Low Trypsin Inhibitors and Chymotrypsin Inhibitors in Winged Bean Mutants

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ABSTRACT

In present studies several attempts have been made to reduce the amount of trypsin and chymotrypsin inhibitors in winged bean (*Psophocarpus tetragonolobus*) mutants developed through induced mutation. The low seed TI (trypsin inhibitor) lines comprised long pod-5 and long pod-2 mutant lines. The low tuber TI feature could be noted in the mutant population. Such lines were long pod-5, large leaf/high yield-5, dark green/flat pod-4 and anthostem-3. The CTI (chymotrypsin) content in seed and tuber of mutant lines has revealed a good amount of variation. The low seed CTI lines like flat pod/wingless-2, large leaf/high yielding-2, early maturing-2 and dwarf-5 could be distinctly observed among the varied mutants of winged bean. The seeds and tubers of such low TI and CTI mutant lines are easy for digestion as compared to original germplasm. The winged bean mutants carrying lowered levels of TI/CTI are likely to assume significant importance and immense economic value especially in regard to their nutritional potential. The detailed understanding of the genetics of inhibitors and other antinutritional components present in winged bean mutants would prove immensely helpful to breeders in planning their programmes directed towards qualitative enhancement of winged bean.

Key words: Winged Bean Mutants, TI, CTI.

INTRODUCTION

Now days nutrition has become a critical issue in medical sciences and the knowledge of nutrition helps modulate the physiopathology and intervention of several diseases. Near about 34% of the world's malnourished children live in India. About 50% of all childhood deaths are attributed to malnutrition. Nearly 30% of all new bornes have a low birth weight making them vulnerable to be further malnourished and diseased (Tekale, 2004).

On this background there seems an urgent need of finding and popularizing high protein crops from the unknown legumes. Winged bean (*Psophocarpus tetragonolobus* (L) DC.) is one of the unknown plant systems, which has demonstrated an exceptionally fast rate of dispersal, development and acceptance as a new legume food crop throughout the tropical regions of the world. It has large potential to fulfill the need of staple food that is rich in protein and oil for man and as a fodder for animals. Masefield (1973) was the first person who could highlight the potential utility of this plant.

Despite possessing high nutritional potential as detailed above the winged bean has remained unfamiliar among society because of the presence of high amount of anti-nutritional factors such as trypsin and chymotrypsin inhibitors, amylase inhibitors, lectins, phenols and tannins some undesirable characters of the plant.

Antinutritional factors in winged bean

Like soybean, several antinutritional factors have been found in winged bean as well. Such factors include the trypsin inhibitors (Kortt, 1979), the chymotrypsin inhibitors (Sohonie and Bhandarkar, 1954), the lectins (Renkonen, 1948) and other biotoxic compounds (NAS, 1981).

The substances that specifically inhibit the catalytic activities of proteases are called as protease inhibitors.

Protease inhibitors are widely distributed among a number of plant species particularly legumes. The trypsin inhibitors and chymotrypsin inhibitors comprise the important proteinase inhibitors. The winged bean trypsin inhibitor and chymotrypsin inhibitor belonging to the category of serine protease inhibitors are also described as serpins. The molecular weight of winged bean trypisn inhibitor (WBTI) is approximately 20,000 Da (Kortt, 1979), while that of chymotrypsin inhibitor (WBCTI) is 21,000 Da (Kortt, 1980). It has been noticed that the WBTI and WBCTI activities can be destroyed by autoclaving and boiling water treatments (Kadam, 1987).

For achieving the better quality food out of winged bean plant parts the development of low trypsin inhibitor, chymotrypsin inhibitor, low lectin, low tannin and low phenol lines of winged bean has become crucial. It is understood from literature that such lines have been achieved in few other food legume plants like *Vigna mungo* (Sagade, 2008) and *Glycin max* (George, 2006) through induced mutational approach. The major advantage of the use of induced mutations has been the possibility to correct one or few negative characters or to get new gene combination, which is desirable without changing the major part of the systems total genetic make up. Due to the advantages of mutation breeding many scientists are entering in this field, for the development of new cultivars in different crop plants.

Induced mutational studies in winged bean were initiated by Kulthe (2000) as a part of his doctoral work. He successfully developed different economically important winged bean mutant lines like, large leaf, high yielding, long pod, flat pod, early maturing, and dwarf. Though these mutant lines were morphologically well studied, their biochemical nature, however, was not well characterized. In winged bean the presence of high amount of anti-nutritional factors is posing the major problem. To overcome this situation it was visualized that induced mutation could be the desirable approach.

It is well established that the induced mutational approach not only creates morphological variation but also alters the biochemical features of plants. It was envisaged that by resorting to mutagenesis the minimization of different undesirable biochemical factors present in all edible plant parts of winged bean mutant plant types would become possible. By keeping this end in view the present studies were organized to assess the status of different antinutritional factors from the mutants of winged bean developed through earlier mutation breeding programme. It was believed that such efforts would lead to an understanding of the exact quantum of improvement in the biochemical and nutritional attributes of different morphologically desirable mutant lines of winged bean.

MATERIALS AND METHODS

Fourteen true breeding M_6, M_7 and M_8 mutant lines of variety EC 38955-A of winged bean obtained from the earlier mutation breeding programme (Kulthe, 2003) were taken for the analysis of carbohydrates and reducing sugar.

The list of mutants of winged bean used in the present study is as follows:

1. Long pod
2. Early maturing
3. Flat pod/wingless
4. Large leaf/high yield
5. Flat pod/linear leaf
6. Flat pod/large leaf
7. Anthostem
8. Long pod/large leaf
9. Long pod/black seed
10. Flat pod/long pod
11. Dwarf
12. Wingless/small pod
13. Dark green/flat pod
14. Large Leaf/stiff stem

Standardization of trypsin assay using BAPNA

The trypsin stock solution was prepared by dissolving 10 mg of trypsin in 1 ml protease buffer (0.1 mM Tris-HCL PH 7.8). This stock solution was diluted to prepare 1 mg/ml working solution of trypsin in the protease buffer. 1mM BAPNA (N-α-benzoyl DL—arginine-p-nitroanilide) was prepared by dissolving 10 mg of BAPNA in 0.45 ml DMSO (Dimethyl Sulfoxide) and then mixed in 22.5 ml of protease buffer. The trypsin working solution (1 mg/ml) was taken with 10 to 100 µl in each test tube and volume was made upto 0.5 ml with protease buffer. 1 ml BAPNA solution was incubated in each test tube at 37 0C for exactly 10 minutes. The reaction was stopped with 0.2 ml of 30 % acetic acid after 10 minutes. The absorbance was read at 410 nm. A graph was plotted with absorbance versus concentration of trypsin and according to it, the optimum trypsin concentration to be used for the consumption of BAPNA was determined.

Trypsin inhibitor assay

Trypsin activity was measured by using BAPNA, as described by Erlanger et al. (1961). For the trypsin assay 20 µl (1 mg/ml) was found to be optimum from the earlier standardization, revealing 20 µl trypsin showing 100 % activity, hence 20 µl of trypsin was used. 10-100 µl sample extracts were taken in test tubes and 20 µl trypsin was added in each test tube. The volume was made to 500 µl with protease buffer, so that the trypsin activity could get inhibited upto 40 % to 60 %. 1 ml of 1 Mm BAPNA was added to the reaction mixture and incubated at room temperature. Later the reaction was stopped after 10 minutes by adding 200 µl of 30 % acetic acid. A graph was plotted with concentrations of sample extract versus absorbance and highest inhibition was recorded. From this highest inhibition 50 % was taken into consideration for further calculation.

One inhibitor unit was defined as the amount of inhibitor that inhibitted 1 unit of trypsin activity. It was expressed as TIU/ /g meal.

Standardization of chymotrypsin assay by using GLUPHEPA

Chymotrypsin stock solution was prepared in 1 mM Tris buffer. 1 mg/ ml working solution of chymotrypsin was prepared from the above stock solution in 50 M Tris- buffer P^H 8, containing 1 mM $CaCl_2$. Chymotrypsin was assumed 100 % active. Different concentrations (10 µl to 100 µl) of chymotrypsin (1 mg/ml) were taken in different test tubes and volumes were made up to 1 ml in all test tubes with

50 m Tris buffer, 10 mg/ml GLUPHEPA (n-glutaryl 1-phenylalanine p-nitroanilide) was prepared in dimethyl formaldehyde and 25 µl of it was added to each tube. All the tubes were incubated at 37 ^{0}C. Optical density was read at 405 nm. A graph of O.D. versus concentration of chymotrypsin was plotted and consequently the ideal chymotrypsin concentration to be used for the assay was calculated.

Chymotrypsin inhibitor assay

Chymotrypsin activity was measured using GLUPHEPA (Muller and Weder, 1989). 30 µl of chymotrypsin was found to be optimum for the assay. 30 µl of chymotrypsin was added to each test tube containing 10 to 100 µl of sample extract so as to give 40 % to 60 % inhibition of chymotrypsin activity. The reaction volume was made up to 1 ml with 50 M Tris-buffer. 25 µl of 10mg/ml GLUPHEPA was added to each test tube and incubated for 1 hour at 37 ^{0}C. The reaction was stopped by adding 200 µl of 30 % acetic acid. The optical density was measured at 405 nm and the chymotrypsin inhibitory activity was estimated similarly as that of the trypsin inhibitor assay.

RESULTS AND DISCUSSION

Quantification of trypsin inhibitor content (Table 2&3)

The seed and tuber trypsin inhibitor content of different winged bean mutant lines were quantified by trypsin inhibitor assay. In control seed trypsin inhibitor was noted as 5589.66 TIU/g meal and tuber trypsin inhibitor exhibited 4275.10 TIU/ g meal.

The low seed trypsin inhibitor lines were selected from morphological mutant lines, such lines comprised long pod-5 (1237.18 TIU/ g meal), large leaf/ high yielding-5 (1850.12 TIU/g meal), large leaf/ stiff stem-5 (1876.85) and long pod-2 (1897.21 TIU/g meal). The highest seed trypsin inhibitor content was found in mutant lines long pod/large leaf-3 (10520.11 TIU/g meal) and long pod/large leaf-1 (10019.20 TIU/g meal).

The low tuber trypsin inhibitor lines could be detected in some mutants like long pod-5 (982.201 TIU/g meal), large leaf/high yielding-5 (1022.71 TIU/g meal), dark green/flat pod-4 (1227.67 TIU/g meal) and anthostem-3 (1275.18 TIU/g meal). The highest trypsin inhibitor activity was also demonstrated in case of tubers by mutant plant types such as long pod/large leaf-3 (9905.56 TIU/g meal), flat pod/linear leaf-4 (8495.28 TIU/g meal) and flat pod/large leaf-1 (8075.15 TIU/g meal). In remaining mutant lines lot of variation was observed as compared with control (EC-38955-A).

Quantification of chymotrypsin inhibitor (CTI) content (Tables 2&3)

The screening of different mutant lines showed increased and decreased values as compared with control (EC-38955-A). The seed chymotrypsin inhibitor content in control was 3571.32 CTIU/g meal and it was found to be 2720.00 (CTIU/ g meal) in tuber.

Table 2: The seed low trypsin and chymotrypsin inhibitor winged bean mutant lines.

Sr. No.	Desired mutant line	Trypsin inhibitor (TIU/g meal)	Chymotrypsin inhibitor (TIU/g meal)
1	Control EC-38955-A	5589.32 ± 58.77	3571.32 ± 18.22
2	Long pod-2	1237.18 ± 35.05	1162.57 ± 25.54
3	FP/Wingless-2	4913.71 ± 105.36	1012.37 ± 36.42
4	Long pod-2	1897.21 ± 33.12	2840.72 ± 21.48
5	La.L./ high yield-5	1850.12 ± 16.32	1825.15 ± 12.29
	Mean	3097.51	2082.43
	S.D.	1997.83	1101.04
	S.E.	893.48	492.42
	C.D.	2483.88	1368.91

Table 3: The tuber low trypsin and chymotrypsin inhibitor winged bean mutant lines.

Sr. No.	Desired mutant line	Trypsin inhibitor (TIU/g meal)	Chymotrypsin inhibitor (TIU/g meal)
1	Control EC-38955-A	4275.10 ± 64.34	2720.00 ± 13.52
2	Long pod-5	982.201 ± 22.62	786.12 ± 33.51
3	La.L./ high yield-5	1022.71 ± 71.82	986.55 ± 23.54
4	Dark green/flat pod-4	1227.67 ± 19.53	1390.57 ± 11.91
5	FP/Wingless-2	3972.58 ± 94.22	624.00 ± 32.95
	Mean	2296.05	1301.45
	S.D.	1674.55	843.19
	S.E.	748.90	377.10
	C.D.	2081.95	1048.33

In mutant lines the lower seed CTI could be detected in flat pod/wingless-2 (1012.37 CTIU/g meal), large leaf/high yielding-2 (1038.85 CTIU/g meal), early maturing-2 (4890.17 CTIU/g meal) and dwarf-5 (4545.18 CTIU/g meal).

The tuber CTI content showed sharply decreased amount as compared with control. The low tuber CTI lines were flat pod/wingless-2 (624.00 CTIU/g meal), long pod-5 (785.12 CTIU/g meal) while the high CTI could be noted in dwarf-5 (3039.57 CTIU/g meal) and flat pod/linear leaf-4 (3005.00 CTIU/g meal) mutant lines.

Legume seeds are known to contain several antinutritional components. The most important ones are the trypsin and chymotrypsin inhibitors (Liener and Kakade, 1980). The antinutritional effect of such factors in the irreversible inhibition of digestive enzymes like trypsin/chymotrypsin has been well documented (Leterme *et al.,* 1992). Removal or reducing the quantity of TI/CTI is essential to improve the nutritional quality of crop plants for effectively utilizing their potential.

Some reports are available in regard to mutation and trypisn and chymotrypsin inhibitor genes in plants. Low TI lines have been reported in barley mutants induced by pesticide treatments (Harsulkar, 1994). An extensive study of mutation and trypsin inhibitor genes has been carried out by Kothekar *et al.,* (1996) in winged bean mutants. Recurrent mutagenesis has been found to be highly useful in reducing the trypisn inhibitor content in winged bean by Khandelwal (1996). In winged bean low trypsin and chymotrypsin inhibitor mutants have been identified and isolated from the M_6, M_7 and M_8 population. The recovery of low seed TI/CTI lines in winged bean through mutational approach has comprised a feature of major accomplishment of the present work. Several researchers like, Khadke (2005) in moth bean, Dadke (1999), in winged bean and Sagade (2008) in urd-bean have worked on TI/CTI in induced mutant lines.

The winged bean mutants carrying lowered levels of TI/CTI are likely to assume significant importance and immense economic value especially in regard to their nutritional potential. The detailed understanding of the genetics of inhibitors and other antinutritional components present in winged bean mutants would prove immensely helpful to breeders in planning their programmes directed towards qualitative enhancement of winged bean.

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