3. MATERIALS AND METHODS

3.1 Procurement of seeds

For the present investigation entitled “Evaluation of protease inhibitor on insect proteases and isolation of gene encoding protease inhibitor from rice bean (Vigna umbellata)” the seeds of rice bean genotype BRS-2 were procured from the Department of Crop Improvement, CSKHPKV, Palampur.

Leguminous plants like rice bean are a rich and varied source of secondary plant compounds like protease inhibitors (PI). Among different types of protease inhibitors, serine PIs (serpins) has been shown to inhibit model trypsin like proteins in insects (Roberts et al. 2003). Therefore, in this study, the first objective was to isolate and purify trypsin inhibitor from rice bean.

![Plate 3.1 A Photograph showing dry mature seeds of rice bean (Vigna umbellata)](image)
3.2 Isolation, purification and characterization of trypsin inhibitor

3.2.1 Isolation and purification of trypsin inhibitor

Reagents

- 0.1 M phosphate buffer (pH 7.5)
- Ammonium sulphate
- Bovine trypsin
- Substrate: N-α-Benzoyl-DL-arginine-paranitroanilide (BApNA)
- DEAE-Sepharose fast flow
- Sephadex G-75
- Dialysis membrane

Method

3.2.1.1 Isolation of crude inhibitor preparation

Isolation and purification of protease inhibitor was carried out from rice bean genotype BRS-2. Finely ground seed powder (50g) was homogenized with 150 ml of 0.1 M phosphate buffer pH 7.5, stirred at room temperature (25°C) for 4h and then filtered through muslin cloth. The filtrate obtained was centrifuged at 10,000g for 15min.

3.2.1.2 Ammonium sulphate precipitation

The supernatant obtained was precipitated overnight with 80 per cent ammonium sulphate (Fig 3.1) and collected by centrifugation at 10,000g for 20 min. They were dissolved in the minimal volume of 0.1M phosphate buffer pH 7.5.

Figure 3.1 Process of salting out of proteins
3.2.1.3 Dialysis

The precipitates dissolved in 0.1 M phosphate buffer pH 7.5 were dialyzed thoroughly against 0.05 M phosphate buffer pH 7.5 for 24 h (buffer was changed at least 6 times) (Fig 3.2). The dialysed solution was used for further analysis by chromatographic techniques.

![Diagrammatic representation of dialysis process](image)

**Figure 3.2 Diagrammatic representation of dialysis process**

3.2.1.4 Ion-exchange chromatography

Protein purification is a series of processes intended to isolate a single type of protein of interest from a complex mixture. Separation steps may exploit differences in protein size, physico-chemical properties, binding affinity and biological activity. Separation based on ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to immobilized ion exchange groups of opposite charge (Fig. 3.3).

Ion exchange chromatography was carried out at room temperature (25°C). Lyophilized sample was dissolved in 0.1M phosphate buffer pH 7.5 and loaded into a DEAE-Sepharose column (2×21cm), equilibrated with the same buffer. Stepwise elution was carried out with a linear gradient of 0.1-0.5M NaCl in 0.1M phosphate buffer (pH 7.5). Fractions (3ml) were collected with flow rate of
0.5ml/min. In all fractions protein content and trypsin inhibitory activity was determined. The active fractions with high trypsin inhibitory activity were pooled and were used for carrying out electrophoresis.

3.2.1.5 Estimation of soluble proteins

Protein concentration during the trypsin inhibitor purification steps was determined using the Lowry et al. (1951). Bovine serum albumin (BSA) was used as the standard protein.

Reagents

- 2 per cent Sodium carbonate in 0.1N sodium hydroxide (Reagent A)
- 0.5 per cent Copper sulphate (CuSO₄·5H₂O) in 1 per cent potassium sodium tartrate (Reagent B)
- Alkaline copper solution: Mix 50ml of A and 1ml of B prior to use (Reagent C)
- 1 N Folin-Ciocalteau Reagent (Reagent D)

Procedure

Five millilitre of reagent C was added to 0.1 ml of protein solution and mixed immediately. 0.5 ml of 1N Folin’s reagent was added after 10 minutes and the test tube was shaken vigorously to ensure proper mixing. After 30 minutes, the intensity of color was measured at 660 nm against a reagent blank.

Standard curve

Standard bovine serum albumin solution was used as the standard for all the above estimations. 10 mg of bovine serum albumin was dissolved in 40 ml distilled water (Fig 3.4).

Calculation The amount of protein was expressed as mg/g or 100g sample.
3.2.1.6 Estimation of trypsin Inhibitory activity

The trypsin inhibitory assay was done with a slight modification in the method described by Chitra and Sadasivam (1986).

Reagents

- 30 per cent Glacial acetic acid
- Trypsin: Dissolve 6.25 mg bovine trypsin and make upto 25 ml with 0.001 N HCl. Dilute 2ml of this solution to 25ml for assay.
- Substrate: N α- Benzoyl-D-L-arginine-paranitroanilide (BApNA)
- 0.1 MTris-HCl buffer (pH 8.2)
Estimation

1. 0 to 1ml of the purified fraction was pipetted out in duplicate sets of the test tubes, one to serve as endogenous (E) and the other test (T).
2. Made volume to 2ml with buffer in the endogenous set and 1ml in test set.
3. Added 1ml of trypsin solution (20µg) to each tube in the test set.
4. For each sample separate standard (S) was prepared with 1ml of Tris-Hcl buffer and 1ml of trypsin solution.
5. Incubated all the tubes in a water-bath at 37ºC.
6. After few minutes, 2.5ml of substrate (1mg BApNA) was added to each tube.
7. The reaction contents were incubated for 10 min. at 37 ºC.
8. The reaction was stopped by adding 0.5ml of 30 per cent glacial acetic acid.
9. Read the absorbance at 410 nm

Calculation

One unit of activity corresponds to that amount of trypsin inhibitor in µg protein which gives 50 per cent inhibition of enzyme activity under experimental conditions. The trypsin inhibitor activity is expressed as trypsin inhibitor units (TIU) per gram sample or per mg protein:

\[
\text{TIU mg/g of defatted sample} = \frac{\text{Differential absorption} \times \text{dilution factor}}{0.019 \times 1000}
\]

3.2.1.7 SDS-PAGE (Sodium dodecyl sulphate Polyacrylamide gel electrophoresis)

The purity of the final trypsin inhibitor protein obtained after the course of purification was checked by polyacrylamide gel electrophoresis using 12.5 per cent acrylamide gels and a slight modification in the method given by Lamelli (1970).
Reagents

(i) **Acrylamide stock solution**

- Acrylamide 30.0g
- Bisacrylamide 0.8g

Dissolved in water and the final volume was made 100ml. Filtered the solution through whatman no. 1 filter paper and stored in brown bottle at 4°C.

(ii) **Stacking gel buffer stock (Tris-HCl, pH 6.8)**

- Tris 0.45 g
- 0.5M HCl 7.8 g

Adjusted pH to 6.8 and final volume was made to 100ml with distilled water. Filtered through whatman no. 1 filter paper and stored at 4°C.

(iii) **Resolving gel buffer stock (Tris-HCl, pH 8.8)**

- Tris 15.39 g
- 1.5M HCl 3.69 g

Adjusted pH to 8.8 and the final volume was made 100ml. It was filtered through whatman no. 1 filter paper and stored at 4°C.

(iv) **Reservoir buffer (Tris-glycine, pH 8.3)**

- Tris 3.03g
- Glycine 14.4g
- SDS 1.0g

Adjusted pH to 8.3 and the final volume was made 1 liter with distilled water.

(v) **Polymerising Agents**

Ammonium persulphate 1 per cent (w/v) 0.1g/10ml, prepared freshly before use

TEMED Fresh from refrigerator
(vi) **Staining solution**

Coomassie brilliant blue R-250 0.25g
Methanol 50ml
Glacial acetic acid 10ml

After mixing the above components, the final volume was made 100ml with distilled water. It was filtered to remove undissolved materials and was stored at room temperature.

(vii) **Destaining solution**

Glacial acetic acid 5ml
Methanol 35ml

The above components were mixed and the final volume was made 100 ml with distilled water.

(viii) **Bromophenol blue solution (1 per cent)**

Ten milligram of bromophenol was dissolved in 100ml of distilled water.

(ix) **Sample buffer (5X)**

Tris-HCl buffer pH6.8 1.25 ml
SDS 2.0 ml
Mercaptoethanol 1.0 ml
Bromophenol blue (1 per cent) 1.0 ml
Water to 10 ml

**Sample preparation**

The purified protease inhibitor was mixed with equal volume of sample buffer. The mixture was then boiled for 3 min in a boiling water bath and cooled at room temperature
Preparation of slab gel and electrophoresis of sample

Glass plates were washed with distilled water and oven dried. The plates were placed on gel casting assembly, the sides of the plates were sealed by tygon tubing and were clamped. Various components of stacking and resolving gel were mixed as specified in Table 3.1. About 10-20 µl of protein sample was loaded in the sample wells.

Table 3.1 Composition of gels for SDS-PAGE

<table>
<thead>
<tr>
<th></th>
<th>Stacking gel</th>
<th>Resolving gel</th>
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</thead>
<tbody>
<tr>
<td>5.0 % gel</td>
<td>15 % gel</td>
<td>12 % gel</td>
</tr>
<tr>
<td>Stock acrylamide solution</td>
<td>0.83 ml</td>
<td>20ml</td>
</tr>
<tr>
<td>Tris-HCl (pH 8.8)</td>
<td>-</td>
<td>8ml</td>
</tr>
<tr>
<td>Tris-HCl (pH 6.8)</td>
<td>0.63 ml</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td>3.4 ml</td>
<td>11.4ml</td>
</tr>
</tbody>
</table>

Degas on a water pump for 3-5 min and then add:

<table>
<thead>
<tr>
<th></th>
<th>Stacking gel</th>
<th>Resolving gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium persulphate solution</td>
<td>0.05 ml</td>
<td>0.2ml</td>
</tr>
<tr>
<td>10 per cent SDS</td>
<td>0.05 ml</td>
<td>0.4ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
<td>20µl</td>
</tr>
</tbody>
</table>

The electrophoresis was carried out by maintaining current of 2 mA per well for initial 15 minutes and a constant current of 3 mA per well was maintained until bromophenol blue reached the other end of the slab gel. After completion of run, the power supply was disconnected and the proteins in the gel were visualized by staining in Coomassie Brilliant Blue R-250 (0.25 per cent in
methanol: acetic acid: water, 5:1:4). The gel was destained by frequently changing the destaining solution. The distance travelled by each protein band was recorded.

3.2.1.8 Gel filtration chromatography

In gel filtration, the matrix consists of porous particles and separation is achieved according to size and shape of the molecules (Fig. 3.5). The technique is sometimes also referred to as molecular sieve chromatography or gel permeation or size exclusion chromatography.

Freeze dried TI sample pooled from DEAE-Sepharose column was dissolved in phosphate buffer 0.1M pH 7.5 and applied to a Sephadex G-75 column (1.5× 22.5 cm). Fractions of 2ml/ tube were eluted with 0.1 M phosphate buffer pH 7.5 and collected using a flow rate of 0.5 ml/min. The active fractions were used for carrying out electrophoresis and fractions with trypsin inhibitory activity were pooled.

3.2.1.9 Affinity chromatography

In the last step of purification, the sample obtained after gel filtration was subjected to affinity chromatography. For affinity chromatography, column was prepared with a slight modification in the method given by Paiva et al. (1992) and Chang and Tsen (1981). The column was prepared as per the following steps (Fig. 3.6):


Figure 3.4. An affinity matrix binding to its target protein
1) **Activation of Sepharose 4B:** The matrix was activated to make it reactive towards the functional group of the ligand. 25g of CNBr was dissolved in 50 ml Dimethyl formamide. This CNBr liquid was then added to 5-10 ml of CM-Sepharose. Using 5M NaOH, pH of the solution was adjusted between 10.5 to 11.5 with the help of pH strips.

2) **Washing of activated CNBr Sepharose matrix:** CNBr activated Sepharose was washed extensively with distilled water (4°C) first and then alternately with 0.001N HCl and Borate buffer (0.05N, pH 9.0) to wash impurities from the matrix (Fig. 3.7).

3) **Coupling of ligand:** 1 mg Bovine pancreatic trypsin was dissolved in 5 ml of 0.01N HCl and was added to the above activated CNBr-Sepharose suspension. This mixture was shaken overnight at 4°C and filtered.

4) **Reactivation:** This Sepharose gel was further alternately washed with acetate buffer (0.1M, pH 4) and borate buffer (0.1M, pH 8) several times to remove unbound trypsin. Washings were collected and checked by measuring trypsin absorbance units at 280 nm.

\[
1g \text{ trypsin} = 1,440 \text{ at } 280 \text{ nm}
\]

\[
15g \text{ Sepharose retained} = 1,440 - \frac{252}{1440} = 83 \text{ per cent trypsin}
\]

5) **Final filling and Equilibration:** The activated matrix was then packed in short column (0.9 cm x 14 cm) with bed volume 3ml. The column was equilibrated with Tris buffer (0.05N, pH 7.8) with added 0.1N CaCl₂.
Freeze dried TI sample pooled from Sephadex G-75 column was dissolved in phosphate buffer 0.1M pH 7.5 and applied to CNBr activated Sepharose column (0.9 cm x 14 cm) (Fig. 3.8). Fractions of 1.5ml/tube were eluted with 0.05N Tris buffer pH 7.8 and collected using a flow rate of 0.5 ml/min. The active fractions with trypsin inhibitory activity were pooled and the purity of the final trypsin inhibitor protein obtained was checked by SDS-PAGE.

3.2.2 Characterization of protease inhibitor

3.2.2.1 Study of temperature stability of rice bean trypsin inhibitor

The inhibitor in 0.1M phosphate buffer (pH 7.5) was at first incubated at 20, 40, 80, 90 or 100 °C for 45 min. The inhibitor was then added to the assay mixture and the incubation continued. The trypsin inhibitor activity was measured at 410 nm.

3.2.2.2 Study of pH Stability of rice bean Trypsin inhibitors

Prior to the addition of the inhibitor in the assay medium, the inhibitor was separately dissolved in different buffer systems and pre-incubated for 45 min. The buffer used were: (a) glycine-HCl buffer, pH 2.2; (b) acetate buffer, pH 4.0; (c) phosphate buffer pH 7.5; (d) tris buffer pH 9.0. Aliquots of the inhibitor in the different buffers were added individually to the assay system containing the buffered substrate and the enzyme, before proceeding with the incubation. After the incubation activity was measured at 410 nm against the blank. Appropriate controls were taken and pH optima were determined.
3.3 Extraction of proteases from *Spodoptera litura* and their metabolic interaction with protease inhibitor (PI)

The defensive role of PIs is based on their inhibitory activities towards the digestive enzymes of insects proteases involved in some vital processes, resulting either in a critical shortage of essential amino acids or interfering with important biochemical or physiological processes of insects. A thorough understanding of metabolic interaction of insect gut proteases and protease inhibitors is a prerequisite to plan strategies and sustainable application of PIs. So, preliminary step of this experiment was the extraction of gut proteases from the larvae of *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) to study the potential and metabolic interaction of protease inhibitors of rice bean.

3.3.1 Collection of experimental insects

*Spodoptera litura* larvae from first to fifth instar stage were collected from infected plants of *Solanum lycopersicum* (tomato) and *Capsicum annum* (Capsicum) plants from Vegetable farm and Biological Control Lab CSK HPKV, Palampur during the month of June - July. The population was maintained on castor (*Ricinus communis*) and capsicum leaves in optimum rearing conditions (27±1°C, 60±5 per cent RH).
Plate 3.2  Photographs showing *Spodoptera litura* fifth instar larvae infesting *Capsicum annum* leaves

Plate 3.3  Photographs showing anesthetized (second instar) larvae of *Spodoptera litura*
Plate 3.4  A Photograph showing foregut, midgut and hindgut of dissected *Spodoptera litura* second instar larvae

Plate 3.5  A Photograph showing midgut homogenate of *Spodoptera litura*
3.3.2 Preparation of luminal enzyme extract of *Spodoptera litura* gut proteases (SLGP’s) (Telang et al. 2005)

Larvae at first to fifth instar stage were anesthetized by exposing them to ethanol/diethyl ether for few seconds. They were then carefully dissected out and fat tissues were removed by small brush. A total of 8-10 clean midguts / larval instar stage were extracted by homogenizing the tissue with its contents in equal volume (5ml) of ice cold distilled water. The gut luminal contents were removed by centrifugation at 10,000 rpm for 20 min at 4°C. The resultant supernatant was then filtered, frozen in aliquots and was used to study the metabolic interaction of gut proteases with protease inhibitors.

3.3.3 Metabolic interaction of midgut proteases with purified Protease inhibitors (Patankar et al. 2001; Telang et al. 2003)

Reagents

- Azocasein
- (BAPNA) N-α- Benzoyl-DL-Arginine-paranitroanilide
- (BTpNA) N-α- Benzoyl-DL-tyrosine-paranitroanilide
- IN NaOH
- Bovine trypsin
- Bovine Chymotrypsin
- 0.1 M Tris Buffer (pH 10.0)
- Glacial Acetic acid

3.3.3.1 Protease activity assays

Total gut proteinase activity was measured by using chromogenic substrate azocasein at a final concentration of 2 per cent. For the assay, 60µl of enzyme extract was added to 200 µl of 1per cent azo-casein (in distilled water) and incubated at 37°C for 30 min. The reaction was terminated by the addition of
300 ml of 5 per cent TCA. After incubation at room temperature for 30 min, tubes were centrifuged at 10,000 g for 10 min. An equal volume of 1N NaOH was added to the supernatant and activity was estimated by measuring the O.D. at 450 nm.

The trypsin and chymotrypsin activity were measured with BApNA and BTpNA as synthetic substrate. 1.35 ml of 0.1 M Tris buffer (pH 10.0) was incubated with 30 µl of gut extract for 10 min at 37°C. 200 µl of substrate BApNA (8 mm) and BTpNA (1 mm) was added to this reaction mixture for trypsin and chymotrypsin activity respectively. Tubes were incubated at 37°C for 10 min. The reaction was terminated by addition of 750 µl of 30 per cent acetic acid and O.D. was measured at 410 nm.

Assays were run in triplicates with appropriate blanks. Proteinase activity unit was calculated in terms of micromoles of paranitroaniline produced per minute. The molar extinction coefficient (M⁻¹ cm⁻¹) for pNA (paranitroanilinide) at 410 nm equals to 8800 (Erlanger et al. 1961) was taken into account to calculate trypsin and chymotrypsin-like activity (BApNA/ BTpNA units /mg protein) using the formula:

\[
\text{Activity units} = \frac{\text{Abs}_{410} \times \text{min} \times 1000 \times \text{ml of reaction mixture}}{\text{Extinction coefficient of chromagen - mg protein in reaction mixture}}
\]

3.3.3.2 *Spodoptera litura* gut protease (SLGPs) interaction with inhibitors

To study the interaction of SLGPs with PIs, inhibitory assays were carried out. 1.35 ml purified fraction of rice bean trypsin inhibitor was added to 30 µl of gut extract for 10 min at 37°C. 200 µl of substrate BApNA (8 mm) and BTpNA (1 mm) was added to reaction mixture for trypsin and chymotrypsin inhibitory activity, respectively. Tubes were incubated at 37°C for 10 min. The reaction was terminated by addition of 750 µl of 30 per cent acetic acid and O.D. was measured at 410 nm. The residual proteinase inhibitory activity was estimated as
described above. In all the assays blanks without sample were run simultaneously. One PI unit was defined as the amount of inhibitor which causes inhibition of one unit of proteinase activity under given assay conditions.

3.3.3.3 Protein determination

Protein content of midgut homogenate was determined by using the Lowry et al. (1951). Bovine serum albumin (BSA) was used as the standard protein.

3.3.4 Assessment of inhibitory potential of rice bean flour on Spodoptera litura and Callosobruchus maculatus

Storage of grains, especially at small scale farming levels is limited due to bruchids like Callosobruchus maculatus a serious coleopteran pest and general feeders like Spodoptera. For investigating the efficacy and insecticidal property of rice bean, a feeding experiment was conducted. Lethal and sublethal effects on S. litura larvae and C. maculates adults by rice bean flour were studied in this objective.

3.3.4.1 Collection of experimental insects

Culture stocks of Spodoptera litura (second instar) larvae were collected from Biological Control Lab, CSK HPKV and the stocks of Callosobruchus maculatus adults were collected from infested cowpea (Vigna unguiculata) seeds from Department of Entomology, CSK HPKV, Palampur.

3.3.4.2 Feeding of rice bean flour to Spodoptera litura

Larvae of Spodoptera litura were reared on artificial diet as described earlier by Shorey and Hale (1965). The artificial diet was composed of soaked mungbean (15g), baking yeast (1g), sorbic acid (0.15g), ascorbic acid (0.3g), casein (0.5g), agar (1.4g), vitamin stock (3g/1000ml) (mixture of niacin, calcium pantothenate, thiamine, riboflavin, pyridoxin monohydrochloride, folic acid, biotin and vitamin B12), 40 per cent fomalin and distilled water.
For feeding studies the artificial diet was supplemented with rice bean flour in long beakers each with a capacity of 1 litre. The culture was routinely maintained on rice bean flour based artificial diet at standard conditions, of 27±5°C, 75±5 (%) relative humidity. The diet was prepared with four treatments and a control. The final composition of diet was made 25 per cent, 50 per cent, 75 per cent and 100 per cent (w/w) with rice bean flour and a positive control of artificial diet (i.e. without PIs). The beakers were covered with muslin cloth to maintain proper aeration.

The beakers were cleaned and shaken everyday to improve aeration and to prevent attack of unwanted micro organisms. The experiment was conducted for 96 hrs (4 days) and was started by rearing 20 insects on each diet preparation.

3.3.4.3 Feeding of rice bean flour to *Callosobruchus maculates*

Another feeding experiment was conducted with bruchid *Callosobruchus maculates*. The samples were prepared for four different treatments of 25 per cent, 50 per cent, 75 per cent and 100 per cent (w/w) of rice bean flour with artificial diet. Chemically defined portion of artificial diet (mg/100ml) was prepared with a slight modification in the method described by Rojas et al. (2000). The ingredients of the artificial diet were Vitamins (Biotin, Folic acid, Inositol, Nicotinamide, Riboflavin, Thiamine, Vitamin B12, Vitamin A, Vitamin C and Vitamin E), free amino acids (Histidine, Glutamine, Cysteine, Lysine, Tryptophan, Proline, Tyrosine, Valine and Alanine), Cholesterol, Glucose, Sucrose and 6 M KOH. The experiment was conducted for 144 hrs (6 days).

3.3.5 Data collected and Statistical Analysis

Data was collected included the number of live and alive insects per day and per cent larval mortality was calculated. Data were analyzed using SAS version 9.3 software. Means of three independent determinations was tested by one way ANOVA followed by post hoc testing using Duncan's multiple range test. A significant level of 0.05 was used for all statistical tests, $\alpha = 0.05$.
Plates 3.6 Photographs showing culture stocks of (a) *Callosobruchus maculatus* adults and (b) second instar larvae of *Spodoptera litura*.

Plate 3.7 Photographs showing feeding of rice bean flour to *Spodoptera litura*.
Plate 3.8A Photograph showing feeding of rice bean flour to *Callosobruchus maculates*
3.4 Isolation of gene encoding protease inhibitor from rice bean

For the isolation of gene encoding protease inhibitor, partial sequencing of the trypsin protease inhibitor was done. The steps involved in the gene cloning (Fig 3.8) and isolation of a specific sequence within the gene are:

- Procurement of plant (leaf) material
- Extraction and isolation of RNA
- cDNA synthesis by RT-PCR
- PCR Amplification of cDNA with degenerate primers
- Insertion of the DNA into a cloning vector
- Insertion of the cloning vector into a host organism (such as into \textit{E. coli})
- Identification of the clone carrying the desired DNA
- Further, partial or full length sequencing of the gene

3.4.1 Procurement of Plant material

Rice bean plants were grown in greenhouse under controlled conditions. Healthy leaves of rice bean were wounded mechanically with the help of forceps across the midvein near the center of the leaflets. These leaves were picked after 3 hours of the initial wounding, frozen in liquid nitrogen and stored at -80°C.
Plate 3.9 Photographs showing experimental trial of rice bean plant in greenhouse
3.4.2 RNA Isolation

Three hours prior mechanically wounded leaves of rice bean plant were taken for the isolation of RNA using TRIzol method. (Ambion life technology Invitrogen, USA).

Reagent

- TRIzol® Reagent and Pure Link™ RNA Mini Kit
- Chloroform
- Ethanol (96-100 % ) and 70 per cent (in RNase-free water)
- Microcentrifuge (Eppendorf)
- Rotor-Stator homogenizer
- Lysis buffer- 10µl of 2- mercaptoethanol was added for every 1ml lysis buffer
- 1.5 mL RNase-free microcentrifuge tubes and RNase-free pipette tips

Method

Wounded leaves were crushed in mortar-pestle with liquid nitrogen till it changed into white powder. 2ml Lysis buffer was added to frozen the contents and were centrifuged at 12,000 g for 15 minutes at 4°C. 600 μl of the colorless upper phase (supernatant) containing RNA was added to an equal volume of 70 per cent ethanol. 700 μl of sample was transferred to a spin cartridge (with a Collection Tube). Centrifugation was done at 12,000 × g for 15 seconds at room temperature, flow through was discarded and the spin cartridge was reinserted into the same collection Tube. The receding steps were repeated to process the entire sample. 700 μl of wash Buffer I was added to the spin cartridge and again centrifuged at 12,000×g for 15 seconds at room temperature. The flow-through and the collection tube were discarded. The spin cartridge along with the contents was inserted into a new collection tube and 500 μl Wash Buffer II with ethanol was added to it. Centrifugation was again done at 12,000 × g for 15 seconds at room temperature, flow-through was discarded and the step was
repeated. The spin cartridge and collection tube were centrifuged at 12,000 × g for 1 minute at room temperature to dry the membrane with attached RNA. Collection tube was discarded and the spin cartridge along with the contents was inserted into a recovery tube. 30 μl RNase-free water was added to the center of the spin cartridge and incubated at room temperature for 1 minute. Centrifugation was done again for 2 minutes at ≥12,000 × g to elute the RNA into the recovery tube. The concentration of the purified total RNA was quantified using nanodrop spectrophotometer ($A_{260}/A_{280}$) and was stored at -80°C for further use.

**Quantification of RNA**

RNA was diluted with DEPC-treated autoclaved water and the absorbance was recorded at 260 and 280 nm. Absorbance of 1 at 260 nm corresponds at 40 μg/ml of RNA. For pure RNA, $A_{260}/A_{280}$ is equal to 1.9-2.0. The following formulae were used to calculate the concentration and the yield of RNA

\[
\text{Concentration of RNA (µg/ml) } = A_{260} \times 40 \times \text{dilution factor}
\]

\[
\text{Total yield (µg/ml)} = \text{Concentration} \times \text{volume of stock RNA sample (ml)}
\]

**3.4.2.1 RNA gel preparation**

The conformation of the RNA obtained after the course of RNA isolation was checked by formaldehyde agarose gel electrophoresis (Sambrook and Russel 2001).

**Formaldehyde Buffer (5xF):**

- 3N Sodium Acetate
- 0.2 M MOPS (3- N-morpholino propan sulfonic acid)
- 0.5M EDTA, pH 8.0
13.3 ml of 3N Sodium acetate was added to 750 ml of MOPS. pH was adjusted to 7.0 with NaOH. 10 ml of 0.5 M EDTA was added and final volume was made to 1 litre with DEPC water

**Preparation of running buffer for RNA:-**

- DEPC Water : 390ml
- 5X Formaldehyde (FA) buffer : 100ml
- Formaldehyde : 10ml
- Total : 500ml

**Composition of 2X RNA loading Dye Buffer:-**

- 95 per cent formamide
- 0.025 per cent SDS
- 0.025 per cent Bromophenol blue
- 0.025 per cent Xylene cyanol FF
- 0.025 per cent Ethidium bromide
- 0.5mM EDTA

**Preparation of slab gel**

Washed the electrophoresis tank and gel casting assembly with detergent, rinsed with sterile water, dried with ethanol and filled with solution of 3 per cent H₂O₂. Various components of the RNA gel were mixed as specified in Table 3.2:

**Table 3.2: Composition of RNA gel:**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>500 mg</td>
</tr>
<tr>
<td>DEPC water</td>
<td>32 ml</td>
</tr>
<tr>
<td>5X (FA) buffer</td>
<td>13 ml</td>
</tr>
</tbody>
</table>

Boiled the above contents and then 8 ml formaldehyde was added.
**Gel preparation and electrophoresis:**

Boiled the above contents except formaldehyde in a flask and swirled it properly until agarose was dissolve properly. 8ml of formaldehyde was added when temperature was about 50°C. The gel was casted in casting assembly, comb was placed and left for polymerization. For sample preparation, 4 µl of RNA sample was mixed with 1 µl 2X RNA loading dye. The sample was denatured by incubating at 65°C for 3-5 minutes followed by chilling on ice. Sample was loaded onto 1 per cent formaldehyde agarose gel. Electrophoresis was carried out at 70 V in 5X FA gel running buffer. The gel was viewed on a UV-transilluminator and captured on gel documentation (Alpha digidoc, Sigma) system.

### 3.4.3 cDNA synthesis by RT-PCR (Reverse Transcription-Polymerase chain reaction)

RT-PCR was done to reverse transcribe single-stranded RNA into complementary DNA (cDNA) by using following steps:

#### 3.4.3.1 DNase digestion

**Reagents**

The reaction mix required for DNase digestion was prepared from reagents obtained from Fermentas. The ingredients of the reaction mix used are as under:

**Preparation of reaction mix:**

<table>
<thead>
<tr>
<th>Reaction Mix</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA sample</td>
<td>2.5</td>
</tr>
<tr>
<td>10X DNase I reaction buffer</td>
<td>1</td>
</tr>
<tr>
<td>DNase I Amp grad (Invitrogen; 1U/ul)</td>
<td>1</td>
</tr>
<tr>
<td>DEPC water</td>
<td>5.5</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
</tr>
</tbody>
</table>
Method

DNase digestion was done to remove genomic DNA from RNA. The above ingredients of the reaction mix along with the RNA sample were taken in 0.5 ml PCR tubes. Volume was made up to 10 µl with RNase free H₂O. Reaction mixture was incubated for 15 min at 25°C in PCR and then the reaction was stopped by adding 1µl of 25M EDTA. The mixture was heated for 10 min at 65°C and chilled on ice.

3.4.3.2 First strand cDNA synthesis

Reagents

- Oligo dT12-18 (500ug/ml)
- dNTP mix(10mM)
- 5X first strand buffer
- Dithiothreitol (DTT) (10 mM)
- Reverse Transcriptase III / Superscript III

Method

1µl Oligo dT and dNTP mix (10mM) was added to the reaction mixture obtained after DNase digestion. Reaction mixture was heated for 65°C for 5 min in PCR and was later on placed on ice for 2 min to remove the RNA secondary structure. 4µl of 5X first strand buffer, 2µl Dithiothreitol (DTT)(10 mM) and 1µl of Reverse Transcriptase III/ Superscript III was added and mixed gently. Reaction mix was again incubated at 42°C for 60 min and then stopped by incubating at 70°C for 15 minutes. This cDNA sample was then stored at -80°C further use.

3.4.3.3 Confirmation of cDNA formed

Confirmation of the cDNA formed was done by PCR amplification of cDNA by using primers designed for 26S rRNA. 26S rRNA forward and reverse primers were used as internal control gene primers (Singh et al. 2004a). The reaction mix was prepared as under and amplified in PCR.
Preparation of reaction mix:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>14.75</td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>15mM MgCl₂</td>
<td>1.5</td>
</tr>
<tr>
<td>dNTPs(10mM)</td>
<td>1.0</td>
</tr>
<tr>
<td>Cresol Red</td>
<td>2.5</td>
</tr>
<tr>
<td>26S Forward Primer</td>
<td>1.0</td>
</tr>
<tr>
<td>26S Reverse Primer</td>
<td>1.0</td>
</tr>
<tr>
<td>cDNA</td>
<td>1.0</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25.0</strong></td>
</tr>
</tbody>
</table>

3.4.3.4. **PCR Amplification:** The following PCR amplification conditions were employed for amplification of cDNA by Thermal Cycler (Labnet).

**PCR amplification protocol from cDNA**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>94°C</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94°C 30 sec</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>54°C 40 sec</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>72°C 1 min</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Final extension</td>
<td>72°C 10 min</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Hold</td>
<td>4°C --</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

3.4.3.5 **Agarose gel Electrophoresis**

25 µl of the reaction mixture along with 4 µl of loading dye was loaded onto 1% agarose gel and 100bp DNA ladder was used as marker. Electrophoresis was done at 100V for 25 minutes. The buffer used was 1x TAE at pH 8.0. The DNA bands were visualized and documented using a gel documentation system (Alpha digidoc, Sigma).
3.4.4 Primers used for amplification of rice bean trypsin protease inhibitor (RBPI)

The presence of RBPI gene in rice bean genotype BRS-2 was detected by PCR amplification of its cDNA with a set of degenerate primers designed by using CLUSTAL W and Oligo Calc softwares. The primers sequences designed were:

Forward primer: 5’ ATGATGGTGCTAAAGGTGTGTG 3’(24 mer)
Reverse primer: 5’ CACTCAGCTTGCAAATCTCG 3’ (20 mer)

3.4.4.1 PCR Amplification with degenerate primers(Sambrook et al. 1989)

Ingredients in the reaction mixture and the PCR conditions were standardized to amplify a single sharp amplicon by standardizing primer concentration, annealing temperature and the concentration of dNTPs. The following reaction mixture was used for amplification.

Preparation of reaction mix:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>18.25</td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTPs (10mM)</td>
<td>1</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>1</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>1</td>
</tr>
<tr>
<td>cDNA</td>
<td>1</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25.00</strong></td>
</tr>
</tbody>
</table>
The following PCR amplification conditions were employed for amplification using standard conditions (Table 4.2). PCR products were run on 1 per cent agarose gel in 1X TAE buffer (pH 8.0).

**PCR amplification protocol from cDNA**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>94°C</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94°C</td>
<td>30 sec</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>60°C</td>
<td>40 sec</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>Final extension</td>
<td>72°C</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Hold</td>
<td>4°C</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

3.4.5 Elution of DNA from the Gel

**Material and Reagents**

- Gene JET™ Gel Extraction Kit (Fermentas)
- Ethanol (96-100 per cent)
- Isopropanol
- 3M sodium acetate, pH 5.2
- Microcentrifuge and Microcentrifuge tubes
- Water bath

**Method**

- **Excision of DNA:** The gel slice containing DNA fragment was excised using a sharp sterile scalpel or razor blade. Placed the gel slice into pre-weighed 2.0 ml microcentrifuge tubes and weight of the gel was recorded. Binding buffer in the ratio of 1:1 (v/w) was added to the gel and incubated at 50-60°C for 10 minutes until the gel slice was completely dissolved. To this solubilized gel solution isopropanol was added in the ratio of 1:2 (v/v).
- **Binding of DNA**: Transferred 800 µl of the suloubilized gel solution to the spin column (Fermentas) and centrifuged for 1 min. The flow through was discarded and 100 µl of binding buffer to it. Again centrifugation was done and flow through was discarded.

- **Washing of column**: 700 µl of wash buffer to the above contents of spin column, centrifuged for 1 min and flow through was discarded. The empty spin column was centrifuged for 1 min to completely remove residual wash buffer.

- **Elution of DNA**: The spin column was placed into a fresh 1.5 ml microfuge tube and 20µl of elution solution was added to it. Centrifugation was done for 1 minute and flow through was collected.

3.4.6 Cloning of PCR product

3.4.6.1 Ligation: Insertion of cDNA Into Vector

The purified PCR fragment was ligated to pGEM-T Easy vector (3015 bp). The multiple cloning sequence and sequence reference points of pGEM-T Easy vector has been given in Figure 3.10 and 3.11, respectively. Ligation was done by using Promega ligation kit. For ligation, an optimal molar ratio of ends of vector and insert (1:3) was computed. The components of ligation mix were added to 0.5 ml micro centrifuge tubes and were incubated overnight at 4°C. For 25 µl of ligation reaction, the ingredients provided in the ligation kit were mixed in the order given below:

**Preparation of reaction mix:**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Rapid Ligation Buffer</td>
<td>5 µl</td>
</tr>
<tr>
<td>pGEM®-T Easy Vector (50ng)</td>
<td>1 µl</td>
</tr>
<tr>
<td>PCR product</td>
<td>3 µl</td>
</tr>
<tr>
<td>T4 DNA Ligase (3 Weiss units/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>nuclease-free water to a final volume</td>
<td>15 µl</td>
</tr>
</tbody>
</table>
Figure 3.5 The promoter and multiple cloning sequence of the pGEM®-T Easy Vector

Figure 3.6 pGEM®-T Easy Vector map and sequence reference points
3.4.6.2 Preparation of competent cells (Inoue et al. 1990)

Materials

- Dh-5α cells
- LB (Luria-Bertani) Medium: To 950 ml of deionized H₂O, 10 g Tryptone, 5 g Yeast Extract and 10 g NaCl was added. Adjusted the pH to 7.0 with 5 N NaOH (~0.2 ml) and final volume was made to 1 liter with deionized H₂O. Media was sterilized by autoclaving for 20 minutes at 15 psi (1.05 kg/cm) on liquid cycle
- Transformation buffer (TFB1, TFB2): The composition of TFB1, TFB2 is given below:

Composition of TFB1 (pH 5.8 for 50 ml) and TFB2 (pH 6.8 for 50 ml) buffers:

<table>
<thead>
<tr>
<th>Transformation Buffer</th>
<th>Reagents</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFB1 (pH 5.8 for 50 ml)</td>
<td>100 mM RbCl₂</td>
<td>0.6045g</td>
</tr>
<tr>
<td></td>
<td>50 mM MnCl₂</td>
<td>0.495g</td>
</tr>
<tr>
<td></td>
<td>30 mM K acetate</td>
<td>0.147g</td>
</tr>
<tr>
<td></td>
<td>10 mM CaCl₂</td>
<td>0.0735g</td>
</tr>
<tr>
<td></td>
<td>15 per cent Glycerol</td>
<td>7.5ml</td>
</tr>
<tr>
<td>TFB2 (pH 6.8 for 50 ml)</td>
<td>10 mM MOPS</td>
<td>0.1046g</td>
</tr>
<tr>
<td></td>
<td>100 mM RbCl₂</td>
<td>0.6045g</td>
</tr>
<tr>
<td></td>
<td>70 mM CaCl₂</td>
<td>0.55125g</td>
</tr>
<tr>
<td></td>
<td>15 per cent Glycerol</td>
<td>7.5ml</td>
</tr>
</tbody>
</table>

Method

The traces of Dh-5α cells were taken from the vial with a sterile toothpick or inoculating loop, streaked on LB agar containing 25 µg/ml media and incubated at 37°C overnight. A single colony was picked and inoculated in 10 ml of media grown overnight at 37°C. 1 ml of overnight grown culture was added to 100 ml prewarmed Luria broth in 250 ml flask and incubated in shaker at 37°C until an
O.D. of 0.5 was reached. Then the culture was chilled on ice for 5 min. Transferred it to a sterile round bottom centrifuge tube and centrifuged at 4000 g for 5 min to collect the cells. Supernatant was discarded and cells were kept on ice. The cells were suspended in cold (4°C) TFB1 buffer (300 ml for 100 ml culture) and kept the suspension on ice for 90 min. Again the cells were collected by centrifugation at 4000 g for 5 min. Supernatant was discarded and cells were kept on ice. The cells were resuspended gently in 4ml cold TFB2 buffer. Aliquots of 100-200 µl were prepared in sterile microcentrifuge tubes, freezed in liquid Nitrogen and were stored at -80°C.

3.4.6.3 Transformation of Dh-5α high efficiency competent cells:-

Material

- Ligation product
- LB broth
- Ampicilline (100mg/ml H₂O)
- X-Gal (30mg/ ml of Dimethylformamide)

Method

100 µl freshly prepared competent cells were taken in a chilled micro centrifuge tube and 10 µl of ligation product was added, mixed gently and chilled in ice for 30 min. Heat shock was given by shifting the chilled mixture to 42°C water bath for 3 min and then chilled on ice for 10 min. To this 900 µl of Luria broth was added and incubated at 37°C for 3 hrs to allow bacteria to recover and express the antibiotic marker encoded by the plasmid. 100µl of each transformation culture was spread on Luria agar plates having Ampicillin, X-gal and incubated overnight at 37°C. The recombinant clones were identified by blue/white assay. After incubation, white colonies having recombinant vectors were picked up and streaked on plates having Luria agar with Ampicillin, X-gal and incubated at 37°C overnight, for multiplication.
3.4.7 Colony Lysis

Materials

Colony lysis buffer with the following composition was used:

- 1.75 ml dH₂O
- 200 µl 10 per cent Triton X-100
- 40 µl 1M Tris HCl (pH 8.0)
- 8 µl 0.5 M EDTA (pH 8.0)

Method

50µl of colony lysis buffer and isolated colony were added in a tube. The mixture was boiled for 10 min in water bath set for boiling and then kept on ice for 2 min. Centrifugation was done for 30 sec at 13000 rpm. Supernatant having colony lysate was taken out in a separate tube.

3.4.7.1 Colony PCR

For confirmation of the transformed colonies amplification was carried out by using M13 primers. The reaction mix was prepared as under and amplified in PCR

Preparation of reaction mix:-

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclaved water</td>
<td>14.75</td>
</tr>
<tr>
<td>10xPCR buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTPs (10mM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Forward primer M13 (10µM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Reverse primer M13 (10µM)</td>
<td>0.5</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.5</td>
</tr>
<tr>
<td>Colony lysate</td>
<td>2.0</td>
</tr>
<tr>
<td>Taq polymerase (5U/ µl)</td>
<td>0.25</td>
</tr>
<tr>
<td>Crysol Red</td>
<td>2.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>
**PCR Amplification:** The following PCR amplification conditions were employed and PCR products were run on 1 per cent agarose gel in 1×TAE buffer.

**PCR amplification protocol:**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>94°C</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94°C</td>
<td>30 sec</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>54°C</td>
<td>40 sec</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>72°C</td>
<td>50 sec</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>Final extension</td>
<td>72°C</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Hold</td>
<td>4°C</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

3.4.8 Plasmid Extraction

Plasmid Extraction was done by using Gene JET Plasmid Miniprep Kit (Fermentas)

**Reagents**

- Lysis Solution
- RNase Solution
- Lysozyme
- Wash Buffer Concentrate
- Elution Buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.5)
- Culture Tubes
- Plasmid Spin Column Assembly (Spin Column and Waste Tube) (Fermentas)
- Ampicillin (100mg/ml H₂O)
- LB Broth
3.4.8.1 Preparation of plasmid culture

10 ml LB broth was taken and 0.7µg ampicillin was added to it. White colonies were picked up, taken in the tubes and incubated at 37°C in shaker overnight.

3.4.8.2 Plasmid Isolation

Plasmid culture was taken in eppendorf (2ml) and centrifuged for 30 sec at 13.2 rpm. Supernatant was discarded and the pellet was resuspended in 250µl of resuspension solution. The cells were dissolved completely by vortexing until no cell clumps remained and were lysed by adding 250µl of lysis solution. 350µl of neutralization solution was added and mixed thoroughly to avoid localized precipitation of the cell debris. Centrifugation was done for 5 min at 12000 rpm to pellet cell debris and chromosomal DNA. Supernatant was transferred to the spin column (Fermentas) by decanting and centrifuged for 1 minute. The flow through was discarded and 500µl of diluted wash solution (with 96 per cent ethanol) was added to spin column. Again centrifugation was again done for 1 minute at 12000 rpm and discarded the flow through. This wash procedure was repeated again. The pellet obtained was kept at room temperature for 2 min and 20µl of elution solution was added to for eluting plasmid DNA. The purified plasmid was quantified using nanodrop spectrophotometer and stored at -20°C for further use.

3.4.8.3 Confirmation of clones

The confirmation was done through comparative restriction analysis of selected clones and the control vector for the presence of insert with EcoRI.

Preparation of reaction mix:-

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid (Template)</td>
<td>6.0</td>
</tr>
<tr>
<td>EcoRI</td>
<td>2.0</td>
</tr>
<tr>
<td>Buffer (FD green)</td>
<td>3.0</td>
</tr>
<tr>
<td>Autoclaved water</td>
<td>19.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>30</strong></td>
</tr>
</tbody>
</table>
The reaction mix was kept in water bath at 37°C for 20 minutes and was run on 1 per cent agarose gel in 1X TAE buffer.

3.4.9 Sequencing

3.4.9.1 Sequencing PCR

Primers of vector sequence were used to sequence both the strands. The sequencing PCR was done by using following reaction mix and the PCR conditions as given below:

<table>
<thead>
<tr>
<th>Reaction mix</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid</td>
<td>2</td>
</tr>
<tr>
<td>Primer</td>
<td>1</td>
</tr>
<tr>
<td>RR (reagent reaction) mix</td>
<td>0.75</td>
</tr>
<tr>
<td>5X Buffer</td>
<td>1.25</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
</tr>
</tbody>
</table>

The PCR amplification was carried out under following conditions:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>96°C</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>96°C</td>
<td>10 sec30</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>48°C</td>
<td>40 sec</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>60°C</td>
<td>4 min</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>Final extension</td>
<td>72°C</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Hold</td>
<td>4°C</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

The sample was washed with injection buffer for removing surplus dyes.
3.4.9.2 Cleaning of PCR Product

25µl of injection solution was added to 5µl sequencing PCR product and mixed well. The mixture was poured onto the wells on Millipore Montage sequence 96 plate. The plate was placed onto a vacuum manifold to dry the plate for 3-4 minutes. Again 25 µl of injection solution was added in wells, solution was mixed thoroughly by pipetting (about 25 times) and was taken in sequencing plate.

3.4.9.3 Sequence Analysis

The PCR product containing rice bean trypsin protease inhibitor gene was sequenced using M13 primers employing primer-walking technique. The sequencing was done in AB Hitachi 3130 xl Genetic Analyser (Applied Biosystems, USA) at Molecular Biology lab, IHBT (CSIR) Palampur. Sequence homology search was also carried out against a gene sequence database at NCBI (National Centre for Biotechnology Information; www.ncbi.nlm.nih.gov) using BLAST (Basic Local Alignment Search Tool). The known sequences were analyzed first by BLASTx followed by BLASTn. The significant score was obtained in term of E value, which represent the number of different alignments with scores equivalent to or better than S (alignment score) that are expected to occur in a database search by chance. Lower the E value, more significant the score.