Chapter I

INTRODUCTION

Legumes are the most important source of plant proteins and energy, they are cultivated throughout the world in wide areas. The grain legumes are cultivated in the tropics, sub-tropics and temperate zones. They are amongst the earliest food crops to be cultivated by man, and constitute one of the most important dietary proteins, especially in Asia, Latin America and Africa. They are of paramount importance not only for their value as human food, but also because of the high protein content for livestock and fish feed. Moreover, legumes are endowed with the ability to fix atmospheric nitrogen and thus improve soil fertility. In different countries they are consumed in various forms, such as immature seed, dry grain, as condiments or roasted grain, for flour production and as fermented products (Parpia, 1973).

The high level of lysine makes grain legumes an ideal supplement for cereals, which are deficient in this particular amino acid. Legumes also form a good source of vitamins, thiamine and niacin as well as minerals, calcium, and iron. In addition to these constituents, they possess about 60 percent carbohydrates, mainly in the form of starch.

Pulses are the edible seeds of various leguminous crops such as peas, beans and lentils. For India the most important pulses are pigeonpea, chickpea,
black gram, and mung bean. Pulses are an important component in the vegetarian diet. Together with grains from graminaceae, such as wheat or rice, they provide a balance in essential amino acids, since pulses are rich in basic amino acids and poor in sulfur containing amino acids, while the reverse is true for graminaceous grains. The basic Indian dish is rice with *dal*, a mash made from pulses.

Pulses are sensitive to various biotic stresses such as insects and fungi as well as to abiotic stresses such as drought, flooding and freezing affecting full realization of the yield potential of these crops. There is a need to increase productivity and enhance the nutritional value of these pulse crops. For the farmer, they are therefore "risk-crops". Reducing this risk by providing resistant plants would encourage a more widespread cultivation of pulses and ensure a sufficient supply of these legumes in a healthy vegetarian diet in the poorest regions of India.

Pigeonpea [*Cajanus cajan* (L) Millsp. family, Leguminosae] is the second most important food legume of India and is extremely drought tolerant. It is important for its value as food and fodder and also for its capacity for biological nitrogen fixation in symbiosis with *Rhizobium* sp. Present yield level of pigeonpea is low i.e. the average production is only 583 kg/ha which is due to damages caused by various fungi, bacteria, viruses and insect-pests.

India accounts for 85 percent of the world pigeonpea production and more than 4.0 million hectares are covered under pigeonpea cultivation. Global
production of pigeonpea is in excess of 2.7 million tonnes. Pigeonpea contributes to sustainable cropping systems by nitrogen fixation, and adding organic matter via its deep rooting system. Pigeonpea is unique in that its duration is a continuum of extra-short duration (<90d) to perennial types. This nature of the species allows it to adapt to a wide range of cropping systems, soils and climatic variations. The medium duration (160 – 180 d) pigeonpeas are also cultivated similar to those of long duration group but under more harsh environments with mean annual rainfall of about 700 mm. The early (120 – 140 d) pigeonpeas are of recent origin and are cultivated as sole crop under high density production system.

The crop losses without the use of pesticides and other non-chemical control strategies worldwide are estimated to be about 70 percent of crop production. The world wide pre-harvest losses due to insect pests, despite, the use of insecticides are 15 percent of the total production (Krattiger and Anatole, 1997). Moreover, the insect pests can overcome the effect of toxic materials or bypass the natural resistance.

This problem is more acute in the tropics and sub-tropics, where the climate provides a highly conducive environment for a wide range of insects and necessitates massive efforts to suppress the population densities of different pests in order to achieve an adequate supply of food. Hence, in order to feed the ever increasing population, particularly in the developing countries viz. Africa, Asia, and Latin America the production of food had to be increased manifold.
This increase in food production has to come from increased yields from major crops grown on existing cultivable lands. One practical mean of achieving greater yield is to minimise the pest associated losses, which are estimated at 14 percent of the total agricultural production. In addition to direct losses caused by insects, there are additional costs in the form of pesticides applied for pest control. Pesticide application not only affects the non-target organisms, but also leaves harmful residues in the food, and results in environmental pollution.

As a result, the chemical control of pests is under increasing pressure. Pesticide use in the world is declining. This has necessitated the use of target specific compounds with low persistence, and an increase in emphasis on integrated pest management based on host plant resistance to insect pests. Although the benefits to agriculture from the pesticide use to prevent insect associated losses cannot be overlooked, there is a greater need to develop alternative or additional technologies, which would allow a rational use of pesticides, and provide adequate crop protection for sustainable food, feed and fiber production in the future.

The major objectives in pigeonpea breeding are to increase grain yield, and to produce resistant varieties. It is cultivated in 5.25m ha of land in Asia, Africa and America. Endowed with several unique characteristics, it finds an important place in the farming systems adopted by small holder farmers in a number of developing countries. Conventional breeding methods for genetic improvement of pigeonpea have been restricted due to non-availability of better
genetic resources, presence of strong sexual barriers and incompatibilities among wild relatives. There is need to widen the genetic base and incorporate desirable characters into this crop plant. There is an urgent need to use transgenic technologies for improvement of pigeonpea (Chandra and Pental, 2003). Therefore, tissue culture techniques amalgamated with biotechnology seems to provide some breakthrough in this area.

In crop plants, many of the shoot regeneration protocols have been developed in recent years specifically for exploitation in genetic manipulation experiments (Bohmer et al., 1995). However, several requirements must be fulfilled in order to produce stably transformed plants. Initially, a suitable method is required to deliver foreign DNA to plant tissues, followed by the appropriate procedure for culturing tissues prior to the regeneration of shoots leading to the recovery of transgenic plants.

With the advent of genetic transformation and recombinant DNA techniques, it has become possible to clone and insert genes into the plant genome that confer resistance to insects (Bennett, 1994). Genes from bacteria such as *Bacillus thuringiensis* (Bt) and *Bacillus sphaericus* have been the most successful group of organisms identified for use in genetic transformation of crops for pest control on a commercial scale (Gill and Saxena, 1992; Charles et al., 1996). Protease inhibitors, plant lectins, ribosome inactivating proteins, secondary plant metabolites, vegetative insecticidal proteins from *Bacillus thuringiensis* and related species, small RNA viruses can also be used alone or
in combination with Bt genes to generate transgenic plants for pest control (Hilder and Boulter, 1999).

Recombinant DNA technology offers the possibility of developing entirely new biological insecticides that retain the advantages of classical biological controlling agents. The basic requirements for genetic transformation are: 1) a target genome, 2) a candidate gene, 3) a vector to carry the gene, 4) modification of the foreign DNA to increase the level of gene expression, 5) method to deliver the transgene into the cell, 6) protocols to identify the transformed cell, 7) tissue culture and procedures to recover the viable plants from the transformed cells and 8) characterization of the putative transgenic plants at the molecular and genetic levels. Subsequently, the newly introduced gene(s) must be expressed in transgenic plants and finally, the foreign DNA must be heritable and expressed reproducibly in succeeding seed generations.

The transgenic plants provide season long protection against the target pests, while the pesticides need to be applied several times during the growing season. Also, only the insects feeding on the crop are exposed to the toxin, and this overcomes the difficulty of targeting pesticide application at the site of insect feeding.

1.1 Constraints in pigeonpea production:

Helicoverpa armigera or legume pod borer is the single most important biotic constraint affecting pigeonpea crop. It attacks pigeonpea crop causing
substantial damage and yield loss every year. Intensification of agriculture has exacerbated the pest problems, and farmers are responding by using more toxic pesticides more frequently. It has long been recognized that host plant resistance is one of the most effective management options. However, the levels of resistance in the available germplasm are moderate to low. Given the wide host range of this pest, only relatively low levels of resistance have been identified. Moreover, excessive use of insecticides to control this insect is not only environmentally hazardous, but has also resulted in the development of resistance to various insecticides thus making it more difficult to address this problem through chemical control. Therefore, development of crop cultivars through genetic engineering of pigeonpea complement to *Helicoverpa* control in this crop.

1.2 Genetic variation:

Primary and secondary gene pools of pigeonpea consist of large variation for various qualitative and quantitative traits. To determine the most efficient breeding approach for genetic improvement of pigeonpea information on the genetic parameters of yield and related traits such as flowering, maturity, pods per plant, branches per plant, plant height and seed size is necessary. In pigeonpea, such information is rather limited. The important agronomic characters are primarily controlled by genes with additive effects (Sharma and Green, 1975). Saxena and Sharma (1981) opined that in quantitative breeding
of pigeonpea where phenology is sensitive to environmental influences, the basic problem is that the interpretation of the results of matching designs is complicated by physiological changes associated with phenological differences.

1.3 Genetic engineering for increased resistance to *Helicoverpa armigera*:

Genetic engineering of pigeonpea includes the incorporation of novel genes for insect resistance in the cultivated varieties of pigeonpea by genetic transformation. The gene of immediate importance is those coding for insecticidal crystal protein from *Bacillus thuringiensis* (Bt-Cry genes).

*Helicoverpa* lays its eggs in the newly formed floral buds where the emerging larvae (first instar) feed on the floral organs. The life continues through a sixth instar larvae that bore into green pods and feed on the developing seeds. Hence, it would be most efficient to express the introduced insecticidal genes in these organs for effective control of the insect.

1.4. Genetic engineering of the crop:

Tissue culture offers a potential tool to contribute to the improvement of crop plants through the manipulation of plants at cellular level. The capacity to regenerate plants from cultured cells permits tissue culture techniques to be applied to the development of transgenic pigeonpea plants. In this context the standardization of protocols of *in vitro* plant regeneration and *Agrobacterium*
mediated as well as microprojectile bombardment method of gene transfer is of fundamental importance to future genetic manipulation of this crop. Although many different techniques (electroporation of intact tissues, silicone carbide whiskers, etc.) have been tested for gene delivery to plant cells, two major methods, namely *Agrobacterium*-mediated and microprojectile (particle) bombardment, have been extensively employed for genetic transformation of crop plants. Regeneration *via* callus lends itself easily (compared to explants regenerating directly) to *Agrobacterium*-mediated transformation, while direct regeneration is more amenable for particle bombardment.

As a result of advances in genetic transformation techniques and gene expression during the last decade (Bevan *et al.*, 1983; Horsch *et al.*, 1984), there has been a rapid progress in using genetic engineering for crop improvement, of which protection of crops against the insects is a major goal. The potential of this technology has now been widely recognized (Burke and Thomas, 1997). Once efficient protocols for tissue culture and transformation are developed, the production of transgenic plants with different genes is fairly routine (Sharma and Ortiz, 2000). Most of the insect resistant transgenic plants have been developed by using Bt δ-endotoxins. Development and deployment of transgenic plants with insecticidal genes for pest control will lead to reduction in insecticide sprays, increased activity of natural enemies and IPM of secondary pests. In view of the above, the present study was initiated with the
objective to optimise a suitable protocol for pigeonpea transformation to generate insect resistant pigeonpea plants.

1.5 Objectives:

The main objectives were

→ To standardize callus induction from various explants of pigeonpea \textit{viz.} leaf, cotyledons, embryonic axes of four different genotypes \textit{i.e.} TRA-1, UPAS 120, MSK and AF 239.

→ To standardize a plant regeneration protocol from callus of the above explants and direct plant regeneration from cotyledons and embryos of pigeonpea.

→ To standardize a protocol for \textit{Agrobacterium} mediated transformation and particle bombardment method of transformation for developing pod-borer resistant pigeonpea plants.

→ Molecular analysis of the putative T0 transformed plants.

→ To develop pod borer resistant pigeonpea plant.

Results obtained from this study will help to identify suitable medium for callus induction and plant regeneration from various explants of pigeonpea. This study will also help in standardizing a protocol for plant transformation and obtaining pod borer resistant pigeonpea plants.