CHAPTER 4.

RESULTS AND DISCUSSIONS

4.1 Southern hybridization of genomic DNA with pea lectin probe

4.1.1 Isolation and restriction of genomic DNA of moth bean

Total genomic DNA of etiolated seedlings of moth bean was isolated using CTAB method and then analyzed by agarose gel electrophoresis. The isolated DNA showed no shearing but was contaminated with RNA (Fig. 4.1A). The crude DNA was purified by treating with RNase (100 mg/ml) and then dissolved in 20 μl of TE buffer. The purified DNA was found to be free from protein and RNA contamination and showed no shearing (Fig. 4.1B). The DNA yield was 400 ng/g of etiolated seedlings as estimated spectrophotometrically. cTAB method was also followed by other workers for isolation of DNA from soybean (Wang et al., 2008), pisum (Yadav et al., 2007), common bean (Ochoa et al., 2006) etc. Chakraborti et al., (2006) compared various protocols on different cultivars of chickpea and found that CTAB method produced good quality and high quantity of intact DNA. The isolation of high molecular weight DNA was carried out from etiolated seedlings so that there was maximum yield of genomic DNA, minimum contamination from organelle DNA such as chloroplast or mitochondrial and also from secondary plant products such as phenolics and polysaccharides. The problem of polyphenols and polysaccharides was exacerbated if green, over matured tissue rather than etiolated leaves were taken for DNA extraction (Sharma et al., 2000). Pure DNA is essential for complete digestion of the DNA with restriction endonucleases which in turn is an important prerequisite for successful Southern hybridization. The purified sample was run on 0.8% agarose gel along with standard λ/HindIII marker.

The result revealed that the isolated DNA exhibited A₂₆₀/₂₈₀ ratio of approximately 1.8 indicating that it was of good quality. Further, isolated DNA showed no shearing or presence of RNA contamination when run on 0.8% agarose gel. The yield of DNA estimated spectrophotometrically was found to be around 5 μg/μl. About 10 μg of purified moth bean DNA was used for complete digestion with 20 units each of EcoRI, HindIII and BamHI.

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Fig. 4.1: Agarose gel electrophoresis of isolated DNA from moth bean (*Vigna aconitifolia*)

(A) **Crude genomic DNA isolated from moth bean, Lane M:** λ DNA/HindIII marker, **Lane 1-6:** Crude genomic DNA of moth bean

(B) **Purified genomic DNA , Lane M:** λ DNA/HindIII marker, **Lane 1-6:** Purified genomic DNA of moth bean restriction endonuclease. A very fine smear indicates successful digestion of the DNA with the restriction endonucleases used.

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4.1.2 Preparation of probe

The plasmid clone pLG 4.10 (Fig. 4.2) that contained pea lectin cDNA insert was isolated as described in 3.2.1.4.1. The clone contains an 860 bp pea lectin insert at EcoRI site of pUC 8 vector. From an overnight grown 10 ml culture about 5 μg of plasmid DNA was obtained. The purified plasmid DNA was restricted with EcoRI to excise the cDNA insert. The restricted samples were fractioned on a 0.8% agarose gel along with 1 kb ladder. After restriction, component resolved into an upper vector band and a lower insert band (Fig. 4.3). When compared with DNA marker, the fragment was found to be ~850 bp which clearly resembles the actual size of 860 bp. From 15 μg of the restricted plasmid DNA, 2 μg of fragment was recovered, radiolabelled and used as a probe for Southern hybridization.

Fig. 4.2: Map of pea lectin clone pLG4.10
Fig. 4.3: Agarose gel electrophoresis of restricted pea lectin plasmid DNA clone

Lane M: 1 kb ladder

Lane 1-3: Digested plasmid

4.1.3 Southern blotting and hybridization of the restricted genomic DNA

The presence of lectin gene was confirmed by Southern blotting. The 860 bp fragment representing pea lectin gene was radiolabelled by Hexalable DNA labelling kit (MBI, Fermentas) and used as probe. The restricted DNA samples when separated on 0.8% agarose gel appeared as fine smear when viewed under UV light (Fig. 4.4A). The blot prepared with the restricted genomic DNA of moth bean was hybridized with radiolabelled pea lectin cDNA probe. Though the probe was heterologous, it hybridized very strongly with moth bean genomic DNA at a high stringency (Fig. 4.4B). The stringency used (0.5 X SSC at 60°C for 15 min) was high enough to remove the non-homologous DNA sequences from nylon membrane. This observation suggested a considerable homology between pea and moth bean lectin genes which is due to high degree of sequence homology existing between leguminous lectins. Such kind of intergeneric homologies have also been reported between various species of leguminosae like *Pisum sativum*, *Glycine max* (Yamauchi and Minamikawa, 1990), *Vigna unguiculata* (Datta et al., 2000) and *Lens culinaris* (Qureshi et al., 2007). This experiment was conducted to establish the homology between pea and moth bean lectin gene, and the pea lectin cDNA probe was further used for screening the cDNA library of moth bean for the presence of lectin clones.
Fig. 4.4: (A) Restriction analysis of the genomic DNA isolated from moth bean seedlings
Lane M: 1 kb ladder
Lane 1-3: Purified genomic DNA restricted with EcoRI, BamHI and HindIII enzymes

(B) Southern hybridization of moth bean restricted genomic DNA with pea lectin gene probe

4.2 Construction and screening of cDNA library

In this approach, mRNA was isolated and converted into cDNA using commercial kit. The cDNA was then cloned into a pGEMT Easy vector and the resulting clones were screened for the presence of lectin gene using a radiolabelled probe. The presence and accumulation of relatively high levels (8% to 10% of the total protein) of well-known lectins like phytohemagglutinin, concanavalin A, soybean agglutinin, pea lectin and favin in developing seeds made the seed an ideal tissue to be used for construction of cDNA library which is aimed at isolation of this gene (Chrispeels and Raikhel, 1991; Qureshi et al., 2007). Immature seed were collected from pods harvested at 10 days after flowering (DAF), as this stage was found to contain significantly higher amount of lectin than other stages of seed maturation (Datta et al., 2000; Qureshi et al., 2006).
4.2.1 Isolation of RNA

Total RNA was isolated using the protocol mentioned under section 3.2.2.1. The ratio of A$_{260}$ to A$_{280}$ was found to be 1.9 which indicated that RNA was of good quality. The typical yield of RNA for this method was 5.4 µg/250 mg of developing seeds. The analysis of RNA by standard 0.8% formaldehyde agarose gel electrophoresis revealed distinct RNA bands with no smearing as shown in Fig. 4.5A. The isolation of mRNA from the total RNA was carried out by using the protocol of commercial kit supplied by Qiagen. A smear of RNA corresponding to the size range expected for intact mRNA was observed when analysed by 1.0% formaldehyde agarose gel electrophoresis as shown in Fig. 4.5B. This method was also followed by other workers for isolation of RNA *Vigna radiate*, *Vigna mungo* (Sharma A., 2010) and *Dolichos biflorus* (Rekha Kansal et. al., 2008) for obtaining good quality and high quantity of intact RNA.

4.2.2 Construction and elution of cDNA

The first strand of cDNA was synthesised from mRNA followed by its amplification through LD-PCR using Creator SMART™ PCR cDNA synthesis kit. The Creator SMART™ PCR cDNA synthesis kit provides a novel, PCR-based method for producing high quality cDNA from total or poly A$^+$ RNA. The advantages of Creator SMART protocol include requirement of nanogram amounts of Poly A$^+$ RNA, synthesis of longer cDNA fragments and hence high percentage of full-length cDNA clones, increased efficiency of cDNA synthesis and selective amplification with no contamination by genomic DNA or Poly A$^+$ RNA. Analysis of the synthesized cDNA on 1.2% agarose gel showed a moderately strong smear of cDNA in the size range of 300 bp to 2 kb (Fig. 4.5C). Earlier also SMART™ PCR cDNA synthesis kit by Clontech also proved useful for the construction of cDNA library from soybean by Wang et al. (2008). The cDNA in the size range of 500 bp – 2 kb was excised from the agarose gel and then eluted from the excised gel pieces. The eluted cDNA was found to be of desired size as checked by agarose gel electrophoresis using 1% agarose gel (Fig. 4.5D).
Fig. 4.5: (A) Total RNA isolated from developing seeds of moth bean, 
Lane M: RNA ladder, Lane 1-6: Total RNA

(B) mRNA isolation, Lane M: RNA ladder, Lane 1: mRNA of moth bean

(C) cDNA synthesis, Lane M: 1 kb ladder, Lane 1-2: cDNA of moth bean

(D) Size fractionation, Lane M: 1 kb ladder, lane 1-3: size fractionation of cDNA

4.2.3 Cloning of cDNA and screening of recombinants

cDNA fragments (500 bp-2 kb) were ligated to pGEMT Easy vector (Fig. 4.6) and the ligated mixture was used to transform E. Coli DH5α cells. A background test for blue/white colour selection was done by plating different concentrations of the ligated mixture on plates containing IPTG and X-Gal. After incubating the plates at 37°C for overnight, blue and white colonies appeared which represented non recombinants and recombinants respectively (Fig. 4.7). Since the moth bean DNA was...
inserted within the structural gene of β-galactosidase, therefore, recombinants were unable to produce colour as synthesis of β-galactosidase was disrupted, but non recombinant with functional β-galactosidase were able to hydrolyze X-Gal to produce blue colonies.

**Fig. 4.6: A map of pGEMT Easy vector**

**Fig. 4.7: Blue- white screening of recombinant clones**

The percentages of the recombinant clones were determined by counting the number of blue and white colonies. An increase in the number of blue/white colonies was observed with increase in the volume of transformed sample used for plating.
The result showed that cDNA library contained appreciable number of recombinant colonies. These white colonies which were spotted on LB agar plates supplemented with ampicillin (100 μg/ml) along with X Gal and IPTG, helped in screening out the false positives as about 19 colonies turned blue when kept overnight at 37°C. These colonies were not included in colony hybridization. About 1250 white colonies were altogether spotted and the GM stock (Appendix) of all these colonies were also prepared and stored at -80°C in a deep freezer for future use.

4.2.4 Screening for the recombinant clones

The recombinant clones were screened by colony hybridization for the presence of lectin gene using pea lectin as probe. The colony hybridization was performed as described in 3.2.2.7.

4.2.4.1 Primary screening

After hybridization and autoradiography, the autoradiogram showed black spots which were considered as positive signals. All together 55 colonies from 13 plates of moth bean cDNA library revealed a positive signal. Fig 4.8A shows a representative autoradiogram after primary screening. It was observed that all the primary clones did not give equally intense signal, as some of these clones could have given signal due to nonspecific hybridization, therefore, secondary screening was done to separate out the real positive clones from the mixture of primary clones.

4.2.4.2 Secondary screening

Out of 55 primary clones 40 were subjected to secondary screening. Four representative positive clones showing positive signal were picked up for a final round of confirmation. Fig. 4.8B shows a representative autoradiogram after secondary screening.
4.2.4.3 Southern hybridization of positive clones

The plasmid DNA of four positive clones of moth bean was isolated and restricted with EcoRI. The digested DNA samples were run on 0.8% agarose gel, blotted on to a nylon membrane and hybridized using pea lectin as a probe (Fig. 4.9A). As seen in Fig. 4.9B the autoradiogram showed the presence of inserts.

Fig. 4.8: Screening of recombinants
(A) Primary screening  (B) Secondary screening

Fig. 4.9: (A) Restriction analysis of positive clones
(B) Southern hybridization of positive clones obtained after screening of cDNA library of moth bean seeds

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Positive clones were further analyzed by sequencing. Out of these two clones were obtained having 826 bp and 843 bp of size (Fig. 4.10 & Fig. 4.11).

5’ATGGGCTTCCCTCAAAACCCAAATGATCTCATTCCATCTGATATTCTATCCAT
TCTCTTAACAAACATCTTTTTCTTCAAGGTGAACTCAACAGAAACCACCTTCC
TTTTCATACCAAGTTTACGCCAGACAAAAAACCCTAATCTTCCAAGGAGG
ATGGCTACACACCACAAAAAGGGAAGTGGTGAACACTGACCAAGGATAGAGATA
CAGGTAACGTGGCTAAATTTTGCAACTCTTCACTTTTGTCATAGATGCGCCC
AGCAGTTACGACGTTGGCCGATGGGTTCGTTCTCATTCCACACCTATATATGGGATAGAGATA
CTAAGCCGCAGACTGGCGGTTACCTCGGAGTTTCTCAATAGACAAAGAT
ATGATTTGCTGATAGCTTTTAATGCTGCCACTAATGTGTTAACTGTTACTTTAA
CTTATCCTAAATTCACTTGGAGGAAGAGAATGTAACAGTTATACTCTTAAATGA
AGTTGTGCTTGGATGAGTTTCTGAGTTGGGATGGATGAGGATGTTTCTCGAG
CTACCACTGGAGCAGATTTTGCAGCACATGAGTTCATCCAGTTTACCTTTC
TTCTGAGTTGGGCGGAAACTCAGTTCTAAGCAAGCTGCAGATGCA

Fig. 4.10: Nucleotide sequence of moth bean lectin gene (826 bp)

4.3 Molecular cloning of full-length moth bean lectin cDNA by Rapid Amplification of cDNA Ends (RACE)

The template RNA used for this reaction was the total RNA isolated from developing seeds collected at 10 DAF, this was done by keeping in mind the earlier reports that the
level of plant indigenous gene transcript is relatively higher in comparison to other stages of seed maturation (Hammond et al., 1984; Hilder et al., 1989; Kansal et al., 2008). Primers for RACE were designed according to the cDNA sequence of moth bean.

5'ATGGCTTCCTCACAACCTTTCTTATATCTTTCTCTCTCGTACCCGCTTCTCC
TGTTGTCTCTCACCACAAGCAACTCAAAACACCGTCTTCTTCCAAACTTCA
ATCCTTCCGACCATCCAACTTTATCTCCTCCAAGGTGACCCACCCTCTC
TCCAAGTCGATGGAAGGCTTTCATTTGCTCTCTCTCATCAGGCGCTTAC
GCCGGCCCGCTTACAGGATACAAAGTTAAAGGCAACGGCAAAACCCACCG
TCATCTCTTGGCCGCGCCTTTCTACTCCGCCCCCATCAAATCTGGGACAGCA
CCACCCGACGCTCGCCAGCTCTCGCCTACCTTCTCCTACCTTAAACATCTTC
GCTCTCAACAAATCAGACGCCGCGATGGGTGTGCATTTGCTCTCTGTACCC
GTGTTGTGAGCCCAACCCGGGTCTTCTTTGCTCTTTCAGCAACCCGACAC
CTACGACAGCTCCTTACCAGACTCTTGCTGCTTGAGTTGACACCATCTACTG
AGTCTATACAGATGGACGTCGTGGGGTTTGGGCTAGGGCAACGGCAAAACCC
ATCGACGATGCGACGCTCGCCAGCTCTTTTGCTTAACTCCTTTTCACTTT
ACTCTGGCGCAGCGTCGCCAGCTTGCCACTTTCCTTCACTTTGCTTCCAC
TAGGAGCCAGCAACCCGGGCTTCTTTGCTCTTCTGCAGCAACCCGACAC
CTACGACTCTGTCGAGCGGCTTCTTCTTCTGGGTCAGCAGCGTCGAGGCT
TTCTGCCAGCGAGGCTTCTTCTTCTGTGGCTGTCGAGCGGCTTCTTCTGGG
CGGAGTGGGTGAGCATTGGGTTCTCTGCCACAAAGTTTGCTCCT
GCCGGGGCAACTGAAACCCACGACGTCGCTCTTCTTGGCTTCTTCTCTC
TAGAATCCCTC

Fig. 4.11: Nucleotide sequence of moth bean lectin gene (843 bp)
4.3.1 Amplification of the 5’ end of moth bean lectin cDNA

After dephosphorylation, decapping and adapter ligation to the total RNA, it was reverse transcribed, after which the RT product was subjected to PCR using adaptor primer and gene specific primer (MbL3). The primary PCR product was then used in the nested PCR amplification with gene specific inner primer (MbL4) and 5’ RACE inner primer. Agarose gel electrophoresis of the nested PCR product showed a single band of about 360 bp (Fig. 4.12A). After subsequent cloning and sequencing, the BLAST analysis of the sequence indicated that it had strong similarities with lectin gene from different Leguminosae species, implying that it is probably a part of lectin gene.

4.3.2 Amplification of the 3’ end of moth bean lectin cDNA

Two gene specific primers were designed according to the cDNA sequence (MbL5 and MbL6) and used for the amplification of 3’ end cDNA. After the initial reverse transcription of the template RNA, the RT product was subjected to PCR using adapter primer and gene specific primer (MbL5). The primary PCR product then used in the nested PCR amplification with gene specific inner primer (MbL6) and 3’ RACE inner primer. Agarose gel electrophoresis of the nested PCR product revealed a single DNA band of about 400 bp (Fig.4.12B). After subsequent cloning and sequencing, the BLAST analysis of the sequence indicated that it had strong similarities with 3’ end of lectin gene from different Leguminosae species.

4.3.3 Amplification of the full-length cDNA

The 3’ and 5’ RACE product were confirmed through colony PCR and digestion by EcoRI (Fig. 4.12C & D). After sequencing the 3’ RACE and 5’ RACE product sequences align on Bioedit software the full-length cDNA of moth bean was deduced. Primers MbL7 and MbL8 based on the sequence of the 5’ and 3’ end of the cDNA were designed and synthesized for amplification of the full-length moth bean lectin cDNA. As anticipated the agarose gel electrophoresis of the PCR product showed the amplicon of 1086 bp (Fig.4.14). Amplified product was cloned in pGEMT Easy vector. After cloning and sequencing, the sequences of the fragment obtained were matched with the aligned cDNA sequence and showed complete homology. BLAST with the sequence in the NCBI database showed strong similarities to lectins cDNA from species of Leguminosae family.
Fig. 4.12: Agarose gel electrophoresis of RACE-PCR

(A) 5’ RACE product, Lane M: 100 bp ladder, Lane 1: 5’ RACE product

(B) 3’ RACE product, Lane M: 100 bp ladder, Lane 1: 3’ RACE product

(C) Conformation of 5’ and 3’ RACE-PCR product through Restriction digestion, Lane M: 100 bp ladder, Lane 1-2: 5’ RACE product, Lane 3-4: 3’ RACE product

(D) Conformation of 5’ and 3’ RACE-PCR product through Colony PCR, Lane M: 100 bp ladder, Lane 1-4: 5’ RACE product, Lane 5-8: 3’ RACE product
Fig. 4.13: Agarose gel electrophoresis of full length moth bean cDNA

Lane M: 1 kb ladder, Lane 1: Full length moth bean cDNA
5’CTATGGAAAAACGCCAGAATATCATCACAATCCATCACTGTAAGACCTTACAGCCCATGCTTCTCTTCTCCTATCCATTATCCTTTCTCCTCTCCGTAGCCTCTTTCCTGCTTTACCCATCCTGAGCCACCTTACCCAGCTCTCCCTCCTCCTTCTCCTCTTCCTCTCAACTTCCCAATCTCCCTGACCCATCCAACCTTTACCCAGTTCAAGGCTAAAAGCTAAGGCAACGGCGAACCACGAGC

CTATGGAAAACGCCAGAAAATCATCAACATCCATCACTGTAAGACCTTACAGCCCATG

GCTTCCTCCAACTTCTCCATTATCCTTTCTCCTCTCCGTAGCCCTC

TTCCTGGTGCTTCTCACCCAAGCAAACTCAACCAACGTCTTCTCCTTCAACTTCCAATCCTTCGACCCATCCAACCTTATCCTCCAAGGTGACGCCACCGTCTCAGCTCTCAACAAATCAAGCACCAGCCGATGGGCTTGCATTTGCTCTCGTACCCGTCCGTCTCTCAGCTACAGCTCTTACCAAGACTGTTGCTGAGTTGACTGACACCTACTC

GACTCCTAGGTTGGACCCGGAACCCCGTCCACATTGGCAATTGACGAACTC

CATCGAGTCTATCACAGATGACCTGCTGGGTTGGGCAACCGGCGAAACACG

GGAGATTCTGGACTCAGTACGACGCTGACGAAAGCTCTTGCTTTTGCTTCAACATCGGAGAAGCGAGCTACATCGACTACTGCTTCACTCTGGACAGTTGAGCTGACGACCAGCTCTTCGAGGAGTGGGACCTC

GCTTCACCCCTTCTCAGGAGAAGCGAGCTACATCGACTTGAGAGTGGGACCTCTGCCCAGTTCAAGATCATTCTGACAAAA

3’

Fig. 4.14: Nucleotide (black) sequence of a cDNA sequence encoding moth bean lectin protein. The start and stop codons (red) are also shown.

4.4 *In silico* analysis of isolated gene sequence

The nucleotide sequence obtained was analyzed on URL www.ncbi.nlm.nih.gov. BlastN and search analysis of the sequence on URL http://blast.ncbi.nlm.nih.gov/Blast.cgi showed 100% coverage with homology 98% (Fig.4.15). This indicated that the isolated lectin gene belonged to a legume lectin family. Amino acid sequence was analyzed through BlastP (Fig. 4.16)
Fig. 4.15: BlastN analysis of moth bean lectin gene sequence

Fig. 4.16: BlastP analysis of the deduced amino acid sequence encoding moth bean lectin

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To obtain the information regarding the presence of ORF in this DNA fragment, the gene sequence was analyzed using FGENESH program of Softberry web server. This online bioinformatics tool predicted the number of exones and introns in the genomic DNA and simultaneously depicted the ORF region in the given sequence. Using this tool, an ORF region of 843 bp in length with no introns was predicted. It had translation initiation codon (ATG) at position downstream of the first base and terminated by stop codon (TAG).

![Diagram showing prediction of potential genes in nucleotide sequence encoding moth bean lectin using FGENESH program of Softberry web server](image)

**Fig. 4.17 Prediction of potential genes in nucleotide sequence encoding moth bean lectin using FGENESH program of Softberry web server**
Though lectin gene has been already reported from other crops, the sequence isolated and characterized in the present study has been reported for the first time. The genomic sequence was found to encode 280 amino acid long lectin proteins using the FGENESH program of softberry web server (Fig. 4.17).

A signal peptide of 18 amino acid at the N-terminal end was predicted by SignalP 3.0 server on Expasy web server. A cleavage site was predicted between amino acid 18 and 19 (Fig. 4.18).

![Signal peptide prediction of moth bean lectin using Expasy software](image)

The presence of a signal peptide indicated that the protein should be directed to the endomembrane system for secretion. Signal peptides were involved in the targeting of proteins to specific compartment. Since the available evidence from other legume indicated that lectins were primarily cystolic proteins, no signals should be necessary.

![Percentage of each nucleotide in the full length gene sequences determined by BioEdit software](image)

So far exact function of signal peptide sequence in the lectin could not be assigned (Hilder et al., 1989). The presence of signal sequences had been reported in many lectins such as soybean (Hammond et al., 1984), cowpea (Hilder et al., 1989), pea...
(Domoney et al., 1995), maize (Rohrmeier and Lehle 1993) and alfalfa (McGurl et al., 1995). The nucleotide composition of this sequence was deduced using BioEdit program. The sequence had 52% (G+C) and 48% (A+T) content (Fig. 4.19).

**Fig. 4.20: ClustalW analysis of the deduced amino acid sequence encoding moth bean lectin using BioEdit software**

Therefore, based on the nucleotide composition it was clear that the isolated sequence encoding for lectin protein was GC rich. The amino acid composition of the protein was deduced by using BioEdit software. The sequence was found to be rich in glycine, leucine, asparagine, serine, valine.
Fig. 4.21: Three dimensional representation of the moth bean lectin using software 3Djigsaw available at www.expasy.com.

(A) Labelled backbone form
(B) Spacefill model
(C) Strand form
(D) Ribbon form

Restriction map analysis of lectin sequence generated using software available at www.nebcutter.com. To release the lectin sequence from the clone, EcoRI and NotI enzymes were chosen as they were present at the flanking sites at MCS (multiple cloning sites) region and not internally in the ORF. Clustal W analysis using Bioedit software was done to align multiple sequences of lectin available on NCBI Genbank with moth bean lectin and lined them to identify their similarities and differences (Fig. 4.20).

The three dimensional structure of the moth bean lectin has been predicted as shown on Expasy web server (Fig. 4.21).

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4.5 Expression analysis of lectin in different tissues of moth bean

To investigate the expression of *MbL* in various tissues of moth bean, semi-quantitative RT-PCR analysis was performed. The expression of lectin was analyzed in different tissues of moth bean viz. leaves, roots, flowers, seeds by RT-PCR. Total RNA was isolated from all these tissues and its purity, concentration and integrity were determined. The $A_{260/280}$ ratio of the isolated RNA samples was found to range from 1.8 to 2.1 which is considered as an acceptable index for RNA purity. The typical yield of RNA varied from 2.5 to 6.0 µg/250 mg of tissue. The integrity of the isolated RNA was evaluated by performing electrophoresis of denatured RNA on agarose gel (1%) containing formaldehyde. The presence of two prominent, distinct and intact RNA bands corresponding to 28S rRNA and 18S rRNA with no smearing indicated that RNA was of good quality. The RNA samples were apparently free of polysaccharides, polyphenolics and genomic DNA.

To normalize for sample to sample variation, actin gene was used as internal control (Suzuki et al., 2000). The cDNA reverse transcribed from RNA was subjected to PCR amplification using lectin gene specific primers which were expected to give an amplicon of 843 bp. Hence the presence of 843 bp amplicon would indicate the expression of lectin gene in the tissue. In the present study, 843 bp amplicon was observed in all the tissues viz. leaves, roots, flowers, seeds but with different expression level. As the lectin mRNA was detected in all the tested plant tissues, the moth bean lectin gene was likely to be expressed constitutively. The result revealed that *MbL* mRNA expression was detected in all tested tissues including immature seeds, flowers, roots and leaves, with the higher expression in seeds (Fig. 4.22). The constitutive expression of lectin gene has also been reported earlier like Zhu et al., (1996) showed similar pattern for *Griffonia simplifolia* at the protein level by using immunoblot. The constitutive expression of lectin in various tissues was also reported in *Pinellia cordata* (Lin et al., 2008) and *Amorphophallus konjac* (Fei et al., 2003) through northern blot analysis. Qureshi et al., (2007); Chen et al., (2005) and Chai et al., (2003) had shown similar pattern for the *Cicer arietinum* lectin, *Len culinaris* lectin, *Zingiber officinale* and *Crinum asiaticum* respectively at the transcript level.
4.6 Transformation of *Brassica juncea* cv. Varuna through *Agrobacterium* mediated transformation

![Image of different parts of a plant](image)

**Fig. 4.22: Expression analysis of moth bean lectin by RT-PCR**

(A) Different parts of moth bean plant used for total RNA isolation
(B) Total RNA of different tissues of moth bean plant along with RNA ladder (Lane M)
(C) RT PCR analysis of different parts of moth bean along with 1 kb ladder (Lane M)

An important component in the implementation of biotechnological crop improvement is the use of various gene expression control elements that direct the expression of the introduced genes. Promoter discovery for plant biotechnology has been largely empirical with most of the promoters deployed being isolated from plant pathogens such as viruses or *Agrobacterium* species (Potenza et al., 2004). The *Agrobacterium* T-DNA provided a variety of constitutive promoters such as those from...
the octopine synthase and mannopine synthase gene but they are less active. CaMV was one of the first plant viruses to be sequenced and used as constitutive promoter to drive the transgenes. Nearly all transgenic crops around the world utilize the CaMV 35S promoter (Odell et al., 1985) to drive transgenes. In this report, isolated lectin gene from *V. aconitifolia* by cDNA library was used for transformation.

4.6.1 Construction of plant transformation vector

Fig. 4.23: (A) Amplification of moth bean lectin gene with primers having enzyme sites *KpnI* and *XbaI*

Lane M: 1 kb ladder, Lane 1-6: Amplified moth bean lectin gene

(B) Amplified gene was cloned in pGEMT Easy vector

Lane M: 1 kb ladder, Lane 1: Amplified lectin gene, Lane 2: Lectin gene released from vector through restriction by *KpnI* and *XbaI*

Plasmids were isolated using a QIAprep Spin Miniprep Kit (Qiagen Inc.). Gene specific primers with *KpnI* and *XbaI* restriction sites were designed according to sequence of cDNA library of moth bean. The cDNA sequence of 843 bp was amplified with these primers and cloned in pGEMT Easy vector (Fig. 4.23).
Cloning was confirmed through colony PCR (Fig. 4.24A) and restriction digestion (Fig. 4.24B). In fig. 4.24(B) the gel has been run for a longer period so the $MbL$ fragment appears slightly bigger in size as compared to 4.24(A). The $MbL$ gene was excised with $KpnI$ and $XbaI$ enzymes and directly cloned in the binary vector pBinAR in right orientation under the control of CaMV 35S constitutive promoter with $nptII$ as a selection marker. The construct was named as pBML. Random colonies were selected for plasmid mini-preparations, which were analyzed by restriction analysis (Fig. 4.25). After restriction enzyme analysis, the plasmids containing a fragment of the expected size i.e. 843 bp, was selected.

The binary construct was then mobilized into $A.~tumefaciens$ GV3101 by direct freeze thaw method (Jyothishwaran S. et. al., 2007). The transformed Agrobacterium colonies were chosen on solid LB medium supplemented with 30 mg/ml gentamycin, 50
mg/ml kanamycin and 40 mg/ml rifampicin by keeping for one days at 28°C. Single colonies of transformed *agrobacterium* were analyzed for the *MbL* gene using gene specific primers and a positive clone named pBMLA was used for transformation purpose.

4.6.2 Plant materials and explants

In efficient tissue culture system for high frequency plant regeneration from cultured tissue is a prerequisite for the success of plant transformation mediated by *Agrobacterium-tumefaciens*.

![Diagram of gene construct](image1)

![Gene analysis](image2)

**Fig. 4.25:** (A) Schematic representation of the *MbL* gene construct (pBML)
(B) Lectin gene released from pBML, Lane M: 1 kb ladder, Lane 1: Plasmid (pBML), Lane 2: Lectin gene digested by *KpnI* and *XbaI*
Although *Brassicas* species in general are highly amenable to *Agrobacterium* mediated transformation and transformed *Brassica juncea* plants have been reported by several authors (Barfield and Pua 1991, Mathews et al., 990, Pental et al., 1993), the transformation and regeneration frequency varies widely among cultivars. In the present investigation transgenic plants transformed with an insecticidal lectin gene isolated from moth bean have been reported.

Surface sterilization of seeds with 0.1% mercuric chloride for 2-3 min followed by through washing in water was sufficient to eliminate any seed borne infection. The vertical cut in the stem segment made them highly susceptible to *Agrobacterium* and in better infection by *agrobacterium*. Stem segments of *Brassica juncea* are reported to be amenable for transformation (Sharma et al., 2004). Induction of BAP in the preculture medium was found to promote *agrobacterium* infection. Stem segments from one month old in vitro regenerated shoots (Fig. 4.26A) were used as explant.

### 4.6.2 *Agrobacterium* mediated transformation

For transformation of *Brassica juncea* (cv. varuna) precultured stem segment (Fig. 4.26B) inoculated with the *Agrobacterium* cells harbouring moth lectin gene for different time durations. An incubation period of 16-18 h was optimized for co-cultivation in the subsequent transformation experiments. After co-cultivation, explants were washed twice with sterile distilled water and once with liquid MS supplemented with 250 mg/l cefotaxime, blotted dried & inoculated on SRM having 250 mg/l cefotaxime for 4 days and incubated at 25±2°C under cool fluorescent light with 16 h photoperiod. The positive effect of delayed application of the selection agent in potato (Visser et al. 1989) has suggested that delayed selection permitted the division of transformed cells, which conferred greater protection against the selective agent. This has resulted in enhanced frequencies of transformation in cases where there was slow start of transcription of the selectable marker gene (Van Wordragen et al. 1992). In the present transformation protocol also, infected stem segments were cultured on selection-free medium for 4 days after co-cultivation (Fig. 4.26C). After 4 days, explants were
Fig. 4.26: Genetic transformation of *Brassica juncea* cv. Varuna using moth bean lectin gene (*MbL*)

(A) Differentiation of shoots from cotyledonary node after one month of culture on regeneration media
(B) Stem segments on preculture media
(C) Co-cultivated stem segments on SRM
(D) Shoot differentiation from transformed stem segments on selection media after two months
(E) Rooting of transformed shoots
(F) Hardening of putative transformants
(G) Plants in phytotron
(H) Plants in flowering stage
(I) Seed setting

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transferred on SRM having cefotaxime (250 mg/l) and Kanamycin (25 mg/l) (Fig. 4.26D). Creamish-green callus started differentiating into green, purple or white colour multiple shoots on selection medium after 10-15 days. Explants were subcultured onto fresh selection medium of the same type at 20 days intervals. The transformation frequency was calculated as the percentage of total number of kanamycin selected shoots out of total number of explants transferred on selection medium. An average of 52.26% transformation frequency was achieved from 5 independent batches (Table 4.1). These results further confirm high transformation frequency of stem segments as reported by Sharma M. et al., 2004 in case of *Brassica juncea* cv. Pusa jaikisan. Shoots of 3 to 4 cm in length surviving on selection medium were then transferred to root induction medium consisting of MS salts supplemented with, 0.2 mg/l indole acetic acid solidified with 0.8% w/v agar and containing 25 mg/l Kanamycin and 250 mg/l cefotaxime. Upto 70% success was achieved in rooting (Fig. 4.26E). After proper acclimatization & hardening (Fig. 4.26F), fully developed plantlets (Fig. 4.26G) were transferred to National Phytotron Facility, IARI, New Delhi (Fig. 4.26H).

### Table 4.1: Transformation of *Brassica juncea* cv. Varuna

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>No. of explants in co-cultivation medium</th>
<th>No. of explants survived in selection medium</th>
<th>Total No. of shoots developed</th>
<th>Total No. of green shoots developed</th>
<th>Transformation frequency %</th>
<th>Putative transgenic plants transferred in rooting media</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>85</td>
<td>63</td>
<td>108</td>
<td>35</td>
<td>55.55</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>89</td>
<td>52</td>
<td>170</td>
<td>25</td>
<td>48.07</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>95</td>
<td>45</td>
<td>205</td>
<td>27</td>
<td>60.00</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>93</td>
<td>70</td>
<td>148</td>
<td>35</td>
<td>50.00</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>102</td>
<td>85</td>
<td>96</td>
<td>50</td>
<td>58.82</td>
<td>35</td>
</tr>
</tbody>
</table>

Results and Discussions...
4.6.3 Molecular analysis of transformants

4.6.3.1 PCR and RT-PCR of transformed plants

Since antibiotic selection was found to give a significant number of false positive transformants under the tissue culture conditions used, transgenic plants were screened for the presence of the *nptII* gene (marker gene present in the binary vector) & lectin gene by PCR.

**Fig. 4.27**: PCR analysis of transformed *Brassica juncea*

(A) T0 plants with *nptII* primers: Lane M: 1 kb Ladder, Lane +ve: Positive control (plasmid DNA), Lane -ve: Negative control (non-transgenic), Lane 3-12: Transgenic mustard

(B) T0 plants with gene specific primers: Lane M: 1 kb Ladder, Lane +ve: Positive control (plasmid DNA), Lane -ve: Negative control (non-transgenic), Lane 3-12: Transgenic mustard

(C) T1 plants with *nptII* primers Lane M: 1 kb Ladder, Lane +ve: Positive control (plasmid DNA), Lane -ve: Negative control (non-transgenic), Lane 1-5: Transgenic mustard

(D) T1 plants with gene specific primers, Lane M: 1 kb Ladder, Lane +ve: Positive control (plasmid DNA), Lane -ve: Negative control (non-transgenic), Lane 1-5: Transgenic mustard

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Genomic DNA of putative transformants growing at phytotron was isolated by using CTAB method. PCR analysis of T0 and T1 transformants using gene specific primers and primers specific for \textit{npt}II gene showed expected band size of ~750 bp from \textit{npt}II gene and 843 bp from gene specific primers corresponding to the size of cloned lectin gene (Fig. 4.27A, B, C& D). No amplification was observed in the untransformed control. Total RNA from the leaves of putative transformants and untransformed control was analysed for the presence of lectin transcript. For this, total RNA was isolated from the leaves of putative transformants of T0 and T1 by using Trizol method (Fig. 4.28).

\textbf{Fig. 4.28:} (A) Total RNA of transgenic plants\textbf{T0:} Lane M: RNA Ladder, Lane C: Negative control (non-transgenic), \textbf{Lane 1}-\textbf{9:} Transgenic mustard

\textbf{(B)Total RNA of T1 transgenic plants:} Lane M: RNA Ladder, Lane C: Negative control (non-transgenic), \textbf{Lane 1}-\textbf{5:} Transgenic mustard

Molecular analysis through RT-PCR showed the presence of transgene in to the plant genome and its expression at different levels in different transgenic lines of T0 and T1 plants (Fig. 4.29).
Fig. 4.29: RT PCR Analysis

(A) **T0 plants with gene specific primers:** Lane M: 1 kb Ladder, Lane C: -ve control (non-transgenic), Lane 1-9: transgenic mustard

(B) **T0 plants with actin primers:** Lane M: 1 kb Ladder, Lane C: -ve control (non-transgenic), Lane 1-9: transgenic mustard

(C) **RT PCR Analysis of T1 plants with gene specific primers:** Lane M: 1 kb Ladder, Lane C: -ve control (non-transgenic), Lane 1-9: transgenic mustard

(D) **RT PCR Analysis of T1 plants with actin primers**
Lane M: 1 kb Ladder, Lane C: -ve control (non-transgenic), Lane 1-9: transgenic mustard

### 4.6.4.2 Southern hybridization of transformed plants

In order to ensure the insertion of the *MbL* gene into the *Brassica* genome and determine the number of the integration sites (or loci) in each independent transgenic line, the genomic DNA of the transgenic plants that were *nptII* positive was digested with *EcoRI* and hybridized with a radiolabelled 0.8 kb fragment excised by *KpnI*-*XbaI* enzymes containing the *MbL* gene. Positive control as well as DNA from 4 independent transformed lines of T0 showed stable integration of gene and 5 plants of T1 transformants (Fig. 4.30). Further, it is known that *Agrobacterium* mediated transformation of explants often results in insertion of multiple copies of the transgene (Hobbs et al. 1993) however, in the present investigation Southern analysis of T0 and T1 plants showed the presence of one band that hybridized with the probe, suggesting the integration of the *MbL* gene at one positions in the genome (Fig. 4.30). In Fig. 4.30

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(B) Lane 2 is showing a higher molecular weight band, this may be due to additional fragment of genomic DNA in addition to the lectin gene cloned. This happens sometimes due to methylation of the restriction sites and DNA is cut at some other site giving higher fragment of DNA.

Fig. 4.30: Southern analysis of plants
A. Restriction analysis of genomic DNA of different transgenic T0 plants
B. Southern hybridization of T0 transgenic *Brassica* genomic DNA with moth bean lectin gene probe
Lane +ve: Positive control (plasmid), Lane –ve: Negative control (non-transgenic), Lane 1-4: Transgenic mustard
C. Restriction analysis of genomic DNA of different transgenic T1 plants
D. Southern hybridization of T1 transgenic *Brassica* genomic DNA with moth bean lectin gene probe
Lane +ve: Positive control (plasmid), Lane –ve: Negative control (non-transgenic), Lane 1-5: Transgenic mustard

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4.6.4.3 Northern hybridization

The RNA blot results (Fig. 4.31) using the same probe revealed that the transgene expressed at least in three transformed plants. All the three transformed plants showed a signal at about 850 nt which fairly correlates with the mRNA size of moth bean lectin (Roberson and Strength 1983). Transgenic plants have been used as tools for studying regulation and organization of plant genes (Topfer et al., 1987; Schell, 1987). The present study also shows variation among individual transgenic plants of Brassica juncea in expression of MbL gene. The size of the transcript in all the three transformed plants was about 850 bp. The results clearly indicate that coding regions of legume lectins are fairly small and devoid of any introns.

Fig. 4.31: Northern analysis of T1 plants
(A) Total RNA of different transgenic lines
Lane M: RNA ladder, Lane C: -ve control (non-transgenic)
Lane 1-3: transgenic mustard
(B) Northern hybridization of transgenic Brassica with MbL gene probe

4.6.4.4 Segregation analysis

To develop a rapid and efficient Agrobacterium-mediated transformation method the action of different concentrations of antibiotics that are currently used as selectable markers in plant transformation procedures was assayed. Plants that were transferred to glasshouse grew and flowered normally and set copious seeds like non-transformed plants. T0 seeds obtained from the self pollination of stable primary transformants were surface sterilized with 0.2% w/v HgCl₂ and allowed to germinate on ½ strength MS medium containing 100 mg/l Kanamycin (Table 4.2). Kanamycin 50...
mg/l blocked regeneration from untransformed explants (Fig. 4.32A) as shown by Ahmed et.al 2007.

Fig. 4.32: Segregation Analysis of T1 seeds

(A) Non-transgenic seeds grown on selection media
(B) Transgenic seeds grown on selection media

Segregation analysis showed definitive transgene inheritance in the next generation in a 3:1 Mendelian fashion in certain lines (Fig. 4.32B & Table 4.2). This also confirmed by single copy insertion as revealed by Southern blot analysis. Non-germinated seeds and brown seedlings were considered as sensitive (S), whereas 10 days old green seedlings were scored as resistant (R). The surviving green plantlets were transferred to soil and grown in the greenhouse for further development. Segregation and inheritance of the MbL gene in randomly selected T1 plants is shown in Fig. 4.33.

Table 4.2: Segregation Analysis for the kanamycin resistance trait in T1 progeny of some selected transformed lines of Brassica juncea cv. Varuna

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Plant line</th>
<th>No. of seeds inoculated</th>
<th>Kanamycin resistant-seedlings</th>
<th>Kanamycin susceptible seedlings</th>
<th>Segregation-ratio</th>
<th>Chi-square</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>60</td>
<td>-</td>
<td>60</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>VT₄</td>
<td>57</td>
<td>42</td>
<td>15</td>
<td>2.8:1</td>
<td>0.052</td>
</tr>
<tr>
<td>3</td>
<td>VT₉</td>
<td>63</td>
<td>47</td>
<td>16</td>
<td>2.9:1</td>
<td>0.005</td>
</tr>
<tr>
<td>4</td>
<td>VT₁₂</td>
<td>50</td>
<td>37</td>
<td>13</td>
<td>2.8:1</td>
<td>1.125</td>
</tr>
<tr>
<td>5</td>
<td>VT₂₇</td>
<td>68</td>
<td>51</td>
<td>17</td>
<td>3:1</td>
<td>0.00</td>
</tr>
<tr>
<td>6</td>
<td>VT₃₂</td>
<td>62</td>
<td>47</td>
<td>15</td>
<td>3.1:1</td>
<td>0.02</td>
</tr>
</tbody>
</table>
4.6.5 Bioassay

Lectins have been suggested as one of the promising agents against insects pests and have been engineered successfully into a variety of crops including rice, wheat, tobacco & potatoes. This approach could be used as a part of integrated pest management strategies and caveat pest attack (Sze Kwan Lam & Tzi Bun Ng, 2011). Plant lectins have been reported to have resistance factors against a variety of insect pests. When lectins were added in artificial diet or expressed transgenically in plant systems, they were shown to resist the attack of the sap-sucking insects including aphids, brown plant hopper, and green leafhopper (Dutta et. al. 2005, Wu. et al. 2006, Sadeghi et al 2007). Lipaphis erysimi, commonly known as mustard aphid is a seriously damaging pest of important oilseed crop, Brassica juncea. To confirm resistance developed in transformants against mustard aphid (Lipaphis erysimi) insect bioassay was done on transformants expressing moth lectin gene as described in 3.2.6.2.6. The results of the plant bioassay experiment are presented in Table 4.3.

Bioassays of T0 plants exhibited the efficacy of expressed lectin gene to reduce the survival ability of L. erysimi. These results supports earlier reports, where bioassay by using leaf discs showed that feeding on transgenics expressing chitin-binding lectin from wheat germ induced high mortality & significantly reduced fecundity of aphids (Kanrar et. al. 2002). To study the insecticidal activity of moth lectin expressed in

Fig. 4.33: T1 plants in phytotron facility

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transgenic plants the survival of aphid was monitored with second instar nymphs collected from field of IARI (Fig. 4.34A) at an interval of 24 h on T0 plants. Untransformed plants were used as control (Fig. 4.34B) and placed in germinator (Fig. 4.34C). Difference in insect survival became apparent within 7 days.

These findings are in confirmation by reports of Dutta et.al. (2005) where efficacy of Allium sativum leaf lectin was proved by In-planta bioassays on T0 plants & their progenies resulted in declined mean survival percentage of aphids to 18.66±0.86% (Mean±SE) over 9 days whereas Sadeghi et. al. 2007 demonstrated that the transgenic tobacco plants expressing Garlic leaf lectin (ASAL) & bulb lectin (ASAI) had a significant effect on the reproduction capacity of the resulting adults with a reduction upto 40%.

Mortality percentage of aphids increased from 2.67% in control to 14.67% in transgenic plants. VT0 and VT27 line of T0 plant showed maximum insect mortality of 14.67% (Table 4.3).
Table 4.3: Bioassay of transformed plants

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Plant line</th>
<th>No. of Aphids inoculated</th>
<th>No. of surviving aphids</th>
<th>No. of dead aphids</th>
<th>Mortality percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>75</td>
<td>73</td>
<td>2</td>
<td>2.67</td>
</tr>
<tr>
<td>2</td>
<td>VT₄</td>
<td>75</td>
<td>66</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>VT₉</td>
<td>75</td>
<td>64</td>
<td>11</td>
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<tr>
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<td>VT₁₂</td>
<td>75</td>
<td>68</td>
<td>7</td>
<td>9.34</td>
</tr>
<tr>
<td>5</td>
<td>VT₂₇</td>
<td>75</td>
<td>64</td>
<td>11</td>
<td>14.67</td>
</tr>
<tr>
<td>6</td>
<td>VT₃₂</td>
<td>75</td>
<td>67</td>
<td>8</td>
<td>10.67</td>
</tr>
</tbody>
</table>

As the full sequence of the *MbL* gene is now known, this gene can now be used to construct several expression cassettes for plant transformation. This gene in combination with other insecticidal genes can be introduced into crops to produce stable homozygous lines with resistance to a wide range of insects (Chrispeels and Raikhel, 1991). Being of plant origin, lectin genes have high degree of compatibility with the metabolic system of transgenic host plants and are expected to give sustained protection against sap sucking insects. The present study clearly demonstrates the efficient transfer of foreign genes into *Brassica juncea* using *Agrobacterium tumefaciens* mediated transformation. The present process of transformation is simple and rapid. Selection of appropriate explant and choice of proper *Agrobacterium tumefaciens* strain with suitable media and hormone combinations are the major determinants for the success of any transformation and regeneration system. Based on the data of the present study we can conclude that the reported transformation protocol have high transformation efficiency, and can be easily used to regenerate transgenic *Brassica* plants expressing the genes of our interest. The findings thus pave a way to transfer other agronomically important genes, which are otherwise difficult to transfer via the conventional breeding methods. The development of such transgenic crop plants will be very useful for sustainable agriculture system. It is expected that the transgenic plants with insecticidal proteins like lectins alone or in combination with other insecticidal genes will be a cost-effective option to defend our crop plants against sap-sucking insects and increase the crop productivity.