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In vitro Screening of Guava Plantlets Transformed with *Endochitinase* Gene against *Fusarium oxysporum* f.sp. *psidii*

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Abstract

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Guava wilt disease is a severe threat to guava growers all over the world. It is caused by the soil-borne fungus *Fusarium oxysporum* f.sp. *psidii*. To control the disease, the *Trichoderma-endochitinase* gene was first introgressed into guava (*Psidium guajava* L.). The transgenic plantlets were screened *in vitro* for resistance against the wilt pathogen. Six-months-old genetically transformed plants raised in cocopeat under *in vitro* conditions were inoculated with a 7-days old culture of *F. oxysporum* f.sp. *psidii*. The presence of the pathogen in the cocopeat medium was confirmed by cultural as well as PCR analysis using species-specific primers. The roots of transgenic plants were wounded to facilitate the entry of the pathogen. The histopathological analysis revealed the presence of mycelium in vascular bundles. However, none of the plants showed symptoms of wilt disease during the investigation. *In vitro* pathogen inhibition assay and subsequently spore germination assay revealed that the crude leaf extract of transformed plants inhibited the germination of fungal conidia. The leaf tissue studied for expression of *endochitinase* revealed that two transgenic plants showed very high activity of N-acetyl-d-glucosamine (0.741 and 0.738 μM/min/μg of protein, respectively) which clearly indicated that transgenic plants could not develop any symptoms of wilt disease due to overexpression of *endochitinase*.

Keywords: chitinase; genetic transformation; pathogen inhibition assay; quantification of chitinase; wilt disease

Guava (*Psidium guajava* L.) is an important fruit crop of subtropical countries. It is a hardy crop and is cultivated successfully even in neglected soils. Guava wilt disease is a challenge for the coming millennium as chemical control has been proved unsatisfactory in the field and losses due to this disease are substantial. Wilt is predominantly a threat caused by the species of *Fusarium*, of which *F. oxysporum* f.sp. *psidii* and *F. solani* are the important causal organisms (Misra 2006; Gupta 2012; Mishra *et al.* 2013). Edward (1960) explained that *F. oxysporum* f.sp. *psidii* penetrates either directly through the root piliferous layer of the guava seedlings or through openings caused by secondary roots. Hyphae were found in the xylem vessels of the roots of the inoculated plants. It was also observed that *F. oxysporum* f.sp. *psidii* existed in a variety of forms which differ in cultural and morphological characters (Edward 1960).

Various chemical and biological means have been suggested for the control of this disease. However, none of the control measures was found effective in controlling the wilt disease in guava (Misra 2006). Development of a transgenic guava variety with genes such as *endochitinase* which encodes enzymes which can degrade the fungal cell wall could prove to be a permanent solution to this problem. The technique of expressing the *endochitinase* gene in the plant system to confer resistance against fungal diseases has been suc-

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cessfully demonstrated in apple (Bolar *et al.* 2000), tomato (Girhepuje & Shinde 2011), cotton (Emami *et al.* 2003; Cheng *et al.* 2005), broccoli (Mora & Earle 2001), lemon (Gentile *et al.* 2007) and rice (Lu *et al.* 2004; Shah *et al.* 2009). Chitinases have been shown to possess an antifungal role in disease resistance (Broglie *et al.* 1991; Sela-Buurlage *et al.* 1993). We have developed transgenic guava overexpressing the *endochitinase* gene derived from *Trichoderma harzianum* (Mishra *et al.* 2014). A total of 11 transgenic lines have been developed. These transgenic lines showed differential *endochitinase* expression. Two promising lines (T22 and T20) had high *endochitinase* expression in terms of N-acetyl glucosamine release. These lines are single copy events which have been clonally multiplied using cutting and used in the experiment. This paper elaborates the *in vitro* screening of transgenic guava plants against *F. oxysporum* f.sp. *psidii.*

MATERIAL AND METHODS

In vitro **screening of transgenic plants**. Six-months-old genetically transformed plants of guava raised in cocopeat under *in vitro* conditions were inoculated with 7-days-old culture of *F. oxysporum* f.sp. *psidii* (6×10^6 conidia) (Figure 1). After inoculation, plants were kept at 25 ± 2°C for six months for observations of the appearance of wilt symptoms. Observations were taken periodically. The presence of inoculants in cocopeat was recorded by a traditional serial dilution method using potato dextrose agar media. The causal pathogen of wilt was identified based on the morphological and microscopic observations. The cultures were kept at a temperature of 25 ± 2°C and 55% relative humidity under diffused light (2000–3000 lux) with 16 h photoperiod.

DNA extraction and molecular confirmation. In order to confirm that transgenic guava plants are being grown in the presence of *F. oxysporum* under *in vitro* conditions, the fungal mycelium was plated on potato dextrose agar (PDA) and incubated at 28 ± 2°C for 4–5 days. Total genomic DNA was extracted using the phenol : chloroform : isoamyl alcohol protocol (Sambrook & Russell 2001). The pellets were air dried and resuspended in 100 µl of 1X TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The isolated genomic DNA was kept at –20°C for further molecular studies. The presence of *F. oxysporum* f.sp. *psidii* in cocopeat was investigated with species specific primers BKP-1 and BKP-2 (Mishra *et al.* 2013). Amplification products were resolved by electrophoresis on agarose gel (1.5%) in 1X TBE buffer (pH-8) stained with ethidium bromide and photographed under UV light using a UV tech gel documentation system.

Histopathology of transgenic guava plantlets. Root samples were collected from fungal inoculated and non-inoculated transgenic plants. Roots were cut into 2–4 cm pieces and surface sterilized using 0.1% $HgCl_2$, washed two to three times in sterilized water and the excess water was absorbed on blotting paper. Then samples were kept in a formaldehyde : acetic acid : alcohol (5 : 5 : 90 ml) solution for 48 h. The samples were processed in 30, 50, 70, 80 and 95% alcohol sequentially for 30 min. Then they were processed in alcohol : xylene at 1 : 3, 1 : 1 and 3 : 1 sequentially for 30 min. Finally, the samples were processed in pure xylene for 30 min. The samples were embedded in melted paraffin wax (54–56°C) for 4–8 h in order to completely replace xylene with paraffin wax. Blocks were prepared in paraffin wax and thin sections (10 µm) were cut with microtome (MICROTOM- HM 350 S, Thermo Fisher Scientific, Walldorf, Germany). At least 20 slides were prepared for each sample. The samples were stained in 0.1% aqueous toluidine

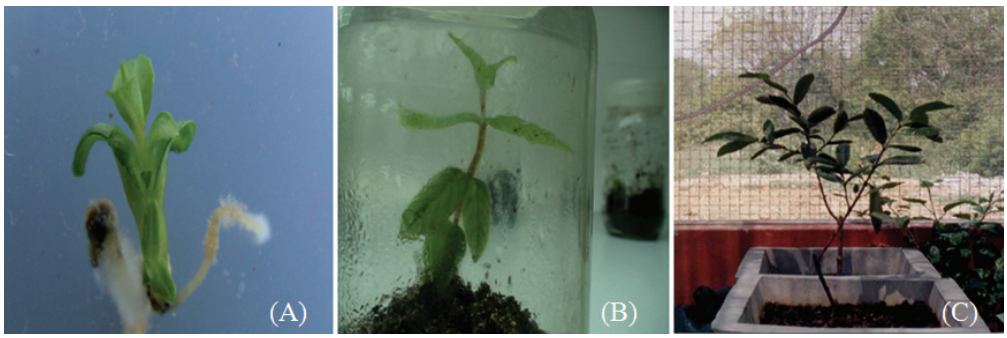

Figure 1. Different stages of growth of transgenic guava plant: plant regeneration in MS + 200 mg/l kanamycin + 2 mg/l BAP + 1mg/l IAA (8 weeks) (A), acclimatization of transgenic plant in cocopeat (16 weeks) (B), establishment of transgenic plant in containment facility (48 weeks) (C)

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blue 'O' and were mounted on 50% (v/v) distrene, plasticiser, xylene (DPX) after bringing down xylene through the alcohol : xylene series and then viewed under a stereoscopic microscope.

***In vitro* pathogen inhibition assay**. The agar disc diffusion and well diffusion techniques have been widely used for antifungal susceptibility testing (Magaldi *et al.* 2004). Leaves taken from both transformed and control guava plants were ground in 2 ml of 50 mM sodium acetate buffer (pH 5.0). The ground mixture was centrifuged at 13 200 g for 10 min at 4°C. The supernatant was pipetted out, filter sterilized and used immediately for bioassay. Holes of 6 mm in diameter were punched with a sterile cork borer aseptically. Filter sterilized control plant and transformed plant extract were introduced into the wells on the same plate. The antibiotic streptomycin was spread on the surface of the plate to avoid bacterial contamination. The disc (6 mm) of the test pathogen (*F. oxysporum*) was taken out from the actively growing plate and placed in the centre of the test plate. The plates were then incubated at 27°C and observed after 3 days. The percentage of mycelial growth inhibition (MGI) was calculated according to the formula:

$$MGI\% = (dc - dt) \times 100/dc$$

where:
dc – fungal colony diameter in control set
dt – fungal colony diameter in treatment set

Spore germination assay. Antifungal activity of plant extracts can be evaluated by spore germination assay using the slide technique. The leaves from transgenic and control plants were ground and centrifuged at 13 200 g for 10 min at 4°C. The supernatant was pipetted out, filter sterilized and used immediately for bioassay. *F. oxysporum* conidial suspension was adjusted to a density of 10^6 conidia in PDB (Difco, New Jersey, USA), mixed at the ratio of 1 : 9 with the leaf extract, and incubated at room temperature in low light. The growth of germinated conidia was assessed after 48 h of incubation period. The assay was replicated at least three times. After incubation, slides were fixed in lactophenol cotton blue and observed microscopically for spore germination.

Chitinase activity assay. All the 11 transgenic lines have been tested for *endochitinase* expression. Crude protein was extracted from transformed and control plants as described earlier for bioassays. Crude protein (0.5 ml) was incubated with 0.5 ml of 0.2% colloidal chitin (in phosphate buffer 0.05 M, pH 5.2) and 1 ml distilled water in a shaking water bath at 50°C for 30 min. Thereafter, 3 ml of dinitrosalicylic acid reagent was added to stop the reaction. The mixture was placed in a boiling water bath for 5 min and after cooling and centrifugation, the developed colour was measured spectrophotometrically at 575 nm, using an APEL PD-303UV spectrophotometer (APEL, Saitama, Japan), and taken as evidence for the quantity of released N-acetylglucosamine (NAG). The amount of NAG was calculated from the standard curve of NAG. One unit (U) of chitinase activity is defined as the amount of enzyme that is required to release 1 µmol of N-acetyl-glucosamine per minute under assay conditions.

RESULTS

Our group developed a genetic transformation system in guava (*Psidium guajava* L.) using *in vitro* grown shoot tips as explants (Mishra *et al.* 2014). A total of eleven transgenic guava plants introgressed with full-length *Trichoderma-endochitinase* gene and *neomycin phosphotransferase* (npt-II*)* gene under the control of CaMV 35S promoter and NOS terminator (Saiprasad *et al.* 2009) were developed. However, two transgenic lines (T20 and T22) which were expressing high *endochitinase* were clonally multiplied and chosen for the study.

Molecular confirmation of *F. oxysporum* f.sp. *psidii* present in the media substrate. The transgenic guava plants were allowed to grow in the sterilized cocopeat supplemented with MS salt solution under *in vitro* conditions. Cocopeat was inoculated with *F. oxysporum* f.sp. *psidii* periodically for six months. The root tips of transgenic plants were wounded to facilitate the penetration of the fungus. *F. oxysporum* f.sp. *psidii* was isolated from cocopeat and PCR amplification of the Internal transcribed spacer (ITS) region of 183 bp was obtained (Figure 2). The result of PCR amplification confirmed that *F. oxysporum* f.sp. *psidii* was present in cocopeat during the period of investigation.

Histopathology of transgenic guava plantlets. The histopathological studies were carried out to investigate the anatomical changes in the root of transgenic guava challenged with *F. oxysporum* f.sp. *psidii* after six months. It was compared with the control plant which was not inoculated with *Fusarium*. The intercellular spaces and the interfaces of contact between neighbouring cells were completely free in the control plant whereas the transverse section (TS) of transgenic plant root indicated disorganization and dark brown discoloration of parenchymatous cells

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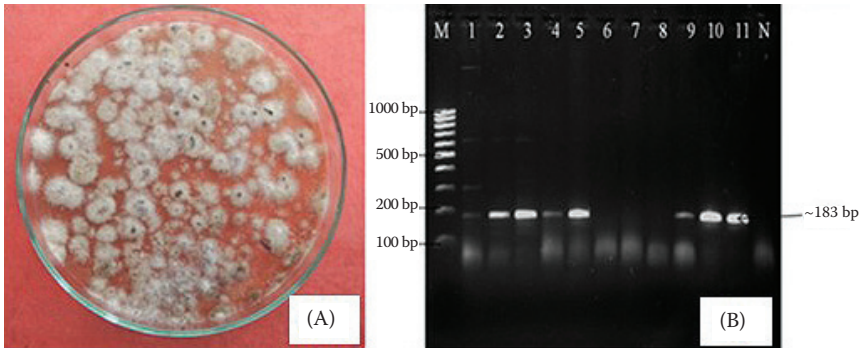

Figure 2. Isolation of *F. oxysporum* f.sp. *psidii* from cocopeat after one month (A), PCR amplification of the ITS region of *F. oxysporum* f.sp. *psidii* using species-specific primers (B); lane M: 100 bp DNA ladder, lane 1–11: isolates from cocopeat, lane N: control (dH_2O)

surrounding vascular bundles. Parenchymatous cells were filled with brown to dark mycelia (Figure 3). The microscopic examination of a section taken from the tissue of Fusarium invaded transgenic plant indicates the ability of pathogenic fungi to invade different tissues causing changes in cells and discoloration of parenchymatous cells. Spores were formed within parenchymatous cells and xylem vessels. However, it is assumed that *Trichoderma-endochitinase* gene in the transgenic plant might have triggered a resistance response.

***In vitro* pathogen inhibition assay**. To screen the transgenic plant against resistance to *F. oxysporum*, an *in vitro* pathogen inhibition assay was utilized. Crude leaf extracts of transformed and control (non-transformed) plants of guava were isolated and a fixed volume of plant crude protein extract was then poured into the bored agar well and a disc of *F. oxysporum* was placed in the centre of the test plate and incubated at room temperature for 3 days. It is evident from our observation (Figure 4A) that the mycelium grew towards the well which was poured with the crude protein extract of control guava leaves. However, the mycelium growth was restricted towards the well which was poured with the crude protein extract of transgenic guava leaves. It is interesting to note that crude protein is able to restrict the growth of the test pathogen up to 28.57% under *in-vitro* assays (Figure 3). This clearly shows the antifungal activity of the leaf extract of transgenic plants which may be attributed to the overexpression of *Trichoderma-endochitinase* gene incorporated in guava plant.

Spore germination assay. The antifungal activity of the crude leaf extract of transgenic guava plants was also assessed by a spore germination assay. Crude protein extracts of transformed and control plants of guava were isolated and mixed at the ratio of 1 : 9 with a conidial suspension of *F. oxysporum* having density of 10^5 conidia in PDB and incubated at room temperature in low light for 48 h. The microscopic examination of samples revealed that the crude protein extract of transgenic guava plant had the strongest inhibitory effects on germination of *F. oxysporum* conidia in comparison with the crude

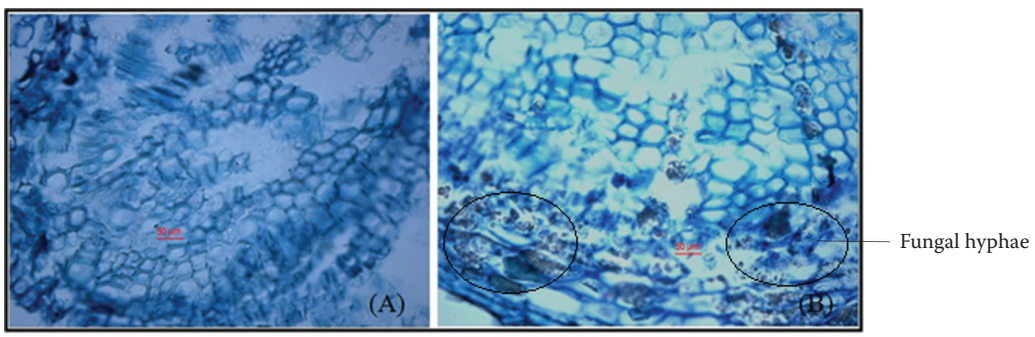

Figure 3. Transverse section of the root of uninoculated transgenic guava plant (A) and *F. oxysporum* f.sp. *psidii* inoculated transgenic guava plant (B)

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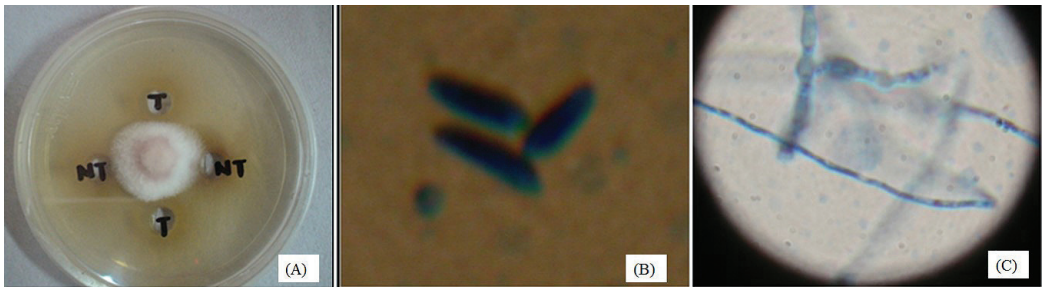

Figure 4. Radial growth of *F. oxysporum* f.sp. *psidii* towards the bored well poured with the crude leaf extract of control (NT) and inhibition of growth towards the transgenic well (A), inhibition of the germination of *F. oxysporum* conidia in the presence of the crude leaf extract of transgenic guava (B) and mycelium growth of *F. oxysporum* in the presence of the crude leaf extract of non-transgenic guava (C)

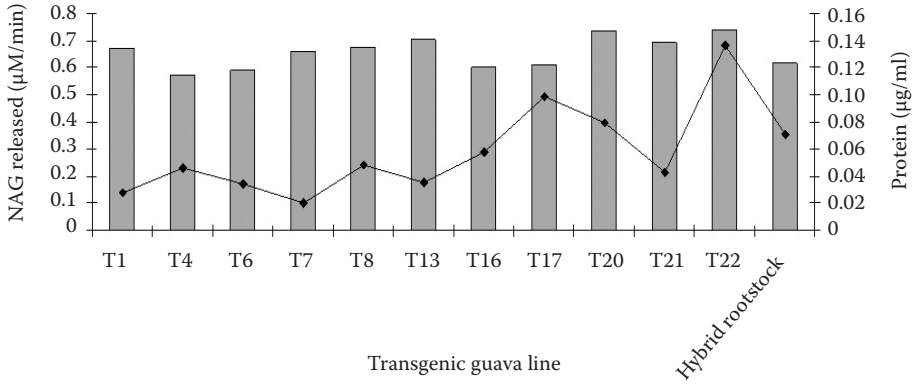

Figure 5. N-acetyl glucosamine (NAG) activity in transgenic guava plants

protein extract of non-transgenic plant. Significant changes in the hyphal morphology were observed in the crude extract of non-transgenic guava whereas no fungal growth was usually detected in the crude extract of transgenic plant (Figures 4B, C).

Expression of *endochitinase* in transgenic guava. *Endochitinase* expression studied in 11 transgenic guava lines revealed that the maximum N-acetyl glucosamine activity was recorded in transgenic guava line-22 (0.741 µM/min/µg of protein) followed by transgenic guava line-20 (0.738 µM/min/µg of protein) over the non-transgenic wilt resistant hybrid rootstock (*P. molle* × *P. guajava*) of guava (0.0875 µM per min/µg of protein), which clearly shows higher *endochitinase* expression in two transgenic lines of guava (Figure 5).

DISCUSSION

Wilt is a major impediment in successful cultivation of guava. Transgenic technology can be gainfully utilized for development of wilt resistant guava. We developed two promising transgenic lines of guava expressing the *Trichoderma-endochitinase* gene (Mishra *et al*. 2014). The transgenic guava plants were screened against *F. oxysporum* f. sp. *psidii* under *in vitro* conditions. Screening of transgenic guava plantlets against resistance to *F. oxysporum* was performed through *in vitro* pathogen inhibition assay, spore germination assay and measuring the NAG activity on a crude leaf extract (transformed and control guava plantlets). PCR was performed with species-specific primers to confirm the presence of *F. oxysporum* in the root zone of transgenic plants in the course of investigation.

The histopathological studies were carried out to investigate the anatomical changes that took place after the invasion of *Fusarium* in transgenic plant. Typical features of the shape and size of tissues were shown in TS of root. The transverse section of root tissues where *Fusarium* has not invaded showed normal vascular bundles. The intercellular spaces and the interfaces of contact between neighbouring cells were completely free whereas TS of transgenic

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root invaded by Fusarium indicated disorganization and dark brown discoloration of parenchymatous cells surrounding vascular bundles. Parenchymatous cells were filled with brown to dark mycelia. Microscopic examination of the section taken from the tissue of Fusarium invaded transgenic plant indicates the ability of pathogenic fungi to enter the root system. Spores were formed within parenchymatous cells and xylem vessels. However, the fungus is unable to colonize the xylem vessels, which may be attributed to higher expression of *Trichoderma-endochitinase* gene in the transgenic plant which might have triggered the resistance response. Mycelium growth was inhibited towards the well poured with the crude extract of transgenic guava leaves during an *in vitro* pathogen inhibition assay, which clearly suggested that the growth of the fungus *F. oxysporum* was retarded by transgenic guava leaves. The crude protein extract from transgenic guava leaves also showed inhibitory effects on the germination of *F. oxysporum* conidia in a spore germination assay and no fungal growth was observed in the slides of transgenic guava plantlets. The leaf extracts from both transformed and control guava plantlets were taken for measuring antifungal activity by a spore germination assay using the slide technique. The microscopic examination of samples revealed that the crude extract of transgenic guava plant had the strongest inhibitory effects on germination of *F. oxysporum* conidia in comparison with the crude protein extract of control (non-transgenic) plant. Significant changes in the hyphal morphology were observed in the slide of non-transgenic guava crude extract whereas no fungal growth was detected in the slide of transgenic plant. It is evident from our observation that mycelium growth was restricted towards the well which was poured with the crude extract of transgenic guava leaves, which may be attributed to the overexpression of *endochitinase* gene incorporated in guava plant. The antifungal activity of chitinase from plants and bacteria has been known for a long time (Yamamoto *et al*. 2000; Pleban *et al*. 1997) but neither single plant nor bacterial chitinase gene has produced an adequate level of resistance. However, *Trichoderma* chitinases have shown substantially higher antifungal activity (Lorito *et al*. 1998; Harighi *et al*. 2007) than any other chitinases purified so far from any other source when assayed under the same conditions. In the present study the inhibitory effect of the crude extract of transgenic guava plant leaves expressing chitinase of *T. harzianum* on the growth of the fungus *F. oxysporum* is clearly manifested. A detached leaf assay has also been used by researchers for measuring antifungal activity in transgenic canola lines (Zamani *et al*. 2012) and transgenic tobacco plants (Hoshikawa 2012) as described by Carstens *et al*. (2003). The size of lesions induced by *S. sclerotiorum* in the leaves of transgenic canola line was significantly retarded as compared to the leaves from non-transgenic canola plants (Zamani *et al*. 2012) while transgenic tobacco plants expressing *amn5A* gene showed enhanced resistance to the infection by the phytopathogenic fungus. After 10 days of inoculation, necrotic lesions of the untransformed plants extended three times of those of transformed plants. This result suggested that transgenic plants could inhibit the growth of *Fusarium* compared to untransformed control plants (Hoshikawa 2012).

The production of transgenic plants overexpressing a chitinase gene has been demonstrated to develop resistance against pathogens. Constitutive overexpression of antifungal genes from microorganisms involved in plant defence mechanisms in agriculturally important plants represents a promising strategy for conferring genetic resistance against a broad range of plant pathogenic fungi. Expression of chitinase encoding genes in plants has been shown to improve their defence response against various fungal pathogens. Chitinases from mycoparasitic *Trichoderma* spp. were already overexpressed in several agriculturally important plants, e.g. lemon, cotton, apple and carrot (Emani *et al*. 2003; Dana *et al*. 2006; Kumar *et al*. 2009). In the present study, *endochitinase* activity was also measured in transgenic guava leaves in terms of N-acetyl glucosamine release. Transgenic guava lines (T20 and T22) showed maximum *endochitinase* activity over the non-transgenic wilt resistant hybrid rootstock of guava. The non-transgenic interspecies hybrid guava rootstock (*P. molle* × *P. guajava*) also expressed high *endochitinase* activity which can be correlated with wilt resistance. Brogue *et al*. (1991) showed an increased ability of tobacco plants to survive in *Rhizoctonia solani* infected soil and delayed development of disease symptoms in tobacco seedlings by expressing chitinase. Dunsmuir *et al*. (1993) also confirmed the bacterial chitinase gene expressed at high levels. Besides the immunity against a fungal pathogen, overexpression of chitinase was also found to be effective to raise resistance of plants against bacterial pathogens, salinity stress and heavy metal stress (Dana *et al*. 2006). Transgenic tobacco (*Nicotiana tabacum*) plants overexpressing chitinases were also shown to be more resistant against other types of abiotic and biotic stresses such as bacterial pathogens, salinity and heavy metals (Dana *et al*. 2006). Chitinase activity in cultures of *Trichoderma*

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strains with chitin was assayed according to Boler *et al*. (2000) using colloidal chitin as a substrate. The released *N*-acetyl glucosamine (NAG) was measured using NAG as a standard. The extracellular chitinase activity of the *T. harzianum* transformants was up to 200-fold greater than that of the wild type in culture with glucose whereas in chitin, the activity of both the transformants and the wild type was similar (Limon *et al*. 1999). Transgenic litchi (*Litchi chinensis* Sonn.) plants were generated by transferring a rice chitinase gene into the zygotic embryos to increase the antifungal response and exhibited higher chitinase activity than the non-transformed plants (Das *et al*. 2012). *Endochitinase* and *exochitinase* from *T. harzianum* have been utilized to confer resistance against the pathogenic fungus causing Scab disease (*Venturia inequalis*) in apple (Bolar *et al*. 2000). Enhanced fungal resistance in cotton against the pathogens *R. solani* and *Alternaria alternata* was obtained after transferring *endochitinase* gene from *T. virens* in cotton (Emani *et al*. 2003) while *endochitinase* gene from *T. virens* was transferred in rice for enhanced sheath blight resistance (Shah *et al*. 2009).

CONCLUSION

Genetically engineered guava plants harbouring *Trichoderma-endochitinase* gene were screened *in vitro* against *F. oxysporum* f.sp. *psidii*. High *endochitinase* activity was found in the leaves of transgenic guava in terms of the release of N-acetyl glucosamine. *In vitro* pathogen inhibition assay and subsequently spore germination assay revealed that the crude leaf extract of transformed plant inhibited the germination of conidia. This preliminary report suggests that transgenic guava plantlets introgressed with *endochitinase* gene might provide resistance against *F. oxysporum* f.sp. *psidii*. The microscopic examination of section taken from the *Fusarium* invaded transgenic plant tissue indicates the ability of pathogenic fungi to invade different tissues causing changes in cells and discoloration of parenchymatous cells. Spores were formed within parenchymatous cells and xylem vessels. However, higher expression of *endochitinase* gene in the transgenic plant might have triggered the resistance response.

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