Introduction
CONTENTS

I. The Genus *Psophocarpus*

II. Reproductive biology

III. Tissue culture

IV. Micropropagation of grain legumes
   1. Shoot tip and nodal segment culture
   2. *De novo* adventitious shoot multiplication
      i. Direct organogenesis
      ii. Indirect organogenesis
   3. Somatic embryogenesis

V. Variation in tissue culture

VI. Scope of the work
The worldwide output of agriculture is unable to meet even the most basic needs of a sizable fraction of humanity suffering from hunger and malnutrition. The number of chronically undernourished people in the world remains alarmingly high, amounting to 850 million people. Half the world, or nearly three billion people live on less than two dollars a day and around 1.1 billion live in extreme poverty on less than one dollar a day. These conditions have caused over one billion children (more than half of those living in developing countries) to suffer from the severe effects of poverty and 674 million (over a third) are living in conditions of absolute poverty (Bourgeois and Susila, 2006).

The development of underutilized species has been perceived as a potential alternative to alleviate these problems. The underutilized plant species are those that appear to have considerable potential for use, yet whose potential is barely exploited, if not totally neglected, in agricultural production. The term “underutilized plant species” covers an enormous range of plants that have the potential to contribute to food and nutritional security, and poverty alleviation. Presently about 7,500 plant species are considered edible. Yet, global food security is increasingly based on a narrowing range of animal and plant species. About 95% of the worlds’ food energy needs is provided by just 30 plant species and 50% of the requirement for protein and calories is met just by maize, wheat and rice (Bourgeois and Susila, 2006).

Underutilized crops make important contributions to the nutrition and health of people in developing countries. These potential benefits provide a good reason for conserving agrobiodiversity, which receives little attention nationally and internationally. As the growing dependence of populations worldwide on a few
staple crops leads to increasing health problems, more attention is being given to biodiversity and the interdependence between human and environmental health. As threats to biological diversity accelerate, the plant genetic resources that guarantee the current and future production of healthy foods, beverages and medicines must be better used to improve the well-being of those whose food security and health is at risk.

The underutilized species can increase food security, especially, if they are adapted to specific marginal agricultural conditions. Many underutilized crops have important nutritional qualities, such as high fat content, high quality proteins (essential amino acids), a high level of minerals (such as iron), vitamins, or other valuable nutrients which have not yet been described satisfactorily. They are therefore a significant complement to the ‘major’ cereals and serve to prevent or combat the ‘hidden hunger’- a diet deficient in vitamins, minerals and trace elements- that is prevalent in developing countries. These crops are capable of supplying both foodstuffs and industrial raw materials, which will offer new opportunities for income generation if their market potential is successfully recognized and developed. These crops also offer potential for the sustainable use of more challenging sites, such as semi-arid or mountain regions. Though these species, like other minor plant species, do not contribute much as basic food, yet they do contribute significantly to diversify the human diet.

A large number of leguminous species that have not yet been exploited or are underexploited have a great potential for contributing to nutritive food, feed and forage needs of the tropical countries of the world. Grain legumes, also known as
pulse crops, by virtue of their pivotal role in human nutrition, are of great economic importance and are next only to cereals as important food sources. The importance of legumes has been known to people by way of its important place in human nutrition as rich sources of proteins, vitamins and minerals.

The protein content of legumes is higher than in other vegetable products and so are nearer to animal flesh in food value. The per-acre production of protein is higher for legumes than for cereals and they provide protein rich food for man and animals (Kochhar, 1981). Protein quality depends upon several factors such as digestibility, balance of essential amino acids and the presence of certain toxic substances. One of the very important aspects of world’s nutritional problem concerns the intake of sufficient quantity of essential amino acids. Humans and other non-ruminant animals like swine and poultry cannot synthesize certain amino acids; their diets must contain these essential amino acids in sufficient quantity for proper growth and maintenance (Widholm, 1979). Legume proteins are rich in lysine and threonine, but poor in sulphur containing amino acids, methionine and cysteine. But the high levels of lysine and threonine make grain legumes an ideal supplement for cereals that are deficient in these particular amino acids. In addition to these they possess about 60% carbohydrates mainly in the form of starch. The caloric yield of pulses is almost same as that of cereals. Besides being rich in proteins, pulses like soybean and groundnut constitute an important source of edible oils. Legumes have also played an important role in development of cropping pattern largely due to the inherent N₂ fixing ability. India is the largest producer of pulses in the world and more than a dozen pulse crops are grown in different parts of the country.
In this era of returning back to nature, the use of legumes as medicines is of great importance. Of late, it has been reported that the legumes is a treasure box of medicines. Most of the legumes are used as medicines and are even used for the treatment of cancer and AIDS. The bean is said to be an untapped medicine chest. *Mucuna* is a great source of a number of chemicals, including ‘daidzein’ – an anti-inflammatory and anti-microbial agent, ‘daidzin’- a cancer preventive and ‘genistein’- an anti-leukemic agent. Velvet bean is a source of ‘dopa’, which the brain converts to the neurotransmitter dopamine, the reduction of which will lead to Parkinson’s disease. This bean also contains ‘serotonin’, a neurotransmitter related with learning, sleep and control of moods. The potential breast cancer fighting chemical known as kievitone is found in hyacinth bean (Morris, 2003).

Since many legumes are self-fertile, they have only a narrow genetic base. The success of crop improvement programme is therefore, primarily dependent on the extent of genetic variability in the base population. Thus the desired goal in grain legume improvement programme can be achieved by introducing new genetic variability into the base population and exploiting them on the execution of complex research programmes (Sandhu, 1992).

*Psophocarpus tetragonolobus* (L.) DC., commonly known as ‘winged bean’ is an underutilized leguminous crop, which has drawn world wide attention of the scientists mainly due to its food value. This plant of high economic importance to the local people and even to the international community, are often neglected due to lack of documented information of the plant. Several characters of wild plants still exists in most of the winged bean accessions, *viz.* viny and indeterminate habit of growth,
photosensitivity, pod shattering (when dry on the vine), presence of toxic substances in the raw dry beans and in the rind of the tubers, uneven germination rate, low yield, and above all, tremendous diversity of form in all characters.

To overcome these problems of this crop, conventional plant breeding techniques and tissue culture techniques can play a significant role. A thorough knowledge of the floral biology of the plant is necessary for successful plant breeding techniques.

I. The Genus *Psophocarpus* Necker ex. A. P. de Candolle

*Psophocarpus* Necker ex. A. P. de Candolle

Subclass: Polypetalae

Series: Calyciflorae

Cohort: Rosales

Family: Leguminoseae [Fabaceae]

Subfamily: Papilionoide

Class: Dicotyledoneae

*Psophocarpus* is a member of the family Leguminoseae and the subfamily Papilionoideae. The genus *Psophocarpus* was first reported by Necker in 1790, but its legitimate publication dates from 1825 (Verdcourt and Halliday, 1979). It is synonymous with other genera described as *Botor*, *Diesingia*, *Psilocarpus* and *Vignopsis*.

The species of *Psophocarpus* are *P. tetragonolobus*, *P. grandiflorus*, *P. palustris*, *P. scandens*, *P. obovalis*, *P. monophyllus*, *P. lecomtei*, *P. lancifolius* and *P. lukafuensis*. According to the FAO (1982) report on Winged bean, *P. tetragonolobus* is the only species in the genus that does not occur in the wild.
**P. tetragonolobus (L.) DC**

**Common names:** Winged bean, Asparagus bean, Asparagus pea, Four Angled bean, Four Cornered bean, Flying bean, Goa bean, Manila bean, Mauritius bean, Princess pea, etc. The name ‘Winged bean’ was first adopted in 1974 at a panel meeting convened by the National Academy of Sciences (NAS, 1975).

The first authentic account of Winged bean was recorded by Rumphius (see Verdcourt and Halliday, 1979) in the 18th century. Loureiro mentioned it in 1790 (see Burkill, 1906). In South Asia, it was mentioned as early as 1814 by Roxburgh in India, although Chandel et al., (1979), reported that it was first introduced in Calcutta in 1799. By the end of the 19th century, its occurrence had been documented right across the humid parts of the Indian subcontinent, Burma, Thailand, Malaysia, Indo-China, Philippines, Indonesia and Masearene Islands.

Although it is an old world species, three widely separated areas have been claimed to be the centers of origin: - Burkill (1906) believes that it originated in the African side of the Indian Ocean, because of the occurrence of other species of the genus in Africa and the Arabic word “botor” commonly used for this plant in Malaysia and Indonesia; Vavilov (1949/50) suggested that it belonged to the Indo-Malayan center, based on his awareness of winged beans present in the North-Eastern hill tracks of Assam in India; Hymowitz and Boyd (1977) believed this plant to have evolved in Papua New Guinea in view of the high genetic diversity occurring in that area.

*Psophocarpus* has recently been acclaimed as a potential crop for the supply of proteins, especially in the hot and humid tropics where heavy rains create
unfavorable conditions to cultivate other food crops and hence is believed to overcome the malnutrition of the third world countries. Although less tolerant to water logging, it is relatively free of pests (Rachie and Roberts, 1974), and is easy to grow on a wide range of soil conditions, in both low lands and high lands.

The plant is a perennial, climbing, herbaceous, short day legume, upto 5 m long, with trifoliate leaves, tuberous roots and pods 4 angled, 15 – 22 cm long and each angle continued into a much crisped and toothed papery wing. This weak stemmed twiner bears leaves with broad, ovate leaflets 7.5 – 15 cm long. Its flowers are large with light blue or white colour, in lax racemes and are cleistogamous. The seeds are nearly globular, 0.6 m across, smooth and yellow, brown or dark brown in colour.

An outstanding feature is that, it contains significant amount of protein in all the parts of the plant, ie. seeds, pods, leaves and roots (National Academy of Science, 1975), all of which are edible. Since nearly every portion of the plant is edible or can be profitably utilized, several superlatives like ‘wonder legume’, ‘miracle plant’, ‘the miracle bean’, ‘wonder bean’ and ‘backyard supermarket’ have often been used for the winged bean. The tubers (Plate 1:10) contain 20% protein in dry weight. This amount is superior to other tubers, such as yam (2%), cassava (1%), potato (2%) and sweet potato (2%). The percentage of crude proteins of the seeds (29.8 - 37.4%), is comparable to soybean seeds (35%), and is higher than that of other legumes (Newell and Hymowitz, 1979). The young leaves are eaten as a vegetable, either raw or steamed. Stem and leaves contain appreciable amount of vitamin A and C and are used as green fodder also. The nutrient content of the
various plant parts are given in Table I. In addition to this, the stem and leaves also contain digestible protein 4.8%; calcium (CaO) 0.37% and phosphorus (P₂O₅) 0.12%. Hydrocyanic acid has been reported in the stem (Teik, 1951), which is an antinutritional factor. The flowers are eaten or added to various dishes to colour them blue. The young pods are harvested before they mature. The young and tender pods are succulent and sweet and are eaten either raw or cooked. They contain appreciable amount of calcium, iron, thiamin and ascorbic acid. Mature pods become tough and fibrous. The edible, tender pods contain: calcium 63%, phosphorus 37%, iron 0.3%, sodium 3.1%, potassium 205, thiamin 0.24%, riboflavin 0.09%, nicotinic acid 1.2%, ascorbic acid 19 mg/100g and vitamin A 595 I.U/100g. The non-protein nitrogen accounts for 44.2% of the total nitrogen in the vegetable. The free aminoacids present are serine, aspartic acid, glycine, glutamic acid, alanine, tyrosine and all the essential aminoacids except histidine and methionine (Kulkarnie and Sohonie, 1956).

*Psophocarpus* is also called “a food pharmacy,” due to its nutritional and medicinal potential. It contains high levels of lectins that are used as diagnostic tools in medical research because they bind to certain blood cells. They also contain erucic acid- an anti-tumor compound and polyunsaturated fatty acids that is used for the treatment of acne and eczema. The winged bean pod is good for blood and is used against diabetes mellitus. The leaves of this plant have been used for the treatment of small pox. The roots have been used in Malaya as a poultice to cure vertigo (Hymowitz and Boyd, 1977). The seeds are rich in tocopherol (Pospisil *et al.*, 1971), an antioxidant that enhances the utilization of
vitamin A in the human body. The best-reported source so far of ‘betulinic acid’ - a triterpene used for the prevention of malignant melanoma, is the edible winged bean. Agmatine and isovitexin, potential combatants of microbial organisms in mammals including humans is found in winged bean (Morris, 2003).

The bean prefers comparatively warm and humid regions and thrives well in the damper parts of India. The seeds are sown early in the rains and the vines may be supported on walls or trellis. The plants flower in a couple of months and continue to grow during the greater part of the year, except during the dry warm months. Where the plants are grown for their tubers on a field scale, as in Myanmar, they are left to spread on the ground.

Even though the plant is a perennial; better results are obtained when planted afresh every season. The young pods are harvested before they mature. Each vine yields about 25 pods every 5 – 6 days and the productivity continues if the vines are liberally manured every 2 – 3 weeks. If green pods are removed periodically, flowers are continuously produced for three months. In tropical areas, it requires short day regimes. According to Masefield (1961), long day conditions and low light intensities are inhibitory for flowering. In subtropical regions, they are considered either as short day plants or day neutral plants. Uemoto et al., (1982) found the critical photoperiod for raceme budding to be approximately 12 hours.

The flowers are cleistogamous, ie. fertilization takes place even before the flower opens and is self-pollinated due to precocious anthesis. However, cross-pollination by the insect Xylocopa aruana Ritz. may account for the low percentage of heterozygozity. Even though the flower is cleistogamous,
Senanayake and Thiruketheeswaran (1978) have reported that the stigma is receptive up to 35 hours after the flowers have opened which could result in a higher degree of out crossing. The varying percentage of out crossing explains heterozygosity observed in natural winged bean populations in Papua New Guinea, Indonesia, Malaysia, Bangladesh (Haq, 1982) and Thailand (Chomchalow and Pongpangan, 1981). However, the extent of heterozygosity in these countries has not yet been established.

In Java, the seeds are eaten after roasting. The seeds are reported to be somewhat indigestible and the dry seeds cook with difficulty. They contain a trypsin inhibitor that is not affected by heating in boiling water and is partially destroyed to about 61% by autoclaving. The oil is similar to soybean oil and can be used for cooking and soap making and the cake can be used as human food as well as stock feed.

Another interesting aspect of the winged bean is the formation of numerous root nodules on the roots without artificial inoculation, when the soil contains compost, peat and sand. It shows symbiotic association with a wide range of tropical *Rhizobium* (Ikram and Broughton, 1978). The yield of tubers varies from 2.5 – 6 tonnes/ hectare. The plant exceeds in nodulation, when compared with most of the other species of leguminous green crops tried, with the calculated weight of 0.75 tonnes/ hectare. In Burma, the sugarcane crop following a crop of winged bean is said to yield half as much more than usual. Therefore, winged bean can be used for its great value as green manure, cover crop, fodder crop or restorative follow crop, particularly in the wet tropics (Masefield, 1957). The root nodule
containing tubers are edible also. In Myanmar, the tuberous roots are eaten as a delicacy. The tuberous roots swell early and by the time roots are ripe, they are stringy and insipid. They have a fairly agreeable flavour when boiled. They are difficult to cook thoroughly because of their hardness. The starch grains of the tubers vary in size and shape, with the longer grains predominating. The roots are rarely infected with *Meloidogyne*, a root knot causing nematode. This infection causes galling of the infected roots (Plate 1: 11) and prevents the formation of tubers. Price and Linge (1979) have reported the biology and epidemiology of root knot disease. Temperatures ranging from 25-30°C appear to favour the organism. Both seed and tuber yields of the plant is reduced due to root knotting. In glass house experiments, the nematode infection was found to cause reduced leaf and shoot growth, indicating substantial potential reductions in the number of pods and seed yield (Price *et al.*, 1981).

Although this is a wonder crop, several difficulties are faced by farmers in cultivation of this crop. The most important factor that affects the farmers is the poor and delayed germination of seeds. Another problem faced by the farmers is the cost of providing support for the plant. If proper support is not provided, stunted growth of the plant is observed. Similarly, reduced flowering and pod formation also occurs. To obtain a good yield proper support must be provided. The cost of providing suitable support in large-scale cultivation is very high. This refrains the farmers from cultivating this crop on a large scale.
II. Reproductive biology

The reproductive biology of flowering plants is important for determining barriers to seed and fruit set, for conservation, and for understanding pollination and breeding systems that regulate the genetic structure of populations.

The developmental stages in reproductive cycle from meiosis to seed formation play an important role in the life cycle of higher plants. For the many species propagated only by seed, effective functioning of these developmental pathways is essential for survival and performance. Even when asexual propagation is possible, the sexual reproduction creates new gene combinations that can result in novel and sometimes superior plant types.

In higher plants, the gametic life cycle is characterized by numerous cell-to-cell (surface-to-surface) contact relationships. In facilitating fertilization, intercellular developments arise from information transfers between the male gamete (the pollen grain and its extending tube) and the cells of the female tissues (the pistil). There is a need to understand how recognition events regulate the cell-to-cell interactions that guide pollen tube development from the receptive surface of the female (the stigma) to the specific termination site in the ovary (the egg).

Evolution of species and the induction of genetic variation solely depend on the interaction of male and female reproductive structures. Pollen-pistil interaction, the most important and vital process in the reproductive cycle, helps in the selection of right pollen for germination and effectively monitors its growth through the sporophytic tissue, to the embryo-sac, which culminates in successful fertilization. Although, the basic events of pollen-pistil interaction leading to
fertilization became known by the end of the nineteenth century, studies remained basically descriptive until 1950. During the past decades, there has been a steady progress in our understanding of the biochemical, genetical and ultrastructural events that occur during pollen-pistil interaction, which owe mainly to the advent of modern techniques.

Pollen-Pistil Interaction

Pollen grains are shed from the anther either in binucleate or trinucleate stage. Often species in whole families have only one form, although variations occur within the same family or even species (Brewbaker, 1957; Corriveau and Coleman, 1988).

Most pollen grains cells are surrounded by a bilayered wall. The outer wall, exine comprises of inert lipid polymer complex, sporopollenin. The morphology of the exine varies from very simple to very complex and this has promoted its use in taxonomy. During the early period, pollen morphology was studied mainly with the use of acetolysed pollen. The wall layers, exine and intine have already been demonstrated to contain large amounts of mobile proteins (Heslop-Harrison et al., 1975; Knox et al., 1975; Malik and Thapar, 1976). The intine proteins are concentrated mainly near the germ pore. In exine, the proteins are located in the sculptured part of the pollen wall. The pollen wall proteins probably discharge several functions in the pollen-stigma interaction and there is ample evidence that some are involved as recognition factors in inter-and intraspecific incompatibility reactions (Heslop-Harrison et al., 1974).
While considerable information exists on the pollen (Stanley and Linskens, 1974) and the ovary (Maheswari, 1950), not much is known about the stigma. Studies on the detailed developmental account of the stigma are scarce. However, from late 1960 onwards, more detailed information regarding the reproductive organs is in vogue.

The pistil is the most vital organ of a flower since it contains the egg deep seated within its ovary and protects the embryo that develops from it after fertilization. Basically it has three main parts, the upper stigma of varied nature and morphology, the middle style, which is long and flexible and the basal ovary that contains the egg. These parts, though morphologically different, serve the purpose of receiving the pollen grain selectively and aiding its growth and allowing the male gametes to make a successful fertilization.

As the stigma is the recipient of the pollen grains, the initial interaction takes place between these two structures. Stigma from more than 100 genera have been analysed by light and electron microscopy (Heslop – Harrison, 1981; Knox, 1984). Traditionally, the stigma is classified into dry and wet types. Wet stigmas are those with copious secretion and dry stigmas are with little or no superficial secretion. Based on the amount of secretion present during the receptive period and the nature of the stigmatic papillae, the two basic types have been further classified as follows (Heslop – Harrison et al., 1975; Heslop – Harrison and Shivanna, 1977) -
Dry stigma (without apparent fluid secretion)

Group I  Plumose with receptive cells dispersed on multiseriate branches (Gramineae). Discontinuities along the receptive surface.

Group II  Receptive cells concentrated in distinct ridges, zones or heads.

A  Surface non-papillate

B  Surface distinctly papillate

   1. Papillae unicellular

   2. Papillae multicellular

      (a) Papillae uniseriate

      (b) Papillae multiseriate

Wet Stigma (Surface secretions present during receptive period)

Group III  Receptive surface with low to medium papillae; secretion fluid flooding interstices.

Group IV  Receptive surface non-papillate; cells often necrotic at maturity, usually with more surface fluid than group III.

In the dry stigma, extracellular proteins in the form of an extracuticular hydrated layer, *i.e.* the pellicle is present (Mattson *et al*., 1974). In the wet stigma, the stigma surface at the receptive stage shows free flowing exudate. Analysis of stigmatic exudate was carried out by many authors (Vithanage, 1984; Sedgley and Blesing, 1985). The composition of the exudate is highly variable and contains varying proportions of lipids, carbohydrates, phenolic compounds and proteins. There is enough evidence to show that stigmatic secretions contain a wide variety of compounds. Some of them are involved in cell recognition reactions between
pollen and stigma (Dumas and Gaude, 1981; Ghosh and Shivanna, 1982; Uwate et al., 1982). In an earlier work on *Lilium longiflorum*, Labarca et al., (1970) reported 99% water in the exudate. Apart from water, 95% is a high molecular weight protein containing a polysaccharide composed of galactose, glucuronic acid and galacturonic acid. Analysis of the stigmatic exudate of *Zea mays* (Martin, 1970) revealed anthocyanin, phenolic compounds, esters of fatty acids and lipophilic compounds. The exudate components which are transferred to the cell wall by endoplasmic reticulum and golgi vesicles are stored within the cell wall of the secretary tissues and secreted from walls directly at the receptive stage (Miki Hirrosige et al., 1987). Extra cellular proteins have been shown to be variably present on the surface of the stigma where pollen grains are received (Mattson et al., 1974; Heslop-Harrison, 1978; Shivanna, 1982). There is a strong circumstantial evidence that the recognition molecule of pellicle is glycoproteins that bind the pollen. The lipid components probably regulate availability of water in the pollen and prevent desiccation of the stigmas and are also nutritive. According to Heslop-Harrison et al., (1975) wet stigma is correlated with gametophytic incompatibility and dry stigma is correlated with sporophytic incompatibility.

The study of its developmental anatomy (Konar and Linskens, 1966) showed that the stigma could be separated into two zones, an upper zone consisting of the epidermis and a lower secretory zone consisting of 1-3 layers of cells. Below this, the lower zone of parenchymatous ground tissue is present. In continuum with
the sub-epidermal region of the upper zone, a central core of transmitting tissue usually occurs. Two provascular tissues traverse through the ground tissue. Styles in angiosperms are of two types: hollow and solid (Shivanna, 1982). In the former, the style has secretory canals. The canal lumen usually is continuous with the stigma and is bordered by glandular cells. The canal cells in the case of open or hollow style are generally secretory and often multinucleate and polyploid. Open style is reported in *Lilium* (Rosen and Thomas, 1970). Monocots mostly have hollow styled pistils (Clarke *et al*., 1977). The stylar canal may be filled with a secretory fluid or remain dry in both types in younger stages. The inner wall of the canal is lined with a layer of cuticle. The stylar secretion contains large number of vesicles and fibrils, (Dickinson *et al*., 1982). Substantial amount of secretion product is deposited on the inner wall of canal cells facing the cavity (Clarke *et al*., 1977; Ghosh and Shivanna, 1982). A core of transmitting tissue traverses the solid style. The transmitting tissue of solid styles has been studied in a number of taxa (Bell and Hicks, 1976; Cresti *et al*., 1976; Herrero and Dickinson, 1979). Transmitting tissue comprises of elongated cells with their transverse walls traversed by plasmodesmata.

Receptivity of the stigma is a critical factor for the successful completion of post pollination events. The development of receptivity of stigmatic surface appears to be a pre-programmed response and may vary widely between species from several days prior to anthesis to several days after the flower opens. In *Acacia retinoides*, receptivity remains for only few hours after flower opening but in *Carraro* variety of vine it lasts for 10 days after the flower opens (Kernick and
Knox, 1989). Receptivity is also influenced by temperature and humidity. Under optimal conditions of temperature and humidity, in Wheat, Rye, and Triticale, the stigma do not show much change in receptivity up to 2 to 4 days after emergence (D’ Souza, 1972). Alterations in the temperature and humidity drastically reduce the period of receptivity of stigma in Lilium (Ascher and Peloquin, 1970).

The recognition of the pollen seems to be established in numerous taxa as a result of interaction between pollen wall proteins and stigma surface proteins (Knox et al., 1976; Shivanna, 1979). Following successful recognition, the pollen grain sends out a tube, which enters the stigma and grows through the style. The pistil provides nutrients to the growing pollen tube. Based on his studies, Knox et al., (1976) suggested that the stigma surface receptors are composed of many components. Some of them being involved in pollen germination and entry of tube and others inhibit the germination and growth of unlike pollen and fungal spores (Martin, 1970c; Martin and Ruberte, 1973).

The adhesion of pollen on the stigma is a primary requirement for successful pollination. It is determined largely by the extent of stickiness of pollen and stigma and on pollen wall sculptures (Woittiez and Willemse, 1979). Wet stigma supports the adhesion of both powdery and sticky pollen. Pollen adhesion on dry stigma is more critical and depends on the extent and composition of the pellicle and the amount of surface coat substances on the pollen. Pollen adhesion often involves specificity between pollen (Stead et al., 1979) and stigma surface proteins (Stead and Roberts, 1980) or morphological complementation between the pollen and the stigma.
Since the rapid growth of pollen tubes is believed to result from high hydrostatic pressure within the cell, it has often been assumed that hydration of the pollen grain is one of the most significant post-pollination events. *In vivo* hydration of the pollen is found to be beneficial to some species like *Petunia* (Gilissen, 1977). It is also found to overcome self-incompatibility in *Brassica oleracea* (Carter and McNeilly 1976; Ockendon 1978).

**III. Tissue culture**

To cope up with the increasing demand imposed by an exploding population, the conventional methods of plant breeding seems insufficient to make any significant breakthrough for a faster solution for developing high yield, good quality, disease resistant cultivars and for which innovative approaches like genetic transformation and tissue culture need to be incorporated. The development of plant tissue culture techniques and related biotechnology target the somatic phase for genetic manipulation, thus helping the breeder to widen the genetic base by generating variability *in vitro* (Bhattacharya *et al*., 1999). The integrated use of genetic engineering along with tissue culture techniques has reinforced biotechnology. With this, plants can be genetically modified by manipulating their genes and also by introducing genes from unrelated plants and other organisms such as bacteria, virus and even animals.

Global biotechnology research is targeted to benefit farmers, food processors and consumers. This new technology can bring gene revolution in many crop plants, including vegetables, bringing improvement in productivity, product quality, resistance to stress, better keeping qualities and to reduce post harvest losses (Swarup, 1999).
The German Botanist, Haberlandt (1902), first proposed the idea of plant cell culture under *in vitro* condition by using an artificial medium. The discovery of the auxins (IAA) and cytokinins (Kinetin) in the mid 1930’s created the greatest opportunity for initiation of *in vitro* culture in higher plants. Most of the modern tissue culture media have been derived from the work of Skoog and co-workers during 1950’s and 1960’s. From that phase onwards, the methods of *in vitro* culture have become more advanced.

Plant cell and tissue culture thus developed into a specialized branch in plant science through the establishment of tissue culture techniques, demonstrating plant cell totipotency, including micropropagation, somatic embryogenesis, *in vitro* androgenesis and somatic cell hybridization (Gautheret, 1985). The science of plant tissue culture and molecular genetics along with conventional plant breeding programs enjoy a pivotal position in the area of agricultural biotechnology.

Over the years, plant tissue culture techniques gained further momentum on commercial application in the field of plant propagation mainly in horticultural species. The success of apical shoot meristem culture has become the backbone of horticultural industry (Yeoman, 1986). Anther and ovule culture provides a pathway for the rapid generation of double haploid lines for use of F1 hybrid formation and targeted breeding programs (Giles and Morgan, 1987). Plant tissue culture has also made it possible to combine distant genotypes through *in vitro* fertilization, somatic hybridization, protoplast fusion and embryo culture techniques. The potential usefulness of callus cultures in propagation lies in the
possibility of adventitious shoot and embryo production. Cell suspension cultures are increasingly utilized for the exploitation of secondary metabolites (Stockigt et al., 1995). Cell line selection is useful in screening cells for increased resistance to biotic and abiotic stress. Other techniques and approaches such as cryopreservation, immobilization and the use of cell cultures for the production of high value compounds for food and pharmaceutical industries are increasingly being exploited. Finally the applications of genetic manipulation both to improve cell lines and to produce more productive plants are also making rapid progress.

IV. Micropropagation of grain legumes

Micropropagation is the true to type propagation of selected genotypes using in vitro culture of shoot meristems or regeneration from somatic cells. Plant regeneration from tissue culture forms the basis of many biotechnological approaches to plant improvement, since they allow not only clonal plant propagation but also specific and directed changes to be introduced into desirable elite individuals by genetic manipulation of somatic cells. Modified individual cells and/or embryos then can be efficiently multiplied in vitro to very high numbers prior to plant development. This approach to genetic improvement bypasses the unwanted consequences of sexual reproduction (mass genetic recombination and required cycles of selection) inherent to conventional breeding technology (Gray, 2000). Micropropagation is thus a crucial aspect of plant biotechnology as it facilitated the production of genetically engineered plants, the release of disease free plants from meristem cultures and the rapid multiplication of difficult to propagate species. Legumes in general are recalcitrant to tissue
culture and are highly genotype specific (Somers et al., 2003). Since the members of the family Leguminoseae contain high levels of phenolics, plant recalcitrance caused by the oxidation of explants is highly prevalent (Anthony et al., 1999). By alleviating the negative effects of this stress, modified regeneration methods are being reported in legumes by various tissue culture routes. Three basic methods used to propagate plants in vitro are: -

i. Enhanced axillary shoot proliferation through shoot tip / node culture.

ii. *De novo* formation of adventitious shoots through shoot organogenesis either directly or indirectly from explants.

iii. Somatic embryogenesis either directly or indirectly from explants.

**1. Shoot tip/nodal segment culture**

Propagation from axillary buds using *in vitro* techniques has proved to be a reliable method for micropropagation of a large number of species. This forms the most frequently used micropropagation method since it provides genetic stability and is easily attainable for many plant species (Kane, 2000). It was Ball (1946) who provided the first detailed procedure for the isolation and production of plants from shoot apex and successful transfer of rooted plantlets into the soil. Later on the role of cytokinins in the inhibition of apical dominance (Wickson and Thimann, 1958) was established which was eventually applied to enhance axillary shoot production *in vitro*. Application of this method was expedited by development of improved culture media that supported the propagation of a wide diversity of plant species (Murashige and Skoog, 1962; Loyd and Mc Cown, 1981). Survey of literature reveals that shoot tips and cotyledonary nodal

2. **De novo adventitious shoot multiplication**

Plant tissue can form shoots from primordials originating *de novo* followed by the initiation of a series of morphogenetic events. Two developmental sequences leading to organogenesis have been suggested. If organogenesis is after a callus phase it is termed indirect and if without callus, it is referred as direct organogenesis (George, 1993).

i. **Direct organogenesis**

The somatic tissues of higher plants are capable, under certain conditions, of regenerating adventitious plants *via* shoot apices and embryos. This phenomenon occurs naturally in many plants and is exploited in conventional propagation by the use of cuttings from various organs such as leaf, stem or root (Benavides and Caso, 1993; Kulkarnie *et al*., 1999). The formation of adventitious shoots however will depend on the reactivation of the genes concerned with the embryogenic phase of development.
In conventional propagation, the main stimulus for adventitious shoot formation arises from the physical separation of the cutting from the parent plant, causing changes in the production and distribution of endogenous hormones. The same applies to explants used for in vitro procedures and in some species, shoot formation may occur spontaneously on medium lacking any growth regulators as in Piper sp. (Sarasan et al., 1993; Bhat et al., 1995).

One of the most influencing factors in adventitious shoot formation is the modulation of exogenous auxin to cytokinin balance (Thorpe, 1980), which stimulate the cell or cells to undergo a number of rapid cell divisions leading to the formation of meristemoids, which are morphogenetically plastic and are capable of developing into shoot primordia. Most species require hormones to evoke shoot formation or to maximize the response in terms of speed and number of shoots. Adventitious regeneration in vitro may be given a much higher rate of shoot production than is possible by proliferating axillary shoots. This is especially true of plants that produce relatively few leaves and their associated axillary meristems and have highly regenerative somatic tissue.

Direct shoot multiplication has been successfully utilized for the micropropagation of a number of pulse crops such as Cajanus cajan (Mohan and Krishnamoorthy, 1998; Jain and Chaturvedi, 2004), Cicer arietinum (Batra et al., 2002), Glycine max (Settu and Kumari, 1998; YingHui and Reichert, 1998), Indigofera tinctoria (Jose, 2002), Phaseolus vulgaris (Santalla et al., 1998) and Vigna radiata (Chandra and Pal, 1995).
ii. Indirect organogenesis

Proliferation of callus from the explants occurs when the levels of growth regulating substances, particularly auxins, are higher than those necessary to stimulate the direct formation of adventitious shoots or embryos (Sarasan and Nair, 1991; Huetteman and Preece, 1993). Callus tissue comprises of a wide range of cell types and characteristically consists of irregularly differentiated, vacuolated cells interspersed with smaller more meristematic cells. The nature of any callus will depend on the tissue or tissues from which it arose and also on the composition of the medium used to induce and to maintain it. Callus may be serially subcultured and grown for extended periods (Sreevastava et al., 2000), but its composition and structure may change with time as certain cells are favoured by the medium and they usually dominate the culture.

Adventitious shoots or embryos are usually formed from the callus, if the concentration of hormones, especially auxins is lowered (Agarwal and Patwardhan, 1994; Basu et al., 1998). Although callus can be obtained from any species, only some plants can be regenerated. Even when totipotent callus has been obtained, extended proliferation by repeated subculture may result in the reduction and eventual loss of regenerative capacity.

*De novo* organ formation from callus increases the possibility of introducing genetic variation in the callus stage that results in both physiological and morphogenic variation in the resulting shoots. Thus callus regenerants are rich sources of genetic diversity and somaclonal variations (Bajaj, 1990). Regeneration of pulse crops are reported in *Cicer arietinum* (Vani and Reddy,

3. **Somatic embryogenesis**

Somatic embryogenesis is the process by which a bipolar structure, resembling a zygotic embryo develops from a non-zygotic cell without vascular connection with the original tissue. *In vitro* regeneration of plants *via* somatic embryogenesis has some distinct features such as single-cell origin, the consequent low frequency of chimeras and the production of a high number of regenerants (Ammirato, 1983; Sato *et al*., 1993). Since the first observation of plant regeneration by somatic embryogenesis from cultured cells of carrot (Steward, 1958), many species have been added to the list of successes. This paved the way for the exploitation of this process as an important biotechnological tool for large-scale plant micropropagation and improvement. The two modes of somatic embryogenesis *viz.* direct and indirect embryogenesis have been studied in detail (Ammirato, 1984). Accordingly, direct embryogenesis in culture initiates from Pre-Embryogenic Determined Cells (PEDC’s) that are already determined for embryogenic development prior to explanting. On the other hand, indirect embryogenesis initiates from Induced Embryogenically Determined Cells (IEDC’s). The process involves the dedifferentiation of differentiated cells, callus proliferation and the development of embryogenically determined state. The delivery of somatic embryos in the form of artificial seeds is a major subject of

**V. Variations in tissue culture**

The success of any crop improvement programme depends on the extent of genetic variability in the base population. There is lack of existing genetic variability in most of the agricultural crops that hampers their improvement through conventional plant breeding methods. Through various tissue culture techniques, it is possible to extend the genetic base, and to provide faster and efficient methods for selection (Bajaj, 1990). The *in vitro* conditions of cell culturing and callus formation act as a stress factor and increase the genetic variation of cells, known as somaclonal variations, which may be often heritable (Larkin and Scowcroft, 1981; Breiman *et al*., 1987). Morphological, cytological and molecular variations may be generated *in vitro* (Larkin *et al*., 1989). The variations may be due to several factors (Vasil, 1987; 1988), such as genotypes used (Breiman *et al*., 1987), pathways of regeneration, and parameters employed for assessing the effect of *in vitro* culture, such as gross morphology and cytology (Swedlund and Vasil, 1985), field assessment and molecular studies (Breiman *et al*., 1989; Shenoy and Vasil, 1992; Chowdhury *et al*., 1994).
Somaclonal variations for several agriculturally important traits have been selected through in vitro selection techniques. These include alterations in plant pigmentation, seed yield, plant vigour and size, leaf and flower morphology, essential oils, secondary metabolites, quality improvement like amino acid over production and resistance to biotic and abiotic stresses (Brown and Thorpe, 1995). Variations have been extensively recorded in crops like wheat, rice, oats, maize, sugarcane, alfalfa, tobacco, tomato, potato, oil seed rape and celery (Karp, 1994). Different types of cultured tissues have been ranked in order of low to high genetic instability as follows: micropropagation from isolated shoot tips and meristem, adventitious shoot formation, somatic embryogenesis and organogenesis from callus, cells and protoplasts (Damasco et al., 1996). One of the major difficulties associated with somaclonal variation has been the need to develop a method whereby materials can be easily and rapidly screened to reveal any genetic differences from the parent plants.

In conventional screening practices there are many limitations like time consuming, the changes might be heterozygous and recessive, and the changes occurred might not be phenotypic. Karyological analysis of regenerated plants even though reveal changes in ploidy levels and structural rearrangements, it does not reveal alterations in individual genes. Isozyme analysis also has limitations of availability of suitable markers. A precise determination of changes in a particular gene sequence resulting from tissue culture can be obtained by using DNA markers. DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers (Soller and
Beckmann, 1983). Several techniques of molecular biology are available, for the
detection of genetic polymorphism at the DNA level (Ferreira and Gratapaglia,
1995; Lanaud and Lebot, 1997). Restriction Fragment Length Polymorphism
(RFLP) has the limitation that the analysis is limited only to those gene sequences
used as a probe. Using the polymerase chain reaction (PCR) in conjunction with
short primers of arbitrary sequence (Williams et al., 1990), Randomly Amplified
Polymorphic DNA (RAPD) markers are shown to be sensitive for detecting
variations among individuals between and within species (Carlson et al., 1991;
Roy et al., 1992). RAPD markers have been used successfully to assess genetic
stability among somatic embryos in spruce species (Isabel et al., 1993; 1996) and
among micropropagated plants of poplar (Rani et al., 1995). By using RAPD, we
can determine the levels of variation in plant material at all stages of culture and
growth, from single protoplasts to regenerated plants (Brown et al., 1993, Al-
Zahim et al., 1999). RAPD analysis offers the markers of choice, since they have
the advantage of being technically simple and cost effective as compared to other
procedures.

The evaluation of genetic diversity and construction of linkage maps would
promote the efficient use of genetic variations in breeding programmes (Paterson
et al., 1991). Molecular changes reflect stable changes in the genome indicating
their usefulness in future crop improvement programmes. RAPD markers have
been used for the identification of cultivars and for assessing the genetic diversity
among cultivars of several crops like bean (Skroch et al., 1992), cowpea
(Mignouna et al., 1998), pea (Hoey et al., 1996), soybean (Brown-Guedira et al.,

**VI. Scope of the work**

*P. tetragonolobus* (L.) DC. requires immediate improvement in characters such as plant architecture and reduction of antinutritional factors in the seeds. Applying conventional plant breeding methods as well as tissue culture techniques can bring about improvement of this plant. There is a need not only to grow this crop on a large scale, but also for its improvement to make it economically attractive to the growers. Plant breeding techniques and genetic manipulation and *in vitro* culture provide a means for the gene pool to be substantially broadened and improving crops in a relatively short period, conserving considerable time and financial resources. In this regard, an understanding of its reproductive biology for formulating the hybridization programme and development of *in vitro* culture techniques for the execution of research programme for increasing variability will help in bringing about improvement in the crop.

A perusal of available literature has revealed that only limited successful tissue culture work has been carried out in this plant. Survey of literature reveals that
studies on the reproductive structures and its dynamics has not been carried out despite its economic importance. Since this is an underutilized backyard crop, there is immense scope for the development of this protein rich, medicinal plant through conventional breeding programmes as well as biotechnological approaches. Hence I have undertaken this work to generate tissue culture protocols for the efficient regeneration as well as the elucidation of certain physiological and reproductive parameters of this plant. The research work was organized with the following specific objectives:

- Determination of the extent of loss of viability of seeds on storage.
- Optimization of scarification strategies to increase the percentage of seed germination.
- Standardization of protocols for the mass propagation of the plant through different *in vitro* techniques.
- Elucidating the biochemical changes taking place during organogenesis/ embryogenesis.
- Analyzing the extent of variation in the regenerated plants.
- Investigation on androgenesis and gynogenesis of the plant.
Table: I. List of contents and quantities in the different parts of ‘Winged Bean’

(Koshy et al., 1999)

<table>
<thead>
<tr>
<th>Source</th>
<th>Moisture</th>
<th>Protein</th>
<th>Fat</th>
<th>Carbohydrate</th>
<th>Fiber</th>
<th>Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf</td>
<td>78.9</td>
<td>6.3</td>
<td>1.0</td>
<td>7.9</td>
<td>4.1</td>
<td>1.8</td>
</tr>
<tr>
<td>Pod</td>
<td>90.4</td>
<td>2.9</td>
<td>0.2</td>
<td>5.8</td>
<td>1.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Seed</td>
<td>8.5</td>
<td>41.9</td>
<td>13.1</td>
<td>31.2</td>
<td>---</td>
<td>5.3</td>
</tr>
<tr>
<td>Tubers</td>
<td>9.0</td>
<td>24.6</td>
<td>1.0</td>
<td>56.1</td>
<td>5.4</td>
<td>3.9</td>
</tr>
</tbody>
</table>

(Koshy et al., 1999)