European Journal of Biotechnology and Bioscience
Online ISSN: 2321-9122, Impact Factor: RJIF 5.44
www.biosciencejournals.com
Volume 4; Issue 5; May 2016; Page No. 40-44

Study of protein and trypsin inhibitors content in some induced mutants of winged bean (*Psophocorpus tetragonolobus* L.) DC

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Abstract
Winged bean (*Psophocarpuste tragonolobus L.)* belong to family Fabeaceae is a nutritious legume in human diet, its mutants like the high seed yield, dwarf, early maturity and low trypsin inhibotor content carrying would assume substantial economic importance. In the present investigation these mutants of Winged bean were biochemically analyzed for different parameters like crude protein content, seed soluble protein content and low trypsin inhibitor content. The viable mutants demonstrated the seed crude protein content in the range of 38% to 54.66%. The dwarf mutant displayed lowest seed protein content of 20.76%, while the highest crude seed protein content could be seen in the high yielding/large leaf p-2 mutant of winged bean. The highest amount of protein content 36.33 µg/ml of defatted seed powder could be seen in high yielding/large leaf p-2 type of mutant in winged bean. The lowest protein content 24.66 µg/ml of defatted seed powder was observable in dwarf mutant of winged bean. The protein polymorphism of different viable mutants revealed a wide variability with respect to the number and mobility of bands. A marginal difference in TI content and iso inhibitor profile was detected in micromutants of winged bean. The lowest TI (272.7 TIU/min/gm meal) could be recorded in dwarf mutant, while the linear leaflet mutant revealed the highest TI content (476.45 TIU/min/gm meal). Out of seven iso inhibitors, four iso inhibitors of trypsin were major, while three iso inhibitors were minor with weak expression as revealed by X-ray film technique.

Keywords: protein and trypsin inhibitors, winged bean, biochemically

1. Introduction
The Winged bean (*Psophocarpus tetragonolobus*), also known as the Goa bean (kacang botol in Malaysia) and Asparagus Pea and Winged Pea (Lotus tetragonolobus), is a tropical legume plant native to Papua New Guinea. It grows abundantly in hot, humid equatorial countries, from the Philippines and Indonesia to India, Burma, Thailand and Sri Lanka. It does well in humid tropics with high rainfall. There are also varieties that can be grown in most areas of the U.S.
The winged bean plant grows as a vine with climbing stems and leaves, 3-4 m in height. It is an herbaceous perennial, but can be grown as an annual. It is generally taller and notably larger than the Common bean. The bean pod is typically 15-22 cm (6-9 in) long and has four wings with frilly edges running lengthwise. The skin is waxy and the flesh partially translucent in the young pods. When the pod is fully ripe, it turns an ash-brown color and splits open to release the seeds. The large flower is a pale blue. The beans themselves are similar to soybeans in both use and nutritional content (being 29.8% to 39% protein).
The plant is one of the best nitrogen fixers with nodulation accomplished by the soil bacterium *Rhizobium*. Because of its ability to fix nitrogen from the atmosphere, the plant requires very little or no fertilizers.
Being a tropical plant, it is sensitive to frost. It will not flower if day length is less than 12 hours. The seeds have a hard coat and it helps to presoak the seeds before planting to hasten germination. The plant grows very quickly, reaching a length of four meters in a few weeks.
This bean has been called the "one species supermarket" because practically all of the plant is edible. The beans are used as a vegetable, but the other parts (leaves, flowers, and tuberous roots) are also edible. The tender pods, which are the most widely eaten part of the plant (and best eaten when under 1" in length), can be harvested within two to three months of planting. The flowers are often used to color rice and pastries. The flavor of the beans has a similarity to asparagus. The young leaves can be picked and prepared as a leaf vegetable, similar to spinach. The roots can be used as a root vegetable, similar to the potato, and have a nutty flavor; they are also much more rich in protein than potatoes. The dried seeds can be useful as a flour and also to make a coffee-like drink. Each of these parts of the winged bean provide a source of vitamin A, vitamin C, calcium, iron, and other vitamins.

1.1 Composition and nutritive value

Table 1: showing composition of different parts of Winged bean in gm/100gm.

Sr.no.	Components	Immature pods	Seeds	Tubers	Leaves	Flowers
1	Water	76-92	6.7-24.6	54.9-65.2	64.2-77.7	84.2
2	Protein	1.9-2.9	29.8-37.4	12.2-15	5.7-15	5.6
3	Fat	0.2-0.3	15-20.4	0.5-1.1	0.7-1.1	0.9
4	Carbohydrate	3.1-3.8	28-31.6	27.2	--	--
5	Fiber	1.2-2.6	5-12.5	17	--	--
6	Ash	0.4-1.9	3.6-4	9	--	--

These two groups of crops namely, cereals and pulses have a complementary relationship as regards their amino acid composition and their combined intake can compensate to a great extent for their mutual amino acid deficiency. The composition of Dal-chawal (pulse-rice) or dal-roti (pulse-unleavened wheat bread) is well recognized as the standard combination in the average Indian diet. Modern science provides strong support for this practice, for it is now recognized that the chemical score (i.e. biological value) improves greatly when wheat or rice is combined with one of the pulses, because of the complementary relationship of their essential amino acids.

Winged bean crops have immense nutritive value. Its production and productivity is quite good among some areas of our country as well as in the world. Besides these good qualities of this crop it possesses some shortcomings like climbing habit which is problematic in the agriculture point of view and long duration. Mutation breeding is a potent tool for creating variability particularly in species where hybridization is difficult or naturally existing variability has exhausted.

The importance of legume grains as a source of proteins has already been recognized and hence immense efforts are being made all throughout the world to increase their nutritional quality. However, few ant nutritional components are limiting their wide scale use.

Most of the nutritional and biochemical studies have been carried out on seed proteins of legumes by involving their quantitative/qualitative aspects. The seeds in legumes comprise storehouses of different biochemical substances like proteins, lipids, carbohydrates and several other materials of considerable importance. Now a day, there is a demand for legumes due to high nutritional quality. The seed proteins of legumes are mainly composed of the globulins and albumins. These storage proteins are generally made up of two major fractions, namely, the 'vicilin' and 'legumin' (Derbyshir *et al.* 1976) [5]. The vicilin and legumin have the molecular weights of 180 kD and 330 kD, respectively (Danielsson, 1949) [3].

Due to the economic importance of the storage proteins of legumes, they have been intensively studied. Logically, the seed proteins became the focus of studies as regards the plant gene expression patterns because they represent the abundant gene products which are produced during different stages of seed development. The seed proteins also offer to basic plant biologists a model for temporal and tissue specific regulation studies during seed development stages. In most legumes, the cotyledons make up at least 80% of the mature seed weight. The differences in content and quality of legume cotyledonary proteins are due to numerous biochemical processes that are responsible for the protein accumulation (Muller, 1983) [21].

The values of protein content mostly depend on the genetic background of the plant system (Gottschalk, 1975) [9]. Dhaliwal has proposed a minimum of 60-70 and a maximum of 360-420 structural genes, involved in determining the seed protein content (Gottschalk, 1975) [9]. Because of this polygenic influence, the quantitative characters are easily influenced by environmental factors. The qualitative differences on the other hand are more or less caused by the action of single gene.

It has been recognized for many years that the nutritive value and protein digestibility of legumes would always be very poor unless subjected to cooking/some sort of heat treatment (Liener, 1992). This depression in protein value and digestibility have been generally attributed to the presence of protease inhibitors which are the common constituents of most of the edible legumes (Liener, 1969) [19].

The adverse dietary effects of the protease inhibitors have been studied by many workers (Liener, 1969; Gunn *et al.* 1980; Krogdahl and Holm, 1981 and Higuchi *et al.* 1983) [19, 10, 16, 13]. The protease inhibitors have been identified, isolated and characterized from different legume systems (Hanovar *et al.* 1962; Hova and King, 1979; Kortt, 1979; de Lumen and Salamat, 1980; Tsukamato *et al.* 1983a) [11, 14, 15, 4, 31].

In the present investigation biochemical analysis of some mutants of winged bean were carried out. These mutants were screened for the different biochemical parameters like crude protein, seed soluble protein content and antinutrinal factor content. The importance of the nutritional quality of legume seeds has two aspects viz. reducing or eliminating the concentration of toxic and other undesirable materials, and improving protein content and its quality.

2. Material and Methods

2.1 Extraction of seed proteins

Mature seeds were washed with water, dried and ground to make fine powder. The mature seed powder was defatted with hexane, air-dried and stored at 4^0C. Seed powder was kept for extraction in 1:6 proportion of distilled water with 1% PVP (Polyvinyl Polypyrrolidone). The suspension was centrifuged at 12,000 rpm at 4^0C for 20 minutes to remove the particulate matter and clear supernatant was used for protein estimation, the trypsin inhibitor assay and the native PAGE.

Similarly, the protein extracted in 10mM Tris-HCl buffer, P^H 8.0 containing 1mM EDTA, 1% SDS and 25% Glycerol was used for protein estimation and SDS-PAGE.

2.2 Protein estimation

The protein estimation was carried out by the Folin-Lowery method.

2.3 Standardization of Biuret assay using BSA

The stock solution of BSA was prepared in distilled water. The protein was estimated by measuring the absorbance at 660nm and by using a molar absorption coefficient (E) for BSA of 6.6. The above stock solution was diluted to prepare 500µg/ml working solution of BSA in distilled water. Folin-Lowrys reagent was prepared by dissolving 2% of sodium carbonate in 0.1N in NaOH. (Reagent A).0.5% copper sulphate in 1% potassium sodium tartarate (reagent B).Mix 50 ml reagent A and 1ml reagent B (reagent C).and Folin cio-calteau (reagent D).

BSA (20µg-200µg) was taken and the volume was made up to 1ml with distilled water. 0.5 ml of Folin reagent was added and incubated at room temperature for 30 minutes in dark. The absorbance was read at 660nm. A graph of absorbance versus concentration of BSA was plotted and this graph was used for estimating the protein concentration in sample. Suitable quantity of extract was assayed by Folin-Lowrys method and respective protein value was determined from BSA standard graph and the protein value was expressed in µg/ml.

2.4 Standardization of trypsin assay by using BAPNA

Trypsin stock solution was prepared by dissolving 10mg of

trypsin in 1ml (0.1mM Tris-HCl $P^{H}7.8$) buffer A. This stock solution was diluted to prepare 1mg/ml working solution of trypsin in the protease buffer.10 to 50µl trypsin was taken and the volume was made up to 0.5ml with protease buffer. 1mM BAPNA solution was prepared by dissolving 10g of BAPNA in 0.45ml DMSO (Dimethyl Sulphoxide) and then mixed in 22.5ml of protease buffer. 1ml of BAPNA solution was incubated with 150µl of trypsin in Eppendorf tubes at $37^{0}C$ for 10 minutes. The reaction was stopped with 0.2ml of 30% acetic acid after 10 minutes. The absorbance was read at 410nm. A graph was plotted with absorbance versus concentration of trypsin and according to it, the optimum trypsin concentration to be used for the assay was determined.

2.5 Trypsin inhibitor assay

Trypsin activity was measured by using the synthetic chromogenic substrate BAPNA, as described by Erlanger *et al.* (1961). For trypsin inhibitor assay 20µg of trypsin was found to be optimum from the earlier standardization. Trypsin inhibitor activity was determined by mixing suitable quantity of protein extract containing inhibitor with 20µg of trypsin in a volume of 30µl of buffer A so that trypsin activity could get inhibited up to 40% to 60%. 1ml of 1mM BAPNA was added to the reaction mixture, the incubation was conducted at room temperature and later the reaction was stopped after 10 minutes by adding 200µl of 30% acetic acid. The residual trypsin activity was measured at 410nm. One inhibitor unit was defined as the amount of inhibitor that inhibited 1 unit of trypsin activity.

2.6 Analysis of mutants for trypsin inhibitors

The 7 macromutants lines were analyzed for the trypsin inhibitor study. The seeds were powdered and defatted with hexane. The defatted seed powder was kept for extraction in 1:6 proportion of distilled water with 1% (PVP) for overnight and centrifuged at 12000 rpm at $4^{0}C$ for 20 minutes to collect the supernatant. This seed extract was used for the analysis of trypsin inhibitors by electrophoretic detection and TI assay as described above.

2.7 Electrophoresis

The water soluble proteins were analyzed by using vertical slab polyacrylamide gel electrophoresis (PAGE) apparatus.

a) Non- denaturing discontinuous PAGE

It was performed by using Davis (1964) system. The composition of buffer system described in the following protocol pertains to non-denaturing type since it did not contain any detergent/other denaturing agents.

3. Result

The seven viable mutants were biochemically studied regarding the parameters like seed crude protein content, seed soluble protein content and trypsin inhibitor content besides the electrophoretic characterization of seed proteins and trypsin inhibitors.

Estimation of seed crude protein content: (Table-2)

The viable mutants demonstrated the seed crude protein content in the range of 38% to 54.66%. The dwarf mutant displayed lowest seed protein content of 20.76%, while the highest crude seed protein content could be seen in the high yielding/large leaf p-2 mutant of winged bean.

Table 2: Crude protein estimation

S. No.	Name of mutant	Conc. of crude Protein micro gm/ml	Shift in mean	S.D.	±S.E.	C.V.
1	Long pod	43.33	5.33	0.26	0.09	0.072
2	Anthostem	39.66	1.66	1.65	0.62	2.74
3	Dwarf	38.33	0.33	2.28	0.86	5.21
4	Linear leaflet	43.00	5	0.39	0.14	0.154
5	HY/LL P-1	51.33	13.33	2.75	1.04	7.59
6	HY/LL P-2	54.66	16.66	4.01	1.52	16.11
7	Control	38.00	---	2.03	0.76	4.134

Estimation of seed soluble protein content

A good amount of variability as regards the soluble seed protein content could be evidently noticed in the viable mutants of winged bean. The highest amount of protein content 36.33 µg/ml of defatted seed powder could be seen in high yielding/large leaf p-2 type of mutant in winged bean. The lowest protein content 24.66 µg/ml of defatted seed powder was observable in dwarf mutant of winged bean (Table-3).

Table 3: Soluble protein estimation

S. No.	Name of mutant	Conc. of protein in mg/ml (mean)	Shift in mean	S.D.	±S.E.	C.V.
1	Long pod	25.33	1	1.27	0.48	1.63
2	Anthostem	27.33	3	0.52	0.37	0.272
3	Dwarf	24.66	0.33	1.52	0.57	2.34
4	Linear leaflet	30.00	5.67	0.48	0.18	0.237
5	HY/LL P-1	33.00	8.67	1.62	0.61	2.629
6	HY/LL P-2	36.33	12	2.87	1.09	8.29
7	Control	24.33	---	1.65	0.62	2.74

Seed protein characterization by Native PAGE

The protein polymorphism of different viable mutants revealed a wide variability with respect to the number and mobility of bands. The band profiles of viable mutants

exhibited minimum eight bands and maximum twelve bands. The least number of bands were present in lane 2, 4, 5 and 11, while nine bands could be seen in lane 3, 7 and 12, ten bands in lanes 6 and 8 and the maximum number of bands could be recorded in lane 9, 10 and 13.
The presence of A, B, C, D, E, H, J and M bands were evidently demonstrated by all the mutants in their protein profiles while the protein polymorphism could be conspicuously seen in regard to their bands. The band 'I' could be observed in lane 4, 10 and 13 only, while the bands 'L' and 'K' was seen in lane 9 and 10.

Seed protein characterization by SDS-PAGE: (Fig.)
Lot of variability was observed in polypeptide profiles of viable mutants of winged bean. The polypeptide polymorphism was almost similar in region of polypeptides with low molecular weights. Nearly all the variability that has been observed was noticeable between the Ist and IIIrd region of polypeptide of the winged bean mutants.

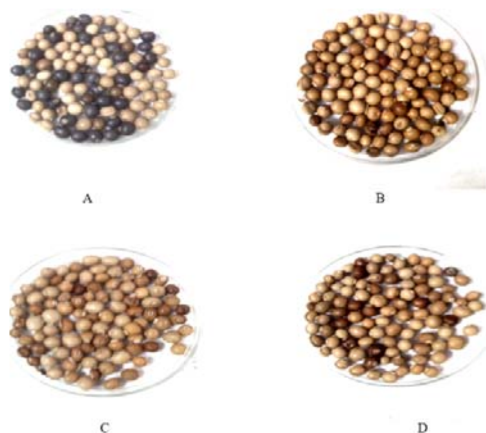
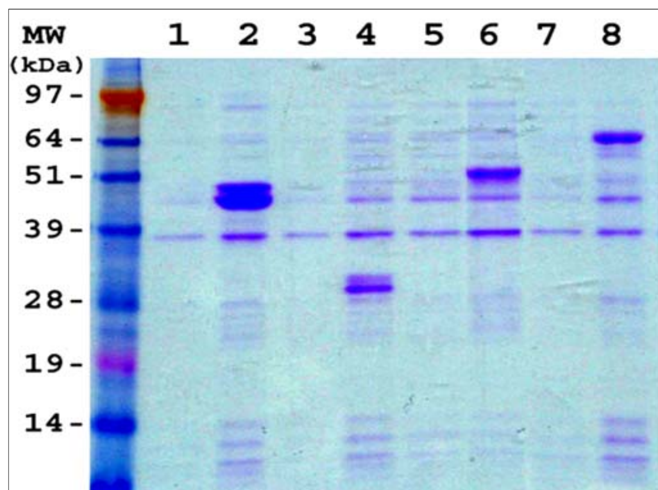


Fig 1: A= Anthostem, B= Control, C= High yield large leaflet P-1 and D= High yield large leaflet P-2



Lane: 1= Control, 2= Long pod, 3= Anthostem, 4= Linear Leaflet, 5= Dwarf, 6= HY/LL P-1, 7= HY/LL P-2, 8= HY/LL P-1.

Fig 2: SDS-PAGE protein profile of viable mutants

Trypsin inhibitor in winged bean mutants: (Table-4)
About seven viable mutants were analyzed for trypsin inhibitor (TI) by electrophoresis and quantification. A marginal difference in TI content and iso inhibitor profile was detected in micromutants of winged bean. The lowest TI (272.7 TIU/min/gm meal) could be recorded in dwarf mutant, while the linear leaflet mutant revealed the highest TI content (476.45 TIU/min/gm meal). Out of seven iso inhibitors, four iso inhibitors of trypsin were major, while three iso inhibitors were minor with weak expression as revealed by X-ray film technique.
In comparison with the control TI profile, some viable mutants showed significant changes. These mutants also showed 25-45% reduction in TI content by quantitation. The quantitation of seed proteins of viable mutants showed significant variation and changes.

Table 4: Trypsin inhibitor content in different mutants Of Winged bean

Sr. No.	Name of mutant	Protein mg/gm of detected seed Powder (mean)	±S.E.	TIU/min/gm of detected Seed powder
1	Long pod	25.33	4.42	356.2
2	Anthostem	27.33	7.15	437.5
3	Dwarf	24.66	16.38	272.7
4	Linear leaflet	30	15.28	476.45
5	HY/LL P-1	33	1.66	398.74
6	HY/LL P-2	36.33	2.97	408.3
7	Control	24.33	10.32	316.04

4. Conclusions
The results obtained decisively demonstrated the usefulness and the effective potential of the induced mutational approaches in genetic improvement of winged bean for recovering superior mutant plant types having high seed yield, dwarf, early maturity besides low trypsin inhibitor content.
As winged bean is a nutritious legume in human diet, its mutants like the low trypsin inhibotor content carrying would assume substantial economic importance. Such material would require lesser heat processing to nullify the trypsin activity possessed by them. All such features can be used in conventional breeding programme which help to develop high yielding, dwarf, early maturing, low trypsin inhibitor carrying winged bean varieties.

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