EFFORTS TO ACCELERATE DOMESTICATION OF WINGED BEAN (*PSOPHOCARPUS TETRAGONOLOBUS* (L.) DC.) BY MEANS OF INDUCED MUTATIONS AND TISSUE CULTURE

Promotor: Dr. Ir. E. Jacobsen
Hoogleraar in de plantenveredeling, in het bijzonder de genetische variatie en reproductie

Co-promotor: Dr. Ir. A.M. van Harten
Universitair hoofddocent plantenveredeling

George.Y.P.Klu

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Proefschrift
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Cover: A proposed "ideal" winged bean plant for grain and tuber production on a background of the winged bean plant as depicted by Rumphius (1747).

Propositions (Stellingen)

1. Induced mutations can be used to accelerate the domestication of winged bean (*Psophocarpus tetragonolobus* (L.) DC). (This Thesis).

2. In the genetic improvement of winged bean (*Psophocarpus tetragonolobus* (L.) DC, chimerism is much more of an asset than a problem. (This Thesis).

3. Legumes, including winged bean, are the cheapest and often the most easily available source of protein for the under- privileged person. Efforts into legume improvement constitute a lasting investment for the people in the poverty-striken parts of the world. (This Thesis).

4. Development of effective selection schemes towards specific traits in a particular crop plant is a contribution to the reduction of the "it is by chance" stigma labelled against plant breeding by induced mutations. (This Thesis).

5. Seed coat colour mutants in winged bean are an indirect way of obtaining plants with an altered nodulation. (This Thesis).

6. The number of people involved in farming in a country is not a good yardstick for determining the significance of the contribution of farming to the economy of that country.

7. Genetic improvement of crops and their multiplication for Africa must be carried out in Africa by Africans.

8. Sandwiches, particularly those of McDonald's, are universally enjoyed. However, there is a special type of sandwich that requires hard work, commitment and good strategy to give satisfaction to the consumer. This refers to the "sandwich" that can be found on the plate of the many PhD students in Wageningen Agricultural University.

9. "Reinforcement of ....patent rights should stimulate investment in research and development and accelerate the creation of promising biotechnology inventions". However, as much as patenting rewards the biotechnology companies, it may be a serious draw-back to the potential end-user who may be financially handicapped.

10. Modern soccer has positively evolved tremendously in different ways. However, it gives reminiscence of the dark periods of human trade in the history of Africa.

George Y.P. Klu.
1996

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I have been deeply touched by the life of Georgius Everhardus Rumphius (1628-1702), who worked as a merchant and a botanist in Amboina. Rumphius became blind during his profession but visual disability was not a handicap to him. He worked hard as a blind man to produce a document that was published (1741-1747) as "Het Amboinsch kruidboek". His illustration of "De vierkante Peul-Plant" - the winged bean - formed the background of the cover design of this thesis.

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Bibliographic Abstract: This thesis describes mutation breeding and tissue culture techniques developed for accelerated domestication of winged bean (*Psophocarpus tetragonolobus* (L.) D C.). The tissue culture techniques, which are the first steps towards genetic transformation of the crop, include: (1) direct adventitious shoot formation from the axes of cotyledon explants; (2) direct simultaneous regeneration of adventitious shoots and somatic embryos; and (3) direct somatic embryogenesis on the wounds of cotyledon explants. An optimised mutation breeding technique for economic significance, based on the early selection of chlorophyll mutations generated from gamma-radiated seeds, has been developed. The use of this scheme has resulted in the recovery of seed coat colour mutants which have succesfully served as an indirect method for selecting changes in tannin content and nodulation. A desired mutant with reduced tannin content and improved nodulation was selected.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

The main task of agriculture is to ensure adequate supply of enough food - quantitatively and qualitatively - for a still growing world population. Despite efforts and successes globally, still millions of people, particularly in Africa and Asia, are undernourished. Reduction in population growth is not the means of solving the problem in the short term; rather, food production seems to be a more realistic solution. Food production can be augmented by increasing the area of crop production (though there are limits) and/or by increasing yield (calories, total protein, essential amino acids, vitamins etc) per hectare. The latter goal can be achieved by improving agronomic techniques and by growing genetically improved cultivars of a wide range of crops.

In addition, when there are possibilities to increase the range of crops, this should be done for several reasons: to avoid risks of famine, to supply certain food components (eg. proteins), to improve food quality in certain regions, etc. As to this latter point, there is room for introduction of "new" crops. Domestication is needed in such cases.

Plant domestication, in fact, is a sympatric evolution (Zeven and de Wet,1982). This implies that populations inhabiting the same geographic range become reproductively isolated. This process, therefore, can be described as a process of species formation (King and Stansfield, 1990; Rieger *et al.*, 1991). Domesticates (i.e. genetically adapted wild plants that are more or less regularly grown as crops) and their wild progenitors differ in phenotype and, therefore, in adaptation but remain sufficiently and genetically related to enable crossing that would yield fertile hybrids (Zeven and de Wet, 1982). Domesticated plants may, among other things, flower and fruit simultaneously, lack shattering of pods and loose dispersal mechanisms, change from perennial to annual plants, and may have acquired better palatability and better chemical composition (Zeven and de Wet, 1982). Smartt (1989) has quoted de Wet as having noted that the domestication of species could be an important factor in meeting food crisis in drought prone areas and could also serve as a base for agricultural self-sufficiency in developing economies. This can be carried out by aiming at new domesticates or by the development of new modes of exploitation of the existing cultigens (Smartt, 1989).

"Underexploited" crops, may be useful in contributing to the afore mentioned goals. This is a group of plants which have received little research and cultural attention, with the implication that although they are potentially useful, they have remained under utilised (Anon,1975;1979). The "underexploited" crops includes a number of "pulses" or "grain legumes" (terms which have been defined by van der Maeson and Somaatmadja (1989) as edible seed legumes). These include winged bean (*Psophocarpus tetragonolobus* (L.) DC), pigeon pea (*Cajanus cajan* (L.) Millsp), Kerstings groundnut (*Macrotyloma geocarpum* (Harm) Marechal & Baudex), the horsegram (*Macrotyloma uniflorum* (Lam.) Verda), hyacinth bean (*Lablab purpureus* (L.) Sweet), the jack bean (*Canavalia ensiformis* (L.) DC) and the sword bean (*Canavalia gladiata* (Jacq.)

DC). Grain legumes offer a very realistic means of eradicating protein malnutrition in the developing countries; however, most of them still have low production levels and, therefore, deserve attention (Rao *et al.*1975). The Food and Agriculture Organization estimated that the average yield of pulses taken together in developing countries is 637 kg/ha as against 1494 kg/ha in the developed world with harvested areas being 5.6×10^7 ha and 1.3×10^7 ha respectively (Anon, 1988).

The practice and acceleration of domestication

The process of domestication, also called sympatric evolution (Zeven and de Wet,1982), has been conditioned by mutations and is driven by selection (Röbbelen and von Witzke,1989). However, only about 200 plant species of a total of about 300 plant species are in use by man for food, fibre and spices have been domesticated (Anon, 1989). This could be due to differences in the domestication process which is species dependent. Some species are much better suited to domestication than others. This can be attributed to differences in ease of reproduction, response to the environment of cultivation and the genetic capacity to respond favourably to breeding methods (Harlan,1956). Mutations involved in domestication are more often recessive and rarely dominant (de Wet,1989) and the reproductive isolation among different groups of domesticated species has been caused by macromutations as well as micromutations (Gould,1980; Stebbins and Ayala,1981;de Wet,1989). For example, chromosomal changes in the form of inversions have been recorded to be the source of differences between some cultivars of soybean (*Glycine max* L. Merr.) and *G. soja* Seb Zucc., the wild progenitor of soybean (Ahmad *et. al.*,1979).

A number of changes may take place in a crop during domestication. These may be morphological, physiological and/or biochemical. Many of the key crop traits that could be improved during domestication include reduced or increased plant size, erect growth habit, modified photoperiod requirements, shorter life cycle, increased content of desired constituents (such as protein, starch and aromatic substances), changes in nodulation and elimination or reduction of undesirable constituents eg, alkaloids, toxins and secondary metabolites (Anon, 1977; Ashri,1989). The use of methods that would accelerate the process of domestication and, therefore, may lead to the improvement of the afore mentioned traits, particularly in the "underexploited" crops with promising economic value, could lead to increased food supply.

In some of the characters mentioned (eg. nodulation and alkaloids in plant parts) flavonoid biosynthesis plays a key role. A majority of legumes is characterised by the ability to live in symbiotic relationship with bacteria of the genera *Rhizobium, Bradyrhizobium* and/or

Azorhizobium. Shortly after germination of their seeds, legumes release plant signals or exudates called flavonoids. Flavonoids constitute a class of secondary metabolites found in the plant kingdom and may serve specific functions. These include plant part protection against uv light damage, plant-microbe interactions for nodulation and nitrogen fixation and contribution to improved plant structure by flavonoid polymers called proanthocyanidins (tannins) which are present in large quantities in seeds coats (reviewed by van der Meer, 1992; Martin and Gerats,1993; Quattrocchio,1994). Flavonoids play roles in both tannin levels in plant parts as well as in legume nodulation. The basic flavonoid structure is composed of two aromatic C6 rings held together by a C3-unit (Fig. 1).

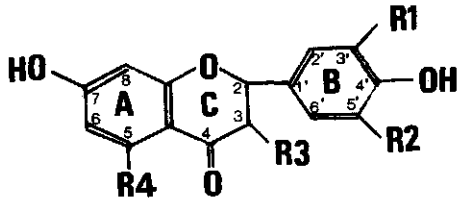


Figure 1. General structure of the flavonoid skeleton (van der Meer, 1991)

Oxidation of the C ring leads to the formation of subclasses including chalcones, flavonones, flavonols, isoflavonoids, flavones and anthocyanins. Substitutions, such as hydroxylation, methylation, glycosylation, acylation or rhamnosylation in these subclasses result in a diversity of flavonoid colours (reviewed by Heller and Forkmann, 1988; Mol, 1993; Koes *et al.*, 1994; Quattrocchio,1994).

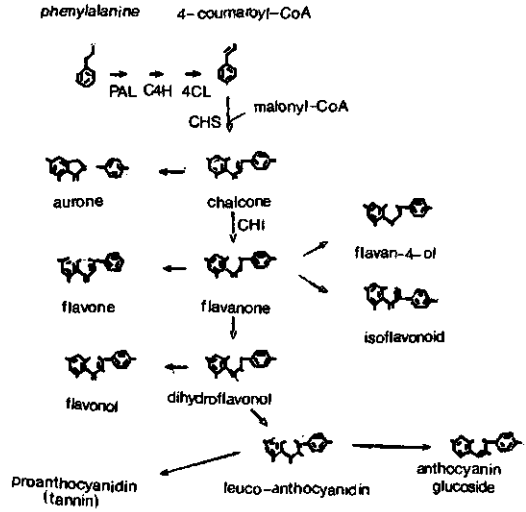


Figure 2. Simplified structure of the flavonoid biosynthetic pathway (Quattrocchio, 1994)

Flavonoid synthesis starts with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA yielding naringenin chalcone. This reaction is carried out by the enzyme chalcone synthase (CHS). This is subsequently isomerised by the enzyme chalcone flavanone isomerase (CHI) to yield a flavanone. From these intermediates, a diversity of flavonoids for specified functions, such as pigmentation (eg. tannin formation) and nodulation, are formed (Fig. 2). Many mutants (eg. in maize, *Zea mays*; snapdragon, *Antirrhinum* spp and petunia, *Petunia* spp), each blocked at different sites along the pathway, could be identified (de Vlaming *et al.*,1984; Martin *et al.*,1987; Dooner *et al.*,1991; Martin and Gerats,1993).

Mutation induction and some other means of genetic modification, like transformation, are tools that may provide variation in some of the plant characters outlined, and hopefully, may lead to the acceleration of domestication in pulses. The last 30 years have shown mutations becoming a useful supplementary tool for the genetic improvement of cultivated plants (Novak and Micke, 1988); and the FAO/IAEA Mutant Varieties Database contains 1737 accessions (Maluszynski *et al.*,1995). The majority of mutant varieties belongs to the cereals, although successes have been recorded in the legumes, vegetatively propagated crops and ornamentals as well (Micke et al.,1987; Maluszynski *et al.*,1995). In the pulses, some of these changes relate to plant architecture and include alteration of a winding plant structure to a non-winding one, reduction or removal of tendrils (Blixt,1972; Blixt and Gottschalk,1975), reduction of leafiness of legumes for production of desired amounts of grains (Rao *et al.*,1975; Rennie,1978). Some of the changes also include increase in seed size, with a probable induction of earlier nodulation in genotypes (Anon, 1982).

Induced mutations, whether by radiation or by chemical mutagens, increase the chances of obtaining desired characters and also could be used to correct one or a few negative characters in an accepted cultivar (Anon,1977; van Harten and Broertjes, 1986). Smartt (1989) has noted that mutagenesis primarily compresses the time scale of domestication. This has been documented by Röbbelen and Witzke (1989). For instance, in the genus *Cuphea* , mutants, such as non-sticky hairiness, monoculm shoot and fasciated stem, which are inherited monogenically have been induced. These features have been induced in several *Cuphea* species, thereby adding to the genetic improvement of *Cuphea* (Röbbelen and Witzke, 1989).

The use of artificial mutagenesis to obtain new genetic variability for improvement of grain legumes has been going on for some time now but advances in their production have been slow; mutants of grain legumes constitute about 12% of all mutant cultivars on record. The improved characters reported include higher grain yield, resistance to shattering, better nitrogen fixing

ability, improved plant architecture, better grain quality, resistance to diseases and tolerance to stress (Micke,1988). Adequate selection techniques for genetic improvement need to be developed and, coupled with mutation induction, can lead to acceleration of the breeding programme (Brock,1965; Konzak *et al.*,1977).

Gene transformation methods, by virtue of their ability to allow the introduction of genetic material, not normally accessible by conventional breeding methods, have potential as well for the domestication of crop plants. In fact, the entire array of genetic discoveries and methodologies should be employed for acceleration of the domestication process (Jain, 1989). The use of plant tissue culture techniques and genetic modification of crop plants for the introduction of new traits into plants and the modification of existing traits must be employed. The success of these techniques is, however, dependent on the availability of reliable *in vitro* regeneration methods. *In vitro* mutant selection has advanced since the early 1970s and this has been stimulated by the availability of haploids and methods for plant regeneration (Maliga,1980; Maliga *et al.*,1981).

A case study

Some crop species although possessing favourable genotypes have very limited genetic variation. Most of these crops which occur mainly in the tropics, have received little research attention (Anon,1989). Of particular significance are the "underexploited" crops, which includes the winged bean (*Psophocarpus tetragonolobus* (L.) DC) as indicated earlier in this chapter. This crop is a semidomesticated legume (Eagleton *et al.*, 1985) which has attracted the attention of many researchers in recent times. Smartt and Hymowitz (1985) have stated, among other things, that knowledge of evolutionary events and processes which have occurred in the development of a crop and also knowledge of the genetic resources available for further improvement of the crop are essential for the formulation of meaningful breeding objectives of a crop. Success of improvement programmes in *P. tetragonolobus* would partly depend on the botany of the crop and the selection methods used. The investigations presented in this thesis are aimed at providing basic tools for the improvement of a crop of the nature described. Knowledge of the botany and other characteristics of the crop would form the basis for formulating tools for its accelerated domestication to serve as an additional source of protein in the neotropical regions of the world. Intraspecific hybridisation should be the ideal method of breeding the winged bean. However, small scale crosses carried out by the author were not successful. Interspecific hybridisation could be an additional source for obtaining the desired traits but no records on interspecific hybrids are known (Smart,1990). Hence, there is the need to find other sources of obtaining an increased genetic variability (Chapter

2). The use of mutation breeding techniques, including *in vitro* methods, could increase the needed genetic variabilitiy. Additionally, somaclonal variation and transformation systems are potentially useful tools for genetic improvement of this crop. However, the development of a reliable *in vitro* regeneration system, in such cases, is required as a prerequisite. Chapter 3 deals with the direct regeneration of adventitious shoots and somatic embryos on cytokinin-supplemented media instead of the usual auxin-supplemented media. This is meant to provide an additional tool for the improvement of this crop; i.e. to make it amenable to both auxin and cytokinin-based regeneration systems. The advantage is that unlike the auxin-based system, the cytokinin-based method avoids the callus phase preceeding regeneration of adventitious shoots and somatic embryos. *P. tetragonolobus* is a viny plant that requires staking for the production of both pods and tubers (Khan,1982). Mutation breeding of such a crop would be very expensive since the relatively large M_2 population normally required for mutant selection, in view of the chimerism of M_1 plants, would need thousands of stakes. In Chapter 4, optimisation of mutant recovery using chlorophyll mutations, aimed at the use for relatively small M_2 populations and thereby, reducing costs is described. The result of this study, which was the identificatioin of areas of highest mutation frequency on M_1 plants, was used for selecting changes in tannin content in seeds by using seed coat colour changes.

This is an indirect method of selecting for changes in tannin content of seeds (Chapter 5). One mutant was obtained with a reduced tannin content. The choice of tannin for this study was based on one of the improvement objectives that requires lines with sufficient nutritional content and a low proportion of antinutritional factors. These mutants were tested for nodulation and some phenotypic traits since components of the flavonoid biosynthetic pathway are involved in tannin synthesis as well as interaction between root hairs and rhizobial bacteria. These mutants developed earlier nodulation than their parents. The details are presented in Chapter 6.

CHAPTER 2

STATUS AND POTENTIAL FOR GENETIC IMPROVEMENT OF WINGED BEAN (*PSOPHOCARPUS TETRAGONOLOBUS* (L.) DC)

G.Y.P. Klu, A.M. van Harten, E. Jacobsen.

SUMMARY

The winged bean, *Psophocarpus tetragonolobus* (L.) DC is a crop with very useful characteristics in that apart from its stem and roots, all parts of the plant are edible and rich in protein, minerals and vitamins. It is, therefore, potentially useful as a source of protein in the diet of the average home in the tropics particularly in Africa, South-East Asia, The Oceania and India. The plant is a climber and requires support for optimum yield of seeds. The requirement of stakes is a major economic setback to the cultivation of the wing bean. Self-supporting, hence short-statured cultivars are needed to transfer this potentially useful pulse from its present position as a backyard crop into an industrial one. In addition, improvement of other traits such as the production of cultivars which are easily dehulled and of high nutritional quality but low in antinutritional factors, such as tannin, are desirable. Interspecific and intraspecific hybridization should offer a tool for obtaining and combining the desired traits, but to date it has not been recorded for *P. tetragonolobus*. The use of other techniques such as mutation breeding, with or without tissue culture techniques and somaclonal variation, for the improvement of this crop have been reviewed. Potentials of these techniques for improvement of the winged bean have been discussed.

INTRODUCTION

Grain legumes constitute the main source of protein in the diet of the average home in Africa, Asia, and South America and the most important ones are cowpea (*Vigna unguiculata*), groundnut (*Arachis hypogaea*) and soybean (*Glycine max*). In addition, a lesser used pulse, the winged bean (*Psophocarpus tetragonolobus*) may be useful. The winged bean produces edible fresh pods, seeds, leaves and root tubers which are all rich in protein and minerals. The presence of high protein and mineral contents in various parts of the plant bestow economic importance on this crop, to which until the advent of three publications of the National Academy of Sciences of the U.S.A. (Anon, 1975; 1979; 1981) to highlight its importance, little attention was paid. In recent times, this underexploited grain legume has been subjected to more detailed studies, the result of which will be unfolded in this chapter.

TAXONOMY, ORIGIN AND DISTRIBUTION

The genus *Psophocarpus* includes two subgenera, *Psophocarpus* and *Vignopsis* (Fig.1). The former has two Sections (Sect. *Psophocarpus* and *Unifoliatae*), which include seven species: *P. scandens* (Endl.) Verd., *P. grandiflorus* Wilczek, *P. tetragonolobus* (L.) DC., *P. monophyllus* Harms and *P. lecomtei.* The subgenus *Vignopsis* has two species, *P. lukafuensis* (De Wild) Wilczek and *P. lancifolius* (Verdcourt and Halliday, 1978). All the nine species except the cultivated species, *Psophocarpus tetragonolobus*, have been found in the wild and appear to be indigenous to Africa, Madagascar and the Mascarene Islands in the Indian Ocean (Hymowitz and Boyd, 1977; Pickersgill,1980; Smartt, 1980; Maxted, 1989. These nine

listed species are indigenous to tropical Africa (Maxted, 1989) and may contain genes for disease and pest-resistance which could be useful in the improvement of *P. tetragonolobus* (Harder *et al.*, 1990). This latter species, which is the only cultigen ie. cultivated species, is widely distributed in South-East Asia, whereas its cultivation in parts of Africa is reported to be of recent occurrence (Khan, 1982). The pattern of distribution of members of the genus *Psophocarpus* makes the origin of *Psophocarpus tetragonolobus* uncertain (Verdcourt and Halliday, 1978). On one hand, Africa has been proposed (Burkhill, 1935; Purseglove, 1968; Smartt, 1980, 1990; Harder and Smartt, 1992; 1995) and on the other hand, Asia (Vavilov, 1951; Cobley, 1956) and Papua New Guinea (Khan, 1976; Hymowitz and Boyd, 1977) have been mentioned to be the source. There is an apparent absence of wild forms of *Psophocarpus* in Asia where it is widely distributed. This is a possible reason for proposing that the winged bean could have been transported from one region to the other and therefore, considering it to be a transdomesticate (Smartt, 1980).

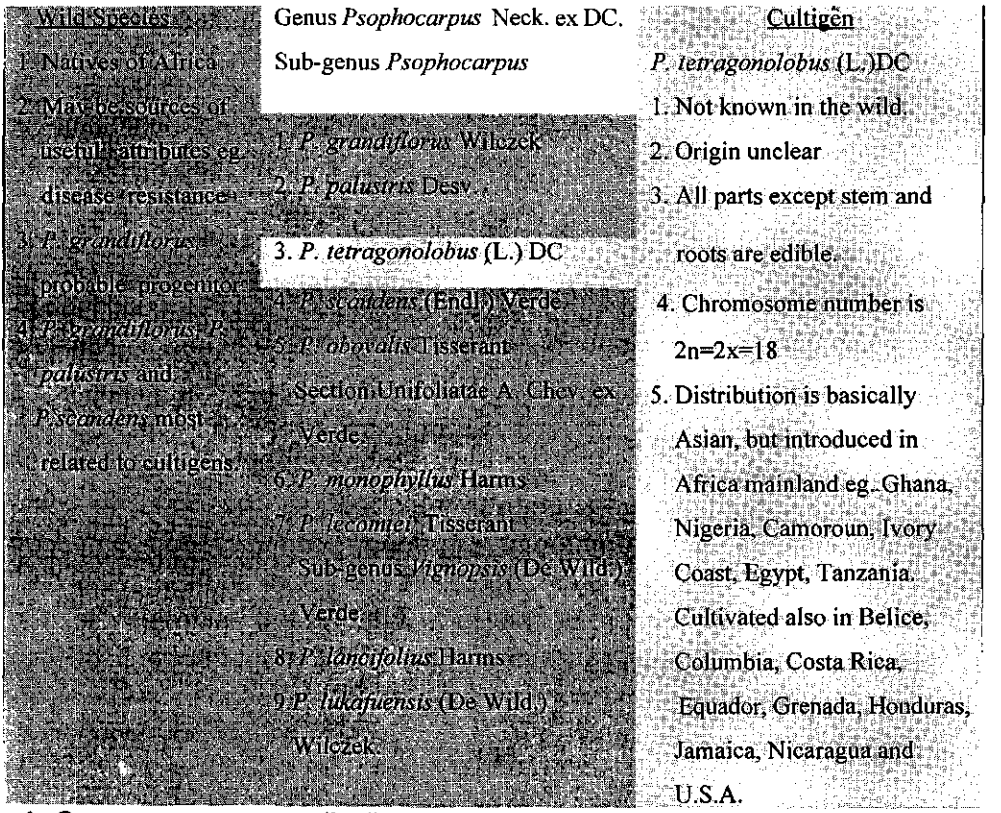


Figure 1. Summary, taxonomy, distribution and some characters of some members of the Genus *Psophocarpus* Neck. ex DC (Based on Verdcourt and Halliday,1978; Smart,1990).

THE PLANT

The winged bean plant is twining, perennial and grows as an annual herb of up to 3 to 4 m long. The stem is glaborous with colour ranges from green to deep purple. The leaves are trifoliate and the leaflets are typically broadly rhomboid and up to 10 cm long. There are 2-10 flowers in axillary racemes which are up to 15 cm long; and the pods are more less square in section, 6-30 cm or longer with longitudinal ridges or "wings" (Fig. 2) (Verdcourt and Halliday, 1978; Duke *et al*, 1981). The seeds are predominanly brown and black, with shades of brown and tan being most common. A dark ring around the hilum and specks on the seed coat have been noted in some materials (Newel and Hymowitz, 1979). Early and late flowering, day-neutral, fungi resistant and non-shattering types have been identified (Vietmeyer, 1978).

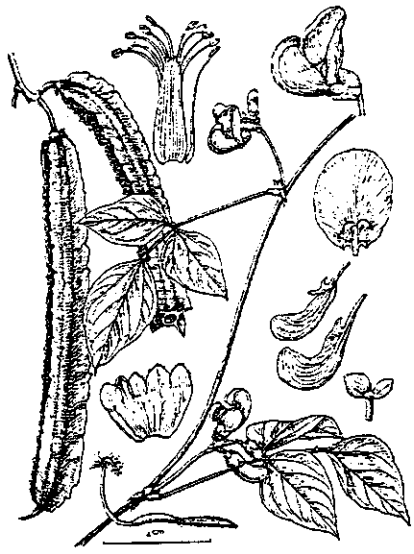


Figure 2. The winged bean, *Psophocarpus tetragonolobus* (L.) DC (From Duke *et al.*, 1981)

Some cultivars of the winged bean, under appropriate environmental conditions, develop root tubers. Most traditional tuber growing areas are found at high altitudes (Khan, 1982). This viny plant requires staking for optimum yield of both seeds. It has been reported that seed yield increased from 2 to 10 fold in staked vines as against unstaked ones. The beneficial effect of plant support is related to an appropriate canopy structure leading to greater leaf area index, dry matter production and nodulation. Most winged bean cultivars are short-day plants and flower when day length is shorter than 12 hours. In addition, light intensity, temperature, and genotype differences affect flowering in this pulse (Khan,1982).

PESTS AND DISEASES

The winged bean has been described as a crop generally free of insect pests. However, it has been reported that a wide range of insects belonging to Hemiptera, Thysanoptera, Diptera, Coleoptera, Lepidoptera, Acarina and Orthoptera have been identified as pests on winged bean,mainly in the South-East Asian countries. The most important ones include *Maruca testulalis* and *Haliothis armigera* which cause pod and flower damage. *Leucoptera psophocarpella* has been found to cause extensive leaf damage. *Mylabris afzelii* and *M. pustulata* cause extensive flower damage (Anon,1981; Khan,1982). *Meloidogyne incognita*, *M. javanica* and *M. arenaria* have been identified to be responsible for nematode disorders in the winged bean. Viral and fungal diseases have also been identified in this crop. Necrotic mosaic virus, ring spot mosaic virus and leaf curl diseases have been recorded (Khan, 1982). False rust caused by *Synchytrium psophocarpi*, leaf spot caused by *Pseudocercospora psophocarpi* , powdery mildew caused by *Erysiphe cichoracearum* (Price, 1978) and collar rot caused by *Macrophomina phaseolina, Fusarium semitectum, F. equiseti, F. moniliformae and Rhizoctonia solani* and leading to high seedling mortality have been identified as the major fungal diseases of the winged bean. In addition, the witches broom, a mycoplasm disease, has also been identified on this crop (Khan, 1982).

CYTOLOGY AND GENETICS

Inconsistencies in chromosome counts in the genus *Psophocarpus* have been recorded in the literature. Chromosome counts of $2n = 2x = 18$ have been recorded for *P. tetragonolobus* (Tixier, 1965; Khan, 1976; Haq and Smartt, 1977). However, in earlier publications, Miège (1960) and Ramirez (1960) had reported $2n = 22$ and 26 respectively (ploidy level not indicated). Studies of meiosis and mitosis of some *P. tetragonolobus* accessions indicate that the chromosomal set up is made up of 6 short and 12 long chromosomes. This supports the view that $2n = 18$ (Khan, 1976; Newel and Hymowitz, 1979; Pickersgill, (1980). Smartt (1990) suggested that a chromosome numerical polymorphism might have existed in the genus and within the species. Winged bean is an inbreeder (Pickersgill, 1980) since pollen is shed in the night before the flower opens (Erskine and Bala, 1976; Senanayake and Thruketheswaran, 1978). It has been observed that outcrossing is possible due to the longer receptivity of the stigma (up to 34 hours after the flowers have opened) and the pollen remaining viable for 24 hours (Aminah-Lubis, 1978; Senanayake and Thruketheswaran, 1978). A low frequency of outcrossing has, incidentally, been recorded under various environmental conditions and also when pollinators such as bumble-bees (*Bumbus* spp) and carpenter bees (*Xylocopa* spp) are available (Erskine, 1978; Khan, 1982; Harder and Smartt, 1995).

NUTRITIVE VALUE AND DIETARY USE

The winged bean is a crop with a considerable merit in that, apart from its stem and roots, all parts of this plant are edible and of high nutritional value since they are usually rich in protein, minerals and vitamins (Tables 1 and 2). The wide range of values could be attributed to the various sampling and analytical methods used, as well as differences between environments and varieties (Claydon, 1978). The protein and amino acid composition of its dry mature seeds are comparable with those of the soybean (*Glycine max* L.) (Hamilton, 1955; Bailey, 1968; Kapsiotis, 1968; Pospisil and Cerny,1968; Cerny *et al.*, 1971; Rachie and Roberts, 1974; Brouk, 1975; Claydon, 1975, 1978; Khan, 1975,1982; Anon, 1975,1981; Wong, 1975; Jaffe and Korte, 1976; Gillespie and Blagrove, 1977; Ekpenyong and Borchers, 1978; Duke *et al.*, 1981). The similarity of the amino acid compositions of the winged bean and soybean seeds lies in the limiting values of the sulphur-containing amino acids, methionine and cysteine. The winged bean seed is, however, higher in lysine and leucine than soybean (Cerny *et al.*, 1971; Kantha and Hettiarachchy, 1981). Research in Ghana (Cerny and Addy, 1973; Kordylas *et al.*, 1978; Plahar and Hoyle, 1987), Ivory Coast (Ravelli *et al.*, 1978) and the former Czechoslovakia and Vietnam (Cerny *et al.*, 1981) shows that the mature dry seeds have the potential of being processed into weaning foods for infants and toddlers, high protein products and animal feed. Root and tuber crops, although limited in nutritive value, form the main food source for most people in the tropics. In the winged bean, however, the high carbohydrate level of 30.5% in the root tubers is accompanied by a considerably high level of 11.4% protein in the same storage organ (Table 3). The digestibility of the winged bean seed like some other pulse crops such as soybean (*Glycine max*), lima bean (*Phaseolus lunatus*) and chick pea (*Cicer arictnum*) are adversely affected by the presence of several antinutritional factors. These include anti-trypsin and anti-chymotrypsin inhibitors (Anon, 1975; Poulter, 1982; Fernando and Bean, 1985, 1986) and lectins in the seeds and tubers (Poulter, 1982). Various levels of tannin have been reported in whole seed meals and tubers of the winged bean (Anon, 1975). Tannins interact with and precipitate protein and, therefore, reduce food protein quality (Tan *et al.*,1983; Cabrera and Martin, 1986).

NODULATION, PLANT NUTRITION AND TUBERIZATION

A majority of the Leguminosae are characterised by the development of root nodules in symbiotic relationship with soil bacteria of the genera *Rhizobium, Bradyrhizobium* and/or *Azorhizobium*. These bacteria can infest the roots of specified host plants and induce the formation of nodules, which develop from newly-formed meristem in the root cortex. It is within these specialised organs called nodules that the bacteria inhabit for fixation of

Table 1. Proximate composition, mineral and vitamin content of different parts of the winged bean expressed per 100g fresh weight.*

	Flowers	Immature pods	Unripe seeds	Leaves	Tubers	Mature dry seeds
Proximate figurest(g)						
Water	8.4-87.5	75.9-93.0	35.8 -88.1	62.2-85.0	51.3-67.8	8.5 - 24.6
Protein(crude)	2.5-5.6	1.9-4.3	4.6 -10.4	5.0-7.6	3.0-20.0	29.8-42.9
Fat	0.5 -0.9	0.1-3.4	0.7-10.4	0.5	2.5	0.1- 1.1
Carbohydrates	3.0 - 3.1	1.1-7.9	5.6 -42.1	3.0-8.5	27.2- 30.5	23.9 -42.0
Fibre	-	0.8-3.1	1.0 - 2.5	3.0 -4.2	1.5 -17.0	3.7 -16.1
Minerals(mg)						
Potassium	-	205 - 381	-	80-436	550	370-1800
Phosphorus	-	26.69	-	52-98	30-64	200-610
Sulphur	-	-	-	-	180-360	380
Magnesium	-	58.0	-	54.0	23.64	110-255
Calcium	-	53.0 - 330	-	113- 260	25-40	80-370
Iron	-	0.2- 12.0	-	2.0-18.0	0.5-70.6	2.0-37.0
Sodium	-	3.0- 3.4	-	2.5-18.0	33.0	3.0-64.0
Manganese	-	2.2-10.0	-	1.5	3.0-10.0	3.9-25.0
Zinc	-	0.2-1.3	-	1.4	3.4-4.4	3.1-5.0
Copper	-	0.6	-	0.5	0.6-6.8	1.3-1.6

*Based on Anon, 1979;1981; Hamilton, 1955; Bailey, 1968; Cerny *et al*, 1971; Pospisil *et al*, 1971; Watson,1971; Claydon,1975,1978; Khan, 1982; Jaffe and Korte,1976; Ravelli *et al*, 1978; Ekpenyong and Borchers 1978; Duke *et al*. 1981.

Table 2. Nutritive values of some food legumes (Values per 100mg for edible portion except minerals which are expressed in mg/100mg). Modified after Watson, 1971, 1977.

Food Stuff	Protein	Fat	Carbohydrates	Fibre	Ash	Minerals (mg.)		
						Ca	P	Fe
Winged Bean *(Psophocarpus tetragonolobus)*								
a. brown seed	32.4	16.5	3.2	6.0	3.4	240	370	3.0
b. fresh pods	2.6	0.6	6.8	3.2	0.6	45	36	15.0
Soybean *(Glycine max)*								
dry seed	32.5	19.2	29.2	4.6	4.8	234	220	2.0
Cowpea (*Vigna unguiculata*)								
a. brown seed	19.0	1.1	60.6	5.0	3.0	70	270	11.0
b. white seed	22.5	1.5	61.5	2.5	4.0	40	460	14.0
Groundnuts (*Arachis hypogaea*)								
a. raw seed with skin	20.5	48.5	20.0	2.6	2.4	22	305	4.5
b. roasted seed	21.8	50.5	20.2	1.9	3.3	44	380	4.5
Bambara groundnut (*Vigna subterranea*)								
a. white seed	19.2	5.6	54.5	5.3	3.5	108	195	9.7
b. red seed	17.2	6.6	62.6	4.5	3.6	71	185	9.4
Pigeon pea *(Cajanus cajan)*								
dry seed	19.8	1.2	55.0	7.8	3.2	145	200	8.0
Mung bean *(Phaseolus sp)*								
a. green seed	23.0	1.3	53.3	3.8	3.4	108	430	8.0
b. black seed	22.6	0.4	56.5	4.7	3.8	135	360	3.5
Yam bean *(Sphenostylis stenocarpa)*								
seed	19.1	0.5	61.6	5.2	2.4	45	-	1.5

Table 3. Nutritive values of some tropical root and tuber crops (Values per 100mg of edible portion)*

Food stuff	Protein	Fat	Carbohydrate (g)	Fibre (g)	Ash (g)	Minerals (mg)		
						P	Ca	Fe
Winged bean (*Psophocarpus tetragonolobus*)	11.4	0.2	30.5	6.1	3.7	25	-	0.5
Cassava (*Manihot esculenta*)	0.7	0.2	42	0.9	0.7	28	60	3.3
Cocoyam (*Xanthosoma sp*)	2.7	0.2	36	0.7	1.4	12	28	3.7
Potato, Salaga (*Coleus dysentericus*)	1.9	0.5	24.7	1.4	1.5	80	90	2.0
Potato, Sweet (*Ipomea batatas*)	0.8	0.2	35.7	0.8	0.8	16	56	3.0
Yam, Water (*Dioscorea alata*)	2.6	0.2	31.8	0.7	1.7	1.2	70	0.5
Yam, White (*Dioscorea rotundata*)	1.8	0.2	33.2	0.7	1.2	6	61	1.5

*Based on Watson, 1971; Claydon, 1975; Anon, 1979; Anon, 1981.

atmospheric nitrogen. Masefield (1957;1973) and Harding *et al.*, (1978), as reviewed by Iruthayathas *et al.*(1985), have observed a higher capacity for nodulation and nitrogen fixation in winged bean than in any other tropical legume such as cowpea (*Vigna unguiculata)*, common bean (*Phaseolus vulgaris*), groundnut *(Arachis hypogaea)*, soybean (*Glycine max*) and bambara groundnut *(Vigna subterranea)*. This nodulating ability has been found to be irrespective of innoculation with unspecified species of rhizobia (Masefield, 1973). However, variations in the effectiveness of different *Rhizobium* species and also in nodulation and nitrogen fixation among different selections of winged bean have been documented (Harding *et al.*,1978; Ikram and Broughton, 1979; Iruthayathas and Herath, 1981; Iruthayathas and Vlassak, 1982). The high nodulation and nitrogen fixing rates have probably contributed to the exceptionally high level of protein in the various parts of the plant (Anon, 1975). Some cultivars of the winged bean develop root tubers and it has been observed that environmental changes as well as the genotype strongly influence this character. Short days induce tuberization (Lawhead, 1978; Harder, 1994), temperatures around 22/18^{o}C favour tuber formation (Ruegg, 1981). Therefore, a decrease in temperature by mulching is favourable in this respect (Bala and Stephenson, 1978). In fact, most traditional tuber producing areas are found at high altitudes. It has also been recorded that tuberization is enhanced by in winged bean by reproductive prunning, which is the periodic removal of flowers and pods. Complete sterility would promote tuberization, since the latter may create self-prunned plants.

GENETIC IMPROVEMENT: OBJECTIVES AND STRATEGIES

Production of green pods with less stringiness is desired if the crop is to be used as a vegetable. However, if it is to be grown as a grain legume, and hence as a field crop, the ideal plant must be early maturing with compressed flowering period, a large number of relatively small pods, dwarf bush type with a few side branches and robust stem (Stephenson, 1978; Eagleton *et al.*, 1985) coupled with reduced internode length and/or reduction in the number of nodes (Smart, 1990). In line with these objectives, the International Council for the Development of Under-Utilized Plants proposed some priorities to govern winged bean genetic improvement. These include the development of self-supporting determinate cultivars for single harvest, high yielding cultivars with high nutrional quality, cultivars with pods that have low pod wall fibre so that pods of large size remain edible and, consequently, useful as green vegetable, cultivars with good tuber develpment and cultivars that are disease resistant (Lazaroff, 1989).

Intraspecific hybridisation should be the normal way of breeding by recombining existing genetic variation in order to select for new varieties. However, small scale crosses carried out by the first author were not succesful. Only one report on intraspecific crossing has been encountered (Erskine and Bala, 1976). Interspecific hybridisation could be an additional source of obtaining the desired traits like disease resistance. *P. scandens,* a wild species widely distributed in Central Africa and reported to be resistant to a number of diseases eg. *Synchytrium psophocarpi* (Khan, 1982) could be useful in such a programme. So far, no conspecific wild relatives have been identified and no cross-compatible wild species producing viable interspecific hybrids are known (Smart, 1990). There is no record on embryo rescue. In view of this, mutation breeding, eventually in combination with tissue culture techniques and also genetic engineering, have been proposed to meet the desired objectives. These approaches appear promising due to the low genetic variability resulting from the high level of self-polination (Erskine, 1978).

Mutation breeding

The use of induced mutagenesis for improvement of this crop has been undertaken (Khan and Brock, 1975; Jalani, 1978; Kesevan and Khan, 1978; Armachuelo and Bernardo, 1981; Klu, 1985; Klu *et al.*,1991). Details of materials and mutagens used and the results obtained are summarised in Table 4. Gamma radiation doses up to 300 Gy on dry seeds yielded mutants for earliness (Veeresh and Shivashankar, 1987), higher tuber yield (Armachuelo and Bernardo, 1981), dwarf and bush type mutants (Anon, 1982; Jugran *et al.*, 1986) and seed protein increases (Klu *et al.*, 1991). Doses of 100 Gy to 300 Gy of Gamma radiation yielded these mutants which were selected in M_2 and M_3 generations (Table 4). Normally, one would not expect to select characters like nodulation, number of pods per plant, number of seeds per pod and tuber yield in the M_1 generation as reported by Jalani (1978) and Armachuelo and Bernardo (1981). Possibly the nomenclature of the generations were misplaced. The use of chemical mutagens, like ethyl methyl sulphonate, have been reported but without any significant effects except for visible changes in leaf colour (Khan and Brock, 1975; Kesevan and Khan, 1978 Armachuelo and Bernardo, 1981). *In vitro* mutation breeding studies using 100 to 200 Gy gamma radiation have also been reported but without any recorded mutants (Table 4) (Chow and Subha, 1986).

Table 4: Summary of induced mutation studies in winged bean (*Psophocarpus tetragonolobus*).

Treated material	Mutagen	Response	Reference
1. Seed	EMS	Germination reduced by 21-25	Khan and Brock,1975
	Gamma rays	Doses above 250 Gy were lethal; 100-250 Gy used for raising M_1 plants, no mutants obtained	
2. Seed	Gamma rays	Germination, plant height, nodfulation, total dry weight, number of pods per plant, pod length and number of seeds per pod in M_1 reduced with increased radiation from 100 to 300 Gy. No mutants reported	Jalani, 1978
3. Seed	Gamma rays	150-200 being optimum doses	Kesevan and Khan,
	EMS	Effective at low concentrations (0.5-2%, 8-12 hr treatment and 24 hr postwash). Mutants for leaf colour, shape, reduced internodes and early flowering in M_2.	1978
4. Seed	Gamma rays	Doses of 150-250 Gy produced higher tuber yield in M_1.	Armachuelo and Bernado,1981
	EMS	No significant effect of EMS on any yield parametre. Flower colour changes in M_1.	

continued

Table 4 continued

Treated material	Mutagen	Response	Reference
5. Seed	Gamma rays	Bush type mutants and and varying degrees of earliness were obtained by using 120 Gy.	Anon, 1982
6. Seed	Gamma rays	Mutants obtained in M_3 by using 200 Gy of radiation. Mutants include single eret stem mutant, a multiple branch, and extra long podded mutant	Acquaah and Klu, 1983
7. Seed	Gamma rays	Early dwarf mutants selected in M_2 and M_3 by using 100 Gy	Shivashankar and Reddy, 1984
8. Seed	Gamma rays	Flowerless mutant with tuber obtained in M_2 when seeds were irradiated with 300 Gy.	Klu, 1985.
9. Seed	Gamma rays	Three dwarf mutants (one fertile) selected in M_2 with 100 Gy treatment.	Jugran *et al.*, 1986
10. Cotyledons, epicotyl, leaves and stem (*in vitro*)	Gamma rays	A dose of 100 Gy reduced callus formation.	Chow and Subha, 1986
11.Seed	Gamma rays	Eleven early mutants were selected in M_1 following 100 Gy treatment	Veeresh and Shivashankar, 1987
12. Seed	Gamma rays	Mutants for improved protein content obtained by using 100 - 400 Gy	Klu *et al.*, 1991

Table 5. Summary of cell, tissue and organ culture studies in winged bean (based partly on Tran Thanh Van *et al.*, 1986 and Venketeswaran, 1990)

Explant source	Explant	Medium*	Growth response	Reference
Leaves	i.Protoplasts	a.B5 (Modified) MS + NAA + BAP (Various combinations)	Callus, plantlets	Zakri, 1983.
		b.MS + 2,4-D(2) + KIN(0.25) MS + NAA(0.2) + BAP(2.0) MS + NAA(0.2) + BAP(1)	Callus,shoots buds and plantlets	Wilson *et al.*, 1985.
	ii.Primary leaves	a.Nitsch's medium + NOA(0.5) + 2iP(10) + CCC(0.1)	Shoots and plantlets	Blackmon *et al.*, 1980
		b.Nitsch & Nitsch + NOA(0.5) + 2iP(10) + CCC(0.1)	Buds and shoots	Blackmon and Reynolds, 1982.
	iii.Young leaves	a.MS + IAA(10^{-5}M) + BAP (10^{-5}M)	Callus and plantlets	Lie-Schricke and Tran Thanh Van, 1981.
		b.MS + IAA(10^{-6}M) + BAP (10^{-5}M)	Callus and buds	Trinh *et al.*, 1981.
	iv.Mature leaves	a.MS + NAA(0.02) + BAP(2) MS + NAA(2) + BAP(0.02) MS + IAA(0.2) + BAP(2)	Callus roots plantlets	Gregory *et al.*, 1980.
		b.MS + NAA(0.2) + BAP(2) MS + IAA(0.2) + BAP(2) MS + NAA(0.4) + BAP(2)	Callus buds shoots and plantlets	Evans *et al.*, 1981
Epicotyl	i.Protoplasts	KAO + 2,4-D(0.1) + BAP(0.5) + N-Z amine	Callus	Cuddihy and Bottino, 1981; 1982.
	ii.Fragments	a.B5 + 2,4-D(0.5) B5 + KIN(0.3) + IAA(5)	Callus roots	Bottino *et al.*, 1979.
		b.Nitsch's medium + NOA(0.5) + 2iP(10) + CCC(0.1)	Plantlets	Blackmon *et al.*, 1980.
		c.MS + 2,4-D(1) + NAA(1) + KIN(0.1)	Callus,embryoids plantlets	Venketeswaran, 1981.
		d.MS + IAA(10^{-6}M) + BA(10^{-5}M)	Direct bud formation	Trinh *et al.* 1981.

Table 5 continued

Explant source	Explant	Medium*	Growth response	Reference
		e.Nitsch & Nitsch +NOA(0.1) +2iP(20)+CCC(0.1)	Bud regeneration	Blackmon and Reynolds,1982.
		f.MS Or B5 + 2,4-D(1) + KIN(1)	Callus	Venketeswaran
		MS or B5 + KIN(1) + /or BAP(0.1)	shoots	*et al.*, 1985.
		MS or B5 + IAA(1)	plantlets	
		g.MS + 2,4-D(0.1-1.0) + NAA (0.1-1.0)	Callus	Venketeswaran, 1990.
		MS + BA(1-5) ± KIN(0.1)	Shoots	
		MS + IAA(1) ± KIN(0.1)	plantlets	
		MS(hormone free)	somatic embryos	
	iii.Fragments plus section of cotyledon	MS + NAA(0.5) + BAP(2)	Compact green callus	Venketeswaran, 1984.
	iv.Thin cell	a.MS + IAA(10^{-5}M) + BA(10^{-6}M)	Direct root formation	Lie-Schricke and Tran Thanh Van, 1981.
		b.MS + IAA(10^{-6}M) + BA(10^{-5}M)	Direct bud formation	Trinh *et al.*,1981.
Stem	i.Fragments	a.B5 + NAA(5 x 10^{-6}M)	Callus	Mehta and Moham Ram,1981.
		b.MS + IAA/NAA(0.2) + BAP(2)	Callus	Brunnel *et al.*, 1981.
		c.MS or B5 + 2,4-D(0.5)	Callus	Venketeswaran *et al.*, 1984.
		d.MS or B5 + 2,4-D(1) + KIN(1)	Callus	Venketeswaran
		MS or B5 + KIN(1)	shoots	*et al.*, 1985.
	ii.Thin cell	MS + IAA(10^{-5}M) + BA(10^{-6}M)	Callus,roots and buds.	Lie-Schricke and Tran Tranh Van, 1981; Trinh *et al.*, 1981.

continued

Table 5 continued

Explant source	Explant	Medium*	Growth response	Reference
Cotyledon	i.Young	MS + IAA(10^{-6}M) + BA(10^{-6}M)	Direct bud	Trinh *et al.*1981
	ii.Mature	a.MS + KIN(0.1) + NAA(0.1) MS (hormone free)	Callus and roots plantlets	Venketeswaran and Huhtinen 1978.
		b.B5 + BAP(10^{-5}M) + IBA(10^{-5}M)	Callus and roots	Mehta and Moham Ram, 1981.
		c.MS + 2,4-D(1) + NAA(1) + KIN(0.1)	Callus,shoots embryoids	Venketedswaran, 1981.
		d.MS + 2,4-D(1) + KIN(1) MS + BAP(1) + KIN(1) MS + IAA + IBA(1)	Callus shoots plantlets	Venketeswaran *et al.*, 1985.
		e.MS + 2,4-D(1) + NAA(1) MS + NAA(1) + BAP(1) MS (hormone free)	Callus shoots embryoids	Venketeswaran *et al.*, 1990.
Seedlings	Whole	B5 + BAP(5 x 10^{-6} - 3 x 10^{-5}M) BA + NAA(10^{-6} - 10^{-7}M)	Shoots plantlets	Mehta and Moham Ram,1981
Shoot tip	-	Nitsch's + NOA(0.5) + 2iP(10)	Plantlets	Blackmon *et al.*, 1980.
Embryo	Segment	MS or B5 + 2,4-D(1) + KIN(1) MS or B5 + KIN(1) + BAP(0.1) MS or B5 + IAA(1)	Callus shoots rooting	Venketeswaran, 1985.
Peduncle	-	Nitsch's + NOA(0.5) + 2iP(10) + CCC(0.1)	Shoots	Blackmon *et al.*, 1980.
Anthers	(Not specified)	a.B5 + 2,4-D(10) + 2iP(5) + KIN(5) + BAP(1)	Callus	Moham Ram, *et al.*,1982.
		b.MS + NAA(0.5) + BAP(2)	Callus	Venketeswaran, 1984.

*Concentrations in mg/L or in Molar where indicated. Abbreviations: MS - Murashige and Skoog(1962), B5 - Gamborg's salts (Gamborg et al.,1968), KAO - Kao(1975), IAA - Indole acetic acid, NAA - Napthalene acetic acid, KIN - kinetin, BA - benzyl adenine, BAP - benzylaminopurine, 2,4-D - 2,4-dichlorophynoxyacetic acid, GA_3 - gibberelic acid, NOA - 2-naphthyoxyacetic acid, CCC-chloroethylammonium chlorine, IBA-indole butyric acid.

Cell, tissue and organ culture studies

The earliest reports on winged bean tissue culture were by Tran Thanh Van and Trinh 1978) and Venketeswaran and Huhtinen (1978). Their reports stimulated the attention of a number of workers, who have used various explants including leaves (protoplast, primary, young and mature leaves), epicotyl (protoplast, fragments, thin cell layer), stem (internode, fragments of adult plants, thin cell layer), cotyledon (young and mature), shoot tips, whole seedlings, embryos, peduncles, anthers and pollen grains (Table 5). In most cases, plantlet regeneration was acomplished via callus; however, there are records of direct regeneration from epicotyl fragments and thin cell layers (Trinh *et al.*, 1981; Lie-Schricke and Tran Thanh Van, 1981) and from young cotyledons (Trinh *et al.*, 1981). Blackmon *et al.* (1980) and Blackmon and Reynolds (1982) developed shoots and plantlets from primary leaves, epicotyl fragments, cotyledons and peduncles. Somatic embryo development has also been reported on epicotyl and cotyledon explants (Venketeswaran *et al.*, 1990) and from pollen (Trinh *et al.*, 1981). Protoplast isolation and culture, callus establishment and regeneration have been carried out for four winged bean varieties (Venketeswaran, 1990).

CONCLUSION

The high level of self-polination in grain legumes results in low genetic variability (Erskine, 1978; Bajaj and Gossal.,1982). Whereas interspecific hybridisation involving *P. tetragonolobus* has not been successful yet, the improvement of this crop would require the use of other approaches which could increase the variability for selection. Some promising ones are mutation induction, with or without tissue culture techniques, as well as genetic engineering. Throughout the years, more than 100 mutant cultivars of grain legumes obtained through the use of mutagens have been released. These mutants have improved characters including yield, plant architecture, maturity time, seed quality, resistance and tolerance (Micke, 1988; Sigurbjörnsson, 1991). This approach should be applicable to the improvement of the diploid winged bean. Available data on mutagenesis in winged bean indicate a potential in meeting the improvement objectives (Table 4). The breeding potentials of genetic modifiaction seem worthwhile to be used in this crop. Application of these techniques would, in no doubt, be useful in the improvement of various traits in the winged bean.

CHAPTER 3

TISSUE CULTURE TECHNIQUES FOR IMPROVEMENT OF WINGED BEAN (*PSOPHOCARPUS TETRAGONOLOBUS* L. DC): DIRECT ORGANOGENESIS AND SOMATIC EMBRYOGENESIS IN MATURE COTYLEDON EXPLANTS

G.Y.P. Klu, C.J.J.M.Raemakers, E. Jacobsen, A.M. van Harten.

SUMMARY

As an alternative to the usual auxin-supplemented media for indirect regeneration, cytokinin-induced direct adventitious shoot development and somatic embryogenesis were studied using mature cotyledons of UPS 122 and Kade 6/16, cultivars of the winged bean (*Psophocarpus tetragonolobus* (L.) DC). These explants were cultured on two basic media, MS and B5, containing varying concentrations of BAP and 2iP. Changes in orientation of positions of explants in media contributed to differences in response. The highest numbers of adventitious shoots were developed on explants cultured on MS media containing cytokinin mixtures. An average of 9.6 and 8.6 adventitious shoots per explant were obtained for cvs Kade 6/16 and UPS 122 respectively on MS medium containing 11.1 μM BAP and 12.3 μM 2iP. There was also a simultaneous direct regeneration of adventitious shoots and somatic embryos on the adaxial surface of explants cultured with their abaxial surfaces on the medium. The embryos were found mainly towards the distal ends of the explants, while the adventitious shoots had their regions of concentration towards the axes of the explants. Wounding of the explants and the part of explant in contact with the MS media containing high concentrations of BAP stimulated direct somatic embryogenesis. Somatic embryos were regenerated mainly on the wounds at the proximal end of the explants.

INTRODUCTION

The winged bean, a grain legume with high nutrient value has been described as "a possible soybean for the tropics" (Anon, 1981). It has the potential of meeting the dietary needs of many people in the tropical and adjoining regions of the world. All parts of the plant, except the stem and roots, are edible and rich in proteins, minerals and vitamins (Klu *et al.*, Chapter 2). In order to maximize this desired impact, the winged bean requires improvement in various characters, such as growth habit, and the level of antinutritional factors in the seeds. Genetic engineering technologies, among others, can be employed in such a programme if efficient *in vitro* regeneration systems are available for this crop.

Plant regeneration can either be accomplished by adventitious shoot formation or by somatic embryogenesis. In both modes of regeneration, two extreme types can be distinguished: direct and indirect. Indirect adventitious shoot regeneration, i.e via callus phase, has been accomplished in the winged bean (Venketeswaran and Huhtinen, 1978; Blackmon *et al.*, 1980: Gregory *et al.*, 1980; Lie-Schricke and Tran Thanh Van, 1981; Trinh *et al.*, 1981; Blackmon and Reynolds, 1982; Zakri, 1983; Venketeswaran, 1985; Wilson *et al.*, 1985). The only records on direct shoot formation are connected with young (Trinh *et al.*, 1981) and mature (Dias *et al.*, 1986) cotyledon explants. The regeneration frequencies, however, have not been reported.

Direct somatic embryogenesis proceeds from already predetermined embryonic cells. Indirect

somatic embryogenesis, on the other hand, develops from cells which require redifferentiation before they can express embryogenic competence, and as a consequence, callus preceeds formation of the embryos (Sharp *et al.*,1980; Evans *et al.*,1981b). In somatic embryogenic systems, high numbers of regenerants can be obtained originating from a few or single cells. To reach this goal, in the majority of crops, auxins have been used. However, in some instances cytokinins have also been used (Williams and Maheswaran,1986: Merkle *et al.*,1990; Raemakers *et al.*,1995). In the winged bean, most of the reports are on indirect somatic embryogenesis induced by auxins, particularly, 2,4-dichlorobenzene (2,4-D) and naphthalene acetic acid (NAA) Venketeswaran (1990), Venketeswaran *et al.*(1990). No records on cytokinin-induced somatic embryogenesis have been encounted for the winged bean.

To our knowledge, this is the first report in winged bean on cytokinin-induced direct simultaneous adventitious shoot regeneration and on somatic embryogenesis from mature cotyledon explants. The use of mature cotyledons ensures the application of plant material at a definite developmental stage; thus avoiding the problem of knowing the exact stage to be used in case immature cotyledons are employed. Some conditions controlling this process are presented.

MATERIALS AND METHODS

Plant material and explant sterilization

Dry seeds of two winged bean (*Psophocarpus tetragonolobus* (L.) DC) cultivars UPS 122 and Kade 6/16 were surface sterilized for 20 minutes in 20% Blue Ram Bleach (a commercial bleach containing 4.5% chlorine) with a drop of Tween 20 and then rinsed several times with sterile, double distilled water. The seeds were then soaked in sterile, double distilled water for 24 hours to imbibe, after which the testa were removed.

Culture conditions and culture media.

Cotyledon explants were cultured in baby food jars containing solid (3g/L phytagel) regeneration media based on Murashige and Skoog (MS) salts and vitamins (1962) or Gamborg's (B5) salts and vitamins (Gamborg *et al.*,1968) supplemented with 30 g/L sucrose, plus various concentrations of N^6-benzylaminopurine (BAP) and 6(γ,-γ-dimethylallymino)-purine (2iP). After adjusting the pH to 5.8 by using NaOH, media were autoclaved for 15 minutes at 121°C and 1.05kg/cm. All cultures were incubated in a growth room regulated at

25°C and 16 hours with white fluorescent light at 40 $\mu Em^{-2}s^{-1}$. Cultures were refreshed every 14 days.

Effect of cotyledon explant orientation on the regeneration of adventitious shoots and somatic embryos on whole and wounded explants

Cotyledon explants were cultured with their distal ends on the different media. An individual treatment consisted of 5 replicates, each made up of 5 jars containing 3 cotyledon explants. This experiment sought to determine the optimum concentration of BAP and/or 2iP required for adventitious shoot and somatic embryo regeneration in MS and B5 basal media.

In another experiment, whole cotyledon explants (W) were cultured with their abaxial surfaces on the different media. Another set of explants, which were transversely sliced into proximal (P) and distal (D) or into proximal (P), distal (D) and middle (M) pieces were also cultured in the same way. Responses of each of the explants to the regeneration of adventitious shoots and/or somatic embryos were recorded.

Plantlet regeneration and establishment

Adventitious shoots developed from these processes were transferred into rooting medium made up of MS supplemented with 0.49 μM indole butyric acid (IBA) and 3g/L phytagel.

Histological investigations

Cotyledon explants bearing adventitious shoots and embryos were fixed in formol acetic acid and dehydrated in a series of ethanol and methyl propanol solutions and then embedded in paraffin. Serial sections were cut using Bright microtome 5030 and stained with Delafield's Haematoxylin and Eosin Y before examining under an "Olympus-IMT2" microscope.

RESULTS

Effect of medium composition on the regeneration of adventitious shoots on the axes of the explants cultured with their distal ends on medium

The response of cotyledon explants of winged bean cultivars UPS 122 and Kade 6/16 to adventitious shoot development at the proximal axes are presented in Table 1. The number of explants that regenerated adventitious shoots at the axes differed with the basal media type

as well as the concentrations of BAP and/or 2iP used. A higher percentage of the explants developed adventitious shoots in MS and B5 media supplemented with cytokinin mixtures than on media supplemented with BAP or 2iP alone. However, responses on B5 media were lower than those on MS media (Table 1). The cv UPS 122 had its highest regeneration response of 89.5% and 79.2% of explants in media containing 11.1μM BAP plus 12.3 μM 2iP and 5.6 μM BAP plus 6.2 μM 2iP respectively. A similar trend was observed for cv Kade 6/16 where the highest responses of 90.4% and 86.4% were obtained in 11.1 μM BAP plus 12.3 μM 2iP and 5.6 μM BAP plus 6.2 μM 2iP respectively (Table 1).

Number of adventitious shoots per axis of explants

A similar trend was observed for the number of adventitious shoots developed at the axes. The higher numbers of adventitious shoots per explant for cvs UPS 122 and Kade 6/16 were obtained on media containing cytokinin mixtures than on media supplemented with BAP or 2iP (Table 1). Additionally, explants cultured on B5 media with the same combinations of BAP and 2iP produced lower numbers of regenerants than those on MS media. The highest mean numbers of 9.6 and 8.6 adventitious shoots per explant were regenerated on MS media supplemented with 11.1 μM BAP and 12.3 μM 2iP for cvs UPS 122 and Kade 6/16 respectively. Certain concentrations of the phytohormones caused an enlargement of the proximal region of the explants into a "hump" on which the shoots developed (Table 1, Fig 1). The "hump" was formed mainly on media supplemented with BAP. Its size decreased with lower concentrations of BAP and was minimal on BAP and 2iP combinations or on 2iP alone.

Structure of adventitious shoots on "hump" and plantlet regeneration

A longitundinal section of the adventitious shoots developed on the proximal axes of the explants and the "hump" is shown in Fig 1. The cells in the adventitious shoots seem to be differently structured from those of the enlarged structure which has been called the "hump". All the adventitious shoots did not require an auxin-enriched medium for rooting. Shoots transferred into full strength MS medium solidified with 3 g/L phytagel rooted easily. However, the frequency of plantlet regeneration was lower for shoots developed on "humps" than those on axes without this swelling. Media containing 0.49 μM IBA caused rooting but with callus formation.

Table 1. Development of adventitious shoots on the axes of winged bean cotyledon explants of the cultivars UPS 122 and Kade 6/16 cultured with their distal ends on media containing various cytokinin mixtures

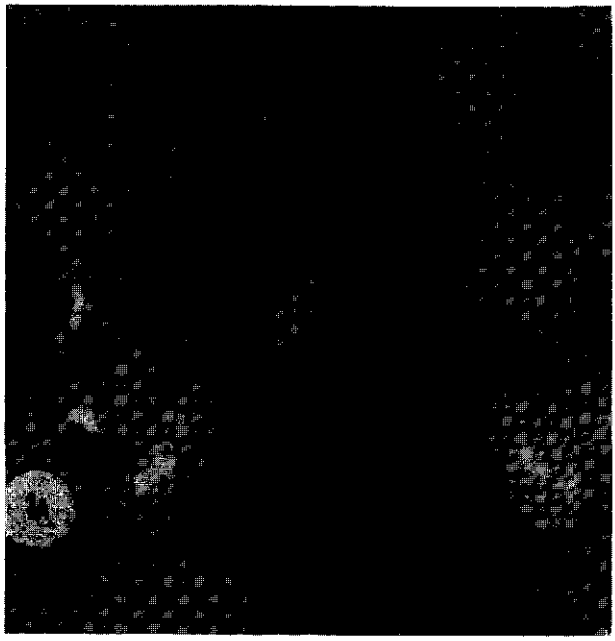
Winged bean cultivar	Basal medium	Cytokinin combination (μM) BAP	2iP	% Response	"Hump" formation*	Mean number of adventitious shoots explant^{-1}
		0	0	0.0±0.0	-	0.0± 0.0
		5.6	0	10.4±2.8	+	1.5± 0.4
		11.1	0	59.2±4.9	+ +	2.0± 0.6
		22.2	0	60.8±7.5	+ + +	5.0± 0.6
		44.0	0	62.4±4.3	+ + +	5.0± 1.1
	MS	0	6.2	46.3±8.2	-	1.3± 0.3
		0	12.3	44.0±7.5	-	2.2± 0.8
		0	24.6	44.8±4.5	+	3.0± 0.6
		0	49.0	63.0±8.2	+	4.2 ±1.0
		5.6	6.2	79.2±3.2	-	7.4± 0.8
		11.1	12.3	89.5±4.5	-	9.6± 0.8
		22.2	24.6	69.6±2.8	+	6.2± 0.4
UPS 122		44.0	49.0	52.3±5.8	+	6.3± 0.2
		0	0	0.0 ± 0.0	-	0.0 ±0.0
		5.6	0	3.5 ± 3.5	+	0.7± 0.3
		11.1	0	25.6±3.2	+ +	1.8± 0.4
		22.2	0	52.8±5.5	+ + +	3.0± 0.6
		44.0	0	51.2±4.8	+ + +	3.0± 0.6
	B5	0	6.2	44.3±5.6	-	1.5± 0.2
		0	12.3	27.2±4.7	-	1.8± 0.8
		0	24.6	36.8±5.8	+	0.8± 0.4
		0	49.0	48.0±6.8	+	3.6 ± 1.1
		5.6	6.2	78.4± 5.2	-	6.6 ± 0.5
		11.1	12.3	67.2±6.3	-	7.2 ± 0.2
		22.2	24.6	67.4±5.4	+	4.6 ± 0.5
		44.0	49.0	50.1±4.8	+	3.4 ± 0.5

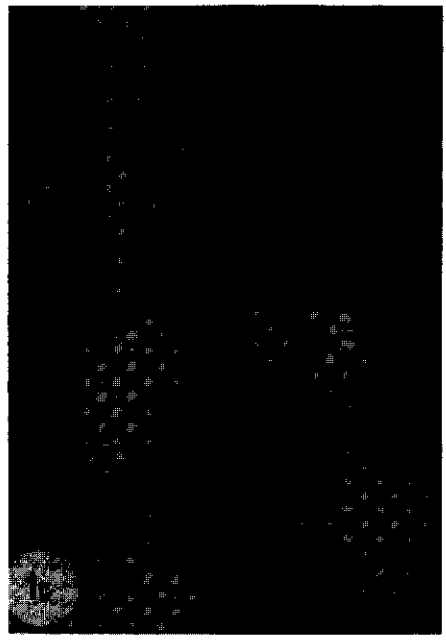
continued

Table 1 continued

Winged bean cultivar	Basal medium	Cytokinin combination (μ M) BAP	2iP	% Response	"Hump" formation	Mean number of adventitious shoots explant^{-1}
		0	0	6.1 ± 2.1	-	0.2 ± 0.9
		5.6	0	20.4 ± 4.5	+	1.3 ± 0.5
		11.1	0	51.2 ± 5.2	+	2.0 ± 0.3
		22.2	0	43.2 ± 4.5	+ + +	5.0 ± 0.6
		44.0	0	63.6 ± 3.2	+ + +	4.8 ± 0.5
		0	6.2	60.3 ± 5.2	-	1.7 ± 0.3
	MS	0	12.3	44.0 ± 3.5	-	2.4 ± 0.5
		0	24.6	52.0 ± 4.5	+	3.6 ± 0.5
		0	49.0	62.4 ± 3.2	+	4.6 ± 0.8
		5.6	6.2	86.4 ± 5.2	-	6.6 ± 1.1
		11.1	12.3	90.4 ± 4.8	-	8.6 ± 0.1
		22.2	24.6	48.8 ± 5.5	+	6.6 ±1.9
		44.0	49.0	62.5 ± 4.5	+	5.4 ± 2.1
Kade 6/16						
		0	0	2.3 ±2.3	-	0.1 ± 0.4
		5.6	0	8.4 ±5.1	+	0.2 ± 0.2
		11.1	0	42.4 ± 3.2	+ +	1.8 ± 0.8
		22.2	0	50.4 ±5.5	+ + +	3.4 ± 0.5
		44.0	0	45.6 ± 5.6	+ + +	4.2 ± 0.4
		0	6.2	44.3 ± 4.5	-	2.5 ± 0.3
	B5	0	12.3	32.0 ± 3.5	-	2.6 ± 0.5
		0	24.6	44.0 ± 4.2	+	3.2 ± 0.8
		0	49.0	42.4 ± 5.2	+	4.6 ± 0.5
		5.6	6.2	65.6 ± 3.8	-	4.4 ± 0.5
		11.1	12.3	62.4 ± 4.5	-	5.4 ± 0.5
		22.2	24.6	39.2 ± 4.2	+	5.2 ± 0.4
		44.0	49.0	3.1 ± 3.2	+	5.2 ± 0.4

* - Absent , + Low level, + + High level, + + + Very high level.





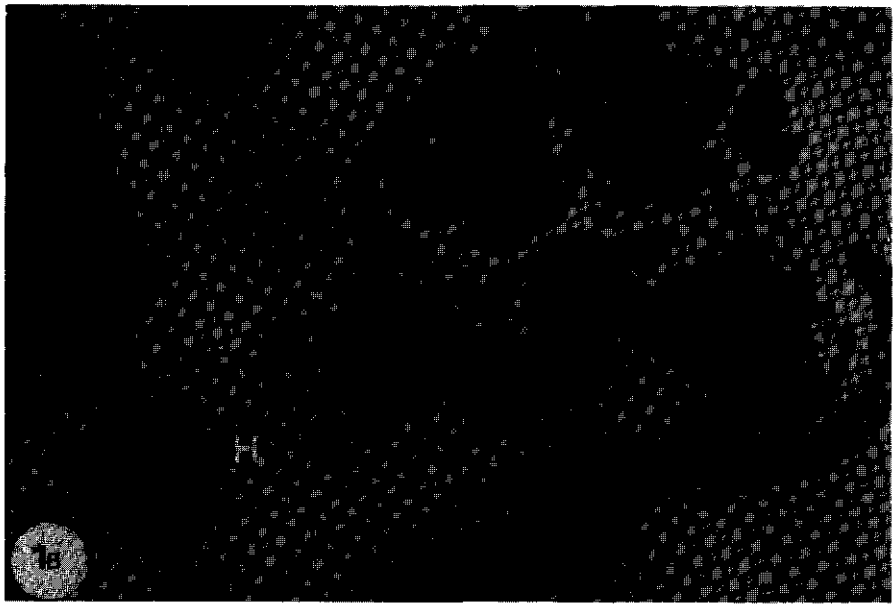


Figure.1. Adventitious shoots developed on mature cotyledon explants of winged bean cvs UPS 122 and Kade 6/16. E-explant, H-"Hump". 1A. Adventitious shoots developed on "Hump" at axis of cotyledon explant. 1B. Longitudinal section of "hump" plus adventitious shoots. 1C. A plantlet developed from an adventitious shoot.

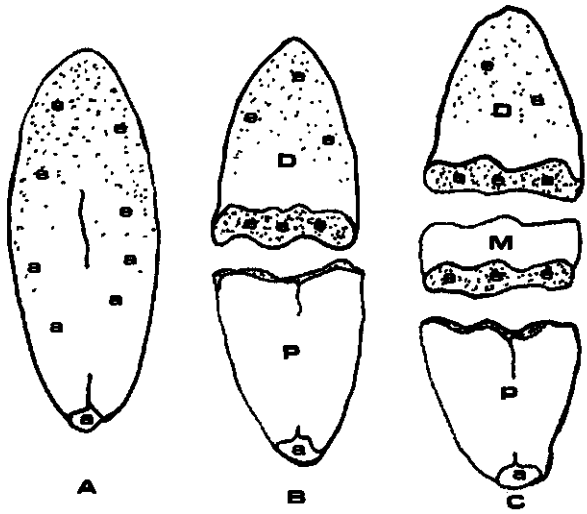


Figure 2. Regions of BAP induced adventitious shoots and somatic embryos on the adaxial surface and on wounded edges of mature cotyledon explants of winged bean cultured with the abaxial surfaces on medium. A-whole cotyledon piece, B and C - wounded cotyledons with proximal (P), middle (M) and distal (D) piece explants. a-adventitious shoots, e - embryoids.

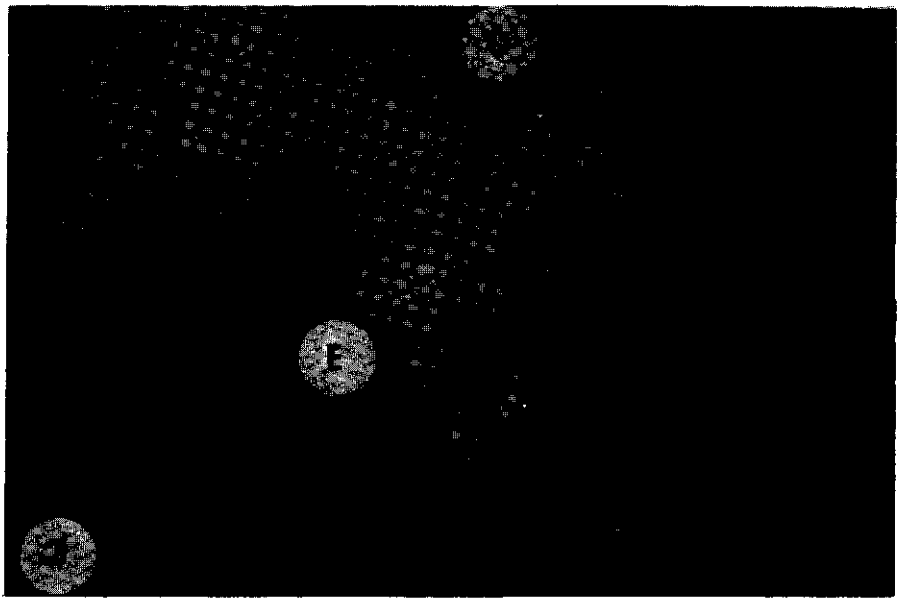


Figure 3. BAP induced somatic embryos on the wounded edges of mature cotyledon explants of winged bean. E- explant, e- embryoids, C- wounded surface.

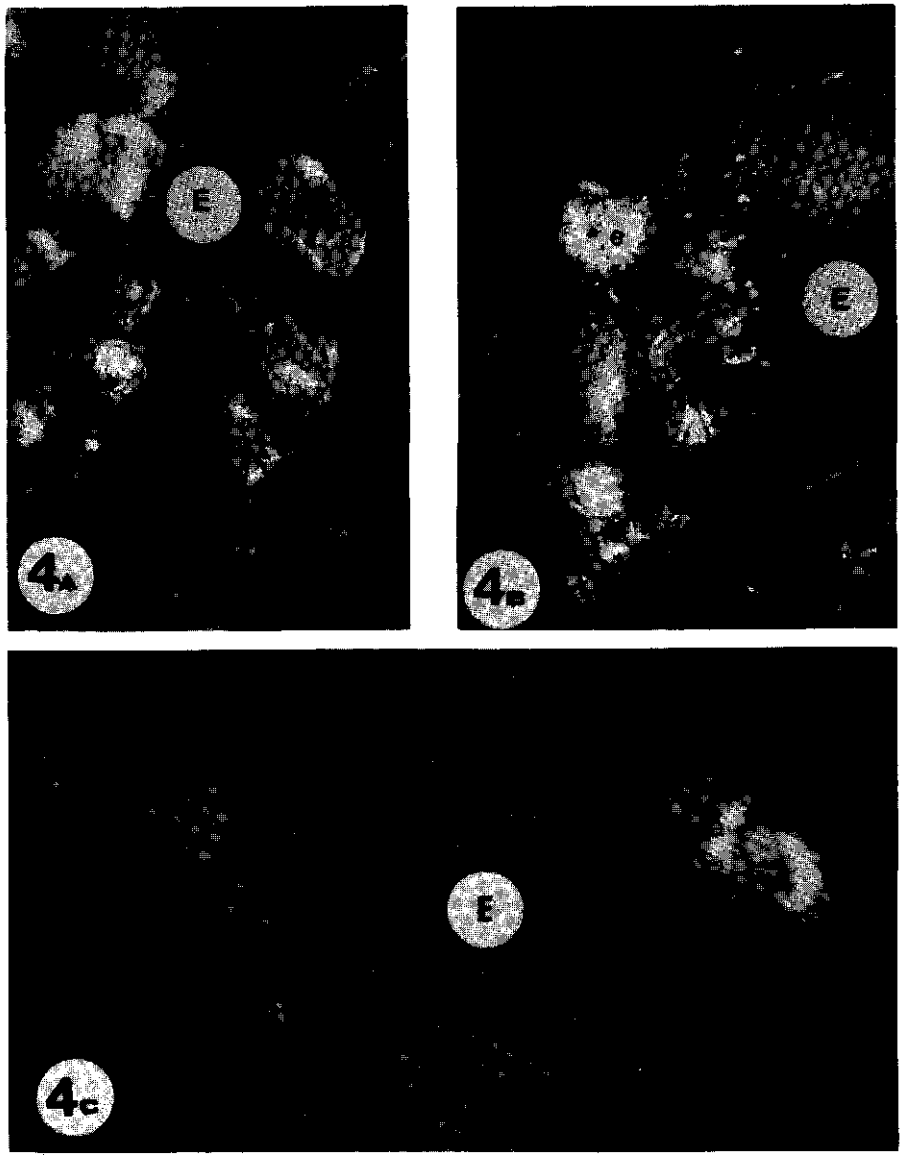


Figure 4. BAP induced adventitious shoots and somatic embryos regenerated on the adaxial surfaces of mature winged bean cotyledon explants. A, B and C, explants with adventitious shoots and somatic embryos on surfaces of explants, E- explant, a- adventitious shoot, e, embryo.

Effect of cotyledon explant orientations and wounding on the development of adventitious shoots and somatic embryos

Fig. 2 shows a schematic representation of the responses observed on explants cultured with their abaxial surfaces on media. The explants which had proximal axes, ie whole (W) and proximal (P) pieces, formed adventitious shoots at the proximal axes; the distal (D) and middle (M) piece explants did not form adventitious shoots at the proximal ends. The highest number of adventitious shoots developed per explant at the axes was 6 with 11.1 μM BAP plus 12.3 μM 2iP in MS medium. Higher concentrations of BAP reduced the delopment of adventitious shoots at the proximal ends as shown for explants cultured vertically (Table 1). On the other hand, these high concentations of BAP in MS medium induced both adventitious shoots and somatic embryos on the adaxial surfaces of the explants within 4 weeks after culture (Fig 3). The adventitious shoots were located mainly on the proximal (P) pieces and towards the proximal ends of the whole (W) explants. The embryoids were located mainly on the distal ends of the W explants and on the whole of the D explants (Fig 2). These adventitious shoots and somatic embryos were not obtained on explants cultured on media containing BAP and 2iP combinations and also 2iP alone.

Wounding of the explants also stimulated embryogenesis. Globular stage embryoids were generated on the wounded edges of the explants cultured on MS medium containing 44 μM BAP (Figs 2 and 3). The embryoids were developed mainly on the wounds at the proximal ends of the M explants, and also on the wounds of the D explants than on those of the P explants. After about 28 days of culture some of the globular embryos developed into torpedo shaped ones.

DISCUSSION

Adventitious shoot regeneration in winged bean cultures has been observed to occur via callus induction (Gregory *et al.*,1980; Wilson *et al.*,1985; Venketeswaran *et al.*,1985; 1990). Plants regenerated from callus sometimes show variability (Larkin and Scowcroft,1981) which may be useful in breeding programmes. On the other hand, the use of direct organogenic and embryogenic systems is preferred in situations in which genetic variation is to be avoided. Cotyledons have exhibited a high potential for plant regeneration in a number of crops including soybean, *Glycine max*, (Mante *et al.*,1989), *Brassica juncea* (Sharma *et al.*,1991), *Helianthus annuus* (Knittel *et al.*,1991) and *Pisum sativum* (Özcan *et al.*,1992). Most available records on direct adventitious shoot induction are from juvenile explants. In our investigations, adventitious shoots were obtained from mature explants. The results obtained

showed that a mixture of 11.1μM BAP and 12.3μM 2iP in MS medium yielded 9.6 and 8.6 shoots per explant for cvs Kade 6/16 and UPS 122 respectively. Although higher BAP levels increased the number of adventitious shoots it also promoted formation of the earlier reported "hump". "Hump" formation is caused by vitrification of shoots ie. hyperhydricity of shoots (Debergh *et al.*,1992). It is preferable to use lower concentrations of cytokinin to obtain adventitious shoots which are able to develop into plants at higher frequencies. Induction of adventitious shoots using BAP has been reported for many other crops. McKently *et al.* (1990) used 122μM 2iP on cotyledon explants of peanut (*Arachis hypogaea*) in which a structure similar to the "hump" recorded here was reported. Similar reports on the use of high BAP concentrations have been documented but without any records of the reported swelling of the proximal axes as observed in our investigations. For example, Qureshi and Saxena (1992) used 20μM BAP for the induction of adventitious shoots and somatic embryos on intact seedlings of seed geranium (*Pelargonium hortorum*). While Malik *et al.*,(1992) used 50μM BAP for direct adventitious shoot regeneration on epicotyl explants of *Lathyrus cicera, L. ochrus* and *L. sativus,* Malik and Saxena, (1992 a,b) used 50-80μM BAP for inducing regeneration of adventitious shoots and somatic embryos on intact seedlings of *Phaseolus acutifolius, P. aureus* and *P. coccineus, P. wrightii* and *P. vulgaris.* In order to avoid "hump" formation from the high concentrations of the BAP, combinations BAP and 2iP in MS are, therefore, recommended for routine adventitious shoot regeneration from the cotyledonary nodes of mature winged bean cotyledons.

Changes in orientation of the explant in the medium caused differences in response. Embryoids and adventitious shoots were observed on the adaxial surface when the abaxial surface of the explant was placed on the medium. Direct embryoid formation was predominant at the distal ends of the explants. This corresponds with the observation in sunflower by Ceriani *et al.*, (1992) that explants derived from distal and proximal regions of cotyledons develop differently. Direct somatic embryogenesis has been documented in some legumes including peanut, *Arachis hypogaea* (Hazra *et al.*, 1989), white clover, *Trifolium repens* (Maheswaran and Williams, 1984; 1985; 1987), soybean, *Glycine max* (Finer, 1988), red bud, *Cercis canadiensis* (Trigiano *et al.*, 1988) and *Albizzia lebbech* (Gharyal and Maheshwari, 1981). In these reports auxins were used for the induction of embryogenesis, whereas in the present report on winged bean cytokinins were used. Somatic embryogenesis had previously been obtained in winged bean indirectly by using auxins (Venketeswaran, 1990; Venketeswaran *et al.*, 1990). This legume is, hence, amenable to both auxin and cytokinin-stimulated somatic embryogenesis.

The role of the high concentration of BAP in the embryogenic process is not clear. Sharma

et al., (1991), however, have suggested its linkage with a diffusible growth factor promoting somatic embryogenesis emanating from the explants. Such a growth factor has been shown to reside in the radicular halves of the cotyledons of *Cassytha filiformis* (Rangaswamy and Rangan, 1971). Raju and Mann (1970) have also observed that cells with the potentials for shoot formation in *Escheveria elegans* are restricted to the proximal end. This "diffusible" factor has been suggested to be an auxin-like substance which, in the presence of a cytokinin, activates the totipotent cells for bud formation in *Glycine max* (Cheng *et al.*, 1980), *Cucinus sativus* (Gambley and Dodd, 1990) and *Brassica juncea* (Sharma *et al.*, 1991). Development of adventitious shoots in the cotyledonary explants studied in this investigation indicates that an auxin-like substance might be present in these explants to cause induction of buds and shoots in the proximal end of the explants. The role of this substance in relation to both adventitious buds and embryoids following the use of high BAP concentrations is unclear. Nevertheless, this factor might have contributed to the process of embryogenesis in both the intact cotyledons and the wounded pieces. This is more probable in the latter case since wounding of tissues may foster or allow the release of endogenous hormones (Smith and Krikorian, 1990). The flow and transportation of this hypothetical "auxin-factor" may be basipetal and gets trapped at the wounds where, with the BAP, it aids embryogenesis (Smith and Krikorian,1990; Terzi and Loschiavo,1990). The concentration of BAP and the endogenous factor may be crucial in the induction of embryogenesis. This may explain the lack or minimal response on the distal wounds to embryogenesis (Fig.2).

Wounding has also been reported to disturb the ability of explant tissues to regulate K^+ exchange leading to increased osmotic potential of cells and, consequently, to the generation of an electrical field across the explant; this in turn controls embryogenesis (George and Sherrington, 1984; Rathore *et al.*,1988). Subsequent studies to identify the "auxin-factor" and its mechanism of regulation would provide further information on somatic embryogenesis.

This report is on the use of cytokinins for direct adventitious shoots and somatic embryo initiation from mature cotyledon explants. It increases the number of successful regeneration procedures for this important crop.

CHAPTER 4

OPTIMISATION OF MUTANT RECOVERY FROM PLANTS OBTAINED FROM GAMMA-RADIATED SEEDS OF WINGED BEAN (*PSOPHOCARPUS TETRAGONOLOBUS* (L.) DC)

G.Y.P. Klu, A.M. van Harten, E. Jacobsen

SUMMARY

Dry seeds of winged bean (*Psophocarpus tetragonolobus* (L.) DC) cvs UPS 122 and Kade 6/16 were treated with acute radiation doses of 150 Gy and 250 Gy at a dose rate of 737.32 Gy/hr from a Cobalt-60 gamma source for studies in optimisation of mutant selection in M_2 and M_3 populations. Mature dry pods were harvested at four different locations on each M_1 plant viz. 0.5, 1.0, 1.5 and 2.0 metres from the ground. M_2 seedlings were screened for different groups of chlorophyll deficiencies and their frequencies. Reduction in chlorophyll mutation frequency from the first formed seeds to the latest ones within the M_1 pods has been observed for both cultivars studied. The high degree of chimerism recorded in the M_2 seedlings present in the first-formed seeds in the M_1 pods provides a clear indication that these seeds constitute a zone from which seeds for the M_2 generation have to be harvested in order to give the highest probability for obtaining different types of mutants. On the other hand, significant differences in mutation frequency were not obtained in M_2 seedlings from pods harvested at the various positions on the M_1 plants. M_1 pods can be harvested at any height on the M_1 plant, but it is preferable to use the earliest mature ones to save time and labour. The zones identified on M_1 plants in this investigation coupled with the use of the "spare" or "remnant" seed selection method, should provide an improved method for mutation breeding in a viny legume like the winged bean.

INTRODUCTION

The winged bean (*Psophocarpus tetragonolobus* (L.) DC) is a pulse crop with high nutritonal value. Apart from its stem and roots, all parts of the plant are edible and rich in proteins, amino acids and vitamins. The mature seeds contain 20 - 46 % protein and 17 - 22 % oil, but it also have antinutritional factors like anthocyanidins. This crop has not received much research attention untill about two decades ago (Anon,1981). In order to make optimal use of the winged bean, it needs to be genetically improved to meet particular needs. For the purposes of developing this crop into a grain legume, cultivars which are bush type with annual growth habit are required. In addition, these cultivars must have synchronous maturity of pods which contain seeds with high nutritional quality (Eagleton *et al.*,1985; Lazaroff,1989). Intraspecific hybridisation should offer recombination of traits and interspecific hybridisation the introduction of new traits like resistances. However, almost no intraspecific and interspecific hybridisation successes have been encountered in literature. So far, no conspecific wild relatives producing viable interspecific hybrids are known (Smart,1990). There is also no record of embryo rescue. Mutation breeding, therefore, could offer an alternative means of obtaining the desired traits in the existing varieties of this self-fertilising, diploid crop.

The success of a plant breeding programme depends on various factors. These include the

amount of genetic variation available in the breeding population, recombination of traits by crossing and the effectiveness of the selection technique and method used (Brock,1965). In winged bean, a mutation breeding programme leading to the production of improved and well-adapted cultivars that would meet agricultural needs could be a suitable approach. In such breeding programmes, management of the M_1 and M_2 populations determine the effectiveness and efficiency of mutant selection (Konzak *et al.*, 1965; Brock, 1979; Dellaert, 1979). The effectiveness, i.e. the number of mutations produced and the efficiency, i.e. the extent of obtaining desirable effects (Konzak *et al.*, 1965) of a mutation breeding programme can be estimated by studying the frequencies of chlorophyll deficient mutations or other easily recognisable phenotypic characters in M_2 seedlings (Brock and Micke, 1979).

Additionally, the techniques that can be applied, in turn, are dependent on the crop species being studied and on the trait that has to be altered. This is because the M_1 plant is chimeric. Earlier views on the fate of a chimeric tissue in the M_1 plant indicate that the growth of mutated and unmutated cell progenies may lead to competition between the groups and a gradual elimination of the mutated cells. This process, referred to as intrasomatic or diplontic selection, is supposed to lead to a reduction in the frequency of sexual offspring plants which are solid mutants (Kaplan, 1951; Gaul,1961; D'Amato,1965; Anon,1977). On the other hand, some other studies have led to the observation that there is no intrasomatic selection against mutated sectors and that chimerism is not a hindrance to mutant selection but may even be desirable (Lindgren *et al.*,1970; Harle,1972; 1974; Ukai and Yamashita, 1974; Cassells and Periappuram, 1993; Cassells *et al.*, 1993). It has further been noted that ontogenesis in a chimeric tissue in a dicotyledonous plant would lead to a generation of mutated and non-mutated branches or sectors. This process referred to as "diplontic drift" (Balkema,1972) would therefore, provide a high probability of obtaining several types of induced mutants (Micke *et al.*, 1987). In considering the cost of mutation breeding involving the chimeric regions, one would need information on the management of the M_1 population. For example, the cost of sowing the M_1 and M_2 generations is taken into account when one or a few seeds per harvested M_1 branch or sector are sown and "spare seed" is kept from each sector and advanced as M_3 lines, if the offspring from an M_1 plant branch or sector segregates for a desired mutant (Dellaert, 1979; 1983). The most suitable method of harvesting the M_1 plants would also depend on the specific properties, such as plant architecture and breeding system, of the plant being studied. It means that each breeding plan must be backed by appropriate selection tools.

The winged bean plant is viny and perennial; it grows up to three to four metres on stakes and is usually cultivated as an annual herb. This pulse has trifoliate leaves; the leaflets are

typically broadly rhomboid, up to 10cm long and have 5 to 15cm long peduncles (Verdcourt and Halliday, 1978). There are 2-10 flowers in axillary racemes, up to 15 cm long. The pods are more or less square in section, 6-30cm or longer with 4 longitudinal ridges or "wings" (Duke *et al.*, 1981). The winged bean with a chromosome number of $2n = 2x = 18$, is a diploid self pollinator since pollen is shed in the night prior to opening of the flower (Pickersgill, 1980; Harder and Smartt, 1995). In the present study, mutation induction studies aimed at broadening the genetic base for selection and the development of an economic selection scheme in the M_1, M_2, and M_3 generations were undertaken. These studies involved the determination of chlorophyll deficient mutations at various pod locations on the M_1 plants and at the seed position within M_1 pods. The usefulness of these observations in mutant selection is discussed.

MATERIALS AND METHODS

Plant material and mutagenic treatment.

Seeds of the winged bean (*Psophocarpus tetragonolobus* (L.) DC) cvs UPS 122 and Kade 6/16 obtained from the Agricultural Research Station of the University of Ghana at Kade were used. Dry seeds of uniform size were equilibrated to 13% moisture content by keeping them in a dessicator containing 65% glycerol for four days. Seed samples were acutely irradiated at total doses of 150 Gy and 250 Gy (at a dose rate of 737.32 Gy/hr) by using a Cobalt-60 Gamma Cell 220 at the Ghana Atomic Energy Commission, Kwabenya, Ghana. These doses were selected from a preliminary experiment carried out by the first author to determine the dose response of winged bean seed samples (data not shown) and from reports in the literature on winged bean mutation breeding programmes that have yielded some mutants (Shivashankar and Reddy, 1984; Jugran *et al.*, 1986; Veeresh and Shivashankar, 1987). Treated seeds and their controls were sown immediately in the field at a planting distance of one metre between plants, and the plants were supported on 3-metre stakes.

Study on effect of pod location on M_1 plants and M_2 seed location within M_1 pods on mutation frequencies

Mature dry pods were harvested at four different locations on each M_1 plant (0.5, 1.0, 1.5 and 2.0 metres from the ground). The M_2 seeds from these pods - separately per pod - were sown in the field in the order in which they appeared in the pods, taking the seed nearest to the stalk as the first. M_2 seedlings were screened for chlorophyll deficient mutations by using

descriptions based on Gustafsson (1940) and Blixt and Gottschalk (1975). These include albina types which are characterised by white leaves; alboviridis types with leaves that have a white top and a yellowish green base; chlorina with yellowish-green leaves; chlorotica which are depicted by greenish-yellow leaves; variegata with yellowish-white spots on leaves; viridis types with uniform light green leaves; and xantha types with straw-yellow seedlings. The frequency of the occurrence of a chlorophyll deficient mutant was expressed as percentage of the total number of M_2 plants obtained from a particular position on the M_1 vine.

RESULTS

M_2 chlorophyll mutation frequency based on the pod location on M_1 plants and on M_2 seed position in M_1 pods

No chlorophyll-deficient mutations were recorded in the untreated material itself and in its sexual offspring. Records on M_1 plant progenies segregating for chlorophyll deficient mutations among M_2 seedlings, related to pod and seed positions in the two winged bean cultivars UPS 122 and Kade 6/16, are presented in Fig. 1 and Table 1. The frequency of chlorophyll deficient M_2 mutants was between 30% and 63%. The mean chlorophyll mutation frequency at 150 and 250 Gy treatments were 46.77 and 51.13 % respectively for cv UPS 122 and 54.88 and 45.73 % respectively for cv Kade 6/16. There seemed to be no difference between the cultivars in the mutation frequencies. In addition, the two doses applied did not seem to show marked differences (Table 1). No difference was observed in the chlorophyll mutation frequencies among the M_2 seedlings obtained from the various pod locations on the M_1 plants. However, the chlorophyll mutation frequency among the M_2 seedlings related to the seed position in the M_1 pods, decreased from the first-formed seeds to the latest ones in both cvs UPS 122 and Kade 6/16 irradiated with 150 Gy as well as 250 Gy (Fig.1,Table 1). No chlorophyll mutations were obtained beyond the 12th seed position in the M_1 pods. In addition, samples of cv UPS 122 treated with 250 Gy had no chlorophyll mutations beyond the 9th seed position (Table 1). In cv Kade 6/16, at the same dose, a similar situation has been recorded except that chlorophyll mutations obtained beyond the 9th seed position were recorded only in pods positioned at about 2 metres from the ground. The chlorophyll deficiency mutation frequency did not seem to differ at the various pod locations on M_1 plants. Within the M_1 pods, the frequency of these mutations were much higher among the seedlings derived from the M_2 seeds nearer to the pod stalk than from those farther away from the stalk (Table 1, Fig.1). In Table 2, the chlorophyll mutation frequency is related to seed position within individual pods. It clearly shows that the mutation frequency in both treatments is highest at the seed positions 1 to 3, and decreasing gradually to zero at seed

positions 13 to 15. In conclusion, the highest levels of chimerism in M_1 plants have been located within the M_1 pods but not between the various pod positions. Seeds for the M_2 generation, if taken from the first formed seeds, in individual pods, would provide a higher number of mutants than if taken from the later-formed ones. In addition, it is preferable that the earliest mature pods are used to save time and costs.

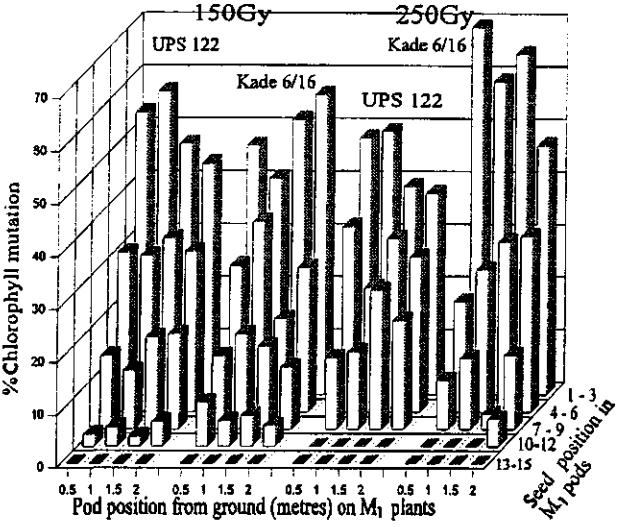


Figure 1. M_2 seedlings segregating for chlorophyll mutations related to pod location on M_1 plants and seed location in M_1 pods of irradiated seeds of winged bean cvs UPS 12 and Kade 6/16.

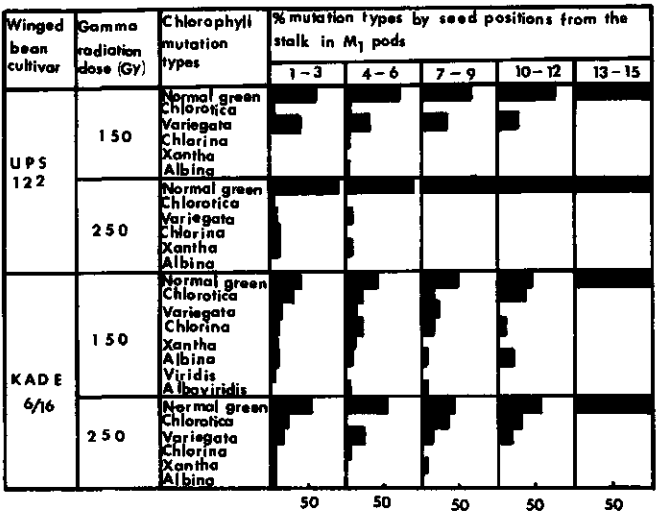


Figure 2. Chlorophyll mutation spectrum among M_2 seedlings of winged bean cvs UPS 122 and Kade 6/16

Table 1. Analysis of M_1 plants based on M_2 seedlings which were segregating for chlorophyll mutations

Winged bean cultivar	Gamma radiation dose (Gy)	Pod location on M_1 plants☼	Total No. of seed-lings	No. of M_2 seedlings segregating for chlorophyll deficient mutations (Loc)*					Total No. of mutants	% of mutants
				1-3	4-6	7-9	10-12	13-15		
UPS 122	0	A	63	0	0	0	0	0	0	0.00
		B	104	0	0	0	0	0	0	0.00
		C	90	0	0	0	0	0	0	0.00
		D	85	0	0	0	0	0	0	0.00
	150	A	75	23	13	7	1	0	44	58.67
		B	116	31	16	6	3	0	56	48.60
		C	208	49	34	19	1	0	103	49.52
		D	337	46	32	19	5	0	102	30.27
	250	A	75	18	13	5	0	0	36	48.00
		B	60	17	8	5	0	0	30	50.00
		C	123	30	25	21	0	0	76	61.79
		D	76	13	10	11	0	0	34	44.74
Kade 6/16	0	A	65	0	0	0	0	0	0	0.00
		B	96	0	0	0	0	0	0	0.00
		C	82	0	0	0	0	0	0	0.00
		D	95	0	0	0	0	0	0	0.00
	150	A	74	17	10	5	5	0	37	50.00
		B	97	25	22	11	3	0	61	62.87
		C	108	27	9	8	4	0	48	44.44
		D	82	29	14	6	2	0	51	62.20
	250	A	79	30	9	4	0	0	43	54.43
		B	92	22	10	5	0	0	37	40.22
		C	79	22	11	1	0	0	34	43.04
		D	126	27	19	8	3	0	57	45.24

☼ A - 0.5, B - 1.0, C - 1.5, D - 2.0 metres from the ground

* Seed location within M1 pod (1 nearest to pod stalk)

Table 2. Chlorophyll deficient mutation frequency among M_2 seedlings related to seed location within M_1 pods of irradiated seeds of winged bean cvs UPS 122 and Kade 6/16 .

Winged bean cultivar	Gamma radiation dose (Gy)	Seed location in M_1 pods	M_2 seedlings				
			No. per seed location	Total No. per treatment	No. with chlorophyll deficient mutants	Total No. of mutants per treatment	% chlorophyll deficient mutants per treatment
UPS 122	0	1- 3	285	705	0	0	0.00
		4- 6	224		0		0.00
		7- 9	108		0		0.00
		10-12	56		0		0.00
		13-15	32		0		0.00
	150	1- 3	383	764	154	257	20.16
		4- 6	248		48		6.28
		7- 9	87		38		4.97
		10-12	40		14		1.87
		13-15	6		3		0.39
	250	1- 3	105	300	13	20	4.33
		4- 6	62		7		2.33
		7- 9	53		0		0.00
		10-12	57		0		0.00
		13-15	23		0		0.00
Kade 6/16	0	1- 3	317	711	0	0	0.00
		4- 6	191		0		0.00
		7- 9	111		0		0.00
		10-12	45		0		0.00
		13-15	47		0		0.00
	150	1- 3	154	367	90	200	24.52
		4- 6	112		63		17.17
		7- 9	55		31		8.45
		10-12	31		10		2.72
		13-15	15		6		1.64
	250	1- 3	225	385	99	21	25.71
		4- 6	110		49		12.73
		7- 9	39		21		5.46
		10-12	7		3		0.78
		13-15	4		0		0.00

Chlorophyll mutation spectrum among M_2 seedlings recorded by M_2 seed location within M_1 pods

As expected, no chlorophyll mutations were obtained among the untreated material (Tables 1 and 2). The spectrum of chlorophyll mutations obtained is presented in Fig.2. They included variegata (56.7 %) and chlorotica (26.6 %) types, which were most frequently found. Chlorina (6.6 %), Xantha (5.8 %) and albina (3.9 %) types were found in much lower frequencies. There were also the alboviridis (0.5 %) and viridis (0.2 %) types.

DISCUSSION

Chlorophyll formation in plants is the ultimate result of a chain of biochemical activities in which many gene loci are involved. The inhibition or blocks in chlorophyll formation in mutants like xantha and albina have been related to changes in chromosomal (Gustafsson,1979) and plastid genes (von Wettstein *et al.*,1971;1978). The chlorophyll mutants recorded in the present experiment include chlorina, xantha and albina, as well as viridis and alboviridis, for which several loci have been established in pea, *Pisum sativum* (Blixt and Gottschalk,1975). The other groups of chlorophyll mutants viz. chlorotica and variegata were most frequent in our experiment. In pea, the occurence of the mutant type, chlorotica, appeared to be associated with a considerable reduction in seed set; for the mutant type variegata, a chromosomal inheritance has been reported to be involved, as well as a cytoplasmic inheritance (Blixt and Gottschalk,1975). The mutants obtained in our investigations could be based on chromosomal as well as extra-chromosomal mutations.

Studies to optimise mutant selection at a reduced cost in an M_2 population following seed irradiation in a viny plant, like the winged bean (*P. tetragonolobus* (L.) DC), were carried out. This is important in view of the large M_2 population generally needed for mutant selection due to the presence of chimerism in M_1 plants. In sexually propagated plants, several procedures for sampling seed progenies as a means of handling the induced chimerism have been proposed. Dellaert (1979, 1983) took into account the costs of growing the M_1 and M_2 generations by proposing the sowing of one or a few seeds in a harvested part on the chimeric M_1 plant. From each zone "spare seed" was stored and used to raise a larger M_2 population if the offspring of a zone segregated for the desired mutant. This has been investigated for okra (*Abelmoschus esculentus*) in which the first fruit on the main stem of each M_1 plant was identified as the propagule to be harvested. In okra, each fruit can yield sufficient seeds to raise an entire M_2 population as single fruit progenies (Bhatia and Abraham, 1983). Hermelin *et al.*, (1983) made a similar study for Faba bean (*Vicia faba*), pepper (*Capsicum annuum*) and

flax (*Linum usitatissimum*). For *Vicia faba* the pattern of chimeric distribution indicated that the highest recovery of M_2 mutants can be obtained from M_2 seeds located on the second and/or third pod-bearing node(s). Fruits of the main bifurcation on the M_1 plant or those from each main branch in *Capsicum annuum* have been identified as the preferred propagules to be harvested. In *Linum usitatissimum* it has been recorded that mutants may be more readily recovered from seeds at the top position of the stem. In the listed crops, as in other seed propagated plants, it is usually advantageous to harvest seeds from the most chimeric part of the plant since this would provide the highest probability of obtaining many differently induced mutants (Micke *et. al.*,1987). This is in line with the principle of diplontic drift which calls for the harvest of seeds from different M_1 plants (Balkema,1972).

Results obtained in this experiment are useful for viny plants which require staking and, hence, demand a high cost of producing an M_2 population for selection. Both the numbers and types of chlorophyll mutants were higher in the first formed seeds than in the later-formed ones within the M_2 pods. This was recorded for UPS 122 and Kade 6/16, the two winged bean cultivars studied, and at the two doses of 150 Gy and 250 Gy of gamma radiation applied (Tables 1 and 2; Figs 1 and 2). Large differences in the response of the seeds in the various positions in the M_2 pods were recorded. The seeds nearer to the pod stalk in the M_1 pods had a much higher level of chimerism than the later-formed ones. This part of the pod constitutes the prefered zone from which seeds for the M_2 generation should be harvested in order to give the highest probability of obtaining different kinds of mutants. On the other hand, significant differences in mutation frequency were not obtained for the various pod positions on the M_1 plant (Table 2). This indicates that in principle, pods can be harvested at any position on the M_1 plant for M_2 seeds. However, it will be more advantageous to harvest the earliest mature M_1 pods since it will reduce the time needed to harvest sufficient M_2 seeds. Additionally, the findings of Verkerk (1971) in tomato (*Lycopersicon esculentum*) indicated that the upper parts of the M_1 plant showed less chimerism than the lower parts and that seeds should be obtained from fruits harvested from the lower plant parts to select the largest possible number of mutants. Similarly, records on identified mutant sectors in *Vicia faba, Abelmoschus esculentus, Capsicum annuum* and *Linum usitatissimum* (Bhatia and Abraham, 1983; Hermelin *et al.*, 1983) provide further attestation to harvesting the earliest seed-containing fruits.

CHAPTER 5

INDUCED MUTATIONS FOR IMPROVEMENT OF WINGED BEAN (*PSOPHOCARPUS TETRAGONOLOBUS* L. DC) TOWARDS AN IMPROVED IDEOTYPE FOR GRAIN PRODUCTION WITH LOW TANNIN CONTENT

G.Y.P. Klu, E. Jacobsen, A.M. van Harten.

SUMMARY

Four mutants of winged bean (*Psophocarpus tetragonolobus* (L.) DC) with altered tannin content were selected among the M_3 seeds present on M_2 plants following mutagenic treatment of seeds of cvs UPS 122 and Kade 6/16 using gamma radiation from a Co^{60} gamma source. These mutants were selected from the most chimeric parts of the M_1 plant, which had earlier been identified to be the first mature pods on the M_1 plant and the earliest formed M_2 seeds in the M_1 pods. The indirect selection of tannin mutants was based on searching for seed coat colour changes in M_3 seeds. Cv UPS 122 yielded the mutants 3/1-10-12 and X22 from 1958 and 1883 M_2 plants respectively. Cv Kade 6/16 yielded the mutants 3/9-0-12 and 3/4-10-7 from 1442 and 1011 M_2 plants respectively. One of the mutants, 3/4-10-7, which was the only desirable one, had a reduced level of tannin of about 75% compared to the wild type cv. Kade 6/16. The other three mutants had increased tannin levels.

INTRODUCTION

Leguminous plants, which produce the bulk of protein requirements of humans, are in general too leafy for grain production and possess some plant parts such as tendrils which compete for energy. Some of them also have winding shoots, indeterminate growth and shattering pods as compared to cereals (Micke, 1983). Reduction or elimination of these parts, leading to the development of a different ideotype, would enhance the economic value of leguminous plants. Donald (1968) described an ideotype as a biological model of a crop that is expected to perform in a predictable manner within a defined environment. Blixt and Vose (1984) defined it as a specified model of a plant expected to produce a desired product in a required amount and quality in a way that minimizes the effect of an environmental variability.

The winged bean (*Psophocarpus tetragonolobus* (L.) DC), a diploid self-fertilising leguminous crop with diverse use, can be grown as grain legume, green vegetable, tuber crop or as a forage and cover crop (Anon, 1981; Khan, 1982). This crop requires an appropriate ideotype for each use. The expected ideotype as a grain legume would require a plant that is early maturing, with a large number of relatively small pods, dwarf type with side branches coupled with reduced internode length and/or reduction in the number of nodes (Stephenson, 1978; Smartt, 1990). In addition, such a plant should produce grains with optimal nutritional quality (Lazaroff,1989). Seeds of winged bean, as of many other legumes, contain toxic and pharmacologically active compounds, such as trypsin and chymotrypsin inhibitors, haematoglutins, cyanogenic glucosides and amylase inhibitors, all of which have been closely examined in this crop (Anon, 1981).

There are other unfavourable compounds like tannins (proanthocyanidins), which have been reported to be present in seeds of the winged bean (de Lumen and Salamat, 1980; Tan *et al.*, 1983; Kantha *et al.*,1986). Tannins, which are polyphenolic compounds and are either

hydrolysable or condensed, interact with and precipitate protein and, therefore, reduce food protein quality (Tan *et al.*,1983; Cabrera and Martin,1986). Tannins form part of the end products of the flavonoid biosynthetic pathway. Flavonoids are phenolic compounds composed of two aromatic C6 rings held together by a C3-unit. Oxidation of the C3 unit leads to the formation of subclasses of flavonoids. These include flavonols, flavonones, isoflavonoids and anthocyanins. Further modifications such as hydroxylation, methylation, acylation, glycosylation resulting in the various kinds of flavonoid colours, take place (Heller and Forkmann,1988; van der Meer,1992; Martin and Gerats,1993; Mol,1993; Koes *et al.*,1994).

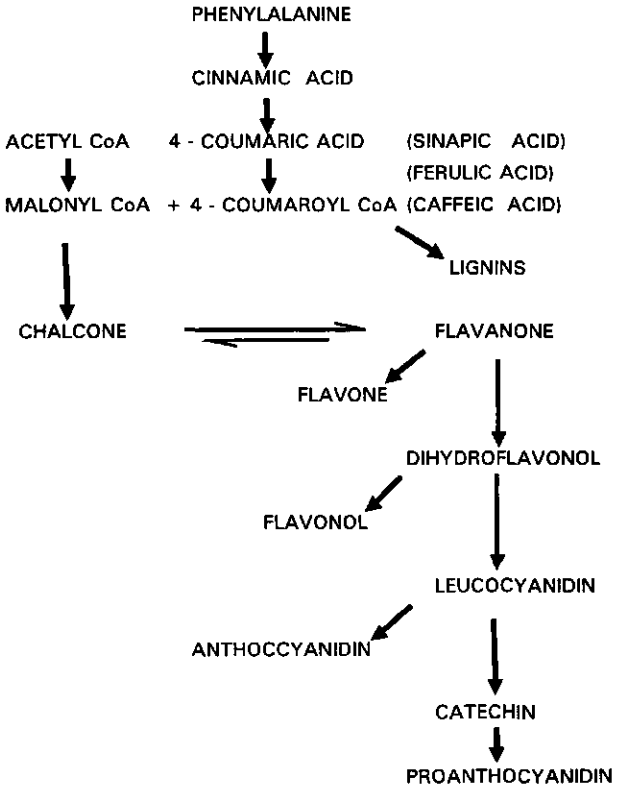


Figure 1. Biosynthetic pathway of proanthocyanidins, anthocyanidins and related compounds (after von Wettstein *et al.*, 1985).

Tannins are important for the reinforcement of plant tissues. Proanthocyanidin-free mutant seeds of snapbean have been reported to be more sensitive to mechanical and water stress than wild type seeds (Moore,1972). Mutations in the flavonoid biosynthetic pathway, that

lead to the formation of proanthocyanidins in the winged bean, could generate changes in the tannin level in the testa of seeds. An example has been described in soybean. A mutable allele in soybean (*Glycine max*) affected seed coat colour. The dominant allele, I, inhibited the production of proanthocyanidins (tannins) that were present in the pigmented recessive, i, genotypes of soybean (Todd and Vodkin,1993).

In breeding winged bean as a grain legume, the quality of grains, in terms of antinutritional factors like tannins, is important as has already bean indicated. A good and a reliable selection scheme for reduced tannin content in *Vicia faba* and *Pisum sativum* could be related to flower colour (Bond,1976; Cabrera and Martin,1986;1989). A similar system of correlation between tannin content and such an easily distinguishable character would offer a useful indirect breeding tool in winged bean as well. Possible characters in this respect could be changes in stem, pod, seed coat and/or flower colour.

Evidence from the literature indicates that changes towards improved nutritional quality, as listed above, are obtainable through induced mutations (Micke, 1983; 1988). This approach has already been used to generate a number of other desired characters in the winged bean. These include dwarf and bush type mutants (Anon,1982; Shivashankar and Reddy,1984; Jugran *et al.*,1986), mutants for earliness (Veeresh and Shivashankar,1987) and flower colour changes (Armachuelo and Bernardo, 1981).

In this report, results of studies on determining a reliable indirect selection scheme for altered tannin content in the winged bean are presented. A reduced tannin level should contribute to the breeding of winged bean to serve as a more acceptable grain legume.

MATERIALS AND METHODS

Control plant material

Two winged bean (*Psophocarpus tetragonolobus* (L.) DC) cultivars, UPS 122 and Kade 6/16 were used. Cv UPS 122 is a Papua New Guinea cultivar, while cv Kade 6/16 is reported to have been developed from a South East Asian introduction at the Agricultural Research Station of the University of Ghana at Kade (Khan,1982). The seeds of cv Ups 122 are black with a testa tannin content of about 18 mg catechin equivalent (CE) per gram while those of cv Kade 6/16, are brown, with a lower testa tannin content of about 13 mg CE per gram. There were, therefore, two seed sources with different colours and tannin content as starting material.

Mutagenesis and multiplication of selected plants

Dry seeds of these winged bean cultivars with uniform sizes and moisture content regulated to 13% in a dessicator containing 65% glycerol for four days were acutely irradiated at 150 Gy and 250 Gy. These doses which were selected from an earlier experiment on optimisation of mutant recovery in the winged bean, were administered using a Cobalt-60 Gamma Cell 220 operating at a dose rate of 518.96 Gy/hr at the Ghana Atomic Energy Commission, Kwabenya, Ghana. Six hundred and 700 seeds treated at 150 Gy and 250 Gy respectively for each cultivar were immediately sown in the field at one metre planting distances. Fifty untreated seeds were sown as controls. After germination and flowering, mature dry pods were harvested at four locations (0.5, 1.0, 1.5 and 2.0 metres above the ground) on M_1 plants (Fig.1). The first three formed M_2 seeds in 2 of the 5 harvested mature pods per location on the M_1 plants were sown to raise an M_2 population (Klu *et al.*, Chapter 4). The remaining pods per position were kept as "spare pods" as earlier recommended by Dellaert, (1979;1983). Then, the five first mature pods were harvested per location on the M_2 plants; and 2 of them were shelled to provide the M_3 seeds for identification of seed coat colour changes (Fig. 2).

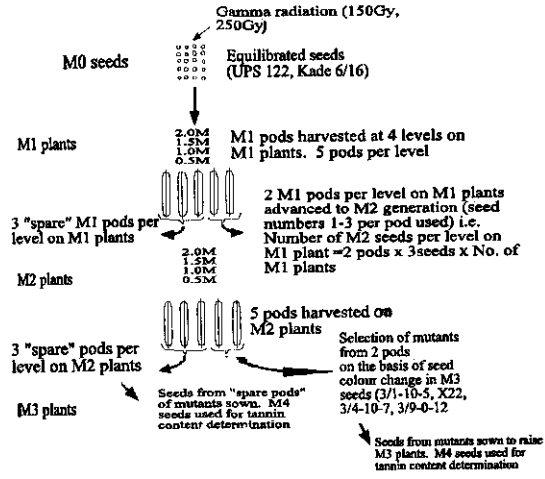


Figure 2. Scheme for induction, selection and evaluation of seed coat colour mutants of winged bean (*Psophocarpus tetragonolobus* (L.) DC)

Phenotypic selection

Mutant selection was carried out on the basis of changes in seed coat colour among the M_3 seeds since the seed coat is a maternally derived tissue and it reflects the genotype of the

previous plant generation and not of the embryo within it. In April, which was the onset of the major raining season, seeds of the selected variants were sown with their parents at 1x1 metre spacing with 2 metre interplot spacing to raise an M_3 population for evaluation of the selected variants. This also served to raise M_4 seeds for investigation of true breeding of these variants and for tannin content determination. In later experiment, the seeds from the "spare pods" from the M_2 plants from which the potential mutants were selected were also sown to raise more material of the mutants for breeding purposes (Fig. 2).

Determination of tannin content

Tannin content of testa and cotyledons was determined at the Grain Legume Unit of the Food Research Institute (Ghana) by using the vanillin-hydrochloric acid method of Price *et al.*, (1978). Mature dry seeds stored at -5^{0}C and manually dehulled were used. Blank corrections were applied and extractions were performed 4 hours after milling. Tannin content was expressed in terms of catechin equivalents (CE). Since the tannin content assay in whole seeds does not give reliable results due to the presence of components that interfere with tannin extraction, the tannin content of the testa was used. An estimate of the whole seed tannin content was obtained by the formula (Plahar and Swanson, 1990):

$$T_s = \frac{T_t H_c}{100}$$

where T_s represents tannin content of the whole seed while T_t and H_c represent tannin content of the seed coat and the percentage seed coat respectively. Tannin analyses were carried out on samples obtained from M_4 seeds harvested on the M_3 mutant progenies and also on the M_4 seeds obtained from the "spare" M_2 pods of the M_1 plants that yielded the mutants. Samples were taken from 5 pods per plant of the mutant progenies.

RESULTS

Mutant selection among M_3 seeds.

Control material

M_1 seeds and their controls were germinated and out of 50 control seeds of cv UPS 122 and cv Kade 6/16 each, 45 and 38 plants were obtained respectively (Table 1). Pods were harvested at the four earlier indicated positions on each plant. Totals of 162 and 150 second

Table 1. M_1 and M_2 populations with seed coat colour mutants among M_3 seeds following gamma radiation of seeds of two winged bean cultivars.

Winged bean cultivar	Gamma radiation dose (Gy)	No. of M_1 plants	Pod location on M_1 plants ☼	M_2 lines		M_2 plants			
				Total No.	No. producing altered seed coat colour	Total No.	No.of pods harv-ested	No. Of pods screen-ed*	No. of plants producing an altered seed coat colour
UPS 122	0	45	A	360	0	40	200	80	0
			B			45	225	90	0
			C			39	195	78	0
			D			38	190	76	0
	150	327	A	2616	1	1961	9805	3922	0
			B			1948	9740	3896	0
			C			1958	9750	3916	5
			D			1960	9800	3920	0
	250	315	A	2520	1	1885	9425	3770	0
			B			1884	9420	3768	0
			C			1890	9450	3780	0
			D			1883	9415	3766	3
Kade 6/16	0	38	A	304	0	39	195	78	0
			B			40	200	80	0
			C			36	180	72	0
			D			35	175	70	0
	150	224	A	1792	1	1440	7200	2800	0
			B			1443	7215	2886	0
			C			1442	7210	2884	3
			D			1440	7200	2800	0
	250	263	A	2104	1	1128	5640	2256	0
			B			1125	5625	2250	0
			C			1050	5250	2100	0
			D			1011	5055	2022	2

☼ A - 0.5, B - 1.0, C - 1.5, D - 2.0 metres from the ground

* Elaborated on in Table 2

generation plants of cvs UPS 122 and Kade 6/16 respectively, were obtained. From these plants, totals of 710 and 750 pods of cvs UPS 122 and Kade 6/16 were harvested, respectively (Table 1). Seeds from these pods were screened for seed coat colour changes. All seeds of cv UPS 122 remained black and those of cv Kade 6/16 brown. The cv UPS plants maintained the purple stem and pink flowers while the cv Kade 6/16 plants kept their green stems and white flowers.

Offspring of irradiated cv UPS 122

Out of 600 and 700 M_1 seeds of cv UPS 122 irradiated at 150 Gy and 250 Gy, 327 and 315 M_1 plants were obtained, respectively (Table 1). From these, 2616 and 2520 M_2 lines with 7827 and 7542 M_2 plants, respectively were raised and their M_3 seeds were tested for seed coat colour changes. The M_3 seeds originating from M_2 families of two M_2 plants segregated for an altered seed coat colour. These were made up of the line 3/1-10-2 in the 150 Gy treatment with 5 plants; this variant was selected from material at 1.5 metres on one M_1 plant, and the line X22 in the 250 Gy treatment with 3 plants having a variant selected from material at 1.5 metres on another M_1 plant (Table 1). All the M_3 plants of both variants, raised from seeds with altered seed coat colour, produced only seeds with altered seed coat (Table 2). These observations give a strong indication that recessive mutations were involved in both mutants.

Offspring of irradiated cv Kade 6/16

Similarly, mutant selection was carried out among irradiated materials of cv Kade 6/16. Out of 600 and 700 M_1 seeds treated at 150 Gy and 250 Gy, 224 and 263 M_1 plants were raised, respectively. These M_1 plants yielded 1792 and 2104 M_2 lines with 5765 and 4314 M_2 plants from the 150 Gy and 250 Gy treatments, respectively (Table 1). The M_3 seeds were screened for changes in seed coat colour. They comprised one segregating line each from the two treatments used. The variant 3/1-0-2 was selected among three M_2 plants which took their origin from 1.5 metres high on the same M_1 plant. Similarly, another variant, 3/4-10-7 was selected among the M_2 lines arising from the pods harvested from a height of 2 metres on the same M_1 plant (Table 1). All the M_3 plants raised from seeds with an altered seed coat colour produced only mutant seeds (Table 2). This again provides an indication that recessive mutations were involved.

Description of the phenotypes of the two winged bean cultivars and their mutants

A description of the seed coat colour and other plant parts of the selected mutants and their parents is presented in and Table 3. The plants of the parent cv UPS 122 are purple and the

Table 2. Investigations of the seed coat colour of M_4 seeds harvested on M_3 plants of winged bean cultivars and their mutants following gamma radiation of seeds.

Winged bean line	No. of M_3 plants		No. of M_3 plants confirmed for seed coat colour changes	
	Screened material	M_2 "spare pod" material	Screened material	M_2 "spare pod" material
UPS 122 (Parent)	37	-	37	-
3/1-10-2 (Mutant)	155	154	155	154
X22 (Mutant)	137	94	137	94
Kade 6/16 (Parent)	45	-	45	-
3/4-10-7 (Mutant)	21	63	21	63
3/9-0-12 (Mutant)	42	99	42	99

Table 3. Phenotypic description of plants and seeds of two winged bean cultivars and their mutants

Winged bean line	Plant colour	Pod colour	Flower colour	Seed coat colour
UPS 122 (Parent)	Purple	Purple	Pink	Black
3/1-10-2 (Mutant)	Green	Green	Light pink	Brown with dark hilum
X22 (Mutant)	Green	Green	Pink	Light brown
Kade 6/16 (Parent)	Green	Green	White	Brown
3/4-10-7 (Mutant)	Green	Green	White	Light brown
3/9-0-12 (Mutant)	Green	Green	White	Brown with black saddle-shaped region around hilum

Table 4. Records on tannin content and weight of the component parts of cv UPS 122 and cv Kade 6/16 seeds and of M_4 seeds of winged bean samples seed coat colour mutants.

Winged bean sample		Seed weight (g) weight	Seed coat weight as % of seed Seed coat	Tannin content (mg CE/ g sample) Cotyledon	Whole seed	(estimated by calculation)
UPS 122 (Parent)	a	0.35	8.82	18.26 (17.15-19.36)*	0.51 (0.58-0.65)	1.79
	b	0.31	9.51	17.16 (15.91-18.41)	0.50 (0.25-0.74)	1.64
X22 (Mutant)	a	0.31	10.46	23.91 (21.52-26.58)	1.78 (1.67-1.88)	2.50
	b	0.29	10.25	22.36 (19.41-25.31)	2.16 (2.02-2.30)	2.29
3/1-10-2 (Mutant)	a	0.31	9.74	20.01 (18.81-21.20)	0.07 (0.05-0.08)	196
	b	0.30	10.15	18.76 (18.41-19.10)	0.07 (0.05-0.09)	1.90
Kade 6/16 (Parent)	a	0.32	10.26	13.24 (13.07-13.41)	0.24 (0.20-0.28)	1.36
	b	0.30	10.12	13.73 (12.41-15.04)	0.26 0.21-0.30)	1.39
3/4-10-7 (Mutant)	a	0.28	10.85	3.42 (2.83-4.02)	0.01 (0.01-0.01)	0.37
	b	0.29	9.91	4.43 (3.41-5.45)	0.10 (0.09-0.11)	0.44
3/9-0-12 (Mutant)	a	0.37	8.91	25.24 (24.62-25.86)	1.87 (1.82-1.93)	2.20
	b	0.40	7.98	25.78 (24.51-27.04)	1.98 (1.90-2.04)	2.06

a - Samples from selected mutants

b - Samples from M_4 seeds of "spare pods" on M_2 plants

CE/g - Catechin equivalent per gram

* - Range

flowers are pink; the fresh pods are purple and the dry seeds black. On the other hand, cv Kade 6/16 has green plants, fresh green pods, white flowers and brown seeds. Plant and pod colour changes from purple to green were observed in the cv UPS 122 mutants (Table 3). No flower colour changes were observed except for a reduction in the shade of the pink flower in the mutant 3/1-10-2. The seed coat colour changed from black to brown with dark hilum. No colour changes were observed on the plants, fresh pods and flowers of the cv Kade 6/16 mutants. However, changes in seed coat colour were observed. One of the mutants (3/4-10-7) had a light brown seed coat while the other one (3/9-0-12) had a brown seed coat with a black saddle-shaped region around the hilum.

Tannin content, testa proportion and seed weight

Records on tannin content are provided in Table 4. Tannin content of cv UPS 122 was 18.26, 0.51 and 1.79 mg catechin equivalents (CE) per gram of testa, cotyledon and whole seed, respectively. Cv Kade 6/16 had only 13.24, 0.24 and 1.36 mg CE per gram of testa, cotyledon and whole seed, respectively. The black seeds of cv UPS 122 had about 1.5 times higher tannin values than the brown coloured seeds of cv Kade 6/16. Although the seed weights of both cultivars were about the same, they differed slightly in the seed coat weight expressed as percentage of the seed weight (Table 4).

The mutant, 3/4-10-7, possessed a lighter brown testa than its parent cv Kade 6/16, and it had the lowest tannin content in the seed coat, cotyledon as well as the whole seed of all the mutants. This mutant had only 25% tannin as compared to its parent and about 20% of that of cv UPS 122. The other mutant from cv Kade 6/16, 3/9-0-12, with a brown seed coat and a black saddle-shaped region around the hilum, had increased tannin contents of 25.24, 1.87 and 2.20 mg CE per gram of testa, cotyledon and whole seed, respectively. This provides an increase of about 90% in the testa. Mutations in cv Kade 6/16, therefore, produced testa colour changes accompanied with an increased, as well a as a decreased tannin content of the seeds. It is also noteworthy that although the seed weight of cv Kade 6/16 and its mutants were not much different, the ratio of testa to seed weight appeared to be the lowest in the mutant 3/9-0-12 which had an increased tannin content (Table 4). Both the brown coloured mutant, 3/1-10-2 and the light brown coloured mutant, X22, originating from cv UPS 122, had an increased tannin content in all samples except in the cotyledon of the mutant 3/1-0-2, in which there was a decrease of about 44%. These mutants, like those of cv Kade 6/16, had the same seed size as their parents but differed by having a higher ratio of testa to seed weight. In all the mutants and their parents the tannin content was highest in the testa as compared to the cotyledons and whole seeds. The light brown coloured

mutant, 3/4-10-7, with the lowest tannin content appeared to be the desired genotype.

"Spare pod material"

Results on the testing of the breeding behaviour of the mutants, using the selected material and the M_2 "spare pods" material are presented in Table 2. All the M_3 seeds of the altered M_2 "spare pods showed, as expected, the altered seed coat colour. This is an additional indication that the selected mutants were always true breeding. Selection of more plants with the desired alteration resulting from the same mutation in which meiotic recombination occured can be made by the growth of additional wild type M_2 and M_3 plants which are heterozygous for the mutation involved. In the next generation more mutants resulting from meiotic recombination between mutated and non-mutated parts of chromosomes can be found, enabling the removal of undesirable mutations.

Selected M_2 line, 3/4-10-7, as a basis for a new variety

Crosses were not sucessful, since all emasculated flowers dropped after pollinations were made. Further breeding of the mutant, 3/4-10-7 towards selection of the most optimal recombinant has to be based on the use of the above described "spare pod " material. In the heterozygous plants meiotic recombinations occur enabling the removal of undesired mutations. This programme is presently on-going.

DISCUSSION

Métz *et al.,* (1992) have suggested that putative anthocyanin mutants might be used as an earlier marker for changes in tannin content in *Vicia faba*. It has further been suggested that seedling and flower colour, as well as tannin content, can be correlated because they all depend on biosynthesis of anthocyanins or their derivatives (Hahlbrock,1981). In *P. tetragonolobus,* as in *V. faba,* purple seedling colour is dominant over green (Erskine and Khan,1977; Erskine,1978; Metz *et al.*,1992). A relationship between seedling colour, flower colour and tannin content in *P. tetragonolobus,* therefore, could not be ruled out. The first positive indication for such a relation was found in the comparison of the parental lines cv Kade 6/16 with brown seeds, green seedlings and white flowers and cv UPS 122 with black seeds and pink flowers. They had clearly different tannin contents(Table 4). The second proof was found in our mutation experiment using cvs UPS 122 and Kade 6/16 as basic material. Four mutants with seed coat colour changes were selected and investigated for tannin content. Results showed that mutants with both decreased and increased tannin

contents were obtained. The mutant 3/4-10-7 (light brown) with a tannin content of 0.37 mg CE per gram of whole seed showed a decrease of about 75% compared to the parental cultivar Kade 6/16 (brown) which had already the reduced tannin content of 1.36 mg CE per gram of whole seed. The other mutant, 3/9-0-12 (brown with black saddle-shaped region) from the same cultivar had an increased tannin content of 2.20 mg CE per gram of whole seed. This dark ring has been foud in some winged bean varieties (Newel and Hymowitz,1979). The other parental cultivar, UPS 122 (black), produced the mutants, 3/1-10-2 (brown) and X22 (light brown) which both had increased tannin contents in their seed samples except the cotyledon of 3/1-10-2 in which a decrease of about 40% was found. The seed coat colour and tannin content changes obtained give the clear indication that some of the mutants, with respect to tannin content, had partially regained the wild type values.

The presence of white flowers of cv Kade 6/16 and mutant 3/4-10-7 appeared to be connected with reduced tannin levels but not to a total lack of tannins. Studies on the relationships between the flower colour, seedling colour and seed coat colour for determination of tannin content in winged bean seeds need to be carried out more extensively. Particularly the alterations in the different enzymatic steps that might have occurred in the flavonoid biosynthetic pathway. This will add to the present selection scheme for tannin content changes in this pulse crop. This means that more knowledge is needed on the mutants about possible changes in the polymerisation of the leucoanthocyanidins into the proanthocyanins (Fig.1). There could also have been obtained changes in an earlier stage of the flavonoid biosynthetic pathway. For example, a mutation in the activity of the enzyme, chalcone flavanone isomerase (CHI) could also affect other activities in the pathway. This has been documented in barley and petunia. Proanthocyanidin-free barley mutants were used to detect mutants with genetic blocks in the biosynthesis of cathechins and proanthocyanidins (von Wettstein *et al.*,1977). An introduced chimeric chalcone synthase gene in anthocyanin biosynthesis in petunia resulted in somatic reversions of plants with white flowers to phenotypically parental violet flowers (Napoli *et al.*,1990).

The frequency of seed coat colour mutants is relatively high (0.4%), based on M_1 plants. Despite irradiations no gross accompanying alterations have been observed. This could mean that mutations based on transposable elements could be involved. Tagging genes with transposable elements would help to isolate the genes involved in these mutants. Records on the minimum amount of tannin in the winged bean that could cause negative effects after consumption are not available (Tan *et al.*, 1983). Nevertheless, since it has been noted that heating has very little effect on tannin content in the testa (de Lumen and Salamat,1980) there is the need to either obtain lines with reduced tannin content in the testa or the testa

must be removed as a means of avoiding ingestion of the high tannin in the seed coat.

Genetic studies involving the mutants were attempted, but no success was made in the hybridisation programme. However, selection of more plants with the desired phenotype resulting from the same mutation in which recombination occured, was carried out. This was done by sowing M_3 seeds originating from the particular M_1 plants. Several of the wild type M_3 plants produced wild type M_4 seeds or only altered ones. This confirms the recessive nature of the selected trait since among the unsegregated M_3 seeds more offspring populations of Aa would produce a 3:1 segregation. In heterozygous M_1 or M_2 plants meiotic recombinations occur enabling the removal of undesired mutations by genetic segregation. Selection of more plants with only the desired alteration could then be carried out by additional M_2 and M_3 plants heterozygous for the mutations involved.

The breeding of winged bean as a grain legume requires the developement of an improved ideotype which, among other things, has grains with the highest nutritional content and lowest antinutritional factors (Lazaroff,1989). This is even more urgent now than before in the light of an ever increasing population in regions that depend mainly on legumes for protein and, therefore, requiring genetic improvement in both quality and yield of pulses. The use of the scheme based on the selection of mutants in the most chimeric parts of the M_1 plant, as presented in Chapter 4 of this thesis, has been identified to be the earliest mature pods on the M_1 plant and the first formed seeds in such pods. One desired mutant, 3/4-10-7, with a reduced tannin content among 1129 M_1 plants was found. This mutant provided a reduction of about 75% tannin in the parent cv Kade 6/16. There is an on-going breeding programme based on the "spare pod" method towards the selection of the most optimal line within the M_2 line, 3/4-10-7.

CHAPTER 6

TESTING OF INDUCED MUTANTS OF WINGED BEAN (*PSOPHOCARPUS TETRAGONOLOBUS* (L.) DC) FOR NODULATION AND PHENOTYPIC PERFORMANCE

G.Y.P. Klu, F.K. Kumaga, E. Jacobsen, A.M. van Harten.

SUMMARY

Four seed coat colour mutants, which were accompanied with changes in tannin content, were earlier selected from M_3 seeds following gamma radiation of dry seeds of two winged bean (*Psophocarpus tetragonolobus* (L.) DC) cultivars. These mutants were X22 and 3/1-10-2 obtained from cv UPS 122 and 3/4-10-7 and 3/9-0-12 from cv Kade 6/16. They were investigated for nodulation behavoiur following inoculation with *Bradyrhizobium*. The two mutants, 3/4-10-7 and 3/9-0-12 produced more nitrogen-fixing nodules per plant than their parent at 76 days after sowing of seeds. Mutant X22 produced a lower number than its parent, cv UPS 122, whereas mutant 3/1-10-2 produced the same number of nodules. For mutant X22, the peak of nodule production seemed to have been reached already at 45 days after sowing of seeds. Nodulation of the parental cultivars was slower than in the mutants at 45 days after sowing but recovered and was relatively more at flowering time, 76 days days after sowing. Nodule dry weight followed a similar trend with the parents producing a lower amount of nodule tissue than the mutants at 45 days after sowing. Significant differences ($P = 0.05$) were recorded for the number of nodules per plant but not for the nodule dry weight. Earlier nodulation and changes in the number of nodules per plant observed in the mutants can be attributed to mutations in the flavonoid biosynthetic pathway that also influenced seed coat colour. The desired mutant, 3/4-10-7 with a low tannin content clearly showed an improved nodulation.

INTRODUCTION

A majority of leguminosae are characterised by development of root nodules in symbiotic relationship with soil bacteria of the genera *Rhizobium, Bradyrhizobium* and/or *Azorhizobium*. These bacteria can infest the roots of a specified host plant and induce the formation of nodules which are developed from newly-formed meristems in the root cortex. It is within these specialised organs, called nodules, that the bacteria inhabit for fixation of atmospheric nitrogen. The rhizobial nodulation genes required for the induction of the nodulation process, the *nod* genes, and the plant genes that are induced during the nodule formation, the *nodulin* genes, have partly been identified (Fisher and Long,1992; Schlaman *et al.*,1992; Long and Staskewics,1993). The *nod* genes are in turn induced by flavonoids which are a group of aromatic rings held together by a C3 unit. Synthesis of the flavonoids, in the presence of the enzyme chalcone synthase 4 (CHS), starts with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA to produce naringenin chalcone (Fig.1). Isomerisation of the narigenin chalcone by chalcone flavonone isomerase (CHI) yields naringenin flavanone. In addition to these *nod* gene inducing flavonoids, there are other flavonoids that inhibit *nod* gene expression (Firmin *et al.*,1986; Djordjevic *et al.*,1987; Long, 1989); for example, the isoflavone daidzein induces *nod* gene expression in *Bradyrhizobium japonicum* but it is an inhibitor in *Rhizobium trifolii* and *R.leguminosarum* (Reviewed by Quattrocchio,1994). The composition of the mixture of flavonoids in exudates released by roots varies between legumes (Peters and Long,1988), and therefore, the induction step by the flavonoids may determine host plant specificity of nodulation (Recourt,1991).

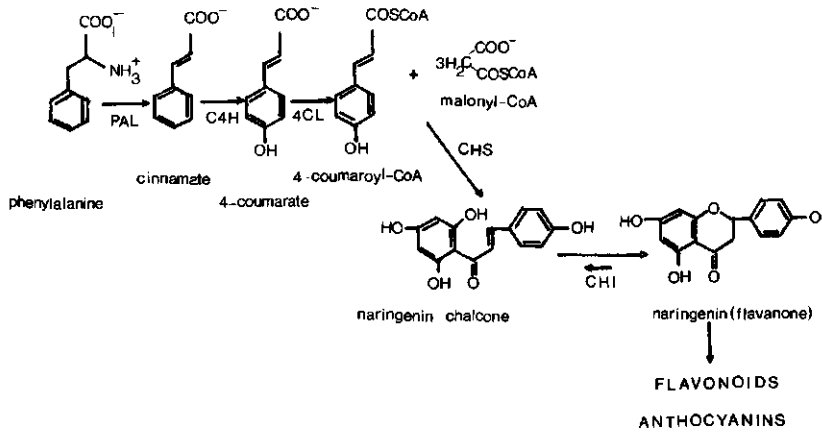


Figure 1. A simplified diagram of the flavonoid biosynthetic pathway (van Tunen *et al*, 1988)

Winged bean (*Psophocarpus tetragonolobus* (L.) DC) is a climber with four-angled pods which have an average length of about 20 cm but can grow to about 60 cm long in some varieties (Anon,1981). The individual pods contain 5 to 20 seeds. Genotypic variation has been recorded for plant growth, flowering time and maturity time (Reviewed by Khan, 1982). Winged bean has been described as the best nodulating legume (Anon,1981). This crop effectively forms a symbiotic relationship with a wide range of bacteria within the *Bradyrhizobium* spp (Ikram and Broughton,1980; Broughton *et al.*,1984). Masefield (1957) recorded that winged bean produced greater numbers as well as higher weight of nodules than other legumes including *Vigna unguiculata, Phaseolus vulgaris, Arachis hypogaea, Glycine max, Pisum sativum, Phaseolus aureus, Pachyrhizus erosus, Canovalis gladiata, Doliches lablab* and *Vigna subterranea*. Harding *et al.* (1978) have also reported that greater numbers of nodules and heavier dry weights of nodules have been found on winged bean roots than on other legumes when inoculated or grown in soils with no previous record of legume cultivation.

This not withstanding, there have been seemingly contrasting reports on winged bean nodulation. Whereas Masefield (1973) noted that good nodulation was obtained wherever the crop had been grown irrespective of inoculation of the seeds, Rachie and Roberts (1974) have reported poor nodulation or lack of efficient rhizobia in parts of Nigeria. These differences have been attributed to the observation that different legume genotypes respond differently to nodulation and nitrogen fixation (Caldwell and West,1977; Herath *et al.*,1978; Iruthayathas and Herath,1981; Nutman,1984).

Natural populations and induced mutations have provided genetic variation in host plants for an altered symbiotic interaction (Postma *et al.*,1988). The use of induced mutations in this regard have been documented. For example, a supernodulating mutant (Jacobsen and Feenstra, 1984) and nodulation resistant mutants (Jacobsen, 1984) have been selected after mutagenic treatment of seeds of *Pisum sativum* cv Rondo with ethyl methyl sulphonate. Supernodulating mutants of *Glycine max* have also been documented (Carroll *et al.*,1985).

The symbiotic behaviour of the host plant can be modified by induced mutations (Jacobsen,1984). Mutant lines for changes in tannin content of seeds have earlier been selected in a winged bean mutation breeding programme (Klu *et al.*, Chapter 5). Tannins are end products of the above mentioned flavonoid biosynthetic pathway. Therefore, it is worthwhile to test them for their nodulation ability. The objective of this study, therefore, was to examine the nodulation in some winged bean seed-coat-colour mutant lines and some other more general phenotypic characters.

MATERIALS AND METHODS

Plant material

Seeds of winged bean (*Psophocarpus tetragonolobus* (L.) DC) cultivars UPS 122 and Kade 6/16 and the M_4 seeds of seed coat colour mutants, 3/1-10-2 and X22 from cv UPS 122 and 3/4-10-7 and 3/9-0-12 from cv Kade 6/16 were used (Chapter 5). The colour changed from black in UPS 122 to shades of brown in the mutants 3/1-10-2 and X22; and from brown to light brown in mutant 3/4-10-7 and light brown with a saddle-shaped region around the hilum in mutant 3/9-0-12. The parents, cv Kade 6/16 was white flowering with green seedlings and cv UPS 122 was pink flowering with purple seedlings. The tannin content of the whole seeds of cv UPS 122 was 1.7 mg CE /g and that of cv Kade 6/16 was 1.36 mg CE/g. The mutants have also been tested for tannin content in their seeds. The mutant 3/4-10-7 had a reduced tannin content of 0.37 mg CE/g seed sample. The other mutants had higher tannin contents than their parents, varying from 1.96 to 2.50 mg CE/g.

Experimental conditions

All the nodulation experiments were set up in a plastic house in which the mean day and night temperatures were 35°C and 23°C respectively. Plants were sown in Rhondic Nitisol soil (local name, Adenta series) with pH(1:1 soil:water) being 5.0, a total nitrogen content of 0.04% and available phosphorus (Bray 1) of 5.5 ppm was used. The soil was air dried and

sieved through a 5mm mesh sieve. Plastic pots (with holes at the bottom) which have a height of 16.0 cm, a width of 18.0 cm at the top and 11.0 cm at the base were each filled with 3 kg soil and were watered regularly. A mixture of two local *Bradyrhizobium* isolates, (labelled LWB3 and LWB8) obtained from the winged bean cultivars UPS 122 and Kade 6/16 growing in the field were used. Each isolate was grown separately in yeast extract mannitol (YEM) broth for 7 days to a cell density of about 10^9 ml^{-1} on a rotary shaker operating at 1000 rpm.

Nodulation of seedlings

Each pot was seeded with four seeds of a winged bean line listed earlier. Each pot of soil was innoculated with 30ml. of a 1:1 ratio mixture of the *Bradyrhizobium* isolates just after sowing of the seeds. Seedlings were thinned to one, a week after emergence. Plants were watered daily with tap water and once a week with 50ml. Hoagland nutrient solution (Hoagland and Aron, 1938). Each winged bean line was replicated four times and pots were arranged in a randomized complete block design on raised benches. Plants were harvested 45 days after sowing of seeds. In another experiment, plants were harvested 76 days after emergence which was when all plants were flowering. Roots were washed free of soil and the nodules removed, counted and dried at 70°C and weighed.

Phenotypic performance of the mutants

The mutants and their parents were also sown in the field at 1x1 metre spacing with 2 metre interplot spacing to raise an M_4 population. The seeds were sown in April which was the time for the onset of the major raining season. The plants were supported on 2 metre wooden stakes and records were taken on flowering time, maturity time, the lengths of dry pods harvested from 5 plants randomly selected on each plot, number of seeds per pod harvested from a plant and weights of sets of 100 seeds per 10 plants randomly selected on a plot.

RESULTS

Nodulation

Records on nodulation of the winged bean cultivars UPS 122 and Kade 3/9-0-12 and their mutants are presented in Table 1. The parental cultivars, UPS 122 and Kade 6/16 produced mean nodule numbers of 12.01 and 6.91 per plant, respectively, at 45 days after sowing of seeds. These numbers were increased three fold at 76 days after sowing to 35.51 and 18.07

nodules for cvs UPS 122 and Kade 6/16. There was a significant difference (P=0.05) in nodulation between the cultivars at maturity with the pink flowering cv UPS 122 being the better nodulator (Tables 1 and 2). In comparison with 45 days after sowing, there were seven and five fold increases in the nodule dry weight at 76 days but there was no significant difference between the cultivars.

The mutants, 3/1-10-2 and X22, which originated from cv UPS 122 produced more nodules per plant than their parent at 45 days after sowing. However, after 76 days the situation changed. These two mutants differed both from each other and from their parent in one way or the other. The nodule number per plant for mutant 3/1-10-2 increased about 1.75 times to 36.0, a final number of nodules that was not significantly different from the value of the parent (Table 1). The mutant, X22, on the other hand, seemed to reach its peak of nodulation earleir at 45 days. The two mutants are, therefore, quicker nodulators than their parent but X22 had the lowest number of nodules per plant at 76 days. However, the dry weights of the nodules at this stage of plant growth did not clearly differ between the mutants and their parent (Table 3).

The observations among the mutants, 3/4-10-7 and 3/9-0-12 and their parent cv Kade 6/16 followed a similar trend. After 45 and 76 days of sowing of seeds, the number of nodules per plant for the mutants were higher than those recorded for the parent (Table 1). Increases in nodule number between 45 and 76 days were about 1.5 times to 29.01 and 42.01 for 3/4-10-7 and 3/9-0-12, respectively, while it was 3 times for the parent. These mutants appeared also to be quicker and heavier nodulators than their parent cv Kade 6/16 (Tables 1, 2 and 3). The desired mutant, 3/4-10-7, with a low tannin content, showed in comparison with cv Kade 6/16, an improved nodulation.

Shoot dry weight

Seedlings of the parental cultivars were, at the beginning, relatively more vigorous than their mutants. However, at 45 and 76 days after sowing of seeds no major phenotypic differences were observed among the plants of all genotypes. Records on shoot dry weight at 76 days after sowing are presented in Tables 1 and 4. There were no statistical differences in this trait among the mutants and their parents; although cv Kade 6/16 and its mutants seemed to have higher shoot dry weight than cv UPS 122 and its mutants (Table 1).

Phenotypic performance

Phenotypic data on winged bean cvs UPS 122 and Kade 6/16 and on M_4 populations of their mutants are presented in Table 5. The number of days to the opening of the first flower seemed to be about the same for both parent cultivars. Although there seemed to be no difference in this trait among the winged bean parental lines, all mutants seemed to flower a little later. Pod maturity in the mutants X22 and 3/1-10-2 followed the same trend as observed for flowering. However, pods of the mutant 3/4-10-7, which had the lowest level of tannin, matured about two to six days earlier than its parent cultivar Kade 6/16 and the other mutant 3/9-0-12 (Table 5). Pod length and the number of seeds per pod did not seem to differ in the winged bean lines. The heaviest seeds were recorded for the mutant 3/9-0-12. The mutant 3/4-10-7, although it had about the same seed weight as its parent, had a lower tannin content. Seeds of cv UPS 122 were heavier than those of its mutants, 3/1-10-2 and X22.

Table 1. Number of nodules, nodule dry weight and shoot dry weight per plant for four winged bean mutants and their parents*

Winged bean line	Mean values at 45 days after sowing of seeds		Mean values at 76 days (flowering time)		
	Nodule No. per plant	Nodule dry weight (g) per plant	Nodule No. per plant	Nodule dry weight (g) per plant	Shoot dry weight (g) per plant
UPS 122 (Parent)	12.01 bc	0.09 d	35.51 xy	0.70 e	6.93 f
3/1-10-2 (Mutan)t	20.61 ab	0.21 d	36.01 xy	0.81 e	6.08 f
X22 (Mutant)	21.81 ab	0.28 d	22.81 yz	0.68 e	6.22 f
Kade 6/16 (Parent)	6.91 c	0.14 d	18.01 z	0.50 e	7.53 f
3/4-10-7 (Mutant)	21.41 ab	0.24 d	29.01 xyz	0.60 e	7.58 f
3/9-01-2 (Mutant)	30.91 a	0.26 d	42.01 x	0.72 e	8.01 f

*Means with the same letter in the same column are not significantly different from each other (P=0.05) by Duncan's multiple range test

Table 2. ANOVA for number of nodules per plant for four winged bean mutants and their parents at (a) 45 and (b) 76 days after sowing of seeds.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F values
a.Replication	3	307.25	102.42	1.06 ns
Winged bean line	5	1411.33	282.27	2.91 *
Error	15	1455.25	97.02	
Total	23	3173.83		
b.Replication	3	290.79	96.93	0.95 ns
Winged bean line	5	1624.21	324.84	3.17 *
Error	15	1538.96	102.60	
Total	23	3453.96		

ns not significant

* significant at P=0.05

Table 3. ANOVA for nodule dry weight for four winged bean mutants and their parents at (a) 45 and (b) 76 days after sowing of seeds

Source of variation	Degees of freedom	Sum of squares	Mean squares	F values
a.Replication	3	0.07	0.02	1.37 ns
Winged bean line	5	0.10	0.02	1.26 ns
Error	15	0.25	0.17	
Total	23	0.42		
b.Replication	3	0.12	0.04	1.06 ns
Winged bean line	5	0.24	0.05	1.21 ns
Error	15	0.59	0.04	
Total	23	0.95		

ns not significant

Table 4. ANOVA for shoot dry weight of four winged bean mutants and their parents at 76 days after sowing of seeds

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F values
Replication	3	4.10	1.37	0.61 ns
Winged bean line	5	16.61	3.32	1.48 ns
Error	15	33.61	2.24	
Total	23	54.32		

ns not significant

Table 5. Phenotypic data on the performence of M_4 lines of winged bean mutants and their parents

Winged bean line	Flowering time		Maturity time		Pod length (cm)	No. of seeds per pod	100 seed weight (g)	Tannin content of whole seed (mg CE/g sample)
	No.of days for first plant to flower	No.of days for 50% of plants to flower	No.of days for first pod to mature	No.of days for 50% of plants to mature				
UPS 122 (Parent)	97.9	98.2 ±5.9	108.8 ±4.9	153.2 ±6.1	14.6 ±0.8	12.8 ±0.6	31.6 ±0.5	1.8
X22 (Mutant)	99.5	103.6 ±4.9	115.0 ±5.8	158.2 ±4.8	13.4 ±0.8	11.8 ±0.8	29.5 ±0.2	2.5
3/1-10-2 (Mutant	101.3	109.2 ±1.5	112.4 ±1.5	159.4 ±3.4	14.9 ±0.9	10.9 ±0.8	29.9 ±0.2	2.0
Kade 6/16 (Parent)	98.2	102.8 ±6.4	112.6 ±5.2	155.2 ±7.2	14.4 ±0.9	14.9 ±0.8	28.6 ±0.5	1.4
3/4-10-7 (Mutant)	102.2	105.2 ±3.4	110.5 ±3.4	160.5 ±4.2	14.5 ±0.7	13.4 ±0.4	28.5 ±0.1	0.4
3/9-0-12 (Mutant)	101.2	103.2 ±3.3	116.0 ±4.9	159.2 ±4.3	14.5 ±0.8	12.2 ±0.8	34.6 ±1.0	2.2

CE/g - Catechin equivalent per gram

DISCUSSION

Variability in nodulation among different winged bean accessions has been documented by Iruthayathas and Herath (1981) and Iruthayathas and Vlassak (1982). It has also been recorded that nodulation in this crop commences two weeks after sowing of seeds and that after three weeks nodules begin to reach their bacteroid stage and by four weeks after sowing of seeds, considerable numbers of fully developed nodules are formed (Iruthayathas and Herath, 1981). The nodulation studies reported here were carried out 45 days after sowing and at flowering time. These are, accordingly, appropriate times for examination of nodulation potentials of the winged bean cultivars and their mutants. The parents had significant differences ($P=0.05$) with respect to the number of nodules formed per plant. The comparison with the mutants clearly indicates that whereas the parent winged bean cultivars, UPS 122 and Kade 6/16 seem to be slower in nodule development but their number increased gradually with time, the mutants were early nodulators. In cv UPS 122 the increase from 12.01 nodules per plant at 45 days to 35.51 nodules per plant at flowering time exceeded increases from 20.61 and 21.81 to 36.01 and 22.81 nodules per plant, respectively, for the mutants 3/1-10-2 and X22. Similarly, a three fold increase from 6.91 to 18.01 nodules per plant for cv Kade 6/16 was more than the increases from 21.41 and 30.91 to 29.01 and 42.01 nodules per plant, respectively, for the mutants 3/4-10-7 and 3/9-0-12 (Table 1). The differences in nodulation between the parental lines was partly due to the observation within different legume species that genotypes can respond differently to nodulation and nitrogen fixation (Caldwell and West,1977; Herath *et al.*,1978; Iruthayathas *et al,* 1984). This phenomenon has earlier been documented in 80 cultivars of soybean (*Glycine max*) which have exhibited considerable variation in nodulation (Graham and Temple,1984; Okereke and Unaegbu,1992); and also in bean (*Phaseolus vulgaris*) in which over 600 cultivars were examined for this phenomenon (Graham and Rosas, 1977; Graham, 1981; Graham and Temple,1984).

Flavonoids are involved in the induction of *nod* genes for nodulation (Chappel and Hahlbrock, 1984; Peters *et al.*,1986; Remond *et al.*,1986; Zaat *et al.*,1987). However,there are also certain flavonoids which inhibit bacterial *nod* gene action (Firmin *et al.*,1986; Djordjevic *et al.*,1987; Peters and Long,1988; Long, 1989). Mutations in the flavonoid biosynthetic pathway could automatically affect the actions of the inducers and/or inhibitors, leading to changes in the signals of the root exudate of the host plant to the rhizobial bacteria (Recourts,1991).

Accumulation of naringenin is highly reversible and not inhibited by the presence of other

flavonoids (Recourt,1991). However, mutations in the structure of the naringenin flavone could offset such a system, and ultimately, *nod* gene activity. Mutations could also affect the enzymatic steps in the biosynthetic pathway. The changes in nodulation, as described for the mutants, could be attributed to the possible mutations outlined, since mutations alterting nodulation may be root and/or shoot specific (Delves *et al,*1986). Host plant genes are directly or indirectly involved in nodule formation and nodule functioning (Postma, 1990). The successful use of induced mutations to create variability in the host plant for differences in nodulation has been described in several legumes. These include *Pisum sativum* (Jacobsen 1984; Jacobsen and Feenstra, 1984), *Vigna radiata* (Micke,1984) and *Glycine max* (Carrol *et al.*,1985). To our knowledge, the variability created for nodulation by mutations in our investigations is the first reported one in winged bean. It is remarkable that all our selected mutants with altered seed coat colour showed this pleiotropic effect. There are not many examples described in the literature in which mutants with an altered seed coat colour and tannin content were systematically investigated for their nodulation behaviour. Our studies showed that selection for seed coat colour changes can be used as means to select for altered symbiotic performance. The mutated host plant genes involved in this process in the winged bean need to be investigated. Colour mutants are an indirect way for obtaining plants with an altered nodulation in which the flavonoid biosynthetic pathway is particularly involved.

Although the winged bean mutants were slow growers as compared to their parents at the seedling stage (data not shown), no dramatic phenotypic differences were observed in the mature plants. The mutants seemed to flower and mature later than their parents. On the other hand, the mutant 3/9-0-12, which had a lower number of seeds per pod than the parent, had heavier seeds and an increase in tannin content. The major effect recorded among the mutants is that the mutant 3/4-10-7, which had a reduced tannin content had an increased nodule number as compared to the parent. Further comparative studies are necessary and will be performed. Agronomcic studies at various locations and seasons may provide more information on any further differences between the winged bean lines studied.

CHAPTER 7

GENERAL DISCUSSION

Investigations were carried out on a number of techniques that could be used for the genetic improvement of the winged bean (*Psophocarpus tetragonolobus* (L.) DC). To begin with, the basic knowledge and the extent of research in this crop had to be explored. This review is presented in Chapter 2.

The winged bean, and a case for increased genetic variation

The winged bean, a semi domesticate (Eagleton *et al.*, 1985), is a legume of high potential to meet the dietary needs in the tropical and neotropical regions of the world, but it has been "underexploited" and is, therefore, underutilised (Chapter 2). However, in recent times, it has been receiving some more research attention. This will, in the long run, transfer it from its present state of just a backyard pulse into an industrial one. In this regard, some of the characters that have been receiving research attention include development of self-supporting determinate cultivars for single harvest, cultivars with pods and seeds of high nutritional quality and cultivars with high tuber yield (Lazaroff, 1989).

This crop is a climber and has a chromosome number of $2n = 2x = 18$. It is an autogamous crop and intraspecific hybridisation should be the normal way of breeding by recombining existing genetic variation in order to select for new varieties. However, successful artificial crosses are difficult to be realised. Interspecific hybridisation could be an additional source of obtaining the desired traits; however, no interspecific hybridisation successes involving *P. tetragonolonus* have been encountered (Smartt, 1990). Mutation breeding and other techniques, such as genetic transformation offer alternative means of generating the required variation for selection.

Tissue culture techniques for genetic variation

Tissue culture techniques could provide valuable tools for the genetic improvement of this crop (Chapter 3). Indirect adventitious shoot regeneration has been established for the winged bean by several researchers (Venketeswaran and Huhtinen, 1978; Blackmon *et al.*,1980; Gregory *et al.*,1980; Lie-Schricke and Tran Than Van, 1981; Wilson *et al.*,1985). The only records on direct adventitious shoot formation are connected with young (Trinh *et al.*,1981) and mature (Dias *et al.*,1986) cotyledon explants in which auxins were used. Reports on somatic embryogenesis are on indirect somatic embryogenesis on media containing auxins, particularly, 2,4-D and NAA (Venketeswaran, 1990; Venketeswaran *et al.*,1990). In our investigations, instead of the usual auxin-containing media for direct organogenesis and somatic embryogenesis, cytokinin-supplemented media for direct organogenesis and somatic

embryogenesis, as well as simultaneous direct regeneration of adventitious shoots and embryoids on mature cotyledon explants were used. This was meant to avoid the callus phase preceeding the regeneration of adventitious shoots and somatic embryos and also to add another technique to the auxin-supplemented systems already established for winged bean (Chapters 2 and 3). In our experiments, four factors influenced the regeneration of adventitious shoots and somatic embryos on the mature cotyledon explants. These were, the various orientations of the explants on the medium, wounding of the explants and the concentration, as well as, the type of cytokinin and the basal medium used. Whole cotyledon explants, which were vertically positioned with their distal ends on the medium, led to the regeneration of adventitious shoots at the axes of the explants. On the other hand, embryoids and adventitious shoots were regenerated on the adaxial surface when the explants were cultured horizontally with their abaxial surfaces on the media. Horizontal positioning also induced adventitious shoots at the axes, but at a lower frequency than when cultured vertically. Wounding of the explants, by slicing them transversely, also induced embyogenesis (Chapter 3). This was predominantly at the distal wounds of the explants. Combined concentrations of 11.1 μM BAP and 12.3 μM 2iP in MS medium produced the highest number of adventitious shoots. The function of the cytokinin, BAP in the induction of adventious shoots and somatic embryos is not clear. However, its role could be associated with an auxin-like, difusible substance which, in the presence of the cytokinin, activates the totipotent cells (Raju and Mann,1970; Cheng *et al.*, 1980; Gambley and Dodd, 1990; Sharma *et al.*, 1991). Wounding of the explant would release the difusible growth substance to flow basipetally and, therefore, get trapped at the wounds for embryogenesis (Smith and Krikorian 1990; Terzi and Loschiavo,1990). Additionally, it has been suggested that wounding disturbed the explant tissues to regulate K^+ exchange, leading to increased osmotic potential of the cells and, therefore the regeneration of an electric field accross the explant. This in turn, controlled embryogenesis (George and Sherrington, 1984; Rathore *et al.*,1988). These suggested processes need to be investigated in the winged bean. The succsessful use of the cytokinins BAP and 2iP to regenerate adventitious shoots and/or embryos, provides an additional tool in the use of *in vitro* culture techniques for genetic improvement of the winged bean, through genetic transformation.

With regards to regeneration, our investigations have provided three important observations that can be used in the transformation of this crop (Chapter 3). These are: direct adventitious shoot formation from the axes of the cotyledon explants, direct simultaneous regeneration of adventitious shoots and somatic embryos, probably from epidermal or subepidermal cells of adaxial surfaces of the cotyledon explants, and direct somatic embryogenesis on the wounds of the cotyledon explants. In winged bean, somaclonal variation as a means of creating

genetic variation is not needed. As earlier mentioned, winged bean is a diploid self fertilising crop. The successfully developed mutation breeding method based on radiation of seeds, described in Chapter 4, does not show the need for investigating regenerants from tissue culture for somaclonal variation as an additional source of genetic variation.

Induced mutations for improvement of winged bean

The use of induced mutagenesis leads to chimerism in M_1 plants. There was, therefore, the need to develop a system for optimisation of mutant induction and selection, in view of the large M_2 population normally required for mutant recovery in a viny plant plant like *Psophocarpus tetragonolobus*. This type of optimisation has been established for a number of crops including *Lycopersicon esculentum* (Verkerk, 1971), *Abelmoschus esculentus* (Bhatia and Abraham, 1983), *Vicia faba, Capsicum annuum* and *Linum usitatissimum* (Hermelin *et al*, 1983). In all these cases, mutant selection was carried out in the most chimeric parts of the M_1 plant, since it would provide the highest probablity of obtaining sufficient genetic variation (Micke *et al.*, 1987). Chlorophyll mutations can be used as an indication of the effectiveness of a mutagenic treatment (Brock and Micke, 1979). In our investigations, the highest chlorophyll mutation frequencies were obtained among the M_2 seedlings raised from M_2 seeds located close towards the pod stalk. On the other hand, no difference was found in chimerism at the various heights of pods on the M_1 plants (Chapter 4). In order to have the highest probability of obtaining different induced mutations, the first mature pods on the M_1 plants following irradiation of seeds with gamma radiation must be used. The first mature pods are often at lower heights on the vines. The first formed seeds, preferably seeds numbered 1 to 3 in the M_1 pods, should be used. This also saves time as one does not have to wait till the whole plant matures. The use of these identified areas for optimal selection, coupled with the use of the "spare' or "remnant" seed selection method developed by Dellaert (1979, 1983), provides an improved method for mutant recovery in a viny legume like the winged bean.

Induced mutagenesis, as a means of creating the required variation in winged bean, has been in use (Kesevan and Khan,1978; Shivashankar and Reddy,1984; Jugran *et al.*,1987; Veeresh and Shivashankar,1987; Veeresh *et al.*,1992). However, the costs of such a programme, aimed at obtaining potentially useful characters must be taken into account. The cost per mutant is related to the cost of growing the M_1 and M_2 generations for selection. The size of the treated M_1 population is directy related to the probability of obtaining a desired mutant. In a viny plant, like the winged bean, thousands of stakes would be needed for supporting the large number of M_2 plants required for selection. The identification of zones of M_1 plants to

be harvested and progeny tested reduces costs; while providing the required variability for selecting a desired mutant (Chapter 4).

The use of zones with the highest chlorophyll mutation frequency has led to the recovery of four seed coat colour mutants which could be investigated for changes in tannin content. These investigations showed that this selection procedure has served as an indirect method of obtaining mutants with tannin content changes (Chapter 5). The mutant, 3/4-10-7 (light brown), with a tannin content of 3.42 mg CE per gram of testa was obtained. This provides a reduction of about 75 % from the parent cultivar, Kade 6/16 (brown), which had a tannin content of about 13.24 mg CE per gram of testa. Another mutant, 3/9-0-12, from the same cultivar had a brown seed coat and a black saddle-shaped region around the hilum. This mutant had tannin content of 25.24 mg CE per gram of testa. This gives an increase of about 90 %. The other parent, cv UPS 122 (black) produced the mutants 3/1-10-2 (brown) and X22 (light brown) which also had increases in tannin content. The indirect selection of changes in tannin content by using seed coat colour alterations has produced mutants with both increased and decreased tannin contents (Chapter 5).

The four seed coat colour mutants with changes in tannin content also showed differences in nodulation (Chapter 6). The parent cultivars seemed to be slower in nodule development and the nodule number increased gradually till flowering time. All four mutants seemed to be early nodulators and two of them had increased numbers of nodules. Apart from the observation that in legumes, such as *Glycine max* (Graham and Temple,1984;Okereke and Unaegbu,1992) and *Phaseolus vulgaris* (Graham and Temple,1984) genotypes respond differently to nodulation, there seems also to be a relationship between seed coat colour changes and nodulation. This needs further investigations. The variability created for nodulation in this study are the first ones described in the winged bean.

Mutants for changed tannin content and nodulation

Flavonoids, which are the products of the flavonoid biosynthetic pathway, have a two fold action on nodulation. They induce *nod* gene action for nodulation (Chappel and Hahlbrock,1984; Remond *et al.*,1986; Zaat *et al.*,1987), and there are some others which inhibit *nod* gene action (Firmin *et al.*, 1986; Djordjevic *et al.*,1987; Peters and Long, 1988,1989). Proanthocyanidins (tannins), which are also products of the flavonoid biosynthetic pathway, accumulate in plant parts (such as seeds and flowers) for various functions including reinforcement of plant tissues (Quattrocchio,1994). Our mutants with changes in tannin content and early nodulation are expected to be under the control of genes

linked to the flavonoid biosynthetic pathway. Mutations on single or multiple enzymatic steps along the pathway could effect changes in the regulatory genes for nodulation and proanthocyanidin expression. In barley, more than 600 induced mutants have been found in which the biosynthesis of proanthocyanidins has been genetically blocked (von Weittstein *et al.*, 1985; Jende-Strid, 1991; 1994). Chalcone synthase (CHS) is regarded as the key enzyme of flavonoid biosynthesis. Isomerisation of this chalcone by the enzyme chalcone flavone isomerase (CHI) form naringenin flavanone. It is from these two central intermediate products that the biosynthesis branches into various ways. If mutations should occur in steps preceeding the formation of these two intermediates, nodulation and proanthocyanidin expression could both be affected. Additionally, polymerisation of the leucoanthocyanidin into proanthocyanidins, which is at a later stage of the biosynthetic pathway (Fig. 1) could also be a target of mutations. The seed coat colour mutants with changes in tannin content and early nodulation have not been studied biochemically for these mutations and there is the need to do so.

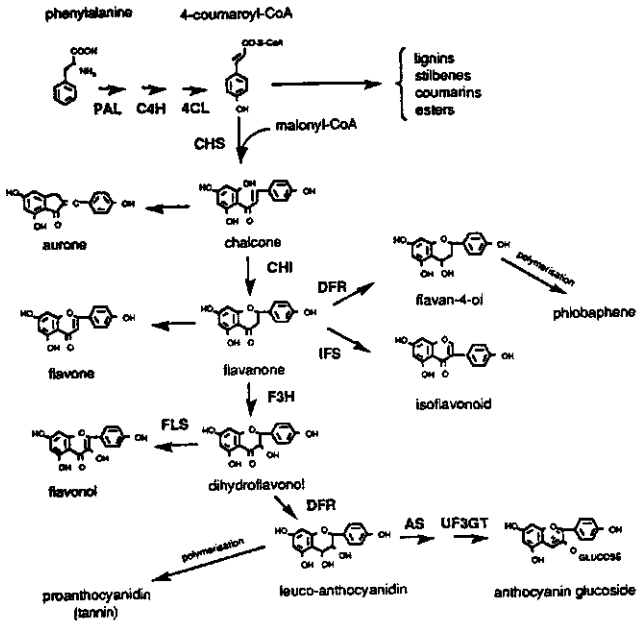


Figure 1. Simplified diagram of flavonoid biosynthetic pathway (Quattrocchio, 1994).

The desired mutant, 3/4-10-7.

Mutant, 3/4-10-7 with tannin contents of 3.42, 0.01 and 0.37 mg catechin equivalent (CE) per gram of testa, cotyledon and whole seed respectively, was selected from cv Kade 6/16 which had tannin contents of 13.24, 0.24 and 1.36 mg CE per gram of testa, cotyledon and whole seed respectively. This gives a reduction of about 75 % tannin in the testa. This tissue has the highest concentration of tannin in winged bean seeds. This mutant also has earlier nodulation than its parent and forms the basis for a new variety. The use of seed coat colour changes has successfully served as an indirect selection method to reduce tannin content and to improve nodulation.

SUMMARY

Food production - both quantitatively and qualitatively - needs to be augmented to meet the demands of a still increasing world population in certain parts of the world, particularly in Africa and Asia. In addition to the improvement of agronomic techniques and the use of genetically improved seeds in these areas, there may be the need to introduce so-called "new crops". This group, in fact, often refered to as "underexploited crops" have potentials of meeting dietary needs in these regions of the world but have been under-researched and under-utilised (at least in certain regions). One member of the group is the winged bean (*Psophocarpus tetragonolobus* (L.) DC).

The winged bean is a semidomesticated crop and it is unique, in that apart from its stem and roots, all parts of the plant are edible, and are good for human nutrition because they are rich in proteins, minerals and vitamins (Chapter 2) . The mature seeds contain 20-46 % protein and 17-22 % edible oil. The amino acid profile is like that of soybean (*Glycine max* L). The winged bean relates appropriately with soybean and groundnut (*Arachis hypogaea* L.) in having a favourable combination of proteins and oil. The fatty acid profile in the seeds is even better than that of groundnut. The average crude protein content of the green pod is 2.4 g per 100 g fresh weight. The root tubers have a total protein content of 8-20% (dry weight) compared with 1-5% for cassava (*Manihot esculenta* Crantz) and potatoes (*Solanum tuberosum* L.). Additionally, the winged bean has a higher capacity for nodulation and nitrogen fixation than most other tropical legumes. However, this crop contains some antinutritional substances such as proanthocyanidins (tannins).

Further exploitation of these potentials is limited seriously by the climbing habit of the winged bean. Staking, which is required for seed yield, is too expensive for most farmers in developing countries. Genetic improvement, at least for the aforementioned aspects of this crop, is needed to transfer it from its present position of just a backyard crop into an industrial grain legume. In this regard, there is the need to develop bush type cultivars with annual growth habit and synchronous maturity of pods which should contain seeds with reduced tannin concentrations. Cultivars with increased tuber yield are also desirable. All these things together imply speeding up the domestication of this crop. Domestication in this context implies the adaptation of a wild plant to anthropogenic environments (Chapters 1 and 2).

Winged bean is a self fertilising crop plant. Intraspecific specific and interspecific hybridisation should be the routine methods for obtaining and combining the desired traits. However, crossing in general has proven not to be easy. No records of interspecific hybridisation are

known. Consequently, the application of other means of creating genetic variability needs to be explored in this crop (Chapter 2). Tissue culture, combined with transformation as well as mutation breeding, could be used as tools to speed up domestication of this crop. Genetic engineering techniques, in addition, enable the introduction of "alien" genetic material.

In this thesis; (1) tissue culture techniques as a first step towards genetic transformation have been developed; (2) an optimised mutation breeding technique has been developed; and (3) mutants with altered tannin content have been selected and the nodulation behaviour of these mutants has been compared with their parents.

In winged bean, the available regeneration methods to date, have been mainly via callus through auxin-supplemented media (Chapter 3). In our investigations, the use of media supplemented with cytokinin for direct organogenesis and somatic embryogenesis on mature cotyledon explants were carried out. This is meant to make winged bean responsive to media containing both auxin and cytokinin for regeneration systems. Four factors were found to have major impact on the regeneration processes. They are; (1) the orientation of the positions of the cotyledon explants on the media; (2) wounding of the explants; (3) the concentrations and the type of cytokinin; and (4) the basal medium used. Whole cotyledon explants which were vertically positioned with the distal ends on the media, led to the regeneration of adventitious shoots at the axes of the explants. On the other hand, embryoids and adventitious shoots were regenerated on the adaxial surface when the explants were cultured horizontally with their abaxial surfaces on the media. Wounding of the explants, by slicing them transversely, induced somatic embryos at the wounds. With respect to regeneration, these investigations have resulted in three major observations that should be taken into account in the transformation of this crop: (1) direct adventitious shoot formation from the axes of the cotyledon explants; (2) direct simultaneous regeneration of adventitious shoots and somatic embryos (probably originating from epidermal or subepidermal cells of the cotyledon explants); and (3) direct somatic embryogenesis on the wounds of the explants.

Mutagenic treatment of seeds leads to chimeric M_1 plants. Hence, there is the need to develop a system for optimisation of mutant induction and selection in view of the large M_2 population normally required for mutant recovery (Chapter 4). Through our investigations on chlorophyll mutations using gamma radiation, a selection scheme for obtaining the highest probability of recovering different induced mutations at a reduced cost has been developed in the winged bean. Harvesting of the first mature pods and, the first-formed seeds (for instance seeds numbered 1 to 3 nearest to the pod stalk) in the M_1 pods would ensure the highest chance of obtaining (desired) mutants. Taking the early mature pods also saves time,

as one does not have to wait till the whole plant matures.

The use of this scheme has led to the recovery of four seed coat colour mutants. More detailed investigation of these mutants showed, because alterations in the flavonoid biosynthetic pathway are involved, additional changes in tannin content (Chapter 5) and improvement of nodulation (Chapter 6). Consequently, alterations in seed coat colour have served succesfully, as an indirect method of selecting for changes in these two traits. One of the four mutants, 3/4-10-7, was desired with reduced tannin contents of 3.42, 0.01 and 0.37 mg catechin equivalent (CE) per g of testa, cotyledon and whole seed respectively. This mutant was obtained from cv Kade 6/16 which had tannin levels of 13.24, 0.24 and 1.36 mg CE per g of testa, cotyledon and whole seed respectively. This provides a tannin content reduction of about 75% in the testa. Testa tissue has the highest concentration of tannin in winged bean seeds. Additionally, this mutant produced earlier nodulation and more nodules per plant than its parent. This may increase the nitrogen fixation ability and, consequently, lead to additional crop improvement. The on-going breeding programme with this mutant is based on the "spare pod" method using heterozygotes for this mutation in the M_2- and M_3-generations. This provides the possibility to remove, by meiotic recombination, additional mutations with negative effects which occurred simultaneously during mutagenic treatment of the particular M_0 seed.

SAMENVATTING

Teneinde te voldoen aan de behoeften van een groeiende wereldbevolking, in het bijzonder in Afrika en Azie, is het noodzakelijk dat het niveau van de voedselproduktie zowel kwantitatief als kwalitatief verder wordt verhoogd. Naast verbetering van de toegepaste landbouwkundige technieken en het gebruik van genetisch verbeterd uitgangsmateriaal, zou het ook zinvol kunnen zijn over te gaan tot introductie van zgn "nieuwe gewassen". Het gaaat in dit verband vaak om op zich bekende gewassen die in potentie een bijdrage zouden kunnen leveren aan de oplossing van het voedselvraagstuk in genoemde gebieden, maar die tot nu toe onvoldoende zijn onderzocht op hun mogelijkheden en die nog slechts op beperkte schaal worden geteeld. Zo'n gewas is "winged bean" (*Psophocarpus tetragonolobus* (L.) DC).

"Winged bean" is een half-gedomesticeerd gewas met als bijzondere eigeenschap dat, afgezien van stengel en wortel, alle plantdelen gegeten kunnen worden en een hoogwaardige voedingsbron vormen vanwege het hoge gehalte aan eiwitten, mineralen en vitamines. De rijpe zaden bevatten 20-46% eiwit en 17-22% olie geschikt voor menselijke consumtie. De aminozuursamenstelling komt overeen met die van de soyaboon (*Glycine max* L.), terwijl het gewas de gunstige combinatie van eiwitten en plantaardige olie deelt met soyaboon en aardnoot (*Arachis hypogaea* L.). De vetzuursamenstelling van de zaden is zelfs gungstiger dan die van aardnoot. Het gemiddeld ruw eiwit gehalte van groene peul bedraagt 2.4 g per 100 g vers gewicht. De knollen bevatten 8-20% eiwit (op basis va droge stof), vergeleken met slechtts 1-5% voor cassava (*Manihot esculenta* Crantz) en aardappel (*Solanum tuberosum* L.) Daarnaast beschikt de "winged bean" over een groter vermogen tot nodulatie en stikstofbinding dan de meeste andere tropische vlinderbloemigem. Daartegenover staat echter dat in de "winged bean" enige antinutritiële stoffen voorrkomen, zoals proanthocyanidines (tannines).

Een verdere benutting van de hiervoor genoemde nuttige eigenschappen wordt echter vooral in de weg gestaan door het feit dat "winged bean" een klimplant is. Het gebruik van staken is nodig om tot een goede zaadopbrengst te komen en dit is te kostbaar voor de meeste boeren in ontwikkelingsladen. Genetische verbetering, tenminste met betrekking tot eerdergenoemde ongunstige factoren, is noodzakelijk om "winged bean" te veranderen van een achtergebleven gewas tot een peulvrucht die op industriële basis kan worden geteeld. Daartoe is het nodig dat eenjarige rassen worden ontwikkeld met een gedrongen plantbouw, met gelijkkijdig afrijpende peulen en met zaden die een lager gehalte aan tannine bevatten dan de huidige cultivars of selecties. Cultivars met een hogere knolopbrengst zouden eveneens zeer gewenst zijn. Dit alles hout in dat een versnelde domesticatie van het gewas nodig is.

Onder domesticatie wordt in dit verband verstaan: de aanpassing van de wilde plant aan antropogene milieus (Zie de Hoofdstukken 1 en 2).

Psophocarpus is een zelfbevruchtend gewas. De basis voor het verkrijgen en combineren van gewenste eigenschappen zou moeten bestaan uit het maken van intraspecifiieke en interspecifieke kruisingen. In de praktijk blijkt echter het maken van kruisingen in dit gewas niet eenvoudig. Voorbeelden van geslaagde interspecifieke kruisingen zijn tot nu toe zelfs helemaal niet bekend. Het is daarom noodzakelijk andere methoden voor het verkrijgen van genetische variatie op hun geschiktheid te onderzoeken (Hoofdstuk 2). Zowel weefselkweek - in combinatie met transformatietechnieken - als mutatieveredeling kunnen dienen als methoden om een versnelling van de domesticatie van "winged bean" te bewerkstelligen. Daarnaast kan via genetische modificatie ook nog "soortvreemd" genetisch materiiaal worden ingerbracht.

In dit proefschrift zijn (1) zijn weefselkweektechnieken ontwikkeld als een eerste noodzakelijke stap voor genetische transformatie; (2) is een verbeterde methode voor mutatieveredeling in *Psophocarpus* opgezet; en (3) zijn mutanten met een ge wijzigd tannine gehalte geselecteerd die vervolgens met hun ouders zijn vergeleken voor wat betreft hun nodulerend vermogen.

Regeneratie in "winged bean" heeft tot op heden voornamelijk plaatsgevonden via callusvorming op basis van media die zijn aangevuld met auxine (Hoofdstuk 3). In het huidige werk is aandacht besteed aan het gebruik van media verijkt met cytokinine ten behoeve van onderzoek naar directe organogenese en somatische embryogenese, waarbij is uitgegaan van explantaten van rijpe cotylen. Het achterliggende doel is om zowel te beschikken over regeneratiesystemen met auxine als met cytokinine voor regeneratiedoeleinden. De regeneratieprocessen bleken door vier factoren te worden beinvloed. Dit zijn: (1) de wijze waarop explantaten van de cotylen op de media worden gebracht; (2) het beschadigen van de explantaten; (3) de gebruikte concentraties en het type cytokinine en (4) het (basis) medium dat werd gebruikt. Wanneer volledige cotylen werden gebruikt als explantaat en deze met het distale eind verticaal op het medium werden gezet ontstonden adventiefscheuten langs de assen van de explantaten. Embryoiden en adventiefscheuten ontstonden echter aan het adaxiale oppervlak wanneer de explantaten horizontaal met hun abaxiale vlak op het medium werden gebracht. Beschadiging van de explantaten door het aanbregen van dwarse insnijdingen, leidde tot het ontstaan van somatische embryos op de wondvlakken. Het huiige regeneratie-onderzoek heeft geleid tot drie hoofdconclusies waarmee rekening kan worden gehouden bij de transformatie van dit gewas: (1) het rechtstreekse ontstaan van adventiefscheuten langs de assen van de explantaten van cotylen; (2) de rechtstreekse en gelijktijdige regeneratie van adventiefscheuten en somatische embryo's (mogelijk uit

epidermale of subepidermale cellen van de explantaten van de cotylen); en (3) rechtstreekse somatische embryogenese op wondvlakken van de explantaten.

Mutagene behandeling van zaden resulteert in chimere M_1 planten, om geinduceerde mutaten in handen te krijgen is meestal een grote M_2 populatie vereist. Het is daarom zeer gewenst het systeem van inductie en selectie van mutanten te optimaliseren (Hoofdstuk 4). Op basis van gegevens die werden verkregen via analyse van door gamma stralen geinduceerde chlorophyll mutaties, is voor "winged bean" een systeem opgezet om met lage kosten zoveel mogelijk verschillende mutaten in handen te krijgen. Oogsten van de eerste rijpe peulen en de eerst-gevormde zaden (bijv. de eerste drie zaden geteld vanaf de peulaanhechting) op M_1 planten biedt de meeste kans om de (gewenste) mutanten in handen te krijgen. Dit systeem werkt bovendien tijdbesparend omdat niet hoeft te worden gewacht tot de hele plant is afgerrijpt.

Toepassing van dit systeem heeft vier mutanten voor zaadhuidkleur opgeleverd. Een verdere analyse van deze mutanten toonde aan dat daarnaast, als gevolg van veranderingen in de biosynthese van flavonoiden, het gehalte aan tannine was gewijzigd (Hoofdstuk 5) en dat mutanten met een verbeterd nodulerend vermogen waren verkregen (Hoofdstuk 6). Via selectie voor gewijzigde zaadhuidkleur kon zodoende dus met succes indirect worden geselecteerd voor veranderingen in beide laatstgenoemde eigenschappen. In één van de vier bestudeerde mutanten, 3/4-10-7, werd per gram zaadhiud, zaadlobweefsel en volledig zaad een (gewenst) lager tanninegehalte van resp. 3.42, 0.01 en 0.37 mg catechine equivalent (CE) gevonden. Deze mutant was afkomstig van cv. Kade 6/16 waar, voor de overeenkomstige plantdelen, 13.24, 0.24 en 1.36 mg CE werd aangetroffen. De reductie van het tanninegehalte in de zaadhiud, het gedeelte met het hoogste tanninnegehalte, bleek dus 75% te bedragen.
Deze mutant bleek daarnaast eerder te noduleren en meer stikstofbindende knolletjes te bevatten dan het ouderras. Dit zou kunnen inhouden dat het stikstofbindend vermogen van de mutant is verhoogd en, dientengevolge, kunnen resulteren in een verdere verbetering van het gewas. Het lopende selectieprogramma met deze mutant is gebaseerd op een "reserve peul method", waarvoor heterogoot materiaal iut de M_2 en de M_3 generatie wordt gebruikt. Dit biedt de mogelijkheid om ongewenste mutatie, die eventueel tijdens de mutagene behandeling zijn geinduceerd, via meiotische recombinatie te verwijderen.

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CURRICULUM VITAE

George Y.P.Klu was born on 12th July 1945 in Peki, Ghana. He obtained his B.Sc. and M Sc. degrees at the University of Science and Technology, Kumasi, Ghana and the University of Cape Coast, Cape Coast, Ghana respectively. He was employed as a research officer by the Ghana Atomic Energy Commission in 1977. Other training programmes took him to ENEA, Cassaccia, Rome, Italy; International Atomic Energy Agency (IAEA) Biotechnology Laboratory, Seibsersdorf, Austria; International Institute of Tropical Agriculture, Ibadan, Nigeria; and Wye College, University of London, Wye, England. He has served as Counter-part for a number of IAEA Technical Co-operation Projects in Ghana. In 1991 he started a Sandwich PhD programme in the Department of Plant Breeding, Wageningen Agricultural University under a scholarship awarded by this University. He carried out the research leading to this thesis at the Ghana Atomic Eneryg Commision, Ghana under the supervision of Prof. Dr. Ir. E. Jacobsen and Dr. Ir. A.M. van Harten. In 1994 he was appointed the Director of the newly-established Biotechnoloy and Nuclear Agriculture Research Institute, Kwabenya, Ghana.