Over-expression of Trigonella foenum-graecum defensin (Tfgd2) and Raphanus sativus antifungal protein (RsAFP2) in transgenic pigeonpea confers resistance to the Helicoverpa. armigera

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Research Article

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Abstract

Pigeonpea is an important food legume crop cultivated in tropical and sub-tropical regions around the world wherein the Indian subcontinent accounting for over 90 % of global production. It is a rich source of protein and is an important component of a well-balanced diet for the majority of Indians. Its productivity is affected by many insect pests among which Helicoverpa. armigera is the most significant pest in producing severe yield loss. Non-availability of resistant genes in germplasm and constraints with traditional breeding induce the application of a genetic engineering approach to generate insect resistance in pigeonpea. Expression of plant defensins in various crops provided enhanced resistance towards a variety of pests and pathogens. In the current study, two defensins Trigonella foenum-graecum defensin 2 (Tfgd2) and Raphanus sativus antifungal protein 2 (RsAFP2) integrated by a linker peptide was transferred into pigeonpea as a fusion gene by Agrobacterium mediated transformation. Putative transgenic lines were confirmed through PCR and the promising lines were identified in the following generations based upon integration, expression and bioefficiency of the fusion gene.Leaf bioassay conducted against H. armigera larvae showed increased levels of insect resistance compared to the control, where six T2 plants were identified as superior lines showing less than 25 % of leaf damage.Our findings illustrates that Tfgd2–RsAFP2 fusion protein is efficient in imparting protection against the insect pest and the transgenic lines developed in this study could be used for further pigeonpea improvement projects.

Key Message

Over-expression of *Tfgd2-RsAFP2* fusion gene conferred enhanced insect resistance against *Helicoverpa armigera* in transgenic pigeonpea plants.

Introduction

Pigeonpea (*Cajanus cajan* (L.) Millisp.) is one of the significant versatile grain legume crop that serves as a backbone of poor farmers in tropical and sub-tropical regions of Asia, Africa and Latin America. Pigeonpea has social, economic and medicinal significance in developed countries (Ramdas etal. 2015). It is the second-largest pulse crop in terms of area and yield, following chickpea and is cultivated in 6.97 million hectares around the world, with yield and production of 724 kg per hectare and 5.05 million tonnes respectively (FAO STAT, 2016). On the other hand, pigeonpea possess a unique position in Indian agriculture as it accounts for over 71.5 % of the global yield, occupying an area of over 5.40 Mha and yield of 4.78 MT affording to 20.9 % of total pulse yield. In the year 2017, worldwide pigeonpea is cultivated mostly in 7.02 Mha in Asia, Latin America, Eastern & Southern Africa at an average production of 0.97 t/ha (FAOSTAT, 2017). It can be cultivated in different climatic conditions all over the year owing to its adaptive nature. This adaptability nature reduces the cost of cultivation, resulting in higher profits for marginal farmers (FAOSTAT, 2014). High protein content of pigeonpea makes it as a potential constituent of diet mainly among the Indian vegetarian population. Its seed contains 20 - 22 % protein, where sulphur containing amino acids such as methionine and cysteine are present in nearly three-fold

times higher than cereals (Srivastava, 2013). Additionally, seeds also consists of other important substances like crude fiber (1.2 - 8.1 %), lipids (0.6 – 3.8 %) and carbohydrates (57.3 – 58.7 %) (Sinha, 1977) etc. Global yield speculates the pigeonpea yield in India where its yield is declined over 0.70 tonnes/ha over the past few years. As it is primarily a rainfed crop, poor rainfall causes moisture loss leading to lesser yield. Irrespective of its major requirement, pigeonpea production has increased by only 1 % during the previous years. This resulted in a severe shortage of this pulse, primarily in India (Jhoshi et al. 2001).The primary reasons for their low yield are due to various abiotic and biotic stresses, as well as absence of proper crop management practices. *Helicoverpa armigera*, a lepidopteran pest, is amongst the most severe biotic stresses in pigeonpea cultivation (Ghosh et al. 2017; Choudhary et al. 2013). It is difficult to control this pest due to its high reproductive potential and potent migratory nature giving rise to yield loss of nearly 85 %. Its larvae attack green colored parts like pods, flowers and leaves of the plants causing significant loss of around 40 – 50 % and yearly yield loss of 400 million US $ globally (Kaur et al. 2016). The insect's broad host range, higher level of migration, random application of pesticides by farmers and insect's innate immunity in developing resistance against pesticides have attained this insect as a major pest (Tripathi et al. 2001; Vishwadhar et al. 2008). To control this, prime insect resistant pigeonpea varieties can be developed through traditional breeding methods, however it's not been successful because of the inconsistency with wild species and confined genetic diversity in the cultivated germplasm (Nene and Sheila 1990). Moreover, evaluating over 14,000 cultivated pigeonpea accessions has illustrated average or low rates of resistance against the pest (Reed and Lateef 1990; Rana et al. 2017). As a result, introducing the pod borer resistant trait is essential in pigeonpea crop development program. In recent times, plant genetic engineering techniques have shown promising results in overcoming this type of challenges.

In various plants, insect pest resistant genes have been successfully incorporated (Dunwell, 2000; Vasavirama K and Kirti PB 2013) to develop resistance. As a result, transgenic techniques for the generation of insect resistant varieties have become a viable option to the integrated pest management programme (Rao and Shanower 1999; Sharma et al. 2006). To incorporate the novel traits and to generate successful transgenic pigeonpea plants effective regeneration & transformation mechanisms are required (Nirmala Nalluri and Vasavi Rama Karri 2019; Sarkar et al. 2019; Yadav et al. 2016; Srivastava J 2013; Rao SK et al. 2008; Singh ND et al. 2002). There has already been some progress in the generation of pod borer tolerant pigeonpea and chickpea varieties enunciating insecticidal genes like *cry*1Ab, *cry*1E-C, *cry*1Ac and chimeric cry1AcF (Kaur et al. 2016; Ramu et al. 2012; Singh et al. 2018). But, there is insufficient evidence in substantiating the transgenic pigeonpea activities in respect of stability in gene expression and insect mortality rates (Ghosh et al. 2014). Apart from these early achievements, more promising events involving effectual toxic gene expression resulting in major effect on pod borer tolerant plants under natural conditions are needed.

Genetically modified crops were usually produced by using a single gene with enhanced insect, viral and fungal resistance (Shin et al. 2002; Khanna and Raina 2002; Horvath et al. 2003; Lentini et al. 2003; Zhu et al. 2012; Ghag et al. 2012). On the other hand, long - lasting antifungal resistance could be obtained by integrated generation of antifungal proteins with various ways of action (Jayaraj

and Punja 2007; Chen et al. 2009), which even needs the assimilation of different transgenes into the plant genome and their symphonious expression in transformed plants. There are several methods for developing genetically modified plants expressing different transgenes. One of the routinely preferred alternatives is to incorporate each transgene separately through different transformation activities and to cross the individual single transgene expressing lines (Bizly et al. 2000). One of the drawbacks of this method is that different transgenes in the succeeding lines were integrated at separate loci constraining the successive breeding. Additionally, this approach is not suitable to the many ornamental plants and fruit trees which are cultivated vegetatively. Francois et al. (2004) stated that polyprotein made of two specific proteins, viz *DmAMP1* isolated from *Dahlia merckii* seeds (Osborn et al. 1995) and *RsAFP2* isolated from seeds of *Raphanus sativus* fused with a linker peptide of IbAMP polyprotein precursor from *Impatiens balsamina* (Tailor et al. 1997) has been transferred into plants with good level of gene expression. Both *Tfgd2* and *RsAFP2* are efficient anti-fungal proteins belonging to the antimicrobial peptide family. *Tfgd2* was proved to show *in vitro* anti-fungal activity against plant pathogens like *Rhizoctonia solani* and *Fusarium moniliformae* (Olli et al. 2007). Likewise, *RsAFP2* was also proved as a potent anti-fungal protein and its constitutive expression showed increased resistance to *Altenaria longiceps* in tobacco (Terras et al. 1995) against *Alternaria solani* in tomato (Parashina et al. 2000) and antagonistic to fungi like *Rhizoctonia cerealis* and *Fusarium graminearum* in wheat (Li et al. 2011). Further, Vasavirama and Kirti (2013) expressed fusion gene made up of two defensins *Tfgd2* and *RsAFP2* in transgenic tobacco which showed disease and insect resistance against *Phytophthora parasitica* var. *nicotianae* & *Rhizoctonia solani* fungi and *Spodoptera litura* larvae. In view of these reports, an effort has been made to generate an insect resistant transgenic pigeonpea expressing the fusion gene made up of the two defensins *Tfgd2* and *RsAFP2* associated together with a linker peptide isolated from *Impatiens balsamina* seeds to acquire resistance against the pod borer *Helicoverpa. armigera*.

Materials And Methods

Preparation of *Agrobacterium tumefaciens* strain with fusion gene construct

The fusion gene (GenBank accession number: KF498667) (Vasavirama and Kirti 2013) consisting of *Tfgd2* and *RsAFP2* genes associated with each other by linker peptide sequence was cloned by directional cloning of 1.2 kb synthetic gene cassette at *Hin*dIII position of pBI121 vector. This fusion gene was controlled by CaMV35S promoter and nos terminator. pBI121 binary vector bearing synthetic gene construct was transferred to *Agrobacterium tumefaciens* (EHA 105 strain) through freeze thaw method. The disarmed rifampicin and kanamycin resistant *Agrobacterium* strain was cultured at 200 rpm till the OD reaches 0.6 - 0.8 and was further used for co-cultivation.

Generation of transgenic pigeonpea plants harboring the *Tfgd2-RsAFP2* fusion gene

The sterilized seeds of pigeonpea ICP 8863 cultivar were germinated on MS basal media (Murashige and Skoog 1962) and maintained under at 28 ± 1^{o} C for 16 h of light. Afterwards 7-day-old leaf petioles were

collected and utilized as explants for *Agrobacterium* mediated transformation and regeneration was done following the protocol of Nirmala Nalluri and Vasavi Rama (2019). The transformed regenerated plants were selected on MS media consisting of 50 mg/l kanamycin as a selective agent. Further, the rooted plantlets were transferred to 1:1 ratio of soil and vermiculate amalgam and were acclimatized in culture room and subsequently transferred to the green house. Afterwards, transgenes integration and expression in putative transgenic pigeonpea plants and segregation analysis of T_0 progenies was performed.

Segregation analysis of putative progenies harbouring *Tfgd2-RsAFP2* fusion gene

It is important to identify the inheritance pattern of the fusion gene in the T_0 progenies to find out the stability of the transgenes integrated. Based on the analysis, PCR positive putative transgenic lines were selected to analyze the segregation motif of the fusion gene by kanamycin sensitivity test. Selection was performed by inoculating the overnight imbibed seeds for 5 h in MS media consisting of 50 mg/l kanamycin and was later sown on autoclaved soilrite. After three weeks, germination response was noted in both sensitive and resistant T_1 seeds, where healthy seedlings were considered as kanamycin resistant (Kan^R) whereas the non germinated seedlings were considered as kanamycin sensitive (Kan^S). Segregation analysis for T_1 plantlets was done by $\chi 2$ test and further these plants were analyzed for transgenes integration, expression and insect resistance.

Molecular analysis T_1 and T_2 transgenic lines

PCR screening was done for kanamycin resistant T_1 transformants in order to find out the inheritance motif of the fusion gene integrated. For this study, genomic DNA from the tender leaves of kanamycin resistant (T_1) and control plants were isolated through CTAB method (Murray and Thompson 1980) and PCR analysis was conducted to check the amplification of the fusion gene using fusion gene specific primers under appropriate PCR conditions.

Southern analysis and RT PCR

To analyze the copy number by southern hybridization, 5-10 µg of genomic DNA was isolated from PCR positive T_1, T_2 and non-transgenic pigeonpea plants and were overnight digested with *HindIII* restriction enzyme. These restriction digested DNA fragments were separated on 1 % agarose gel electrophoresis and were transferred onto Hybond-N+ nylon membrane through capillary blotting. Further, the PCR amplified *nptII* fragment was radiolabeled employing α-32P-dATP probe. Further, the membrane was rinsed with 0.1 % SDS, 1 X SSC for 15 minutes and consequently with 0.1 % SDS, 0.1 X SSC at 65^0 C for 10 minutes twice. The radioactivity count was calculated by the means of GM counter. Further, RT-PCR was conducted to evaluate the fusion gene expression. To carry out this experiment, total RNA was isolated from one month old leaves of PCR positive and untransformed plants utilizing TRI reagent(Bangalore Genei) as per the manufacturer's guidelines. For RT-PCR, RNAase free water was utilized to prevent the contamination of RNase. The first cDNA strand was produced from 5 µg of total

RNA utilizing cDNA synthesis kit (Bangalore Genei). About 1/20thvolume of the primary strand cDNA reaction was utilized to carry out the PCR amplification reactions for fusion gene.

Insect bioassay of transgenic pigeonpea plants

For *in vitro* bioassay *H. armigera* egg masses were acquired from NBAIR, Bangalore and were hatched on castor leaves by placing them in well aerated boxes. Completely expanded trifoliate leaves of 50-60 days old transgenic and control plants were taken and placed in petri dishes containing one layer of wet cotton and double layer of wet tissue paper to maintain moisture. On each trifoliate leaf, five number of second instar larvae were released. The experiment was repeated thrice and the larval growth, mortality and intensity of damage produced on the leaves were recorded after 72 h of larval release. The petri dishes were placed in an incubator at 25 ± 2°C temperature and 70 % relative humidity. Likewise, detached pod assay was also conducted, where two weeks old pods were taken for evaluating the efficacy against third instar *H. armigera* larvae by releasing single larvae on each pod.

Stastical analysis

The experiment was conducted in a Completely Randomized Design (CRD) and the experimental results in both the generations were graphically represented using sigma plot 14.5 software. Chi-square ($\chi 2$) test was done in both T_1 and T_2 generations to evaluate the segregation ratio (3:1) of the fusion gene depending on the performance in kanamycin sensitivity test.

Results

To acquire long-term pest resistance against devastating insect pest *Helicoverpa armigera*, *Tfgd2-RsAFP2* fusion gene was expressed in pigeonpea ICP 8863 cultivar through genetic transformation. Under laboratory conditions, the fusion protein was expressed and evidenced its insecticidal efficiency towards *H. armigera.* The findings are illustrated in the below section.

Generation of transgenic pigeonpea lines and transformation efficacy of putative transformants

To produce transgenic pigeonpea, multiple shoots were initiated from co-cultivated leaf petiole explants of ICP 8863 pigeonpea variety using the standardized protocol. Out of 90 explants cultured in three batches, 81 shoots were identified to be kanamycin positive and among them 35 plants were established well in the green house. PCR analysis performed for these plants showed 500 bp band with fusion gene primers (Fig.1) and 700-bp band with *nptII*-specific primers (Fig. 2) respectively in 12 plants confirming the presence of fusion gene. The transformation efficiency obtained was 16.66 %, which is calculated as the percentage of total number of rooted shoots versus fusion gene and *nptII* positive plants.

Analysis of T_0 and T_1 progenies for the inheritance of the T-DNA

Out of 12 PCR positive putative transformants, six healthy plants were used for segregation analysis. All six transgenic plants showed normal development and the results analyzed by the chi square test

showed 3:1 ratio ($p \geq 0.05$) (Table 1). These were further subjected to PCR analysis. In the same way, the RT PCR positive and insect resistant positive T_1 progeny seeds were also subjected to segregation analysis (Table 2).

Molecular analysis of the T_1 transgenic plants for stable integration and inheritance of fusion gene

PCR screening was performed to 48 well established T_1 plants out of 71 kanamycin resistant plants and among them 16 showed strong PCR amplification (Fig. 3) and were further subjected to southern analysis. Amongst the 16 PCR positive plants, 13 plants were southern positive, where 10 plants showed single copy insertion and 3 plants showed two copy insertions (Fig. 4). These southern positive plants were maintained in the green house and the seeds collected from them were used to generate T_2 generation for further analysis.

Improved resistance of T_1 transgenic pigeonpea plants expressing the fusion gene antagonistic towards *Helicoverpa. armigera*

Insect leaf bioassay was conducted in ten southern positive T_1 transgenic plants against second instar *H. armigera* larvae which exhibited significant variance in degree of leaf damage and larval mortality (Fig. 5). The immensity of leaf damage was evaluated after 72 h of leaf feeding in both transgenic and control plants. The plants that exhibited high mortality showed lesser leaf damage and the larval mortality varied between 20 to 86 % (Table 3). It was observed that the area of leaf damage in untransformed after 72 h was noticed as 24.0 cm^2, while in transgenic plants it varied from 3.6 to 8.06 cm^2 respectively (Table 4). In the current investigation, it was noticed that, more than 80 % of the T_1 transgenic plants showed less than 25 % damage. Larvae fed on the transgenic pigeonpea plants were stunted in growth with darkening and shrinking of body color, whereas larvae fed on the control plants showed weight gain after 72 h of leaf feeding (Fig. 6, Fig. 7). In this generation, eight lines were selected as superior based on their percentage of mortality and leaf damage (9-5, 2-4, 2-1, 14-10, 8-14, 14-1, 8-3, 9-8) (Fig. 8). Further, the selected plants were progressed to T_2 generation for analyzing the stable integration of T-DNA and their stable potency.

RT-PCR assay of T_1 transgenic plants for the expression of fusion gene

Eight single copy transgenic lines of T_1 generation that are identified as superior in leaf assay were selected to analyze the level of fusion gene expression through RT-PCR analysis including control. This revealed the expression of fusion gene in all the eight transgenic lines while no expression was noticed in the control (Fig. 9). RT-PCR analysis signified that the fusion gene was easily visible in the transgenic plants. This analysis represented that the level of expression was different in the transgenic resistant lines and their level of expression was higher in pigeonpea transgenics compared to the control, which was related with the enhanced pod borer resistance in these transgenic plants. **Improved insect resistance of T_2 transgenic pigeonpea plants against *H. armigera***

T_2 transgenic lines were subjected to *in vitro* leaf bioassay to determine their efficiency against the insect pest *H. armigera*, where they exhibited improved efficiency which is correlated to high larval mortality and less leaf damage. Variance in larval mortality rate was noticed in transgenic plants (40 to 86 %) (Table 5); furthermore the plants that displayed high mortality rate showed lesser leaf damage (Fig. 10). The leaf damage rate was identified as 25.0 cm^2 in control and in case of transgenic plants it varied between 5.1 to 6.96 cm^2 respectively (Table 6). The bioassay finally stated that the transformants selected not only have the fusion gene stably integrated in their genome but also showed improved ability in resistance against *H. armigera* as represented in the histogram indicating the percentage of leaf damage and larval mortality rate (Fig. 11). Moreover, it was noticed that, 100 % of the T_2 transgenic plants displayed < 25 % of leaf damage. In the current study, difference in larval physiology was observed in regards to dramatic decrease in larval weight and increase in larval mortality after feeding on the transgenic leaves (Fig. 12, 13). But, larvae fed on the control leaves portrayed normal physiology and development.

Detached pod assay

Two week old pods of single copy transgenic lines were analyzed for efficiency towards third instar larvae of *Helicoverpa armigera*. Decrease in pod consumption was noticed in transgenic plants (0.21 g) compared to the control (0.92 g) and further complete pod was damaged after 24 h of feeding in the case of control (Fig. 14, Table 7). In the present study, it was clearly implied that the functionality of the transgenic lines displayed a clear stability in efficiency of the transgenic lines to resist larval attack with decreased damage.

Discussion

The loss of agricultural produce owing to insect pest damage is a serious concern all over the world, which has already taken a lot of time and effort to resolve. Immense use of synthetic pesticides to combat these losses not only endangers the environment but also costs a lot of money. Marginal farmers in developing countries are frequently unable to afford this cost. These diseases not only lead to productivity loss but even decrease the crop quality. Many scientific ways to achieve long-term disease resistance in pigeonpea cultivars have been developed, concentrating on the objective of stacking numerous R-genes or introducing broad-spectrum insect resistance genes into cultivated lines. Application of transgenic technologies is advantageous and one amongst the crucial components in integrated pest management (IPM) program to generate wide range of insect and disease tolerance in plants (Meiyalaghan et al. 2011). Concurrently, it reduces the necessity of insecticides and is effective than traditional breeding methods. Despite the controversy surrounding GM or Bt crops, biotechnologically generated plants have gained importance over those developed through traditional crop improvement like breeding, particularly for the development of insect resistant crops (Tamiru et al. 2015). As a result, compared to normal traditional breeding techniques, transgenic technology which takes less time would be the ideal choice for plant enhancement to address the rising issue of food scarcity. Among the effective techniques to generate insect resistant plants, generation of transgenic plants expressing the insecticidal protein of *Bacillus thuringiensis* is the important one providing

resistance against insect pests. Employing the *Bt* toxin gene, more than 30 insect resistant crops have been produced so far.

Many reports stated that, defensins are the essential elements of the innate immune system in plants. These defensins present in majority of plant parts display wide array of *in vitro* antimicrobial function and currently, there are various records depicting the generation of transgenic plants showing constitutive expression of foreign defensins. They possess a wide array of biological functions like insecticidal, antimicrobial, as protein synthesis inhibitors, as abiotic stress mediators, zinc tolerance and as digestive enzymes inhibitors (Carvalho and Gomes 2009, 2011). Thomma et al. (2002) reported that, over 80 defensins genes were sequenced from various plant species. Primarily, Terras et al. (1992a) identified two anti-fungal defensins from radish seeds, that are *RsAFP1* and *RsAFP2* and their activity was assessed towards various fungi such as, *Phomabetae*, *Cercospora beticola* and *Pyricularia Oryza* and concluded that these two defensins constrained their growth (Terras et al. 1992b). *Rs-AFP1*, *Rs-AFP2*and *Rs-AFP3/4* were the most extensively investigated defensins isolated from *Raphanus sativus* seeds (Carvalho and Gomes 2009). Genetically modified peanut plants expressing *SniOLP* and *RsAFP2* genes displayed increased disease tolerance to *P. personata* (Vasavirama and Kirti 2012). Further, transgenic apple plants with *RsAFP2* gene displayed enhanced resistance to *F. culmorum* (De Bondt et al. 1999). Constitutive expression of *RsAFP2* gene in GM tomato plants displayed resistance to many fungal phytopathogens like *A. tenuis, R. solani, A. solan* and *P. Infestans* (Parashina et al. 2000). Vijayan et al. (2013) has stated that the GM plants expressing TvD1 defensin isolated from the *Tephrosia villosa,* a weedy legume enhances both insect and disease resistance in transgenic tobacco plants. Previously, it was also reported that, synchronous use of two defensins may lead to enhanced insecticidal and antimicrobial activity than using a single gene (Vasavirama and Kirti 2013; Bezirganoglu et al. 2013; Guler et al. 2014). So, we chose a polyprotein type of gene expression with an aim that *Tfgd2* - *RsAFP2* fusion gene linked by a linker peptide could impart enhanced insect resistance in transgenic pigeonpea. In accordance with this, genetic transformation in pigeonpea was performed to analyze the insect resistance in fusion gene expressing pigeonpea plants.

The *Agrobacterium* mediated transformation of the *Tfgd2* - *RsAFP2* fusion genes resulted in the generation of 35 putative transgenic plants (T_0) and among them 12 plants were observed to be fusion gene and *nptII* positive by PCR analysis. The transformation efficiency obtained was 16.66 %, which is measured as the frequency of fusion gene and *nptII* positive plants versus total number of rooted shoots. In other transgenic pigeonpea studies, 15 % PCR positive T_0 plants were attained by GV2260 *Agrobacterium* strain harboring pPK202 vector (Surekha et al. 2005) and with C58 *Agrobacterium* strain harboring pHS723 vector 1 - 50 % transformation frequency was obtained (Sharma etal. 2006). Whereas Krishna et al. (2011) reported transformation frequency of 44.61 % with *A. tumefaciens* strain GV3101 carrying the pPZP211 binary vector and Dayal et al. (2003) reported transformation potency of 50 % using leaf explants by biolistic methods. The variance in the efficiency of transformation may be due to explant and genotypic variations in pigeonpea, co-cultivation method and the genetic backdrop of the *Agrobacterium* strains (Surekha et al. 2005, 2007). The survival of T_0 progenies in kanamycin containing

media exhibited the segregation of fusion gene according to the 3:1 Mendelian ration and was transmitted to the following generation. Further, fusion gene integration in the T_1 and T_2 progenies was confirmed through PCR and southern blotting and the expression of the fusion gene was evaluated by RT-PCR assay. In our study, southern positive lines at T_1 and T_2 generations were studied as stably integrated transgenic lines without deviation.

Finally, an *in vitro* leaf bioassay was carried out with *H. armigera* second instar larvae, which displayed that *Tfgd2 - RsAFP2* fusion gene present in the transgenic pigeonpea leaves provided protection against the insect pest damage compared to the control untransformed lines. There was a significant relation between the expression level of the fusion gene and the level of resistance against *H. armigera* in the transgenic plants. In this experiment, high percentage of mortality and lesser leaf damage showed by the transgenic lines represents that all the transformants could confront the insect damage to certain degree. Small variations in the percentage of larval mortality and area of leaf damage in all the transformants may be featured due to the variations in the expression levels of the fusion gene. The T_2 transgenic plants 9-5-5, 9-5-2 and 2-4-9 showed highest percentage of larval mortality ranging from 80 - 86 % within three days of incubation. Gosh et al. (2017) reported 80-100 % mortality towards second instar *H. armigera* larvae in transgenic plants transformed with *Cry1Ac* and *Cry2Aa* insecticidal genes. In acceptance to the earlier findings, the difference in the percentage of larval mortality from our observations could be attributed due to the type of gene employed and the period of data collection (Kranthi and Kranthi 2004; Lacey and Kaya 2000). The current insect bioassay experiment displayed a significant decrease in the larval weight resulted by increased percentage of mortality related to the control plants, representing the expression of sufficient amount of the protein. Detached pod assay against third instar larvae of *H. armigera* conducted for the pods collected from the six transgenic lines of T_2 generation showed decreased pod consumption compared to the control. This was supported by the findings of pod assay done by Das et al. (2016) against 7-day-old *H. armigera* which showed 90 to 100 % larval mortality in T_4 and T_5 generations. In addition, Kaur et al. (2016) reported 97.78 % mortality of third instar larvae fed on the pods of T_2 transgenic lines after 48 h and Singh et al. (2018) reported less than 5-10 % of pod damage by the *H. armigera* second instar larvae. Based on these findings, it was observed that the fusion gene was effective in imparting insect resistance against *H. armigera* larvae in the gene expressing pigeonpea plants.

Conclusion

In conclusion, superior lines with high expression and efficiency of fusion gene against the pod borer were identified through molecular analysis and insect bioassay. The method used and the promising lines developed in T_2 generation could be a significant contribution to the community trying to minimise the damaging pest through gene transfer. Overall, the present results conclude that over expression of *Tfgd2-RsAFP2* fusion gene imparts resistance to *H. armigera* in pigeonpea plants carrying the fusion gene.

Declarations

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Author contributions

V K designed and guided N N to work on this specific topic to acquire data. N N prepared manuscript according to guidelines under the supervision of V K. Finally the manuscript was checked and corrected by V K for submission in favor of publication. Both worked hard in the analysis of data to complete this investigation. All authors have read and approved the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest

Ethical approval: Not applicable.

Consent for publication: Not applicable.

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Tables

Table 1 Seeds of six T_0 pigeonpea lines showed 3:1 segregation conforming to the chi square test ($p \geq 0.05$)

T_0 plants	T_1 seeds	Kan^R	Kan^S	Observed ratio	$\chi 2$ value	*P*-value
T-2	24	13	11	1.18:1	5.556	0.018
T-8	27	15	12	1.25:1	5.444	0.020
T-9	18	10	8	1.25:1	3.63	0.057
T-14	20	12	8	1.5:1	2.4	0.12
T-28	16	9	7	1.28:1	3.0	0.083
T-32	22	12	10	1.2:1	4.909	0.027

Table 2 Seeds of six T_1 pigeonpea lines showed 3:1 segregation conforming to the chi square test ($p \geq 0.05$)

T_1 plants	T_2 seeds	Kan^R	Kan^S	Observed ratio	$\chi 2$ value	*P*-value
9-5	24	13	11	1.18:1	5.556	0.018
2-4	23	11	12	0.91:1	9.058	0.0026
2-1	20	8	12	0.66:1	13.067	0.0003
14-10	15	8	7	1.14:1	3.756	0.053
8-14	19	9	10	0.9:1	7.737	0.0054
14-1	16	9	7	1.3:1	3	0.083
8-3	25	15	10	1.5:1	3	0.083
9-8	19	10	9	1.37:1	2.965	0.085

Table 3 *In vitro* leaf assay depicting the larval body weight and mortality percentage of *H. armigera* second instar larvae fed on the trifoliate leaves of T_1 transgenic pigeonpea plants and untransformed. The data are mean of three replicates ± SE

Transgenic Plants	Larval body weight after 72 h (mg) Mean ± SE	Percentage of larval mortality (%) Mean ± SE
9-5	6.63±0.10	86.66±8.16
2-1	7.73±0.14	73.33±8.16
8-3	14.63±0.10	66.66±8.16
14-10	14.23±0.18	60±0.00
2-4	7.63±0.10	86.66±8.16
8-14	14.36±0.11	73.33±16.32
28-6	22.36±0.10	20.00±14.14
32-3	19.2±0.14	40.00±14.14
9-8	14.53±0.04	66.66±8.16
14-1	14.33±0.10	60 ±0.00
Control	43.33±0.08	0±0.00

Table 4 *In vitro* leaf assay of T_1 transgenic pigeonpea plants depicting the area of leaf consumed by the *H. armigera* second instar larvae in three days and their percentage of leaf damage. The data are mean of three replicates ± SE

Transgenic Plants	Total leaf area (cm^2)	Leaf area consumed by 72 h (cm^2) Mean ± SE	Percentage of leaf damage (%) Mean ± SE
9-5	29	3.6±0.14	12.66±0.81
2-1	30	6.01±0.14	21±0.71
8-3	28	7.03±0.18	24.66±0.40
14-10	29	6.63±0.10	21.66±0.40
2-4	28	5.4±0.14	19.33±1.08
8-14	29	7.06±0.15	24.66±0.82
28-6	28	8.5±0.19	31 ± 0.70
32-3	29	8.06±0.22	27.66±1.08
9-8	29	7.23±0.15	24.66±0.40
14-1	28	6.33±0.10	23.33±1.08
Control	29	24.23±0.18	83.66±1.08

Table 5 *In vitro* detached leaf assay depicting the larval body weight and mortality percentage of second instar

larvae fed on the trifoliate leaves of T_2 transgenic pigeonpea plants and control after 72 h. The data represented are mean of three replicates ± SE.

Transgenic Plants	Larval body weight after 72 h (mg) Mean ± SE	Percentage of larval mortality (%) Mean ± SE
9-5-2	7.36±0.10	86.66±8.16
2-1-1	13.63±0.10	66.66±8.16
2-4-9	8.33±0.10	86.66±8.16
9-5-5	7.63±0.10	80 ±0.00
14-1-8	18.23±0.18	40 ±14.14
14-10-1	13.73±0.15	60 ±0.00
Control	40.66±0.82	0.00±0.00

Table 6 *In vitro* leaf assay conducted in T_2 transgenic pigeonpea plants representing the area of leaf consumed and percentage of leaf damage by *H. armigera* second instar larvae compared to the control after 72 h. The data

are mean of three replicates ± SE.

Transgenic Plants	Total leaf area (cm^2)	Leaf area consumed by 72h (cm^2) Mean ± SE	Percentage of leaf damage (%) Mean ± SE
9-5-2	28	5.1±0.07	18.33±1.08
2-1-1	28	6.23±0.10	22.00±0.71
2-4-9	29	5.73±0.21	19.00±0.71
9-5-5	28	4.5±0.21	16.66±0.82
14-1-8	28	6.96±0.14	25.0 ±0.70
14-10-1	28	6.06±0.21	21.33±1.08
Control	29	25.06±0.21	86.33±0.08

Table 7 Comparison of pod damage in control and T_2 transgenic pigeonpea lines in *in vitro* insect assay against

third instar *H. armigera* larvae. The data are mean of three replicates ± SE

Transgenic Plants	Primary pod weight (gm)	Average pod tissue consumed (g/larva) Mean ± SE
9-5-2	1.208	0.21±0.01
2-1-1	0.905	0.32±0.02
2-4-9	0.918	0.26±0.01
9-5-5	1.105	0.22±0.01
14-1-8	1.200	0.35±0.02
14-10-1	0.920	0.29±0.02
Control	0.910	0.92±0.02

Figures

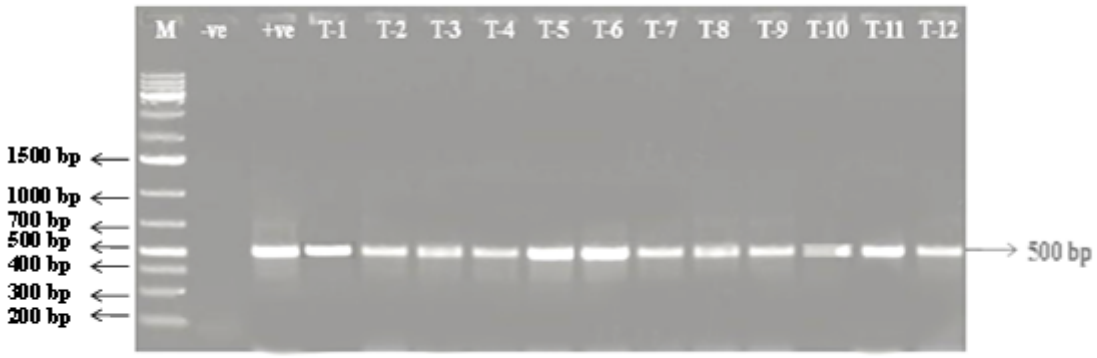

Figure 1

Primary confirmation of putative pigeonpea plants by PCR amplification of *Tfgd2-RsAFP2* transgenes. M represents 1kb plus DNA ladder, Lane '-ve' represents negative control (Non-transformed plant), Lane '+ve' represents positive control, Lanes T-1 to T-12 represents the amplification of 500 bp *Tfgd2-RsAFP2* gene fragment

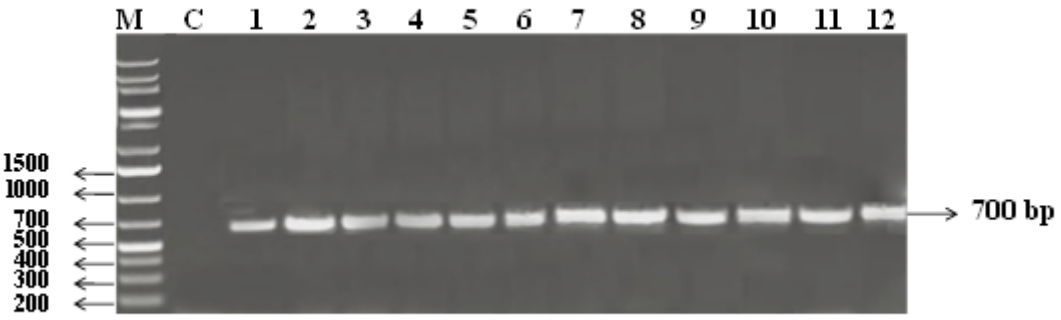

Figure 2

Confirmation of the *npt*II gene in putative transgenic lines. Lane M represents 1kb plus DNA ladder; Lane 2 represents negative control (Non-transformed plant); Lanes 1 to 12 represents T_0 transgenic plants showing the presence of the *npt*II gene

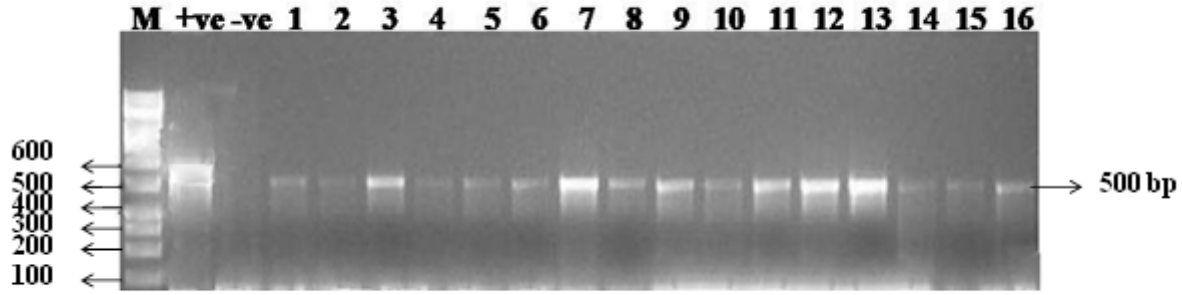

Figure 3

Confirmation of T_1 transgenic lines through PCR amplification of *Tfgd2-RsAFP2* transgenes. M indicates 100bp DNA Ladder, Lane '+ve' indicates positive control, Lane '-ve'represents negative control, Lanes T-1 to T-16 (2-1, 2-4, 2-10, 8-3, 8-10, 8-14, 9-5, 9-8, 14-1, 14-3, 14-10, 28-2, 28-6, 28-8, 32-3 & 32-7) indicates the amplification of 500 bp *Tfgd2-RsAFP2*gene

Figure 4

Southern blotting analysis of T_1 transgenic plants. 10 µg of genomic DNA was digested with *HindIII* restriction enzyme and probed with 700 bp *nptII* gene fragment. Lane '-ve' represents non-

transformed plant, Lanes 1-13 represents southern positive transgenic pigeonpea plants (2-1, 2-4, 8-3, 2-10, 8-10, 8-14, 9-5, 9-8, 14-1, 28-8, 14-10, 28-6 & 32-3). Lanes 1, 2, 3, 5, 6, 7, 8, 9, 11, 12 & 13 exhibited single copy insertion whereas Lanes 4, 5 & 10 showed double copy insertions

Figure 5

Different stages of T_1 transgenic plants established in green house

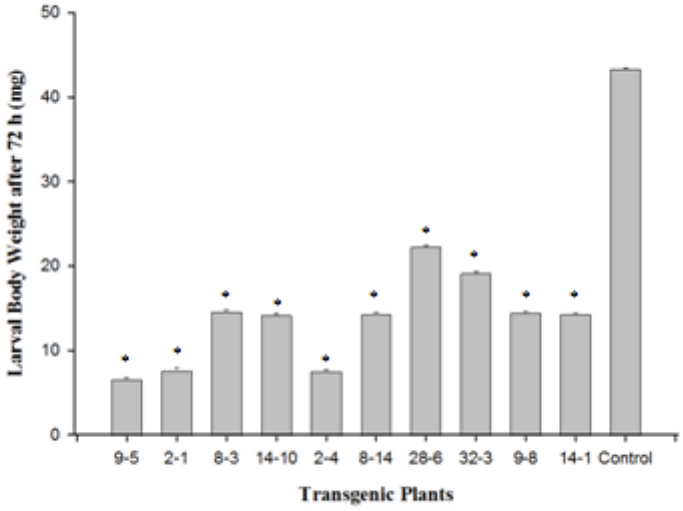

Figure 6

A bar graph depicting the comparative study of larval body weight of 2nd instar larvae of *Helicoverpa armigera* fed on T_1 transgenic and control plants leaves of pigeonpea after three days. *Represents significant variance in body weight gained among transgenic and control pigeonpea plants applying Student's t test ($P \leq 0.05$)

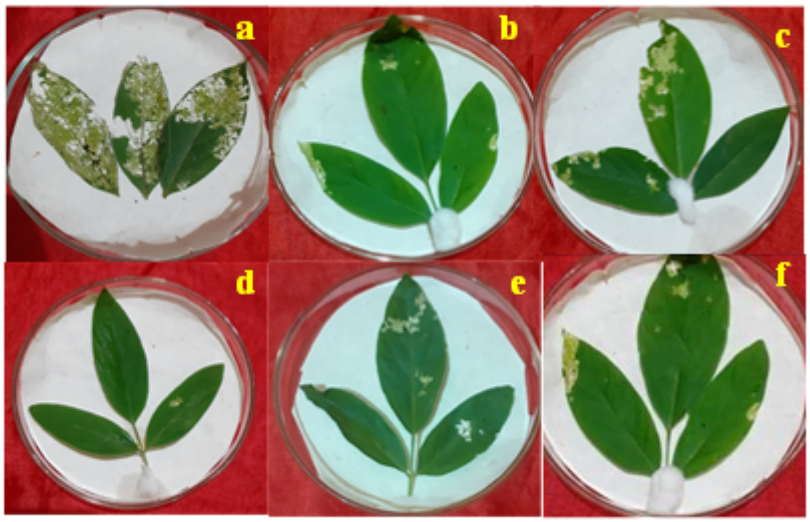

Figure 7

In vitro leaf bioassay of T_1 transgenic pigeonpea plants and control with second instar larvae of *H. armigera* after 72 h of feeding. Leaf assay was done by placing pigeonpea leaves on two layers of moist tissue paper in an autoclaved petri dishes. Five second instar larvae of *H. armigera* were released onto the leaves and were enabled to feed for 72 h and were placed in an incubator at 25 ± 2°C temperature. (a) Control leaf, (b) to (f) Transgenic plants leaves

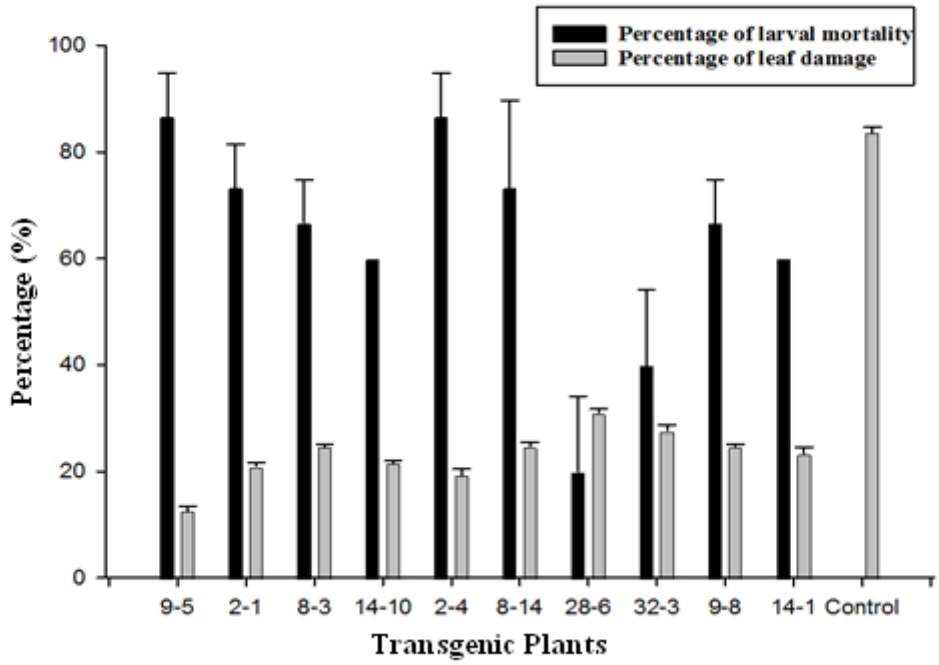

Figure 8

A histogram representing the variance in the performance of T_1 transgenic pigeonpea plants and control in the *in vitro* leaf bioassay depicting the percentage of mortality and percentage of leaf damage by the *H. armigera* second instar larvae

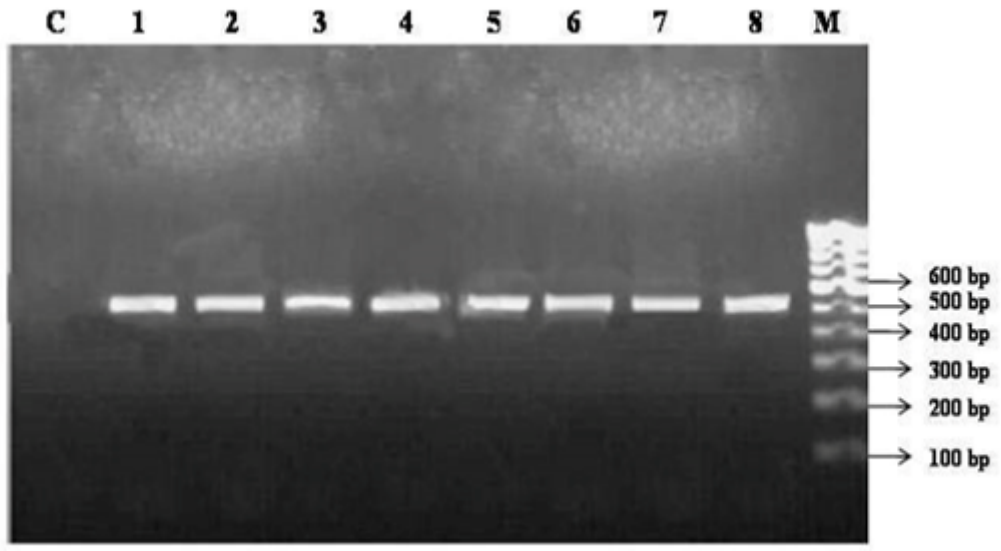

Figure 9

RT PCR analysis of control and T_1 transgenic pigeonpea plants for fusion gene transcript. Lane - C represents the control, Lanes 1-8 represents the eight transgenic lines (9-5, 2-4, 2-1, 14-10, 8-14, 14-1, 8-3, 9-8), M represents the 100 bp DNA Ladder. All the transgenic plants (lanes 1-8) amplified a distinct band of 500 bp size and no band was observed in the control

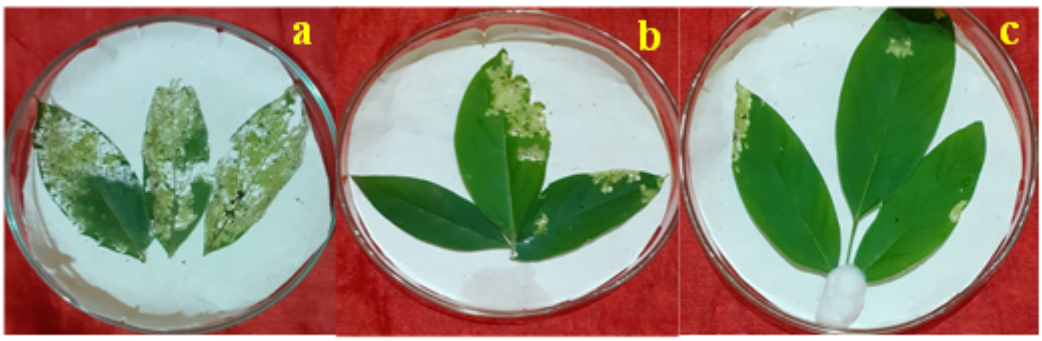

Figure 10

Performance of the T_2 transgenic pigeonpea lines against second instar larvae of *H. armigera* in the leaf bio-assay experiment (a) Control, (b) & (c) Transgenic

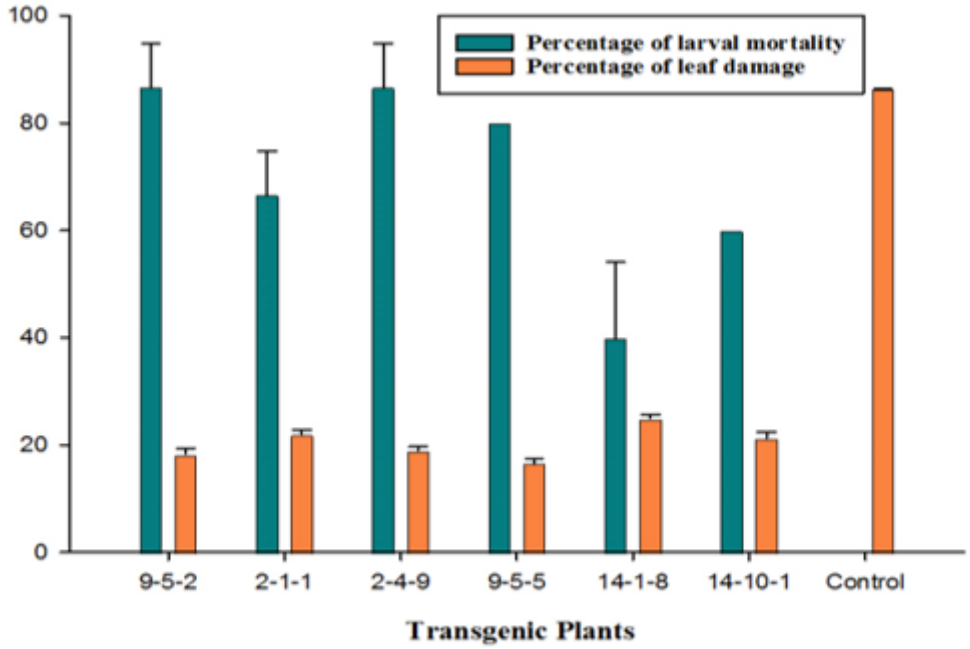

Figure 11

A histogram representing the variance in the performance of T_2 transgenic pigeonpea plants and control in the leaf bio-assay depicting the percentage of larval mortality and percentage of leaf damage

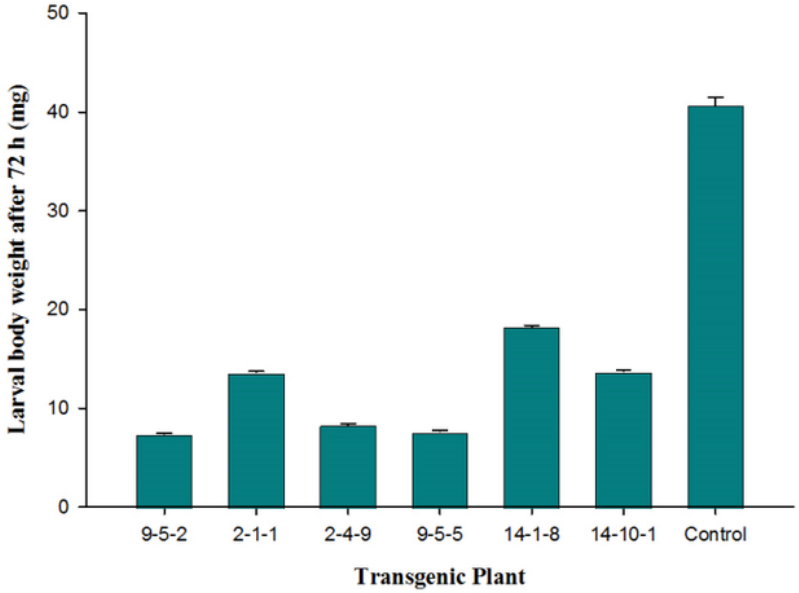

Figure 12

A bar graph depicting the comparative study of weights of 2nd instar *Helicoverpa. armigera* larvae fed on the leaves of control and T_2 transgenic pigeonpea plants after three days. *Represents significant variance in body weight gained among transgenic and control pigeonpea plants applying Student's t test ($P \leq 0.05$)

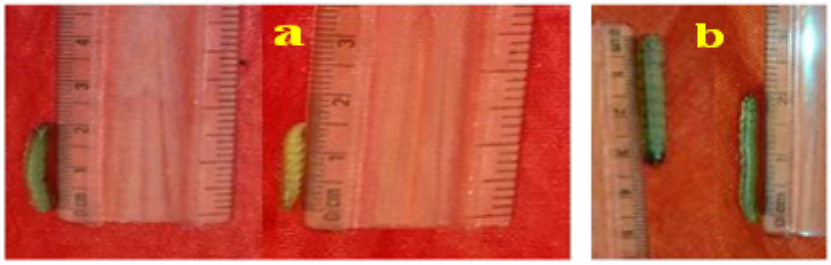

Figure 13

Larval morphology of second instar *H. armigera* larvae feeding on transgenic and untransformed leaves. (a)Transgenic, (b) Untransformed

Figure 14

In vitro pod assay done in the pigeonpea pods collected from T_2 transgenic plants against third instar *H. armigera* larvae. (a), (b) Transgenic, (c), (d) Control