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Ectopic Expression of the *Leptochloa fusca* and *Allium cepa* Lectin Genes in Tobacco Plant for Resistance against Mealybug (*Phenococcus solenopsis*)

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Abstract

Insecticidal toxicity of the *Allium cepa* and *Leptochloa fusca* lectin genes was tested against sucking insect pest mealybug (*Phenococcus solenopsis*). Both genes were cloned under *2XCaMV35S* promoter and transformed into tobacco plants using *Agrobacterium* mediated plant transformation method. Integration of the lectin genes was confirmed by southern hybridization and expression of the transgene was confirmed by real time PCR. Leaves of the transgenic plant were used for insect bioassays under laboratory and glasshouse conditions. In the case of laboratory experiment, the mealybugs could not survive or multiply on leaves detached from both types of the transgenic tobacco plants. The insect mortalities 87.50% and 90% were observed on the transformed and non-transformed plants respectively. Similarly, in the case of greenhouse experiment, 81.25% and 87.5% insect mortalities were recorded when nymphs of the mealybugs were released to feed on the alive transgenic and non-transgenic tobacco plants. These results suggest that lectin genes can be effectively transformed in the major for the control of mealybugs.

Keywords: Lectin; *Allium cepa*; *Leptochloa fusca*; *Phenococcus solenopsis*; Insect resistant transgenic crops

Introduction

Sucking insect pests like aphid, jassid, whitefly and mealybug are major threats for the economically important crops. Sucking insect pests attack the phloem and suck phloem sap. The phloem sap contains all essential nutrients like sugar, proteins and amino acids that are accumulated in high concentrations [1]. Mealybug (*Phenococcus solenopsis, Hemiptera*, *Pseudococcidae*) is one of the significant sucking pests that feed on phloem tissues. During last few years, mealybug has become major insect pest of tobacco, tomato, ladyfinger, cotton and many other important field crops. In Pakistan, mealybug for the first time was reported on cotton and other important crops in 2005 [2]. Mealybug crawls and reaches to the growing tips of plants to sucks the sap from tender leaves [3]. It is a polyphagous insect that feeds on various plants species including vegetables, field crops and fruits. Mealybus also attacks on ornamentals plants of the *Leguminaceae*, *Malvaceae* and *Solanceae* families [4,5]. In addition to sucking the phloem sap, mealybugs secret honeydew that induces fungal (black sooty mold) contaminations [6] and disturb plant photosynthesis activity [7-9] During off-season, it survives on alternate hosts [10]. Mealybugs cause injury, curling, drying of leaves and drastically decrease the crop yield. These insects have ability to build populations within shoots and apexes. Mealybugs have waxy secretions on their body surface that's why it is very difficult to control with foliar applications of pesticides [11]. Due to presence of natural enemies and predators of mealybugs, serious problems had not reported in the countries of their origin. The sever epidemics were often reported when mealybugs had introduced to new locality in the absence of their natural enemies [12,13]. These insects not only cause damage to crops but also act as vector for various plant viruses [14,15]. The use of some biotechnological approaches like *Bt* has been successful in controlling chewing pests like bollworms [16] but so far, no effective control has been identified for sucking insects like mealybug. There is an urgent need to test various *B.t* d-endotoxins, VIPs, proteinase inhibitors, lectins, etc for their toxicity to insect pests [17]. Novel insecticidal toxin genes have been used for insect control strategy [18]. The Mannose binding plant lectins have been reported as promising toxins to control insect pests of *Hemipteran* order due to their insecticidal activity as well as their complementation to protease inhibitors and *Bt* toxins [19]. Plant Lectins are carbohydrate (well defined sugars) binding proteins that bind glycolipids, glycans of glycoproteins, or polysaccharides with great affinity [20]. Lectins probably inhibit the absorption of nutrients by coming in contact with glycoproteins of the intestinal tracts of insect/predators [21,22]. Plant lectins are considered significant against sap sucking insects. Lectin expressing transgenic plants even expressing partial resistance against a sap-sucking insect pest [23] can be useful in designing strategies for integrated pest management [24]. When lectins are transformed, the transgenic plants develop adequately effective resistance against sap sucking insect pests [25-28]. Such resistance in transgenic plants against sucking insect would find acceptance in agriculture sector and could be used in integrated pest management [23,29]. Plant lectins have been reported to have insecticidal activity against a wide range of insect orders including *Coleoptera, Diptera, Homoptera* and *Lepidoptera* [30]. Some plant lectins are potent toxins (potential anti-insect compounds) that bind to the midgut surface of the insect, cross epithelium and pass into the hemolymphs of a range of insects [31]. Among different lectins, *ASAL*, a mannose binding lectin from *Allium sativum* was reported very effective against sap sucking insect pests i.e., Cowpea aphid (*Ahis craccivora*) [32]. *A. sativum* leaf agglutinin (*ASAL*) lectin gene from garlic plants has been tested to develop resistance against sap-sucking insect pests like white backed plant hopper (WBPH), brown plant hopper (BPH) and green leaf hopper (GLH) [33]. Expression of the *Allium sativum* leaf lectin (*ASAL*) gene reduced the population of sap sucking mustard aphid (*Lipaphis*

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erysimi) [27]. Transgenic plants expressing three monocot mannose binding lectin genes garlic *Allium sativum* L leaf agglutinin (*ASAL*), *Glanthus nivalis* L agglutinin (*GNA*), onion *Allium cepa* L agglutinin (*ACA*) and fusion lectin of *ASAL* and *ACA* genes were tested against mustard aphid which is a major sap sucking pest of oilseed crops [34]. Ectopic expressions of *ACA* and fusion proteins in mustard plants developed resistance against aphids in transgenic mustard and *ACA* was found more efficient than *ASAL* and *GNA* against mustard aphid [34]. *Galanthus nivalis agglutinin* or *GNA* was first lectin reported to be found against sucking pests at developmental stages [35]. Studies on transgenic potato, rice, tobacco, tomato, and wheat plants have revealed that ectopically expressed *GNA* have deleterious effects on leaf hoppers, plant hoppers and aphids [14,36-38]. Although lectins are naturally present in many crop plants like wheat, rice, potato, tomato, soybean, grasses and beans but they express at low level under their natural promoters. Their expression can be enhanced by expressing them under constitutive double promoters like *2XCaMV35S* and transferred into economically important crops. In view of these objectives, present study was designed to test the toxicity of *Allium cepa agglutinin* (*ACA*) and *Leptochloa fusca agglutinin* (*LfA*) lectin genes under the control of *2XCaMV35S* promoter in tobacco plants for control of sap-sucking insect pest mealybug (*P. solenopsis*) and their potential applications in developing insect resistance in economically important transgenic crops like cotton.

Materials and Methods

Plasmid gene constructs

Lectin genes were amplified from onion (*Allium cepa var. Phulkara*) and kallar grass (*Leptochloa fusca* L, Local land race) cDNAs library. Fresh leaves of onion were used for isolation of total RNA by Trizol® method (MRC, Cat # TR118). The first strand of cDNA was synthesized by using oligo dT primers (revert Aid H- cDNA synthesis kit, Fermentas®, Cat # K1631). The *Allium cepa agglutinin* lectin gene (*ACA*) was amplified through reverse transcriptase PCR by using lectin gene specific primers (forward: 5'-GGCAAGCTTATGAGAAACGTATTGGTGAA-3' and reverse 5'- GGCGAATTCTCATTTCCTGTACGTACCAGTAGACCA-3') designed from the known sequence of *Allium cepa* (Database ID: L12171) with additional *Hind* III and *Eco*R1 restriction sites at 5' and 3' ends respectively. The following PCR profile was used: denaturation at 94°C for 5 min (cycle 1) followed by denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min (35 cycles). Final extension was performed at 72°C for 10 min (1 cycle). The PCR product was resolved on 1% agarose gel and desired PCR amplicon was eluted from the agarose gel using JET quick gel extraction spin kit (GENOMED®, Cat # 420050). The eluted PCR product was cloned in T/A cloning vector pTZ57R/T (Fermentas®; Cat # K1214).

The *Leptochloa fusca agglutinin* lectin (*LfA*) gene was amplified from *Leptochloa fusca* cDNA library [39] by using a set of primers as given above. Plasmid DNA was isolated (Gene Jet™ plasmid miniprep kit; Fermentas®, Cat # K0503) and confirmed by sequencing from Macrogen, Korea (www.macrogen.com). The *LfA* gene sequence was also confirmed by similarity searches at ClustalW and submitted at NCBI GenBank (Accession # JF342239). Both lectin genes were sub-cloned in plant expression vector pGreen0029 under *2XCaMV35S* promoter (Figure 1). These new recombinant clones were named as *pLec1* and *pLecII* respectively. Recombinant clones were transformed in *E. coli* and Plasmid DNA was isolated and stored at -20°C.

Tobacco plant transformation

Two plant transformation vectors; *pLec1* and *pLecII* harboring *ACA* and *LfA* lectin genes cloned under *2X35S* promoter were transformed in *Agrobacterium* strains LBA4404. The tobacco (*Nicotiana tabacum L.* cv. Samson and Spade 28) leaf discs were used as explant for *Agrobacterium* mediated plant transformation. Seed were sterilized and grown on MS medium [40] in jars under control condition. Leaf discs were inoculated with recombinant *Agrobacterium* cultures and placed at 25 ± °C on co-cultivation medium containing 1 mgL^{-1} BAP. After 2 days leaf discs were transferred to regeneration and selection medium containing 1 mgL^{-1} BAP, 50 mgL^{-1} Kanamycin, 250 mgL^{-1} Cefotaxime and 0.1 mgL^{-1} NAA. Regenerated plantlets were shifted to jars containing MS_0 for the development of roots. Plantlets having well developed roots were transferred to earthen pots. Leaf tissues of putative transgenic tobacco plants were used for DNA extraction by CTAB method [41]. DNA was used as template for PCR amplification of transgenic tobacco plants.

Molecular analysis of transgenic plants

Expression of the lectin genes in transgenic tobacco plants was determined through real time PCR. Total RNA was isolated from meristematic leaves of transgenic tobacco plants. Total RNA was used for synthesis of cDNA. SYBER Green chemistry was employed for real time PCR through standard curve method and curve was obtained from the serial dilution of reference plasmid, recombinant plasmid having lectin gene [42]. The data was analyzed by the BIO-RAD iCycler software (version 3.06070). The reaction mixture consisting of 2 ng/ul cDNA of each transgenic and non-transgenic plant in 96-well optical plate was used. All reactions were performed in triplicate using iCycler iQ5 Real Time PCR machine (BIO-RAD, USA). The PCR profile was used as: 94°C for 5 min at cycle 1, 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec for 35 cycles followed by a melt curve analysis. Cloned reference gene (serially diluted recombinant plasmid having lectin) was also employed on the same plate to produce the appropriate standard curve for relative quantification.

Characterization of transgenic plants for mealybugs resistance

Insect bioassays under laboratory conditions: Mortality of mealybug was analyzed on leaves of transgenic tobacco plants to perform insect bioassays at the laboratory scale. For that purpose mealybugs were reared in the glasshouse in cages to be used in subsequent experiments. Leaves from transgenic and non-transgenic (wild type control) tobacco plants were placed in petri plates on clammy blotting paper to keep them fresh and suitable to be consumed by mealybugs. Four 2nd instar nymphs of mealybug were placed on each leaf of transgenic and control tobacco plants. Insect mortality was documented after every 24 hours up to 240 hours after releasing into petri plates.

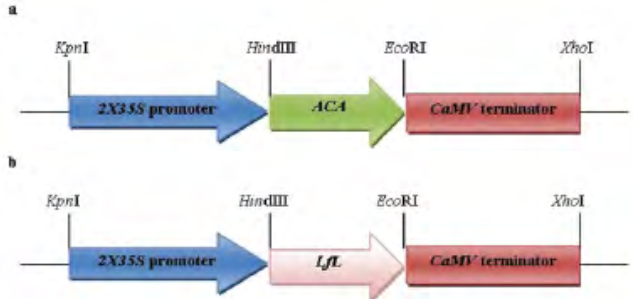

Figure 1: a) Map of *pLecI* vector consisting of *Allium cepa* lectin gene (*ACA*) cloned under *2XCaMV35S* promoter in pGreen0029 plant expression vector. b) Map of *LecII* vector consisting of *LfA* lectin gene cloned under *2XCaMV35S* promoter in pGreen0029 plant expression vector.

Insect bioassays under glasshouse conditions: Insect bioassays under glasshouse conditions were accomplished with mealybug on transgenic tobacco plants. Transgenic and wild type control tobacco plants were kept in separate woody cages of 2 feet (wide) × 2 feet (Length) × 3 feet metric units (Height) size. Ten 2nd instar nymphs of mealybugs were placed on each transgenic and wild type control tobacco plant in separate cages. The data of insect mortality was recorded from 24-240 hours of exposure after every 24 hours. To statistically analyze the mortality data chi-square test was implemented to monitor significance or insignificance of difference in insect mortality on transgenic and wild type plants in each combination.

Results

Production of transgenic tobacco plants

Out of the 100 inoculated leaf discs in three different batches, 24 independent transgenic lines were produced. The regenerated plants grew normally, survived on selection medium and attained maturity. Four to five transgenic plants from each clone were subjected to PCR analysis and insect bioassays.

Molecular verification of transgenic plants

For confirming the integration of inserted lectin gene in transgenic tobacco plants, PCR analysis was performed by using a pair of primers as described earlier. The PCR amplified product was resolved on 1% agarose gel and visualized under UV light. Expected PCR product of ~335 bp of *ACA* and *LfA lectin* genes were confirmed in transgenic plants (Figure 2). The results of the southern blot analysis showed successful integration of the transgenes into tobacco plants. The Transgenic tobacco plants expressing onion lectin gene under 2X35S promoter indicated that lane 4 have two insertions of the transgene while Lanes 1, 2 and 3 showed insertion of transgene at single loci.

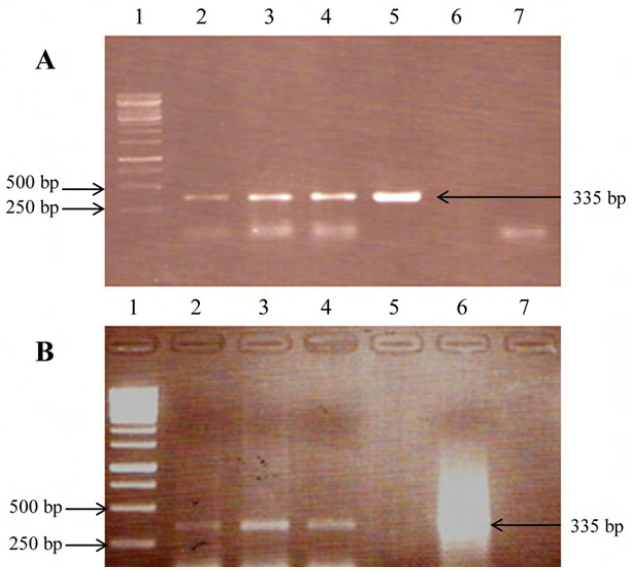

Figure 2: A) Confirmation of the transgenic tobacco plants expressing *ACA* lectin gene under *2XCaMV35S* promoter by PCR. Lane 1 represents 1kb DNA ladder. Lanes 2-5 represent amplified *lectin* gene from transgenic plants. Lane 6 and 7 represent negative positive control respectively. B) Confirmation of the transgenic tobacco plants expressing *LfA* lectin gene under *2XCaMV35S* promoter by PCR. Lane 1 represents 1kb DNA ladder. Lanes 2-4 represent amplified bands from transformed plants containing *Leptochloa fusca lectin* gene. Lane 5 and 6 represents negative and positive control respectively. Lane 7 represents empty well.

Transgenic plants expressing kallar grass lectin gene under 2X35S promoter indicated that lane 8 have two insertions of transgene, while lanes 5, 6 and 7 showed single insertion of transgene (Figure 3).

The results of real time PCR confirmed the overexpression of *ACA* lectin gene in various transgenic tobacco plants under 35S promoter. The maximum level of expression was recorded in transgenic line 2 (0.0018 ngμg^{-1} RNA) followed by 0.000854 ngμg^{-1} RNA, 0.000546 ngμg^{-1} RNA and 0.000208 ngμg^{-1} RNA in transgenic lines 1, 4 and 3 respectively. No expression of *lectin* gene was observed in control (wild type) tobacco plants (Figure 4A). Expression of *LfA lectin* gene was also confirmed in different transgenic tobacco lines. The highest level of expression (0.00098 ngμg^{-1} RNA) of *LfA lectin* gene was verified in line 2, followed by 0.00057 ngμg^{-1} RNA and 0.00038^{-1} ngμg^{-1} RNA in transgenic line 1 and 4 respectively. Minimum expression level (0.00026 ngμg^{-1} RNA) of *LfA lectin* gene was detected in line 3. While in case of wild type control plants no expression of *LfA lectin* gene was recorded as shown in Figure 4B.

Insect bioassays

Detached leaf method: Bioassay on leaves of transgenic tobacco plants expressing *ACA* lectin gene showed resistance against mealybugs. More than 87% insect mortality was observed on transgenic tobacco leaves up to 240 hours (Table 1; Figure 5A). Transgenic plant leaves expressing *LfA* lectin gene also showed resistance against nymphs of mealybug and about 81% insect mortality was recorded till 240 hours (Table 1; Figure 5B). No mortality of mealybug was recorded on leaves of non-transgenic tobacco plants and insects continued multiplication and feeding (Table 1; Figure 5C).

Following formula given by Schneider-Orelli was used to calculated corrected insect mortality:

$$Corrected(\%) = \frac{Mortality\ (\%)\ in\ transgenic\ plants\ -\ Mortality\ (\%)\ in\ Wild\ type\ plants}{100 - Mortality\ (\%)\ in\ Wild\ type\ plants}$$

Attach leaf method: Transgenic tobacco plants having *ACA* lectin gene driven by 2X35S promoter exhibited high level of resistance and about 90% insect mortality was observed in various transgenic

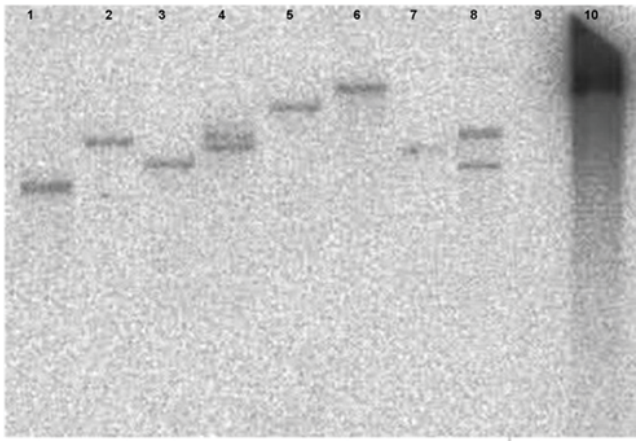

Figure 3: Confirmation of the transgenic tobacco plants by Sothern blot analysis. The lanes 1-3 indicate *ACA* expressing transgenic tobacco plants (*ACA*-T1 to *ACA*-T3) having single gene copy, the lane 4 indicates *ACA*-T_4 plant have two insertions of the transgene. The lanes 5-8 indicate transgenic plants expressing *LfA lectin* gene. The lanes 5-6 indicate single gene insertion in transgenic plant *LfA*-T1, *LfA*-T2 and *LfA*-T3 while lane 8 indicates double gene insertion in *LfA*-T4 plant. The lanes 9 and 10 indicate negative and plasmid control.

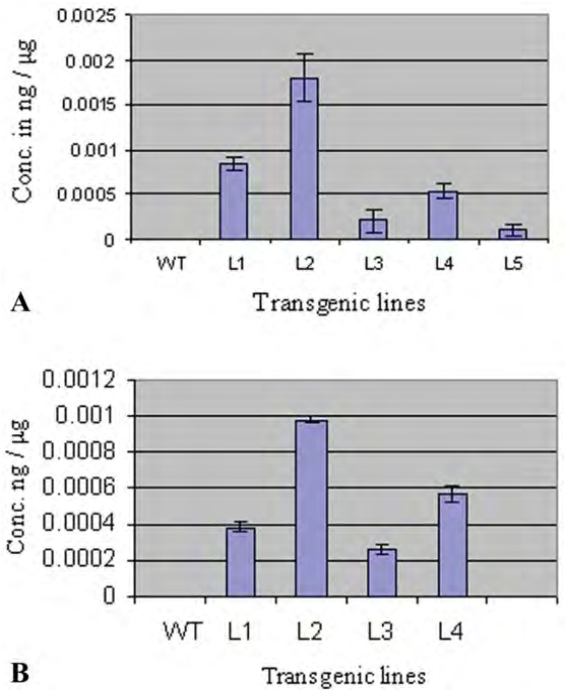

Figure 4: Expression analysis lectin genes determine by qPCR. The experiments were performed with three repeats. A) Expression analysis of the *ACA* lectin gene under *2XCaMV35S* promoter B) Expression analysis of the *LfA lectin* gene under 2XCaMV35S promoter.

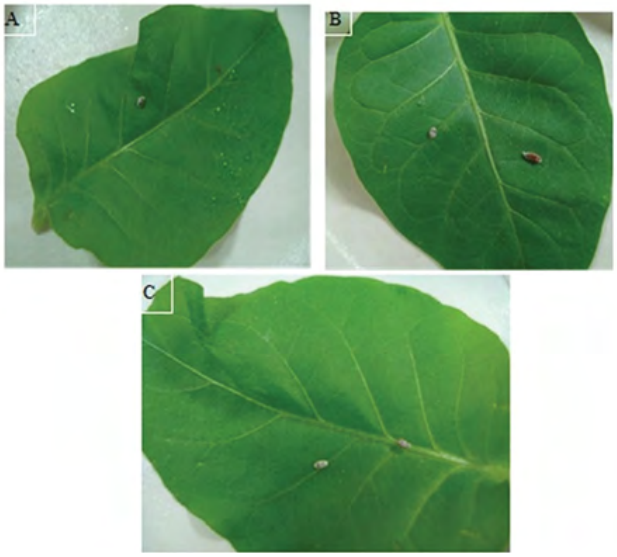

Figure 5: Insect bioassays with mealybug (*Phenococcus solenopsis*) on detached leaf of transgenic tobacco plants: A) Detached leaf from transgenic tobacco plants expressing *ACA lectin* gene under *2XCaMV35S* promoter. B) Detached leaf from the transgenic tobacco plants expressing *LfA lectin* gene under *2XCaMV35S* promoter. C) Detached leaf from the non-transgenic WT plant.

lines for 240 hours of insect (Table 1; Figure 6A). Mealybugs placed on transgenic plants expressing *LfA lectin* gene under 2XCaMV35S promoter representing high resistance level and more than 87% insect mortality was noted in diverse transgenic lines up to 240 hours (Table 1; Figure 6B). No mortality was recorded on leaves of wild type (control) tobacco plants. Insects continued feeding and multiplying even after one month of exposure (Table 1; Figure 6C and 6D). Insect mortality was calculated as mentioned earlier.

Table 1: Average mortality percentage of mealybug released on detached leaves and lives transgenic tobacco plants expressing *Allium cepa* and *Leptochloa fusca* genes under *2XCaMV35S* promoter.

Treatment	Mortality % on detached leaves	Mortality % on live transgenic plants in green house
Control	0%	0%
2X35S+OLG	87.50%	90%
2X35S+KLG	81.25%	87.5.%

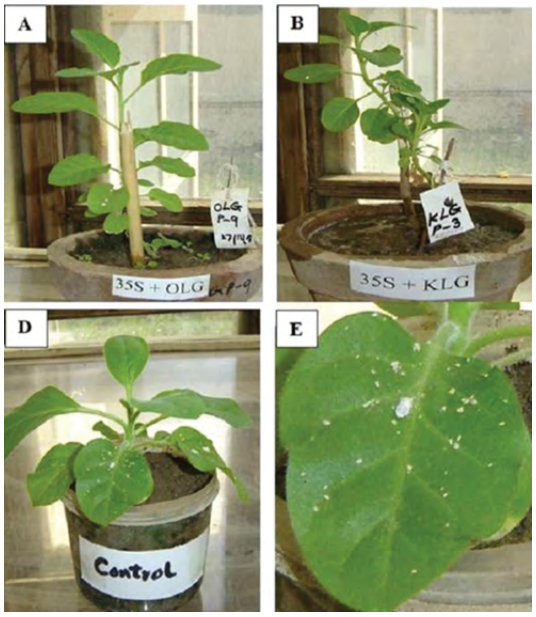

Figure 6: Insect bioassays with mealybug (Phenococcus solenopsis) on intact transgenic tobacco plants: A) Live transgenic tobacco plants expressing Allium cepa lectin gene (ACA) under 2XCaMV35S promoter. B) Live transgenic tobacco plants expressing LfA lectin gene under 2XCaMV35S promoter. C) Non-transformed tobacco plant D) Non-transformed tobacco plant after one month of exposure. Mealybugs grew and multiplied on these plants (Close up picture).

Statistical analysis: Chi-square analysis data indicated that there was significant difference in insect mortality among transgenic and non-transgenic tobacco plants at $P<0.05$ in case of both lectin genes tested on detached leaves and intact tobacco plants in cages.

Discussion

Insecticides are toxins are they cause acute and chronic effects on human health. Organophosphorous compounds cause inhibition of AChE enzymes through process of phosphorylation [43]. Alternative to chemical control of insect pests are the biotechnological applications. Many insecticidal proteins are available in nature which is effective against agriculturally important insect pests. A number of different novel insecticidal toxin genes isolated from diverse organisms are employed in plant transformation to develop insect resistance in transgenic plants. This includes introduction of genes conferring insect resistance in crops. Through transgenic approaches, agricultural crops may be saved from insect pests. The most common organism *Bacillus thuringiensis* (Bt) has been used as a bio-insecticide which codes for the insecticidal Cry proteins [44]. Another approach to insect

resistance is the expression of serine protease inhibitors. The transgenic tobacco plants producing high abundance of this protein conferred insect resistance [45]. Mannose binding plant lectin genes have been reported as good candicate to be used as insecticide [19]. The lectin genes have significant role as insecticidal [14,46], as antifungal [47] and as anti-viral [48]. Plant lectins are being used for the development of transgenic plants against sap sucking insect pests. The transgenic plants expressing lectin genes even with partial resistance are considered very effective against sap sucking insect [29]. Transgenic plants expressing insecticidal toxins were also tested against sap sucking insect mealybug. Mealybugs attack on cotton and other economically important crops. This problem still persists in the field especially in *B.t* cotton fields where farmers do not spray for bollworms. Management and chemical controls are expensive and difficult. Companies imported additional quantities of pesticides to cover the shortage. Mealybugs possess a waxy coating that protects them from insecticides and there is no known pesticide that can completely kill this insect. Mealybugs have a high reproductive rate and have the ability to hide in the soil and plant debris. Although some predators are known but their effectiveness is not sufficient to keep mealybugs under control. In this scenario, it is highly desirable to develop insect resistant genetically modified crops. Sucking insect pests target the phloem tissues and suck the cell sap. The *lectin* can be used for the control of sucking insects as use of other insecticidal toxins have not been significantly effective against sucking insect pest. The over expression of lectin gene in transgenic plants shows resistance against sucking insects [23] and could be effective in integrated pest management strategy [24]. Insertion of *lectin* gene in plant genome have been proved to be very effective against insect pest attack [26-28,49-51]. In another study, Hossain et al. [34] reported that use of *Allium cepa* L agglutinin (*ACA*) is more efficient against mustard aphid than *ASAL* and *GNA*. The *ACA* lectin gene showed higher activity than *ASAL* and *GNA*. Similarly Chakraborti et al. [32] reported the control of phloem sap-sucking *Hemipteran* insect *Aphis craccivora* by expressing *ASAL* lectin gene in chickpea under CaMV35S and RolC promoters. The bioassay of *A. craccivora* showed 22-42% decrease in their survival rate on transgenic plants as compared to wild type which exhibited more than 80% survival. They suggested that plant lectins are potent anti-feedant for sap sucking insect. This study also revealed that expression of lectin gene from *Allium cepa* and *Leptochloa fusca* in transgenic tobacco plants showed enhanced resistance against phloem sap-sucking insect *P. solenopsis* and adversely affect the growth and development of the insects [52,53].

The *Allium cepa* lectin (*ACA*) yield highest resistance in transgenic tobacco plants against sucking insect mealybug (*P. solenopsis*). Transgenic plants expressing *LfA lectin* under constitutive promoter also showed enhanced resistance against insects and more than 81% mortality was observed after 240 hours on detached leaves and transgenic plants. Lectins are naturally occurring in plants, possess biopesticidal properties and are more acceptable for generating insect resistance in transgenic crops. These are expected to be safe for mammals and humans. Lectins were tested in model tobacco system and gave encouraging results. These findings could be used for developing insect resistant economically important transgenic crops.

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Conflicts of Interest

This statement is to certify that all authors have seen and approved the manuscript being submitted. The manuscript is original, has not been published before, and is not being considered for publication elsewhere in its final form neither in printed nor in electronic format. All authors contributed significantly and are in agreement with the content of the manuscript and there is no conflict of interests with any one.

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