Studies on the in vitro culture and genetic transformation of pigeonpea (*Cajanus cajan* (L.) Millsp.) for induced resistance to fungal pathogens

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Doctor of Philosophy

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2003

Dedicated to my

Loving Parents...

CERTIFICATE

Certified that the entire work embodied in this thesis entitled **"Studies on the in vitro culture and genetic transformation of pigeonpea (*Cajanus cajan* (L.) Millsp.) for induced resistance to fungal pathogens"** has been carried out by Ms. G. Suneetha, for the degree of Doctor of Philosophy, under our supervision in the Genetic Transformation Laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. This work is original and has not been submitted so far, in part or in full, for the award of any degree or diploma of any University.

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DECLARATION

I hereby declare that this thesis entitled **"Studies on the in vitro culture and genetic transformation of pigeonpea (*Cajanus cajan* (L.) Millsp.) for induced resistance to fungal pathogens"**, comprises of my own work except where specifically stated to the contrary, and it is not substantially the same as my thesis that has been submitted for any degree to any other university.

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ABBREVIATIONS

µg/L	microgram per liter
µl	microliter
µM	micromole
2,4-D	dichlorophenoxyacetic acid
2-iP	2-[isopentenyl] adenine
BA	N^6-benzyladenine
bp	base pair
cDNA	complementary deoxyribonucleic acid
cm	centimeter
d	day (s)
DNA	deoxyribonucleic acid
DNTPs	deoxyribonucleotide triphosphates
EDTA	ethylenediamine tetra acetic acid
GA_3	gibberellic acid
GUS	β-glucuronidase
ha	hectares
HCl	hydrochloric acid
hr	hour (s)
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
kb	kilo base pair
kg	kilogram
kinetin	kinetin (6-furfuryl aminopurine)
L	litre
LB	Luria Broth
LD_{50}	lethal dose at 50% mortality
M	molar
mg/L	milligram per liter

min	minutes
ml	milliliter
mm	millimeter
mol wt	molecular weight
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog medium
NAA	α-naphthaleneacetic acid
NaOH	sodium hydroxide
*npt*II	neomycin phosphotransferase
^{O}C	degree centigrade
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	negative logarithm of H^{+} ion
psi	pounds per square inch
RChit	rice chitinase gene
RIM	root induction medium
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDM	shoot development medium
SDW	sterile distilled water
Sec	second (s)
SEM	shoot elongation medium
SIM	shoot induction medium
SSC	sodium chloride and sodium citrate
TAE	Tris acetate-EDTA
T-DNA	transfer DNA
TDZ	thidiazuron [1-phenyl-3-(1,2,3-thidiazol-5- yl) urea]
TE	Tris EDTA
Ti plasmid	tumour inducing plasmid

*uid*A	β-glucuronidase gene
uv	ultraviolet
vir	virulence
Vol	volume
w/v	weight per volume
wk	week
wt	weight
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide

1. INTRODUCTION

1. INTRODUCTION

Fabacea is one of the largest families of flowering plants and is composed of 690 genera and 18000 species comprising of three subfamilies *Mimosoidae, Caesalpinanoidae* and *Pappillinoideae Papilionoideae* is the most important of them and contains more than 12000 tropical, sub-tropical, and temperate species They are characterized by flowers with butterfly keel shaped petals and pod type fruits The grain legumes commonly known as pulses belong to this sub-family (Le Houerou, 1991)

Pulses: yield, distribution, productivity and importance

Pulses have been indispensable constituents of the Indian diet where, pigeonpea stands second in terms of cultivation and yield amongst the pulse crops grown in India India is a major pulse growing country in the world, sharing 35 to 36 per cent of total area and 27 to 28 per cent of the production of these crops, respectively Over the past three decades, the area of pulse cultivation has been fluctuating between 22 to 24 million ha The production of pulses per annum has been around 10 to 14 million tonnes and the annual productivity of pulses has been fluctuating between 475 to 544 kg ha^{-1}, respectively (Yadav, 1991) According to the Center for Monitoring Indian Economy (2003), in India, the annual production of pulses in the year 2001 to 2002 is about 13 10 million tonnes, occupying a total area of 38 7%.

Pulses are unique crops capable of fixing atmospheric nitrogen in their root nodules and thus meet their nitrogen requirements to a great extent. These crops can utilize soil moisture very efficiently and can be grown in areas left after satisfying the

demand for cereals, since even in moisture deprived conditions pulses give better returns. Pulse crops improve soil fertility and physical structure of the soil and fit well in mixed or inter cropping systems, crop rotations and dry farming while providing green pods as vegetables and nutritious fodder to the cattle. Pulses constitute a rich diet of the predominantly vegetarian masses of India by virtue of being high in seed protein content and also in possessing several essential amino acids. The ease with which they fit into crop rotation, restoration of soil fertility and with their capacity for producing minimum yield even under marginal and most neglected conditions with minimum inputs, pulses have become popular with Indian farmers (Hulse, 1991).

Pigeonpea: geographic distribution

Pigeonpea [*Cajanus cajan* (L.) Millsp.] with chromosome number of 2n=22, is an important grain legume crop of rainfed agriculture in the semi-arid tropics of the Indian sub-continent and is also widely grown in eastern and southern Africa, Latin America and the Caribbean (Nene and Sheila, 1990). It is a member of the sub-tribe *Cajaninae*, tribe *Phaseoleae,* and family *Fabacea*. Pigeonpea is so called because of its appeal to wild pigeons. It has been reported to occur in wild state in the upper region of Nile river and the coastal districts of Angola in Africa. Various concepts have been put forward regarding the origin of pigeonpea. Van der Maesen (1980) stated pigeonpea to be of Indian origin, from where it might have been distributed to Africa by two millennia BC at the latest and also suggested that Africa is the secondary center of origin.

Early wild species of pigeonpea were reported in China and Indochina by Watt (1908), while de Candolle (1885) stated that the wild ancestors grew in Africa from the coast of Guinea to Zanzibar, and in the Nile valley Though measurable quantities are cultivated in the Caribbean, Southeast Asia and eastern Africa, about 90% of total world cultivation is in the Indian sub-continent (Hulse, 1991)

Pigeonpea grows well in sub-tropical and tropical environments, extending between latitudes of 30 °S to 30 °N, at elevations of up to 2000 m from mean sea level India accounts for more than 90% of the world's pigeonpea production and area World-wide, the crop is cultivated on about 3 4 million ha of land with an annual production of 2 7 million tonnes at an average yield of 790 kg ha^{-1} (Nene and Sheila, 1990) According to the Center for Monitoring Indian Economy (2003), in India pigeonpea is cultivated on about 3503 4 thousand ha of land with an annual production of 2 3 million tonnes Among all the states in India, Andhra Pradesh stands third in cultivation occupying 429 8 thousand ha of land, with an annual production of 154 7 thousand tonnes, standing sixth with an average yield of 360 kg ha^{-1}

Constraints to the productivity of pigeonpea

Pigeonpea production is constrained by poor cultivation practices, less productive land-resource allocation, excess water or drought stress and losses caused by diseases and pests The long-duration of the crop of 160 to 250 d contributes to the constraints include inadequate support for technology generation and its transfer, the lack of stress-resistant, high-yielding genotypes, modern agronomic management, new production systems, and lack of utilization of research results and promotion

Non-availability of high yielding and disease and insect resistant varieties has been the greatest bottleneck for high productivity among pulses With the increase in the world's population, human needs have also increased at rapid rates, which have become major challenges for food production (Reddy *et al.*, 1998)

Pigeonpea is susceptible to many diseases and insect pests It's productivity is constrained by over 100 pathogens (Nene *et al.*, 1989) The diseases of considerable economic importance at present are sterility mosaic (SM), a viral disease, caused by *Leveillula taurica* (Reddy *et al.*, 1994), wilt caused by soil borne fungus *Fusarium oxysporum F. udum* (Butler) Synder and Hansen (Butler, 1906), *Phytophthora* blight (PB) caused by fungus *Phytophthora dreschleri* (Williams *et al.*, 1968), *Macrophomina* root rot caused by *Macrophomina phaseolina* (Williams *et al.*, 1968) and *Helicoverpa armigera* (legume pod borer), which is a major insect pest Of all *Fusarium* wilt is the most important soil borne disease of pigeonpea

Fusarium wilt

Wilt disease in India was discovered in the early 1900s (Butler, 1906), where the causal pathogen was described as *Fusarium udum* (Butler, 1910) Synder and Hansen (1940), named the soil borne fungus as *F. oxysporum* Schlecht *F. sp. udum* (Butler) It is the oldest and most widespread disease of pigeonpea and has been reported from all major pigeonpea-growing countries in Asia and Africa (Nene *et al.*, 1996)

The first indication of the wilt is the occurrence of patches of dead plants in the field that appear usually at the flowering or pod-setting stage of the crop (Reddy *et al.*,

1990) Isolated wilted plants are also noted about a month after sowing The most characteristic symptom in adult plants is a purple color extending upwards from the base of the main stem This band is more easily seen in juvenile branches of pigeonpea than the main stem Partial wilting of the plant is a definite indication of wilt and distinguishes this disease from termite damage, drought, and *Phytophthora* blight, which also kills the plant Partial wilting is associated with lateral root infection, while total wilt is a result of taproot infection (Reddy *et al*, 1990) The intensity of browning or blackening decreases from the base to the tip of the plant Sometimes, branches especially lower ones are affected even when there is no band on the main stem These branches show die back symptoms with a purple band extending from the tip downwards, and intensive internal xylem blackening When young (1 to 2 months old) plants die from wilt, they usually do not show external banding, but have obvious internal browning or blackening Plants infected by *F udum* also exhibit loss of leaf turgidity, interveinal clearing and chlorosis before death Wilting in the infected plants occurs due to the intrusion of the pathogen into the root tissues, especially the xylem vessels leading to the blockage of the water conduction system (Reddy *et al*, 1998)

Application of chemicals to overcome the disease

The crop protection chemicals are used to overcome plant diseases caused by the invasion of insects and pests The pesticides include various organic compounds that contain lead, antimony, arsenic, mercury, selinium, sulphur, thallium, zinc and fluorine as active compounds although not toxic to the insects, were very persistent, for instance, sprayed crops sometimes retain sufficient arsenical residues potentially harmful to the

consumers and crops were damaged by residues which accumulate in the soil. Fungicides containing sulphur, copper and mercury have been another source of persistent chemicals; residues. The pesticide residues are significant due to their toxicological properties and persistence in the environment. Their significance become more pertinent when they accumulate in the environment, get converted into more toxic metabolites, exhibits toxicity towards non-target organisms and mammals (Ray, 2003). In comparison, the use of resistant cultivars involves minimum cost thereby forgoing purchase of pesticides (Robinson, 1996)

Recombinant DNA technology and genetic transformation

Improvement of crops by breeding involves accumulating genes for higher productivity and other desirable characteristics such as resistance to pathogens and pests. Introgression of genes from distantly related or unrelated donors is seriously hampered by a variety of incompatibilities including sexual barriers. Realizing the fact that the traditional varieties of pulses are not only poor at yield but also highly vulnerable to common diseases, the major emphasis in varietal improvement has been therefore laid on disease resistance breeding. Although research on the disease has been carried out for the past 90 years, limited information on methods of management other than the development of resistant/tolerant cultivars is available. Loss of resistance has been reported in some cultivars in India, suggesting the need for more concentrated efforts on understanding and identifying the pathogen, and breeding for stable resistance (Reddy *et al.*, 1998).

Recombinant DNA and genetic transformation technologies can circumvent taxonomic limitations to the gene pool for pathogen resistance even to the extent that plants need not be the only sources for disease resistance traits. Further, molecular biological techniques provide capabilities to engineer host plant resistance that is effective both against specific as well as a broad spectrum of pathogens and are genetically stable. Further, these techniques permit to locate, clone and sequence individual genes (DNA fragments) from a complex DNA sequences (Yun *et al.*, 1997).

The development of plant transformation techniques has been a major breakthrough in overcoming constraints to achieve precision of genetic manipulation. With the establishment of vector-mediated and direct gene transfer systems, the applicability of transformation technology has advanced rapidly in recent years, and genes from taxonomically distant and or unrelated donors have been incorporated into plants. Genetic transformation technology promises to overcome not only the major agronomic problems not yet solved through recombinational breeding due to the non-availability of relevant genes within the accessible primary or secondary gene pools, but also to introduce and express genes from unrelated sources. Recent advances in tissue culture and recombinant DNA technology have opened new avenues of transformation of crop plants to produce transgenic plants with new genetic properties (Yun *et al.*, 1997), including specific applications for the semi-arid tropics (Sharma and Ortiz, 2000).

Plant chitinases

In order to survive, plants have to withstand many adverse conditions of the environment The conditions that affect a plant's homeostasis are caused by environmental factors such as temperature, water, salt, mechanical damage (wounding), chemicals, UV light, and the interaction with pathogenic organisms, which forms an important biotic stress Plants have their own networks of defense against plant pathogens that include a vast array of proteins and other organic molecules produced prior to infection or during pathogen attack Not all pathogens can attack all plants and a single plant is not susceptible to the whole plethora of plant pathogenic fungi, viruses, bacteria or nematodes Following pathogenic attack, some plants activate the expression of defense-related genes (Bowles, 1990) Some defense-related genes encode enzymes involved in phenyl-propanoid metabolism (Hahlbrock and Scheef, 1987), hydrolytic enzymes such as chitinases and β-1,3 glucanases(Boller, 1987) and proteinase inhibitors (Ryan, 1988) Chitinases belong to the "pathogenesis-related" (PR) proteins, which are coded by a subset of defense-related genes and have been extensively characterized due their potential involvement with disease resistance (Muthukrishnan *et al*, 2001)

Chitin, a β-1,4-linked polymer of N-acetylglucosamine, is a structural component in a diverse array of organisms, including fungi, insects, various crustaceans, and nematode eggs (Gooday, 1990, Flach *et al*, 1992, Roberts, 1992, Ruiz-Herrara, 1992, Cohen, 1993) The enzyme chitinase poly glycanhydrolase [1,4-(N-acetyl-b-d-glucosaminide], hydrolyzes the chitin polymer to release N-acetyl glucosamine oligomers, following either endo or exo cleavages of the β-1,4 bond

One of the roles attributed to chitinases in higher plants is a defense mechanism against attack by pathogens, especially fungi, because the expression of chitinases is significantly enhanced upon infection Furthermore, chitinases have antifungal activity and cause hyphal tips to lyse in vitro (Shapira *et al.*, 1989, Sela Buurlage *et al.*, 1993, Mauch *et al.*, 1998) The expression of chitinases could therefore be speculated to have a defensive role during both the early and the late stages of the infection process, depending on the levels of constitutive enzyme and the affectivity of induction Much of the evidence for the suggested roles of chitinases in plant defense has been based on dramatic and rapid enhancement of enzyme levels in hypersensitive reactions, during induced host resistance (i e , in association with several other pathogenesis-related [PR] proteins), and in tissues following infection by a pathogen Recombinant DNA technology allows the enhancement of inherent plant responses against a pathogen by either using single dominant resistance genes not normally present in the susceptible plant (Keen, 1999) or by choosing plant genes that intensify or trigger the expressions of existing defense mechanisms (Bent and Yu, 1999, Rommens and Kishore, 2000) The availability of techniques in molecular biology now permits the isolation of specific genes and their reintroduction into plants, providing a powerful tool to elucidate the roles of specific enzymes in plants (Muthukrishnan *et al.*, 2001)

The imminent cloning of disease resistance genes, further molecular dissection of stress signal perception and transduction mechanisms and identification of genes that affect symptom development will provide attractive new opportunities for enhancing crop protection (Lamb *et al.*, 1992) Hamilton (1980, 1985) first suggested that virus

resistance could be obtained by introducing viral genes into plant, the possibility of viral genes functioning as resistance genes has been demonstrated in many virus-plant systems The advantage of using PAP (pokeweed antiviral protein), a ribosomal-inhibiting protein found in the cell walls of *Phytolacca americana*, in transgenic plants for virus resistance is resistant to abroad spectrum of plant viruses by expression of a single gene Tavladoraki *et al.*, (1993) showed that constitutive expression in transgenic plants of a single-chain Fv (scFv) antibody, directed against the plant icosahedral tombus virus artichoke mottled crinkle virus, caused reduction of infection incidence and delay symptom development Voss *et al.*, (1995) showed a similar results with TMV Tobacco plants expressing full-length Mab against TMV produced fewer numbers of necrotic local lesions when challenged with TMV

Plant and microbial chitinases introduced into plants under the control of constitutive promoters, resulted in the enhancement of resistance of the host plant to fungal pathogens (Broglie *et al.*, 1991, Vierheilig *et al.*, 1993, Lin *et al.*, 1995) The results of genetic engineering for fungal resistance has been encouraging (Garner *et al.*, 1992, Ward *et al.*, 1994) Chitinases have shown to possess antifungal role in disease resistance (Pegg and Young, 1981, Mauch *et al.*, 1984, Broglie *et al.*, 1991, Sela-buurlage *et al.*, 1993) The genes encoding chitinases have been introduced into plants by genetic engineering programs aimed at developing disease resistant varieties. These genes have originated from both plants (Broglie *et al.*, 1986, Shinshi *et al.*, 1987, Gaynor, 1988, Nishizawa and Hibi, 1991) as well as bacteria (Jones, 1986). The bacterial chitinase gene isolated from *Serratia marcescens* (*chi-A*) was transferred into

tobacco plants by Jones (1988) and Suslow *et al*, (1988), and the transformants showed significantly higher chitinase activity and more resistance to *Alternaria longipes* than that of the control plants Broglie *et al*, (1991) generated transgenic tobacco plants using the cauliflower mosaic virus (CaMV) 35s promoter and the bean *CH5B* gene The homozygous transformants showed an increased ability to survive in soil infected by *Rhizoctonia solani* Elevated field tolerance to fungal pathogens was first reported in transgenic *Brassica napus* plants that contained a chimeric chitinase gene from bean (Grison *et al*, 1996) Resistance to blackleg (*Leptosphaeria maculus*) disease in transgenic canola was reported by Wang *et al*, (1999) Terakawa *et al*, (1997) reported the antifungal activity to transgenic tobacco using fungal chitinase gene from *Rhizopus oligosporus* Extensive review on chitinases in disease resistance has been worked by Graham and Sticklen (1994), and Does and Cornelissen (1996) Enhaced levels of *Fusarium* resistance achieved in transgenic tomato lines accumulating multiple isoformss of chitinases and β-1,3-glucanases, were shown to result largely from the simultaneous expression of a class I chitinase and a class I β-1,3-glucanase gene (Jongedijk *et al*, 1995)

Transgenic rice expressing rice endochitinase gene, driven by the 35s promoter also showed enhanced resistance to sheath blight (Lin *et al*, 1995) Transgenic tobacco harboring rice endochitinase gene (Nishizawa *et al*, 1993) expressed resistance against powdery mildew (*Erysiphe cichoraccrum*) Rice plants induced with hydrolases such as chitinases and β-1,3-glucanase resulted in moderate field resistance to sheath blight caused by *R. solani* (Anuratha *et al*, 1996) Tabei *et al*, (1998) obtained transgenic

cucumber plants harboring a rice chitinase gene that expressed enhanced resistance to grey mold (*Bortytis cinnerra*) The resistance was stably inherited in the progenies, though one of the cucumber strains allowed the penetration of hyphae, invasion of the infected hyphae was restricted Sorghum plants belonging to genotype 607E expressed resistance to green bug, *Schizaphis graminum* Rodani, an amphid that attacks sorghum with the induction of chitinase protein (Krishnaveni *et al.*, 1999) Transgenic sorghum plants constitutively expressing a gene encoding a class I rice chitinase (*chiII*) were generated by biolistic transformation of scutellum-derived embryogenic calli with a plasmid DNA carrying the bar gene as the selectable marker and the rice chitinase gene under the control of a cauliflower mosaic virus 35S promoter showed increased resistance to the stalk rot causing fungus, *Fusarium thapsinum* (Krishnaveni *et al.*, 2001). Therefore, chitin metabolism forms an excellent target for selective pest control strategies (Kramer and Koga, 1986, Cohen, 1987, Kramer *et al.*, 1997) This strategy was also adopted for the present studies

Objectives

The present work was undertaken to develop plant regeneration and genetic transformation protocols for pigeonpea with the following objectives

- To develop an efficient and high frequency plant regeneration system in pigeonpea
- To study the ontogeny of shoot bud differentiation from the selected leaf explant for better understanding of the system

- Optimization of efficient transformation protocol and genetic transformation of pigeonpea with *RChit* gene by using microprojectile-mediated gene transfer.
- Characterization of T_0 and T_1 generation transgenics of pigeonpea through molecular and genetic methods for the confirmation of gene transfer and expression.

2. REVIEW OF LITERATURE

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2.1 Transgenic technology: Current status

The manipulation of the structures and function of genes at the sequence level, which aims to benefit the human race, is referred to as gene revolution that led to the development of recombinant DNA technology. Recombinant DNA technology involves the manipulation of genes, which include their isolation, identification, cloning and transfer into various organisms (Old and Primrose, 1994a). Traditional plant breeders followed conventional methods to improve various traits in crops such as yield, stress tolerance, etc. A number of desirable cultivars have been developed through recombination and mutation breeding methods. However, due to the limitations involved, crossing the species barrier was not possible. Plant tissue culture methods were later used to develop variability and also to produce a few interspecific hybrids. Introgression of foreign genes into desirable cultivars was a Herculean task through conventional breeding methods (Toenniessen, 1993). Therefore, non-conventional breeding methods, which include recombinant DNA technology, have come to the rescue of the plant breeder in the improvement of specific traits pertaining to the yield stability, disease and pest resistance, and product quality. Therefore genetic transformation technology has succeeded in overcoming the barriers of sexual incompatibility, which limits the genetic pool available for crop improvement (Kung, 1993). Plant genetic manipulation focuses on the cellular level of organization and involves the interfacing of all aspects of cell biology; molecular biology and gene transfer procedures (Sharp *et al.*, 1984). The most important aspect of biotechnology

comprises the transfer of specifically constructed assemblies through various transformation techniques, which constitutes genetic engineering. The plants obtained through genetic engineering contain a gene or genes usually from an unrelated organism and such genes are called as transgenes and the plants containing transgenes are known as transgenic plants (Birch, 1997).

According to Birch (1997), the major components in a gene transfer system for the production of transgenic plants are the development of reliable and efficient regeneration systems, availability of gene constructs, transformation with suitable vectors, methods to introduce DNA into the regenerable cells, selection and regeneration of transformed plants, molecular and genetic characterization of transgenic plants for stable and efficient gene expression.

2.2 Tissue culture and regeneration

The development of an efficient regeneration system is a pre-requisite for genetic transformation of plants and the transformation efficiency is generally been proportional to the efficiency of the tissue culture and gene transfer systems (Birch, 1997). The term tissue culture in a wide sense includes the in vitro culture of various kinds of explants ranging from cells to tissues and organs. With the availability of suitable conditions, each living cell of a multicellular organism is capable of independent development (White, 1963). The basic concept of in vitro plant regeneration comprises the response of individual somatic plant cells or protoplasts, capable of proliferation and organizing into tissues, and eventually developing into a

complete plant or organism, which constitutes the term totipotency (Ritchie and Hodges, 1993). The term totipotency was coined by Morgan (1901) that denotes the capacity of cell to develop into an organism by regeneration and was experimentally expressed at single cell level in the model species like tobacco (Vasil and Hildebrandt, 1965), and carrot (Normura and Komanine, 1985). All the cells in an individual explant are capable of expressing totipotency (Raghavan, 1986; Ammirato, 1987; Ranch and Pace, 1988; Vasil, 1988; Potrykus, 1990).

Cellular competence

Several factors such as morphological, cytological, biochemical and molecular aspects might play a vital role in distinction between totipotent and non-totipotent cells. The mixture of cells comprising the tissue explants vary physiologically, biochemically and developmentally (Lindsey and Yeoman, 1985), that led to the cellular heterogenicity in which certain cells in an explant are "competent" enough to respond to the in vitro culture conditions (Halperin, 1969; Vasil and Vasil, 1986; Potrykus, 1990). The capability of a cell or group of cells responding to an induced stimulus for a developmental process refers to the term "competence", which further represents a series of each of the genetic, epigenetic and physiological processes of the responding cell. The ability to induce differentiation in tissue culture is recognized as morphogenesis and the culture should comprise of a population of cells that are physiologically and biochemically uniform and express synchronized pattern of cell division, enlargement and differentiation (Meins and Binns, 1979).

Factors affecting in vitro cultures

Three vital factors affecting in vitro plant regeneration are genotype, explant source and culture conditions (including culture medium and environment) Variations in the above three factors led to the development of an in vitro regeneration system (Halperin, 1986, Ranch and Pace, 1988)

Effect of genotype

In order to regenerate a plant through in vitro culture, the choice of the genotype forms the most important factor (Ritchie and Hodges, 1993) The response of particular cultivars within a species responds differently to in vitro culture (Brown and Thorpe, 1986), which further illustrate that the genetic component is highly influential on success of in vitro culture and plant regeneration (Bingham *et al.*, 1975, Koorneef *et al.*, 1986, Hodges *et al.*, 1986) However, Vasil and Vasil (1986) stated that the genotypic effect could be solved by the use of correct combination of explant and culture conditions used Further the development of genotype-independent methods can provide efficient transformation procedures (Sharma and Anjaiah, 2000)

Source of explant

The type of explant largely affects plant regeneration Immature organs, meristematic and undifferentiated tissues, and young tissues form the most responsive and reliable explant sources (Green and Phillips, 1975, Kamo *et al.*, 1985, Vasil and Vasil, 1986) Genetic, epigenetic or physiological changes that occur in mature cells might be the reason for stage specific response (Halperin, 1986, Vasil, 1988, Lee and

Phillips, 1988). Other factors of the explant to be considered include size, orientation in culture, pretreatment and inoculation density (Brown and Thorpe, 1986) Seedling explants form the most feasible explants for in vitro regeneration studies for their yearlong availability, uniformity and availability in large numbers (Bean *et al.*, 1997, Geetha *et al.*, 1998)

Culture conditions

Regeneration of complete plant from a single cell in controlled environment or in vitro was first attempted by Vasil and Hildebrandt (1965) Components of a medium include inorganic macro and micronutrients, reduced nitrogen, carbon source, vitamins and growth regulators (Gamborg and Shyluk, 1981)

Of these components, the concentration and ratios of the growth regulators have proven to be most critical for culture initiation and morphogenesis (Skoog and Miller, 1957, Vasil, 1988) In addition to the nutritional value, reduced nitrogen (Halperin, 1966) and sugar component (Brown and Thorpe, 1986) further affect morphogenesis Many researchers have put forward various compositions of a nutrient medium for the growth of plant tissue (White, 1942, Murashige and Skoog, 1962, Linsmaier and Skoog, 1965, Gamborg and Eveleign, 1968). Inorganic salts are supplied in two groups as macro salts or nutrients and as micronutrients The salts needed in higher amounts are called macronutrients, which include nitrogen, phosphorus, sulphur, magnesium, calcium and potassium Nitrogen is mostly provided in two forms as nitrates and ammonium compounds The salts needed in trace amounts are called as micro salts, which include boron, zinc, molybdenum, manganese, copper and iron etc Carbohydrate

is supplied usually as sucrose The most commonly used amino acid is glycine Apart from nitrogen sources present in the inorganic salts, amino acids and amides are used in plant tissue culture media (Huang and Murashige, 1977) The most widely used amino acids are L-aspartic acid, L-asparagine, L-glutamic acid, L-glutamine and L-arginine Vitamins are required in trace amounts as they catalyze the enzyme system of the cells Vitamin B1 (thiamine) is the most commonly used vitamin for all plant tissue cultures Other groups of vitamins, which stimulate growth, are niacin (nicotinic acid), vitamin B2 (riboflavin), vitamin B6-pyridoxin, vitamin C (ascorbic acid) and vitamin B12-cyanocobalamine (Gamborg *et al*, 1976, Huang and Murashige, 1977)

Murashige and Skoog (1962) first reported the use of MS medium It is the most preferred medium for tissue culture based studies on wide plant species The medium was formulated with the entire essential macro and micro mineral elements and hence has become the first complete medium designed for in vitro culturing of diverse plant species To date, MS medium is the most widely used culture media in various tissue culture practices such as micropropagation, callus induction, regeneration etc However, specific media for specific plant groups are available, for example, B5 and L2 media have been used for legumes (Thu *et al*, 2003)

Growth regulators

Several growth regulators are known to stimulate the biological activity in cultured plants Shoot bud differentiation was first reported by Skoog (1944) in in vitro cultured tobacco pith Cytokinins promote cell division and regulate growth, and development that include kinetin, a cytokinin that was discovered by Miller *et al*,

(1955). Root-shoot differentiation in the above system was proposed by Skoog and Miller (1957), and further stated that it is regulated by auxin-cytokinin ratio. The most widely used cytokinins are adenine, kinetin, zeatin, benzyladenine and auxins are IAA (indole-3-acetic acid), NAA (α-naphthaleneacetic acid), IBA (indole-3-butyric acid) and 2,4-D (2,4-dichlorophenoxyacetic acid) etc. The hormones are physiologically active and also determine the acidity and alkalinity of the culture media to a small extent.

Based on the constituents, culture media are of two types, chemically defined and chemically undefined. In a chemically defined medium, the composition and concentration of all the constituents is known and it is prepared from inorganic and organic chemicals. Where as, in a chemically undefined medium, the concentration of the constituents is unknown due to the addition of natural products such as coconut milk, casein hydrosalate etc. A medium can be solid, semisolid, or liquid. Further, culture media is influenced by physical factors such as light, pH and temperature (Evans *et al.*, 1981

2.2.1 Pathways of regeneration

Various protocols for plant tissue culture have been developed during the past five decades and any plant species can now be regenerated in vitro through several pathways. Various pathways have been put forward to depict the regeneration processes such as organogenesis and embryogenesis. The rate of plant regeneration in tissue culture varies greatly from one species to another. Various cells, tissues and organs

from numerous plant species can be successfully cultured aseptically to regenerate whole plants

2.2.1.1 Organogenesis

Organogenesis is the development of adventitious organs or primordia from undifferentiated cell mass or directly from the explant in tissue culture by the process of differentiation In vitro plant regeneration by organogenesis usually involves induction of shoot buds leading to the development of shoots from the explant tissue, with or without an intervening callus stage, followed by transfer to a root induction medium for root formation and developmen Alterations of the auxin and cytokinin ratios can control morphogenesis in tobacco was reported by Skoog and Miller (1957). The developmental stage and physiological state of the explant at the time of culture would affect the ability to induce direct organogenesis and differentiation with out an intervening callus stage (Thomas and Davey, 1975) The hypothesis of organogenesis was advanced by Torrey (1966), who suggested that organogenesis via callus starts with the development of a group of meristematic cells i e , meristemoids that can respond to the factors within the system to initiate a primordium depending on the kinds of factors inducing either root, shoot or embryoid For the induction of organogenesis via callus on a particular medium, the medium should cause dedifferentiation (callus induction), attainment of competence, induction for the organogenic pathway and determination for the pathway, and should not interfere with the morphogenic expression of the developmental pathway (Christianson and Warnick, 1985) Therefore, organogenesis

was found to be the most reliable pathway for the regeneration of transgenic plants Organogenesis is of two types 1 Direct organogenesis and 2 Indirect organogenesis Organogenesis by direct rather than indirect pathway is preferred due to the problems with somaclonal variations confronted in callus cultures (Ritchie and Hodges, 1993)

Direct and indirect organogenesis

In direct organogenesis, either the shoot or root is induced directly from the pre-existing cells in the explant without undergoing an intervening callus phase (Brown and Thorpe, 1986, Christianson and Warnick, 1988) Direct organogenesis in vitro was reported in pigeonpea (Shiva Prakash *et al.*, 1994, Geetha *et al.*, 1998, Mohan and Krishnamurthy, 1998, Geetha *et al.*, 1999, Dayal *et al.*, 2003), in pea (Bean *et al.*, 1997), and in peanut (Sharma and Anjaiah, 2000 Indirect organogenesis involves an intervening phase of callus proliferation and growth, followed by shoot or root induction Indirect organogenesis was reported in pigeonpea (Eapen and George, 1993, George and Eapen, 1994, Ramesh and Baldev, 1994, Eapen *et al.*, 1998, Thu *et al.*, 2003, Dolendro *et al.*, 2003)

2.2.1.2 Embryogenesis

The developmental pathway in which embryos have been induced to form from a somatic cell or group of somatic cells is referred to as somatic embryogenesis. Reinert and Stewart first reported development of somatic embryos in 1958-1959. The auxin (2,4-D), which is provided in the initiation phase of embryogenic cultures, generally

leads to the induction of cellular proliferation (callus induction) along the embryogenic pathway of development (Evans *et al.*, 1981, Normura and Komamine, 1985) Somatic embryogenesis is of two types 1 Direct and 2 Indirect somatic embryogenesis

Direct somatic embryogenesis

Somatic embryogenesis, which occurs directly from cells of the explant tissue without an intervening callus phase, is stated as direct somatic embryogenesis (Conger *et al.*, 1983, Raghavan, 1986) Direct embryogenesis was initially reported in *Glycine max* (Lazzeri *et al.*, 1985, Finer, 1988), and *Zea mays* (Armstrong and Green, 1985, Vain *et al.*, 1989)

Indirect embryogenesis

Indirect embryogenesis is a common pathway in which somatic embryos get induced and develop from proliferated callus (Mc William *et al.*, 1974, Williams and Maheswaran, 1986) The explant most often used in indirect embryogenesis is the immature zygotic embryo (Merkle *et al.*, 1990, Finer, 1994) Adventitious embryony has been reported in increasing number of plants, including cereals (Vasil, 1988), woody plants (Von Arnold and Wallin, 1988) and recalcitrant tropical crops such as banana (Novak *et al.*, 1989)

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2.3 Genetic transformation of plants

2.3.1 Marker genes

Genetic transformation of plants requires markers, which play a vital role to ascertain and confirm the presence of transformed DNA, introduced into the plant cells (Walden, 1989, Klein *et al.*, 1989b) Selection of few transformed cells from the untransformed cells constitutes the key step in gene transfer methods. Screening offers an alternative in which selection pressure is not imposed on cells or shoots, but instead offers a provision to visibly assay them

Selectable genes comprise selectable markers which are used for stable transformation, allowing living cells, tissues or whole plants to grow under conditions that prevent the growth of untransformed tissues or confer a phenotype which allows transformed and untransformed tissues to be distinguished unequivocally (Bowen, 1993) These marker genes exhibit dominant phenotype, and are usually of microbial origin and placed under the control of a strong constitutive expression of a eukaryotic promoter often of viral origin (Birch, 1997, Sharma and Ortiz, 2000)

The most commonly used selectable marker genes include those conferring resistance to antibiotic such as kanamycin [*npt*II, encoding neomycin phosphotransferase, (Herrara-Estrella *et al.*, 1983, Bevan *et al.*, 1983)], hygromycin [*hpt* encoding hygromycin phosphotransferase isolated from *E. coli* (van den Elzen *et al.*, 1985, Waldron *et al.*, 1985)], and herbicides such as phosphinothricin [*ppt* encoding phosphinothricin acetyltransferase that was isolated from *Streptomyces hygroscopicus*, an anaerobic soil actinomycete (De Block *et al.*, 1987, Haughn *et al.*, 1988)

List of selectable markers used in gene transfer methods

Gene	Enzyme encoded	Selective agent (s)	Reference
hpt	Hygromycin phosphotransferase	Hygromycin B	Van den Elzen *et al.*, 1985
*npt*II	Neomycin phosphotransferase	Genticin (G418)	Bevan *et al.*, 1983
		Kanamycin	Herrera- Estrella *et al.*, 1983
ppt	Phosphinothricin acetyltransferase	Phosphinothricin (Bialophos)	De Block *et al.*, 1987
als	acetolactate synthase	Chlorosulphuron Imidazolinones	Haughn *et al.*, 1988

Scorable markers called as "reporter genes" are the screenable and or selectable genes of bacterial origin, which are detected through enzyme assays (scorable reporter genes) or through expression of resistance to a toxin (selectable reporter genes) Some of the common reporter genes used in plant transformation are *cat* (chloramphenicol acetyl transferase) Herrera-Estrella *et al.*, (1983), *lux* (Luciferase) Ow *et al.*, (1986), *uid*A (β-glucuronidase) Jefferson (1989), and GFP (green fluorescent protein) Chalfie *et al.*, (1994)

List of Screenable marker (reporter) genes used in transformation systems

Gene	Enzyme encoded	Reference
cat	Chloramphenicol	Herrera-Estrella *et al.*, 1983
lacZ	β-Galactosidase	Helmar *et al.*, 1984
*npt*II	Neomycin phosphotransferase	Reiss *et al.*, 1984
lux	Luciferase	Ow *et al.*, 1986
*uid*A	β-Glucuronidase	Jefferson *et al.*, 1987

Criteria for choosing selectable markers

Plant cell transformation frequencies are generally low and to ensure the survival of transformed cells in the presence of a selective agent, usually markers are used in stable transformation which confer a dominant phenotype on transformed cells, and result in adding up of a new trait not normally associated with untransformed cells The frequency of untransformed cells or plants surviving at different levels of the selection pressure is initially recorded and the suitable levels are selected for the culture of transformed ones If the frequency of escapes (surviving untransformed cells) is high, an alternate selection is sought In order to ascertain the above strategy, LD_{50} was taken into consideration (i e , the level at which 50% growth inhibition is observed) and higher levels are then set up for the culture of transformants (Nutter *et al.*, 1987, Hauptmann *et al.*, 1988, Dekeyser *et al.*, 1989) Christou *et al.*, 1987 used antibiotics such as kanamycin and genticin (G418) for selection of transformed friable callus cultures of legume plants with neomycin phosphotransferase (*npt*II) gene However, the effectiveness of the antibiotic selection depends on the explant source Kanamycin enriched the growth of the transgenic cotyledonary nodal explants of soybean (Hinchee *et al.*, 1988), whereas it was too harsh and unsuitable for soybean meristem organ cultures (Mc Cabe *et al.*, 1988) Hygromycin was originally tested for tobacco transformation (Waldron *et al.*, 1985), and has more recently been used for the transformation of several plant species like maize (Walters *et al.*, 1992) The efficacy of hygromycin or kanamycin as a selectable marker differs in a transformation system, for example in peas the shoot organogenesis was reproducibly induced on hygromycin

resistant calli but not on the calli selected for kanamycin resistance (Puonti-Kaerlas *et al.*, 1990)

2.3.2 Vectors for gene transfer

Plant genetic transformation vectors have certain essential features such as multiple unique restriction sites, which facilitate cloning the gene of interest, bacterial origin of replication, selectable markers that allow the recognition of untransformed cells, selectable marker gene for plasmid selection and maintenance in *E. coli* (Old and Primrose, 1994b) Two strategies have been adopted to obtain a vector suitable for the transfer of genes into plants such as the use of co-integrative pTi (Fraley *et al.*, 1985) and binary vectors (Hoekaema *et al.*, 1983) Initially developed vector systems were of the cointegrate type and made use of *Agrobacterium* strains with non-oncogenic Ti plasmids, e g Vector pGV3850 (Zambryski *et al.*, 1983) *Agrobacterium*-mediated gene transfer system has become more efficient with the advent of disarmed vectors or plasmids that replace wild type plasmids normally present in *Agrobacterium* In these vectors, the genes encoding growth regulators have been deleted to avoid interference with the regeneration of normal plants and have been replaced with dominant scorable and selectable markers (Bevan *et al.*, 1983, Fraley *et al.*, 1985, Bevan, 1984)

2.3.3 Efficient techniques for transformation

Transgenic plant research depends on the availability of procedures for plant transformation. Amongst the several techniques developed for transformation of plants two types of methods for plant transformation are more popular (Potrykus, 1990). The use of *Agrobacterium* as a biological vector for foreign gene transfer and direct gene transfer techniques, in which DNA is introduced into cells by the use of physical means i.e.; biolistics or microprojectile bombardment.

2.3.3.1 *Agrobacterium*-mediated gene transfer

The dawn of plant molecular biology and genetic engineering was basically the consequence of the discovery and study of the plant pathogen *Agrobacterium tumefaciens* (Gheysen *et al.*, 1998). *A. tumefaciens*, a gram-negative soil bacterium, causes crown gall tumors at the wound site of many dicotyledonous plants (Smith and Townsend, 1907). The crown gall formation is due to the transfer of a specific DNA fragment called the T-DNA (transfer DNA) from tumor inducing (Ti) plasmid of the bacterium (Zaenen *et al.*, 1974) to the plant cell. The transfer of T-DNA and its integration into the plant nuclear genome leads to crown gall phenotype (Chilton *et al.*, 1977; Schell *et al.*, 1979). T-DNA contains genes, which encode for the production of auxins (Akiyoshi *et al.*, 1984), and cytokinins (Barry *et al.*, 1984). Further, these genes encode for the production of enzymes involved in the synthesis of opines (Ellis and Murphy, 1981; Dessaux *et al.*, 1992). *Agrobacterium* strains were classified based on opines encoded by Ti plasmids as octopine, nopaline, agropine, succinamopine or

chrysopine strains (Dessaux, 1992, Vandequin-Dransart *et al.*, 1995) The transfer of T-DNA into plants requires three bacterial genetic elements, which constitutes, 1 25 bp imperfect repeats flanking T-DNA (Van Haaren *et al.*, 1988) acting in cis-orientation (Zambryski *et al.*, 1983), 2 Virulence genes (*vir* genes) encoded by Ti plasmid that is involved in processing of T-DNA and its transfer from the bacterium to the plant cell (Hooykaas and Beijersbergen, 1994), and 3 Bacterial elements consisting of a number of bacterial chromosomal genes that are necessary for T-DNA transfer and for the attachment of bacterium to the plant cell

2.3.3.2 Direct gene transfer

Agrobacterium-mediated gene transfer has been the most commonly used method of gene transfer in plants, but the cereals (monocots), comprising the most important food crops are not easily amenable to this method of gene transfer, further some of the dicots were recalcitrant to in vitro manipulation thus making inevitable to adopt direct gene transfer methods that are genotype independent in terms of DNA delivery, but however their efficiency is influenced by the type of target cells and their utility for the production of transgenic plants Direct gene transfer involves physical means to introduce DNA into intact cells (Gheysen *et al.*, 1998)

2.3.3.2.1 Microprojectile bombardment or biolistics for gene transfer

The invention of the direct gene transfer technique of microprojectile bombardment (Sanford *et al.*, 1987) was a major development in plant genetic manipulation as it has enabled the transformation of many plants that were not amenable to *Agrobacterium* or protoplast based gene transfer techniques. Microprojectile bombardment is the most widely used plant transformation method after *Agrobacterium* and has been applied to a wide range of crops (Barcelo and Lazzeri, 1998).

DNA delivery into plant cells is carried out with heavy microparticles (tungsten or gold) coated with DNA of interest, which are accelerated at a very high velocity, 1,400 ft per second (Russell *et al.*, 1993a). The microprojectiles are generally 1 to 3 μm in diameter, which are carried by a macroprojectile or the bullet and are accelerated into target cells such as pollen, cultured cells, cells in differentiated tissue and meristems, by penetrating cell walls of intact tissue. Particle gun, the PDS-1000He, uses helium for particle propulsion. In this gun, a burst of helium gas, released by the rupture of a plastic disc calibrated to break at a known pressure, accelerates a macrocarrier upon which microscopic gold or tungsten particles (microcarriers) coated with DNA get precipitated (Russell, 1993b). The term "biolistics" (biological ballistics) was coined to describe the nature of the delivery of foreign DNA into living cells or tissue through bombardment with a biolistic (gene gun) device (Weissinger, 1992).

Factors affecting microprojectile-mediated gene transfer

Physical factors

Physical factors affecting particle velocity and its penetration into target cells are particle acceleration, pressure and the distance of particle insertion into the target cells. Particle velocity used to penetrate the DNA into the target cells should cause minimum damage to the cells (Birch and Franks, 1991).

Biological factors

Biological factors taken into consideration in the gene transfer method is the capacity of the bombarded cells that undergo bombardment to proliferate and regenerate into plants which subsequently depends on factors such as growing conditions of the donor plants, explant, physiological and developmental stage of the explant, culture conditions of the explants and selection agents and selection intensity used (Weissinger, 1992).

Application of microprojectile bombardment for the production of transgenic plants

Following microprojectile bombardment, foreign genes have been delivered and expressed in both dicots and monocots including economically important crop species such as soybean, wheat, corn and rice (Wang *et al.*, 1988; Wu, 1989; Cao *et al.*, 1990). Biolistics has proven to be the most effective means of transformation of plant organelles (Svab *et al.*, 1990; Daniell *et al.*, 1991). Expression of the introduced genes was quantified by studies based on transgene regulation in specific plant cells or tissues

(Cao *et al.*, 1991; Lee *et al.*, 1991; Goldfarb *et al.*, 1991; Seki *et al.*, 1991a). Transgenic plants were produced from wide range of crops through microprojectile bombardment, that include the transgenic plants produced from bombardment of shoot apices of maize, (Zhong *et al.*, 1996); immature zygotic embryos of wheat (Becker *et al.*, 1994); immature inflorescence of Tritordeum (Barcelo *et al.*, 1994), leaves of tobacco (Tomes *et al.*, 1990) roots of *Arabidopsis* (Seki *et al.*, 1991b); anthers of rubber tree (Arokiaraj *et al.*, 1994), pollen grains of tobacco (Stoeger *et al.*, 1995) and embryogenic cell suspension of soybean (Finer and McMullen, 1991). The first bombardment experiment was carried out on Chlamydomonas that could stably integrate transgenes in the chloroplast genome (Boynton *et al.*, 1988) and the same approach was subsequently used by Svab *et al.*, (1990) to produce tobacco plants with stably transformed chloroplasts. Morrish *et al.*, (1993) and Varsha *et al.*, (1996) emphasized the potentiality of microprojectile bombardment in transforming plant cells to produce transgenic plants. Christou *et al.*, (1989) reported first stable transformation of soybean, subsequently in maize (Kamm *et al.*, 1990), *Glycine max* (McCabe *et al.*, 1988; Christen *et al.*, 1989; Takano *et al.*, 1996; Hadi *et al.*, 1996; Hu *et al.*, 1999; Santarem and Finer, 1999; Aragao *et al.*, 2000), *Gossypium hirsutum* (McCabe and Martinell 1993); *Phaseolus vulgaris* (Russell *et al.*, 1993c; Ozcan 1995; Aragao *et al.*, 1996; Kim and Minamikawa, 1996; Zhang *et al.*, 1997); *Arachis hypogea* (Schnall *et al.*, 1993; Brar *et al.*, 1994; Livingstone and Birch, 1995); *Zea mays* (Lowe *et al.*, 1995), *Hordeum vulgare* (Ritala *et al.*, 1994), sorghum (Devi and Sticklen, 2001) and pearlmillet (Devi and Sticklen, 2002).

Advantages of microprojectile bombardment and limitations of microprojectile bombardment

Some of the advantages of microprojectile bombardment in gene transfer are: 1. It is relatively easy to handle, 2. One shot yields many hits and 3. The total survival of cells from the intrusion of particles (Monti, 1992). Though many reports concerning varied crops expressed the efficiency of DNA delivery, but stable transformation frequencies were low. This might be due to low frequency of transgene integration and intrinsically low regeneration capacity of target cells and finally the tissue damage by bombardment. However the above problems could be solved by slightly modifying the parameters affecting DNA delivery (Weissinger, 1992).

Comparative studies between microprojectile bombardment and Agrobacterium-mediated gene transfer methods

Physical methods of gene transfer are fundamentally different from *Agrobacterium* transformation. For microprojectile bombardment, the most important is to first examine the available tissue culture systems, determine the modes of regeneration and identification of potential explants, location of the cells involved, optimize tissue culture conditions to increase the number or accessibility of cells and develop conditions for non-lethal transfer of DNA into large number of such cells per bombardment. In *Agrobacterium* transformation, genetic sequences are introduced into disarmed Ti plasmids, which carry essential genetic elements required for the DNA transfer. The plasmids are then reintroduced into defined strains of *Agrobacterium*. DNA transfer occurs as part of modified pathogenesis process,

involving complex and highly evolved interactions between the target plant cell and the bacterium. A defined segment of DNA is cut from the Ti plasmid molecule, transferred into the recipient cell and integrated into the plant chromosome Where as physical method for gene transfer do not involve any intermediate vector organism

Because no vector organism is involved, physical gene transfer techniques are not limited by any constraints characteristic of *Agrobacterium*-mediated transformation Genetic sequences can be incorporated into virtually any plasmid, which provides the sequences necessary for replication and selection in bacteria Other sequences, such as the *"vir"* or border sequences of the Ti plasmid are not essential In order to facilitate the above purpose, the plasmid construction for DNA delivery by physical means is simplified and plasmids tend to be smaller in size (Pietrzak *et al.*, 1986) since large plasmids (>10kb) will be subjected to greater fragmentation during microprojectile bombardment Further, physical introduction of DNA is not dependent upon any bacterial/host cell interaction In addition to this no *Agrobacterium* infection occurs during DNA transfer by physical procedures, removal of *Agrobacterium* cells from the resulting transgenic tissue or plants by antibiotic treatment is not required, which simplifies and expedites the transformation process The absence of *Agrobacterium* also eliminates the possibility of accidental release of recombinant bacteria into the environment, reducing the possibility of unintentional movement of alien genes to non-target plants

2.4 Molecular characterization, screening of transgenic plants and inheritance of transgenes

Preliminary screening of the putative transformants was carried out by PCR analysis PCR (polymerase chain reaction) is an in vitro enzymatic method of amplifying specific DNA sequences conceived by Mullis (Mullis, 1990) The simple concept of the PCR relies upon the repeated synthesis of the targeted DNA by the enzyme DNA polymerase (Phillip and Stewart, 1992) A breakthrough has come in PCR with the introduction of the thermostable enzyme Taq DNA polymerase (Lawver *et al* 1989) from the thermophilic bacterium "*Thermus aquaticus*" which is resistant to high temperature thus doing away with replenishment during the PCR process (Erlich *et al*, 1988

The pre-requisites for proving stable transformation are 1 A tight correlation between molecular data on integration of foreign genes (Southern blot) and phenotypic expression of integrated genes (enzyme assays of reporter genes), 2 Transmission and expression of integrated foreign genes in several offsprings and 3 Use of appropriate controls in various assays to rule out false positives and combination problems often encountered in experimentation (Yang, 1993)

It is generalized that *Agrobacterium* produces simpler integration patterns than direct gene transfer, but both approaches result in a similar range of integration events, including truncations, rearrangements and various copy numbers and insertion sites Further, the frequency, distribution of inserts and rearrangements vary with transformation parameters in both gene transfer methods Majority of the reports

suggests T-DNA insertions by *Agrobacterium*-mediated transfer are stably inherited in normal Mendelian fashion (Budar *et al*, 1986, Chyi *et al*, 1986, Wallroth *et al*, 1986, Feldmann and Marks, 1987) T-DNA copy number vary considerably in transformed plants (Thomashow *et al*, 1980, Zambryski *et al*, 1982, Spielmann and Simpson, 1986, Jones *et al*, 1987) and two unlinked T-DNA elements can integrate into the same plant cell (Depicker *et al*, 1985, De Framond *et al*, 1986, Petit *et al*, 1986) T-DNA junctions can vary from tandem inverted repeats, direct repeats or single copy insertions (Zambryski *et al*, 1982, Spielmann and Simpson, 1986, Jorgensen *et al*, 1987) A rearrangement of target plant sequences at the site of insertion has been observed by Gheysen *et al*, (1987) The presence of both multiple copy insertions and inverted tandem structures suggests some differences in the mode of DNA uptake from direct DNA transformation of mammalian and plant cells Direct DNA transfer and incorporation in cells often results in large direct repeated arrays of introduced DNA (De Jonge and Bootsma, 1984, Potrykus *et al*, 1985)

2.5 In vitro regeneration and genetic transformation in pigeonpea

2.5.1 Current status of tissue culture and regeneration studies in pigeonpea

The availability of an in vitro regeneration protocol is a pre-requisite for the application of most biotechnological techniques, such as production of synthetic seeds, transgenic plants and somaclones (Parrott *et al*, 1992) Development of protocols of high regeneration is an inevitable pre-requisite for the production of transgenic plants Considerable work has been done till date ShamaRao and Narayanaswamy (1975),

obtained callus from hypocotyl explants from seeds exposed to gamma irradiation and organogenesis was induced in fifty percent of the callus initiated on MS medium supplemented with 2,4-D and kinetin Gosal and Bajaj (1979) observed the formation of multiple shoots from differentiated immature embryos cultured on MS medium supplemented with 2,4-D and casein hydrolysate in which globular to early heart shaped embryos directly developed into plantlets Mehta and Mohan Ram (1980) have used in vitro grown seedlings as the source for culturing leaf, stem, epicotyl and root explants cultured on MS medium fortified with B_5 + BA + GA_3 + NAA Callus was initiated from these explants, which was friable initially but later became necrotic and compact Shoot bud formation resulted from leaves and cotyledonary callus cultured on Blaydes medium supplemented with BA, NAA and GA_3 (gibberillic acid). Kumar *et al.*, (1984) observed that cotyledons are the better explants for regeneration, nodal halves of the cotyledons developed more shoots when compared to distal halves on MS medium with 2,4-D and BA Kumar *et al.*, (1985) used immature embryos as explants that resulted in direct plantlet formation when cultured on MS and B5 medium supplemented with 2,4-D However, it was observed that B_5 medium to be superior in giving more results than MS medium Subramanyam *et al.*, (1988) obtained callus from anthers cultured on MS medium fortified with 2,4-D, however it failed to differentiate on various combinations of media. Reena *et al.*, (1992) obtained large number of shoot buds from callus. Cotyledons from mature seedlings were taken as explants, produced green compact calli, which further resulted in shoot bud formation, and later elongated on addition of GA_3. Eapen and George (1993) regenerated shoot buds from leaves cultured on MS

supplemented with BA and IAA Shoots buds resulted from callus after 45 d of culture on shoot induction medium Baldev and Ramesh (1994) reported the formation of callus from leaf and epicotyl explants cultured on B_5 medium with various combinations of phytohormones such as BA, kinetin and NAA They observed the callusing response from leaf explants was better than epicotyl region due to the presence of meristematic cells in leaf that could be induced to multiply at an accelerated rate and the formation of healthy callus was obtained in NAA, at relatively higher concentration Shiva Prakash *et al*, (1994) obtained multiple shoot formation from cotyledonary nodal region of seedlings germinated on MS medium supplemented with BA and the shoot buds elongated on MS basal medium (hormone free medium) Proliferation of the shoot initials resulted with topical application of IAA The resulted elongated shoots rooted efficiently on MS fortified with IBA Geetha *et al.*, (1998) obtained direct shoot bud differentiation and multiple shoot induction from seedling explants such as epicotyl, hypocotyl, leaf, cotyledon and cotyledonary nodal segments cultured on MS medium augmented with varied concentrations of BA/kinetin Elongation of multiple shoots resulted when placed on MS medium fortified with BA in combination with NAA and GA_3 Maximum frequency of shoot formation resulted from the cotyledonary node explants and minimal regeneration from leaf explants They observed BA to more effective cytokinin for shoot organogenesis The elongated shoots rooted on MS supplemented with IBA Eapen *et al.*, (1998) observed the formation of shoot regeneration from primary leaf segments cultured on MS medium supplemented with TDZ with IAA Tiny protuberances were produced from the cut end of the leaf

segments within 14 to 21 d of culture, which became distinct as shoot buds after 45 to 50 d The initial shoot meristems (5 to 10%) elongated on medium supplemented with BA, GA_3 and IAA They observed TDZ induced high frequency regeneration from leaf discs of pigeonpea Sreenivasu *et al* (1998) obtained plant regeneration through somatic embryogenesis using cotyledon and leaf explants of 10-d-old seedlings cultured on MS medium fortified with TDZ Subsequent withdrawal of TDZ from the induction medium resulted in the maturation and growth of the embryos into plantlets on MS basal medium Mohan and Krishnamurthy (1998) observed the formation of shoot buds from the distal cotyledonary segments cultured on MS medium fortified with different combinations of BA, kinetin and adenine sulfate Shoot buds elongated on transfer to MS medium containing BA and NAA, which later rooted on IBA Distal halves of the cotyledons turned green after 2 to 3 wk of culture which subsequently produced small, green, dome like structures, and later developed into shoot buds after 3 to 4 wk Geetha *et al*, (1998) used 7-d-old seedling explants of epicotyl, hypocotyls, leaf, cotyledon and cotyledonary nodal segments Number of shoots per explant was more when cultured on MS medium supplemented with BA in combination with NAA Geetha *et al*, (1999) used shoot tips for the regeneration of transgenic pigeonpea Seedlings raised from decoated seeds produced clusters of leafy structures, when cultured on low concentration of TDZ (0 05 to 1 0 µM), whereas the same explants when cultured on high concentration of TDZ (10 0 to 20 0 µM), induced somatic embryos (Dolendro *et al*, 2003) Germinated seedlings grown on B5 medium supplemented with BA (10 mg/L) induced differentiating callus formation in the cotyledonary node region The

calli when transferred to B5 medium containing reduced concentration of BA (0 2 mg/L) resulted in the formation of shoots (Thu *et al*, 2003) Dayal *et al*, (2003) reported high frequency regeneration from leaf explants of aseptic seedlings Primary leaf explants of 4 to 5-d-old exhibited adventitious shoot regeneration from the proximal cut end of the leaf when cultured on MS medium fortified with BA and kinetin

2.5.2 Genetic transformation of pigeonpea

Most of the legumes that include pigeonpea are recalcitrant in nature Till date there is no appropriate regeneration protocol in pigeonpea that can be exploited for the production of transgenics, either due to long callus phase or due to non-reproducibility of the protocol The use of wild types of *A tumefaciens* strains (Rathore and Chand, 1997) induced tumor formation in several cultivars (Bahar and Pant A-106, H82-1, UPAS 120 and ICPL 151) of pigeonpea Sagore *et al*, (1997) reported *A tumefaciens*-mediated transfer of pigeonpea embryo axis, using reporter gene Arundhati (1999) reported *Agrobacterium*-mediated transfer of leaf disks of pigeonpea cultivar ICPI 5164, using LBA4404 pBAL2 *Agrobacterium* strain and further studied the transient expression in leaf disks For *Agrobacterium*-mediated gene transformation, Geetha *et al*, (1999) have utilized shoot apices and cotyledonary nodal explants and the mode of regeneration was direct organogenesis Lawrence and Koundal (2001) have used embryonal axes for *Agrobacterium*-mediated gene transfer method and they have observed indirect regeneration resulting from callus produced through embyronal axes

Thu *et al.*, (2003) reported the production of transgenic pigeonpea using colyledonary nodal region through microprojectile bombardment as well as *Agrobacterium*-mediated gene transfer method. Transgenic pigeonpea using leaf explant was produced by Dayal *et al.*, (2003).

3. MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Tissue culture

3 1 1 *Plant material*

Different genotypes of pigeonpea (*Cajanus cajan* (L) Millsp) were obtained from the gene bank of ICRISAT (International Crops Research Institute for the Semi-Arid Tropics), Patancheru, India These genotypes include ICPL 87, ICPL 88039, ICPL 87119, ICPL 85063, ICPL 88009, ICPL 87091, ICPL 2376, ICPL 87051, ICPL 91011, ICPL 332 and ICPL 84031 Unless mentioned other wise, all the experiments were carried out with the cultivar ICPL 88039 (Appendix 1)

3.1.2 Explant preparation and shoot regeneration

The seeds of pigeonpea cultivar ICPL 88039 were surface sterilized with 70% ethanol for 2 min, and washed with 0 1% (w/v) aqueous mercuric chloride containing 1 to 2 drops of Tween-20 for 8 min on a rotary shaker and rinsed four to five times in sterile water and soaked in autoclaved water for 4 hr The seed coat was removed from pre-soaked seeds and germinated on semi-solid MS (Murashige and Skoog, 1962) basal medium (Appendix 2)

Primary leaves from 4 to 5-d-old aseptically grown seedlings were used as explants for culture While preparing leaf explants, care was taken to excise the petiolar region sufficiently away from the axillary meristem, so as to completely eliminate any preformed meristematic tissue The explants were placed on SIM (shoot induction

medium) consisting of MS basal medium augmented with 5 μM BA in combination with 5 μM kinetin. Once the shoot initiation was observed, the leaf explants forming shoot bud were placed on ½ strength SIM [or shoot development medium (SDM)] consisting of MS medium having 2.5 μM BA and 2.5 μM kinetin after removing half the lamina. The explants with differentiated and proliferated multiple shoot buds were transferred to SEM (shoot elongation medium) consisting of MS medium supplemented with 0.58 μM GA_3. The shoots over 3 cm in length were transferred to rooting medium (RIM) consisting of MS basal medium with reduced sucrose concentration (1% w/v). Pulse treatment was given to the elongated shoots with 11.42 μM IAA solution. First pulse treatment was given to the cut shoots, which were directly dipped into the IAA (11.42 μM) solution for 30 to 60 sec and later transferred to the tubes containing MS medium devoid of any growth regulators in which the sucrose concentration was reduced to 1% (w/v). Second pulse treatment was given to those unrooted shoots, which were unable to root in the first pulse treatment. The in vitro regenerated plantlets with well-developed roots were transferred to small pots for hardening, which consisted of autoclaved sand and small amount of thiaram (fungicide). The plantlets were kept in the pots (3 inches) covered with polythene bags for 5 d. Initially these plantlets were kept in culture room for one day at a temperature of 26 °C and relative humidity of 40%. Later the pots were transferred to the greenhouse. Within these 5 d various steps were involved for the acclimatization of the rooted plantlets. In the initial phase of the acclimatization, small holes were punched on the sides of the polythene bag. After 3 d top portion of the polythene bag was totally cut, with eventual removal of the bag after

7 to 8 d The plants from the smaller pots (6 inches) were transferred to much bigger pots (13 inches) which consisted of autoclaved sand and soil in 1 1 ratio supplemented with small amount of manure and di-ammonium phosphate (DAP). During the initial transplantation process, the plants were covered with same polythene bags (with top cut portion) and small sticks were used to give support to the plant. After 3 d, the plants were totally uncovered and are exposed to withstand various climatic conditions. The surviving plants were maintained in the glasshouse until maturity and harvest.

Various parameters effecting shoot regeneration like growth regulators, sucrose concentration, age of the explant and genotype were dealt in detail in the following section (3 1 3)

3.1.3 Culture medium and conditions

MS basal medium with 3% sucrose was used for all the experiments except, rooting experiments pH of the medium was adjusted to 5 8 prior to the addition of 0 8% agar and was autoclaved at 15 psi pressure for 15 min. The regeneration experiments were carried out in 90 mm X 16 mm sterile disposable plastic petri-plates sealed with Parafilm®, while elongation and rooting experiments were conducted in 15 mm X 25 mm long test tubes plugged with non-absorbent cotton plugs wrapped in one layer of cheese cloth. Cultures were maintained at 26±1 °C with 16 h photoperiod provided by white cool fluorescent lamps having 60 $\mu Em^{-2}S^{-1}$ light intensity and 8 hr of dark period.

Growth regulators

TDZ and IAA: TDZ in combination with IAA was tested for shoot bud induction in shoot regeneration experiments on pigeonpea Leaves from 2, 3, 4, 6, and 8-d-old aseptically grown seedlings were taken as explants for culture A total of 24 explants of 3 replicates were taken and observations were recorded based on the explant response for shoot bud induction and change in the morphology of the explants in response to the above growth regulators was observed Data was recorded after 3 wk of culture MS medium supplemented with three different levels of TDZ and IAA were tested (TI, TI-1, TI-2) and the explant's response for shoot bud induction was observed In the first combination, 9 0 μM TDZ in combination with 0 57 μM IAA (TI) was taken In the second combination, the concentration of TDZ was 9 08 μM and IAA 1 14 μM (TI-1) and in the third, TDZ concentration was 11 35 μM in combination with 1 14 μM IAA (TI-2)

BA and NAA: Earlier reports on pigeonpea stated the combination of cytokinin and auxin to be effective for shoot bud induction Therefore experiments were conducted using leaves from 4, 5, 6, 7, 9, 11, 13, 15-d-old aseptically grown seedlings were used as explant MS medium containing various combinations of BA + NAA (BN1, BN2, BN3, BN4) were tested The effect of combination of cytokinin and auxin on the explant for response to the formation of shoot buds was observed Four combinations of the medium were prepared A total of 24 explants of 3 replicates were taken for culture and the experiments were repeated thrice and the data was scored In the first combination, BA concentration was 4 44 μM in combination with 0 54 μM

NAA (BN1), in the second combination, concentration of BA was 11 09 µM and NAA 1 07 µM (BN2), in the third combination, concentration of BA was 15 53 µM with 1 16 µM NAA (BN3) and in the fourth combination 22 18 µM BA in combination with 1 07 µM NAA (BN4)

BA, kinetin and 2-iP: As cytokinins are useful for shoot organogenesis, various combinations of BA, kinetin and 2-iP were supplemented with MS medium The effect of these cytokinins on the explant in response to the formation of multiple shoots was tested Four to 5-d-old aseptically grown leaf explants were cultured on MS medium augmented with various levels of BA, kinetin and 2-iP (C1 to C6, K1 to K6, P1 to P6 and I1 to I6) Four combinations of the medium were prepared In the first combination, BA concentration ranged from 0, 2 5, 5, 7 5, and 10 µM, in which, kinetin concentration of 5 µM was kept constant (C1 to C6), in the second combination, concentration of BA and kinetin was vise versa (K1 to K6), and in the third, the concentrations of kinetin ranged from 0, 2 5, 5, 7 5, and 10 µM and 2-iP concentration of 5 µM was kept constant (P1 to P6) In the fourth combination, the concentration of kinetin (5 µM) was kept constant and with different concentrations of 0, 2 5, 5, 7 5, and 10 µM, 2-iP (I1 to I6)

Type of explant

Various explants like epicotyl, hypocotyl, embryonal axes, leaf lamina, leaf with half cut lamina and petiole from in vitro raised aseptic seedlings were taken to compare their morphogenic potential, and the observations were recorded based on the explants response for shoot induction The explants were placed on MS medium supplemented

with 5 μM BA and 5 μM kinetin The explant, which responded well, was selected for all the consecutive experiments

Genotype

To study the effect of genotype of the explant donor seedlings, eleven genotypes of pigeonpea belonging to different maturity groups were selected (Table 5) These genotypes include ICPL 87, ICPL 88039, ICPL 87119, ICPL 85063, ICPL 88009, ICPL 87091, ICPL 2376, ICPL 87051, ICPL 91011, ICPL 332 and ICPL 84031 The leaf explants of 4 to 5-d-old in vitro germinated seedlings were cultured on SIM and their response for shoot regeneration was compared

Age of explant

For efficient organogenesis, the age of the explant was taken as one of the important parameter Explants from different ages of 2, 3, 4, 5, 6, 7, 9, 11, 13 and 15-d-old aseptically germinated seedlings were taken A total of 60 explants with three replicates were taken and cultured on SIM Age of the explant was optimized based on the shoot induction response from leaf explant obtained from seedlings of different ages

Size of the leaf lamina

To study the role of lamina, different portions of the lamina were surgically removed so as to have explants with full, ½, ¾ portions of the lamina and without lamina along with the petiolar cut end The explants were cultured on SIM with the abaxial surface of the lamina in contact with the medium

Effect of GA_3 on shoot elongation

Different steps were involved for the elongation of the multiple shoots A total of 24 explants of three replicates were taken for culture Initially the explants with differentiated shoot buds were placed in SIM for elongation In the second combination, the explants with shoot buds were placed in ½ strength SIM and in the third combination, the explants with multiple shoot buds were cultured in MS medium supplemented with various levels of GA_3 (0 58, 1 51, 1 71, 2 31 and 2 89 µM) Explants cultured on MS medium supplemented with 2 5 µM BA and 2 5 µM kinetin were taken as controls The concentration of GA_3 was optimized based on the shoot bud response for the elongation of healthy and multiple shoots

IBA and IAA for rooting

Elongated shoots (upto 3 cm) were transferred to RIM (root induction medium containing MS basal medium supplemented with various concentrations of IBA) MS medium with different concentrations of IBA such as 0 9, 1 97, 2 95, 3 94 and 4 9 µM with reduced sucrose concentration (1% w/v) were tested and the response of the elongated multiple shoots for root initiation was observed

Based on the response of the elongated shoots on IBA, IAA was tested for root initiation in the elongated shoots MS basal medium having different concentrations of IAA, which include 1 14, 2 82, 3 42, 4 57, 5 71, 6 85, 7 56 and 11 42 µM was used for pulse treatment of rootable shoots

Sucrose for rooting

Sucrose starvation experiments were carried out on elongated shoots placed on RIM consisting of 1.14 µM IAA. Various percentages of sucrose from 1, 2 and 3% were tried for rooting and sucrose percentage was optimized based on the response of the shoots rooted.

3.1.4 Sterilization of BA, kinetin, GA_3 and IAA

Different sterilizing methods were used for BA, kinetin, GA_3 and IAA, for different stages of regeneration studies involving SIM, SDM, SEM and RIM, was carried out and the effect of autoclavable and filter sterilized phytohormones on shoot regeneration was observed.

3.2 Ontogeny studies on shoot bud differentiation

To study the morphology and shoot bud development from petiolar region of the leaf, the leaf explants from in vitro raised aseptic seedlings and cultured on SIM and the petiolar region from which the regeneration occurred was used for ontogeny studies. The explants of different ages from 0, 2, 4, 6, 8, 10, 12 and 14-d-old were taken and processed for paraffin sectioning as described by Johansen (1940). Steps involved in processing the tissue are fixation, dehydration, infiltration, embedding, sectioning, deparafinising, staining, and mounting were as follows:

Fixation

Petiolar region of the leaf explant was fixed in a solution of acetic acid 95% ethanol (1 3) The samples were infiltrated under vacuum and maintained at 4 °C for 4 to 6 hr

Dehydration

The fixed samples were vacuum infiltrated and washed thrice with distilled water (30 min each) and then dehydrated with alcohol dilution series (10, 30, 50 and 70%) for 30 min each and finally stored in a fresh solution of 70% alcohol at 4 °C

Infiltration

The samples in 70% ethanol were taken and placed in oven maintained at 52 to 60 °C temperature (melting point of paraffin wax) The lid of the vial was opened and at an interval of 30 min, a rice grain size of paraffin wax was added to the vial and the lid was replaced tightly and retained in the hot air oven This process was continued until the material was infiltered in wax and the extra wax starts occupying the glass vial by dissolving in the 70% ethanol At this stage the lid of the vial was opened and wax addition was continued till the entire vial was filled with wax, which was taken out of the oven and placed at room temperature

Embedding

The vial with the material infiltered completely with wax was once again placed in the oven to bring the wax into melting state Plastic boats were taken and placed in a tray with water, and the melted wax in the vial along with the sample was poured into

the boat along with some fresh melted wax so that the material was completely embedded in wax to the brim of the boat (Appendix 3)

Sectioning

The material embedded in the paraffin wax was trimmed to remove the extra wax in and around the tissue and the boat was placed in the racket of the microtome and sections of 10 µm thin were cut using Leica RM 2155 [TM], India microtome device and later were slided onto a microslide

Deparafinising and staining

The slides were dried and then deparafinised by passing through a series of solutions and a total time period of 16 hr is required for the above process to be carried out and staining of the sections was done using Eosin stain The slides with the sections were serially processed as given in Appendix 4

Mounting

A drop of DPX mount was taken on a cover slip and the slide reversed on to the cover glass with the section falling in the center of the cover glass and the slide was turned to the correct position The cover glass was tapped gently to remove air bubbles and the slides were air dried for 15 to 20 min and then stored with proper labeling

Observations and photography

All the observations were made by using a compound microscope under 4, 10, 20 and 40X magnifications and photographed with Kodak 100 ASA black and white films

3.3 Genetic transformation

3.3.1 Effect of the kanamycin (antibiotic selection) on untransformed control leaf explants

Initial experiments were carried out to study the effect of antibiotic selection for shoot regeneration using untransformed leaf explant The explants were placed on shoot induction medium Once the shoot bud initiation was noticed, the explants with half cut lamina were placed on ½ strength SIM Various concentrations of kanamycin (25, 50, 75, 100, 125 and 150 mg/L) were tested on leaf explant and observations were carried out on the morphological changes and response of the leaf explant for shoot regeneration About 72 explants were taken and the experiments were repeated thrice The efficiency of the antibiotic on the explant cultured was observed after 2 to 3 wk

3.3.2 Microprojectile bombardment for gene transfer

3.3.2.1 Bacterial strain and plasmid

The *RChit* gene was kindly provided by Dr S Muthukrishnan of Kansas State University, USA The *RChit* gene was cloned in the plasmid pRTT99GUS in the Genetic transformation laboratory, ICRISAT (K K Sharma and coworkers) The plasmid construct pRT99GUS RChit (Fig 6) carrying chitinase expression cassette was maintained in *E. coli* strain DH5α The plasmid pRT99GUS RChit has marker genes such as *npt*II and *uid*A, and *RChit* as candidate gene under the control of a constitutive promoter CaMV 35S and polyA terminator

3.3.2.2 Isolation of plasmid DNA

Plasmid DNA was isolated according to the procedure reported by Sambrook *et al.*, (1989). Various steps in the procedure were as follows -

Components

- GTE buffer 50 mM Glucose, 25 mM Tris (pH 8 0), 10 mM EDTA (pH 8 0)
- Lysis buffer 0.2 N NaOH and 1% SDS
- 5 M Potassium acetate, pH 5 2
- RNase (10 mg/ml)
- Chloroform
- Isopropanol
- 70% ethanol
- TE buffer 10 mM Tris (pH 8 0) and 1 mM EDTA (pH 8 0)

Method

Plasmid construct pRT99GUS RChit was maintained in *E. coli* strain DH5α and grown on LB (Appendix 5) agar plates containing 100 µg/ml ampicillin Single isolated colonies were grown in 10 ml of LB medium overnight at 37 °C on a rotary shaker at 220 rpm and 10 ml bacterial suspension was pelleted by centrifuging for 10 min at 6000 rpm The bacterial pellet was suspended in 600 µl of GTE for 5 min at room temperature (can be placed even in ice), in order to maintain the osmoticum To the resuspended bacterial solution, 1200 µl of freshly prepared lysis buffer was added and the samples were placed on ice for 5 min After 5 min, 900 µl of 5 M potassium acetate was added to the lysed bacterial solution and the samples were mixed well by inverting the tubes slowly and the mixture was placed back on ice for 5 min. The solution was centrifuged for 10 min at 14,000 rpm and the supernatant was transferred to fresh tubes

to which 3 to 5 µl of RNase (10 mg/L) was added, to remove RNA, and incubated at 37 °C for 30 min Equal volumes of phenol chloroform was added to remove proteins present in the DNA mixture and a brief spin was given Further, to the aqueous phase equal volume of chloroform was added and the sample solution was centrifuged for 30 sec The top aqueous phase was collected into fresh tubes and to this 0 8 volume of isopropanol was added to precipitate nucleic acids and stored at –20 °C for 30 min The sample was centrifuged for 10 min at 10,000 rpm / 4 °C and the pellet was washed with 70% ethanol and air-dried The dried plasmid DNA pellet was finally dissolved in 30 µl of TE

3.3.2.3 Preparation of plasmid DNA for microprojectile bombardment

Plasmid pRT99GUS RChit consisting of *RChit* gene along with *uid*A and *npt*II genes was used for microprojectile bombardment (Fig 6) Microprojectile bombardment was performed using PDS-1000He (Bio-Rad, Hercules, Calif)

3.3.2.4 Particle preparation

A total of 30 mg of 1 µm tungsten (Bio-Rad, Hercules, Calif) particles were sterilized with 500 µl of 70% ice-cold ethanol The particles were vortexed for 3 to 5 min and incubated at room temperature for 15 min, later centrifuged for 5 sec in microfuge to pellet the microparticles The microparticles were then washed and resuspended in sterile water Particles were coated with DNA according to Sanford (1990) with some modifications

Particle suspension (tungsten) of 50 µl was mixed with 5 to 7 µl of plasmid DNA (1 µg/ml), and to the above suspension 50 µl of $CaCl_2$ (2 5 M) and 20 µl of 0 1 M spermidine was added for the precipitation of the plasmid DNA (Russell *et al.*, 1993b) The mixture was vortexed for 3 min and a brief spin of 5 sec was given Later, the supernatant was discarded and the pellet was washed with 70% ethanol followed by 100% ethanol DNA coated particles were resuspended in 60 µl of absolute ethanol Particle-DNA mixture of 6 µl was placed in the center of the nylon microprojectile screen The target explants were placed at a distance of 6 and 8 cm from the stopping plate and covered with a stainless steel mesh Chamber pressure of 25 mm Hg was maintained For bombarding the explants, two different distances of 6 and 8 cm from the stopping plate were tested and two different pressures of 1100 and 1300 psi pressure were also tested individually to bombard the DNA into the target cells A total of 400 explants of three replicates were taken and bombarded Based on the regeneration potential of the bombarded explants, the above parameters were standardized

3.3.2.5 Bombardment of particles

Macrocarriers were dipped in isopropanol and placed on a sterile filter paper for drying Tungsten particles coated with 6 µl of DNA were loaded on each macro carrier Rupture disc was loaded into the acceleration tube to which petri-plate with the sample was placed at an optimum distance and the door was closed. Vacuum switch was kept pressed till it reached 23 to 25 inches Hg and immediately the vacuum hold switch was pressed. Later the fire switch was pressed and the rupture disk bursts once the helium

gas fills up and the pressure to increase to the required limit as. Fire switch was released immediately after the rupture disk bursts and the gun fired onto the macrocarrier, which was stopped by the stopping screen, while the DNA coated micro projectiles were shot onto the target tissue

Explants (50 to 60) in number were placed in each petri-plate in a circular manner without leaving any space in between and the position of the explants was in such a way that the cut end of the petiolar region of the explant where the target cells competent for regeneration was facing towards the center (Fig 7) Parameters like size of the tungsten particle (1 μm) and particle density were kept constant throughout the experiments

3.3.2.6 Plant regeneration and selection

Initially 74 explants were taken in each experiment The explants were pre-cultured on SIM at the time of bombardment The bombarded explants were culture in SIM for 24 hr, and later the explants were transferred to fresh plates consisting of SIM with small amount of cefotaxime to avoid contamination, and the plating density of 10 explants was maintained The bombarded explants were placed on SIM With the onset of multiple adventitious shoots from the proximal cut end of the petiole of the leaf explant, the explants with half cut lamina was transferred to the selection medium, consisting of ½ strength SIM with 25 mg/L kanamycin The explants were cultured in the above medium for a week, and later the explants were transferred to SEM with 50 mg/L kanamycin The elongated shoots cultured in the above medium were

subjected to continuous selection pressure of 50 mg/L kanamycin and in the following 2 to 3 sub-cultures of 2 wk each on the shoot elongation medium, the concentration of kanamycin was increased upto 100 mg/L for stringent selection of transformed shoots The selected shoots were transferred to the RIM The elongated putative transformed shoots were subjected to pulse treatment for rooting (pulse treatment given, is similar to that of the treatment given to the in vitro regenerated plantlets)

Acclimatization and transplantation of putative transgenic plants

The in vitro selected plantlets with well-developed roots were transferred to small pots (6 inch) for hardening, which consisted of autoclaved sand and pinch of thiaram (fungicide) The plantlets were kept in the pots of 3 cm covered with polythene bags for 5 d Initially for one day these plantlets were kept in culture room at a temperature of 26 °C and relative humidity of 40% Later the pots were transferred to the glasshouse Within these 5 d various steps were involved for acclimatization of the rooted plantlets In the initial phase of the acclimatization, small slits were made on sides of the polythene bag After 3 d top portion of the polythene bag was totally cut

The plants from the smaller pots were transferred to bigger pots (13 inch) which consisted of autoclaved sand and soil in 1 1 ratio supplemented with small amount of manure and di-ammonium phosphate (DAP) Even in bigger pots, the plants were covered with polythene bags and small sticks were used to give support to the plant After 3 d, the plants were totally uncovered

3.4 Analysis of transgeinic plants

Molecular and biochemical analysis of putatively transformed plants was carried out for two reasons First, to determine whether the material is transgenic and second, to characterize the material (e g to determine copy number and/ or the complexity of the DNA insert, and evaluate transgene expression)

3.4.1 Histochemical analysis of putative transformants

Transient expression of a β-glucuronidase (*uid*A) marker gene was assayed by the histochemical procedure of Jefferson (1987) The substrate used to study the *uid*A gene expression was 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc)

GUS reaction

The enzyme β-glucuronidase cleaves the substrate X-Gluc into Glucuronic acid and indoxyl derivative The indoxyl derivative dimerizes and is then oxidized to form insoluble, highly colored indigo dye (dichloro-dibromo indigo, CIBr indigo) Although atmospheric oxygen stimulates the dimerization of of indoxyl derivate the oxidation catalyst potassium ferricyanide and ferrocyanide present in a reaction mix, accelerates the reaction EDTA added in the reaction mix leads to the partial inhibition of the enzyme by oxidation catalyst

The procedure followed for GUS assay was as follows putative transformed tissue either leaf, petiole or stem sections were incubated in GUS histochemical staining solution in 1 5 ml eppendorf, covered with aluminum foil Later the plant tissue kept for

GUS assay was vacuum aspirated for 10 min and later incubated in dark at 37 °C overnight The tissue was rinsed in 70 and 100% ethanol for 30 min till the chlorophyll comes out from the intercellular spaces of the plant tissue expressing intense blue Explants after 2 d of bombardment, as well as the explants (leaf, petiole) after subsequent sub-cultures after bombardment were stained with GUS histochemical staining solution (Appendix 6)

3.4.2 Molecular analysis of putative transgenics

3.4.2.1 Extraction of genomic DNA

Genomic DNA from the putative plants was extracted according to the protocol reported by Porebski *et al.*, (1997)

Solutions

- Extraction buffer (Appendix 7)
- Poly vinyl pyrrolidone (PVP 40,000)
- Chloroform octanol (24 1)
- 5 M NaCl
- 95% ethanol, 70% ethanol, absolute ethanol (100%)
- TE buffer 10 mM Tris-HCL + 1mM EDTA, pH 8 0
- RNase A 10 mg/L
- Proteinase K 1 mg/L
- Chloroform-Phenol saturated in TE
- 3 M sodium acetate pH 4 8

Genomic DNA was isolated from leaf material of glasshouse grown putative transformants Leaf tissue (500 mg) was ground in liquid nitrogen with a mortar and pestle To the powdered leaf tissue, 5 ml of extraction buffer was added and transferred to 30 ml polypropylene tubes The contents were mixed well by inverting the tubes 3 to 5 times and incubated at 60 °C for 45 min To the incubated mixture, 6 ml of chloroform octanol solution (24 1 ratio) was added and centrifuged at 6000 rpm for 20 min The supernatant was collected and adding ½ volume of 5 M NaCl and double the volume of 95% ethanol precipitated the DNA, and incubated at -20 °C for 20 min DNA pellet was collected by centrifuging at 6000 rpm for 6 min The pellet was washed with ice-cold 70% ethanol, air-dried and dissolved in 500 µl of 10 mM Tris and 1 mM EDTA, pH 8 0 To the dissolved pellet 10 µl of RNase (10 mg/L) was added to degrade RNA by incubating at 37 °C After 30 min, 3 µl Proteinase K was added and incubated further at 37 °C in water bath for another 30 min Later equal volumes of phenol chloroform (1 1) was added to the DNA solution The vial consisting of the solution was inverted slowly for three to four times and centrifuged for 10 to 15 min at 14,000 rpm To the clear supernatant 1/10 volume of 3 M sodium acetate and equal volume of 100% ethanol was added and placed and incubated at -80 °C In order to recover more DNA, to the bottom layer, 100 to 150 µl of TE was added and a 10 min spin was given at 14,000 rpm To the supernatant 1/10 volume of 3 M sodium acetate and equal volume of absolute ethanol was added and incubated at -80 °C (preferably overnight) Samples were centrifuged at 14,000 rpm and the pellet was washed in 70% ethanol, air dried and later dissolved in TE

Rapid preparation of genomic DNA using plant DNAzol® reagent (Invitrogen U.S.A.) for quick PCR analysis

Plant DNAzol is an extra strength DNAzol® reagent, specifically formulated for the isolation of genomic DNA from plants The plant DNAzol procedure is based on the use of a novel guanidine-detergent lysing solution, which hydrolyzes RNA and allows the selective precipitation of DNA from a variety of plant tissues The whole protocol can be carried out at room temperature and time involved in the extraction of the DNA is very less and the method is very efficient and quick

Components

- DNAzol® reagent
- Chloroform
- DNA Zol® + ethanol mix (1 0 75)
- 70 and 100% ethanol
- TE buffer 10 mM Tris-HCL + 1 mM EDTA, pH 8 0

Young leaf tissue of 100 mg was taken and ground in liquid N_2 to a fine powder To the ground tissue 300 µl of DNAzol® reagent was added and incubated at 25 °C with shaking for 5 min After 5 min 300 µl of chloroform was added to the above mixture and vortexed for few sec The mixture in the eppendorf was further incubated for 5 min at 25 °C, which was later centrifuged for 10 min at 12,000 rpm The clear supernatant was transferred to the fresh tubes and to it two volumes of 100% ethanol was added The samples were mixed well by inverting the tubes for 6 to 8 times and were stored at room temperature for 5 min After incubation the samples were centrifuged at 5,000

rpm for 4 min. The supernatant was discarded and to the pellet 300 µl DNAzol® + ethanol mix was added to precipitate DNA and further vortexed and stored at room temperature for 5 min. The samples were centrifuged for 4 min. The supernatant was discarded and the pellet was washed with 70% ethanol and the samples were spinned at 5,000 rpm for 4 min. The ethanol solution was discarded and the pellet was air or vacuum dried and dissolved in 70 µl of TE.

3.4.2.2 PCR analysis of putative transformants

Putative transformants were screened by PCR (polymerase chain reaction) for the presence of *npt*II, *uid*A and *RChit* genes in plants transformed with plasmid pRT99GUS:RChit vector.

The 700 bp region of *npt*II and 1.2 kb fragment of *uid*A genes was amplified by using 22-mer oligonucleotide primers (Hamill *et al.*, 1991).

Upper primer I: 5^1-GAG GCT ATT CGG CTA TGA CTG -3^1

Lower primer II: 5^1-ATG GGG AGC GGC GAT ACC GTA - 3^1

*uid*A gene of 1.2 kb region was amplified using oligonucleotide primers (*gus I gusII*),

Upper primer I: 5^1 - GGT GGG AAA GCG CGT TAC AAG -3^1

Lower primer II: 5^1 - GTT TAC GCG TTG CTT CCG CCA -3^1

RChit gene of 525 bp region was amplified using 21-mer oligonucleotides primers, designed in Genetic transformation laboratory, ICRISAT.

Upper primer I 5^1- TCT GCC CCA ACT GCC TCT GCT -3^1

Lower primer II 5^1- CCC CGC GGC CGT AGT TGT AGT - 3^1

PCR reaction was performed with 50 µl of a total reaction mixture containing 25 ng of genomic DNA, 5 µl of 10X PCR buffer (- $MgCl_2$), 1 5 µl of 50 µM $MgCl_2$, 1 µl of 10 mM dNTP mix, 1 µl of 10 µM Primer I, 1 µl of 10 µM Primer II, and 0 25 µl of 1 25 units of Taq DNA polymerase The total volume was made upto 50 µl with sterile distil H_2O The control without template DNA was used in each set of reactions with each primer Using TECHNE PHC3 thermocycler programmed for 32 cycles performed the amplification reaction

Amplification reactions were carried out by using eppendorf thermal cycler using the following conditions denaturation at 94 °C for 60 sec, annealing at 59 °C (*uidA*), 58 5 °C (*nptII*), 63 °C (*RChit*), for 45 sec, extension at 72 °C for 90 sec for 32 cycles and final extension at 72 °C for 4 min (one cycle). The amplified products were assayed by electrophoresis on 1 2 % agarose gel.

3.4.2.3 Southern blot analysis

Steps involved in Southern blotting are Digestion of the genomic DNA with an appropriate restriction enzyme, Gel electrophoresis of the restriction fragments, Transfer of the DNA to a nylon or nitrocellulose membrane, Hybridization of the Southern blot with labeled probe, and autoradiography to detect the signal

Materials

- Denaturation buffer 1 5 M NaCl and 0 5 M NaOH
- Neutralization buffer 1 5 M NaCl and 0 5 M Tris, pH 7 5
- 20X SSC NaCl→175 3 gm/L and Na Citrate→88 2 gm/L
- 250 mM HCL
- Blotting tray
- 3M Whatman paper
- Nylon membrane, Hybond-N^+ membrane (Pharmacia)
- UV cross linker (Pharmacia)
- Paper towels
- Parafilm®
- 500 gm weight
- Pasture pipette

Digestion of genomic DNA with restriction enzymes

Genomic DNA from putative transformants transformed with the plasmid pRT99GUS RChit was digested with enzyme *Hind*III which produces double cut within the plasmid to release the 1 2 kb *RChit* gene, and enzyme *Xba*I was used to restrict the genomic DNA which produces single restriction site with in the plasmid to know the copy number of the inserted gene

Genomic DNA	10 to 15 µg
10 X restriction buffer	5 µl
Enzyme	2 5 µl
Sterile distil H_2O	7 5 µl
The entire set up was for	50 µl

Gel electrophoresis of the restricted fragments

Genomic DNA of 10 to 15 μg was extracted from the putative transformed plants as described above was digested and restricted fragments were electrophoretically separated on 0.8% agarose gel casted in 1X TAE and electrophoresis was performed at 40 volts for 3 to 4 hr.

Transfer of the DNA to a nylon or nitrocellulose membrane

Restriction of the genomic DNA was carried out overnight by placing the samples at recommended temperature (preferably at 37 °C). Untransformed genomic DNA was taken as control sample. The agarose gel, resolving the restricted DNA fragments, was placed in a plastic tray and depurinated in 250 mM HCl with gentle shaking at 10 to 20 rpm for 15 min. Later HCl was drained off and the gel was rinsed with distilled water and denaturated in solution consisting of 1.5 M NaCl and 0.5 M NaOH for 15 min with gentle shaking. The above denaturation step was repeated with fresh solution for another 15 min. Denaturation solution was drained off and the gel was gently rinsed twice with distill water and the gel was placed in neutralization solution (1.5 M NaCl and 0.5 M Tris, pH 7.5) for 15 min and the neutralization step was repeated again with fresh solution for another 15 min. Nylon membrane and 3 sheets of 3M Whatman filter paper were cut to the exact size of the gel to be transferred. The nylon membrane was soaked in 20X SSC. A blotting tray was taken and a glass plate was placed on the blotting tray. A filter paper was placed in such a way that both ends of the filter paper wick were immersed into the 20X SSC placed in blotting tray. A

sheet of 3M Whatman filter paper of gel size was placed onto the blotting paper. The gel was placed carefully on top of the filter paper starting from one side and with the well side facing down with two additional sheets of filter papers. On this, pre-soaked nylon membrane was placed on the gel with marked side facing the gel. Air bubbles from the gel were removed by gently rolling a glass pipette over the gel and a sheet of Whatman paper cut to the size of the gel was placed over the gel and the area all around the gel was covered with Parafilm® to prevent contact of the top paper towels with the wick. A stack of paper towels was placed on top of the Whatman paper placed over the gel and a weight of 500 gm was kept on top of the papers. The blotting was performed overnight for complete capillary action. Later the blot was dried at room temperature for 10 min and the DNA was cross-linked by placing the blot DNA side down on a UV transilluminator for 3 to 6 min.

DNA hybridization with non-radioactive probes

The hybridization was carried out by using the commercially available Alkphos Direct labelling and Detection System (Pharmacia), a non-radioactive electrochemiluminiscent system. The probe was labeled with alkaline phosphatase enzyme that reacts with the added substrate, CDP-Star™ (Pharmacia) and emits photons in the form of signals that can be identified on an X-ray film.

Temperature in the hybridization oven was set at 55 °C. The Blot was placed in the hybridization bottle. Pre-heated pre-hybridization solution was added to the blot and kept for hybridization. Probe DNA of 10 µl was taken in an eppendorf tube and heated at 100 °C by placing the sample in boiling water bath for 5 min and immediately the

sample was placed on ice for another 5 min to denature the DNA The sample was briefly spinned down prior to use To this denatured DNA, 10 µl of reaction buffer, 10 µl of cross linker (2 µl of cross linker added to 8 µl of distilled water), 2 µl of enzyme labeling reagent was added and a brief spin was given Subsequently the sample was placed in water bath set at 37 °C for 30 min The blots were probed with non-radioactive labelled (Alkphos Direct labelling and Detection System, Pharmacia), PCR amplicons

The above-labeled solution was added to the pre-hybridization buffer (Appendix 8) present in the hybridization bottles without touching the membrane Hybridization was performed overnight at 55 °C After overnight hybridization, the used probe was discarded and primary wash (Appendix 9) was given at the same temperature for 10 min and this step was repeated for another 10 min with fresh primary wash buffer Later secondary wash was given for 5 min at room temperature and at this step, the blot can be placed for 30 min in secondary wash buffer (Appendix 10) prior to autoradiography

Autoradiography and X-ray film development

Drops of chemiluminescent detection (CDP-Star™) was added on the blot and excess solution of CDP-Star™ was drained off by touching the tip of the blot on the tissue paper where excess droplets can be soaked Blot was placed in a saran wrap and an X-ray film was placed over it in an exposure film cassette in a dark room Initially the film was exposed after 45 min of placement For intensification, the blot was placed for another 4 hr

X-ray film development

X-ray film was removed from the cassette and was placed in a tray containing the X-ray Kodak GBX developer for 60 to 120 sec. Later the film was transferred to another tray containing water and rinsed for 30 sec. The film was further placed in a tray containing the Kodak GBX fixer and the film was incubated for 60 to 120 sec. The film was rinsed with water for 2 min followed by air-drying.

3.5 RT-PCR analysis

Reverse transcription followed by the polymerase chain reaction (RT-PCR) leading to the amplification of specific RNA sequences in cDNA form, is a sensitive means for detecting RNA molecules, a means for obtaining material for sequence determination, and a step in cloning a cDNA copy of the RNA. Various strategies that can be adopted for first strand cDNA synthesis are the reverse transcriptase reaction that can be primed by the down stream PCR, primer annealed to the RNA or by random hexamers or by an oligo dT primer at the polyA tail of mRNA (Kawasaki, 1990).

RT-PCR analysis of the putative transformants growing in the glasshouse was carried out using the Thermoscript RT-PCR system (Pharmacia). RT-PCR analysis was carried out on 18 independent transformed plants of T_0 generation (P1 to P18). Total RNA from the putative transformants was isolated (Appendix 11) using TRIzol reagent

(Invitrogen) according to the manufacturer's protocol and the amplified products were separated on 1.2% agarose gel .

3.6 Inheritance of transgenes in T_0 and T_1 generation

T_O plants were selfed to get T_1 progeny. A total of 40 seeds, 5 seeds from each primary independent transformed plant (P1, P2, P3, P5, P6, P7, P8, P13) were sown for further inheritance studies in T_1 generation (a total of 40 T_1 lines were taken). Five T_1 lines each (P1→1-4, P2→1-4, P3→1-4, P5→1-4, P6→1-4, P7→1-4, P8→1-4, P13→1-4), of 8 independently transformed T_0 plants corresponding to T_1 generation obtained through microprojectile bombardment were tested. Inheritance studies was carried out based on PCR analysis for the presence of *uid*A gene in plants transformed with the plasmid pRT99GUS:RChit. PCR positive plants were further tested for Southern blot analysis. Self-fertilized progeny of T_1 plants were germinated and raised to maturity to get T_2 progeny. Four seeds of 2 independent transformed plants of T_1 generation (P1and P8) were sown and the replicates (P1→1→1-4, P8→1→1-4) were tested for segregation studies, which were carried out based on the PCR positive results and analysis of transgene integration was further confirmed through PCR analysis for *uid*A, *npt*II and *RChit* genes. For Southern analysis of *RChit* gene, 16 lines (P1→1→1-4, P8→1→1-4, P9→1→1-4, P10→1→1-4) of 4 independent transgenic plants (P1, P8, P9 and P10) representing the T_2 generation were tested.

3.7 Statistical analysis

All the experiments were replicated thrice and data was scored and analyzed based on mean and SE values (Standard error).

4. RESULTS

4. RESULTS

A highly efficient regeneration protocol is a pre-requisite for the development of transgenic plants In the process of optimizing such a regeneration protocol in pigeonpea, various factors for in vitro culture were successfully optimized to develop a shoot regeneration protocol that is reproducible and efficient These include genotype effect, role of growth regulators, culture medium, and other physical factors that were dealt in detail to optimize the regeneration protocol Most efficient response was obtained from the petiolar region of the leaf explant It was amenable for microprojectile-mediated gene transfer for the production of a large number of transgenic pigeonpea plants over relatively short periods of time The bombarded explants were grown to maturity and the shoots were grown on the culture media with selection to ensure their transgenic nature The putative transformants were grown till maturity and the later generations raised (T_0, T_1 and T_2 generations) were further confirmed through PCR, Southern blot analysis and RT-PCR

4.1 Tissue culture and Plant regeneration

The pathway for shoot regeneration in the present study was through organogenesis Leaf explant from 4 to 5-d-old in vitro grown seedlings of pigeonpea variety ICPL 88039 was taken for all the experiments The explants were cultured on MS basal medium supplemented with 5 µM BA + 5 µM kinetin (SIM) by placing their abaxial surfaces in contact with the medium (Fig 1A) After 5 d of culture, swelling of

the petiole was observed (Fig 1B) and small protuberances emerged out of the petiolar region after 7 d (Fig 1C) Initiation of the shoot buds was restricted towards the proximal cut end of the petiole With the onset of shoot bud initiation at 7 d, half of the distal portion of the leaf lamina was removed and further cultured on the shoot development medium (SDM) consisting of 1/2 strength SIM ie, MS basal medium supplemented with 2 5 μM BA + 2 5 μM kinetin (Fig 1D) Formation of multiple shoots was observed after 14 d of culture (Fig 1E) Prolonged culturing of the explants on SDM led to the formation of multiple shoots after 21 d (Fig 1F) Therefore, the explants of 21 d with multiple shoots were cultured on MS basal medium fortified with 0 58 μM GA_3 After 3 d of culture, shoots elongated upto 1 cm (Fig 2A) Multiple shoots elongated rapidly after 7 d of culture on SEM (Fig 2B) These shoots were separated and placed individually on MS basal medium containing 1% sucrose for rooting Adventitious root formation was observed in the pulse treated (pulse treatment was given to the cut shoots, which were directly dipped into the IAA (11 42 μM) solution for 30 to 60 sec) elongated shoots (Fig 2C) The rooted explants were transferred to pots containing sand for their hardening and transplantation to the glasshouse

Over 90% of the rooted plants were transferred to the pots containing autoclaved sand and soil in 1 1 ratio for hardening The rooted plants covered with polythene bags (to overcome the excessive loss of moisture due to transpiration and to maintain high humid conditions) acclimatized upon transfer to pots resulting in the formation of well-developed shoots and leaves with a survival rate of over

95% (Fig 5J) The regenerated plants were similar phenotypically to those of normal pigeonpea plants and the time taken for the complete regeneration of the plants under in vitro conditions was 45 to 50 days Following this protocol over 200 plants were produced that in turn produced fertile seeds (Fig 5K)

Sequential events of shoot regeneration were summarized in Fig 3A-K

The results of specific media constituents are as follows

Role of phytohormones

Effect of TDZ and IAA on shoot bud induction

The use of TDZ for shoot induction was earlier reported in pigeonpea (Eapen and George, 1993, Eapen *et al.*, 1998, Thu *et al.*, 2003, Dolendro *et al.*, 2003) In the present study, efficacy of TDZ in combination with IAA on shoot bud induction was studied by using the leaf explants Leaves from 2, 3, 4, 6 and 8-d-old of in vitro raised aseptic seedlings were taken as explants Various concentrations of TDZ (9 0, 9 08 and 11 35 μM) in combination with IAA (0 57 and 1 14 μM) were tested However, the explants failed to respond to shoot bud induction when cultured on TI, TI-1 and TI-2 media consisting of the above concentrations of TDZ and IAA Instead the explants turned pale yellow (Table 1)

Effect of BA and NAA on shoot bud induction

Leaves from 4, 5, 6, 7, 9, 11, 13 and 15-d-old in vitro raised seedlings were taken as explants and tested on various concentrations of BA in combination with NAA for shoot induction. The explants cultured on BN2, BN3 and BN4 media, enlarged in

size, but no shoot formation was observed Instead, callus formation was observed from 3 wk onwards Two types of calluses were observed, a) light green and friable, b) dark green, hard and compact A combination of 4 44 μM BA and 0 54 μM NAA (BN1) resulted in the shoot bud formation in 2 explants out of 24 explants cultured through intervening callus phase (Table 2)

Effect of BA, kinetin and 2-iP on shoot bud induction

Earlier reports on shoot regeneration suggest that a combination of two cytokinins was required for the regeneration of shoot buds from leaf explants Hence, various combinations of cytokinins such as kinetin and BA and kinetin with 2-iP were tested to induce shoot bud differentiation from leaf explants of pigeonpea In general, BA along with kinetin was found to be more efficient in inducing multiple shoot buds when compared to the combinations of kinetin and 2-iP where only single shoots were formed at low frequencies A combination of 5 0 μM BA and 5 0 μM kinetin was found to be the best for shoot bud induction medium (Table 3)

Effect of explant on shoot regeneration

Various explants such as epicotyl, hypocotyl, embryonal axis, leaf lamina, leaf with half-cut lamina and leaf with intact lamina and petiole was cultured on SIM The developmental pathway of each explant was observed after one week of culture Only callus formation was noticed after 2 wk of culture from epicotyls, hypocotyls and embryonal axes explants Five explants out of a total of 24 with half lamina showed the formation of shoots from intervening callus, after 6 wk, while the remaining explants developed only callus Leaf with intact lamina and petiole expressed a high frequency

of totipotency resulting in the formation of shoot buds which were restricted to the proximal cut end of the petiole in 95% of explants (Table 4) Basal on these observations, leaf explants were considered as ideal for obtaining adventitious shoot buds in pigeonpea

Effect of lamina tissue on regeneration from leaf explants

In studies on role of the lamina tissue in regeneration from the petiolar cut end of the leaf explant, it was found that the leaf explant containing intact lamina was essential for regeneration response while the shoot bud induction declined with reduced lamina tissue Hence, whole leaf explants from 4 to 5-d-old seedlings were used for all the subsequent experiments (Table 6)

Effect of age of the explant donor seedlings on shoot regeneration

Effect of age of the explant donor seedlings was determined to study the effect on shoot regeneration In general, younger seedlings (5 d) provided explants that were highly regenerative while the regeneration potential declined with the age thereafter Leaf explants from 4 to 5-d-old seedlings exhibited the highest frequency of multiple shoot regeneration where over 90% of the explants responded (Fig. 4)

Effect of orientation of the explant on culture medium

The orientation of the leaf explant played a vital role in shoot bud formation Culturing the leaf explant with the abaxial side in contact with the medium promoted the initiation of shoot buds During shoot formation, the margins of the petiole of leaf explant expanded towards the adaxial side resulting in lifting of the cut end of the

petiole away from the medium leading to the drying of the explant as well as hindering further growth of the shoot buds Physical contact of the petiole with the culture medium was essential to elicit a complete response Therefore, constant observation of the cultures was done to overcome the above problem

Effect of genotype on shoot regeneration

To study the effect of genotype on shoot regeneration potential by using the optimized regeneration protocol, 11 genotypes of pigeonpea belonging to different maturity groups (extra short, short and medium duration types) were tested In general, there was no significant variation amongst the genotypes belonging to different maturity types Moreover, the frequency of explants producing shoot buds and the variation in regeneration frequencies between the maturity groups was as great as that within the maturity groups (Table 5)

Effect of GA_3 on shoot elongation

The shoot buds that developed on leaf explants on SIM failed to elongate when placed on the same medium Further, differentiation and formation of rosette shoots buds occurred when transferred to ½ strength SIM Hence various concentrations of GA_3 were tested to induce shoot elongation Higher concentrations of GA_3 ranging from 1 51 µM to 2 89 µM when added to MS medium resulted in the formation of weak and thin shoots with curved tips The control explants cultured on MS medium fortified with 2 5 µM BA and 2 5 µM kinetin resulted in rosette shoot buds However, rapid elongation of multiple shoots occurred on 0 58 µM GA_3 Hence the shoot elongation

medium was formulated by using MS with 0 58 μM GA_3 on which over 88% shoots underwent elongation within 3 to 5 days (Table 7)

Rooting of elongated shoots

Various concentrations of IBA (the rooting hormone) were tested for inducing adventitious roots on the elongated shoots The elongated shoots (>3 cm in length) were transferred to rooting medium consisting of IBA While a high concentration of IBA (2 95, 3 94 and 4 9 μM) proved to be detrimental, only 2% of shoots produced roots on medium containing 1 99 μM IBA (Table 8)

Therefore, IAA was tested for the induction of root on elongated shoots Of the various concentrations tested (1 14, 2 82, 3 42, 4 57, 5 71, 6 85 and 7 56, μM), pulse treatment given to the elongated shoots with 11 42 μM IAA resulted in maximum percentage of shoots producing roots Considering this concentration as optimum, pulse treatment (by placing the cut ends of the shoots in 11 42 μM IAA solution for 30 to 60 sec) was given to the freshly cut elongated shoots, which resulted in 52% of the shoots undergoing root formation in the first sub-culture Root initiation was observed within 6 d of the pulse treatment In the second sub-culture, the shoots already pulse treated were subjected to second pulse treatment that resulted in the rooting of additional 66% shoots By the end of the third sub-culture, above 87% of the elongated shoots had rooted The amount of phenols produced by the explant was very high which if neglected hindered further growth of the shoots Hence, the unrooted shoots were sub-cultured on to the fresh medium (RIM) for every 10 d (Table 9)

Effect of sucrose concentration on rooting

To study the effect of sucrose concentration on rooting, cultures were initiated with RIM supplemented with 1, 2 and 3% sucrose Maximum percentage of rooting (over 45 8%) was observed in MS basal medium supplemented with 1% sucrose (Table 10)

4.2 Effect of media sterilization method on shoot induction

Method used for sterilizing the culture media for regeneration from leaf explant greatly influenced the frequency of shoot regeneration stages that included induction, differentiation, elongation and rooting In each of the medium tested, filter sterilized phytohormones were nearly 3 times more effective than autoclaved phytohormones (Table 11)

4.3 Ontogeny of adventitious shoot differentiation from the petiolar region of leaf explants

Leaf explants from 4 to 5-d-old seedlings exhibited high frequency morphogenesis in over 90% of cultures and the shoot differentiation was restricted to the proximal cut end of the petiolar region of the leaf explant To study the morphological and differential development pathway, histological analysis of the explant undergoing morphogenesis was carried out to determine the developmental pathway of the competent cells capable of morphogenesis, and to confirm the adventitious nature of the regenerated shoots. For ontogeny studies, 4 to 5-d-old leaf

explants (0 d) cultured on shoot induction medium for different periods (2, 4, 6, 8, 10, 12 and 14 d) were taken for tissue processing Transverse section of 0 d explants showed epidermis, cortex and vascular parenchyma cells, which were isodiametric in nature (Fig 5A), and longitudinal section of the cut end of the petiole exhibited starch filled cells in the epidermal region (Fig 5B)

Two-day-old explants exhibited actively dividing cells in the cortical region (Fig 5C), followed by anticlinal and periclinal cell divisions in the dividing cells at the cut end (Fig 5D) The formation of starch grains was observed in the dividing cells at the petiolar cut end in 4-d-old explants (Fig 5E and 5F), whereas cells in the cortical region were highly starch filled after 2 d and a clear demarcation of dividing and non-dividing cells was observed in 6 d culture (Fig 5G) Sixth-day culture showed the formation of meristematic zones in the vascular parenchyma region (Fig 5H) and the formation of meristematic zones were observed in the peripheral cells of the cut end of the petiole of 8-d-old, leaf explant (Fig 5I) The formation of mitotic divisions was maximum in the epidermal and sub-epidermal region of the petiole (Fig 5J) Eventually, the cells in the meristematic zones started withering and the cells in the cortical region became highly vacuolated (Fig 5K) by 10 d The initiation of the leaf primordium was observed at the cut end of the petiolar region that was very prominent in 10-d-old explants (Fig 5L) Localized meristematic activity at the proximal end resulted in the formation of multiple nodular structures of 12-d-old explants (Fig 5M) which further resulted in the clear demarcation of the withering cells and cells getting collapsed in the meristematic nodular region (Fig 5N) The nodular structures resulting

from the meristematic activity proliferated and gave rise to well-defined shoot buds by 14 d of culture of the leaf explant in ½ strength SIM (Fig 5O) The shoot buds formed started differentiating and led to the formation of the leaf primordia, and the formation of the multiple shoots was visible after 14 d of culture (Fig 5P)

Based on the ontogeny studies, the multiple shoots differentiated from the proximal cut end Cells of the petiole were adventitious in origin and developed through organogenesis The cells underwent various developmental stages to give rise to shoot buds and leaf primordia Initially, the epidermal, cortical and vascular parenchyma cells of the petiolar region were filled with rich starch grains and highly cytoplasmic in nature During the developmental stages, the cells underwent mitotic divisions and the formation of shoot buds involved the consumption of starch that can be observed with the vacuolation of cortical, epidermal and vascular parenchyma cells and further led to the formation of the meristematic cells at the peripheral cells of the petiole The meristematic cells gave rise to the nodular structures at the cut end with subsequent culturing of the explants in the shoot induction medium During the formation of the shoot buds, the cells near the meristematic zones completely collapsed By 14 to 15 d of culture the formation of multiple shoot buds and leaf primordia resulted through the proliferation of shoot buds at the proximal cut end of the petiolar region of the leaf explant

4.4 Genetic transformation

After standardizing the various parameters for in vitro plant regeneration in pigeonpea, cut end of the leaf explant proved to be efficient in expressing a high regeneration frequency of over 90% Hence the leaf explant was used for producing transformants by using microprojectile bombardment

4.4.1 Effect of antibiotic selection (kanamycin) on shoot regeneration from untransformed control leaf explants cultured on shoot induction medium

Since the plant selection marker gene for kanamycin resistance is present in the bombarded construct, various concentrations of kanamycin were tested on shoot regeneration potential of unbombarded control explants Results were considered based on visual observations after 3 wk Explants cultured on 25 mg/L kanamycin did not exhibit any morphological change or hindrance to shoot bud formation The explants responded well to kanamycin selection upto 50 mg/L and expressed optimum regeneration frequency However, about 20% of the explants cultured on 75 and 100 mg/L of kanamycin expressed bleaching The explants subjected to higher concentrations of the antibiotic (125 to 150 mg/L) did not respond well and expressed a low regeneration frequency Therefore, kanamycin at 100 mg/L was considered as a stringent selection for all the subsequent experiments (Table 12)

4.4.2 Microprojectile bombardment for gene transfer

4.4.2.1 Microprojectile bombardment , Plant selection and regeneration

Plasmid pRT99GUS RChit consisting of *RChit* gene along with *uid*A and *npt*II genes was used for microprojectile-mediated gene transfer (Fig 6) Different parameters for microprojectile bombardment were used including different pressures of helium gas and different bombardment distances to find the most effective ones Later, the bombarded explants were cultured on selection medium containing kanamycin to recover and regenerate the putative transformants

Pressures, of 1100 and 1300 psi of helium gas used for bombarding the target cells were found to be effective since there was minimal damage to the tissue and the bombarded explants regenerated well on SIM In addition to this, the use of bombardment distances, of 6 and 8 cm resulted in similar response and there was no significant variation (Table 13)

After a recovery period of 14 d, the explants subjected initially to a low concentration of kanamycin (25 and 50 mg/L) for a week proved to be effective without effecting their regeneration potential (Table 14) Later, the explants were subjected to stringent selection with 100 mg/L kanamycin for 3 wk Multiple shoots developed from more than 80% of the explants However, bleaching was also observed in less than 25% of the shoots This observation suggested that in pigeonpea although kanamycin did not result in efficient visual selection of the transformants, it did play a selective role on the suppression of shoot bud induction from the untransformed cells of the explants

4.4.2.2 Elongation, rooting and acclimatization of transformants

As the shoot buds differentiated, the explants with proliferated shoot buds were cultured on SEM containing 50, 75 and 100 mg/L kanamycin The multiple shoots after attaining a length of 3 cm were transferred to RIM without any antibiotic selection However, prior to the transfer, the elongated shoots were pulse treated with 11 42 μM IAA Over 50 putative transformed plantlets produced through microprojectile bombardment were transferred to pots containing autoclaved sand covered with polythene bags This method helped the plant to overcome excessive loss of water through transpiration and addition of small amount of thiaram rescued the plant from fungal contamination during acclimatization Later, the plantlets were transferred to the potting mix containing autoclaved sand and soil in 1 1 ratio The plants could overcome hardening process and the survival rate was over 95%

4.5 Analysis of transgenics

4.5.1 Histochemical analysis

Initial screening of the putative transformants was carried out through GUS assay, which was performed on the explants that were subjected to bombardment Transient expression of GUS was studied after 48 hr of bombardment and stable expression was studied after 3 wk of bombardment The target tissue, the proximal cut end of the petiolar region of leaf explant, and leaf lamina was taken for in vivo GUS histochemical assay However, untransformed control plants expressed faint blue (Fig

8A), whereas the transformed explants expressed intense blue coloration (Fig 8B) Hence based on these results, GUS assay was not considered ideal for screening the putative transformants of pigeonpea

4.5.2 Molecular analysis of transgenics

By using the optimized transformation protocol based on microprojectile bombardment, 50 putative independent transgenic plants were transferred to the glasshouse (Fig 9A, B) Eighteen (P1 to P18) independently transformed plants in T_0 generation were analysed by PCR, Southern blot analysis, and RT-PCR PCR analysis was carried out for the amplification of coding regions of *uid*A (Fig 10A), *npt*II (Fig 10B) and *RChit* genes (Fig 11A) gene RT-PCR was carried out on all the 18 independent transformed plants (Fig 11B) Out of 18 plants, 17 of them showed the expression of *uid*A gene Out of 17 plants (P1 to P17) tested for the PCR analysis for *uid*A gene, 16 plants expressed the amplification of 1200 bp fragment of *uid*A gene and only 1 plant (P5) was found to be negative Eighteen plants (P1 to P18) were tested for the amplification of 700 bp *npt*II gene fragment and all the plants showed the amplification of the respective gene For the amplification of 525 bp fragment of *RChit* gene, 10 plants (P1, P2, P3, P6, P7, P8, P9, P10, P20 and P23) were tested and 9 plants showed the positive amplification of the gene and 1 plant (P7) was found to be negative Based on the above results, it was concluded that 90% of the plants of T_0 generation expressed the amplification of the expected size of the respective gene fragments. Fidelity of the amplified gene fragments was verified by subjecting the PCR gels to

Southern blotting which resulted in positive Southern hybridization with respective *uid*A (Fig 12A) and *RChit* gene (Fig 12B) probes Gene integration pattern in the nuclear genome of the putative transformed plant for *RChit* gene was verified through Southern blot analysis Out of the 11 independent transformed plants of T_0 tested (P16, P17, P18, P19, P20, P23, P24, P25, P26 and P27), 6 plants (P16, P17, P18, P20, P23 and P26) expressed the presence of the *RChit* gene Out of 6 plants, 2 plants (P17 and P23) showed single copy inserts, while rest of the plants showed double inserts (Fig 13A) Further Southern blot analysis of *npt*II gene confirmed the transgenic nature of 5 other plants (P1, P3, P4, P7 and P8) out of a total of 8 plants (P1 to P8) Plants (P1, P3 and P4) among the Southern (*npt*II) positives showed single gene inserts and the rest (P7 and P8) showed double inserts (Fig 13B) Eighteen positive T_0 plants (P1 to P18) were selected and tested for the expression of the inserted gene through RT-PCR of *uidA* gene Out of 18 plants, 17 plants were found to be positive Based on the results from PCR and Southern blot analysis, a high transformation efficiency of 50% was concluded

Of the 50 independently transformed plants of T_0 generation, 8 plants (P1, P2, P3, P5, P6, P7, P8 and P13) of T_0 generation were advanced to T_1 generation and later, to T_2 generation In order to study the Mendelian inheritance pattern in the T_1 progeny, 5 seeds of each T_1 lines (P1→1-5, P2→1-5, P3→1-5, P5→1-5, P6→1-5, P7→1-5, P8→1-5, P13→1-5) were sown and PCR analysis was performed on 40 T_1 plants for the amplification of *uid*A gene Of 40 plants, 25 plants (P1→1, 4, 5, P2→3, 4, 5, P3→1, 2, 3, 4, 5, P5→3, 5, P6→2, 3, 4, 5, P7→2, 4, 5, P8→1, 3, 4, 5, P13→1) were PCR

positives expressing the 1 2 kb of *uid*A gene fragment (Fig 14A) gene following the Mendelian ratio of 3 1 (Table 15) The inheritance of the *RChit* gene in T_1 generation was analyzed on randomly selected T_1 PCR positive plants through Southern blot hybridization (Fig 14B) and the following (16) plants were analysed for Southern blotting of *RChit* gene ((P1→1, 4, P3→1, 3, P5→1, 3, 4, P6→2, 4, P7→2, 3, 4, P8→3, 4, P13→2, 4), in which 3 plants (P1→4, P8→2, 4) expressed the *RChit* gene integration in T_1 generation plants tested for the same

The PCR positive T_1 plants (from the progeny of P1 and P8, i e., P1→1-4, P8→1-4) were further processed for T_2 generation Four seeds of each T_1 plant (P1→1-4, P8→1-4) were sown for further study of T_2 generation Of 8 T_2 lines of 2 independent transformants of T_0, 7 plants (P1→1→1-4, P8→1→1-3) showed the amplification of *uid*A (Table 16) gene fragment (Fig 15B) and 7 plants were tested positive (P1→1→2-4, P8→1→1-4) for *npt*II gene (Fig 15A), which was further confirmed through Southern blot analysis of PCR amplicons (Fig 16A)

PCR amplification of *RChit* gene in 16 T_2 plants (P1→1→1-4, P8→1→1-4, P9→1→1-4, P10→1→1-4) resulted in the positive amplification of *RChit* gene in only three plants (P8→1→1, P9→1→1, 2) produced from 2 independent transgenic lines (Fig. 15C) Because of the high stringency in the annealing temperature (63 °C) for the PCR analysis, the % of positive plants was found to be less. Ultimately, 16 T_2 plants belonging to 4 (P1, P8, P9 and P10) independent transgenic plants of T_0 generation of 4 replicates each (P1→1→1-4, P8→1→1-4, P9→1→1-4, P10→1→1-4) were taken up

for the Southern blot analysis of *RChit* gene Out of 16 lines, 8 plants (P1→1→1, 2, 4, P8→1→1, 2, P9→1→1, 4, P10→1→1) were found to be positives indicating the presence of *RChit* gene and thereby inheritance of the gene from T_o and T_1 generation (Fig 16B)

The results of the molecular analysis of the transformants produced through microprojectile-mediated gene transfer method are clearly depicted in Table 17 All the transgenic plants thus showed the Mendelian inheritance (3 1) of the introduced genes in the T_1 and T_2 generation These plants also exhibited stable gene expression based on RT-PCR analysis of the respective genes

5. DISCUSSION

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5.1 Tissue culture of pigeonpea

One of the pre-requisites for successful gene transfer to plants is the availability of suitable protocols for plant regeneration and genetic transformation In vitro plant regeneration protocols in pigeonpea have been reported from hypocotyl (ShamaRao and Narayanswamy, 1975), cotyledons (Mehta and Mohan Ram, 1980), epicotyl (Kumar *et al*, 1984), leaf (Eapen and George, 1993, Ramesh and Baldev, 1994, George and Eapen, 1994, 1998, Geetha *et al*, 1998), distal halves of the cotyledons (Patel *et al*, 1994), cotyledonary node (Shiva Prakash *et al*, 1994, Geetha *et al*, 1998), distal cotyledonary segments (Mohan and Krishnamurthy, 1998), shoot tips (Geetha *et al*, 1999), and through somatic embryogenesis from cotyledons and leaf explants (Sreenivasu *et al*, 1998) ShamaRao and Narayanswamy (1975) reported that the hypocotyl segments obtained from the seeds irradiated with gamma rays produced profuse callus that regenerated shoot buds and plantlets in 50% of the cultures, while shoot regeneration was not observed in seeds, that were not irradiated, or in seeds which were exposed to higher dosage of gamma rays Kumar *et al*, (1990) reported a low frequency of shoot formation from callus initiated from leaf explants Patel *et al*, (1994) reported regeneration through somatic embryogenesis from cotyledonary explants in which the induction, maturation and germination of somatic embryos took 45 to 50 d George and Eapen (1994), observed organogenesis via callus from the primary leaves and cotyledons, where only 36% of the callus cultures regenerated shoot buds Further somatic embryogenic regeneration from diverse

explants such as immature embryonal axes, stem and roots was observed, but the somatic embryos developed from these explants failed to produce shoots Eapen and George (1993) reported high frequency shoot bud formation directly from leaf explants, which were initiated after 20 to 25 d and subsequent formation of shoot buds resulted after 45 d Shiva Prakash *et al.*, (1994) observed the formation of multiple shoot buds from cotyledonary node However, these shoot buds failed to elongate Eapen *et al.*, (1998) reported the formation of shoot buds after 45 d of induction of primary leaf segments cultured on media supplemented with TDZ In the cotyledonary segments, although the shoot bud formation was observed in 88% of the explants, only 18% of them developed shoots (Mohan and Krishnamurthy, 1998) It is observed that in pigeonpea, regeneration from explants other than cotyledons occurred at low frequencies (ShamaRao and Narayanswamy 1975, Kumar *et al.*, 1984, George and Eapen, 1994, Shiva Prakash *et al.*, 1994, Geetha *et al.*, 1998) However, in all the above neither the presence of the embryonic axis stimulated the formation of buds on cotyledons nor the axillary meristems proliferated, producing multiple shoots. In the present study however, an efficient system for the regeneration of multiple shoots was observed in pigeonpea where over 90% of cultures underwent differentiation of adventitious shoot buds from the petiolar cut end of the leaf explants that are devoid of any pre-existing meristems This was confirmed in our studies on the ontogeny of shoot bud histogenesis from the petiolar cut end By understanding the events associated with the formation of shoot bud, histogenesis can provide important clues into the ontogeny of shoot formation Ontogeny studies showed the adventitious nature of the shoot buds

by revealing the pattern of the formation of actively dividing cells that led to the formation of distinct meristemoids, at the proximal cut end of the petiole. These meristemoids progressively developed into shoot apices, each with an apical meristem in the center and leaf primordia surrounding it. Similar observations were found in the ontogeny studies of *Echinocloa* (Samantaray *et al.*, 1995). Histological sections in the present study revealed that shoot bud differentiation took place quite early, which was similar to that of the shoot development in jute (Saha *et al.*, 1999). Ontogeny studies of the present pigeonpea revealed the presence of epidermal cells, parenchyma, vascular elements and meristematic cells. Similar observations have been earlier reported in *Populus* (McCown *et al.*, 1988) and *Pinus* (Aitken-Christie *et al.*, 1984). Observations during the ontogeny studies of *Eucalyptus* (Herve *et al.*, 2001), revealed that protuberances originated from the cambial cells of the leaf's vascular system suggesting that the regeneration occurred from the adventitious shoot buds rather than pre-existing meristematic cells. This observation agrees with the present study that confirmed the regenerated shoots had an adventitious origin the present study also emphasizes the epidermal or sub-epidermal origin of the adventitious buds which originated from the peripheral layers of leaf-derived protuberances where, the leaf-derived protuberance formation involved the leaf's vascular system. The overall adventitious shoot formation in *Crinum* was similar to the present studies in pigeonpea where shoot formation was not preceded by callus, but developed adventitiously from the floral stem epidermis (Slabbert, 1995).

Regeneration potential is affected by explant origin and culture maintenance conditions (Pierik, 1987) and age of the explant (Welander, 1988, Sharma *et al.*, 1990a) The physiological age of the explant affects shoot regeneration and determines the morphogenic pathways of adventitious shoot formation (Saini *et al.*, 2002) The juvenile explants from in vitro-raised seedlings often have a better regenerability than explants derived from mature tissue (Thorpe, 1993, Harding *et al.*, 1996) and younger tissue has been found to more responsive in tissue culture than the older tissue (Brown and Thorpe, 1986) In the present study age of the donor seedlings greatly influenced the differentiation of shoot buds from the petiolar region of the leaf explants where younger leaves from 4 to 5-d-old seedlings exhibited a greater regeneration potential that declined with the increasing age of the explant donor seedlings. Similar observations were recorded in the cotyledonary explants of *Brassica juncea* (Sharma *et al.*, 1989, Sharma *et al.*, 1990a) Younger leaves of cowpea were reported to regenerate significantly with larger average number of shoots per explant than the older leaves (Van Eck and Kitto, 1992). In oat, age of the seedlings was found to be an important parameter for regeneration from the leaf base segment (Gless *et al.*, 1998) The age of the cultured explants was found to be the crucial factor in affecting the regeneration ability of geranium (Chang *et al.*, 1996) and Chinese cabbage (Choi *et al.*, 1996) Shoot organogenesis was observed from cotyledons of immature raised seedlings of *Medicago trunculata* (Trieu and Harrison, 1996) Immature cotyledons have found to be more responsive in multiple shoot bud formation in pea (Grant *et al.*, 1995) Immature zygotic cotyledons of soybean exhibited higher embryogenic potential than the mature

cotyledons (Yan *et al.*, 2000) Young purple red laminate explants provided better nodular callus formation than the older or larger ones (Te-Chato and Lim, 1999).

Age dependent variation in in vitro responses was linked to difference in endogenous auxin levels (Cassels *et al.*, 1982) or endogenous cytokinin levels (Josephina *et al.*, 1990) Such differences have been ascribed to the interaction of endogenous levels of auxins and cytokinins in the explants and those applied exogenously (Niederwieser and Van Staden, 1990) Further, Eapen and George (1993), Eapen *et al.*, (1988), Geetha *et al.*, (1998), reported a higher regeneration from primary leaves The number of regenerated shoot buds from cotyledonary explants generally decreased as the seedlings aged in Douglas fir (Goldfarb *et al.*, 1991) Young immature embryos exhibited a higher regeneration frequency in case of sunflower (Hunold *et al.*, 1995) While a similar response was also observed in monocots such as *Alstroemeria* in which the younger stalk cultures gave maximum response (Lin *et al.*, 1998) Transformation and regeneration rates were significantly reduced using older epicotyl segments of Washington navel orange (Bond and Roose, 1998)

Explants excised from 2-d-old seedlings showed higher transient transformation rates than those excised from 4 or 6-d-old seedlings This substantiates the earlier finding that younger explants are more susceptible to *Agrobacterium* than older explants (Jaiwal *et al.*, 2001)

One of the important features of regeneration is a polarized regeneration response where some of the tissues of an explant have a greater regeneration potential. Similar observations were made on the leaf explants of wild pear (Caboni *et al.*, 1999),

leaves of cowpea (Muthukumar *et al*, 1995), cotyledonary petioles of *Brassica juncea* (Sharma *et al*, 1990b, 1991), leaf explants of *Piper columbrium* (Kelkar and Krishnamurthy, 1998), cotyledons of peanut (Sharma and Anjaiah, 2000) and leaf explants of *Paulownia* sp (Kumar *et al*, 1998) In the present study, regeneration was restricted to the proximal cut end of the petiolar tissues with an increasing gradient in the morphogenic ability from the leaf tip towards the base of the leaf This might be due to the increased density of the vascular bundles present in the proximal region of the leaf that could also play a role in inducing the higher morphogenic response Such polarity in the formation of shoots from the petiolar region of leaf explants was also observed in carrot petiole segments (Ammirato, 1985), petiole explants of *Vitis* (Cheng, 1989), petiole explants of papaya (Hossain *et al*, 1993) and the petioles of the leaf explants of peanut (Kanyand *et al*, 1994, Sharma and Anjaiah, 2000) With respect to the differentiation of shoot buds from leaf lamina, they were polarized at the cut end of the leaf explants of peanut (George and Eapen, 1994), while a higher morphogenesis was detected towards the petiole rather than at the apex area in apple (Yepes and Aldwinkle, 1994) Polarity appeared to be a factor in case of Japanese pear leaf explants, because regeneration occurred at the same position on most of the leaves regenerated Cutting the leaf explants may also alter the polarity of regeneration from intact leaves (Lane *et al*, 1998) The callus derived from the leaf base had a greater embryogenic potential than that derived the leaf-tip in *Echinochloa* (Samantaray and Das, 1997) Basal portions of the shoot tip explants of cocoyam were more responsive compared to

the shoot tip (Nyochemberg and Garton, 1998). Leaf explants from the terminal part of the in vitro derived shoots were more responsive and produced more shoots than explants from the basal part of the shoot (Economou and Maloupa, 1995).

Leaf segments without petiole or petiole segments alone did not form adventitious shoots in suggesting the existence of a petiole-derived endogenous substance (s), whose distribution and accumulation may be important for polar development of adventitious shoots (Akaracharanya *et al.*, 2001)

In the present study, leaf with intact lamina and petiole enhanced the formation of shoot buds in over 90% of the explants. Although the regeneration of shoots occurs from the petiolar cut end in leaf explants in the present study, both the tissues, viz., petiole and lamina play an important role in organogenic response and a direct correlation exists between the amount of laminar tissue and shoot regeneration response (Dayal *et al.*, 2003). This suggests the possible involvement of hormones and/or metabolites provided by the lamina tissue in expression of cellular totipotency of the petiolar tissue that may be provided by the lamina tissue (Sharma *et al.*, 1991; Kumar *et al.*, 1998).

The size of the cultured leaf explant played a vital role in the present study of pigeonpea, where higher percentage of regeneration occurred in cultured explants with full lamina and similar increased regeneration was obtained from the smaller sized explants viz: in soybean where smaller zygotic cotyledon explants were found to have a higher embryogenic potential (Yan *et al.*, 2000); the leaflets of peanut

(Baker and Wetzstein, 1992), the length of floral stems of *Crinum* (Slabbert *et al.*, 1995), the leaves of melon (Yadav *et al.*, 1996)

Composition of the culture medium with respect to phytohormone types and levels was found to be an important factor for in vitro responses Further, the growth regulators used during tissue culture and plant regeneration can have a lasting effect on plant performance (Bhat and Srinivasan, 2002) Cytokinins in general are required to induce shoot buds from cultured tissues (Thorpe, 1993) The auxin/cytokinin balance is one of the important factors that determine the pattern of morphogenesis from petiole explants in order to increase the number of vegetative shoots These combinations therefore may have encouraged the interactions that resulted in specific morphogenetic responses in the formation of adventitious shoot buds from petiolar region of leaf explants in the present study, which was similar to the observations found in African violet (Sunpui and Kanchanapoom, 2002) Shoot buds developed under the influence of TDZ, which was histologically proved in case of African violet (Mithila *et al.*, 2003) The increased regeneration in the cut mid rib portion of Japanese pear leaf explants was more than compared to the intact leaves, might be due to a change in the balance of endogenous growth regulators resulting from the wounding treatment (Lane *et al.*, 1998), implying the role of endogenous growth regulators in regeneration

Although, TDZ has been used effectively for shoot bud formation in leaf explants of pigeonpea (Eapen *et al.*, 1998), it proved to be toxic for the young immature pigeonpea leaves in the present study where the explants became necrotic A combination of BA and kinetin was however found to be beneficial for pigeonpea leaf

explants. Similar observations were found in *Picea omarika,* where a combination of BA and kinetin showed maximum meristematic activity in embryos and seedling shoot tips (Pletikapic and Buturovic'-Derick, 1995) and in elephant apple (Hossain *et al.,* 1994). Differences in the requirements of growth regulators might be due to the differences in the type of explants and in the cultivars used. BA was found to be a more effective cytokinin in inducing multiple shoot buds when compared to TDZ in Douglas-fir cotyledons (Goldfarb *et al.,* 1991). The effect of BA in promoting multiple shoot buds was highlighted in several pigeonpea shoot regeneration protocols (Mehta and Mohan Ram, 1980; Eapen and George, 1993; George and Eapen, 1994; Shiva Prakash *et al.,* 1994; Mohan and Krishnamurthy, 1998; Geetha *et al.,* 1998). BA has been reported to be more effective than other cytokinins in promoting maximum number of shoots in pea (Bean *et al.,* 1997), peanut (Cheng *et al.,* 1996), and *Vigna mungo* (Karthikeyan *et al.,* 1996). Further, the morphogenic response varied with different concentrations of BA in pearl millet in terms of multiple shoots or in vitro flowering (Devi *et al.,* 2000). The level of organogenic competence of the explants was defined by concentration of BA in *Piper columbrimum* (Kelkar and Krishnamurthy, 1998), where lower concentrations of BA of 5 μM was more effective without directly creating a stress on juvenile explants. BA was found to be very effective in enhancing shoot bud formation in leaf explant of pigeonpea in the present study where kinetin further supported the growth of the multiple shoot buds. Kinetin was found to be an effective cytokinin in inducing organogenesis in chickpea (Fontanna *et al.,* 1993). Regeneration was much quicker and frequency was high or because of the specific

combination of growth regulators, which resulted in high shoot organogenesis from leaf explants of cassava (Guohua, 1998) High frequency regeneration was obtained from leaves and petioles supplemented with plant growth regulators of BA and kinetin in African violet (Sunpui and Kanchanapoom, 2002) The use of BA and kinetin for long duration (3 to 4 wk) in the present study led to the formation of healthy shoots, which synchronously increased in number However, the cytokinin failed to initiate the elongation of shoots, resulting in rosette shoots

Besides the cytokinins and auxins, the other growth regulators such as GA_3 can also play a vital role in the production of shoots in vitro In the present studies, multiple shoot buds produced were treated with GA_3 for further development and elongation. GA_3 is known to have stimulatory effect on stem elongation in different plants (Phinney, 1984) In the present study, shoots elongated rapidly when transferred to MS basal medium supplemented with GA_3 A combination of BA and GA_3 induced optimum for the best shoot elongation and plant uniformity in pineapple (Escalona *et al.*, 1999) Similar effect of GA_3 has been observed earlier in pigeonpea (Geetha *et al.*, 1998), in castor (Sujatha and Reddy, 1998) and in *Phaseolus* (Mallik and Saxena, 1992). Shoots elongated well, when cultured on MS basal medium supplemented with GA_3 in *Aristolochia* (Manjula *et al.*, 1997). GA_3 is used for shoot elongation in pea (Sanago *et al.*, 1996) and in *Lonicera* (Palacios *et al.*, 2002). It has been suggested that GA_3 promotes cell division and elongation in the sub apical zone of the shoot primordium of leaf explant (Eapen and George, 1993). Shoot buds developed when exposed to cytokinins, and subsequent removal of cytokinin led to shoot

elongation, which was earlier, observed in Douglas fir cotyledons (Goldfarb *et al*, 1991) Addition of GA_3 to the transient expression medium enhanced the elongation and recovery of the shoots regenerating from the explants of *Helianthus* (Malone-Schoneberg *et al*, 1994)

Orientation of the explant on the culture medium can play an important role in plant regeneration (Sharma *et al*, 1990c) Orientation of the explant in the present study was found to be critical for obtaining higher regeneration from the leaf explant Placing the leaf explant with the abaxial side in contact with the medium and adaxial side faced up has promoted the initiation of shoot buds Similar observations were reported in the case of the cotyledonary node explants with 1 to 2 mm split embryonal axis of *Medicago trunculata* (Trieu and Harrison, 1996) and the leaf explants of *Alstroemeria* (Lin *et al*, 1998) Orientation of the floral stem explants of *Crinum* played an important role in the initiation of shoots, where most of the shoots could be regenerated, when the discs were placed with their basal ends touching the medium (Slabbert *et al*, 1995) Melon cotyledons were highly regenerative when cultured adaxially than when cultured abaxially (Yadav *et al*, 1996) Changing the orientation of the explants of *Vigna* from vertical upright position to horizontal position drastically decreased the frequency of shoot induction as well as the length of the shoot indicating that orientation appears to interact with polarity to affect shoot regeneration (Saini *et al*, 2002) Orientation of the leaf base segments had considerable influence on shoot bud development of *Echinocloa*, where a greater number of shoot buds developed on the adaxial side of the explant surface than on the abaxial side (Samantaray *et al*, 1995) Cocultivation of the

stem segments with *Agrobacterium tumefaciens* placed in a horizontal position proved to be critical for efficient transformation in *Brassica* (Kuvshinov *et al.*, 1999)

Genotype of the explant donors has been shown to play a major role in the regeneration of shoots in several species including legumes (Pederson, 1986, Chen *et al.*, 1987, Meijer and Brown, 1987, McKently *et al.*, 1989, 1991, Sellars *et al.*, 1990) Since the development of an ideal regeneration system should be genotype independent, the choice of a suitable genotype is critical in such studies (Jain *et al.*, 1988, Shoemaker and Counche, 1986) Shoot production in a particular explant donor explant genotype depends on the culture conditions and principally to the interaction between the genotype and the culture medium (Flores *et al.*, 1999) The plant regeneration protocol reported in the present study has been found to be applicable across a wide range of pigeonpea genotypes tested, which belonged to different maturity groups Among all the genotypes tested the genotype ICPL 88039 was found to respond better and hence was used in the present work Uniform observations were reported in all the three cultivars of gerbera in terms of efficient shoot bud regeneration with slight variation and therefore, the regeneration protocol used was genotype independent (Orlikowska *et al.*, 1999) Genotype independent protocols were reported in guineagrass (Chen *et al.*, 2002), in melon (Ficcadenti and Rotino, 1995, Yadav, 1996), in gypsophila (Ahroni *et al.*, 1997), and in *Vitis* (Torregrosa and Bouquet, 1996) Genotype dependent response was reported in all the varieties of onion (Luthar and Bohance, 1999)

Therefore, a highly efficient regeneration protocol has been optimized in the present study, which is genotype independent and the time involved in raising the

plantlets was less that can be further used for the production of transgenic plants with genes of agronomic importance

5.2 Genetic transformation of pigeonpea

The most important aspect of successful genetic transformation is the induction of a larger number of regenerable cells that are accessible to the gene transfer methods, and which continue to proliferate transformed cells under stringent selection conditions Further, the gene transfer into potentially regenerable cells may not allow recovery of transgenic plants if the capacity for efficient regeneration is short lived (Birch, 1997) Transformation efficiency is proportional to the efficiency of the tissue culture and gene transfer systems (Hiei *et al.*, 1994, Ishida *et al.*, 1996, Bower *et al.*, 1996, Li *et al.*, 1996) In the present study, an efficient regeneration and transformation protocol was optimized by using the petiole region of the leaf explant for high frequency production of multiple shoots at a rapid rate, from the bombarded (microprojectile bombardment) target cells, without any intervening callus phase

It is reported that the length of the callus phase is negatively correlated with regeneration ability, where the somaclonal variations can influence the phenotype of the regenerated shoots (Fontanna *et al.*, 1993) The rate of plant regeneration using the present protocol (>90%) is highly efficient and reproducible and is applicable to a wide range of pigeonpea genotypes tested

The cut end of petiole of leaf explant, site at which there is high potential for differentiation, was found to be an efficient target tissue amenable to microprojectile bombardment As the age of the explant played a vital role in the transformation, primary leaf explant of 4 to 5-d-old used for bombardment was proved to be efficient During this period, the cells at the proximal region of the petiole divided actively

Preliminary trials were carried out to determine the LD_{50} concentration of kanamycin for plant survival by culturing untransformed explants on shoot induction medium containing varying concentrations of kanamycin The explants could tolerate a concentration of 50 mg/L, but higher concentrations of 75 and 100 mg/L caused bleaching of plantlets Hence it was decided to employ a concentration of 100 mg/L kanamycin for the selection of transformed plants after microprojectile bombardment

With the onset of shoot bud formation, the explants were placed in the selection with a lower concentration of kanamycin Formation of the buds or the meristematic nodules would have been suppressed, if the explants were placed before the formation of the shoots Hence the 14 -d-old explants were more feasible for selection Therefore after microprojectile bombardment, a recovery period of 10 to 14 d was given so that the explants could recover from bombardment shock The use of dominant selectable marker is an integral part of a transformation strategy The sensitivity of plant cells to a selectable marker depends on the genotype, the physiological condition, size and type of the explant and tissue condition If organogenesis is the pathway for subsequent regeneration of transgenic plants, then the risk of producing chimeric plants exists (Kar *et al*, 1996) However, application of selection pressure in the initial shoot bud

formation leads to the suppressed growth of untransformed plants In an organogenic pathway there is a higher chance for escapes Avoiding the use of antibiotics during early stages of organogenesis seems to be important for quick and healthy growth of the shoots, since they interfere with development of transformed cells (James *et al.*, 1989, Michelmore *et al.*, 1987)

Concentration of the antibiotic selection also played a vital role in recovery of putative transformants Presently, a higher dose of selection in the initial stages of the shoot bud induction (from the explant) was detrimental This was also recorded in case of immature zygotic cotyledons of soybean (Yan *et al.*, 2000) Hence, the explants were subjected to lower concentrations of the antibiotic in the initial phase, and later subjected to a gradual increase in the selection concentration Similar selection regime was followed for pea transformation in which the concentration of the selection agent was gradually increased (Bean *et al.*, 1997), for groundnut transformation (Cheng *et al.*, 1996), *Vigna* transformation (Karthikeyan *et al.*, 1996) and in papaya transformation (Fitch *et al.*, 1990) In the present transformation protocol, great emphasis was laid on gentle selection regime in the initial stages, which was vital for the successful recovery of transformants and was further found to be crucial

The absence of selection in the RIM facilitated the development of healthy roots, which was found to be crucial This was similar to reports on other crops such as apple (James *et al.*, 1989), *Brassica* (Moloney *et al.*, 1989) and chickpea (Fontanna *et al.*, 1993)

In the present study, preculturing the explants prior to bombardment proved the transformation frequency similar to woody fruit plants, such as plum (Mante *et al.*, 1991) The number of putatively competent cells for transformation was greatly increased by a preculture treatment on a medium rich in auxins in *Arabidopsis thaliana* (Sangwan *et al.*, 1992) and in embryogenic suspension cultures of cassava (Watad *et al.*, 1998)

Leaf explants were found to be efficient for regeneration and transformation through microprojectile bombardment as in the case of tobacco (Horsch *et al.*, 1985), *Vigna* (Karthikeyan *et al.*, 1996), and groundnut (Cheng *et al.*, 1996 and Baker and Wetzstein, 1992)

In case of the bombarded explants, they could with stand substantial pressures of 1100 and 1300 psi and their regeneration ability was retained The target cells used in the present transformation procedure were highly regenerable, and were so chosen because direct transformation of totipotent tissues would minimize somaclonal variations (Sanford, 1990) Further, the bombardment distance of 6 and 8 cm did not impair the regeneration capacity of the bombarded explants Several workers maintained similar distances viz zygotic embryos of peanut (Schnall and Weissinger, 1993), and in bombarded shoot tip clumps of sorghum and pearl millet (Devi and Sticklen, 2001, 2002) A dramatic effect of the increase in number of blue spots was observed in the embyrogenic suspension cultures of cassava, when the optimal bombardment pressure (1100 psi) and the optimal particle size

(1 0 μm) were used (Schopke *et al.*, 1997) Distance of 6 cm resulted in highest transient expression levels of *uid*A gene in lily calli (Watad *et al.*, 1998)

Transient expression resulted was recorded in more than 90% of the bombarded explants Microprojectile bombardment negatively affects long-term cell survival and hence the number of stable transformation events Although, transient expression may be quite efficient (Hunold *et al.*, 1995, Devi and Sticklen, 2001, 2002), the choice of the explant, type and culture conditions were very important for high expression levels In case of microprojectile bombardment, a successful regeneration event requires that a cell not only survives the bombardment and stably integrates the foreign gene into its genome, but also is a part of cell population induced to regenerate into a whole plant In the present study with petiolar region of leaf explants, transient expression resulted in more than 90% of the bombarded explains But not only the bombarded explants, but also untransformed control explants were also stained blue Such endogenous activity has been reported in some species (Hu *et al.*, 1990) Though kanamycin was found to be a better selective agent in screening the putative transformants after microprojectile bombardment, it however did not allow the visual selection of the putative transformants, which was also observed in the case of groundnut transformants (Sharma and Anjaiah, 2000)

The stable transformants resulted through microprojectile bombardment was found to be 50% positive In Southern blot analysis of the stable transformants resulted (*RChit* gene T_0 Southern blot), out of 11 independent transformants 6 plants were found to be positive and all the plants except 2 plants had double insertions, which is more

common in direct gene transfer methods (Sanford, 1990) However most of the Southern positive *npt*II plants showed single inserts The hybridizing band therefore reflects the integration of the presence of the gene inserted in the genome and provides an estimate of the number of copies of the gene integrated The difference in the size of the bands also indicated that the events were independent and the integration was random In case of wheat the number of hybridizing bands indicating the presence of bar gene varied from 1 to 4 (Khanna and Daggard, 2003) and multiple copies of the gene insertion was observed in carrot (Hardegger and Sturn, 1998) Further, of the 6 putatively transformed plants, one plant contained high number of transgene copies in *Festuca* (Bettany *et al.*, 2003), one or two copies each in the genome of strawberry (Schaart *et al.*, 2002) and in mung bean (Jaiwal *et al.*, 2001) Most of the transformants expressed single gene copy inserts and only one plant showed two copies in pear (Gao *et al.*, 2002) and in *Phaseolus*, out of 10 plants 4 plants showed single copy inserts, whereas other plants expressed 2 or more (De clereq *et al.*, 2002) The positive signals obtained in the above analyses confirmed the integration of the transgene into the genome of all the regenerated plants The copy number of both the transgene and the rearranged fragments is often highly variable in plants transformed by microprojectile bombardment, similar to the observations in rose (Pawlowski and Somers, 1996) Transgene rearrangements are particularly common among the transgenics derived from biolistic delivery of DNA (Jorgenson *et al.*, 1996)

The transformation efficiency in the present study was 50% indicating that the microprojectile bombardment was an efficient gene transfer method, which

subsequently relies on the target tissue that is competent enough to combat the bombardment shock Even though limited numbers of plants were tested, the results suggest a Mendelian inheritance of the transgene which was evident in the T_1 and T_2 generations

Although many papers have been published on pigeonpea transformation, most of the regeneration protocols used in producing the transformants were not reproducible, either due to long callus phase and inefficiency of the explants to produce shoots at a higher frequency Geetha *et al.*, (1999) and Lawrence and Koundal (2001) have reported genetic transformation in pigeonpea The transformation efficiency reported by Lawrence and Koundal (2001) was only 1 2%, although the explants which were used by them produced calli, but out of 213 calli produced (23 8%) only 11 calli (1 2%) showed shoot bud initiation and 6 calli developed suggest the shoots indicating the poor efficiency of regeneration and transformation potential of the explants In case of transformation protocol put forward by Geetha *et al.*, (1999) only 3 plants were found to be positive In the present study, selection of explant has become an important criterion and as such regeneration resulted from the cut end of the petiole, without any callus phase Though the pathway for regeneration was through organogenesis, high stringent selection of the shoots led to the development of transgenics with above 50% efficiency and the transgenic plants showed positive gene integration events

Molecular analysis of T_0 plants through PCR and Southern blot analysis proved the existence of transgenes in the transformants PCR analysis resulted in the amplification of respective gene fragments such *as uidA*, *nptII*, and *RChit* genes in

more than 90% of the putatively transformed plants Southern blot of the 5 T_0 plants positive for *npt*II gene revealed the presence of the gene in a single insert in 3 plants In Southern blot analysis of T_0 plants for *RChit* gene, out of 11 independent transgenic lines, 6 plants expressed prominent signals indicating the presence of the gene and 2 plants had a single insertions

Further amplification of the marker as well as candidate genes in T_1 and T_2 generation proved the simultaneous transfer of both the genes All the transformants produced were morphologically normal and fertile and there was no significant phenotypic changes observed in all the plants belonging to T_0, T_1 and T_2 generations In T_1 generation, 8 independent transformed plants of T_0 generation with 5 replicates were taken Out of 5 lines of each of the independent transformants of T_0, the number of PCR positives for *uid*A gene was either 3 or 4 indicating the segregation pattern following Mendelian inheritance ratio of 3 1

To verify the stable integration of the *RChit* gene, Southern blot analysis was carried out on 16 T_2 generation plants representing 4 independent transformants of T_0 generation, which resulted in positive gene integration in 8 plants This further proved the transformation protocol that was used for the production of transformed plants led to the highest transformation frequency

In conclusion, a well established and a relatively efficient method of plant regeneration compatible to the genetic transformation of pigeonpea through microprojectile bombardment has been developed, that allows stable integration and

expression of the transgene, as confirmed by histochemical and molecular analysis, as well as the studies on heritability of the transgene

SUMMARY AND CONCLUSIONS

SUMMARY

The present study deals with the production of transgenic pigeonpea resistant to wilt disease (caused by fungus, *Fusarium oxysporum F udum* (Butler) Synder and Hansen) This was achieved by using microprojectile-mediated gene transfer method for the introduction of *RChit* gene into pigeonpea cultivar ICPL 88039 Development of a reliable tissue culture regeneration system is a pre-requisite for the development of transgenic plants In the present study, a rapid and reliable in vitro regeneration protocol was developed by using 4 to 5-d-old leaf explants obtained from in vitro raised aseptic seedlings The regeneration protocol exhibited a high frequency (more than 90%) of multiple shoot development Experiments were carried out to optimize the plant regeneration protocol amenable to genetic transformation for the development of transgenic pigeonpea The experiments involved studies on tissue culture, and genetic transformation aspects

Tissue culture studies

1 The method for shoot bud regeneration describes in this thesis for the development of high frequency adventitious multiple shoots from the petiolar region of leaf explant, (which is devoid of any pre-existing meristematic cells)

2 Primary juvenile leaf explants from 2 to 15-d-old donor seedlings were tested for their regeneration efficiency, and the explants from 4 to 5-d-old seedlings showed the highest response The regeneration potential declined with increase in the age of the explant

3 Various plant growth regulators such as BA, kinetin, 2-iP, TDZ, IAA, and NAA supplemented in the MS medium were tested for shoot bud differentiation The explants cultured on MS supplemented with BA in combination with kinetin showed the best response Whereas, BA proved to be a potential cytokinin for the induction of target cells and multiple shoot buds, kinetin supported the further growth of the multiple shoots TDZ (a phenyl urea), was found to be detrimental to the leaf explant culture in the present study Auxins such as NAA and IAA in combination with cytokinins were found to be futile for shoot induction An interesting observation was that there was a three-fold increase in the efficiency of shoot bud regeneration that was induced by the use of filter-sterilized phytohormones compared to the autoclaved phytohormones

4 Based on the genotypes tested, it was observed that the regeneration protocol put forward was applicable across a wide range of pigeonpea genotypes ICPL 88039, which has a moderate level of resistance to the legume pod borer was used for all the experiments for the production of transgenic pigeonpea

5 The formation of shoot buds exhibited polarity by restricting their formation to the cut end of the petiole of the leaf explant Better response was obtained with the petiole with intact lamina, for shoot induction

6 The orientation of the leaf explant played a vital role in enhancing morphogenesis Placing the abaxial side of the explant in contact with the culture medium gave better results

7 The explants exhibited adventitious shoot bud formation when cultured on MS medium supplemented with 5 0 μM BA and 5 0 μM kinetin The shoot buds were initiated within a week of induction Differentiation of shoot buds was visible after 8 to 10 d Differentiation and further proliferation of the multiple shoot buds occurred when transferred to shoot development medium comprising of MS basal medium supplemented with 2 5 μM BA in combination with 2 5 μM kinetin

8 The formation of multiple shoots and leaf primordia was observed from 14 d onwards Histological analysis of the regenerated explants revealed that the ontogeny of shoot buds was adventitious in origin The shoot buds differentiated into shoots in the shoot development medium The differentiated shoots could be elongated when cultured on MS medium supplemented with 0 58 μM GA_3 and the shoots elongated within 6 to 7 d

9 For rooting, various concentrations of IBA (0 98 to 9 0 μM) and IAA (1 14 to 11 42 μM) and MS medium with reduced sucrose concentration (1% w/v) were tested individually Freshly cut shoots were pulse treated by dipping the shoots in 11 4 μM IAA solution before the transfer to the culture tubes containing MS medium fortified with 1% sucrose (w/v) and devoid of any growth regulators Pulse treatment enhanced the formation of adventitious roots to in

80% of the rootable shoots, while the adventitious root formation was observed after 6 d of root initiation Sugar starvation of 1% by adding only sucrose was also found to be effective in the successful induction of rooting

10 The rooted shoots could undergo the hardening process with a survival rate of 95 to 100% The use of polythene bags to cover the in vitro raised plantlets during the hardening process helped the plantlets to withstand the minimal loss of water through transpiration and by maintaining high humid conditions The plants were later transplanted into regular pots with sand and soil in 1 1 ratio Addition of small amount of thiram (fungicide) helped in the acclimatization of the plant to overcome fungal infection if any

Genetic transformation studies

1 The method of microprojectile bombardment was used for gene transfer by using leaf regeneration system developed in the present study

2 The plasmid construct used for bombardment of the explains was pRT99GUS RChit, carrying chitinase expression cassette was maintained in *E. coli* strain DH5α The plasmid pRT99GUS RChit contained marker genes viz *uidA* and *nptII* besides the *RChit* gene under the control of a constitutive promoter CaMV 35S and polyA terminator

3 In the present study, the target cells amenable for bombardment were identified as the cells residing in the proximal cut end of the petiolar region of the leaf

explant Since the plant selection marker gene *npt*II was used presently, which imparts the plant cells resistance to kanamycin, initial tolerance levels of kanamycin were standardized on untransformed explants and a concentration of 100 mg/L was selected for the culture of bombarded explants

4 In microprojectile-mediated gene transfer method, the bombardment distance and the pressure of the helium gas generally play a vital role Pressures of 1100 and 1300 psi of helium gas were found to be optimum for the penetration of the plasmid coated particles into the target cells and the optimum bombardment distance was 6 and 8 cm

5 The explants were transferred to fresh culture plates 24 hr after bombardment A recovery period of 14 d was found to be ideal before transfer to selection medium Kanamycin selection was gradually increased from 25, 50, 75 to 100 mg/L The explants were subjected to a selection pressure of 25 mg/L for 2 wk and later transferred to a selection of 50 mg/L for 1 wk and further placed in a medium consisting of 75 mg/L for a wk The explants were stringently subjected to 100 mg/L selection for longer period's upto 3 wk, that screened the untransformed shoots produced from untransformed cells

6 Exclusion of selection from root induction medium was found to be effective in the formation of roots The transfer of the plants to the potting mix for the acclimatization consisted of sand and soil in 1 1 ratio and the plants were covered with polythene bags to retain high humidity

7 Transient expression of *uid*A gene is generally assayed through histochemical analysis of GUS, where X-Gluc was used as a substrate The explants subjected to bombardment were tested for GUS assay The transformed tissue expressed intense blue, while the untransformed control tissue expressed faint blue Since the untransformed control tissue also expressed blue coloration, transient expression based on GUS assay was found to be unreliable for the present study on pigeonpea

8 Fifty independent transformed (T_0) plants were transferred to the glass house PCR analysis was carried out on 17 (T_0) plants for *uid*A gene amplification, 10 (T_0) plants for *RChit* gene amplification and 18 (T_0) plants for *npt*II gene amplification

9 Fidelity of the PCR amplification was confirmed through Southern blotting of the PCR amplicons, where 90% of the transfromants revealed the presence of the transgene

10 Further, for gene integration and copy number analysis, Southern blotting for *RChit* gene was carried out on 11 randomly selected plants Out of the 11 independent transformed plants of T_0 tested (P16, P17, P18, P19, P20, P23, P24, P25, P26 and P27), only 6 plants (P16, P17, P18, P20, P23 and P26) expressed the presence of the *RChit* gene Out of the 6 plants, 2 plants (P17 and P23) showed single copy inserts, while rest of the plants showed double inserts Further, Southern blotting for *npt*II gene was carried out on first 10 plants and 5 plants (P1, P3, P4, P7 and P8) were found to be positives. Three plants (P1, P3

and P4) from among the Southern (*npt*II) positives showed single gene inserts and the rest (P7 and P8) showed double gene inserts

11 Self-fertilized first 8 plants of T_0 generation were advanced to T_1 generation for inheritance studies Five replicates were taken from each independent transformant Forty T_1 plants (P1→1-5, P2→1-5, P3→1-5, P5→1-5, P6→1-5, P7→1-5, P8→1-5, P13→1-5) were analysed through PCR for inheritance of *uid*A gene and 25 plants (P1→1, 4, 5, P2→3, 4, 5, P3→1, 2, 3, 4, 5, P5→3, 5, P6→2, 3, 4, 5, P7→2, 4, 5, P8→1, 3, 4, 5, P13→1) were PCR positives, expressing the 1 2 kb of *uid*A gene fragment

12 Southern blotting for *RChit* gene was carried out on 16 T_1 lines of 8 independent transformed T_0 plants (P1→1, 4, P3→1, 3, P5→1, 3, 4, P6→2, 4, P7→2, 3, 4, P8→3, 4, P13→2, 4), in which only 3 plants (P1→4, P8→2, 4) expressed the *RChit* gene integration in T_1 generation that confirmed the presence of the gene The self-raised plants of T_1 generation were grown to maturity to raise T_2 generation

13 A total of 8 T_2 plants from 2 independent T_0 transformed plants (4 replicates each) were subjected to PCR analysis of *uid*A and *npt*II genes. Seven plants (P1→1→1-4, P8→1→1-3) showed the amplification of *uid*A gene fragment and 7 plants were tested positive (P1→1→2-4, P8→1→1-4) for *npt*II gene

14 For confirmation of integration of *RChit* gene in the T_2 transformants, 4 replicates were taken of 4 plants and tested through Southern blotting Out of 16 lines, 8 plants (P1→1→1, 2, 4, P8→1→1, 2, P9→1→1, 4, P10→1→1) were

found to be positives indicating the integration of *RChit* gene and thereby inheritance of the gene from T_0 and T_1 generation

CONCLUSIONS

In the present study on the development of in vitro plant regeneration system in pigeonpea and establishment of an efficient genetic transformation protocol using microprojectile bombardment, the following conclusions were drawn,

1 An efficient plant regeneration system using the leaf explant was standardized The regeneration system developed was rapid, reliable, reproducible, efficient and capable of producing plants independently (without any intervening callus phase) through organogenesis

2 Primary juvenile leaf explants from 4 to 5-d-old plants exhibited the highest regeneration response of 90% Target cells at the proximal cut end of the petiole were found to be amenable for microprojectile bombardment

3 MS basal medium fortified with 5 0 μM BA and 5 0 μM kinetin was found to be highly effective for the initiation of shoot buds from the petiolar region of leaf explant

4 Histological studies of the regenerating explants revealed the adventitious origin of multiple shoot buds

5 Shoot development medium consisting of 2 5 μM BA and 2 5 μM kinetin was found to be the most suitable for shoot bud differentiation Incorporation of GA_3 in the MS medium enhanced shoot elongation

6 Efficient rooting could be induced in MS basal medium supplemented with IAA containing a reduced sucrose concentration of 1% Shoots pulse treated with IAA developed more number of adventitious roots The survival rate of the transplanted and hardened plantlets was more than 90%

7 An efficient transformation protocol was standardized by using the petiolar region of the leaf explant as the target tissue for microprojectile bombardment Pressures of 1100 and 1300 psi and distances of 6 and 8 cm were found to be ideal for bombardment of the target leaf explants The selection regime of 100 mg/L kanamycin was found to be ideal for screening of the putative transformants

8 Fifty T_0 putative transgenic plants were regenerated, rooted and transferred to the glasshouse

9 Of the 18 T_0 plants analysed for *uid*A and *npt*II genes almost all the plants expressed the amplification the respective gene fragment Of 10 T_0 plants analysed for *RChit* gene, 9 plants expressed the presence of the gene

10 Eleven T_0 plants were tested for *RChit* gene that confirmed the presence of the gene in 6 plants and single copy inserts were found only in 2 plants Southern blot analysis was carried out on 8 T_0 plants for the integration of *npt*II gene, 5 plants

were characterized for the presence of the gene Single copy inserts were observed in 3 plants

11 Of the 40 T_1 plants analysed for the amplification of *uid*A gene by PCR, 25 plants showed the amplification of the above gene

12 Eight T_2 plants were tested for the PCR amplification of *uid*A gene and 7 were found to be positives, and the same result was observed for the amplification of *npt*II gene Sixteen T_2 plants were tested for PCR amplification of the *RChit* gene and only 3 plants were found to be positive probably due to the high stringent conditions applied The segregation and inheritance of the transgene followed Mendelian 3 1 ratio

13 Further confirmation of the T_2 plants for the presence of the *RChit* gene was carried out Out of the 16 T_2 plants, 8 plants were positive for *RChit* gene integration

14 Based on the results presented above, it is concluded that the transformation efficiency was over 50%

15 The regeneration and transformation protocol reported here for pigeonpea is efficient for the production of high frequency of transformants and the system has been successfully utilized to produce transgenic plants with genes of agronomic importance Therefore, this system could be generally applicable for the biotechnological improvement of pigeonpea

APPENDICES

APPENDICES

Appendix 1. Pigeonpea variety ICPL 88039

Released name/Year	ICPL 88039
Parentage	ICPL 161-H2-HB-HB-HB-H1-HB-HB
Yielding	2 0 tonnes ha^{-1}
Cropping systems	Suitable for sole crop/Inter-cropping
Sowing time	Kharif in June
Seed rate	15 to 20 kg ha^{-1} (Kharif)
Duration	Early (short-duration)
Growth habit	Non-determinate (NDT)
Resistance	Maruca resistant
Seed size	Medium size 10 5 (gm)/100 seed
Days of flowering	77 d
Days to maturity	111 d
Plant height	182 cm
Protein dhal	22 03%
Dhal yield	82 80%

Appendix 2. **Murashige and Skoog (1962) medium**

Salt	Conc (mg/L)	Stock (per L)	Use (per L)
MAJOR SALT (X 50)			
NH_4NO_3	1650	33 0 g/200 ml	10 ml
KNO_3	1900	38 0 g/400 ml	20 ml
KH_2PO_4	170	3 40 g/200 ml	10 ml
$CaCl_2$	440	8 80 g/200 ml	10 ml
$MgSO_4$ $7H_2O$	370	7 40 g/200 ml	10 ml
MINOR SALTS (X 100)			
H_3BO_3	6 20		
KI	0 83	6 20 mg/L)	
$MnSO_4$ H_2O	22 3	83 0 mg/L)	
$ZnSO_4$ $7H_2O$	8 6	2230 mg/L)	
NA_2MoO_4 $2H_2O$	0 25	860 mg/L)	5 0 ml
$CuSO_4$ $5H_2O$	0 025	25 mg/L)	
$CoCL_2$ $6H_2O$	0 025	2 5 mg/L)	
IRON (X 100)	37 3	2 5 mg/L)	
Na_2EDTA $2H_2O$	27 8	3 73 g/L)	
$FeSO_4$ $7H_2O$	40	2 78 g/L)	
OR		2 0 g/500ml	
$FeNA_2EDTA$			
ORGANICS (X 100)			
GLYCINE	2 0	200 mg/L)	10 ml
NICOTINIC ACID	0 5	50 mg/L)	10 ml
THIAMINE HCL	0 1	100 mg/L)	10 ml
PYRIDOXINE HCL	0 5	50 mg/L)	10 ml
m-Inositol	100	5 0 g/500 ml	

Appendix 3 Embedding of the petiolar region of the leaf explant for ontogeny studies

Station no	Solvent	%	Time
1	Isopropyl alcohol	80	1hr
2	- do -	90	- do -
3	- do -	95	- do -
4	- do -	95	- do -
5	- do -	absolute	- do -
6	- do -	- do -	- do -
7	- do -	- do -	- do -
8	chloroform	-	- do -
9	- do -	-	- do -
10	- do -	-	- do -
11	Paraffin - 1	-	3hr
12	Paraffin - 2	-	3hr

Appendix 4 Staining the deparafinised sections of the petiolar region of the leaf explant for ontogeny studies

Station no	Reagent	Time in min
1	Xylene (sulphur free)	7
2	Xylene	7
3	Acetone (absolute)	2
4	Ethanol 95%	2
5	Tap water (wash I)	5
6	Distilled water	3
7	Hematoxylin	12
8	Tap water (wash II)	12
9	Distilled water	2
10	Eosin	1
11	Ethanol 95%	1/60
12	- do -	1/60
13	- do -	1/60
14	Acetone (absolute)	1
15	- do -	2
16	Acetone and xylene	2
17	Xylene	2
18	- do -	2

Appendix 5. Preparation of LB medium (Sambrook *et al.*, 1989)

Preparation for 1 L

Chemical	Wt in gm
Bacto–peptone	10
Yeast extract	5
Sodium chloride (NaCl)	10
Agar	15
pH	7

Appendix 6. Preparation of GUS histochemical staining buffer for 5 ml (Jefferson 1987)

Chemical	Stock conc	Working conc
X-GLUC	10 mg	Dissolve in 100 µl of dimethyl formamide
Tris-HCL	1 M	100 mM (100 µl from 1 M stock)
NaCl	5 M	50 mM (100 µl from 5 M stock)
Potassium Ferricyanide	200 mM	2 mM (100 µl from 200 mM stock)
Triton X	1%	0 1% (100 µl from 1% stock)
Sodium azide	50 mg/ml	0 2% (400 µl from stock)
Distilled H_2O		8 2 ml

The buffer was filter sterilized and stored in 1 5 ml eppendorfs at 4 °C

Appendix 7. Composition of DNA extraction buffer (Porebski *et al.*, 1997)

Component	Stock Conc	Working conc for 100ml
Tris	1 M	20 ml
Nacl	5 M	56 ml
EDTA (pH 8 0)	100 mM	40 ml
CTAB	10 %	40 ml
β- mercaptoethanol	0 3 %	300 µl (added just before use)
H_2O		40 ml

Appendix 8. Preparation of Pre-hybridization buffer

Hybridization buffer (Alkaline phosphatase) - 25 ml

0 5 M NaCl - 0 73125 gm

Blocking reagent - 1 gm

Add NaCl of 0 73125 gm and blocking reagent of 1 gm to 25 ml of hybridizatıon buffer and keep it for thorough mixing on a magnetic stirrer for 1 hr

Note Pre-heated buffer (55 °C) to be added to the blots

Appendix 9. Preparation of Primary wash buffer for 500 ml

Chemical	Chemical to be added for 500ml
Urea- 2 M	60 gm
SDS-0 1%	500 mg
0 5 M $NaPO_4$ - 50 mM	pH 7 (50 ml)
NaCl- 150 mM	4 32 g
$MgCl_2$ -1M	500 µl
Blocking reagent	1 gm

Appendix 10. Preparation of 20X Secondary wash buffer for 500 ml

1 M Tris base-60 5 gm

2 M NaCl-56 gm

pH set at 10

Appendix 11. Extraction of RNA through TRIzol method

- Grind the leaf tissue in liquid N_2 (100 mg). Homogenize the tissue in 1 ml of TRIzol reagent.
- Incubate the sample for 5 min at 15 to 30 °C.
- Add 200 µl of chloroform per 1 ml of TRIzol reagent.
- Shake vigorously for 15 sec and incubate at 15 to 30 °C for 2 to 5 min.
- Centrifuge at 14000 rpm for 15 min at 2 to 8 °C till three phases' form.
- Collect the upper aqueous phase (3/4 vol only) into fresh tubes and precipitate it with 500 µl of isopropanol.
- Incubate the samples at 15 to 30 °C for 10 min and later centrifuge at 14000 rpm for 10 min at 2 to 8 °C.
- Decant the supernatant.
- Wash the pellet with 70% ethanol (1 ml)/ vortex it.
- Spin at 10000 rpm for 5 min at 2 to 8 °C.
- Dry the pellet in vacuum drier for 5 min.
- Dissolve the pellet in RNase free (30 µl) of water.

Preparation of chemicals and buffers useful in genetic transformation studies

1. Preparation of 2 N NaOH, mol wt - 40 gm

Constituent	Wt in gm	Vol
NaOH	8	Dissolve in 100 ml of SDW

2 Preparation of 5 M Potassium acetate pH 5 2 mol wt- 98 14 gm

Constituent	Wt in gm	Vol
Potassium acetate	49 07	Dissolve in 100 ml SDW

Make up the pH to 5 2 with acetic acid

3 Preparation of 5 M NaCl, mol wt - 58 44 gm

Component	Wt in gm	Vol
NaCl	29 22	Dissolve in 100 ml SDW

4 Preparation of 0 5 M Sodium phosphate, mol wt – 138 gm

Constituent	Wt in gm	Vol
Sodium phosphate	6 9	Dissolve in 100 ml of SDW

Adjust the pH to 7 with 5 M HCL

5 Preparation of 1 M Tris pH 8, mol wt - 121 14 gm

Constituent	Wt in gm	Vol
Tris-HCL	12 1	Dissolve in 100 ml of SDW

Adjust the pH to 8 with 1N NaOH

6 Preparation of 100 mM EDTA pH 8, mol wt - 372 24 gm

Component	Wt in gm	Vol to be added
EDTA	3 72	Dissolve in 100 ml of SDW

Adjust the pH to 8 with 1N NaOH

7 Preparation of 10% CTAB

Dissolve 10 gm of CTAB in 100 ml of SDW

8 Preparation 10% SDS

Dissolve 10 gm of SDS in 100 ml of SDW

9 Preparation of 2 5 M $CaCl_2$, mol wt - 147 gm

Chemical	Wt in gm	Vol
$CaCl_2$	36 75	Dissolve in 100 ml of SDW

10 Preparation of 3 M Sodium acetate pH 4 8, mol wt - 82 03 gm

Chemical	Wt in gm	Vol
Sodium acetate	24 61	Dissolve in 100 ml of SDW

Adjust the pH to 4 8 with acetic acid

11 Preparation of 50X TAE

Chemical	Wt in gm	Vol to be added
Tris-HCL	242	
Na_2 EDTA $2H_2O$	372 2	
Acetic acid		57 1 ml

Adjust the pH to 8 5, make up the volume to 1L with SDW

12 Preparation of 1X TAE for 5 L

Chemical	Vol
50X TAE	100 ml

Make up the volume to 5 L with SDW

13 Preparation of varied % of ethanol

Component	Working %	Vol of ethanol to be added (ml)	Final vol
Ethanol	10	10	Make up the vol to 100 ml with SDW
Ethanol	30	30	- do -
Ethanol	50	50	- do -
Ethanol	70	70	- do -

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TABLES

Table 1. Shoot bud induction from leaf explant of pigeonpea when cultured on MS medium containing TDZ and IAA*.

Culture media**	No of explants cultured	No explants forming shoots (mean±SE)	% explants forming shoots	Morphology of the explant
0 (Control)	24	0 0	0 0	Green
TI	24	0 0	0 0	Pale yellow
TI – 1	24	0 0	0 0	Pale yellow
TI – 2	24	0 0	0 0	Pale yellow

* The results were recorded at the end of 3 wk

* * MS basal medium supplemented with TDZ and IAA

T I (9 0 μM TDZ) + (0 54 μM IAA)

T I-1 (9 08 μM TDZ) + (1 14 μM IAA)

T I-2 (11 35 μM TDZ) + (1 14 μM IAA

Table 2. Effect of cytokinin in combination with auxin on shoot bud induction from leaf explant*.

Culture media**	No of explants cultured	Fresh wt of the callus (mg)	Dry wt of the callus (mg)	No of explants with callus (mean±SE)	No of shoots per responding explant (mean±SE)	% explants responding for shoot formation	Morphology of the calluses
0 (Control)	24	0 0	0 0	0 0	0 0	0 0	Callus was not formed
BN1	24	0 2863	14 1	23 3±0 2	**0.66±0.4**	**2.7**	Light green friable
BN2	24	0 1664	8 1	23 3±0 4	0 0	0 0	Light green friable
BN3	24	0 1361	25 6	23 7±0 2	0 0	0 0	Dark green, hard compact
BN4	24	0 5116	12 2	24 0±0 0	0 0	0 0	Dark green, hard compact

*The results were recorded at the end of 4 wk

**MS medium supplemented with various concentrations of BA and NAA

BN1 = BA (4 44 μM) + NAA (0 54 μM)

BN2 = BA (11 09 μM) + NAA (1 07 μM)

BN3 = BA (15 33 μM) + NAA (1 61 μM)

BN4 = BA (22 18 μM) + NAA (1 07 μM)

Table 3. The effect of BA, kinetin and 2-iP supplemented to the MS medium on shoot bud induction from leaf explants of in vitro germinated seedlings of pigeonpea*.

Media	Growth regulators (μM)			No of explants cultured	Explants producing shoots (mean±SE)	% explants forming shoots
	kinetin	BA	2-iP			
0 (Control)	0 0	0 0	0 0	30	0 0	0 0
C1	5 0	10 0	0 0	30	15 0±0 7	50 0
C2	5 0	7 5	0 0	30	20 5±0 4	68 3
C3	5 0	5 0	0 0	30	28 5±0 4	**95.0**
C4	5 0	2 5	0 0	30	26 5±0 4	88 3
C5	5 0	1 0	0 0	30	22 0±0 4	73 3
C6	5 0	0 0	0 0	30	24 5±0 4	81 7
K1	10 0	5 0	0 0	30	15 0±0 7	50 0
K2	7 5	5 0	0 0	30	23 5±0 4	78 3
K3	5 0	5 0	0 0	30	27 0±0 7	90 0
K4	2 5	5 0	0 0	30	20 5±0 4	68 3
K5	1 0	5 0	0 0	30	22 0±0 4	73 3
K6	0 0	5 0	0 0	30	23 5±0 4	78 3
P1	10 0	0 0	5 0	30	25 5±0 4	85 0
P2	7 5	0 0	5 0	30	21 0±0 7	70 0
P3	5 0	0 0	5 0	30	18 5±0 4	61 7
P4	2 5	0 0	5 0	30	17 5±0 4	58 3
P5	1 0	0 0	5 0	30	21 5±0 4	71 1
P6	0 0	0 0	5 0	30	20 5±0 4	68 3
I1	5 0	0 0	10 0	30	11 0±0 7	36 7
I2	5 0	0 0	7 5	30	21 0±0 4	70 0
I3	5 0	0 0	5 0	30	23 5±0 4	78 3
I4	5 0	0 0	2 5	30	10 5±0 4	35 0
I5	5 0	0 0	1 0	30	13 5±0 4	45 0
I6	5 0	0 0	0 0	30	21 5±0 4	71 7

* The results were recorded at the end of 3 wk

Table 4. Shoot bud formation from different explants, cultured on shoot induction medium consisting of MS + 5.0 μM BA + 5.0 μM kinetin*.

Explant	No of explants cultured	No of explants developing callus (mean±SE)	No of explants with shoot buds (mean±SE)	% explants developing shoots
Epicotyl	24	24 0±0 0	0 0	0 0
Hypocotyl	24	23 7±0 2	0 0	0 0
Embryonal axes	24	23 3±0 2	0 0	0 0
Leaf lamina with out petiole	24	24 0±0 0	0 0	0 0
Leaf with half cut lamina and petiole	24	23 0±0 3	1 7±0 5	6 9
Leaf with lamina and petiole	24	0 0	**23.0±0.3**	**95.83**

* The observations were recorded at the end of 3 wk

Table 5. Shoot bud regeneration from leaf explants of the in vitro germinated seedlings of various donor genotypes of pigeonpea after culture on shoot induction medium comprising of MS + 5.0 μM BA + 5.0 μM kinetin*.

Genotype	Duration type	No of explants cultured	No of explants producing shoot buds	% explants producing shoot buds
ICPL 91011	Extra short	39	24	61 5
ICPL 88009	Short	39	16	41 0
ICPL 84031	Short	42	25	59 2
ICPL 87091	Short	50	31	62 0
ICPL 87	Short	73	52	71 2
ICPL 88039	Short	54	45	**83.3**
ICPL 2376	Medium	77	34	44 2
ICPL 87051	Medium	57	31	54 4
ICPL 332	Medium	35	19	54 3
ICPL 85063	Medium	69	44	63 8
ICPL 87119	Medium	35	23	65 7

* The observations were recorded at the end of 2 wk

Table 6. Influence of the size of lamina on shoot bud induction from the petiolar region of the leaf explant of in vitro germinated seedlings of pigeonpea, cultured on shoot induction medium consisting of MS + 5.0 μM + 5.0 μM kinetin*.

Size of the lamina	No. of explants cultured	No. of explants producing shoot buds	% explants producing shoot buds
Leaf with full lamina	30	27	**90.0**
Leaf with ½ lamina	30	26	86.6
Leaf with ¼ lamina	30	22	73.3
Leaf with no lamina	30	6	20.0

*The observation on response of the explant to shoot bud formation was recorded after 12 d

Table 7. Effect of GA_3 on the elongation of adventitious shoot buds. MS was used as a basal medium.

Growth regulators (μM) BA	kinetin	GA_3	No of explants cultured	No of explants with elongated shoots (mean±SE)	% explants with elongated shoots	Morphology of the elongated shoots
Control•			24	0 0	0 0	Rosette shoots
2 5	2 5	2 89	24	2 7±0 6	11 11	Thin, weak shoots and tip curled
-	-	2 89	24	2 7±0 2	11 11	Thin, weak shoots and tip curled
-	-	2 31	24	3 0±0 3	12 5	Thin, lean, weak shoots
-	-	1 71	24	3 7±0 5	15 3	Weak shoots
-	-	1 51	24	9 0±0 3	37 5	Weak shoots
-	-	0 58	24	**23.3±0.5**	**88.9**	Healthy strong shoots

*The observations on explant with elongated shoots were recorded after 1 wk

• MS basal medium supplemented with 2 5 μM BA + 2 5 μM kinetin

Table 8. Effect of IBA on the regeneration of adventitious roots, on elongated shoots. MS was used as basal medium *.

IBA (μM)	No of elongated shoots cultured for rooting	No of shoots forming roots (mean±SE)	% shoots forming roots
0 (Control)	24	0 0	0 0
4 90	24	0 0	0 0
3 94	24	0 0	0 0
2 95	24	0 0	0 0
1 97	24	0 7±0 2	2 7
0 98	24	**2.0±0.4**	**2.7**

*The observation for the root development was recorded after 3 wk

Table 9. Effect of IAA on the regeneration of adventitious roots. MS was used as basal medium *.

IAA (μM)	No. of elongated shoots placed for rooting	No. of shoots forming roots (mean±SE)	% shoots forming roots
0 (Control)	24	0.0	0.0
7.56	24	0.0	0.0
6.85	24	0.0	0.0
5.71	24	0.3±0.2	0.0
4.57	24	1.0±0.3	1.3
3.42	24	1.0±0.3	4.2
2.28	24	2.3±0.2	4.2
1.14	24	4.3±0.2	9.7
**11.42 with pulse treatment	24	12.7±0.8	18.1
11.42 second subculture	24	16.0±0.3	52.8
11.42 third subculture	24	**21.0±0.3**	**87.5**

*The observations on elongated shoots developing roots were recorded at the end of 15 d.

**Pulse treatment was given to the freshly cut elongated shoots by placing the shoots in 11.42 μM IAA solution for 30 to 60 sec and later transferring them to MS basal medium with 1% sucrose.

Table 10. Effect of sucrose on rooting of the regenerated shoots cultured on MS basal medium with 1.14 μM IAA*.

% of sucrose	No of shoots placed for rooting	No of explants forming roots (mean±SE)	% shoots forming roots
0 (control)	24	0 0	0 0
3	24	4 3±0 2	18 1
2	24	6 7±0 2	27 8
1	24	**11.0±0.3**	**45.8**

*The observation on the shoots for rooting after first sub-culture was recorded at the end of 3 wk

Table 11. Effect of the method of sterilization of BA, kinetin, GA_3 and IAA for the production of in vitro regenerated plantlets*.

Culture medium**	Stages of regeneration	Method of sterilization	No of explants cultured	No of explants responded (mean±SE)	% explants responded
SIM	Shoot induction	Autoclaved	24	17 7±0 2	73 6
		Filter sterilized	24	**23.7±0.2**	**98.6**
SDM	Shoot bud differentiation	Autoclaved	24	16 0±1 0	66 7
		Filter sterilized	24	**23.3±0.4**	**97.2**
SEM	Shoot elongation	Autoclaved	24	14 3±0 7	59 7
		Filter sterilized	24	**21.3±0.5**	**88.9**
RIM	Root induction	Autoclaved	24	4 3±0 2	18 1
		Filter sterilized	24	**21.0±0.3**	**87.5**

*The observations were recorded at the end of 2 wk

**MS basal medium supplemented with the following combinations of growth regulators

SEM (MS + 5 0 µM BA + 5 0 µM kinetin)

SDM (MS + 2 5 µM BA + 2 5 µM kinetin)

SEM (MS + 0 58 µM GA_3)

RIM [MS basal medium with 1% sucrose (shoots were pulse treated with 11 42 µM IAA)]

Table 12. Effect of kanamycin on explants surviving selection. Untransformed leaf explant cultured on MS medium containing 5.0 μM BA + 5.0 μM kinetin*.

Kanamycin (mg/L)	No of explants cultured	No of explants surviving selection	% explants surviving selection
0 (control)	72	72	100
25	72	70	97 2
50	72	67	93 3
75	72	62	86 1
100	72	57	79 2
125	72	36	50 0
150	72	26	30 0

*The observations were recorded based on the phenotypic response of explant to kanamycin after 3 wk

Table 13. Effect of different parameters for microprojectile-mediated gene transfer on the recovery of putative transformants*.

Parameter	No of explants bombarded	No of explants responded for shoot regeneration			% explants producing shoots
		Rep1	Rep2	Rep3	
Bombardment distance (cm)					
Control (not bombarded)	400	396	394	398	99 00
6	400	386	390	383	96 58
8	400	384	396	384	97 0
Pressure of helium gas (psi)					
Control (not bombarded)	400	397	393	398	99 00
1100	400	393	387	382	96 83
1300	400	396	385	382	96 92

The observations were recorded at the end of 3 wk

*The plasmid used for bombarding the explants was pRT99GUS RChit

Table 14. Effect of selection on kanamycin for the recovery of putative transformed shoots after microprojectile bombardment*.

Kanamycin (mg/L)	No of explants cultured	No of explants which could not regenerate after selection (mean±SE)	% explants which could not regenerate after selection
25[a]	74	1 3±0 4	5 5
50[b]	70	3 3±0 2	13 9
75[c]	57	4 3±0 2	18 1
100[d]	40	7 0±0 3	29 2

The observations were recorded at the end of 3 wk

*The plasmid used for bombarding the explants was pRT99GUS RChit

a→ 1/2 strength shoot induction medium consisting of MS + 5 μM BA + 5 μM kinetin supplemented with 25 mg/L kanamycin

b→MS basal medium containing 0 58 μM GA_3 supplemented with 50 mg/L kanamycin

c→MS basal medium containing 0 58 μM GA_3 supplemented with 75 mg/L kanamycin

d →MS basal medium containing 0 58 μM GA_3 supplemented with 100 mg/L kanamycin

Table 15. Inheritance of *uidA* gene in T_1 generation of transgenic pigeonpea plants

Plant No.[1]	No of T_1 plants tested	PCR analysis of *uidA* gene		3 1 segregation*
		No of plants +ve	No of plants -ve	$\chi 2$
P1	5	3	2	0 60
P2	5	3	2	0 60
P3	5	5	0	1 67
P5	5	2	3	1 65
P6	5	4	1	0 03
P7	5	3	2	0 60
P8	5	4	1	0 03
P13	5	1	4	4 03*

[1] P1, P2, P3, P5, P6, P7, P8, P13 are independent T_0 plants and seeds of 5 replicates were taken from the above independent transgenic plants for inheritance studies in T_1 generation

*Significant at 5 % probability at 1 Degrees of freedom, where tabulated χ^2 value is 3 841

Table 16. Inheritance of *uid*A gene in T_2 generation of transgenic pigeonpea plants

T_1 plant*	No of T_2 plants tested	PCR analysis of *uid*A gene		3 1 segregation
		No of plants +ve	No of plants -ve	$\chi 2$
P1	4	4	0	1 33
P8	4	3	1	0 00

*1 and 8 are independent T_2 plants, 1 to 4 plants are the replicates of the above independent transgenic plants

Table 17. Summary of the molecular analysis of the putative transformants of pigeonpea belonging to T_0, T_1 and T_2 generations produced through microprojectile-mediated gene transfer method

	PCR			Southern blot analysis	
	*uid*A	*npt*II	*RChit*	*npt*II	*RChit*
No of T_0 plants analysed (P1 to P18)	17	18	10	8	11
No of +ve T_0 plants	16	18	8	5	6
No of –ve T_0 plants	1	0	2	3	5
No of T_1 plants analysed (Progeny of P1 to P8 and P13)	40	-	-	-	16
No of +ve T_1 plants	25	-	-	-	3
No of –ve T_1 plants	10	-	-	-	13
No of T_2 plants analysed (Progeny of P1, P8, P9 and P10)	8 (P1 and P8)	8 (P1 and P8)	16 (P1, P8, P9 and P10)	-	16 (P1, P8, P9 and P10)
No of +ve T_2 plants	7	7	3	-	8
No of –ve T_2 plants	1	1	13	-	8

FIGURES

Figure 1. Different stages of shoot regeneration from the proximal cut end of the petiolar region of leaf explant of pigeonpea (arrows indicate the differentiation of the shoot buds at the petiolar region leaf explant).

A. Leaf explants cultured on MS basal medium supplemented with 5 μM BA + 5 μM kinetin with abaxial side of the explant in contact with the medium.

B. Swelling of the petiolar region after 5 d.

C. Small protuberances from the proximal cut end of the petiole after 7 d.

D. Leaf explant with half cut lamina in shoot development medium.

E. Formation of multiple shoot bud from the petiolar region after 14 d.

F. Multiple shoot formation after 21 d of culture.

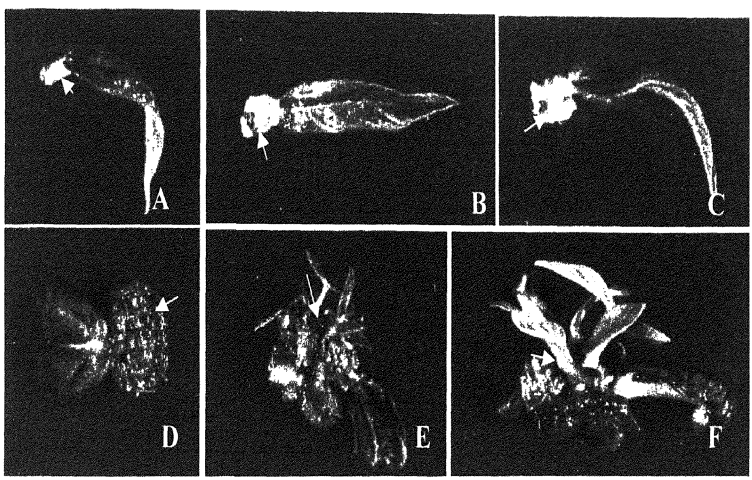

A
B
C
D
E
F

Figure 2. Elongation and rooting of shoots obtained from leaf explants of in vitro raised seedlings of pigeonpea after culture on MS basal medium containing GA_3 and IAA.

A. Multiple shoot development from leaf explants cultured on MS medium fortified with 0.58 µM GA_3.

B. Elongation of individual shoots cultured on MS medium supplemented with 0.58 µM GA_3

C. Formation of adventitious roots from elongated shoot after culture on MS basal medium supplemented with 1% sucrose.

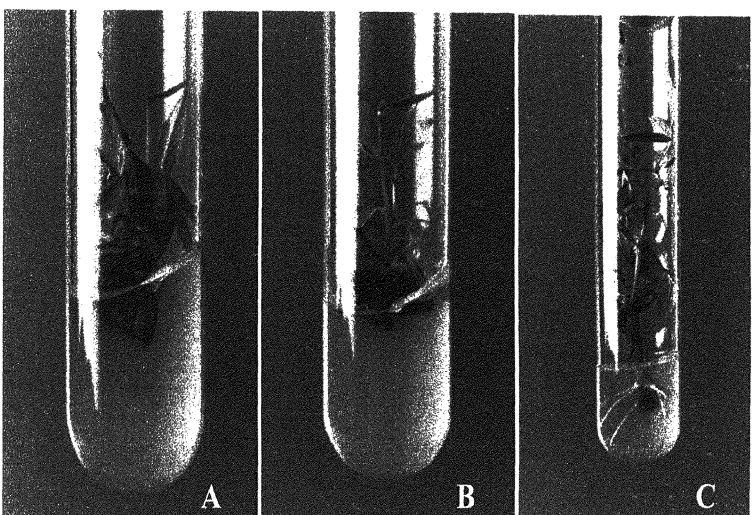

A
B
C

Figure 3A-K. Sequential events in the regeneration of multiple shoots from leaf explants derived from in vitro germinated seedlings of pigeonpea, (arrows indicate the petiolar cut end undergoing shoot bud differentiation).

A. Leaf explants at d 0 cultured on MS medium supplemented with 5.0 μM BA and 5.0 μM kinetin (SIM). **B.** Enlargement and swelling of the petiolar region within 5 d of the culture on SIM. **C.** Initiation of adventitious shoot buds from leaf explants from the swollen tissue of the petiolar cut end observed after 7 d on SIM. **D.** Differentiation of multiple shoot buds after 8 d from the petiolar cut end. At this stage, the explants with reduced lamina are ready for transfer to reduced SIM for shoot development. **E.** Proliferation of the shoot buds after 10 d on SIM. **F.** Development of shoot buds into shoots after 12 d on reduced SIM. **G.** Formation of multiple shoots after 2 wk on reduced SIM. **H.** Explant bearing multiple shoots placed on shoot elongation medium (SEM) containing MS supplemented with 0.58 μM GA_3 for shoot elongation after 7 d. **I.** A rooted pigeonpea plant on MS containing 1.14 μM IAA (RIM) after 3 wk ready for transplantation. **J.** A well-established and hardened plant successfully transplanted to the glasshouse at 4 wk. **K.** In vitro produced plants after 2 months in the glass house showing growth, flower production, and pods that contain viable seeds.

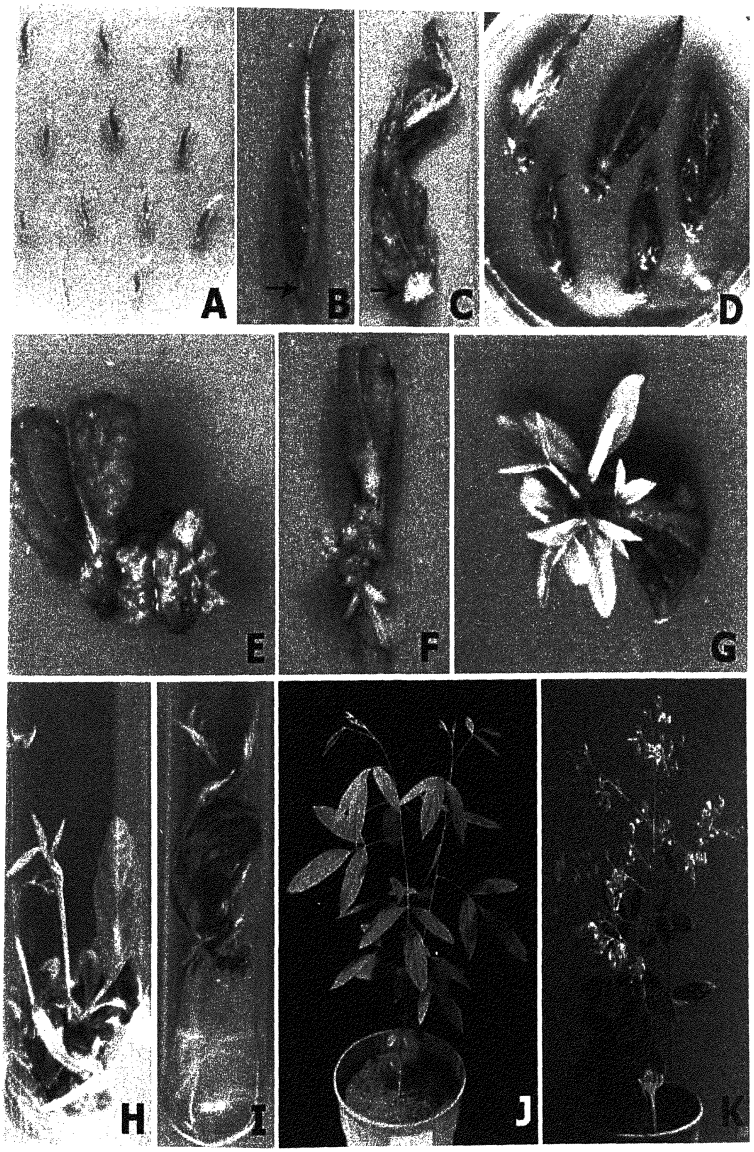

A
B
C
D
E
F
G
H
I
J
K

Figure 4. Effect of the age of leaf explant donor seedlings on the regeneration of multiple adventitious shoots in pigeonpea. Sixty explants were cultured on shoot induction medium containing MS basal medium supplemented with 5 μM BA + 5 μM kinetin.

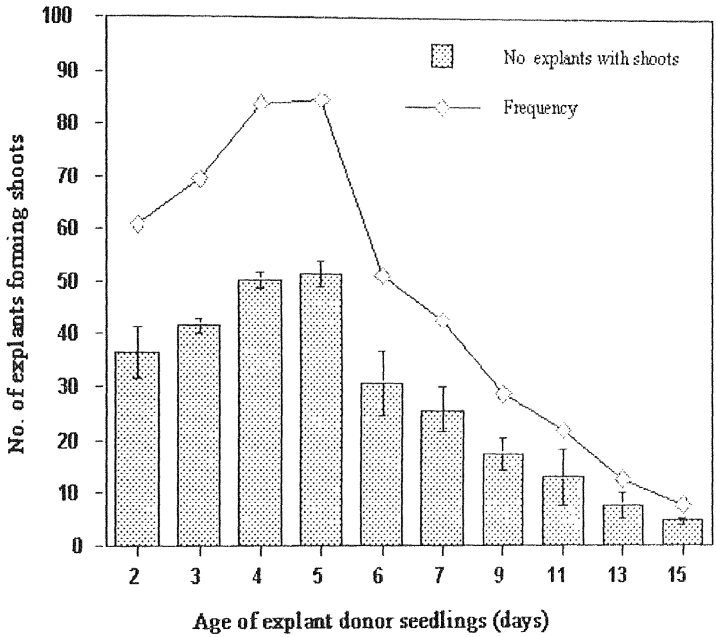

100
90
80
70
60
50
40
30
20
10
0
No. of explants forming shoots
No explants with shoots
Frequency
2
3
4
5
6
7
9
11
13
15
Age of explant donor seedlings (days)

Figure 5A-H. Ontogeny of shoot bud formation from the proximal cut end region of the petiole of leaf explant of pigeonpea during 0 to 6 d on SIM containing MS basal medium supplemented with 5 μM BA + 5 μM kinetin.

A. Transverse section of the petiole 0 d showing epidermis and cortical cells and vascular parenchyma cells (arrow). **B.** Transverse section of the petiole 0 d exhibiting cut end of the petiole (arrow). **C.** Transverse section of the petiole of 2-d-old culture in which the formation of actively dividing cells (arrow) was observed in the cortical region. **D.** Transverse section of the petiole of 2-d-old culture expressing the anticlinal and periclinal divisions in the actively dividing cells (arrow). **E, F.** Transverse section of 4-d-old petiole exhibiting starch filled (arrow) in the dividing cells. **G.** Transverse section of 6-d-old petiole showing the demarcation of dividing and non-dividing cells in the cortical region leading to the formation of meristematic zones (arrow). **H.** Transverse section of 6-d-old petiole expressing the formation of meristemoids (arrows).

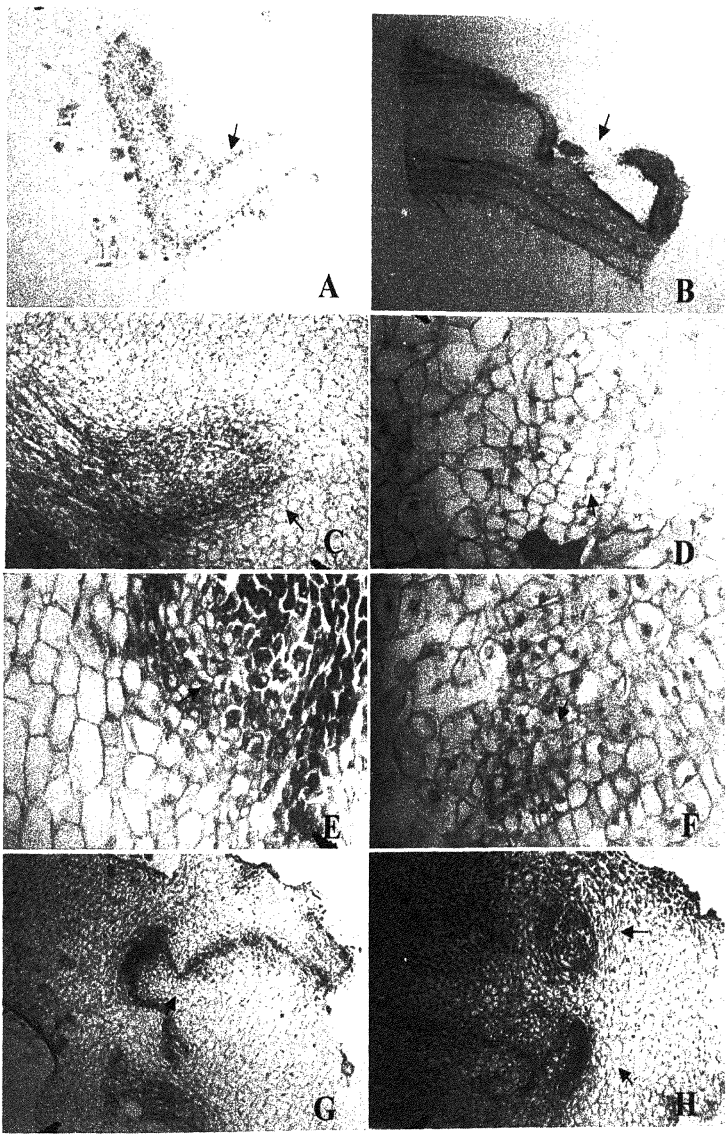

A
B
C
D
E
F
G
H

Figure 5I-P. Ontogeny of shoot bud formation from the proximal cut end region of the petiole of leaf explant of pigeonpea during 8 to 14 d on SIM containing MS basal medium supplemented with 5 μM BA + 5 μM kinetin.

I. Formation of the meristematic nodules at the cut of the petiolar region (arrow) of the transverse section of 8-d-old petiole. **J.** Formation of the actively dividing cells in the epidermal and sub epidermal region (arrow) of cut end of the petiole. **K.** Withering of the cells (arrows) observed near the meristematic regions of the transverse section of 10-d-old petiole. **L** Transverse section of 10-d-old petiole expressing the initiation of the leaf primordium (arrows) at the proximal cut end of the petiole. **M, N.** Transverse section of 12-d-old petiole exhibiting the collapsing of the cells (arrows) in the meristematic nodular region. **O, P** Transverse section of 14-d-old petiole showing clear differentiation of leaf primordium (arrows).

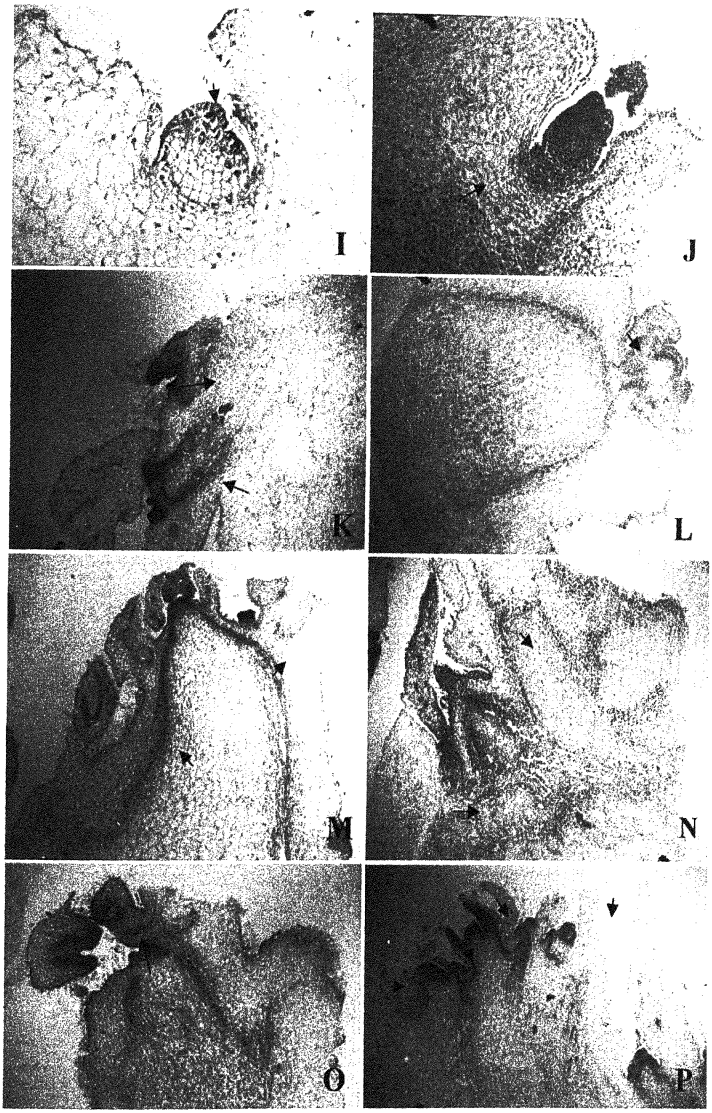

I
J
K
L
M
N
O
P

Figure 6. Restriction map of the plasmid used for microprojectile-mediated transformation of leaf explants from in vitro germinated seedlings of pigeonpea. Plasmid construct pRT99GUS:RChit used for microprojectile bombardment has marker genes such as *npt*II and *uid*A, and *RChit*, as candidate gene under the control of a constitutive promoter CaMV 35S and polyA terminator.

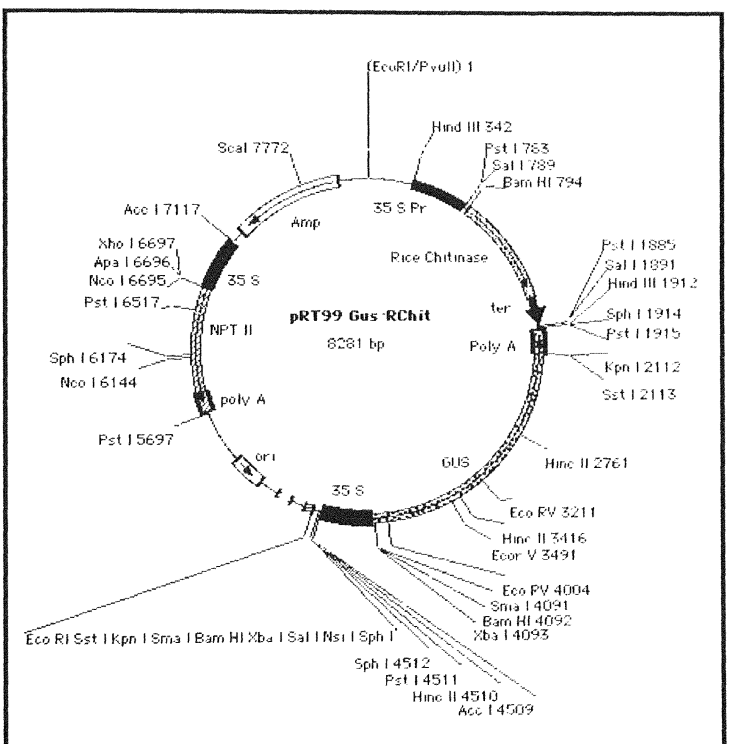

(EcoRI/PvuII) 1
Hind III 342
Pst I 783
Sal I 789
Bam HI 794
35 S Pr
Rice Chitinase
ter
Pst I 1885
Sal I 1891
Hind III 1912
Sph I 1914
Pst I 1915
Poly A
Kpn I 2112
Sst I 2113
Hinc II 2761
GUS
Eco RV 3211
Hinc II 3416
Ecor V 3491
Eco PV 4004
Sma I 4091
Bam HI 4092
Xba I 4093
Eco RI Sst I Kpn I Sma I Bam HI Xba I Sal I Nsi I Sph I
Sph I 4512
Pst I 4511
Hinc II 4510
Acc I 4509
35 S
ori
Pst I 5697
poly A
Nco I 6144
Sph I 6174
NPT II
Pst I 6517
Nco I 6695
Apa I 6696
Xho I 6697
35 S
Amp
Acc I 7117
ScaI 7772
pRT99 Gus-RChit
8281 bp

Figure 7. Arrangement of the leaf explant for microprojectile bombardment on a petri-plate

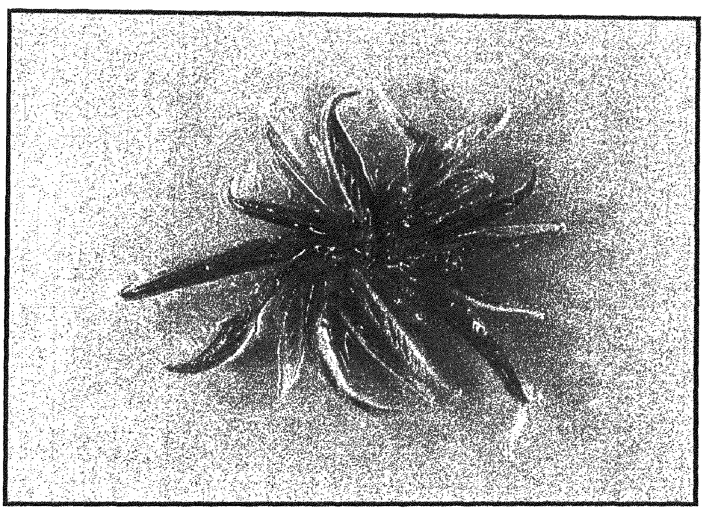


Figure 8A-B. Endogenous GUS activity in untransformed control and transformed bombarded pigeonpea leaves.

A. Untransformed control leaf showing faint blue.

B. Putative transformed leaf showing intense blue.

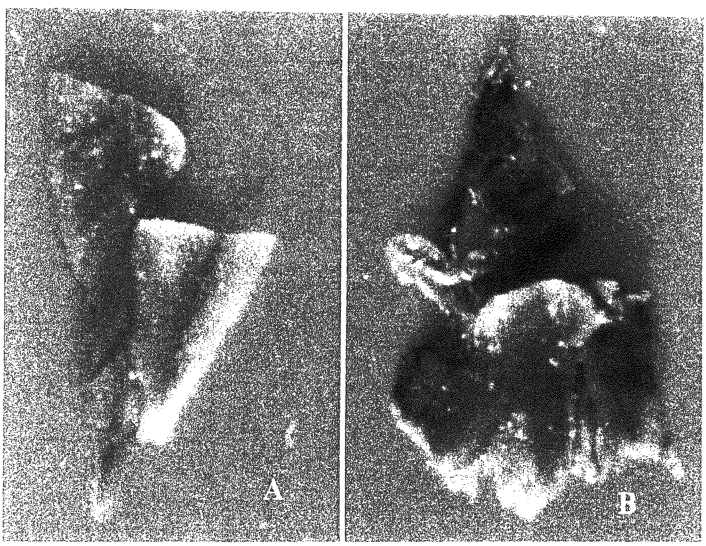

A
B

Figure 9A-B. Transgenic plants grown in the glass house [Pre-field screening, greenhouse facility (P_2 level), ICRISAT]

A. Ten-day-old T_1 generation seedlings grown in the glasshouse

B. Well established putative transgenic plants (T_1 generation) showing normal growth in the glasshouse

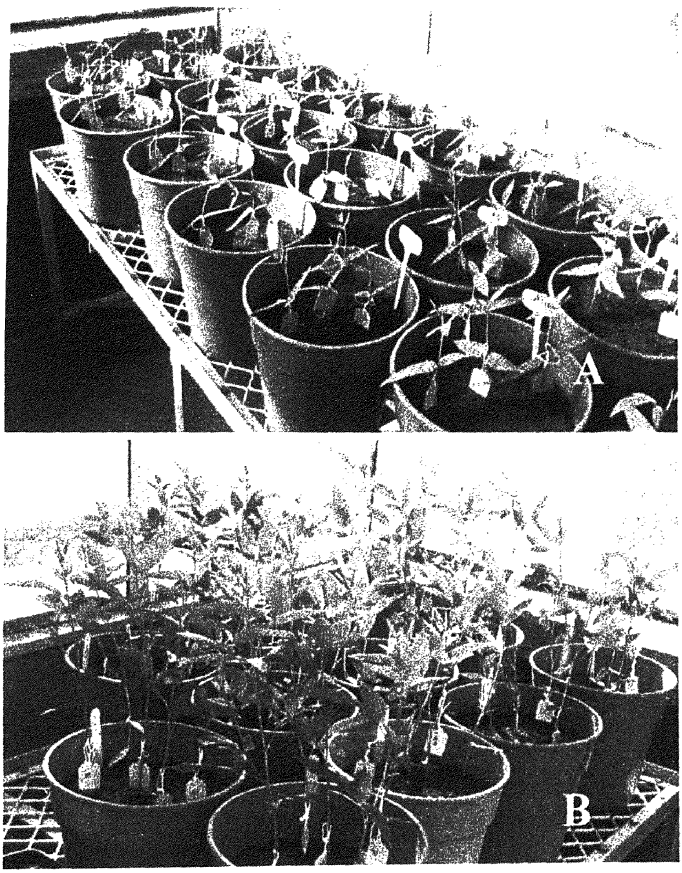

A
B

Figure 10A-B. Molecular analysis of putative transformants (T_0) of pigeonpea obtained after microprojectile bombardment using plasmid pRT99GUS:RChit. The lanes 1-17 carry genomic DNA from putative transformants; Lane 18 is untransformed control; Lane 20 is λ DNA restricted with *Hind*III.

A. PCR amplification on the genomic DNA showing amplification of the 1200 bp fragment of *uid*A gene. Lane 18 is DNA from putative transformed control; Lane 19 is DNA from pRT99GUS:RChit.

B. PCR amplification on the genomic DNA showing amplification of the 700 bp fragment of *npt*II gene. Lane 18 is DNA from untransformed control; Lane 19 is DNA from pRT99GUS:RChit

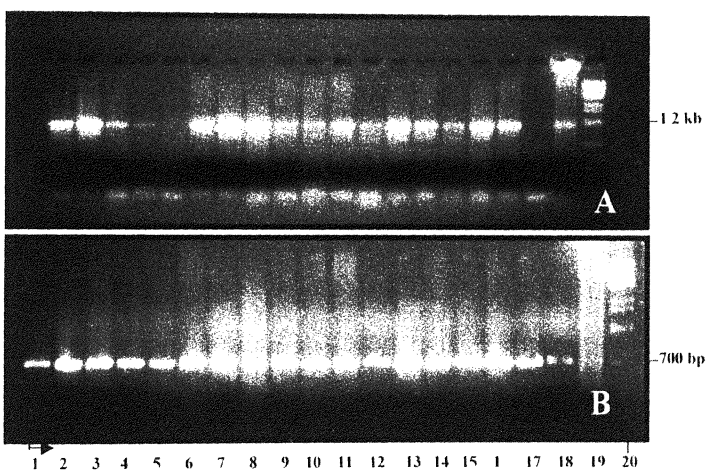

1.2 kb
A
700 bp
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 1 17 18 19 20

Figure 11A-B. Molecular analysis of putative transformants (T_0) of pigeonpea obtained after microprojectile bombardment using plasmid pRT99GUS:RChit.

A. PCR amplification of the genomic DNA showing amplification of the 525 bp fragment of *RChit* gene after microprojectile bombardment using plasmid pRT99GUS:RChit

Lane 11 is DNA from putative transformed control; Lane 12 is DNA from pRT99GUS:RChit. Lane 1 to 8 carry genomic DNA from plants transformed by microprojectile bombardment using plasmid pRT99GUS:RChit.

B. RT-PCR of the cDNA showing amplification of the 1200 bp fragment of the *uidA* gene Lane 18 is cDNA from untransformed control; Lane 19 is DNA from pRT99GUS:RChit. Lane 20 is λ DNA restricted with *Bst*I.

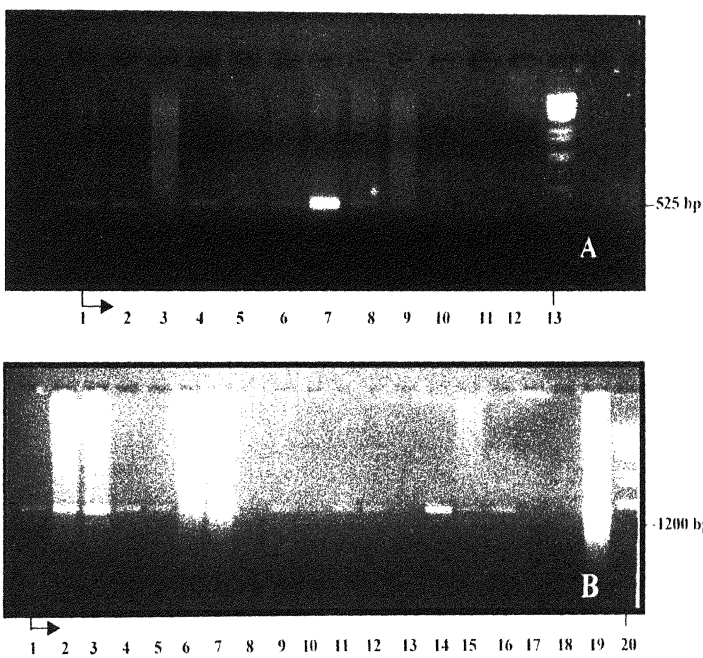

525 bp
A
1 2 3 4 5 6 7 8 9 10 11 12 13
1200 bp
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

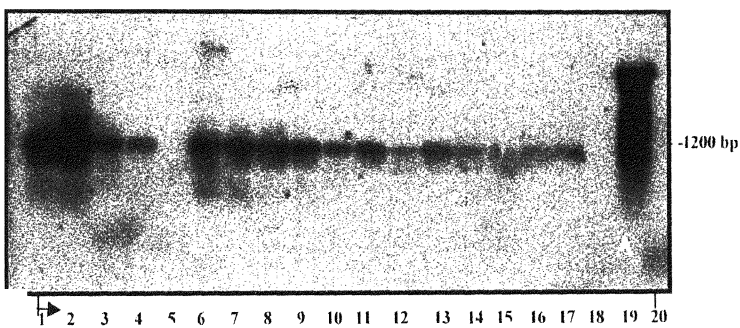
Figure 12A-B. Southern blot hybridization of PCR amplicons in the genomic DNA from putative transformants of T_0 generation. The blots were probed with non-radio Alkphos (Amersham UK.) labeled PCR amplified gene fragments.

A. Lane 1-17 shows PCR amplified products of genomic DNA from the putative transformants. Lane 19 is untransformed control, Lane 10 carries DNA fom the plasmid pRT99GUS:RChit.

PCR amplification of 1200 bp fragment of *uid*A coding region. The PCR products were resolved on 1.2% agarose gel and probed with non-radio Alkphos-labeled *uid*A gene from plasmid pRT99GUS:RChit restricted with *Hind*III .

B. Lane 1-11 show PCR amplified products of genomic DNA from the putative transformants. Lane 12 is untransformed control, Lane 13 carries DNA fom the plasmid pRT99GUS:RChit.

PCR amplification of 525 bp fragment of *RChit* gene coding region. The PCR products were resolved on 1.2% agarose gel and probed with non-radio Alkphos-labeled *RChit* gene from plasmid pRT99GUS:RChit restricted with *Hind*III .


~1200 bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

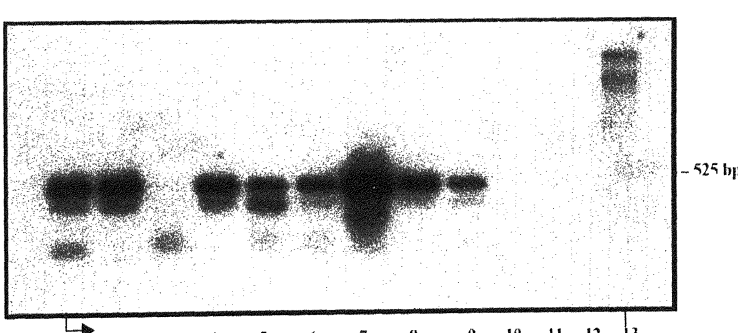
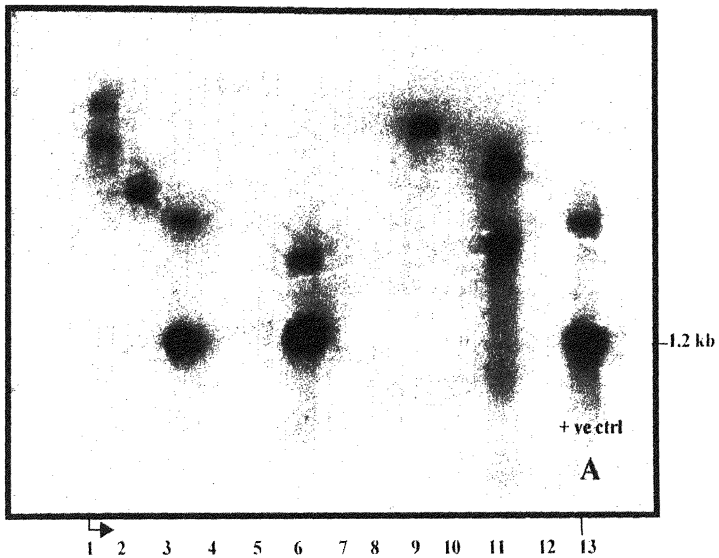

~ 525 bp
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 13A-B. Molecular analysis of putative transformants (T_0) of pigeonpea obtained after microprojectile bombardment using plasmid pRT99GUS:RChit.

A. Southern blot hybridization of *RChit* in the genomic DNA from putative transformants of T_0 generation. The DNA was digested with *Xba*I to provide a single restriction site within the plasmid DNA. The blot was probed with non-radio Alkphos labeled 525 bp PCR amplified *RChit* gene fragment to test the copy number. Lane 1-8 carry genomic DNA from the putative transformants transformed by microprojectile bombardment. Lane 9 is untransformed control, Lane 10 is plasmid pRT99GUS:RChit restricted with *Hind*III to release the 1.2 kb fragment of *RChit* gene.

B. Southern blot hybridization of *npt*II gene in the genomic DNA from putative transformants of T_0 generation. The DNA was digested with *Xba*I to provide a single restriction site within the plasmid DNA. The blot was probed with non-radio Alkphos labeled 700 bp PCR amplified *npt*II gene fragment. Lane 1-8 show genomic DNA from putative transformants. Lane 9 is untransformed control, Lane 10 is plasmid pRT99GUS:RChit restricted with *Hind*III to release the 700 bp *npt*II gene fragment.


1.2 kb
+ ve ctrl
A
1 2 3 4 5 6 7 8 9 10 11 12 13

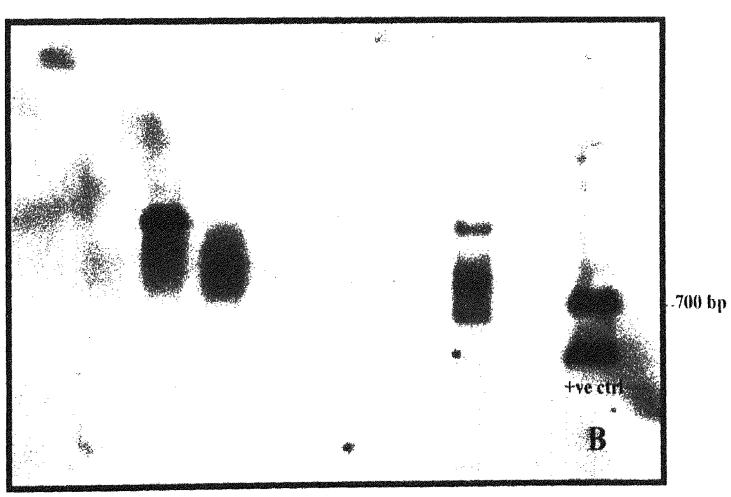
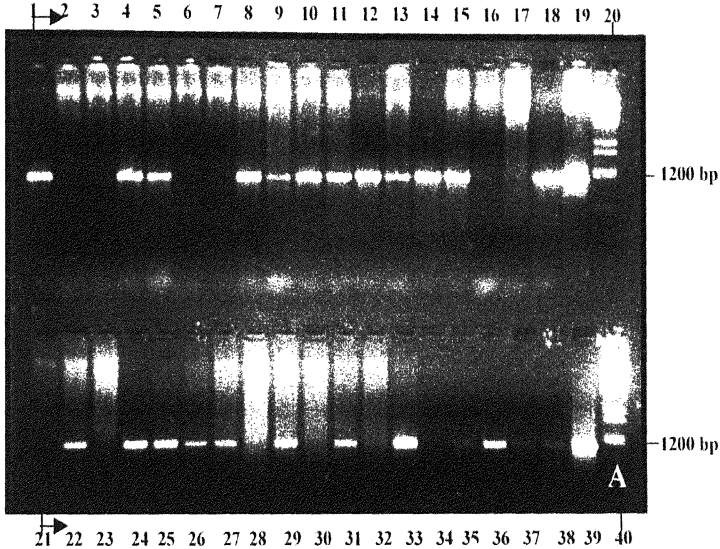

700 bp
+ve ctrl
B
1 2 3 4 5 6 7 8 9 10

Figure 14A-B. Molecular analysis of putative transformants (T_1) of pigeonpea obtained after microprojectile bombardment using plasmid pRT99GUS:RChit.

A. PCR amplification of the genomic DNA of 40 T_1 lines representing 10 independent transformed plants of T_0, showing amplification of the 1200 bp fragment of *uid*A gene. Lane 1-18 and lane 21 to 38 carry genomic DNA from the putative transformants of T_1 generation. Lane 19 and lane 39 is DNA from pRT99GUS:RChit. Lane 20 and 40 is λ DNA restricted with *Bst*I.

B. Southern blot hybridization of *RC'hit* gene in the genomic DNA from putative transformants of T_1 generation. The DNA was digested with *Xba*I to provide a single restriction site within the plasmid DNA. The blot was probed with non-radio Alkphos labeled 525 bp PCR amplified fragment of *RC'hit* gene. Lane 1-16 carry genomic DNA of 16 T_1 lines representing 10 independent transformed plants of T_0. Lane 18 is untransformed control, Lane 19 is plasmid pRT99GUS:RChit restricted with *Hind*III to release the 1.2 kb *RC'hit* gene fragment (arrows indicate the positive signal for gene insertion).


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1200 bp
1200 bp
A
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40

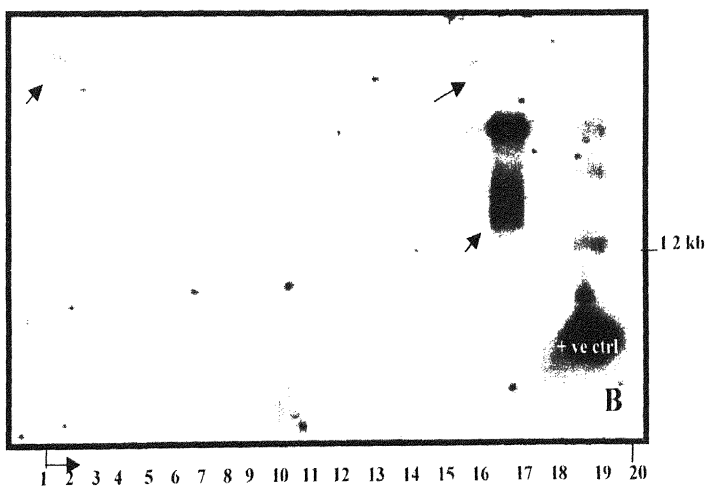

1 2 kb
+ ve ctrl
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 15A-B. Molecular analysis of putative transformants (T_2) of pigeonpea obtained after microprojectile bombardment using plasmid pRT99GUS:RChit.

A. PCR amplification of the genomic DNA showing amplification of the 700 bp fragment of *npt*II gene. Lane 1 to 8 carry genomic DNA from 8 T_2 lines representing 2 independent transformed plants, lane 9 is DNA from untransformed control; Lane 10 is DNA from pRT99GUS:RChit. Lane 11 is λ DNA restricted with *Hind*III.

B. PCR amplification of the genomic DNA showing amplification of the 1200 bp fragment of *uid*A gene. Lane 1 to 11 carry genomic DNA from putative transformed plants of 8 T_2 lines representing 2 independent transformed plants control, Lane 12 is DNA from pRT99GUS:RChit, Lane 13 is λ DNA restricted with *Bst*I.

C. PCR amplification of the genomic DNA of 16 T_2 lines from 4 independent transformed plants, showing amplification of the 525 bp fragment of *RChit* gene after microprojectile-mediated gene transfer method using plasmid pRT99GUS:RChit.
Lane 1 to 16 carry DNA from putative transformed plants of T_2, lane 19 is DNA from pRT99GUS:RChit. Lane 20 is λ DNA restricted with *Hind*III.

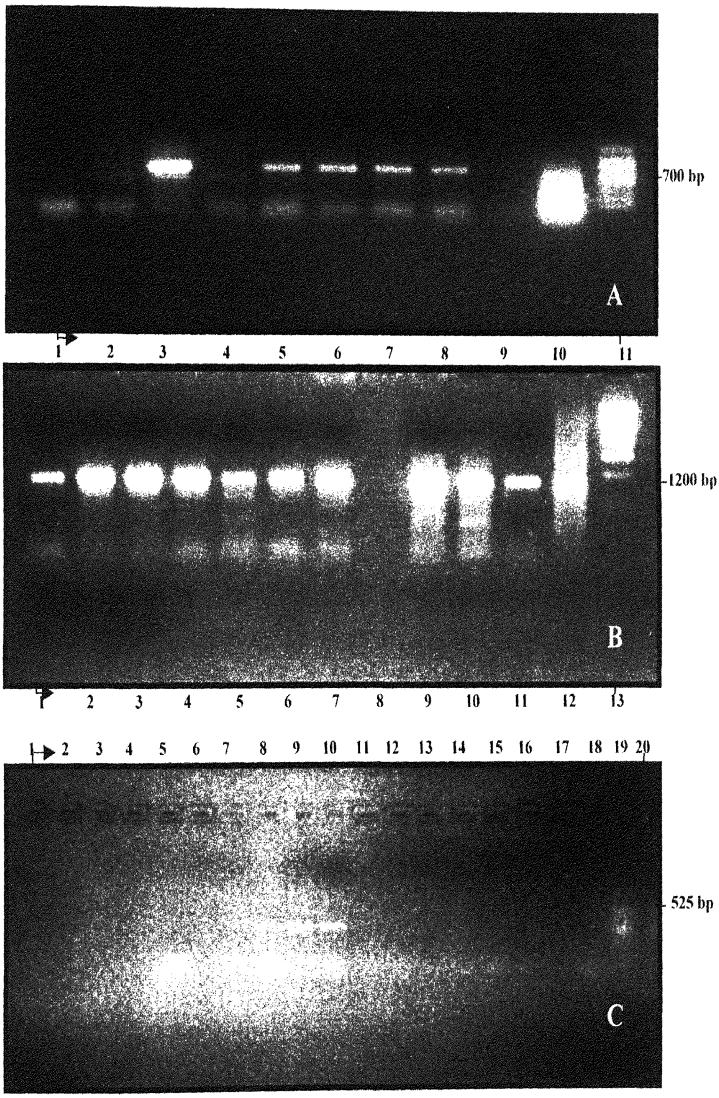
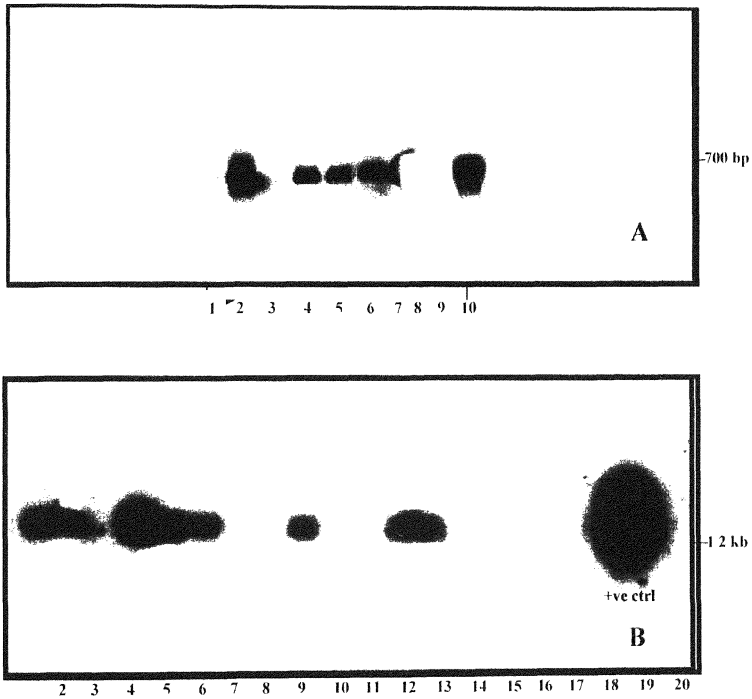

700 bp
A
1 2 3 4 5 6 7 8 9 10 11
1200 bp
B
1 2 3 4 5 6 7 8 9 10 11 12 13
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
525 bp
C

Figure 16A-B. Southern blot hybridization of PCR amplicons in the genomic DNA from the putative transformants of T_2 generation The blots were probed with non-radio Alkphos (Amersham UK) labeled PCR amplified gene fragments.

A. PCR amplification of 700 bp fragment of *npt*II coding region Lane 1-8 show PCR amplified products of genomic DNA from the putative transformants. Lane 9 is untransformed control, Lane 10 carries DNA fom the plasmid pRT99GUS:RChit.

The PCR products were resolved on 1 2% agarose gel and probed with non-radio Alkphos-labeled *npt*II gene.

B. Southern blot hybridization of *RChit* gene in the genomic DNA from putative transformants of T_2 generation The DNA was digested with *Hind*III to provide a double cut within the plasmid DNA to release 1.2 kb fragment of *RChit* gene. The blot was probed with non-radio Alkphos labeled 525 bp PCR amplified *RChit* fragment Lane 1-16 carry genomic DNA of 16 T_2 lines representing 4 independent transformed plants of T_2 generation Lane 18 is untransformed control, Lane 19 is plasmid pRT99GUS:RChit restricted with *Hind*III to release the 1.2 kb *RChit* gene fragment.


700 bp
A
1 2 3 4 5 6 7 8 9 10
1.2 kb
+ve ctrl
B
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Annexure

1. **Dayal, S., Lavanya, M., Devi, P.** and **Sharma, K.K.** (2003). An efficient protocol for shoot regeneration and genetic transformation of pigeonpea [*Cajanus cajan* (L.) Millsp.] using leaf explains. Plant Cell Rep., (in Press).

2. **Dayal, S., Lavanya, M., Devi, P.** and **Sharma, K.K.** (2003). Genetic transformation of pigeonpea (*Cajanus cajan* (L.) Millsp.) by using the rice chitinase gene for the development of resistance to fungal pathogens. Communicated to Plant Science.

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Genetic Transformation and Hybridization

An efficient protocol for shoot regeneration and genetic transformation of pigeonpea [*Cajanus cajan* (L.) Millsp.] using leaf explants

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Abstract A protocol for efficient plant regeneration from leaf explants of pigeonpea [*Cajanus cajan* (L.) Millsp.] was developed for the production of transgenic plants Leaf explants from 4- to 5-day-old in vitro raised seedlings were most efficient in producing multiple adventitious shoots in 90% of the explants on shoot induction medium [Murashige and Skoog (MS) medium +5.0 μM benzyladenine +5 μM kinetin] Shoot buds originated from the petiolar cut end of the explants and elongated rapidly on medium containing 0.58 μM gibberellic acid Over 80% of the elongated shoots rooted well on MS medium containing 11.42 μM indole-3-acetic acid and were transplanted with 100% success The procedure reported here is very simple, efficient and reproducible, and is applicable across diverse genotypes of pigeonpea The usefulness of this system for further studies on the genetic transformation of pigeonpea has been demonstrated in biolistics-mediated gene transfer by using *npt*II and *uid*A as marker genes, where 50% of the selected plants showed gene integration and expression

Keywords Biolistics - *Cajanus cajan* (L.) - Organogenesis - Pigeonpea - Transformation

Abbreviations

BA	N^6-Benzyladenine
GA_3	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
2-iP	N^6-[2-Isopentenyl]adenine
MS	Murashige and Skoog medium
RIM	Root induction medium
RT-PCR	Reverse transcriptase polymerase chain reaction
SEM	Shoot elongation medium
SIM	Shoot induction medium

Communicated by P P Kumar

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To
The Dean
Faculty of Science
Osmania University
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Dated 12 6 2003

From
Ms G Suneetha
Research Scholar
(Regd for Ph D)
Department of Botany
Osmania University

Through Proper Channel

Sir,

This is to inform you that I G Suneetha, a registered Research Scholar (Ph D) vide OU letter no 505/DFSC/98-99 Dated 5th July 1999, have completed my research and presently submitting my thesis for evaluation for the award of Ph D degree I have worked as Project Fellow in the AP-Netherlands Biotechnology Project **"Development of disease resistant transgenic pigeonpea"**, under the supervision of the Principal Investigator Dr B Prathibha Devi and the Co-investigator Dr Kiran K Sharma The Project work involved the use of several methods for genetic transformation of pigeonpea and was carried out mostly in the Genetic Transformation Laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India However, the research concerning the use of microprojectile method of gene transfer has been compiled for my Ph D thesis entitled **"Studies on the in vitro culture and genetic transformation of pigeonpea (*Cajanus cajan* (L.) Millsp.) for induced fungal pathogens"**. This is for your information

Thanking you,

Yours sincerely

G Suneetha

Prathibha Devi
12th June 2003

Signature of Principal investigator

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