the initiation of cell suspension culture by sub culturing this callus in liquid medium and green shoots were obtained from these cell suspensions on medium containing 2-chloroethylphosphonic acid (2-CEPA) (Audley *et al.*, 1975; Audley and Wilson, 1978). Paranjothy and Ghandimathi (1975) described the culture of shoot apices derived from 2 to 3 week old aseptically grown seedlings. They obtained rooted plantlets within 4 weeks of culture in liquid MS medium. However, these shoots failed to grow on solid medium. Later Enjalric and Carron (1982), using shoots derived from 1-3 year greenhouse grown seedlings as explants, developed rooted plants.

The explants derived from elite clones of mature *Hevea* trees are highly recalcitrant and only limited reports are available on successful micropropagation using clonal materials (Thulaseedharan *et al.*, 2004). The major problem with clonal material from mature trees is the failure of producing adequate root system with taproot quality necessary for tree stability (Carron and Enjalric, 1983). The

root systems of axillary bud originated plants of juvenile origin did not contain a tap root, though the tree growth observed in the field was comparable to that of embryo cultured and bud grafted plants which contained tap root systems (Seneviratne *et al.*, 1995). Sinha e*t al.* (1985) succeeded in developing shoots by axillary bud culture from different clones but they failed to obtain rooting. Asokan *et al.* (1988) reported shoot and root development from the shoot tips derived from clonal trees (GT 1) for the first time, on a medium containing (1.5-3.0 mg/l) IAA and (0.5-1.5 mg/l) Kn. The rooted plantlets were successfully transplanted in the field. Seneviratne and Flegmann (1996) obtained multiple axillary shoot production from nodal explants of juvenile origin on a medium containing thidiazuron at (0.02 mg/l) in combination with NAA (0.2 mg/l). Shoot elongation was obtained on a growth regulator free medium. Elongated axillary shoots produced root in the presence of IBA (2.0 mg/l). Mendanha *et al.* (1998) described shoot development from axillary buds cultured on MS medium supplemented with (1.0 mg/l) of each Kn and 2,4-D, 2% sucrose and 4% agar. Shoots developed from axillary buds rooted vigorously when cultured on MS medium provided with NAA, IAA and IBA. Physiological stage of the explants was found to have significant role in micropropagation and detailed studies were carried out by many researchers. Rejuvenated explants by micrografting and buds of nodal explants taken from dormant branches were found to exhibit better *in vitro* response (Seneviratne and Wijisekara, 1997; Lardet *et al.*, 1998). Conditions for an efficient and reproducible system for *in vitro* micrografting for the induction of explant rejuvenation as well as for the rescue of important difficult to root plant materials were standardized by Kala *et al.* (2002).

Although, progress has been achieved on micropropagation using shoot tips and axillary buds, many problems like explant viability, bud sprouting, shoot elongation and difficulty in producing taproot still persists. Even though extensive work has been done over the past few decades on *in vitro* micropropagation of *Hevea*, large scale commercial application of tissue culture techniques is still not available (Thulaseedharan *et al.*, 2004).

Somatic embryogenesis pathway could overcome difficulties with clonal propagation of difficult to root tree species and accelerate the introduction of improved clones into commercial production and provides a rapid and reliable

regeneration system amenable to genetic manipulation studies for alteration of tree genomes (Evers *et al.*, 1988).

2. 2.3. Somatic embryogenesis as a means of micropropagation

Somatic embryogenesis, the formation of embryos from somatic cells has been proved as a promising means of micropropagation. Somatic embryogenesis serve as a potential tool for plant propagation since 1958 when the plant embryos were obtained from somatic tissue of carrot culture *in vitro* (Steward, 1958) and an ever increasing number of species have been induced to form somatic embryos. Somatic embryos derived from the somatic cells closely resemble their sexual counterparts even though they have bypassed genetic segregation and recombination. Consequently, this represents a method of clonal propagation maintaining the genotype of the plant from which the explant tissues are derived (Akins and Vasil, 1988). Thus somatic embryogenesis could be used as a tool in maintaining fidelity among regenerants (Parrot *et al.*, 1991). Plant regeneration via somatic embryogenesis has recently become an attractive tool for the production of transgenic *H. brasiliensis* plants, because of its single cell origin (Sushamakumari *et al.*, 2000).

2.2.4. Somatic embryogenesis in *Hevea brasiliensis*

Experiments on somatic embryogenesis of *Hevea brasiliensis* was initiated by Satchuthananthavale and Irugalbandra (1972) with the aim of obtaining haploid plants by culturing anther. Satchuthananthavale (1973) reported callus formation from anther tissues and pollen grains. Paranjothy and Ghandimathi (1975; 1976) induced callus from segments of epicotyl or hypocotyl and pieces of cotyledons on MS medium with low levels of hormones or in the absence of hormones. However, they failed to develop plantlets from this callus. Chen *et al.* (1978) reported the development of first pollen plant in *Hevea* by pollen culture in MS medium supplemented with 2.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l kinetin (Kn). Wang *et al.* (1980) succeeded in developing and transplanting somatic plants derived from anther wall. Carron (1981) used integument tissue of seed for somatic embryogenesis and subsequent plantlet development. Carron and Enjalric (1982) cultured anther wall for callus induction and subsequent embryoid development. Later Wang *et al.* (1980) developed 130

somatic plants from Chinese clone Haiken 1 and SCATC 88 - 13 clones by anther culture and some of these plantlets were successfully transferred to soil. Gao *et al.* (1982) developed somatic plants from un- pollinated ovule. Shijie *et al.* (1990) developed pollen plants from 13 clones and they have been successfully transplanted in the field. With the available reports dealing with *in vitro Hevea* regeneration, it could be said that the regeneration ability of *Hevea* is highly genotype dependent and a specific protocol must be developed for each clone or cultivar (Shijie *et al.*, 1990). Das *et al.* (1994) developed callus from anthers of several *Hevea* clones on a medium with 6% sucrose. Cold treatment of anthers at 8-10°C for 24h had a promotive effect on callus development. The frequency of embryogenesis was enhanced in the presence of N6 benzyladenine (BA) and 2-isopentyladenine (2ip). Veisseire *et al.* (1994a; 1994b) induced embryogenic calli from integument tissue of immature seeds on medium containing 234 mM sucrose, 9 μM BA and 9 μM 3,4-dichlorophenoxyacetic acid (3,4-D). Abscisic acid (ABA) stimulated embryo development in liquid medium. Wang and Chen (1995) regenerated plantlets through somatic embryogenesis from stamen cultures and a regeneration frequency of 40.5% was obtained with the optimized condition. Cailloux *et al.* (1996) and Linossier *et al.* (1997) studied the role of sucrose and ABA on embryo induction. Blanc *et al.* (2002) assessed the effect of carbohydrate types on somatic embryogenesis.

Extensive experiments were carried out by many researchers to enhance the frequency of somatic embryo induction and plant regeneration. Attempts were made to standardize the optimum growth regulator concentrations and the balance between different growth regulators, the nutritional requirements and the physical factors such as light and temperature for maximum callus proliferation, embryo induction and subsequent regeneration of normal and healthy plantlets. As a result immature anther (before microsporogenesis) (Jayasree *et al.*, 1999) and immature inflorescence (Sushamakumai *et al.*, 2000) were identified as the suitable explants and protocols were developed for high frequency somatic embryo induction and plant regeneration for RRII 105, the most popular Indian *Hevea* clone. Simultaneously attempts were made to isolate single cells as well as protoplasts from different explant sources from *Hevea brasiliensis* (Sushamakumari *et al.*, 1999). They could develop an efficient and reproducible protocol for somatic embryo induction and plant

regeneration from embryogenic cell suspension derived protoplasts of RRII 105 clone (Sushamakumari *et al.*, 2000a).

2. 2. 5 (1). Factors influencing plant regeneration via somatic embryogenesis

Considerable progress has been made in the last decades on somatic embryogenesis so as to make it a micro propagation system and a reliable *in vitro* plant regeneration system for the development of transgenic plants. However, the requirement of essential nutritional, hormonal and physical factors restrict efficient plant regeneration.

In *Hevea*, Chen *et al.* (1978) observed callus induction on MS as well as on MB medium which contained macro elements and iron salt of MS medium, and microelements and organic growth substances of the medium designed by Bourgin for tobacco. Later Carron and Enjalric while developing a protocol from inner integument of seeds observed that the whole process of embryogenesis involved four successive phases: callogenesis, differentiation of embryoids, multiplication of embryoids and finally development of embryos into plantlets (Carron and Enjalric, 1984).

Growth regulators have an important role in developing an *in vitro* plant regeneration system. Auxin was found to be necessary for callus induction from different explants of *Hevea* (Chen *et al.*, 1978; Carron and Enjalric, 1984; Jayasree *et al.*, 1999; Sushamakumari *et al.*, 2000). Extensive examination of the action of auxins (2,4-D, NAA and IAA) revealed that, IAA and 2,4-D had synergistic effect and callus induction was observed on the basal medium supplemented with (0.3 mg/l) 2,4-D and (1.0 mg/l IAA). Induction of embryogenic callus was strongly stimulated either by a decrease in exogenous auxins and cytokinins or by the addition of ABA to the culture medium (Etienne *et al.*, 1991).

Studies on factors influencing callus friability and somatic embryogenesis in different clones of *Hevea* revealed that callus friability was enhanced in PB 260 clone either by reducing the concentration of Kn or 3,4-D (2.0 mg/l - 0.2 mg/l) during first culture or by maintaining high sucrose (351 mM) or calcium (12 mM) levels during subcultures (Montoro *et al.*, 1993). Although, BA was not essential for callus induction, callus proliferation was promoted by the addition of 1-5 mg/l BA (Carron and Enjalric, 1984). Moreover, for the induction of normal embryos it

was necessary to control the hormones of callus induction and proliferation media at a rather low level. Continuous exposure of the callus to auxin containing medium led to loss of morphogenetic potential (Chen *et al.*, 1978). Embryo induction was observed on a medium provided with Kn (0.2-0.3 mg/l) NAA (0.2-0.3 mg/l) and gibberellic acid (GA_3) (0.2-0.6 mg/l). Later Wang *et al.* (1980) succeeded in developing somatic plants from anther. Carron and Enjalric (1982), cultured anther wall for callus induction and developed embryoids.

A detailed investigation on the response of various cytokinins on *in vitro* germination of *Hevea* somatic embryos was carried out using BA, zeatin (Zn), Kn and thidiazuron (TDZ). The results revealed that TDZ was superior to BA and Zn while Kn showed the least response. Maximum germination (80%) and plant development (82%) was obtained when the medium was supplemented with (0.25 mg/l) TDZ (Jayasree *et al.*, 2001a). Effect of GA_3 on embryo induction and germination in *Hevea brasiliensis* (clone RRII 105) was analyzed by Jayasree and Thulaseedharan (2001). They observed that lower levels of GA_3 up to (2.0 mg/l) favoured embryo induction, germination and subsequent development of full plants. Slight reduction in response was observed for both induction and germination of embryos by autoclaving GA_3.

Linossier and co workers (1997) analyzed the use of high concentration of polyethylene glycol (PEG) for imparting osmotic stress for the development of embryos. They reported that the presence of 140 g/l PEG greatly reduced secondary embryogenesis and improved the conversion of pro-embryonic masses into torpedo-shaped embryos, while the addition of exogenous ABA (10 μM) favored only the formation of globular stage embryos (Linossier *et al.*, 1997). Several experiments were carried out by different groups for determining the effect of desiccation, medium osmolarity and abscisic acid (ABA) on the maturation of somatic embryos and conversion into plantlets (Etienne *et al.*, 1993; Cailloux *et al.*, 1996; Linossier *et al.*, 1997). Addition of 351 mM sucrose in presence of 1.0 μM ABA enabled the accumulation of protein reserve and enhanced the individualization of shoot and root meristems thereby improving embryo germination and conversion into plantlets (Etienne *et al.*, 1993). It is reported that incorporation of 234 mM sucrose as an osmoticum in combination with 10.0 μM ABA enhanced embryo maturation (Cailloux *et al.*, 1996).

Effect of different carbohydrates such as sucrose, maltose, fructose and glucose on early somatic embryogenesis was assessed by Blanc *et al.* (2002). They observed that somatic embryogenesis was significantly higher with maltose, rather than with glucose, fructose or sucrose. In the presence of maltose, callus growth was slow and only half that was seen with sucrose and the induction of embryogenesis was uniform and twice as fast as with sucrose supply.

2. 3. Plant genetic transformation

Genetic improvement of tree species by conventional breeding methods is a rather slow and elaborate process. Advances in molecular and cell biology over the past few decades have led to the development of a wide range of techniques for manipulating the genome of any species. Recent developments in molecular biology techniques like gene tagging, gene mapping and other techniques such as RFLP, PCR, and transgenic technology have increased the precision and efficiency of plant breeding. Developments in recombinant DNA technology combined with *in vitro* plant regeneration techniques have paved the way for the successful integration of foreign genes into the genome of many plant species with stable expression of an introduced foreign gene (Bevan , 1984; Zambryski *et al.*, 1983). Genetic engineering enables movement of genes of interest across sexual incompatibility barriers not only among species and genera but also among kingdoms (Paroda, 1999). This technique has led to a quantum leap in the understanding of some basic aspects of plant development, physiology and biochemistry (Birch, 1997).

2. 3. 1. *Agrobacterium tumefaciens*: a natural vector for genetic transformation

Plant genetic engineering started with the discovery that *Agrobacterium tumefaciens* could transfer a set of genes from the Ti- plasmid, into infected plants and this DNA could become co-valently integrated into the genome of the host cell, where it gets expressed (Roberts, 1982). Genetic transformation can be defined as the transfer of foreign genes isolated from plants, animals, bacteria or virus into a new genetic background and it involves several distinct stages, namely insertion, integration, expression and inheritance of the new DNA. Consequent to extensive research conducted in several laboratories (Hoekima *et al.*, 1983;

Caplan *et al.*, 1983; Chilton, 1983; Schell and Montagu, 1983), enough information was accumulated to render the *Agrobacterium* system a sure candidate for the genetic transformation of plants.

Agrobacterium mediated gene transfer has been successfully used for a broad range of plant taxa. The interaction of *Agrobacterium* with higher plant cells to deliver T-DNA seems to be universal. An increased understanding of the mechanisms of gene transfer and integration of transgenes into plant genomes should help to improve the use of this vector for genetic manipulation of elite genotypes of the desired tree species.

Significant variation in virulence has been reported with *Agrobacterium* strains carrying different Ti plasmids in some plant species (Morris *et al.*, 1989; Ellis, 1995). This has led to the selection of strains that were specially suitable for transformation of different groups of plants. The efficiency of *Agrobacterium* mediated transformation can be optimized by using hyper virulent strains by treatments such as wounding, vacuum infiltration of bacteria into the explant, pre-treatment of the explants with vir-gene inducers (e.g. acetosyringone), optimization of temperature and duration of co-cultivation (Bidney *et al.*, 1992; Santarem *et al.*, 1998; Hansen and Chilton, 1999).

Agrobacterium rhizogenes has also been used as a vector for gene transfer in a number of plant species. Its ability to transfer transgene into woody plant cells was demonstrated by Huang *et al.* (1991), and since then this species has been used for transformation of several plants (Tzfira *et al.*, 1996). *Agrobacterium rhizogene* has also been used to aid in the production of adventitious roots on explants of woody species (Magnussen *et al.*, 1994). For long, the inability of *Agrobacterium* to transfer T-DNA to monocotyledonous plants was considered as a major limitation. However, with effective modification, in Ti plasmid vectors and finer modifications of transformation conditions, a number of monocotyledonous plants (rice, wheat, maize, etc) have now been transformed (Hiei *et al.*, 1994; Ishida *et al.*, 1996).

2.3.2. Plant genetic transformation: an over view

Genetic engineering of crop plants integrated with specific genes has been achieved either by direct gene transfer or by *Agrobacterium tumefaciens* mediated genetic transformation. *Agrobacterium* mediated transformation has been widely used because of its efficiency, simplicity and stability of the integrated gene (Joshy and Joshy, 1991: Dale *et al.*, 1993). The method of such transfer, which is chosen, is generally governed by the plant species and its regeneration response in tissue culture. One of the critical factors in the genetic transformation of a particular plant species is the ability of the target cells, once transformed, to develop into complete plants via somatic embryogenesis or organogenesis (Horsch *et al.*, 1985).

The protocol described by Horsch *et al.*, (1985) for the transformation of tobacco leaf discs forms the basis of most modified procedures used with *Agrobacterium tumefaciens*. Later, transgenic plant expressing an agronomically desirable trait was developed. The modification of plant genome through genetic engineering approaches has been perfected to a great extent in several crops and the first major breakthrough for environmental stress was transgenic tobacco with increased low temperature tolerance (Murata *et al.*, 1992).

Significant progress has been made in developing transgenic plants devoid of antibiotic marker genes (*nptII* or *hpt)* or herbicide resistance markers (e.g *bar*) are essential for selectively propagating transformed cells and tissues. Elimination of marker is advocated since the antibiotic resistant genes may be transferred to pathogenic bacteria or the herbicide resistance gene may be transferred to weeds. This can be achieved by co-transformation of multiple T-DNA or by site- specific recombination strategies and deployment of Ac/Ds- based transposition (Veluthambi *et al.*, 2003). Besides marker elimination, it offers the following advantages: 1) it enables the successive rounds of transformation so that, useful transgene can be stacked without crossing 2) retention of promoters integrated along with the selection markers which will lead to the presence of multiple copies of a promoter, thereby activating signals for transcriptional gene silencing. The co-transformation strategy was successfully deployed in rice for engineering

provitamin A biosynthesis. A total of four genes contained in two separate T-DNA cassettes were transferred together in rice (Ye *et al.*, 2000).

Undesirable genes in the plant genome can be selectively silenced in the target tissues by the antisense approach (Bourque, 1995). The antisense RNA gene induced silencing has been successfully used to manipulate physiological process such as fruit ripening, photosynthesis and source-sink relationships. Smith *et al.* (1988) were pioneers in the application of antisense cDNA in genetic transformation of plants. Delayed ripening of tomato fruit was achieved by expression of the antisense ACC synthase or ACC oxidase cDNAs (Oeller *et al.*, 1991; Hamilton *et al.*, 1990). Inhibition of the fruit softening has been achieved by antisense polygalacturonase gene (Gray *et al.*, 1992) and by antisense pectin methylesterase genes (Tieman *et al.*, 1992). The antisense technology allowed the selective accumulation specific intermediates in metabolic pathways by reducing the activity of a single enzyme. For e.g. the modulation of carbohydrate synthesis in potato was achieved by antisense starch synthase (Visser *et al.*, 1991).

Genetic engineering also allows the use of several desirable genes in a single event, and reducing the time required to integrate novel genes into elite background (Sharma *et al.*, 2003b). The demonstration that substantially large stretches of DNA (up to 28 kb) can be transferred in to plant cells by *Agrobacterium tumefaciens* (Hamilton *et al.*, 1996) suggests the possibility of transferring several genes, e.g. those controlling a complete metabolic pathway into plant cells. Placing multiple genes in one T-DNA is technically difficult due to the loss of single restriction sites in successive cloning steps. This limitation was overcome by successfully engineering provitamin A biosynthesis genes in rice (Ye *et al.*, 2000). Thus, it is possible to transform multiple genes of a pathway by co-transformation of multiple T-DNAs.

Significant progress has been made over the past decades in handling and introduction of exotic genes into plants, and has provided opportunities to modify crops for increased yield, impart resistance to biotic and abiotic stress factors and improve nutrition (Sharma *et al.*, 2003b). Although, genetic transformation and stable integration of foreign genes have been successful in many cereal crops and herbaceous annual plants, the success in perennial tree crops is rather limited

(Dandekar *et al.*, 1988; James *et al.*, 1989; Ueno *et al.*, 1996). The main difficulties associated with developing transformed trees are the lack of efficient tissue culture systems. If the plant material could be manipulated in sterile culture, and large number of regenerants could be obtained from single cell, then transformation can be easily achieved in tree crops. But except for a few species, most trees cannot be manipulated in tissue culture with the same ease as herbacious dicots (Thornburg, 1990).

2. 3. 3. Genetic transformation in *Hevea brasiliensis*

The possibility of genetic transformation in *Hevea brasiliensis* was first explored in 1991 (Arokiaraj and Rahaman, 1991) by *Agrobacterium* mediated transformation of calli derived from *in vitro* and *in vivo* seedling cultures. Tumours, developed on the co-cultivated explants produced octopine indicating that transformation has taken place. They screened several wild type strains of *Agrobacterium* of nopaline type (C58 and T37) and octopine type LBA4404 and 547/71. Strain C58 and its disarmed derivative pGV2260, pGV3850 and strains GV2260 and GV3850 constructed with the super virulent plasmid pToK47 worked well.

The major objectives of *Hevea* genetic transformation in different laboratories are (a) to improve the agronomic traits of elite *Hevea* clones and (b) production of pharmaceuticals and other valuable recombinant proteins in the transgenic rubber tree. In the improvement of agronomic traits, the underlying direction is towards development of transgenic rubber tree with increased rubber biosynthesis, timber volume, resistance to diseases, various abiotic stresses etc. (Arokiaraj *et al.*, 2002b; Thulaseedharan, 2002).

The first transgenic *Hevea* plant was produced by the integration of β-glucuronidase (GUS) and neomycin phosphotransferase (*nptII*) genes into callus cultures by particle gun method (Arokiaraj *et al.*, 1994). The transformed callus was subsequently regenerated into complete plantlet. Genetic transformation was confirmed by histochemical staining, fluorometric assay for GUS activity, ELISA for detecting expression of the *nptII* gene. Further, the presence of foreign gene in the transformed callus, embryoids and transgenic plants was confirmed by the

polymerase chain reaction (PCR). Later genetic transformation has also been achieved using *Agrobacterium* strains GV2260 and GV3850 into which the plasmid was introduced for *Hevea* transformation (Arokiaraj *et al.*, 1998). The presence of GUS gene in the transgenic plants was confirmed by Southern blot analysis. They observed GUS expression in the leaves, latex, phloem and laticifers of transformed plants. Todate, *Agrobacterium* mediated method is the preferred technique for *Hevea* genetic transformation.

Attempts were made to over express the recombinant protein, human serum albumin (HSA) in the serum fraction of latex of *Hevea brasiliensis*. *Agrobacterium tumefaciens* mediated transformation of *Hevea* callus was carried out using a binary vector pLGMR.HSA, containing HSA cDNA for isolating human serum albumin in the serum fraction of latex. Sixteen transgenic plants integrated with this gene were developed and established in soil. The presence of HSA cDNA was confirmed by PCR and the 16 plants were positive. The presence of recombinant protein was detected in the latex and leaf extracts of transgenic plants using two site ELISA and by Western blot analysis. The presence and the quantity of HSA in the latex was assayed by ELISA test. The amount of HSA varied from plant to plant and a maximum of 33 ug/ml of latex was obtained. Western blot of latex extracts from transgenic plants showed the presence of a protein band of approximately 68 kDa that was recognized by the monoclonal antibody against HSA, while control plants gave only a very low background labeling (Arokiaraj *et al.*, 2002 a).

The productivity of rubber trees has been increased by conventional breeding and by the application of flow stimulants. Molecular studies have helped to identify several genes for genetic transformation with the aim of improving latex production. While analyzing most of the enzymes involved in rubber biosynthesis in *Hevea*, Lynen (1969) observed that the activity of 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMGR) which catalyzes the formation of mevalonate appeared to be significantly low compared to other enzymes suggesting that the constitutive level of this enzyme may be a limiting factor in rubber biosynthesis (D'Auzac, 1997). This led to the suggestion that rubber biosynthesis may be increased by enhanced synthesis via. transcription and translation of *hmgr*1, involved in rubber biosynthesis and by maximizing the specific activity of the enzyme already *in situ*. With a view to develop transgenic plant with enhanced HMGR activity, Arokiaraj *et al.* (1995) transformed anther

derived callus with the gene coding for *hmgr*1. Transgenic embryos were developed, but they failed to regenerate transgenic plants from the embryoids. Other candidate genes that have control on the rate of biosynthesis of latex, cloned for genetic transformation were rubber elongation factor (REF), proteinaceous stimulator (cIF-5A) and the small rubber particle protein (SRPP) (Arokiaraj *et al.*, 2002 b).

To enhance timber production, several genes have been identified to increase the girthing rate of tree trunk for transformation in *Hevea*. The endogenous levels of gibberellins in hybrid aspen trees were increased by over-expressing a gibberellin synthesis gene from the model plant *Arabidopsis thaliana* (Eriksson *et al.*, 2000). They observed, profound improvements in growth rates and biomass production with longer stem fibers in transgenic trees compared with unmodified wild-type trees. Other important phenotypes altered in the modified trees were shoot elongation and stem diameter. This study opens the way to genetically modify rubber trees to grow faster and produce more biomass simply by increasing endogenous gibberellin levels. It has been reported that a group of enzymes, the 4-Coumarate CoA ligases (4-CLs) are important in lignin synthesis in the developing xylem tissues of hybrid aspen. Transgenic plants with altered levels of a specific 4-CL enzyme displayed modified phenotypes including altered stem diameters, xylem cell size and general wood properties (Hu *et al.*, 1998). Modifying specific enzymes involved in the wood forming process can also potentially influence important tree parameters and hence improve timber production.

2. 3. 4. Control of oxidative stress tolerance in plants

In nature plants encounter a wide range of environmental stresses that detrimentally affect their growth and development. Plants exhibit a variety of responses that enable them to tolerate and survive adverse environmental conditions through physical adaptations as well as metabolic alterations. Some of the common metabolic alterations observed in plants exposed to abiotic stresses are a decrease in net photosynthesis (Dubey, 1997), an increase in respiration (Livena and Levin, 1967), decrease in the activities of some important enzymes of nitrogen metabolism such as nitrate reductase (Srivastava, 1980; Rao and Gnanam, 1990), synthesis and accumulation of non toxic low molecular weight

organic molecules widely known as compatible solutes such as proline, glycinebetaine, mannitol etc. (Mc Cue and Hanson, 1990; Alia and Saradhi, 1993; Hanson *et al.*,1994). Plants exposed to abiotic stresses generate excess of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the most potent oxidant, the hydroxyl radical (OH^-) (Asada and Takahashi 1987; Scandalios, 1993; Foyer *et al.*, 1994). The level of ROS can increase due to various environmental factors such as high light intensity, drought, temperature stress, salinity, heavy metals, pathogens and air pollutants - ozone, SO_2, acid rain and various other redox active compounds. Also during infection by plant pathogens toxic oxygen radicals are involved (Scandalios, 1993; Gressel and Galum, 1994).

Increased levels of ROS or free radicals create a situation known as oxidative stress, which leads to a variety of biochemical and physiological lesions often resulting in metabolic impairment and cell death. These lesions in turn leads to membrane leakage, senescence, chlorophyll destruction and decreased photosynthesis in plants (Dubey, 1997). Oxidative stress is a constant burden to plants resulting from toxic oxygen species such as superoxide, hydroxyl radicals and additional toxic oxygen species. As a challenge to the toxic and potentially lethal effects of active oxygen, aerobic organisms evolved protective scavenging or antioxidant defense systems, both enzymatic and non-enzymatic (Halliwell and Gutteridge, 1985). Non enzymatic mechanisms include the action of carotinoids, which occur in great abundance in higher plants and perform many functions to protect plants from solar radiation damage. Ascorbic acid, vit E or α- tocopherol and ferredoxin are other agents scavenging free radicals in chloroplasts via a redox reaction (Allen, 1975). Enzymatic antioxidant defense include enzymes capable of removing, neutralizing or scavenging oxygen intermediates, eg, superoxide dismutase detoxifies superoxide radical to hydrogen peroxide, ascorbate peroxidase and glutathione reductase, which are believed to scavenge hydrogen peroxide in chloroplasts and mitochondria respectively (Fridovich, 1986; Foyer and Halliwell, 1976).

2. 3. 4 (1). Role of SOD in abating oxidative stress

Superoxide dismutase (SOD) [EC.1.15.1.1] is the first enzyme involved in the detoxifying process of the active oxygen species. It is a family of metallo

enzymes, which are known to accelerate the spontaneous dismutation of superoxide radical to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Fridovich, 1986).

$$2\,(O^{-}{}_2 + 2H^{+}) \xrightarrow{SOD} H_2O_2 + O_2$$

Peroxidase and catalase enzymes eliminate the hydrogen peroxide. Thus the combined action of these enzymes convert superoxide radical and hydrogen peroxide to water and molecular oxygen, thus abating the accumulation of the most toxic and highly reactive oxygen species.

SOD exists in four isoforms, the cytosolic Cu/Zn SOD, the choloroplast residing Cu/Zn SOD, the mitochondrial Mn SOD and the chloroplast residing Fe SOD. All these are nuclear genes but those coding for mitochondrial and chloroplastic SODs contain transit peptides that lead the mature SODs to their destination (Beyer *et al.*, 1991). The genes for the cytosol and chloroplast Cu/Zn SODs were isolated from tomato and their transcription was found to be highly regulated by environmental conditions such as drought, paraquat and ethylene (Treves, 1990). Over expression of superoxide dismutase, catalase and peroxidase has been observed in plants exposed to abiotic stresses (Bowler *et al.*, 1992; Scandalios, 1993; Foyer *et al.*, 1994; 1997). Owing to the importance of these antioxidant enzymes in providing protection against the toxic oxygen species several attempts have been successfully made to introduce the genes for these enzymes in plants to enhance their tolerance to abiotic stresses.

2. 3. 5. Tapping panel dryness (TPD)

In recent years significant yield loss occurred all over the rubber growing countries due to the incidence of tapping panel dryness (TPD), a serious physiological disorder. The first visible syndrome is continued dripping of latex with low dry rubber content. Sometimes partial dryness of tapping panel is developed as the initial symptom and the bark turns light brown. TPD was reported in plantations in Asia since the beginning of the 20th century (Belgrave, 1917; Keuchenius, 1919). It is reported that TPD incidence is 5-10% among seedlings and 15-30% among high yielders, of which RRII 105 clone is very susceptible and no *Hevea* cultivar is completely resistant to TPD (Sreelatha,

2003). Krishnakumar *et al.* (1997) reported that the trans-zeatin riboside levels in the bark samples of TPD tolerant plants are higher compared to susceptible plants. In TPD affected trees, specific organelles in the latex known as lutoids are more prone to damage leading to destabilization of latex. TPD is of economic importance as it renders the trees unproductive.

2. 3. 5 (1). Role of SOD in the prevention of TPD

It has been well documented that TPD is always associated with an accumulation of free radicals. Chrestin (1989), reported that TPD affected trees exhibited high levels of NAD(P)H oxidase activity, which led to the formation of reactive oxygen species like superoxide dismutase (O_2^-) that damaged lutoid membranes resulting in the cessation of latex production. SOD is believed to assist in the maintenance of the lutoid membrane integrity (Chrestin, 1989). This is so because lutoids from dry trees appear to exhibit high NAD(P)H oxidase activity which leads to the release of superoxide and scavenging of such toxic oxygen species by SOD provides protection against damage to lutoid membrane (Chrestin, 1989). In TPD affected bark the free radical accumulation was found to be high with a reduced level of SOD activity (Das *et al.*, 1998). Insertion of cDNA coding for SOD gene may be able to scavenge the excess superoxide effectively thus providing protection against TPD.

2. 3. 6. Development of transgenic plant for environmental stress tolerance

Several reviews discussed the protection of plants against oxidative stress by genetic transformation (Bowler *et al.*, 1992; Gressel and Galun, 1994; Foyer *et al.*, 1994; Allen, 1995). Bartels and Nelson (1994) reviewed some approaches to improve abiotic stress tolerance by molecular- genetic methodologies. One of the most successful approach for enhancing the tolerance of plants to abiotic stresses has been through identification and successful transfer of the genes for various compatible solutes or by the over expression of antioxidant enzymes. The genes for synthesis/ over-production of compatible solutes viz. proline, glycinebetaine, mannitol, trehalose l and fractans which are known to impart stress tolerance in plants (Tarczynski *et al.*, 1993; Kishore *et al.*, 1995; Grover *et al.*, 1999). The key enzymes include superoxide dismutase, catalase and ascorbate peroxidase, glutathione peroxidase etc. which scavenge hydrogen peroxide. Activities of

enzymes associated with ascorbate glutathione cycle viz. ascorbate peroxidase, monodehydroascobate reductase, dehydroascorbate reductase and glutathione reductase, which would play a vital role in the detoxification of toxic oxygen species also increase in plants under stress. In most cases investigators attempted to over express genes coding for superoxide dismutase.

Tobacco plant was transformed with the gene construct harbouring the binary vector that encodes the Mn SOD of *N. plumbaginifolia.* CaMV 35S promoter was included in the gene construct. Transgenic plants that produced Mn SOD in their chloroplasts were tolerant to paraquat (Bowler *et al.*, 1991) and ozone (Van Camp, *et al.*, 1994). Transgenic potato plants over-expressing chloroplast Cu/Zn SOD of tomato showed tolerance to paraquat and such tolerance was also observed in root cultures of transgenic plants over-expressing the alien gene for cytosol SOD (Perl *et al.*, 1993).

Resistance against the combination of high light and chilling as well as paraquat tolerance was observed in transgenic tobacco plants that over-expressed pea chloroplast Cu-Zn SOD (Gupta *et al.*, 1993a). They have further reported that these transgenic plants also had elevated ascorbate peroxidase (APX) activity (Gupta *et al.*, 1993b). The protection against oxygen radicals and its relation to mechanism of stress tolerance was reviewed by Foyer *et al.* (1994). A field trial supported the connection between transient drought resistance and over-expression of SOD. Mc Kersie *et al.* (1996) developed alfalfa transgenic plants that over-expressed the Mn SOD gene either in the chloroplast or mitochondria. They observed that the transgenic plants had less injury from water deficit than control (untransformed) plants. Some examples of transgenic plants integrated with ROS scavenging enzymes are given in Table 2.1. Genetic engineering approaches have now become a very useful technique for transferring the gene for abiotic stress tolerance, but the success was achieved mostly in model plants like *Nicotiana tabacum* or *Arabidopsis thaliana.* Among annual crop plants Japonica rice and Indian mustard have been successfully transformed for abiotic stress tolerance (Xu *et al.*, 1996; Sakamoto *et al.*, 1998; Prasad *et al.*, 2000 a, b).

Table 2. 1.Transgenic plants integrated with AOS scavenging enzymes

Plant species transformed	Gene/source/ gene product	Product synthesized	Enhanced tolerance to:
Medicago sativa	SOD/*N.plumbaginifolia*/ SOD	superoxide dismutase	freezing stress (McKersie *et al.*,1993
N tabacum	*GR/E. coli*/ glutathione reductase	glutathione reductase	Ozone (Aono *et al.*, 1993)
N tabacum	SOD(Cu-Zn)/ Pea superoxide dismutase	superoxide dismutase	chilling and draught (Gupta *et al.*,1993a)
N tabacum	SOD(Cu-Zn)/ petunia/ SOD	superoxide dismutase	no tolerance to ozone (Pitcher *et al.*, 1991)
S. tuberosum	SOD (Cu-Zn)/ tomato/SOD	superoxide dismutase	oxidative stress (Pearl *et al.*, 1993)
M. sativa	SOD (Mn)/*N.plumbaginiofolia* / SOD	superoxide dismutase	drought (McKersie *et al.*, 1996).

Although, work has been done on the genetic transformation of *Hevea brasiliensis* by the integration of foreign genes such as marker genes and genes coding for recombinant proteins (Arokiaraj *et al.*, 1994; 1998, 2002a), significant progress has not been made in the development of transgenic plants integrated with agronomically important traits (Sobha *et al.*, 2003; Jayashree *et al.*, 2003). For the wide scale planting of *Hevea brasiliensis* in various agro-climatic conditions, plants, which can withstand environmental stresses are needed.

3

MATERIALS AND METHODS

3.I. Development of transgenic plants

3. 1. 1. Induction of callus

The explant used for the genetic transformation of *Hevea brasiliensis* was newly formed yellow friable callus derived from immature anther. Young flower buds (Fig. 2) were collected from 15-year-old *Hevea brasiliensis* (clone RRII 105) trees growing in the Rubber Research Institute of India experimental fields. The flower buds were washed thoroughly in running tap water for 10 min to remove dust and other surface contaminants. Surface sterilized with 0.1% (w/v) mercuric chloride solution containing 2-3 drops of Tween-20 for three minutes and washed extensively in sterile distilled water. Immature anther, at diploid stage (before microsporogenesis) were dissected out aseptically under a stereomicroscope. Few drops of sterile ascorbic acid (0.1%) solution were spread over the flower buds during dissection to prevent oxidation and browning of the anther. The dissected anthers (5-6 numbers) were inoculated on sterile callus induction medium reported earlier (Jayasree *et al.*, 1999). The callus induction medium was modified Murashige and Skoog (1962) (MS) medium. MS medium was modified by lowering the NH_4NO_3 concentration to 1.0 gm/l and replacing MS vitamins with B5 (Gamborg *et* al., 1968) vitamins. The composition of the callus induction medium is given in Table 3.1.

The pH of the medium was adjusted to 5. 6 with 1N potassium hydroxide and made up the volume to one liter with double distilled water. Phytagel (synthetic agar, Sigma USA), 2.0 g was added to the medium and heated to boil with constant stirring till phytagel gets dissolved. The melted medium (10 ml) was poured to glass culture tubes (150x25 mm) and plugged tightly with non-absorbant cotton plugs. The medium was autoclaved at 121°C, 15 lb pressure for 15 minutes and stored at 25°C.

Fig. 2: *Hevea* (Clone RRII 105) flower buds for dissection of immature anther

Table. 3.1. Composition of the callus induction medium

Ingredients	**Concentration in (mg/l)** (unless indicated otherwise)
Modified MS major salts	
NH_4NO_3	1000. 00
KNO_3	1900. 00
$CaCl_2$ (anhy)	333. 00
$MgSO_4$ (anhy)	181. 00
KH_2PO_4	170. 00
MS Minor salts	
KI	0. 83
H_3BO_3	6. 20
$MnSO_4. 4H_2O$	22. 30
$ZnSO_4. 7H_2O$	8. 60
$Na_2Mo\ O_4. 2H_2O$	0. 25
$CuSO_4. 5H_2O$	0. 025
$CoCl_2.$	0. 025
$FeSO_4.7H_2O$	27. 80
Na_2 EDTA. $2H_2O$	37.30
B5 vitamins	
Thiamine HCl.	10.00
Pyridoxine HCl.	1.00
Nicotinic acid	1.00
Myo-inositol	100.00
Caseine hydrolyzate	400.00
Sucrose	50.0 g
Coconut water	5.0 %
2,4-Dichlorophenoxyacetic acid (2,4-D)	2.0
Kinetin (Kn)	0.5

3. 1. 2. *Agrobacterium tumefaciens* strain and binary vector.

The *Agrobacterium tumefaciens* strain used for the genetic transformation of *Hevea brasiliensis* was EHA101. The plasmid vector (pDU 96.2412) (Fig. 3), used in this study was developed at Prof. A.M Dandekar's Laboratory, Department of Pomology, University of California, USA in a collaborative research programme with Rubber Research Institute of India and University of California. The vector contains β-glucuronidase (*uid*A) as the reporter gene, neomycin phosphotransferase (*npt*II) as the plant selectable marker gene and *Hevea* Manganese Superoxide dismutase (Hb Mn SOD) gene under the control of Figwort Mossaic Virus (FMV) 34S promoter.

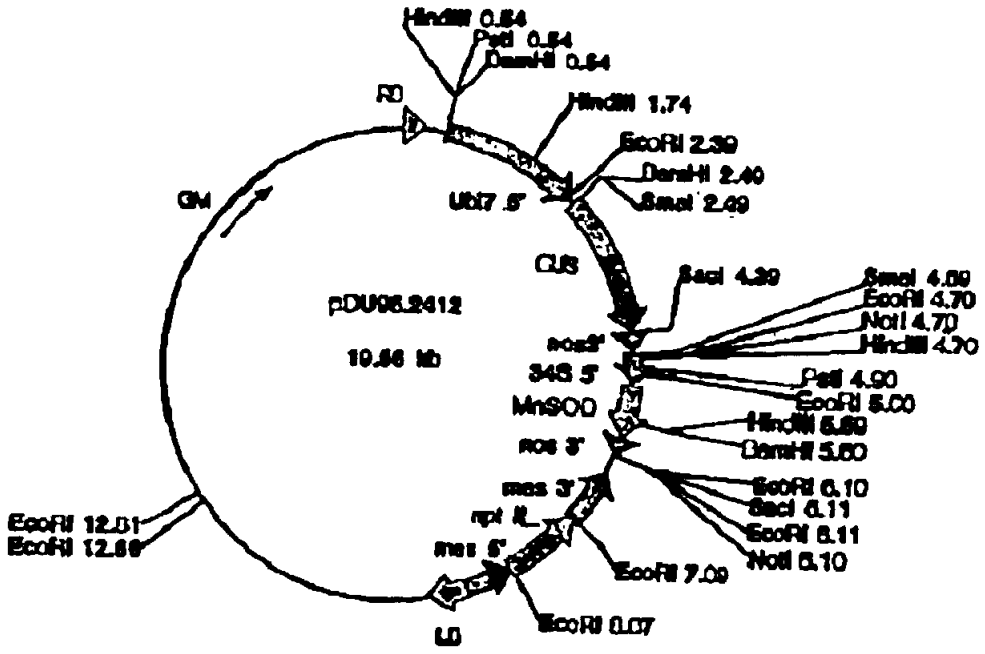


Fig. 3: Binary vector of pDU96.2412 used for genetic transformation

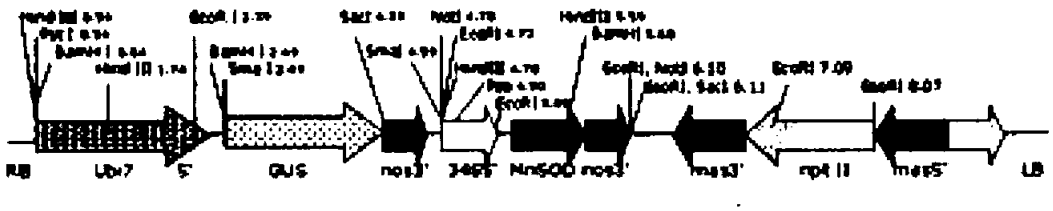


Fig. 4: Details of SOD, GUS and *npt* II genes between RB and LB of T-DNA region of pDU96.2412

The 702 nucleotide Hb Mn SOD cDNA was obtained by RT-PCR of mRNA isolated from bark tissues of *Hevea brasiliensis* using primer sequences corresponding to previously published sequence of *Hevea* Mn SOD (Mia and Gaynor, 1993). The Hb Mn SOD cDNA was cloned into the vector, pCR 2.1 (Invitrogen, CA) to create pTA Mn.SOD7. The 400 bp FMV 34S promoter was PCR amplified from the plasmid pMS 34 obtained from Steve Daubert, University of California at Davis, USA which contained the promoter sequences (Sanger *et al.*, 1990). This fragment was used to create a plasmid pDU 95.1411 that contains a functional expression cassette with 400 bp 34S promoter upstream from a nos 3' site with a *Bam*H 1 in the middle. The Hb Mn SOD coding sequence was removed from pTAMnSOD7 and was ligated with *Bam*H1 digested intermediate vector to facilitate the ligation of Hb Mn SOD fragment downstream from the 34S promoter and upstream from the nos 3' sequence (Fig. 4), creating the binary vector pDU 96.2412. The binary vector was introduced into the *Agrobacterium* strain EHA 101. The *Agrobacterium* strain containing the plasmid vector was stored in glycerol stock at –80°C and is very stable under nonselective conditions.

3. 1. 3. Preparation of antibiotics

The binary vector used in the present study contains the gene sequence for neomycin phosphotransferase (*npt*II) and resistance to gentamycin. Kanamycin (50 mg/l) and gentamycin (20 mg/l) were used for bacterial selection. The *npt*II gene confer resistance to four antibiotics viz. neomycin, kanamycin, paromomycin and geneticin (Galun and Breiman, 1997). All these antibiotics are water soluble and the stock solutions were prepared and sterilized using Millipore filter (0.2-µm. pore size. These antibiotics are relatively stable and could be stored at -20°C for one month without degradation.

3. 1. 4. Antibiotic kill curve tests

Antibiotic kill-curve tests were carried out with two-month old callus with different concentrations of the antibiotics to identify the optimum concentration of the antibiotic that can be effectively used for the selection of transformed cell lines. Two-month old *Hevea* callus clumps were cultured on callus induction medium supplemented with different concentrations of the antibiotics (Table. 3.2).

Ten callus clumps were cultured on each plate with three replications. After three weeks, the calli were transferred to fresh antibiotic containing medium. Three subcultures were made at three weeks interval.

Table 3. 2. Antibiotic concentrations tried for kill-curve test.

Antibiotics	Concentrations tried (mg/l)
Neomycin	200, 300, 400, 500, 600, 700, 800, 900,1000.
Kanamycin	50, 100, 150, 200, 250, 300, 350, 400.
Paromomycin	50, 100, 150, 200, 250, 300, 350, 400.
Geneticin	50, 100, 150, 200, 250, 300.

3. 1. 5. Preparation of *Agrobacterium* culture for infection

The bacterial culture for *Agrobacterium* infection was prepared according to Dandekar *et al.* (1989). A single colony of *Agrobacterium* harbouring the binary vector (pDU96.2412) was streaked on solid AELB medium supplemented with gentamycin (20 mg/l) and kanamycin (50 mg/l) and grown overnight at 28°C. After 24 hours a single colony of this actively growing bacteria was transferred to 20 ml liquid AELB medium containing the above antibiotics in the same concentration.

AELB medium

Tryptone 10 g/l, yeast extract 5 g/l, pH 7

The *Agrobacterium* was allowed to grow over night at 28°C in a rotary shaker at 250 rpm to get a density of 10^8 cells/ml (an optical density of 0.5 at 420 nm.). Ten ml of this culture was centrifuged at 2500 rpm for 10 min for separating the bacteria. Re-suspended the bacteria in 100 ml sterile MS basal medium containing 20 mg/l acetosyringone, 115 mg/l proline and 153 mg/l glycinebetaine to get a bacterial density of 10^8 cells/ml. The bacterial cells were allowed to grow in this medium in a rotary shaker at 250 rpm for four hours at 28°C. This bacterial culture was used for callus infection.

3. 1. 6. *Agrobacterium* infection and co-culture

Initially *Agrobacterium* infection was carried out with different stages of the anther derived callus viz. emerging callus, callus proliferated for 20, 40 and 50 days after initiation for identifying the ideal stage of the callus for getting maximum transformation frequency. Approximately 1.0 g of the yellow friable callus was taken in sterile (30 mm) glass petri plates containing 5 ml of the *Agrobacterium* culture. The callus clumps immersed in bacterial culture (inoculum) were cut into small pieces with sterile scalpel blade and kept in the *Agrobacterium* inoculum for 10 min. The infected calli were then blotted dry with sterile filter paper to remove the excess bacterial suspension and carefully transferred to solid co-cultivation medium.

3. 1.7. Preparation of co-cultivation medium

The basal medium used for callus induction was used as the basal medium for co-cultivation also. After autoclaving the medium was cooled to 50-55°C, filter sterilized (0.2- μm millipore filter) acetosyringone (20 mg/l), proline (115 mg/l) and glycinebetaine (153 mg/l) were added to the medium, mixed thoroughly and poured approximately 30 ml to 90 mm sterile disposable petri plates. The media plates were sealed with parafilm and used as the co- cultivation medium.

Co-cultivation was carried out for 72 hours in the dark at 26 ± 2°C. After 72 hours the calli were blotted dry with sterile filter paper and transferred to selection medium. Except for the antibiotics, the basal media composition of selection medium was same as that of callus induction. For identifying the suitable antibiotic for getting maximum recovery of transformed cell lines with *npt*II selectable marker gene, different antibiotics viz. kanamycin, paromoycin and geneticin were tested. Separate selection media were prepared with the different antibiotics by incorporating the optimum concentration of the antibiotics (identified by the kill-curve test). In the selection medium 500 mg/l cefotaxime was added to prevent the overgrowth of the *Agrobacterium*. Three subcultures were made on the selection medium at three weeks interval for the elimination of escapes.

3. 1. 8. Selection of transformed cell lines by GUS histochemical staining

After 40-50 days culture in the selection medium, antibiotic resistant yellow callus emerged from the *Agrobacterium* infected callus. Approximately 2 mg of the antibiotic resistant callus was tested for GUS histochemical staining. GUS histochemical staining was carried out according to Jefferson (1987) and the GUS positive cell lines were selected.

The GUS staining solution contains the following components

- Sodium phosphate buffer (pH 7.0) 50. 0 mM
- Ethylenediaminetetraacetic acid 10. 0 mM
- Triton X-100 0.10%
- Potassium ferrocyanide 2. 0 mM
- Potassium ferricyanide 2. 0 mM
- X-gluc 1. 0 mM

Approximately 2 mg of the antibiotic resistant proliferating callus samples were collected and placed separately into 5 ml scintillation vials containing one ml of GUS staining solution. The vials were placed in a desiccator and vacuum infiltration was carried out for five minutes. After slow release of the vacuum the samples were incubated at 37°C over night. Visual observations were made for GUS staining.

3. 1. 9. Callus proliferation and embryogenic callus induction

The putatively transformed cell lines selected from independent transformation events were proliferated separately in callus proliferation medium. MS basal medium as well as the basal medium used for callus induction were tested for callus proliferation. The basal media were fortified with, casein hydrolyzate (400 mg/l), glutamine (300 mg/l), sucrose (30 g/l) and 5% (v/v) coconut water. Effect of various growth regulators viz 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA) and N6-benzyladenine (BA) at different concentrations were tested for callus proliferation.

The growth regulator combinations and concentrations tested for callus proliferation were:

2,4-D (0.0- 2.0 mg/l) + NAA (0.0- 0. 5mg/l)

2,4-D (0.2-1.0 mg/l) + NAA (0.0-0.5 mg/l) +BA (0.1 – 1.0 mg/l).

For embryogenic callus formation, the proliferating calli were subcultured on proliferation medium at 30 days interval. Untransformed callus was cultured separately as control.

3. 1. 10. Somatic embryo induction

For embryo induction embryogenic calli were subcultured onto embryo induction medium. Modified MS basal medium was used for embryo induction. The modification was made by changing the concentration of NH_4NO_3 to 825 mg/l, KNO_3 to 950 mg/l and replacing MS vitamins with B5 vitamins. Casein hydrolysate (400. 0 mg/l), coconut water (5% (v/v) and sucrose 30.0 g/l were added. The pH of the medium was adjusted to 5.6 before autoclaving. Effect of various growth regulator combinations (1) gibberellic acid (GA_3), NAA and Kn, (2) GA_3, NAA, Kn and BA on embryo induction was assessed. The combination and concentration of growth regulators used for embryo induction were:

GA_3 (0.2 – 1.0 mg/l) + NAA (0.2 mg/l) + Kn (0.0 - 0. 5 mg/l)

GA_3 (0.3 – 1.5 mg/l) + NAA (0.2 mg/l) + Kn (0.3 mg/l) + BA (0.0 - 0. 5 mg/l)

The cultures were maintained by subculturing every four weeks on this medium. Observations were recorded after 8 weeks of culture.

Effect of water stress, on embryo induction and callus fresh weight in untransformed and transformed cultures were assessed by incorporating different concentrations of phytagel ranging from 0.2% to 1.0%(w/v). In order to study the effect of osmotic stress on embryo induction and callus fresh weight, varying concentrations of polyethylene glycol (PEG, Mol. wt 6000) (2%-12% w/v) were included in the embryo induction medium fortified with NAA (0. 20 mg/l), BA (0. 30 mg/l), Kn (0. 30 mg/l) and GA_3 (0.60 mg/l).

3. 1.11. Embryo maturation

For embryo maturation the globular/torpedo stage embryos were transferred to maturation medium. The basal medium used for embryo induction was used for embryo maturation also except for the hormonal combinations. An

experiment was carried out with GA_3 and BA for identifying the optimum concentration of the growth regulators for getting maximum embryo maturation. Effect of GA_3 and thidiazuron (TDZ) on embryo maturation was also evaluated with different concentration of GA_3 and TDZ.The hormonal combinations tested were:

GA_3 (0.1- 0.6 mg/l) + BA (0.1- 0.5 mg/l)

GA_3 (0.1- 0.6 mg/l) + TDZ (0.0 - 0.5 mg/l)

The cultures were maintained by subculturing every four weeks on the maturation medium. Observations were recorded after 8 weeks.

Effect of PEG and abscisic acid (ABA) on embryo maturation was studied by incorporating different concentrations of PEG (2-12% w/v) either alone or in combination with 0.1 mg/l ABA in the maturation medium. Effect of water stress on embryo maturation was also evaluated by supplementing the maturation medium with phytagel (0.2-1.0% w/v).

3. 1. 12. Embryo germination and plant regeneration

For plant regeneration, mature somatic embryos were transferred to half - strength MS medium lacking hormones as well as on medium fortified with different growth regulators viz. BA, GA_3 and indole-3-butyric acid (IBA). An experiment was carried out with GA_3 and IBA to identify the optimum concentration of the growth regulators required for getting maximum plantlets. Effect of BA on embryo germination was tested with different concentrations of BA (0.0-0.5 mg/l) and GA_3 (0.0-0.5 mg/l) combined with (0.1 mg/l) IBA. The fully developed plantlets were kept for hardening.

3. 1. 13. Culture conditions

Unless otherwise mentioned, the pH of the medium was adjusted to 5.6 with 1N KOH. Phytagel (0.2%) was added as gelling agent before autoclaving at 121°C and 15 lb for 15 min. Also (0.2%) activated charcoal (Sigma) was included in the embryo induction, maturation and plant regeneration medium. For callus proliferation and embryo induction, cultures were maintained at 26 ± 2°C under complete darkness. For embryo maturation and plant regeneration, the cultures were maintained at 26 ± 2°C under 16 h photoperiod (40 $\mu E\ m^{-2}\ s^{1}$).

3. 1. 14. Acclimatization of the plants

The transgenic and untransformed (control) plants were transferred to hormone free half-strength MS medium and maintained in this medium till one to two whorls of mature leaves were developed. The plants were then taken out of the culture tubes and washed in running tap water to remove the agar and transplanted into small polythene bags or in earthenware pots (5"x10") filled with soilrite, soil and sand (1:1:1). These plants were covered with thin transparent polythene bags to maintain a relative humidity of about 90-95% and maintained in glass house at 28 ± 2°C. The plants were kept at this relative humidity for seven days and then gradually reduced the relative humidity to 60-65% by making holes in the plastic cover. After two weeks the protective cover was removed and the plants were maintained in the glass house at 50-55% relative humidity. After transplanting to earthenware pots, the plants were irrigated once in two days and also provided with half-strength Hoagland's solution once in two weeks.

3. 2 . Molecular analysis of transgenic plants

3. 2.1. Isolation of DNA from transgenic and control plants

Total genomic DNA was isolated following the modified protocol of Doyle *et al.* (1990). This modified CTAB procedure consists of the following steps:

- Young, uninfected leaves were collected from three transgenic plants derived from three independent transformation events and one untransformed (control) plant, maintained in the green house.
- The leaves were washed thoroughly in tap water and rinsed with sterile distilled water.
- 2 g leaf tissue was ground to a fine powder in Liquid Nitrogen using a sterile, pre-cooled mortar and pestle.
- The powered tissue was homogenized with 20 ml 2X CTAB extraction buffer.

2x CTAB buffer:

2% CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl pH-8.0,

0.1% Polyvinylpolypyrrolidone (PVPP) and 0.1% β-mercaptoethanol

- The samples were incubated at 60°C for 30 min in a 50 ml centrifuge tube.
- Centrifuged at 8,000 rpm for 10 min, the supernatant was transferred to a new sterile tube and the pellet was discarded.
- Equal volume of Tris-saturated phenol-chloroform-isoamyl alcohol (25:24:1) was added and mixed gently by inverting the tubes.
- The sample was spun at 10,000 rpm for 10 min and the aqueous phase was transferred to a fresh tube. The organic phase containing the denatured proteins was discarded.
- 5. 0 µl of DNAase free RNAase (10 mg/ml stock) was added and incubated at 37°C for 1 h to remove RNA.
- Proteinase K 3.0 µl (20 mg/ml- Bangalore Genei) was added and incubated at 37°C for 1 h to inactivate the RNAase and other residual proteins.
- Equal volume of chloroform:isoamyl alcohol was added to the sample, mixed gently and centrifuged at 10,000 rpm for 10 min.
- The aqueous phase was transferred to a fresh tube and the organic phase containing the lipids and carbohydrates were discarded.
- To the sample equal volume of chloroform: isoamyl alcohol was added, mixed gently and centrifuged at 10,000 rpm for 10 min.
- Aqueous phase was transferred to a fresh tube and the organic phase was discarded.
- To the sample 0.6 volume ice-cold isopropyl alcohol was added to precipitate the DNA and incubated in ice for 20 min.
- The tube was kept in ice for 20 min to precipitate DNA. The precipitated DNA was pelleted by centrifuging at 8,000 rpm for 10 min at 4°C.
- The DNA was washed in 70% ethanol.
    i) The pellet was air-dried and dissolved in 1.0 ml of TE buffer (10:1).
    ii) After checking the quantity and quality in a spectrophotometer, the DNA samples were stored at -20°C.

3. 2. 2. DNA quantification

The quality and quantity of genomic DNA was checked in an UV spectrophotometer (Beckman USA). The quality was checked by measuring the ratio of absorbance at 260 nm and 280 nm (260/280) respectively. A ratio between 1.7:1.8 indicate good quality DNA without protein contamination. DNA quantification was carried out using the following formula:
1 OD at 260 nm = 50 ng of DNA. The OD of each DNA sample was measured and quantified accordingly.

3. 2. 3. Isolation of plasmid DNA

Plasmid DNA was isolated from *E. coli* (DH 5α) carrying the binary vector pDU 96.2412 following the alkaline lysis method (Sambrook *et al.*, 1989).

- An overnight grown single colony of *E. coli* (DH 5α) carrying the binary vector pDU 96.2412 was inoculated into 3.0 ml LB medium containing 20 mg/l gentamycin and (50 mg/l) kanamycin, in a 15 ml tube capped with cotton plugs. Incubated the culture overnight at 37°C in an orbital shaker at 250 rpm.

LB medium
Tryptone 10 g/l, Yeast extract 5 g/l and Sodium chloride 10 g/l, pH 7

- 1.5 ml of the culture was taken in a microfuge tube. Centrifuged at 12,000 rpm for 5 min at 4°C. The medium was decanted off completely from the pellet.
- Re-suspended the bacterial pellet in 100 µl of solution I by vigorous vortexing and the tubes were stored on ice for 5 min.

Solution I
50 mM glucose, 25 mM Tris.Cl pH 8 and
10 mM EDTA pH 8
Autoclaved at 121°C, 15 lb and stored at 4°C

- 200 µl of freshly prepared solution II was added to the tubes and mixed five times rapidly by inversion and stored the tubes at room temperature for 5 min.

Solution II
0.2 M NaOH (freshly diluted from 10 M stock)
SDS 1%

- 150 µl of Solution III was added to the tubes and the contents were vortexed gently to disperse the solution through the viscous bacterial lysate. The tubes were returned to ice for 5 min.

Solution III	
5 M potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

- Centrifuged at 12,000 rpm for 5 min at 4°C to collect the clear supernatant into a fresh tube.
- An equal volume of phenol : chloroform was added to the collected supernatant, kept for 10 min, centrifuged at 8,000 rpm for 10 min at room temperature and collected the upper aqueous phase.
- RNA in the sample was degraded by incubating at 37°C for 1 h after the addition of 1 µl DNAase free RNAase (10 mg/ml stock).
- Plasmid DNA was precipitated by the addition of 2 volume of ethanol and spun at 8,000 rpm for 10 min at 4°C.
- The DNA pellet was washed with 70% alcohol (v/v) dried the pellet then dissolved in 50 µl TE buffer (10:1, pH 8).

3. 2. 4. Polymerase chain reaction

3. 2. 4 (a). Amplification of *npt* II and Hb SOD genes by PCR

For the detection of *npt*II gene, forward (5'-GAGGCTATTCGGCTA TGACT-3') and reverse (5'-AATCTCGTGATGGCAGGTTG-3') primers corresponding the *npt*IIcoding region of the plasmid vector was used. The

transgene (Hb Mn SOD) as well as native SOD gene were amplified with gene specific primers 5'-ATGGCTCTGCGATCAGTGACCC–3'(forward) and 5'-CTAAGAAGAGCATTCTTTGGCAT-3' (reverse). The PCR reactions were carried out in 20 µl volumes containing 100 µM dNTPs, 250 nM of each primer, 1X Taq DNA polymerase buffer containing 1.5 mM $MgCl_2$ and 0.5 U *Taq* DNA polymerase (Bangalore Genei, India) with 20 ng template DNA, in a thermal cycler (Perkin Elmer-480). For the amplification of *npt*II gene, the PCR conditions were; initial denaturation at 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min 30 sec. and 72°C for 2 min. The final elongation was at 72°C for 7 min. For the amplification of Hb SOD native genomic sequence DyNAsyme EXT DNA polymerase (Finnzymes, Finland) was used. Initial denaturation at 94°C for 2 min was followed by 35 cycles of 94°C for 30 sec, 58°C for 1 min and 69°C for 2 min and a final elongation at 69°C for 7 min. Amplified DNA fragments were electrophoresed in 1.5% agarose gels, stained with ethidium bromide and visualized under UV light.

3. 2. 5. Southern hybridization analysis

3. 2. 5 (a). Restriction digestion of genomic DNA

DNA from one untransformed and three transgenic plants obtained from three independent transformation events showing positive signals in GUS histochemical staining and PCR were used for southern hybridization analysis. 10 µg of genomic DNA was digested with *Eco*RI to detect the fragments of predicted size and with *Sac*I to confirm the integration in to the host genome.

Reaction mixture for restriction digestion
DNA 10 µl (10 µg), enzyme buffer 3µl, restriction enzyme 2 µl (20 U) and sterile distilled water 15 µl

The digestion was continued overnight at 37°C and the fragments were size fractionated in 1.0 % agarose gel.

- Prepared 1.0% (w/v) agarose gel containing 0.1% (w/v) ethidium bromide. The restriction digested DNA samples, undigested DNA and plasmid DNA samples were loaded into the slots of the agarose gel.

- Run the gel at 50 V till samples have migrated into the gel and then reduced the voltage to 25.
- Electrophorosis was continued till the dye front migrated to two-third length of the gel.
- Photographed the gel on a UV transilluminator.
- Trim the gel to final size and then marked one corner.

3. 2. 5 (b). Blotting of the DNA fragments

•DNA in the gel was depurinated by soaking in a solution of 0.25 N HCl for 15 min. Then the gel was briefly rinsed twice with distilled water.

•Denaturation of the DNA was carried out by treating the gel in denaturation solution for 25 min with gentle shaking. Then it was rinsed with water

Denaturation Solution
0.2 M NaOH, 1.5 M NaCl

♦Neutralized the gel by soaking in neutralization buffer for 30 min.

Neutralization Solution
1 M Tris-HCl pH 8, 1.5 M NaCl

♦Care was taken to see that gel was completely immersed in all solutions while treatment.

♦ After neutralization the gel was briefly washed in 10X SSC and kept ready.

20 X SSC
3 M NaCl, 0.3 M Sodium citrate pH adiusted to 7

♦DNA was transferred from the treated gel to nylon membrane (Hybond N^+, Amersham, USA) through capillary blotting transfer method (Sambrook *et al*, 1989).

♦A glass tray was filled to a height of 1 cm (about 200 ml) with 10 X SSC. A suitable platform with dimensions bigger than the gel was placed in the tray.

♦The surface of the platform was covered with Whatman No.3 filter paper pre-soaked in 10 X SSC so that the ends of the paper are immersed in the SSC.

♦Three sheets of Whatman No.1 filter paper, being cut exactly to the same dimensions of the gel and presoaked in 10X SSC, was placed on top of the platform. Any air bubbles was removed by rolling the surface with a glass rod.

♦The gel was placed carefully on top of this (upside down) and then a Hybond N+ nylon membrane presoaked in 10X SSC was placed on top of the gel. Any air bubbles were removed by gently rolling a glass rod on the surface.

♦Two sheets of the pre-soaked filter paper was placed on top of this. Three more sheets of clean dry filter paper was then stacked on it over which crude filter papers cut to the gel dimension was stacked to a height of 1. 5 cm. Over this, a suitable weight of around 200 g was placed. The weight should not crush the gel but should be able to compress the papers tightly.

♦The transfer was allowed to proceed for a period of 12-16 hours.

♦After the transfer the assembly was disassembled and the nylon membrane was briefly washed in 5X SSC and air-dried.

♦The membrane was fixed using a UV cross linker (Hoefer, USA) at 12,000 J/cm^2. The membrane was wrapped in Saran wrap and stored between crude filter paper in the refrigerator till use.

3. 2. 5 (c) Preparation of labeled probe

The *npt*II gene probe was radiolabeled using 'Multiprime DNA labeling kit (Amersham, UK). It utilizes random hexa-nucleotides to prime DNA synthesis on denatured template DNA at numerous sites along its length. The labeling was carried out following the manufacturer's instructions.

♦About 50 ng of template DNA was diluted to 5 µl with double distilled water and boiled for 5 min to denature.

♦Immediately chilled on ice for 5 min and centrifuged briefly.

♦11.5 µl of nuclease free autoclaved water was added.

♦Added 2.5 µl of buffer which contained all the dNTPs except dCTP.

♦ 2.5 µl of random primer solution was added.

♦To this 2.5 µl of α-^{32}P labeled dCTP (Sp. Activity ~ 3000 Ci/mMol or 10 µ Ci/µl) was added.

♦Finally 1 µl of the enzyme (Klenow fragment of DNA polymerase I) was added and mixed gently by pipetting up and down.

♦The labeled probe was purified by passing through a Sephadex G-50 column as follows:

♦Sephadex G-50 was added to STE buffer to form the slurry (10 g of dry powder yields around 160 ml of slurry).

♦A little glass wool was placed at the bottom of a 1 ml column and 1 ml of the slurry was added without the formation of air bubbles.

♦The column was spun at 3000 rpm for 3 min in a swinging bucket rotor.

♦More slurry was added until the Sephadex tightly packed up to 1 ml level.

♦The column was then equilibrated first with STE buffer and then with dist. water.

♦The labeled probe was then passed through the column. The eluted fraction was collected in a 1.5 ml micro-centrifuge tube.

♦The column purified probe was denatured by boiling at 100°C for 3 min and immediately chilled in ice. It was stored in the freezer till use.

3. 2. 5 (d). Hybridization

♦Hybridization of the labeled probe to the nylon membrane was performed according to Sambrook *et al*, (1989).

♦The blotted membrane was placed in a hybridization tube and pre-hybridisation solution (0.2 ml/cm^2 of the blot), 25 ml for the 13x10 cm membrane was added.

Pre-hybridization solution
6 X SSC, 5 X Denhardt's reagent and 0.5 % SDS

♦The pre-hybridisation was carried out at 65°C for 1 hr in a hybridization oven (Amersham, UK) with rotary movement at very low speed.

♦The pre-hybridization solution was poured out and the hybridization solution (pre-hybridization solution was used as hybridization solution) was poured into

the tube. Labeled denatured probe was added and incubated with slow rotation for 16 hr at 65°C.

3. 2. 5 (e). Blot washing and autoradiography

After hybridization, the membrane was washed twice at room temperature for 5 and 15 min respectively with solution I

Washing solution I
2 X SSC, 0.1 % SDS

♦The blot was then subjected to high stringent wash at 65°C for 30 min with solution II

Washing solution II
0.1 X SSC, 0.5 % SDS

♦The membrane was then floated briefly in 0.1 X SSC at RT then subjected to autoradiography.

♦The membrane was wrapped in a saran wrap and placed inside the X-ray cassette. An X-ray sheet was placed over it after marking the orientation.

♦An intensifying screen was placed over this assembly and the cassette was closed tightly and placed in -70°C for 2 days. Subsequently the X-ray sheet was removed and developed in the developer solution under safe red light.

♦As soon as the spots develop, the X-ray sheet was cleaned with water and quickly placed in the fixer for a few minutes.

♦ The sheet was extensively washed in water and dried.

3. 3. SOD expression by Northern hybridization.

Northern hybridization was carried out using the RNA isolated from untransformed and transformed embryogenic calli as well as from leaves of transformed and untransformed plants subjected to stress.

3. 3. 1. Induction of stress

Transformed and untransformed (control) embryogenic calli were cultured on hormone free half strength MS medium. Water stress was induced to the callus cultures using phytagel (0.4 % and 0.6% w/v). For the induction of salinity stress, callus samples were subcultured on media containing 100 mM and 200 mM sodium chloride. After stress induction for 15 days, total RNA was isolated from the samples.

To determine the expression of Mn SOD mRNA in untransformed (control) and transformed plants due to environmental stress, leaves were excised from the plants. The leaves were sliced separately into sections (1x1.5 cm) and incubated with 40% (w/v) PEG (Mol. wt 6000) for four hours. The control (no stress) leaf samples were incubated in DEPC treated water. After stress induction for 4 hours, total RNA was isolated from the samples.

3. 3.2. RNA isolation from leaf and callus samples

Total RNA was isolated from untransformed and transformed callus and leaf samples subjected to stress and control (without stress) according to the protocol reported by Venkatachalam *et al.* (1999) after modifications. All the reagents required were prepared in DEPC treated water. The protocol involved the following steps

- One gram each tissue (leaf and callus) were separately ground to a fine powder in Liquid Nitrogen.
- 10 ml extraction buffer was added to the fine powder and the homogenate was transferred to a sterile centrifuge tube.

Extraction buffer
0.2 M NaCl, 0.1 M Tris-HCl (pH 8), 0.01 M EDTA, 1.5% SDS, 0.1% β-mercaptoethanol (added immediately before use) and 0.1% (w/w) insoluble PVPP (added to the homogenate).

- Equal volume of extraction buffer saturated phenol was added, mixed gently and centrifuged at 10,000 rpm for 15 min.

- The upper aqueous phase was transferred to a fresh centrifuge tube and re-extracted with equal volume of chloroform.
- Centrifuged at 10,000 rpm for 10 min and the aqueous phase was collected.
- 1/3 volume 8 M LiCl was added and kept for RNA precipitation overnight at -20°C.
- The RNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C.
- The pellet was washed first with 2 M LiCl followed by 70% ethanol, air-dried and dissolved in 1 ml DEPC treated sterile water.
- For further purity, the RNA was re-precipitated with 0.1 vol 3M sodium acetate (pH 5.2) and 2.5 vol of ethanol
- The precipitated RNA was pelleted by centrifuging the samples at 10,000 rpm for 10 min at 4°C and washed the pellet twice with 70% ethanol.
- RNA pellet was air-dried, re-suspended in 200 µl of sterile DEPC water.
- The isolated RNA was used immediately for subsequent analysis or stored in 3 vol of ethanol at -70 °C.

The quality of RNA was checked in an UV spectrophotometer as described in (section 3. 2. 2) and its quality and DNA contamination, if any, was checked in 1% agarose gel. (The agarose gel electrophoresis was carried out according to the standard protocol (Sambrook *et al.*, 1989).

3. 3. 2 (a). Electrophoresis of RNA

Agarose (1% w/v) was melted in 31 ml of DEPC treated H_2O and cooled to 60°C. In a fume hood, 10 ml of 5X formaldehyde gel running buffer (MOPS) and 9 ml of formaldehyde was added to give a final concentration of 1X and 2.2 M respectively. The gel was allowed to set for at least 30 min at room temperature.

5X Formaldehyde gel running buffer (MOPS buffer)
0.1M MOPS pH 7, 40 mM sodium acetate, 5 mM EDTA pH 8

From leaf samples twenty µg of RNA and from callus fifteen µg of RNA was incubated for 15 min at 65°C along with 4 µl MOPS buffer, 7 µl formaldehyde and 20 µl formamide. After a brief spin, 4 µl of RNA loading buffer

was added and loaded in the gel. The gel was run in 1X MOPS buffer at 50V for 3-4 hours (until the bromophenol blue had migrated to 2/3 of the gel). The gel was photographed and then transferred to nylon membrane.

3. 3.2 (b) RNA blotting

Before transfer to the membrane, the gel was washed thrice in DEPC treated H_2O to remove the formaldehyde. The nylon membrane (Hybond N+, Amersham, UK) was cut into the size of the gel and was presoaked in 10X SSC. Blotting was carried out as described in the Southern protocol [Sec 3.2.5.(b)]. After transfer, the membrane was air- dried and UV cross linked

3. 3. 2 (c) Preparation of labeled probe and hybridization

The SOD probe was prepared and purified as mentioned in (section 3.2.5c &3.2.5d). Hybridization of the labeled probe to the nylon membrane was performed according to Sambrook *et al.* (1989). The pre-hybridization was carried out at 42°C for 3 hrs in a hybridization oven (Amersham, UK) with rotary movement at very low speed.

The pre-hybridization solution was poured out and hybridization solution was added into the tube. Labeled denatured probe was added and incubated with slow rotation at 42°C for 16 hrs.

3. 3. 2 (d) Washing and autoradiography

The membrane was washed first with solution 1 for 15 min at room temperature.

Solution 1
2X SSC, 0.1% SDS

Then two low stringent washing were given with solution II for 5 min each at room temperature. This was followed by a stringent washing in pre-warmed solution II at 42°C for 15 min.

Solution II
0.1X SSC, 0.1% SDS

The membrane was rinsed with 2XSSC and the excess liquid was removed with blotting paper. It was then wrapped in a UV transparent saran wrap and exposed to X-ray film with intesifying screens. The X-ray cassettes were placed at –70°C for two days and the film was washed and fixed as described in Southern protocol [sec 3.2. 5 (e)].

3. 4. Estimation of superoxide dismutase, peroxidase and catalase enzyme activities in transformed callus.

3. 4.1. *Induction of stress*

The transformed embryogenic callus was used for the studies on enzyme activity after induction of stress, keeping untransformed callus as control. Water stress was induced by subculturing the callus on hormone free half strength MS medium solidified with different concentrations of phytagel (0.2-1.0%). Osmotic stress was induced by subculturing the callus on media containing different concentrations of either polyethylene glycol (PEG, molecular weight 6000) mannitol or sorbitol (2-10%) in hormone free half strength MS medium solidified with (0.2% w/v) phytagel. The cultures were maintained in darkness for 30 days. The enzyme assay was carried out after stress induction for one month. For determining the effect of light on SOD enzyme activity the cultures were kept under continuous light for thirty days.

3. 4. 2. Enzyme preparation

Transformed and untransformed embryogenic calli (0.5 g) were homogenized in a sterile pre-chilled mortar and pestle after freezing in liquid nitrogen and the fine powder was re-suspended in 5 ml phosphate buffer (0.1M, pH 7.0). The resulting slurry was filtered through cheese cloth and the filtrate was centrifuged at 8,000 rpm at 4°C for 10 minutes. The supernatant was used for the assay of superoxide dismutase, peroxidase and catalase enzyme activities.

3. 4. 3. Assay of Superoxide dismutase

Superoxide dismutase enzyme activity was estimated following the method of Kakkar *et al.* (1984).

Reagents for SOD assay
Sodium pyrophosphate 0.052 M pH 8.3, phenazine methosulphate 186 µM, nitroblue tetrazolium 300 µM, β-NADH 780 µM, acetic acid 12 N

The assay mixture contained the following components

- Sodium pyrophosphate buffer pH 8.3 — 1.2 ml
- Nitroblue tetrazolium — 0.3ml
- Phenazene methosulphate — 0.1 ml
- Enzyme preparation — 0.05 ml
- Water — 1.15 ml

The reaction was started by the addition of 0.2 ml of β-NADH reagent. Allowed the reaction to continue for 90 sec at 30°C and then stopped the reaction by adding 1ml acetic acid. The reaction mixture devoid of enzyme served as control. Colour intensity of the chromogen in the reaction mixture was measured at 560 nm in a Beckman UV spectrophotometer.

- One unit of enzyme activity is defined as the enzyme concentration required to inhibit the optical density at 560 nm by 50% in one minute under the assay conditions and expressed as specific activity in milli units / min/ mg protein.

3. 4. 4. Estimation of Peroxidase enzyme activity

Peroxidase enzyme activity in the sample was estimated according to Addy and Goodman (1972).

Reagents for Peroxidase assay
Phosphate buffer 100 mM pH 6.5, Guaicol 10 µM, Hydrogen peroxide 20 µM.

The assay mixture contained

- Phosphate buffer — 2.5 ml
- Guaicol — 0.5 ml
- Enzyme preparation — 10 µl

The reaction was started by adding 0.2 ml H_2O_2. The amount of tetraguaicol formed during the reaction was measured in a spectrophotometer at 420 nm for two minutes at 15 seconds interval. Enzyme activity was calculated from the increase in absorbance at 420 nm after the addition of hydrogen peroxide.

One unit of enzyme activity was defined as the change in OD/minute and specific activity was expressed in terms of units/min/mg protein.

3. 4. 5. Catalase enzyme assay

Catalase enzyme activity was estimated by the method of Machly and Chance (1954). The estimation was done spectrophotometrically following the decrease in absorbance at 240 nm.

Reagents for catalase assay
Phosphate buffer 10mM pH 7, hydrogen peroxide 30 µM

The assay mixture contained

- Phosphate buffer 2.5ml
- Enzyme preparation 0.5 ml

The reaction was started by the addition of 0.5 ml H_2O_2. Change in optical density at 0. 30 and after 60 seconds were measured at 240 nm. Specific activity was expressed as milli moles of H_2O_2 consumed/min/mg protein.

3. 4 6. Protein estimation

The protein content in the samples was estimated following Lowry *et al.* (1951).

Reagents for protein assay
Sodium hydroxide 0.1 N, copper sulphate 0. 5%, Sodium carbonate 2 %, Sodium potassium tartarate 1%,
Alkaline copper reagent: prepared by mixing 0.5 ml copper sulphate, 0.5 ml sodium potassium tartarate and 50 ml sodium carbonate. Folin- phenol reagent (2N) stock diluted 1: 1 with double distilled water.

* Standard protein solution (100 µg/ml) was prepared freshly by dissolving 10 mg bovine serum albumin in 100 ml 0.1N sodium hydroxide.

To 0.2 ml of tissue extract, 1.8 ml of sodium hydroxide (0.1N) and 5 ml of alkaline copper reagent were added and kept for 15 min. Then 0.5 ml of diluted Folin-phenol reagent was added, mixed well, kept for 30 min and read the OD at 675 nm. To the blank, 0.2 ml of water and to the standard 0.2 ml of bovine serum albumin standard were added instead of enzyme extract and treated as above.

Statistical analysis

All the enzyme analyses were repeated three times and the data was analyzed statistically.

4

RESULTS

The present study was undertaken to develop a suitable method for the *Agrobacterium tumefaciens* mediated genetic transformation of *Hevea brasiliensis* incorporating agronomically important traits. The gene coding for superoxide dismutase under the control of FMV 34S promoter was selected with a view to develop transgenic *Hevea* plants with increased environmental stress tolerance. Attempts were also made to optimize the conditions for increasing the regeneration frequency of transgenic plants from the transformed cell lines and to assess the over-expression of the integrated gene.

4. 1. Development of Transgenic plants

4. I. I. Induction of callus for *Agrobacterium* infection

Callus induction in *Hevea brasiliensis* (clone RRII 105) from immature anther was first noticed as the swelling of the explants after two weeks in culture followed by callus initiation between 40-50 days in modified MS medium fortified with (2.0 mg/l) 2,4-D and (0.5 mg/l) Kn (Fig. 5). This emerging callus as well as callus proliferated for 20, 40 and 50 days after callus initiation were used for *Agrobacterium* infection.

4.1.2. Identification of ideal explant stage for *Agrobacterium* infection

When emerging callus was used as the explant, the transformation frequency was found to be 4%. With two-month old callus (20 days after callus initiation) 11% transformation frequency was obtained. With aged callus (40-50 days after callus initiation) the transformation efficiency was only 2%. From the optimization experiments it was observed that two-month old (20 days after callus initiation), yellow friable callus was the ideal stage of the explant for *Agrobacterium* mediated genetic transformation and plant regeneration.

4. I. 3. Identification of the ideal antibiotic for the selection of transformed callus

In the kill-curve test, the percentage of cells survived decreased with increasing concentration of the antibiotics. Since the effect of these antibioticc in a freshly prepared medium persists only for 14-15 days, three subcultures were made in fresh antibiotic containing medium at three weeks interval for identifying the optimum concentration. The antibiotic concentration at which no cell proliferation occurs and all the clumps turned brown after three subcultures was selected as the optimum concentration.

Table. 4.1. Antibiotic kill-curve test for the selection of transformed callus

Antibiotic	Optimum conc. identified (mg/l)
Neomycin	-
Kanamycin	300.00
Paromomycin	200. 00
Geneticin	100. 00

Ten callus clumps were cultured for every antibiotic concentration. All the experiments were repeated thrice.

In the kill curve test neomycin was found to be insensitive to *Hevea callus* even at a concentration of 800 mg/l. Therefore, it was not used for the section of transformed callus. Kill-curve test carried out with geneticin revealed that the optimum concentration required for the selection of transformed callus was 100 mg/l. Transformation experiment was carried out twice with geneticin as the antibiotic for selection and several transformed cell lines were obtained but none of these putatively transformed cell lines produced embryogenic callus. The minimum concentration of kanamycin required for the selection of putatively transformed callus was found to be 300 mg/l. The optimum concentration identified for paromomycin was 200 mg/l (Table 4.1). Standardization experiments revealed that paromomycin (200 mg/l) or kanamycin (300 mg/l)) could be effectively used for the selection of transformed callus.

The *Agrobacterium* infected callus, after three sub cultures at three weeks interval in the selection medium, antibiotic resistant yellow friable callus emerged within 45-50 days from those cells in which gene integration have occurred and the untransformed callus became brownish black (Fig. 6).

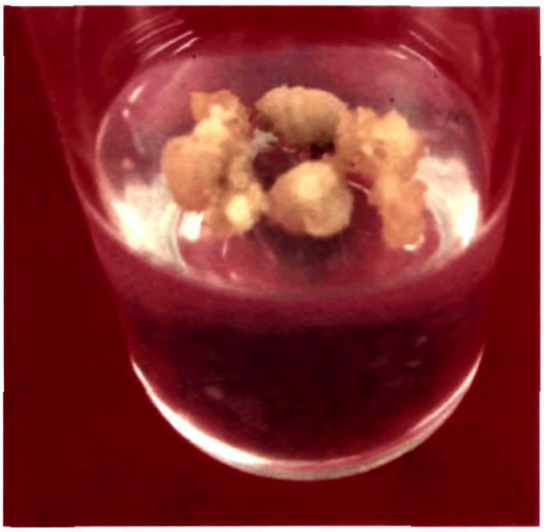


Fig.5: Callus formed two months after inoculation of immature anther

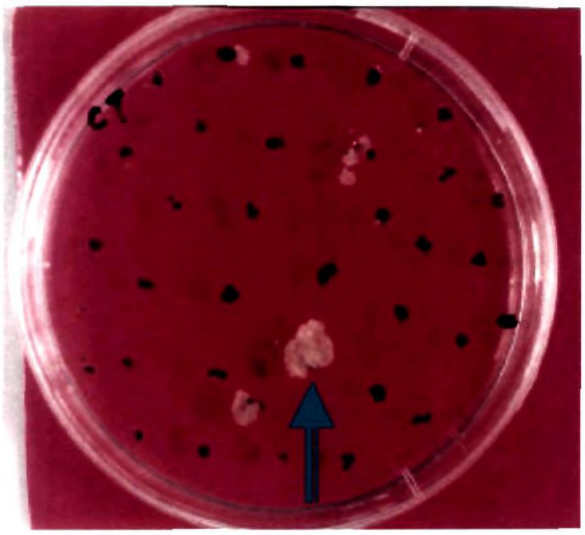


Fig.6: Proliferation of transformed callus in the selection medium (arrow)

Fig. 7: Histochemical staining for GUS expression in the callus
A- Untransformed (yellow), B- Transformed (blue)

4. I. 4. Selection of transformed callus by GUS histochemical staining

The antibiotic resistant yellow friable calli emerged from the infected calli were used for GUS histochemical staining as well as for further regeneration studies after confirmation of gene integration. GUS histochemical staining was carried out with approximately 2 mg of the yellow friable callus. β-glucuronidase catalyses the hydrolysis of the substrate x-gluc and liberates a molecule of 5-bromo-4-chloro-indoxyl (XH) which is in equilibrium with its tautomer, 5-bromo-4-chloro- indoxyl (X). In the presence of oxygen, the soluble indoxyl (X) and (XH) are dimerized to form insoluble diX-indigo and diXH- leuco indigo resulting in the blue coloration. The callus in which gene integration had occurred became dark blue in color (GUS positive) (Fig.7) and these putatively transformed cell lines were selected. From the kanamycin containing medium 18 putatively transformed cell lines were obtained out of 300 explants. A transformation frequency of 6% was obtained with kanamycin. When paromomycin was used for selection 22 transformed cell lines were obtained out of 200 explants, getting 11% transformation frequency. With geneticin 18 putatively transformed cell lines were obtained from 150 explants and a transformation frequency of 12% was obtained.

4. I. 5. Callus proliferation and embryogenic callus induction

The putatively transformed callus and untransformed calli were proliferated in modified MS basal medium fortified with varying concentrations of growth regulators 2,4-D, NAA and BA. A detailed study was conducted to understand the effect of auxin on callus proliferation on modified MS medium fortified with different concentrations of 2,4-D (0.0-2.0 mg/l) and NAA (0.0-0.5 mg/l). No callus induction and proliferation was observed in the absence of 2,4-D and NAA, where as callus proliferation increased with increasing concentrations of the auxins. Callus proliferation was favoured by the addition of 2,4-D (0.5-1.50 mg/l) and NAA (0.1- 0.3) mg/l, while higher concentrations of 2,4-D (above 1.25 mg/l) induced hard, compact and nonembryogenic callus. A synergistic effect of these two auxins was observed. The optimum concentration of 2,4-D and NAA required for obtaining a callus proliferation frequency of around 60% was found to be 1.0 mg/l and 0.2 mg/l respectively (Table 4.2). This

proliferating callus on further subculture onto proliferation medium supplemented with BA produced friable embryogenic callus.

Table. 4.2. Effect of 2,4-D and NAA on callus proliferation

2,4-D (mg/l)	NAA (mg/l)	Callus proliferation* rate
0.00	0.00	-
0.50	0.10	+
0.75	0.15	+ +
1.00	0.20	+ + +
1.25	0.30	+ +
1.50	0.40	+ +
2.00	0.50	+

* Callus proliferation was scored by visual observation. Approximately 100 mg of callus was cultured on the proliferation medium. Callus induction frequency is expressed as + =1-20%, + + = 21-40%, + + + = 40- 60%.
Values are average of 10 replicate samples and the experiment was repeated three times

The combined effect of 2,4-D, NAA and BA on callus proliferation was determined by subculturing the calli on media fortified with different concentrations of 2,4-D (0.20 to 2 mg/l), NAA (0.0-0.5 mg/l) and BA (0.1- 1.0 mg/l). Results indicate that BA in combination with 2,4-D and NAA promoted callus proliferation. Callus proliferation was increased with increasing concentrations of 2,4-D, NAA and BA (Table.4.3). A callus proliferation

Table. 4.3. Effect of 2,4-D, NAA and BA on callus proliferation

2,4-D	NAA	BA	Callus proliferation* rate
0.20	0.00	0.10	+
0.40	0.05	0.20	+ +
0.60	0.10	0.30	+ +
0.80	0.15	0.40	+ + +
1.00	**0.20**	**0.50**	+ + + +
1.25	0.30	0.60	+ + +
1.50	0.40	0.80	+ +
2.00	0.50	1.00	+ +

*Rate of callus proliferation was taken by visual observation by scoring the percentage of callus proliferated in each culture tube, compared to initial callus culture.
Callus induction frequency is expressed as + =1-20%, + + = 21-40%, + + + = 40- 60%, + + + + =60-80%
Values are average of 10 replicate samples and each experiment was repeated three times

frequency of above 70 % was achieved with the hormonal combination 2,4-D (1.0 mg/l), NAA (0.20 mg/l) and BA (0.50 mg/l). On further increasing the auxin and

cytokinin concentrations, a reduction in callus proliferation was observed. Even though callus proliferation was observed above this optimum concentration, this callus on further subculture in the proliferation medium no embryonic cluster formation was observed.

To understand the effect of nitrogen on callus proliferation, MS basal medium as well as MS medium modified by changing the NH_4NO_3 concentration to 1000 mg/l and supplemented with 2,4-D (1.0 mg/l), NAA (0.20 mg/l) and BA (0.50 mg/l) were tested for callus proliferation. Callus proliferation was observed in both MS and modified MS medium supplemented with growth regulators. However, the frequency of callus proliferation was higher in modified MS basal medium than that was observed in MS basal medium.

Yellow, friable translucent calli were developed from all the forty independently transformed cell lines (18 cell lines selected from kanamycin containing medium and 22 cell lines from paromomycin). This callus, on further subculture on to the callus proliferation medium fortified with 2,4-D (1.0 mg/l), NAA (0.20 mg/l) and BA (0.50 mg/l), embryogenic calli were formed. Out of these forty cell lines, embryogenic callus (Fig. 8) was formed only from five independently transformed cell lines.

4.1. 6. Somatic embryo induction

Embryogenic callus, on further subculture onto embryo induction medium, embryonic clusters were formed within four to five weeks which further developed into globular stage embryos (Fig. 9). Effect of Kn and GA_3 in combination with 0.2 mg/l NAA on embryo induction was evaluated. The results indicate that the addition of Kn in combination with NAA (0.2 mg/l) favoured the conversion of embryonic mass to globular/ torpedo stage embryos. The number of globular/ torpedo stage embryos formed per unit mass of callus varied with varying concentration of Kn and the maximum was observed with 0.3 mg/l Kn. An embryo induction frequency of 42.0 % was obtained with the hormone combination NAA (0.2 mg/l), GA_3 (0.6 mg/l) and Kn (0.3 mg/l) (Table. 4. 4).

The beneficial effect of BA and GA_3 on embryo induction was assessed by carrying out an experiment with BA and GA_3 in presence of 0.2 mg/l NAA and 0.30 mg/l Kn. Embryo induction was favoured by the addition of GA_3. Embryo induction frequency increased with increasing concentration of GA_3 and above (0.6 mg/l) embryo induction was decreased indicating that GA_3 concentration had a crucial role

Fig. 8: Transformed embryogenic callus

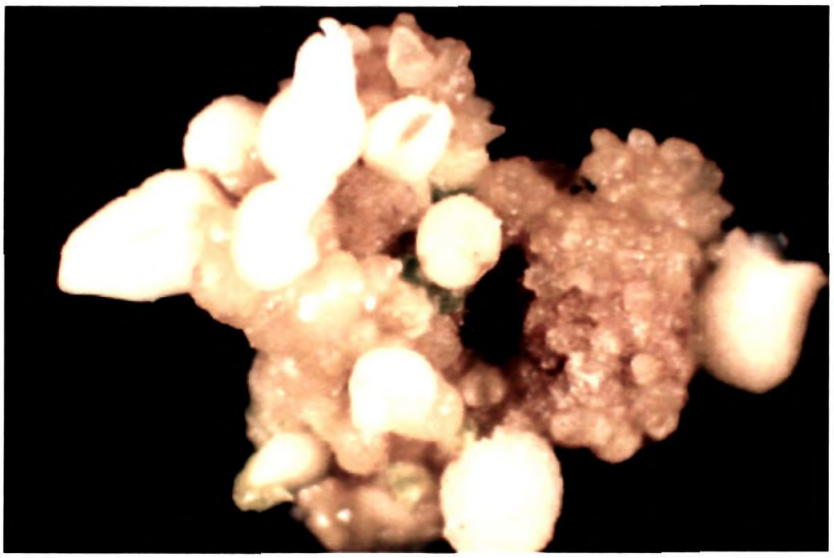


Fig. 9: Cluster of globular embryos emerging from embryogenic callus

in promoting embryo induction. Incorporation of BA (0.30 mg/l) significantly improved embryo induction frequency (Table 4.5). Higher concentrations of BA (above 0.3 mg/l) promoted callus proliferation and thereby embryo induction frequency was decreased. The optimum concentration of growth regulators identified for efficient embryo induction was NAA (0. 20 mg/l), BA (0. 30 mg/l), Kn (0. 30 mg/l) and GA_3 (0.60 mg/l) (Table 4.5). An embryo induction frequency of 52 % was obtained with this hormone combination.

Table 4.4. Effect of Kn and GA_3 in combination with (0.2 mg/l) NAA on embryo induction (%)*

Kn	GA_3				
(mg/l)	0.2	0.4	**0.6**	0.8	1.00
0.00	3.00	5.75	13.00	9.00	8.00*
0.05	5.50	8.50	15.00	11.00	10.00
0.10	8.50	19.00	24.50	19.25	13.00
0.20	12.00	25.00	32.00	27.50	25.00
0.30	18.25	30.00	42.00	31.75	30.00
0.40	16.00	27.00	31.00	24.00	22.00
0.50	8.00	12.50	16.00	10.00	9.00

CD (5%) = 1.64

*Embryo induction frequency was calculated by visual counting the number of globular/ torpedo stage embryos formed from approximately 100 mg of embryogenic callus subcultured on to embryo induction medium and converting into percentage.
Values are average of ten replications and each experiment was repeated four times.

Table 4.5. Effect of BA and GA_3 in combination with (0.3 mg/l) Kn and (0.2mg/l)NAA on embryo induction (%)*

	GA_3 (mg/l)				
BA (mg/l)	0.30	**0.60**	0.90	1.20	1.50
0.00	0.00	11.66	16.66	20.00	5.00*
0.10	18.33	21.66	20.00	15.00	8.33
0.20	26.66	35.00	23.33	18.33	15.00
0.30	43.33	**51.66**	30.00	25.00	15.00
0.40	10.00	21.66	11.66	10.00	6.66
0.50	8.33	15.00	10.00	6.66	5.00

CD (5%) = 4. 71

*Embryo induction frequency was calculated by determining the number of globular/ torpedo stage embryos formed from approximately 100 mg of embryogenic callus cultured on to embryo induction medium and converting into percentage.
Values are average of ten replicates and each experiment was repeated four times.

4.1.7. Effect of water and osmotic stress on embryo induction

Effect of osmotic stress on embryo induction revealed that osmotic stress induced by the addition of high concentration of PEG improved embryo induction frequency of untransformed as well as transformed callus. Embryo induction frequency was increased with increasing concentration of PEG and the optimum concentration identified for obtaining maximum embryo induction in transformed and untransformed callus were found to be 8.0% and 6% (w/v) respectively. Above this concentration callus became deep brown colour, indicating cell necrosis and damage to the tissues. Embryo induction frequency of transformed callus was increased by 10% in medium supplemented with 8.0% (w/v) PEG, where as for untransformed cultures, 8% increase was observed at 6% (w/v) PEG concentration (Table.4. 6).

Table.4.6. Effect of PEG induced stress on embryo induction

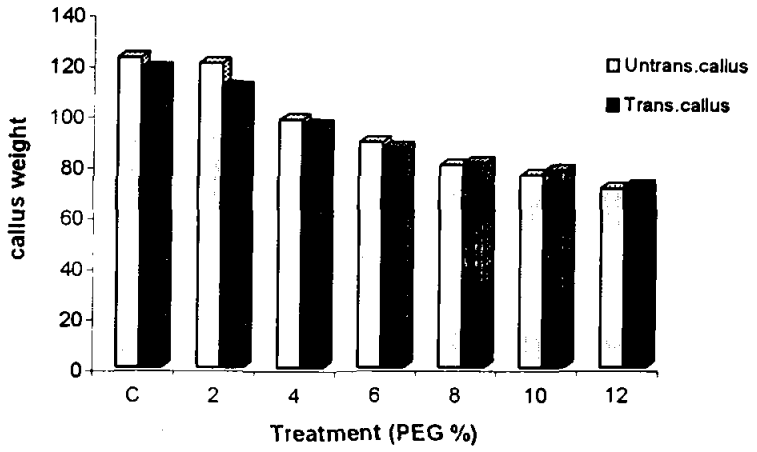
PEG con (%)	Embryo induction (%)*	
	Untransformed callus	Transformed callus
0.00	47.50	51.00
2.00	47.50	52.50
4.00	50.00	55.00
6.00	**56.00**	57.50
8.00	48.50	**61. 00**
10.00	34.00	46. 00
12.00	22.50	34. 00

CD (A) = 1.51, CD (B) = 2.62

*Percentage of embryo induction was calculated as the mean number of embryos formed from unit mass of the callus cultured on embryo induction medium provided with different concentrations of PEG, and converting into percentage.

In order to assess the effect of osmotic stress on callus fresh weight varying concentrations of PEG (2-12%) was included in the embryo induction medium fortified with NAA (0. 20 mg/l), BA (0. 30 mg/l), Kn (0. 30 mg/l) and GA_3 (0.60 mg/l). Results revealed that in the control (no stress) callus proliferation was observed and the callus fresh weight was increased by 22%. Callus proliferation was impaired by the induction of osmotic stress with PEG and the callus fresh weight decreased with increasing concentration of PEG in the untransformed as well as transformed callus (Fig. 10)

Fig.10 Effect of osmotic stress on callus fresh weight



Water stress induced by the addition of phytagel also improved embryo induction. Embryo induction efficiency of transformed callus was enhanced from 51.6% to 62% in medium supplemented with 0.6% (w/v) phytagel thus getting an increase of 10.4% (Table. 4.7).

Table. 4.7. Effect of phytagel induced water stress on embryo induction*

Phytagel Conc. (%)	Embryo induction (%)*	
	Untransformed callus	Transformed callus
0.20	47.50	51.00*
0.40	**55.00**	56.00
0.60	37.50	**62.00**
0.80	21.00	36.00
1.000	12.50	14.00

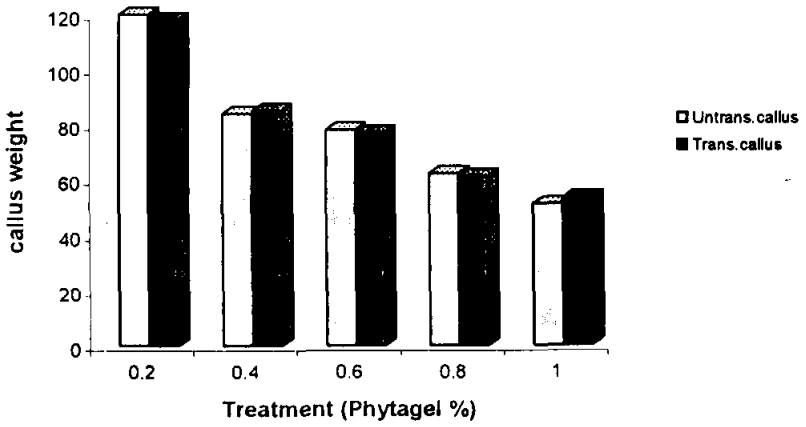
CD (A) = 2. 02, CD (B) = 3.19

*Percentage of embryo induction was calculated as the mean number of embryos formed from unit mass of the callus cultured on stress induction medium and converting into percentage.

Callus proliferation was inhibited by imparting water stress to the callus cultures. Water stress induced by the addition of high concentration of phytagel (above 0.2%) led to reduction in callus weight. This dehydration of the callus favoured embryo induction and the embryo induction frequency was improved

(Table.4.7). As the phytagel concentration was further increased, the water availability was decreased and the callus in contact with the medium first turned brown in colour and then got dried up within 30 days. Callus weight decreased as the phytagel concentration was increased and the maximum reduction was observed at 1.0% phytagel concentration (Fig.11).

Fig. 11 Effect of water stress on callus fresh weight



4. 1. 8. Embryo maturation

Globular/ torpedo stage embryos in the maturation medium, developed further into mature embryos within 40-50 days. Embryo maturation was initiated by the conversion of globular stage embryos to heart, torpedo and finally to cotyledonary stage embryos (Fig. 12 & 13). In the maturation medium, along with transition process from globular to cotyledonary stage, many secondary embryos were also developed from the primary embryos. Effect of growth regulators such as GA_3, BA and TDZ on embryo maturation was evaluated. Embryo maturation was significantly improved by the addition of GA_3 and TDZ. Compared to embryo induction, lower concentration of GA_3 was required for embryo maturation. The optimum concentration of GA_3 required for embryo maturation was (0.4 mg/l). As the GA_3 concentration was increased above 0.4 mg/l, precocious germination of the embryos was observed. An embryo maturation frequency of 43% was obtained with the hormone combination 0.3 mg/l TDZ and 0.4 mg/l GA_3 (Table.4.8). It was also noticed that the percentage of plant regeneration from the embryos matured on

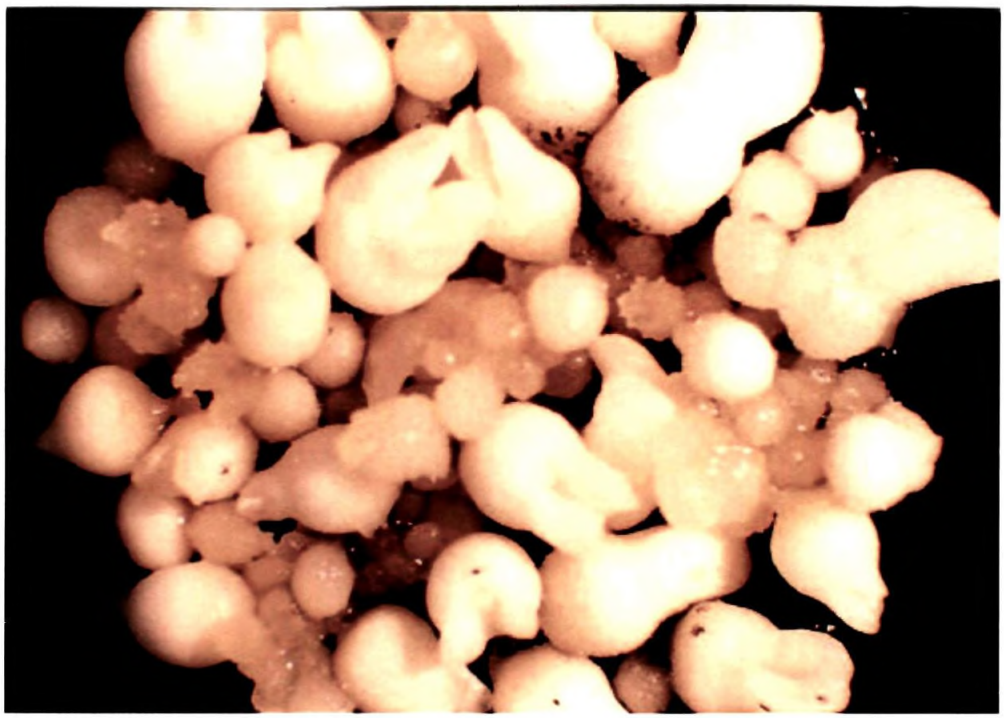


Fig. 12: Somatic embryos under different developmental stages (globular, heart, torpedo and cotyledonary)

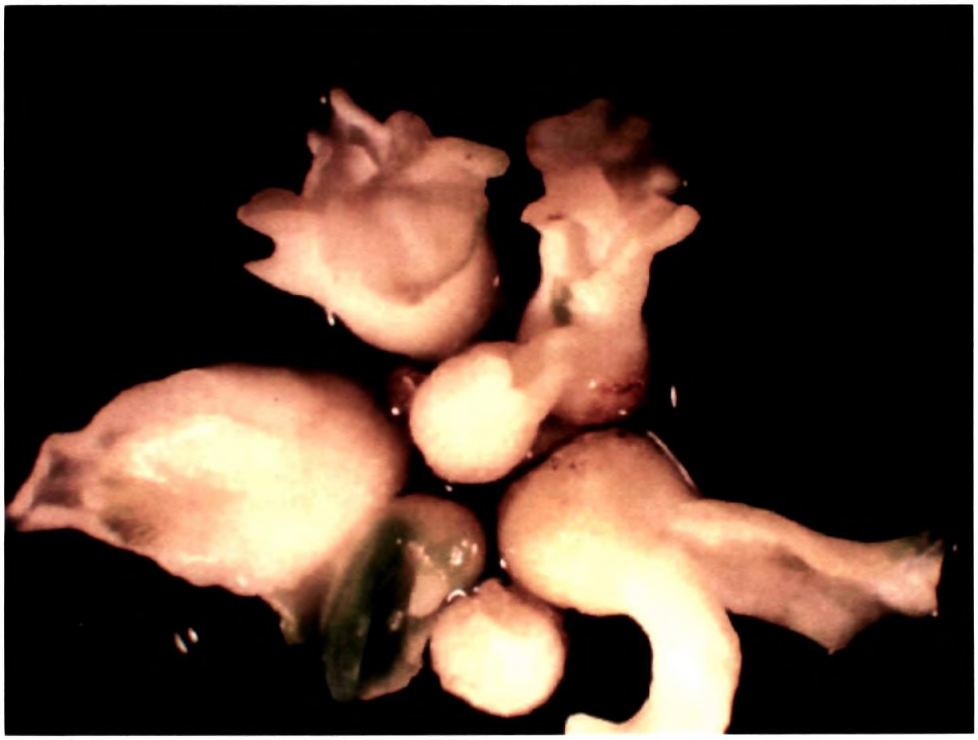


Fig. 13: Mature embryos with well-developed cotyledons

medium containing GA_3 and TDZ was higher than that was observed with GA_3 and BA combinations

Table. 4.8. Effect of GA_3 and TDZ on embryo maturation (%)*.

TDZ (mg/l)	GA_3 (mg/l)					
	0.10	0.20	0.30	**0.40**	0.50	0.60
0.00	0.00	10.66	17.00	13.33	9.00	2.00*
0.10	5.33	13.00	16.66	20.66	14.00	7.00
0.20	10.33	17.00	20.00	31.33	23.00	15.66
0.30	12.00	21.33	29.33	**43.00**	33.66	18.00
0.40	9.00	12.00	18.33	25.00	20.66	13.33
0.5 0	2.66	5.00	6.66	10.00	4.33	2.00

CD (5%) = 1.53

*Percentage of embryo maturation was calculated as the mean number of mature embryos obtained out of 100 globular /torpedo stage embryos subcultured on maturation medium. The experiment was repeated thrice.

Incorporation of BA in the culture medium favoured embryo maturation. The optimum concentration of BA and GA_3 identified for getting a maturation frequency of 35% was found to be 0.3 mg/l and 0.4 mg/l respectively (Table.4.9). Embryo maturation frequency observed with the combination of BA and GA_3 was lower than that was obtained with TDZ and GA_3.

Table. 4.9. Effect of BA and GA_3 on embryo maturation (%)*

BA (mg/l)	GA_3 (mg/l)					
	0.10	0.20	0.30	**0.40**	0.50	0.60
0.1	2.25	5.00	9.25	9.75	6.50	3.00*
0.2	5.00	7.00	12.00	15.50	10.00	5.25
0.3	8.25	12.25	21.0	**35.00**	19.00	11.00
0.4	4.75	11.00	14.25	21.75	15.25	6.50
0.5	2.00	3.00	5.75	7.50	3.50	1.25

CD (5%) = 1. 91

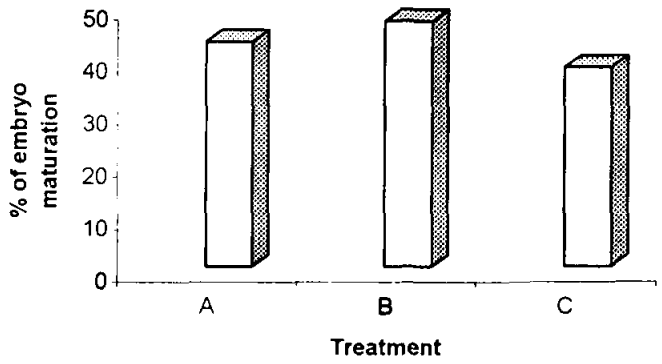
*Percentage of embryo maturation was calculated as the mean number of mature embryos obtained out of 100 globular /torpedo stage embryos cultured on maturation medium.
Experiment was repeated four times.

4. 1. 9. Effect of amino acids on embryo maturation

When casein hydrolyzate was used as the source of organic nitrogen, the embryo maturation was 43%. Supplementation of basal medium with amino acids such as glutamine (200 mg/l), proline (100 mg/l) and glycine (10 mg/l) increased

the maturation frequency to 47%. As the organic nitrogen concentration was further increased by the addition of asparagine (100 mg/l), alanine and arginine (50 mg/l each), the embryo maturation was reduced to 38% (Fig. 14).

Fig. 14 Effect of amino acids on embryo maturation



A – No amino acid; B – Pro (100 mg/l), Gln (200 mg/l) and Gyl (10 mg/l); C – Pro (100 mg/l), Glu (200 mg/l), Gly (10 mg/l), Asn (100 mg/l), Ala (50 mg/l) and Arg (50 mg/l).

4. 1. 10. Effect of water and osmotic stress on embryo maturation

Water stress induced by the addition of phytagel improved embryo maturation frequency. Supplementation of the maturation medium with 0.4% (w/v) phytagel resulted in getting a maturation frequency of 57.5%, which was significantly higher than the conversion frequencies obtained with the other concentrations of phytagel (Table.4.10). Further increase in phytagel concentration caused browning of the embryos after 2-3 weeks and a reduction in the fresh weight of this embryos was also observed and these embryos finally got dried up.

Effect of osmotic stress (PEG) alone and in combination with 0.1 mg/l abscisic acid (ABA) on embryo maturation was evaluated. PEG alone was not effective in promoting embryo maturation. However, addition of PEG in combination with 0.1 mg/l ABA in the maturation medium, significant difference was observed on the number of mature embryos recovered and conversion of embryos into plantlets. Supplementation of the maturation media with 6.0% PEG (w/v) in combination with 0.10 mg/l ABA resulted in 6% increase in maturation

frequency of untransformed as well as transformed embryos (Table.4.11). Embryo maturation was increased at 4.0-8.0%(w/v) PEG concentrations, further increase in PEG concentration caused browning of the embryos and they never germinated.

Table. 4.10. Effect of phytagel induced water stress on embryo maturation

Phytagel Con.(%)	Embryo maturation* (%)	
	Untransformed callus	Transformed callus
0.20	45.50	47.50*
0.40	**52.50**	**57.50**
0.60	32.50	40.00
0.80	15.50	22.50
1.00	5.00	7.50
		CD (A) = 3.63CD (B) = 5.74

*Ten replicate samples were taken for each treatment and the experiment was repeated four times.

Table.4.11. Effect of PEG and 0.1 mg/l ABA on embryo maturation

PEG con. (%)	Embryo maturation (%)*	
	Untransformed callus	Transformed callus
0.00	45.50	47.50*
2.00	46.00	48.00
4.00	48.00	52.00
6.00	**52.00**	**54.00**
8.00	42.00	44.00
10.00	24.00	26.00
12.00	14.00	16.00
		CD (A) = 2.63 CD (B) = 4.56

*Ten replicate samples were taken for each treatment and the experiment was repeated four times.

Another phenomenon observed in transgenic embryo maturation process was that around 15 to 20% of the late torpedo stage embryos in the maturation medium turned into abnormal embryos with swollen cotyledons but without shoot apex. This abnormality may be due to the toxic effect of kanamycin used in the selection medium. These transformed embryos showed β-glucuronidase activity,

as judged from the blue staining observed in histochemical test, indicating the occurrence of transformation (Fig.17).

4. 1. 11. Embryo germination and regeneration of transgenic plants

Embryo germination was initiated by the bipolar differentiation of (root and shoot primodia) the mature embryos (Fig. 15). The mature embryos germinated and developed into plantlets (Fig. 16) after four to five weeks when individual embryos were cultured on half- strength MS medium. Embryo germination was observed on hormone free medium as well as on medium fortified with growth regulators. Embryo germination was enhanced by the addition of GA_3. Although germination was observed in hormone free medium, the plantlets germinated in GA_3 containing medium were having more vigorous growth than that was developed from hormone free medium. Even though embryo germination was observed at higher concentrations of GA_3, it adversely affected further development of the plantlet. Early leaf senescence was observed in plantlets germinated at higher concentrations (above 0.3 mg/l) of GA_3. Addition of IBA (0.1-0.3 mg/l) favoured root elongation. Maximum plant conversion was observed on a medium supplemented with 0.3 mg/l GA_3 and 0.10 mg/l IBA (Table. 4.12).

Table. 4.12. Effect of GA_3 and IBA on embryo germination (%)*

IBA	GA_3 (mg/l)					
(mg/l)	0.0	0.1	0.2	**0.3**	0.4	0.5
0.0	5.25	8.75	11.75	16.50	14.25	9.50*
0.1	7.50	11.00	12.25	**21.50**	16.00	7.75
0.2	8.00	9.25	12.50	20.75	14.75	8.50
0.3	6.75	8.00	10.75	19.00	14.00	7.25
0.4	5.25	7.25	9.00	15.50	11.00	6.25
0.5	3.75	6.75	7.25	13.00.	9.25	6.25

CD (5%) = 1.80

*Ten replicate samples were taken for each experiment and the experiment was repeated thrice.

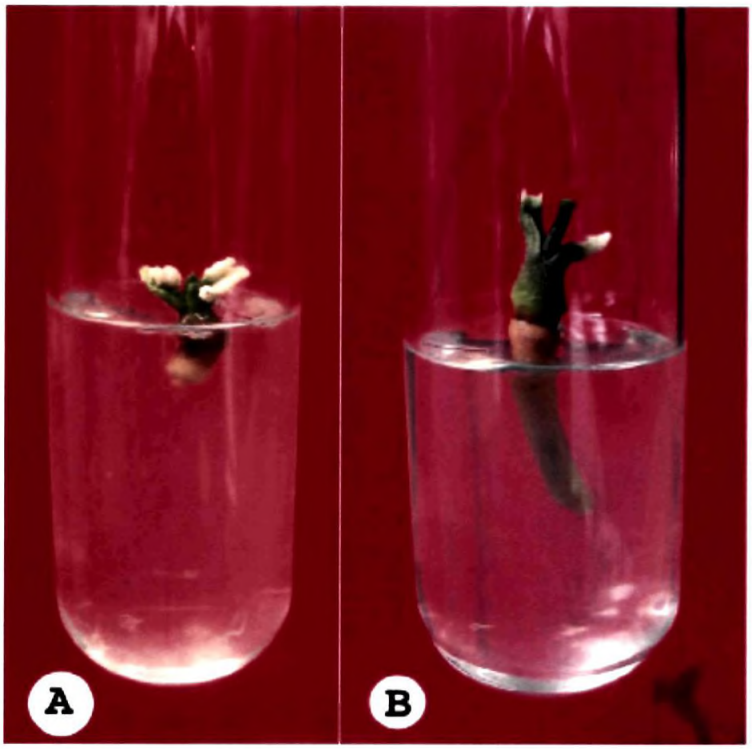


Fig. 15: Germinating embryos with shoot and root primodia

Fig. 16: Fully developed transgenic plantlet in culture tube

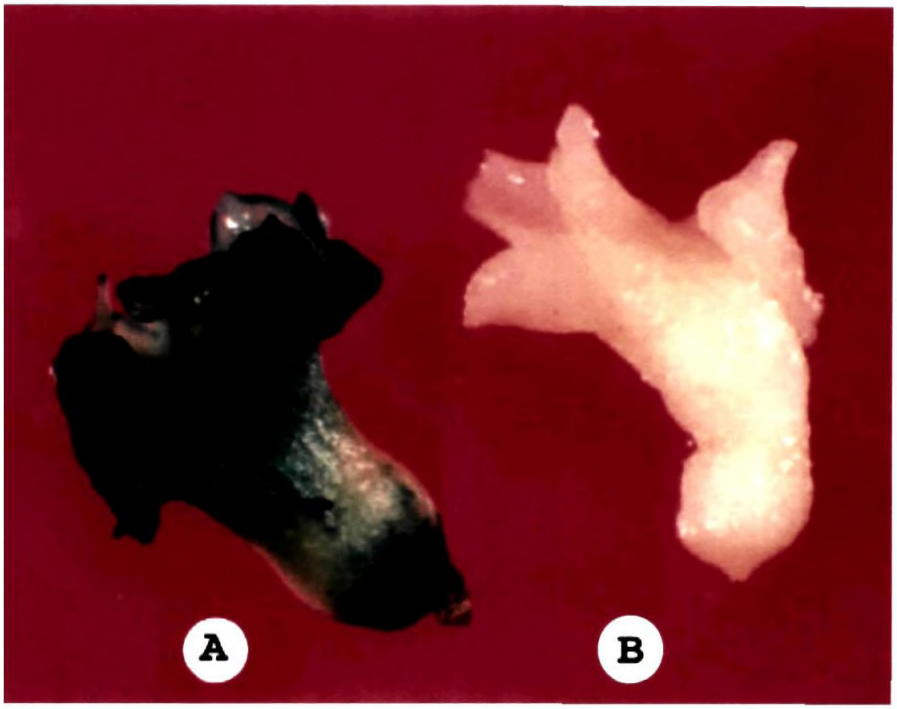


Fig. 17: Transgenic embryo showing GUS expression
A - Transformed (blue), B- Untransformed (yellow)

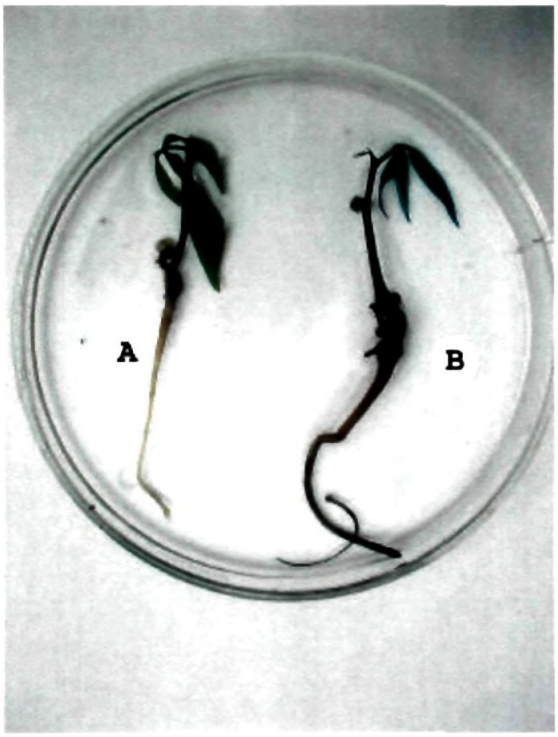


Fig. 18: Transgenic plantlet showing GUS expression
A - Untransformed plantlet, B - Transgenic plantlet

Effect of BA on embryo germination and full plantlet development was evaluated in combination with GA_3 and 0.1 mg/l IBA. Even though BA was not effective in improving embryo germination, addition of BA (0.3 mg/l) in the embryo germination medium favored shoot elongation.

Table. 4.13. Effect of BA and GA_3 in combination with 0.1 mg/l IBA on embryo germination (%)*.

BA (mg/l)	GA_3 (mg/l) + 0.1 mg IBA					
	0.0	0.1	0.2	**0.3**	0.4	0.5
0.0	5.25	8.50	10.25	16.00	14.25	10.00*
0.1	7.50	11.25	11.50	19.25	16.00	7.00
0.2	7.75	11.70	13.00	19.25	14.50	5.50
0.3	6.25	8.50	12.50	**19.50**	15.25	6.00
0.4	4.00	6.00	7.75	18.00	11.50	6.50
0.5	2.50	4.25	5.50	11.00	9.00	5.25

CD (5%) =1.83

*Ten replicate samples were taken for each experiment and the experiment was repeated four times

4. 1. 12. Acclimatization of the plants and transplantation to soil

The *in vitro* raised transgenic and untransformed (control) plants were acclimatized by initially planting in small earthenware pots (5"x10") filled with sterile soilrite, sand and soil. After transplantation relative humidity of 90-95% for six to seven days was required for the survival of the plants. The survival rate during hardening was 40-45 %. The acclimatized plants (Fig. 19) were maintained in the glass house till new leaves emerged. These plants with 2-3 whorls of mature leaves were transplanted to black polythene bags (9½" x 22") filled with soil, sand and cow dung (2:1:1) and maintained in the greenhouse (Fig. 20). During this transplantation 50-55 % of the plants survived.

Fig. 19: Hardened transgenic plants in earthenware pots

Fig. 20: Transgenic plant growing in polythene bag

4. 2. Molecular analysis of transgenic plants

4. 2. I. Polymerase chain reaction.

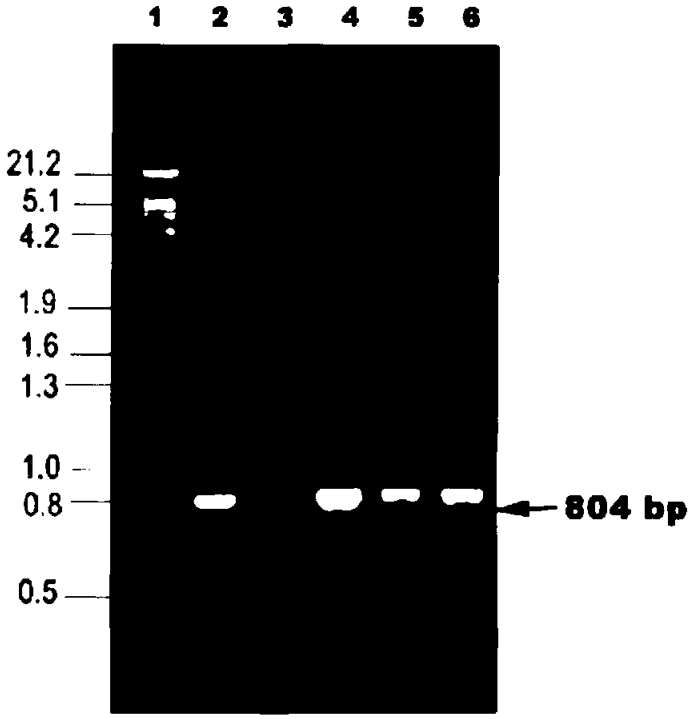
RNA isolated from three transgenic plants developed through three independent transformation events along with one untransformed plant as negative control were used in PCR analysis using primers designed for the amplification of *npt*II and Mn SOD genes. The plasmid vector was used as the positive control. The presence of *npt*II gene was confirmed by the amplification of 804 bp single product in all the three transgenic plants and in positive control. No amplification was detected in the untransformed plant (Fig. 21A). When PCR was performed with Mn SOD gene specific primers, a 702 bp fragment was amplified in all transgenic plants and in the positive control plasmid. This fragment corresponds to the cDNA sequences coding for Mn SOD gene that has been integrated into the plant genome. This 702 bp fragment was absent in the untransformed plant. However, a 3.2 kb native Mn SOD gene present in the transgenic as well as untransformed plants were also amplified in a long PCR (Fig. 21B). This 3.2 kb band, corresponds to the native genomic DNA sequence of Mn SOD which was absent in the plasmid. These results indicate the presence of the transgene in the genome of *Hevea* transgenic plants.

4.2. 2. Southern hybridization analysis

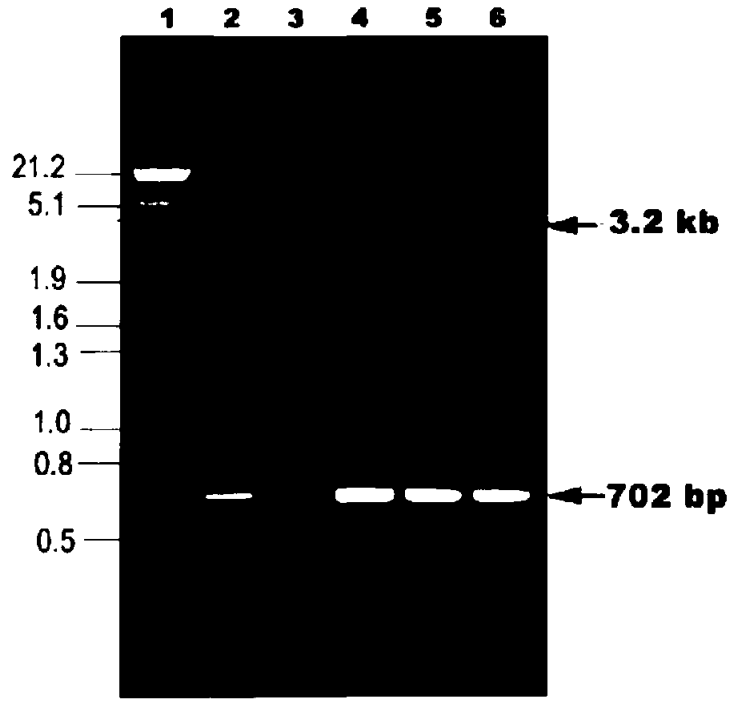
The presence as well as the integration of the T-DNA region in to the genome of the putative transgenic plants was confirmed by Southern hybridization analysis. Genomic DNA was digested with the restriction enzyme *EcoR* I to show the insert of predicted size and with *Sac* I to confirm the integration into the plant genome and the number of insertions. When the DNA of the transgenic plants were digested with *EcoR* I, the integrated *npt*II gene of 980 bp get liberated (Fig. 22 A). The ^{32}P labeled *npt*II gene probe generated a band of predicted size (980 bp) with *EcoR* I digests in all the three tested transgenic plants and in positive control (vector plasmid) (Fig. 22A, Lanes-1,2,4,5&6). No hybridization could be detected in the untransformed plant (negative control) (Lane-3).

The *Sac* I enzyme had a unique restriction site towards the left border of T-DNA. Hybridization of *Sac* I digests with *npt*II probe produced bands with more than 2.5 kb size, representing the DNA fragments containing the *npt*II gene and a part of the plant genomic DNA located outside the left T-DNA border (Fig 22 B). The

Fig. 21: Detection of transgenes by PCR



A. with *npt* II gene specific primers. Lanes: 1- Molecular weight marker, 2 - Plasmid DNA (positive control); 3 - Untransformed plant (negative control); 4-6 Transgenic plants



- with MnSOD gene specific primers. Lanes: 1- Molecular weight marker, 2 - Plasmid NA (positive control); 3 - Untransformed plant(negative control); 4-6 Transgenic plants

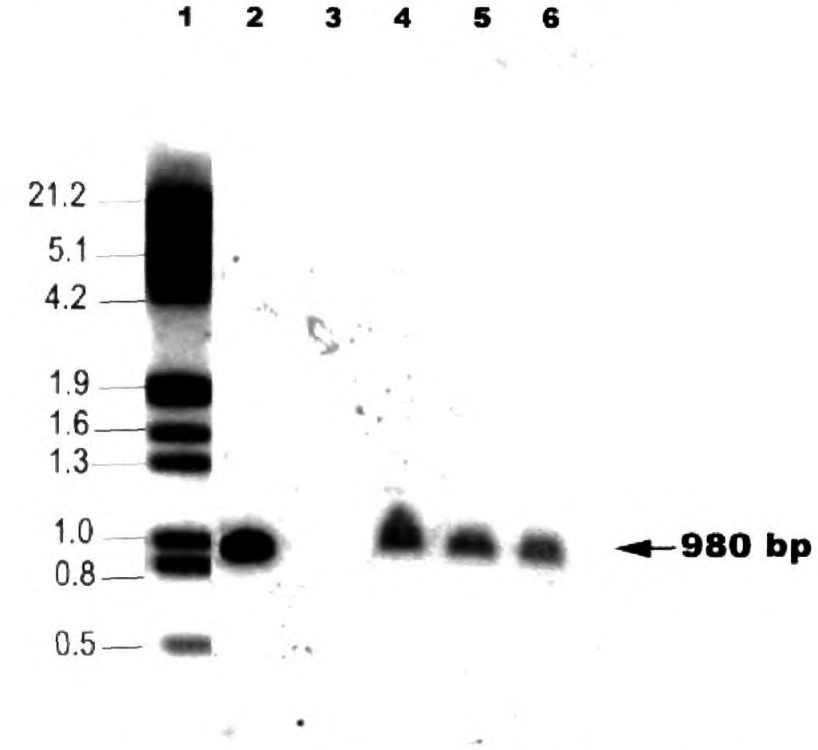
different hybridization patterns observed for transgenic plants indicate random integration and multiple insertion of the T-DNA into the genome of these plants. Each band that hybridized to the *Sac* I digested genomic DNA represents a single copy of the transgene. Although, same amount of digested DNA was loaded in each lane, the intensity of the hybridizing bands in transgenic plants was different, further indicating the difference in T-DNA copy numbers (Fig. 22B, Lanes-4,5&6). No hybridization was detected for the untransformed (negative control) (Lane-3). These results confirmed the successful integration of the foreign gene in *Hevea* through *Agrobacterium* mediated genetic transformation.

4. 3. Northern hybridization analysis

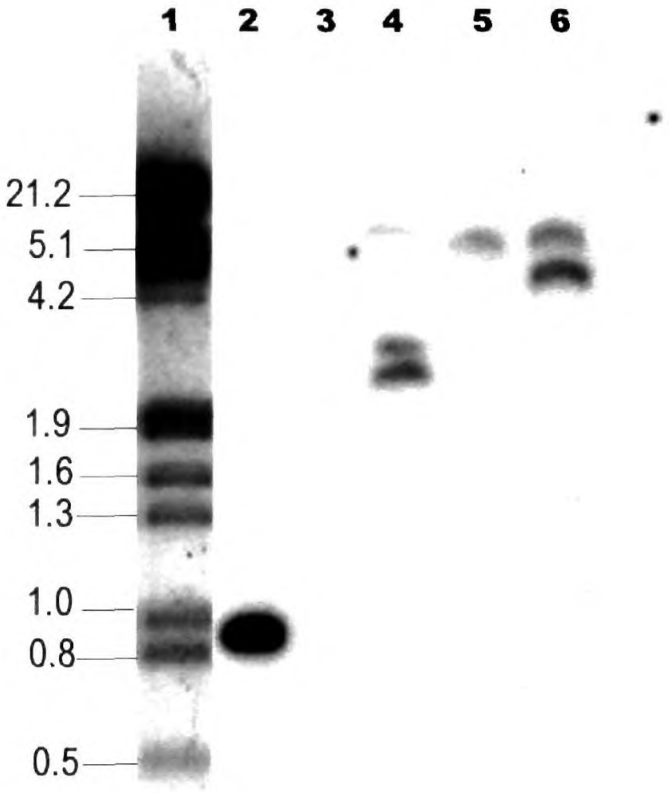
The accumulation of mRNA transcript level of Mn SOD gene in response to water and salinity stress was determined by RNA blot analysis. For Northern blot analysis total RNA was isolated from transformed as well as untransformed callus subjected to water stress with phytagel (0.4 and 0.6%), keeping non-stressed control. Results indicate that mRNA transcript level in the transformed control callus was higher than that was observed for untransformed control callus (Fig. 23 A, Lanes-UTC and TC). Further, the mRNA transcript level in the transformed callus subjected to water stress with 0.4% (w/v) phytagel concentration (T 0.4%) was higher when compared with the transformed callus without stress (TC). It was also observed that the mRNA expression was found to be low in untransformed and transformed callus with water stress at 0.6% phytagel concentration (Lanes- UT 0.6% &T 0.6%).

In the case of salt stress, which was induced by sodium chloride (100 and 200 mM), over-expression of SOD gene was noticed at 100 mM NaCl in normal as well as transformed callus (Fig. 23 B, Lanes- UT100 mM &T100 mM). The mRNA transcript level of transformed callus subjected to stress with 100 mM NaCl was abundant compared with the untransformed callus subjected to same stress (Lanes- UT 100 mM & T 100 mM). Callus samples subjected to stress with 200 mM NaCl, the mRNA transcript level was less in transformed as well as untransformed callus (Lanes- UT200 mM & T200 mM) compared to that observed with 100 mM NaCl.

Fig.22: Southern hybridization of genomic DNA from transgenic plants



A. DNA digested with *EcoR* I showing fragment of predicted size(980bp) after probing with *npt* II gene. Lanes: 1 - Labeled molecular weight marker ; 2- Positive control (plasmid); 3-Untransformed plant (negative control) and 4-6 Transgenic plants from three independent transformed cell lines



B-DNA digested with *Sac*I enzyme. PCR amplified 804 bp *npt* II gene was used as probe. Lanes: 1 - Labeled molecular weight marker; 2- Positive control (plasmid); 3-Untransformed plant (negative control) and 4-6 Transgenic plants from three independent transformed cell lines

Fig. 23: Northern blot analysis after probing with Mn SOD cDNA

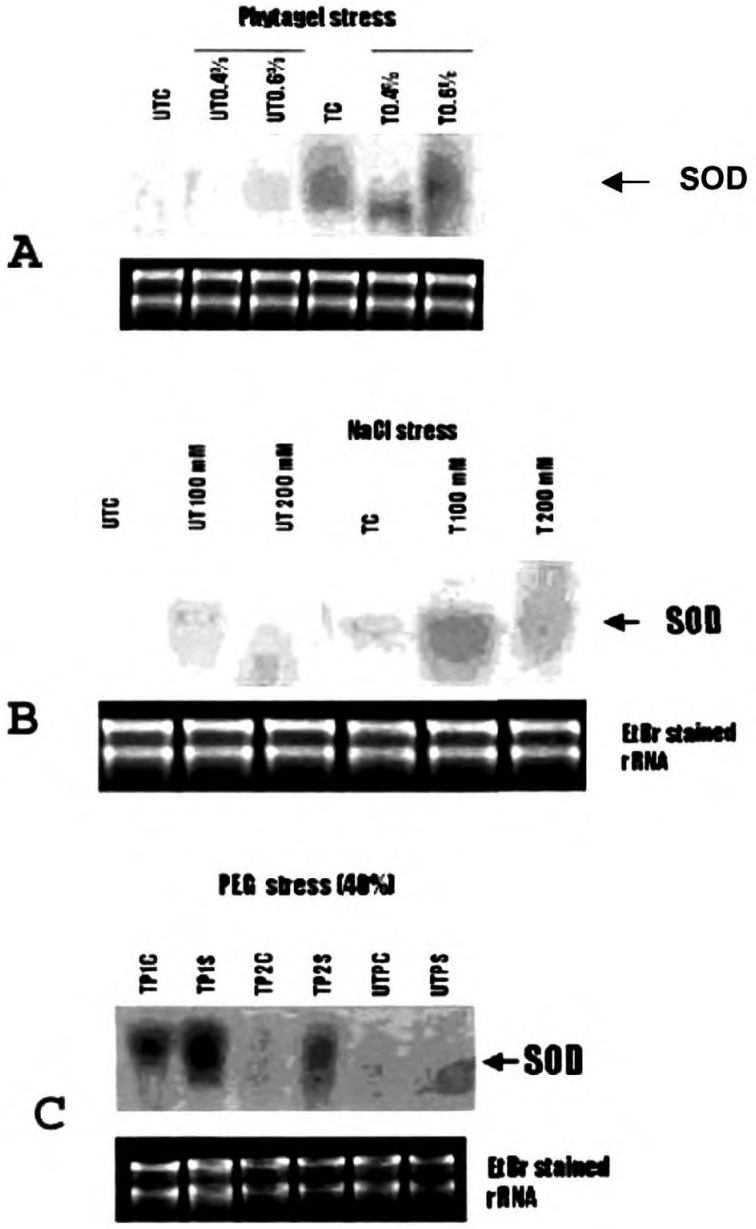


Fig. 23. Northern blot analysis after probing with Mn SOD cDNA

A- Transformed and untransformed callus subjected to water stress with phytagel. Lanes: UTC-Untransformed control; UT 0.4% and UT 0.6% - Untransformed callus subjected to water stress with 0.4% and 0.6% phytagel; TC-Transformed control; T 0.4% and T 0.6%-Transformed callus subjected to water stress with 0.4% and 0.6% phytagel

B- Transformed and untransformed callus subjected to salinity stress with NaCl. Lanes: UTC-Untransformed control; UT100 mM and UT 200 mM- Untransformed callus with 100 and 200 mM NaCl stress; TC-Transformed control; T 100 mM and T 200 mM -Transformed callus with 100 and 200 mM NaCl

C- Leaves from transgenic and untransformed *Hevea* plants subjected to water stress with 40% PEG. Lanes: TP1C -Transgenic plant -1 without stress; TP1S- Transgenic plant-1with stress; TP2C- Transgenic plant-2 without stress; TP2S- Transgenic plant-2 with stress; UTPC-Untransformed plant without stress, UTPS-Untransformed plant with stress

To investigate the extend to which Mn SOD mRNA is over expressed in transgenic plants due to environmental stress, leaves were excised from transgenic and untransformed plants and subjected to water stress induced with 40% PEG (Mol.wt 6000) for four hours. The results showed that mRNA transcript accumulation was more in the transgenic plants than that was observed in the untransformed plant (Fig. 23C, Lanes- TP1C, TP2C & UTPC). The mRNA transcript level was observed to be higher in the stress induced leaves (Lanes – TP1S & TP2S). Even though equal amount of RNA (20μg) was loaded in each lane, significant difference in the accumulation of SOD transcripts was observed between two transgenic plants. The higher mRNA level observed in the first plant compared with the second plant may be due to integration of multiple copies of the transgene (Lanes-TP1C & TP2C).

4. 4. Estimation of superoxide dismutase, peroxidase and catalase enzyme activities in transformed callus.

4. 4.1. Effect of water and PEG stress on SOD enzyme activity

Water stress was induced to the transformed embryogenic callus as well as untransformed embryogenic callus with 0.4% to 1.0% phytagel concentrations. In the control (phytagel concentration 0.2%), the untransformed callus exhibited 9.15 milliunits of enzyme activity and the enzyme activity observed in the transformed callus at this phytagel concentration was 10.07 milliunits/min/mg protein. At 0.4% phytagel concentration, the transformed callus exhibited 16.78 milliunits/min/mg protein. At this phytagel concentration the SOD specific activity in the transformed callus was 50% higher than that was observed at 0.2% phytagel concentration. As the phytagel concentration was increased above 0.4%, SOD activity started declining in transformed as well as in untransformed calli. At phytagel concentration 0.8% browning of the tissues was observed and the enzyme activity was decreased. Compared with the untransformed callus, the transformed callus exhibited a higher SOD activity at all concentrations of phytagel (Table 4.14).

Table. 4.14. Effect of phytagel induced water stress on SOD activity

Phytagel conc. (%)	SOD activity (milli units/min/mg.protein)	
	Untransformed callus	Transformed callus
0.2	9.15	10.07
0.4	**9.88**	**16.78**
0.6	8.42	12.95
0.8	7.23	11.17
1.0	6.59	8.95

CD (AB)=0.89

A similar SOD enzyme activity was observed when osmotic stress was induced with different concentrations of PEG ranging from 2.0 - 10% (w/v). With PEG the maximum enzyme activity (16.27) was observed with 4% PEG, which is more than 40% higher than the control values (Table.4.15).

Table. 4.15. Effect of PEG induced osmotic stress on SOD activity

PEG conc. (%)	SOD activity (milliunits/min/mg.protein.)	
	Untransformed callus	Transformed callus
2	10.77	10.83
4	**11.23**	**16.27**
6	9.38	15.07
8	7.54	12.78
10	5.83	11.80

CD (AB)=0.84

On further increasing the PEG concentration the SOD activity decreased in the transformed as well as untransformed calli. The difference in SOD specific activities of transformed and untransformed tissues due to water and PEG stress were found to be statistically significant. No significant difference in SOD specific activity was observed when different concentrations of mannitol or sorbitol were used to impart stress. Further, no significant difference in the enzyme activities were observed between the cultures maintained in complete darkness or on continuous light for 30 days.

Peroxidase enzyme activity was also found to be higher in the transformed callus over-expressing SOD. The highest level of peroxidase activity was observed at the concentration in which the SOD activity was maximum ie. at

0.4% (w/v) phytagel and 4% (w/v) PEG concentrations (Table 4.16). At this concentration peroxidase activity was found to be 15.83 and 22.18 units/min/mg protein for untransformed and transformed callus respectively. On further increasing the phytagel concentration peroxidase activity decreased in control as well as transformed calli and the lowest enzyme activity was observed at 1.0% phytagel concentration at which SOD activity was also low.

Table. 4.16. Effect of phytagel induced water stress on peroxidase activity

Phytagel conc.(%)	Peroxidase activity (units/min/mg protein)	
	Untransformed callus	Transformed callus
0.2	9.53	11.80
0.4	**15.83**	**22.18**
0.6	11.69	17.71
0.8	11.35	16.34
1.0	9.69	12.23
		CD (AB) =1.62

However, in the untransformed callus a reduction in peroxidase activity was observed at 6% (w/v) PEG concentration (Table 4.17).

Table. 4.17. Effect of PEG induced osmotic stress on peroxidase activity

PEG conc. (%)	Peroxidase activity (units/min/mg protein)	
	Untransformed callus	Transformed callus
2.0	9.72	11.28
4.0	**12.49**	**15.19**
6.0	9.95	14.03
8.0	12.39	12.86
10.0	7.79	8.21
		CD (AB) =1.37

Catalase eliminates hydrogen peroxide by converting it to molecular oxygen and water. In the present study, the trend of catalase activity was similar to that of SOD and peroxidase. In the case of callus subjected to water stress with 0.4% phytagel, catalase specific activity was 0.40 and 0.49 units/min/mg protein for untransformed and transformed callus respectively. At this phytagel concentration SOD and peroxidase activities were also high. For all other concentrations catalase activity was observed to be higher in the transformed

callus compared with untransformed callus at the respective phytagel concentrations (Table 4.18).

Table. 4. 18. Effect of phytagel induced water stress on catalase activity

Phytagel conc. (%)	catalase activity (units/min/mg protein)	
	Untransformed callus	Transformed callus
0.2	0.28	0.37
0.4	**0.40**	**0.49**
0.6	0.30	0.46
0.8	0.26	0.45
1.0	0.22	0.38

CD (AB)=0.028, CD(AB)=0.045

Catalase enzyme activity was increased in untransformed and transformed calli by the induction of osmotic stress with PEG. The maximum enzyme specific activity (0.49 units/min/mg protein) was observed in transformed callus at 4% PEG concentration and for control callus the value was 0.36 units/min/mg protein. For other PEG concentrations the difference was not statistically significant (Table 4.19)

Table. 4.19. Effect of PEG induced osmotic stress on catalase activity

PEG conc. (%)	Catalase activity (units/min/mg protein)	
	Untransformed callus	Transformed callus
2.0	0.29	0.43
4.0	**0.36**	**0.49**
6.0	0.20	0.39
8.0	0.28	0.37
10.0	0.22	0.33

CD (A) = 0.028, CD (B) = 0.045

The results indicate that catalase activity was also increased in transformed as well as untransformed callus and the maximum activity was observed at the concentration at which SOD and peroxidase enzyme activities were maximum.

5

DISCUSSION

Biotechnological approaches have led to tremendous progress in crop improvement in the recent past. The recombinant DNA technology combined with tissue culture technique provides opportunity to introduce novel characters into commercially important crop plants, which cannot be achieved easily by conventional breeding. The modification of plant genome through genetic transformation helps in expressing the desired genes. Transgenic technology has opened new vistas for producing annual as well as perennial tree crops with increased tolerance to high temperature, salinity, disease, insect/pest, drought and other biotic and abiotic stresses.

The present work was undertaken to develop suitable protocols for the *Agrobacterium tumefaciens* mediated genetic transformation as well as to develop transgenic plants with increased tolerance to environmental stress and tapping panel dryness. The gene coding for superoxide dismutase under the control of FMV 34S promoter was selected for this study. Methods for efficient *Agrobacterium tumefaciens* mediated genetic transformation of *Hevea* calli and further plant regeneration was standardized. PCR and Southern analyses were carried out to confirm gene integration. Since, SOD is the first enzyme involved in the detoxification of reactive oxygen species, over-expression of this enzyme may initiate the activation of a chain of free radical scavenging enzymes. Therefore, over-expression of SOD and other related free radical scavenging enzymes such as peroxidase and catalase in response to abiotic stresses were also studied in untransformed as well as in transformed callus cultures.

5. 1. Development of transgenic plants

In order to accomplish successful genetic transformation in plants, it is important to have an efficient and reliable *in vitro* plant regeneration system from the explants/tissues which are amenable to foreign DNA intake and integration into its genome. In the absence of a high frequency *in vitro* plant regeneration

system, it would be difficult to produce large number of transgenic plants from independent transformation events. In *H. brasiliensis*, somatic embryogenesis and subsequent plant regeneration have been reported by different authors from different clones and explants (Chen *et al.*, 1978; Wang *et al.*, 1980; Wan *et al.*, 1982; Carron *et al.*, 1985; Asokan *et al.*, 1992; Jayasree *et al.*, 1999; Sushamakumai *et al.*, 2000). Of the above reported protocols, the recently developed protocols by Jayasree *et al.* (1999) and Sushamakumari *et al.* (2000) are more efficient and reproducible plant regeneration systems for the most popular Indian clone, RRII 105.

It is well documented that, somatic embryogenesis and plant regeneration is highly dependent on the nature of explant, the developmental stage of the tissues and culture conditions. The culture conditions include culture medium as well as environment and the success in plant regeneration is dependent upon the correct combination of all these factors (Minocha *et al.*, 1993; 1995). Therefore, standardisation of the regeneration conditions in a tissue culture environment by choosing the right type of tissue and the developmental stage of the explant is essential. In *Hevea brasiliensis,* immature or juvenile tissues provide the best source of explant for plant regeneration via somatic embryogenesis (Carron and Enjarlic 1984; Gao *et al.*, 1982; Jayasree *et al.*, 1999; Sushamakumari *et al.*, 2000).

In the present study callus induction from immature anther (clone RRII 105) was initiated between 40 to 50 days in modified MS medium fortified with 2,4-D (2.0 mg/l) and Kn (0.5 mg/l). The emerging callus was soft and pale yellow in colour, which on further proliferation for 10-20 days became friable. Callus maintained in the proliferation medium for 40-50 days after callus initiation, its friability decreased and became hard and compact. Earlier Montoro *et al.* (1993) induced callus from the integumental tissues of immature seeds of *Hevea* (clone PB 260) and observed that callus often become brown and degenerating 2-3 months after induction. Therefore, the callus structure and textural characteristics should be taken into consideration before using the callus as the explant for genetic transformation.

Agrobacterium mediated transformation experiments carried out with different stages of *Hevea* callus derived from immature anther helped to identify the ideal stage of the explant for obtaining maximum transformation frequency.

In the current study, low transformation frequency observed with just emerging callus may be due to the lack of friability and a low level of phenolic content. Actively growing friable callus facilitated *Agrobacterium* infection due to callus friability and high regeneration potential. Aged, hard callus responded only very little. This may be due to the loss of meristematic activity and friability leading to the formation of hard and compact callus. Michaux-Ferriere and Carron (1989) reported that during *Hevea* somatic embryogenesis using integumental tissues the culture aging led to decreased meristematic activity, the pro-embryonic cells degenerate and finally the callus became brown and necrotic. It was also reported that aged callus becomes hard, compact and dehydrated (Carron *et al.*, 1992). Earlier reports also shows that maintenance of immature anther derived primary callus in the callus induction medium for a prolonged period leads to the formation of hard and compact callus with loss of morphogenetic potential (Jayasree *et al.*, 2001b). Thorpe (1982) reported that callus maintained in the same medium without subculturing, often undergo changes with time and these changes reduced the regeneration capacity of the tissue. All these reports supported that aged and hard callus was incompatible for *Agrobacterium* infection and regeneration of transgenic plants. In the present study actively growing, two-month old, yellow, friable callus was identified as the ideal stage of the explant for genetic transformation.

In genetic transformation, it is well documented that the transformation efficiency is highly dependent on the molecular size of the binary vector, wounding of the explant, pre-treatment of the explants with *vir* gene inducers (acetosyringone), optimization of temperature, duration of co-cultivation, the type of antibiotics used in the selection medium for inhibiting the bacterial overgrowth after co-culture and for the selection of transgenic cells (Bidney *et al.*, 1992; Santarem *et al.*, 1998; Hansen and Chilton, 1999).

In the present study, the transformation efficiency was improved by pre-treatment with *vir* gene inducers and wounding the explant during *Agrobacterium* infection. Pre-treatment of the *Agrobacterium* inoculum with 20 mg/l acetosyringone, 153 mg/l glycinebetaine and 115 mg/l proline for four hours before *Agrobacterium* infection was found to promote the transformation frequency. Aldemita and Hodges (1996) reported that pre-induction of

Agrobacterium with 400 µM acetosyringone prior to co-cultivation facilitated rice transformation. In *Hevea*, wounding of the callus during the bacterial infection resulted in phenolic exudation at the wound sites, thus promoting *Agrobacterium* infection. Several reports are available indicating that the T-DNA transfer occur at wound sites where accumulation of phenolic compounds takes place (Hooykaas and Schilperort, 1992; Winans, 1992). In the current study the combined effect of wounding of the explant, pre-treatment with acetosyringone and the physiological stage of the explant might have attributed to the high transformation frequency.

The use of a selectable marker gene allows the efficient selection of transformed cells. The most widely accepted selectable marker gene for dicotyledonous plants is *npt*II, which confers resistance to neomycin, kanamycin, geneticin and paromomycin (Galun and Breiman, 1997). Since plant species differ in their response to such phytotoxins, one can choose among these antibiotic compounds that is best for selection, in combination with *npt*II gene.

In the kill-curve test, it was observed that *Hevea* callus was highly tolerant to neomycin (even at 800 mg/l) and sensitive to geneticin, kanamycin and paromomycin. This type of differential tolerance to antibiotics was reported in citrus, rice etc. Citrus embryogenic callus had a relatively high tolerance to kanamycin and to geneticin, but it was sensitive to paromomycin (Vardi *et al.*, 1990). Similarly, kanamycin reduced the regeneration potential when used for rice transformation but geneticin was suitable for the selection of transformed rice integrated with *npt*II gene (Peng *et al.*, 1992). Even though efficient transformation (12%) was obtained with geneticin, the embryogenic competence of the transformed callus obtained was very low or even nil. This indicates that geneticin was more lethal to *Hevea* callus than the other two antibiotics. When paromomycin was used for selection, 11% transformation frequency was observed. This may be due to the reason that, paromomycin may be less toxic to *Hevea* callus and more number of transformed cells have undergone proliferation. With kanamycin, a transformation frequency of 6% was obtained. This result was in concurrence with the earlier report by Rugni *et al.* (1991) that the transformation frequency is dependent on the nature of antibiotic employed after co-cultivation and for the selection of transformed cells.

The GUS positive cell lines were selected and cultured on callus proliferation medium. Callus proliferation and embryo induction frequency was influenced by the composition and concentration of the basal medium and growth regulators used. Nitrogen has a key role in plant growth and development because it has direct effects on rate of cell growth, differentiation and totipotency (Kirby *et al.*, 1987). Nitrates are good sources of nitrogen supply to plants since it is readily taken up and metabolized by the cells and effects on a number of developmental process leading to root branching, bud dormancy and apical dominance (Gould *et al.*, 1991; Shanjani, 2003).

In the present study callus proliferation was influenced by the N content in the basal medium. Modified MS medium containing lower ammonium nitrate concentration promoted callus induction and proliferation compared to MS basal medium containing high NH_4NO_3 content. Similarly in the case of soybean, callus grew better when NH_4NO_3 in MS medium was substituted by $(NH_4)_2SO_4$ of Gamborg B5 medium as a source of reduced nitrogen. Bonga and von Aderkas (1992) reported that, in the somatic embryogenesis of many woody species, the organized cell growth was inhibited by the full strength of MS salts and the toxicity was reduced by lowering the amount of ammonium or total nitrogen.

Auxins have a significant role in somatic embryogenesis. They stimulate cell division and cell enlargement. The auxin, cytokinin ratio controls vascular differentiation, induction of somatic embryogenesis formation of shoot apices or buds etc. Auxins induce rooting also. The most frequently used auxins are 2,4-D, NAA, IBA and IAA. In the current study callus induction and proliferation was observed in the presence of 2,4-D. Lower concentration of 2,4-D produced yellow friable callus with high embryogenic potential and the optimum concentration identified for callus proliferation and further cell differentiation was 1.0 mg/l. Even though callus proliferation was observed at higher concentrations of 2,4-D (2.0 mg/l), this callus on further subculture onto embryo induction medium developed only very few embryos. Moreover, the embryonic cluster formation was adversely affected. It is well documented in *Hevea* that the callus induction and proliferation was favoured by the addition of 2,4-D (Chen *et al.*, 1978; Asokan *et al.*, 1992; Jayasree *et al.*, 1999). Jayasree *et al.* (1999) reported that 2,4-D (2.0 mg/l) induced embryogenic callus from immature anther and above this concentration, even though callus induction was observed, the callus texture and

embryogenic competence was very poor indicating that the concentration of 2,4-D was crucial for callus initiation and further morphogenesis.

It is well documented that in many plant species, callus initiation was facilitated by the addition of 2,4-D in the culture medium. Michalezuk *et al.* (1992) reported that 2,4-D promoted callus induction in carrot cells. They also reported that both exogenous and endogenous auxins were closely involved in the process of somatic embryogenesis, and the competence of the cells to initiate embryogenesis was affected by exposure to 2,4-D. For rice somatic embryogenesis also an auxin treatment was essential to induce embryogenic competence (Heyser *et al.*, 1983; Abey and Futsuhara 1986; Rueb *et al.*, 1994). Padmanabhan *et al.* (2001) reported that, sweet potato somatic embryogenesis exhibited a greater responsiveness to 2,4-D for callus induction and gave rise to greater number of competent embryos.

In *Populus* somatic embryogenesis, 2,4-D was supplemented in the medium in all cases to initiate embryogenic callus (Park and Son, 1988). In citrus, 2,4- D has been frequently used to induce embryogenic calli from nucellar and ovular explants of *C. limon* and *C. volekameriana* (Saad, 1975). All these reports suggested that among the various types of auxins, 2,4-D was mostly used for callus initiation. NAA was also used singly or in combination with 2,4-D for callus initiation and embryo induction. In the present study callus proliferation was improved by the addition of NAA along with 2,4-D. Addition of low concentration of NAA in combination with 2,4-D in the proliferation medium improved embryogenesis due to the synergistic effect of these two growth regulators. Incorporation of NAA (0.2 mg/l) in combination with 2,4-D (1.0 mg/l) resulted in the formation of yellow, friable translucent callus and the callus proliferation frequency was also enhanced. This study also indicate that combination of two auxins was superior to a single auxin in promoting somatic embryogenesis. Similar effects were reported in the somatic embryogenesis of blue pearl where a high frequency callus formation was obtained from leaf explants of blue pearl on media containing 2,4-D and NAA (Suzuki and Nakano, 2001). In table grapes also, the frequency and texture of callus formation was influenced by the incorporation of different auxins in the culture medium (Brown *et al.*, 1994). Martin (2004) assessed the beneficial effect of NAA on callus induction of *Centella asiatica* (L.) and reported that the callus initiated on MS medium fortified with NAA and Kn

favoured somatic embryogenesis and plant regeneration compared to the callus derived on 2,4-D and Kn containing medium.

For embryonic cluster formation the nutrient composition and the hormone concentrations of the culture medium is very important and this varies with each crop and genotype. In the current study, after the induction of callus in an auxin containing medium, the callus was transferred to a medium containing low auxin and cytokinin. It was noted that a stepwise decrease in 2,4-D and addition of BA in the proliferation medium stimulated embryogenic callus formation. In somatic embryogenesis, generally cytokinins are used in combination with auxins for both embryo induction and further plant development. However, the auxin/cytokinin ratio is crucial for every stage of somatic embryogenesis. In the present study, a higher auxin/cytokinin ratio promoted callus proliferation and embryogenic callus induction. In *Populus* somatic embryogenesis also, a higher auxin (2,4-D) to cytokinin (BA) ratio (5:1) resulted in the formation of highest number of globular embryos (Michler and Bauer 1991). In *Medicago*, the combination of 2,4-D with a cytokinin was required for inducing somatic embryogenesis from protoplasts (Arcioni *et al.*, 1982).

In most plant species, callus growth on 2,4-D containing medium for a short period and its subsequent transfer to a 2,4- D free medium brings about differentiation of embryos (Ball *et al.*, 1993). In the present study also, culturing of embryogenic callus on a medium supplemented with GA_3 and devoid of 2,4-D promoted the formation of globular stage embryos from pro-embryonic clusters. Similarly in wheat and barley, although 2,4-D has been used for callus formation and proliferation, reduction or removal of 2,4-D from the regeneration medium was essential for plant development from the callus (Gould *et al.*, 1991; Ball *et al.*, 1993).

In the current study, while analysing the effect of BA on embryo induction, it was observed that incorporation of BA (0.3 mg/l) significantly improved embryo induction. At higher concentrations, embryo induction frequency was reduced due to callus proliferation. Contradictory reports are available regarding the beneficial effect of cytokinin on somatic embryo induction. In *Hevea*, even though BA was not essential for callus induction, incorporation of BA (1-5 mg/l) promoted callus proliferation and embryo

induction (Carron and Enjalric, 1984). Embryogenesis in some *Citrus* species was inhibited by the exogenous application of cytokinins such as BA, Kn and Zea or auxins like IAA, NAA in the culture medium but in some species supplementation of basal medium with cytokinins such as BA, Kn and Zea resulted in embryoid formation (Bhansali and Arya, 1978a; Kochaba *et al.*, 1972; Chadurvedi and Mitra, 1975; Hidaka and Kajiura, 1988).

Incorporation of GA_3 in the embryo induction medium favoured the conversion of pro-embryonic clusters to globular embryos and maximum embryo induction was observed at 0.6 mg/l and above this concentration, the embryo induction frequency was decreased. It was also noticed that the efficiency of embryo induction was more dependent on the concentration of GA_3 as well as the combination of auxin and cytokinin. The combination of GA_3, NAA and Kn was found to be better for embryo induction than the combination of GA_3 along with NAA. Conflicting reports are available regarding the influence of GA_3 on somatic embryogenesis. Addition of autoclaved or filter sterilized GA_3 to the induction medium or embryo development medium increased the number of explants producing embryos in fennel and it was also noticed that the embryogenic frequency of petiole explants of several fennel genotypes, previously considered as poorly responding was highly enhanced by the addition of GA_3 to the culture medium (Hunault and Maatar, 1995). In *Santalum album* L also GA_3 enhanced somatic embryogenesis (Sita *et* al., 1979; Sita, 1986). Higher concentration of GA_3 in combination with IAA in the initiation phase enhanced embryoid formation in spinach (Xiao and Branchard 1993). However, embryo induction was inhibited by the addition of GA_3 in carrot cells (Tisserat and Murashige, 1977), ovule culture of *Citrus sinensis* (Kochaba *et al.*, 1978) and soybean (Phillips and Collins, 1981).

Phytagel (synthetic agar) is normally used as a gelling agent for the preparation of tissue culture medium. The use of high concentration of phytagel (0.4-1.0 % w/v) as gelling agent will make the medium too hard and dry and thereby water availability to the cultures will be reduced. In the present study, when embryogenic callus was cultured on medium containing higher concentrations of phytagel (0.4%-1.0%), the callus proliferation was inhibited and fresh weight of the callus was decreased indicating dehydration of the callus. The beneficial effect of water stress was that the desiccation treatment promoted cell

morphogenesis and thereby favoured the conversion of pro-embryonic mass to somatic embryos. Above 0.6%, the reduction in fresh weight was high indicating that the cells were too much dehydrated. This dehydration led to cell necrosis and loss of embryogenic potential. In control, (without stress) the embryogenic callus was watery and callus proliferation was observed which might have impaired the efficiency of embryo induction. During wheat transformation, it was observed that desiccation of plant tissues by culturing on media containing high concentrations of phytagel before *Agrobacterium* infection enhanced T-DNA delivery and increased stable transformation (Cheng *et al.*, 2003).

The role of PEG in inducing osmotic stress is due to the fact that PEG molecules are too large to move through the cell wall and do not cause plasmolysis and non-plasmolysing osmotica are more effective in promoting somatic embryo maturation in some conifer species (Attre *et al.*, 1991). In the current study, callus growth was observed at low concentrations of PEG (2-4% w/v) and at higher concentrations the callus growth was decreased. The fresh weight decreased with increased PEG concentration and lowest values were noticed at highest (12%) PEG concentration. This reduction in callus growth (up to a concentration of 10% PEG) favoured the conversion of embryogenic callus to pro-embryos. The pro-embryos further developed into globular stage embryos in the same medium, thereby improving the embryo induction frequency in normal as well as transformed callus cultures. At 12% PEG, the embryo induction frequency was decreased due to excessive drying of the callus, which was evident from the reduction in fresh weight of the callus. Such variation in growth due to excessive dehydration was reported in rice (Reddy *et al.*, 1994) and in citrus (Hayyin, 1987).

TDZ is one among the most active cytokinin like substances for woody plant tissue culture. Embryo maturation was favoured by the addition of TDZ in presence of GA_3. The combination of TDZ and GA_3 responded better than the combination of BA and GA_3. The stimulatory effect of TDZ on embryo maturation may be attributed to the fact that TDZ, a substituted phenyl urea, exhibits a strong cytokinin like activity. Although, the precise mode of action of TDZ is not known, TDZ may induce the synthesis or accumulation of endogenous cytokinins (Huetteman and Preece, 1993). TDZ is less susceptible to the plant's degrading enzymes than endogenous cytokinins and is also active at lower concentrations than the aminopurine cytokinins (Mok *et al.*, 1987). Banerjee *et al.* (2004) reported that TDZ induced high frequency

shoot proliferation in *Cineraria marcilima* L. Further, addition of TDZ in the culture medium proved superior to the combined treatments of BA and NAA. TDZ was earlier reported as a potent growth regulator for the induction of somatic embryos in many tree crops like white ash (Bates *et al.*, 1992), eastern black walnut (Neuman *et al.*, 1993) and in *Hevea* embryo germination (Jayasree *et al.*, 2001a).

The different nitrogen sources in the culture medium also determine the nature of growth and morphogenesis of the cells. In MS basal medium NH_4NO_3 and KNO_3 serves as the source of (NO_3^-). Casein hydrolyzate and vitamins serve as the source of (NH_4^+). Amino acids are the primary sources of organic nitrogen for the growth of many eukaryotic cells and they promote communication between cells and tissues within multicellular organisms (Young *et al.*, *1999).* Studies on effect of amino acids on embryo maturation and plant regeneration revealed that addition of some amino acids such as proline, glutamine and glycine favoured embryo maturation. This may be due to the reason that the presence of amino acids in the culture medium increased the level of reduced nitrogen which stimulated the development of somatic embryos. As the organic nitrogen concentration was further increased by the addition of asparagine (100 mg/l), alanine and arginine (50 mg/l each), the embryo maturation was reduced to 38%, due to the absorption of NH_4^+ ions resulting in the liberation of H^+ leading to reduction in pH of the medium. Niedz (1994) studied the close correlation between pH and the NO_3^- to NH_4^+ ratio on citrus callus growth and reported that inorganic N uptake can strongly influence pH since the uptake of NO_3^- and NH_4^+ changes the pH in opposite directions. Absorption of NH_4^+ results in the production of H^+ which are excreted into the medium, lower the pH and further reduce the cation uptake by competitive effects (Kirby and Mengel, 1967). This reduction in pH might have adversely affected the proper maturation of the embryos. It is well documented that the reduction in the level of inorganic nitrogen in the culture medium and supplementation with amino acids such as glutamine and L-proline induced somatic embryogenesis in a number of plant species such as *Medicago sativa* (Lai *et al.*, 1992), rice (Ozawa *et al.*, 1996), white spruce (Barret *et al.*, 1997), and cotton (Benito *et al.*, 1997; Haq and Zafar, 2004). The morphology of regenerated *Indica rice* shoots was strongly affected by the (NO_3^-) / (NH_4^+) ratio (Grimes and Hodges, 1990). In the case of cotton, both source and amount of nitrogen in the medium showed significant effects on cell growth and embryogenesis

(Haq and Zafar, 2004). The positive effect of proline on somatic embryogenesis in *Medicago arborea* was evident from the number of embryos developed (Ozawa *et al.*, 1996). However, the positive effect of proline cannot be extended to all amino acids since this effect was not produced when alanine, serine and tryptophan were added to the culture medium for plant regeneration from suspension cultures of *Iris germanica* (Shimizu *et al.*, 1997). This stimulatory effect of proline was contradictory to the results obtained with peanuts, where the addition of proline to the culture medium reduced the total number of somatic embryos formed (Murch *et al.*, 1999). In most cases certain amino acids provide higher embryo yield and quality (Elkonin and Pakhomova, 2000; Lirio *et al.*, 2001).

The present results also indicate the stimulatory effect of glutamine, proline and glycine on embryo maturation and the adverse effect of complex combination of amino acids. Similar effect was reported in *Pinus strobus* somatic embryo maturation, which proceeded in the presence of glutamine, while complex combination of eight amino acids showed little effect on mature embryo production (Elizabeth *et al.*, 2000).

The importance of water relations in controlling embryo maturation was proposed by Fischer *et al.* (1987), and has been supported by evidence from both zygotic and somatic embryo culture experiments (Xu *et al.*, 1990) and *in situ* studies (Saab and Obendorf, 1989). Attempts to stimulate the *in vivo* environment through modification of the composition of maturation medium showed increased storage compound levels and desiccation tolerance (Finkelstein and Crouch, 1986; Xu *et al.*, 1990).

Desiccation is a natural event observed during the late phases of embryogensis. A severe decrease of moisture content experienced by fully developed seed embryos is a key event required for the termination of the developmental processes in preparation for germination (Kermode, 1995). The imposition of a water stress is also required in culture for increasing the conversion frequency of the embryos (Attree *et al.*, 1991).

Supplementation of maturation medium with 6% (w/v) PEG improved the embryo maturation by 6%. Further increase in PEG concentration impaired embryo maturation. For soybean embryo maturation Saab and Obendorf (1989) monitored water and osmotic potentials in somatic embryo axis, cotyledon, seed coat and pod tissues during periods of seed growth and maturation *in situ* and found that

changes in water relations were limited to the last seven days of maturation. They observed a sharp decline in water content in all of those tissues at that period. In soybean zygotic embryo *in vitro* germination, the rate and degree of desiccation of mature embryos was crucial to their subsequent germination ability (Obendorf *et al.*, 1998). Studies on soybean (Egli, 1990), alfalfa (Xu *et al.*, 1990) and rape seed. (Finkestein and Crouch, 1986) have shown that embryo maturation was frequently associated with a low osmotic potential in tissues or medium surrounding the embryos. All these reports suggested that excessive desiccation of embryos led to tissue necrosis, loss of storage proteins and ultimately led to drying of the embryos.

During zygotic embryo germination also, the process in which the embryo is awakened requires specific physical conditions. Similarly in the case of somatic embryos, although the development is artificial, need precise cultural conditions for initiation and development of shoots and roots leading to complete plantlet formation. Gibberellins have significant influence on dormancy breaking and further growth and development of plants (Hooley, 1994).

Hevea somatic embryo germination was also influenced by GA_3. Although, embryo germination was observed in hormone free medium, incorporation of GA_3 in combination with IBA in the germination medium favoured bipolar differentiation and clearly improved embryo germination. Even though the exact mechanism of the promotive effect of GA_3 on embryo germination is not clear, the ultra structural studies carried out by Choi *et al.* (1999) showed that somatic embryos developed *in vitro* may be dormant after maturing and thus required a dormancy breaking treatment. The beneficial effect of GA_3 on somatic embryo germination has been reported in grape wine (Mullins and Srinivasan, 1976) and in *Panax ginseng* (Chang and Hussing, 1980; Choi *et al.*, 1999). *Citrus sinensis* embryos derived from ovules were germinated by supplementing GA_3 (1.0 mg/l) in the basal medium and the presence of GA_3 in the germination medium stimulated rooting as well as stem elongation (Button and Bornman, 1971). In the growth and development of tomato embryos, it was observed that low concentrations of GA_3 appeared to stimulate growth following germination but IAA was not as effective. Combinations of IAA plus GA_3 were antagonistic resulting in poor growth. Combinations of Kn and GA_3 or Kn and IAA were most beneficial for those embryos (Neal and Topoleski, 1985). GA_3

when used at concentrations from 10^{-8} to 10^{-6} M promoted the growth of caraway somatic embryos and some embryos grown in media with GA_3 exhibited extensive root growth (Ammirato, 1997).

For acclimatization, the plantlets were transplanted in small earthenware pots or polythene bags filled with soilrite, soil and sand, and maintained in glass house at 28 ± 2^0C with a high relative humidity. Covering the plants with a thin transparent polythene bag was effective to maintain relative humidity of 90-95%. High relative humidity was necessary for preventing wilting of leaves due to water loss. Siril and Dhar (1997) reported that covering the *in vitro* raised plants with polythene bags for the first two weeks was necessary for the acclimatzation of chinese tallow tree. In the current study high humidity was needed only for the first six to seven days and extended periods under this humidity led to fungal growth. Therefore a gradual reduction in the relative humidity was essential and this was achieved by making holes in the polythene cover. After two weeks the protective cover was removed. These plants were maintained in glass house for 7-8 weeks to achieve stem elongation and formation of new leaves. Seneviratne *et al.* (1993) also reported that the *in vitro* raised *Hevea* shoot tip derived plants were acclimatized by controlling the ambient conditions, mainly humidity. After acclimatization, the plants were planted in polythene bags filled with soil, sand and cow dung (1:1:1 w/v). The plants were irrigated with water once in two days and Hoagland's mineral solution was applied every alternate week.

5. 2. Molecular confirmation of gene integration

5.2.1. Polymerase chain reaction analysis

The presence of *npt*II gene was confirmed by the amplification of 804 bp single product in all the three transgenic plants and in positive control. When PCR was performed with HbSOD gene specific primers, a 702 bp fragment was amplified in all transgenic plants and in control plasmid. This fragment corresponds to the cDNA sequences coding for a 702 bp SOD transgene of *Hevea* transgenic plants. This 702 bp fragment was absent in the genome of untransformed plant. Therefore, this band was not detected in the untransformed plants. The native SOD gene (genomic sequence) of *Hevea* is about 3.2 kb, which was present in the transformed as well as untransformed plants. However, the 3.2kb genomic fragment in the

transgenic plants was amplified only when extended PCR was carried out. These results indicate the presence of the transgene in the genome of *Hevea* transgenic plants.

5. 2. 2. Southern hybridization analysis

Integration of the T-DNA region in to the genome of the putative transgenic plants was confirmed by Southern hybridization analysis. When the DNA of the transgenic plants were digested with *EcoR* I, the integrated *npt*II gene fragment of 980 bp get liberated. This is due to the presence of the *Eco*R I restriction sites on both sides of the *npt* II sequence. The ^{32}P dCTP labeled *npt* II gene probe generated a band of predicted size (980 bp) with *Eco*R I digests in all the three tested transgenic plants and in positive control (vector plasmid). No hybridization could be detected for untransformed negative control plant. The results confirm the presence of the transgene in the genomic DNA of the transgenic plants.

The *Sac* I enzyme had a unique restriction site towards the left border of T-DNA. Hybridization of *Sac* I digests with *npt* II probe produced bands with more than 2.5 kb size, representing the DNA fragments containing the *npt* II gene and a part of the plant genomic DNA located outside the left T-DNA border. The different hybridization patterns observed for transgenic plants indicate random integration and multiple insertion of the T-DNA into the genome of these plants. Each band that hybridized to the *Sac* I digested genomic DNA represents a single copy of the transgene. Although, same amount of digested DNA was loaded in each lane, the intensity of the hybridizing bands in transgenic plants was different, further indicating the difference in T-DNA copy numbers. No hybridization was detected for the untransformed negative control. These results confirmed the successful integration of the foreign gene in *Hevea* through *Agrobacterium* mediated genetic transformation. This type of multiple insertion has been reported in the genetic transformation of *Pinus radiata* (Walter *et al.*, 1998) and in banana (Ganapathi *et al.*, 2001). Hernandez *et al.* (1998) reported that in the *Agrobacterium* mediated genetic transformation of avacado, Southern blot analysis performed with *EcoR* I revealed the presence of different numbers of bands indicating the integration of T-DNA at different sites.

5. 3. SOD gene expression by Northern blot analysis

Detection of RNA transcript levels was performed by Northern blot analysis. The primers used for the synthesis of the probe for the present study corresponds to Mn SOD cDNA from *Hevea brasiliensis* (Mia and Gaynor, 1993). Mn SOD transcript levels were observed in the untransformed and transformed callus and leaves collected from untransformed and transgenic plants. Induction of water stress using 0.4% and 0.6% led to the accumulation of Mn SOD mRNA. A higher transcript level was observed in the stress induced callus untransformed and transformed callus compared with the controls (no stress). In the case of callus subjected to salinity stress with sodium chloride, the mRNA level was observed to be higher at 100 mM NaCl compared with the control transformed as well as untransformed callus. But at 200 mM concentration the mRNA transcript level was lower than that was observed at 100 mM NaCl concentration due to inhibition of callus growth.

Northern hybridization analysis carried out with untransformed as well as transgenic leaves subjected to water stress with 40% PEG revealed difference in intensity of the bands indicating that the SOD transcript levels were different. The mRNA level was observed to be higher in the transgenic plants compared with untransformed plant. PEG stress induction led to higher mRNA accumulation in the transgenic plants than in untransformed, which was evident from the intensity of the bands. Difference in expression was also observed between the two transgenic plants. This may be due to difference in the site of gene integration and multiple integration.

5. 4. Evaluation of superoxide dismutase, peroxidase and catalase enzyme activities in transformed callus.

Water is required as a medium for biochemical activities for all known life forms. Vegetative growth of plants can only occur at a certain range of water status. Induction of water stress in plants is known to trigger several biochemical changes. Plants respond to this stress in part by modulating gene expression, which eventually leads to the restoration of homeostasis, detoxification of toxins and recovery of growth. Analysis of the enzyme activities of transformed and untransformed embryogenic callus provided interesting insight into the expression of free radical scavenging enzymes, particularly superoxide dismutase enzyme in response to abiotic stress. Water stress induced to the transformed callus with

0.4% (w/v) phytagel exhibited more than 50% increase in the total SOD activity. The role of SOD in protection of plants against oxidative stress is well documented (McKersie *et al.*, 1993). Under drought conditions, the activity of the enzymes that detoxify the ROS may increase and a higher scavenging activity may correlate with enhanced drought tolerance of the plants (Bowler *et al.*, 1992). In transgenic alfalfa it was observed that, over-expression of Fe-superoxide dismutase increased superoxide scavenging capacity and thereby improved the winter survival. The increased Fe-SOD activity in alfalfa was associated with increased winter survival over 2 years in field trials (McKersie *et al.*, 1996).

Maize callus cultures subjected to water stress using PEG, activities of antioxidant enzymes such as SOD, catalase, peroxidase and glutathione reductase were higher, and damage indicated by the levels of hydrogen peroxide and malondialdehyde was lower in the drought resistant callus (Li and Van-Staden, 1998). The role of pea (*Pisum sativum*) chloroplast manganese (Mn) and copper-zinc (Cu-Zn) superoxide dismutase in protecting plant cells from oxidative stress was compared using transgenic plants over expressing the SODs. Exposure of these plants to methyl viologen showed that the Mn SOD over expressing plants had increased protection against oxidative membrane damage even at relatively high concentrations. In contrast, the Cu-Zn SOD over-expressing plants showed significant protection only at lower methyl viologen concentrations (Gupta *et al.*, 1993a). The overproduction of chloroplastic SOD, APX, and GR in tobacco, cotton, and poplar has resulted in an improvement in the protection of photosynthesis during chilling stress (McKersie *et al.*, 2003).

Peroxidases are a large class of enzymes which are very efficient catalysts in oxidative reactions. H_2O_2 is scavenged by the ascorbate peroxidases and catalases of plant cells (Asada, 1994). In the present study peroxidase and catalase were also over expressed proportional to the over expression of SOD. The peroxidase enzyme activity was also found to be higher in the transformed callus over-expressing SOD. This may be due to the toxic levels of H_2O_2 accumulated by the over expression of SOD in the callus undergoing stress. The highest level of peroxidase activity was observed at the concentration in which the SOD activity was maximum ie. at 0.4% (w/v) phytagel and 4% (w/v) PEG. Accumulation of H_2O_2 is potentially harmful since it can lead to oxidative damage and loss of

structure and function. It may, however, also have a regulatory role in signal transduction during low temperature stress (Prasad *et al.*, 1995).

Over-expression of an *Arabidopsis* peroxisomal ascorbate peroxidase gene in tobacco could protect leaves from oxidative stress damage caused by aminotriazole which inhibits catalase activity that is found mainly in glyoxysomes and peroxisomes and leads to accumulation of H_2O_2 in those organelles (Wang *et al.*, 1999).

Over-expression of Mn Superoxide dismutase in maize leaves leads to increased monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase activities. Enhanced SOD activity was accompanied by enhancement of monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase enzyme activities. This suggests that increased recycling of reduced ascorbate was required to compensate for enhanced hydrogen peroxide production in transformed plants (Alison *et al.*, 2000).

Catalase is a tetrameric heme-containing enzyme found in nearly all the aerobic organisms, which converts hydrogen peroxide into water and molecular oxygen in plants. Catalase enzyme also plays an important role in eliminating the harmful and toxic effects produced by the accumulation of free radicals in biological system under various biotic and abiotic stresses. A reduction in the activity of this enzyme can lead to the accumulation of O_2^- and H_2O_2, which in turn helps the formation of hydroxyl radical, which is highly toxic. In the present study, the trend of catalase activity was similar to that of SOD and peroxidase. Catalase activity was higher at water stress induced with 0.4% phytagel and 4% PEG concentrations which indicates that proportionate to the over expression of SOD, catalase activity is also enhanced. There are reports that genes for catalase isoforms in plants are induced by abiotic stress as well as ABA (Guan *et al.*, 2000). The correlation between elevated SOD, peroxidase and catalase enzyme activities suggests that the expression of SOD enzyme also influences the other free radical scavenging enzymes to provide protection against oxidative stress in normal as well as transformed callus cultures. These results demonstrate that SOD is a critical component of the active-oxygen-scavenging system of plants and modification of SOD expression in transgenic plants could improve plant's stress tolerance.

6

SUMMARY AND CONCLUSIONS

Natural rubber present in numerous plant species commonly known as laticiferous plants has been an essential product for mankind. Advances in molecular and cell biology over the past few decades have led to the development of a wide range of techniques for manipulating genomes. The advent of modern biotechnology, particularly those involving the application of genetic engineering, brought about an industrial revolution, which resulted in the production of many value added products that have great impact on health, agriculture and environment. The recent developments in recombinant DNA technology and *in vitro* plant regeneration techniques have paved the way for the successful integration of foreign genes into the genome of many plant species. Susceptibility of *Hevea brasiliensis* to various biotic and abiotic stress factors in the various agro-climatic conditions and susceptibility to tapping panel dryness has been a major concern for crop production. Crop loss due to these factors is very heavy. Development of transgenic plants resistant/tolerant to these factors may contribute enhanced crop production.

In the present work a protocol for *Agrobacterium tumefaciens* mediated genetic transformation of *Hevea brasiliensis* (Rubber tree, clone RRII 105) has been developed and transgenic plants integrated with the gene coding for Mn.SOD under the control of FMV 34S promoter is described. The plasmid vector (pDU 96.2412) used in this study contains β-glucuronidase *uid*A as the reporter gene, neomycin phosphotransferase (*npt* II) as the selectable marker gene and the gene coding for *Hevea* Mn. SOD under the control of FMV 34S promoter.

Hevea brasiliensis (clone RRII 105) callus derived from immature anther was transformed with *Agrobacterium tumefaciens*. The antibiotic resistant callus emerged were selected after *GUS* histo-chemical staining. The putatively transformed cell lines were proliferated in modified MS medium fortified with B5 vitamins, 400 mg/l

casein hydrolyzate and 300 mg/l glutamine, 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2, 4-D), 0.2 mg/l naphthaleneacetic acid (NAA) and 0.5 mg/l 6-benzyladenine (BA).

An embryo induction frequency of 52% was obtained in modified MS medium supplemented with growth regulators NAA (0.2 mg/l), BA (0.3 mg/l), Kn (0.3 mg/l) and GA_3 (0.6 mg/l). Frequency of embryo induction was improved by inducing water or osmotic stress to the cultures. In the case of transgenic embryos, embryo induction frequency of 62% was obtained on a medium supplemented with 0.6% phytagel. Embryo maturation was obtained in modified MS medium containing GA_3 (0.4 mg/l) and TDZ (0.3 mg/l). The mature embryos germinated and developed into plants after four to five weeks, when individual embryos were cultured on half-strength MS medium fortified with growth regulators. Addition of BA (0.3 mg/l) in the embryo germination medium favored shoot elongation, but the germination percentage was not improved. Maximum plant conversion (21%) was observed on half- strength MS medium supplemented with GA_3 (0.3 mg/l) and IBA (0.10 mg/l). Plantlets with 1-2 whorl of mature leaves were planted in small polythene bags or earthenware pots filled with sterile soilrite, sand and soil (1:1:1) and covered with thin transparent plastic covers to maintain a relative humidity of 90-95% and maintained in glass house at 26 ± 2°C. After transplantation, the plants were irrigated every alternate days and provided with half-strength Hoagland's solution fortnightly. The acclimatized plants were then transplanted to big black polythene bags (9½" x 22") filled with soil, sand and cow dung (2:1:1) and maintained in greenhouse.

PCR was performed with Hb Mn SOD gene specific primers, a 702 bp fragment was amplified in all transgenic plants and in the plasmid vector used as positive control. This fragment corresponds to the cDNA sequences coding for SOD gene that has been integrated into the plant genome. This 702 bp fragment was absent in the untransformed plant. However, a 3.2 kb native SOD gene present in transformed as well as untransformed plants were also amplified in an extended PCR. This 3. 2 kb band corresponding to the genomic DNA sequence of native SOD was absent in the plasmid vector. These results indicate the presence of the Hb Mn SOD transgene in transgenic plants.

Integration of the T-DNA region in to the genome of the putative transgenic plants was confirmed by Southern hybridization analysis. Genomic DNA was digested with restriction enzyme *EcoR* I to show the insert of predicted size and with *Sac* I to show the integration into the plant genome and the number of insertions. When the DNA of the transgenic plants were digested with *EcoR* I, the integrated *npt*II gene of 980 bp get liberated. The ^{32}P dCTP labeled *npt*II gene probe generated a band of predicted size (980 bp) with *EcoR* I digests in all the three tested transgenic plants and in positive control (vector plasmid). No hybridization could be detected for non-transgenic negative control plant. The *Sac* I enzyme had a unique restriction site towards the left border of T-DNA. Hybridization of *Sac* I digests with *npt*II probe produced bands with more than 2.5 kb size, representing the DNA fragments containing the *npt*II gene and a part of the plant genomic DNA located outside the left T-DNA border. Each band that hybridized to the *Sac* I digested genomic DNA represents a single copy of the transgene. Although, same amount of digested DNA was loaded in each lane, the intensity of the hybridizing bands in transgenic plants was different, further indicating the difference in T-DNA copy numbers. It is confirmed that the three transgenic lines contain different copy numbers of the integrated gene and that each plant represents an independently transformed line. No hybridization was detected for the non-transgenic negative control. The Southern analysis result with *Sac* I digested DNA confirmed the successful integration of the foreign gene in *Hevea* through *Agrobacterium* mediated genetic transformation.

Over expression of the SOD cDNA in response to abiotic stress was analyzed at the transcript level by Northern hybridization analysis. Water stress was induced to callus cultures using phytagel (0.4% and 0.6%) and salinity stress using 100 mM and 200 mM sodium chloride. After the induction of stress for 15 days, total RNA was isolated and Northern hybridization was carried out using radio labeled Hb Mn SOD probe. The mRNA transcript level was found to be higher in transformed callus subjected to water stress with 0.4% phytagel compared to untransformed callus subjected to water stress. Higher mRNA transcript accumulation was observed in the callus subjected to salinity stress induced with 100 mM sodium chloride. The oxidative stress tolerance of the transgenic plant was also analyzed by Northern blot

hybridization technique. Leaves were detached from one normal and two transgenic plants, osmotic stress was imparted to the leaves using 40% PEG for 4 hrs and for control the leaves were kept in DEPC treated water. RNA was isolated and Northern hybridization analysis was carried out using SOD probe isolated from the plasmid vector. The results revealed higher SOD transcript level in stress induced leaves indicating over expression of the transgene due to stress induction. SOD enzyme activity was also higher in callus subjected to abiotic stress. The transformed callus cultures to which water stress was induced with 0.4% phytagel exhibited more than 50% increase in SOD activity. Higher SOD enzyme activity was also observed in callus subjected to osmotic stress with different concentrations of polyethylene glycol, 40% increase in SOD enzyme activity was observed at 4% PEG concentration. Peroxidase and catalase enzymes were also over-expressed correspondingly in response to stress induction.

Following this biochemical characterization, the most promising transgenic plants will be preserved as source of bud-wood for mass multiplication of transgenic plants by bud grafting. Since rubber trees are amenable to vegetative propagation by bud grafting, once a transgenic plant for a trait is obtained, an unlimited number of such plants can be produced. The original mother plant may be maintained in the green house for long term studies.

The promise of biotechnology as an instrument of development lies in its capacity to improve the quantity and quality of plants quickly and effectively. The application of biotechnology can create plants that are resistant/tolerant to drought and diseases. Plant's physiological characteristics can also be altered for reducing the immaturity period for latex harvest. Engineering of *Hevea* for high yield potential, improvement of wood quality and volume i.e. generation of 'latex timber' clones are other promising future objectives in *Hevea* crop improvement by genetic engineering. It is noteworthy to observe that, with its unique laticiferous system with continuous production of latex *Hevea* genetic engineering may contribute to a new revolution, which will allow to grow trees with desired traits and produce valuable recombinant proteins having pharmaceutical and industrial importance.

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List of Figures

Abbreviations

ABA	Abscisic acid
BA	N6- Benzyladenine
B5	Gamborg B5
β-NADH	β-nicotinamide adenine dinucleotide reduced
CTAB	Hexadecyltrimethylammonium bromide
2,4-D	2,4-Dichlorophenoxyacetic acid
DEPC	Diethyl pyrocarbonate
DNTPs	Deoxynucleotide triphosphates
EDTA	Ehtylenediaminetetraacetic acid
EtBr	Ethidium bromide
FMV	Figwort Mosaic Virus
GA_3	Gibberellic acid
GUS	β-glucuronidase
Hb Mn SOD	*Hevea brasiliensis* manganese superoxide dismutase
HAS	Human serum albumin
IAA	Indole- 3- acetic acid
IBA	Indole-3-butyric acid
Kb	Kilobase
Kn	Kinetin
LB	Luria-Bertani broth
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
NBT	Nitroblue tetrazolium chloride
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMS	Phenazine methosulphate
PVPP	Polyvinylpolypyrrolidone

RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RT-PCR	Reverse transcriptase-polymerase chain reaction
rpm	Revolution per minute
SOD	Superoxide dismutase
TE	Tris- ethtylenediaminetetraacetic acid
TPD	Tapping panel dryness
TDZ	Thidiazuron
v/v	Volume/Volume
w/v	Weight/Volume
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide
Zn	Zeatin