Enhanced Resistance To Fungal Pathogens in Transgenic Peanut (*Arachis Hypogaea* L.) Cultivar L14 by Overexpression of Gene encoding Chitinase 42 kDa from *Trichoderma Asperellum* SH16

Phung Thi Bich Hoa
Hue University
Nguyen Hoang Tue
Hue University
Huynh Thi Quynh Trang
Hue University
Hoang Anh Thu
Hue University
Le Ngoc Huyen Nhung
Hue University
Nguyen Ngoc Luong
Hue University
Nguyen Xuan Huy
Hue University
Nguyen Quang Duc Tien
Hue University
Nguyen Hoang Loc (nhloc@hueuni.edu.vn)
University of Sciences, Hue University https://orcid.org/0000-0002-6387-0359

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Abstract

This study reports the expression of 42 kDa chitinase genes from *Trichoderma asperellum* SH16 in peanut (*Arachis hypogaea*) roots under the regulation of tissue-specific Asy promoter through *Agrobacterium tumefaciens*-mediated transformation. The 42 kDa chitinase genes, including one wild-type sequence (*Chi42*) and two synthetic sequences (*syncodChi42-1* and *syncodChi42-2*) which were optimized for codon usage for plant expression, were incorporated into the peanut genome and successfully expressed in their roots. The investigation revealed that the enzyme chitinase from two synthetic genes had higher activity than that from the wild-type gene, about 901 U/mg (140 U/mL) and 1124 U/mg (197 U/mL) vs about 508 U/mg (87 U/mL). Transgenic peanut roots also exhibited extracellular chitinase activity which was driven by signal peptide of rice amylase 3D gene against the pathogenic fungus *Sclerotium rolfsii* under *in vitro* conditions. The higher chitinase activity of two synthetic genes in peanut roots promises potential applications in the field of transgenic crops against phytopathogenic fungi.

Introduction

Chitinases (EC 3.2.2.14) are enzymes that break down the linear polymer of N-acetyl-D-glucosamine monomers known as chitin (Wang and Yang 2007). Chitinases may be found in a wide range of organisms, including bacteria, fungus, animals, and plants (Ramos and Malcata 2011; Zarei et al. 2011; Hamid et al. 2013; Veliz et al. 2017). Because of their propensity to secrete extracellular chitinases, *Trichoderma* species are widely utilized as biocontrol agents of phytopathogenic fungi with chitin in cell walls (Abdel-lateif 2017; Poveda 2021).

Peanut (*Arachis hypogaea* L.), which belongs to the family Fabaceae (or Leguminosae), is a high-value grain legume crop derived from Central Brazil and widely cultivated in tropical and subtropical regions for its edible oil and seeds (Pal et al. 2014; Singh et al. 2021). It is, however, one of the crops sensitive to serious diseases like stem rot, root rot, and pod rot caused by a variety of soilborne pathogens such *Rhizoctonia solani*, *Aspergillus niger*, and *Sclerotium rolfsii* (Ismail et al. 2007; Gour et al. 2012; Thiessen et al. 2012; Xu et al. 2015).

The method of using *Agrobacterium tumefaciens* to transfer a gene of interest into plant cells, resulting in transgenic plants, is known as *Agrobacterium*-mediated transformation (Gelvin 2003). Because of the advantages of transferring pieces of DNA with defined ends and minimal rearrangement, transferring relatively large segments of DNA, integrating small numbers of copies of genes into plant chromosomes, and the high quality and fertility of transgenic plants, *Agrobacterium*-mediated transformation has become the most widely used method for transferring genes into plants (De La Riva et al. 1998; Pratiwi and Surya 2020).

Even though certain chitinase genes from *Trichoderma* or other species, such as rice and tobacco, have been incorporated into various crops to aid them against fungal infections (Nishizawa et al. 1999;

Takahashi et al. 2005; Gentile et al. 2007; Baranski et al. 2008; Zarinpanjeh et al. 2016; Ojaghian et al. 2018; Ojaghian et al. 2020). To date, there has been no record of chitinase gene transfer from *Trichoderma*, especially *T. asperellum*, into peanuts.

As a consequence, the present study might be the first to employ *Agrobacterium*-mediated transformation to transfer the chitinase gene from *Trichoderma* to peanuts in order to increase their antifungal activity. Different chitinase genes were employed in this study, including one wild-type gene, *Chi42*, from *T. asperellum* SH16, which encodes chitinase 42 kDa, and two synthetic genes (*syncodChi42-1* and *syncodChi42-2*) generated from the *Chi42* gene by optimizing codon usage for plant expression (Luong et al. 2021). The goal of this study is to identify high levels of expression of the two synthetic genes regulated by the tissue-specific promoter (pAsy) in peanut roots, as well as their antifungal efficacy. As a result, peanuts with the *T. asperellum* SH16 42 kDa chitinase gene may be resistant to *S. rolfsii* phytopathogenic fungus.

Materials And Methods

Plant materials

Peanut (*A. hypogaea* L.) cultivar L14 from the Vietnam Academy of Agricultural Sciences' Field Crops Research Institute was utilized in this study (Hoa et al. 2021a). The seed coat was removed after 1 min soaks in 70% EtOH and 10 min sterilization with 65% NaClO/100 µL Tween 20 solution. The embryonal and de-embryonal cotyledons were separated from the seeds longitudinally for use as explants in *Agrobacterium* transformation.

Plant expression binary vector

In the present work, the *Agrobacterium tumefaciens* strain LBA4404 was employed, which contained the pNHL20 vector harboring the chitinase genes *(Chi42, syncodChi42-1,* and *syncodChi42-2)* expressing 42 kDa chitinase (Tue et al. 2021). *Chi42* (HM191683.1) is a wild-type gene from *T. asperellum* SH16 (Loc et al. 2011). Both *syncodChi42-1* (MT083802.1) and *syncodChi42-2* (MT083803.1) are synthetic genes derived from the *Chi42* gene optimized for codon usage for plant expression (Luong et al. 2021). Chitinase genes were driven by the root-specific Asy promoter from peanut (Geng et al. 2014). A signal peptide of amylase 3D gene from rice (Jung et al. 2016) was situated at the 5' end of the genes guides newly synthesized chitinase to the periplasm for extracellular secretion (Fig 1).

Briefly, the COOL program was used to optimize the *chi42* gene for plant expression (Chin *et al*. 2014). The optimization algorithm included maximizing the codon context, ignoring individual codon usage, destabilizing the mRNA secondary structure at 5' termini, and limiting the presence of restriction enzyme sequences. Two sequences with high codon context scores (named *syncodChi42-1* and *syncodChi42-2*) were randomly selected for synthesis (PHUSA Biochem Co).

Agrobacterium transformation

The *Agrobacterium* transformation procedure was followed as reported in our previous study (Hoa et al. 2021b). Briefly, explants were infected for 20 min with *A. tumefaciens* LBA4404 carrying the pNHL20 vector, then co-cultured on TDT medium supplemented with 66.6 µM BAP for de-embryonal cotyledon or 66.6 µM BAP and 9.1 µM 2,4-D for embryonal cotyledon at 25±2°C for 3 days in the dark. TDT medium was made up of 200 µg/mL acetosyringone, 3% sucrose, 0.8% agar, and MS basal medium (Murashige and Skoog 1962). After co-culture, explants were subcultured on the same medium with 100 mg/L kanamycin and 250 mg/L cefotaxime for screening transformants. Shoots were isolated from the screening culture to multiply on MS medium supplemented with 3% sucrose, 0.8% agar, 8.9 µM BAP, 0.6 µM IAA, and 100 mg/L kanamycin. Finally, single shoots from the shoot cluster were grown into whole plants on MS medium with 5.4 µM NAA and no antibiotics. Except for *Agrobacterium* treatment, all *in vitro* cultures were maintained at 25±2°C for 4 weeks at a light intensity of 2000-3000 lux and 16 h of daylight.

PCR amplification

The CTAB method was used to extract genomic DNA from *in vitro* transgenic peanut leaves, as reported by Clarke (2019). PCR amplification was carried out using specific primers for DNA segments that served as chitinase gene indicators (Table 1). The reaction is made up of 20 ng of genomic DNA as a template, 10 pmol of each primer, 1 µL of Master Mix (Thermo Scientific), and water added to a final volume of 12 µL. The PCR settings were as follows: 15 min of genomic denaturation at 95°C, then 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min.

SDS-PAGE and dot blot analysis

The TSP from *in vitro* transgenic peanut roots was extracted with phosphate buffer (pH 7) and quantified using Bradford's assay (1976). 50 µg TSP was denatured for 10 min at 95°C before fractionation by SDS-PAGE. After that, Coomassie blue was used to stain the gel. For dot blot analysis, an equivalent amount of TSP was loaded onto a nitrocellulose membrane (Novex^{TM}-Thermo Fisher Scientific). The non-specific binding on blot was blocked by 5% skim milk (Sigma-Aldrich). The primary antibody was a mouse anti-Ta-CHI42 polyclonal antibody diluted 1:2000 in TBST, Ta-CHI42 is recombinant chitinase 42 kDa derived from fungus *T. asperellum* SH16 (Luong et al. 2021). The secondary antibody was a 1:5000 dilution of goat anti-mouse IgG antibody conjugated with alkaline phosphatase (AbD Serotec-currently Bio-Rad Antibodies). The dot signals on the blot were developed with BCIP/NBT solution (Sigma-Aldrich, Cat No B6404) for 10 min in the dark.

Chitinase assay

The plate assay method was used to *preliminarily* evaluate the chitinolytic activity of plant chitinase. The pre-punched holes on the assay plate containing 1.5% agar and 1.2% colloidal chitin were filled with 50 µg TSP (crude chitinase). Colloidal chitin was made according to Murthy and Bleakley's method (2012). The assay plate was incubated at 4°C for 8 h for diffusing enzyme, and then at 28°C for 6 h for chitinolysis. The plate was colored with 0.1% Lugol's solution after incubation to detect chitin

degradation (Calissendorff and Falhammar 2017). 10 U/mL of bacterial purified chitinase 42 kDa was used as a positive control. 50 µg TSP of the extract from the non-transgenic root was used as a negative control.

The chitinase activity was determined by measuring the absorbance of *p*-nitrophenol at 420 nm (Tsujibo et al. 1998). A reaction mixture containing 50 µg TSP and 15 µL of 2.5 mM pNpGlcNAc (Merck) as substrate was incubated at 45°C for 10 min. After that, the hydrolysis was stopped using 1 mM of 0.2 M sodium carbonate. The amount of chitinase required to release 1 µmol of *p*-nitrophenol from pNpGlcNAc per minute was defined as one activity unit. A *p*-nitrophenol standard purchased from Merck was used to make the calibration curve. The specific activity (U/mg protein) of an enzyme is determined by dividing its total activity (U/mL) by the TSP content (mg/mL).

In vitro antifungal activity

In vitro roots of chitinase transgenic peanuts were transferred in 1/2 PDA medium containing 10^4 *S. rolfsii* spore for evaluating their antifungal activity based on mycelium growth inhibition. Incubation was performed at 28°C for 96 h. 20 µL (10 U/mL) of bacterial purified chitinase 42 kDa was used as a positive control, sterile distilled water and non-transgenic peanut roots were used as negative controls.

Statistics

Experiments were carried out with the tri-replicate. The data were given as the means and the findings were evaluated using one-way ANOVA with Duncan's test at p = 0.05.

Results And Discussion

Agrobacterium transformation

Table 2 shows that after 4 weeks of culture, 303 surviving shoots were obtained from two types of transformed explants on a selective medium containing kanamycin and cefotaxime (Fig. 2). Among them, 207 shoots were from embryonal explants (68 for *Chi42*, 74 for *syncodChi42-1*, and 65 for *syncodChi42-2*) and the rest were from de-embryonal explants (33 for *Chi42*, 36 for *syncodChi42-1*, and 27 for *syncodChi42-2*). However, PCR amplification revealed that there were much fewer shoots with the chitinase gene. In embryonal cotyledons, it was just 48.5% for *Chi42*, 52.7% for *syncodChi42-1*, and 40% for *syncodChi42-2*, whereas in de-embryonal cotyledons, it was 54.5% for *Chi42*, 61.1% for *syncodChi42-1*, and 55.6% for *syncodChi42-2* (Fig. 3).

In a recent study, Iqbal et al (2012) obtained 70% of surviving plantlets on the selective medium from cotyledonary nodes of peanut and 40% of which were putatively *chitinase-3* transgenic plants. Then, Prasad et al (2013) also obtained a total of 65 regenerated shoots from peanut mature cotyledons which were transferred rice *Rchit* gene encoding chitinase. While Sharma and Anjaiah (2000) achieved a significant percentage (55%) of transgenic peanuts cultivar JL-24 from de-embryonal cotyledons through

Agrobacterium transformation. These data seem to suggest that genotype and explant type had an impact on transformation efficiency. Although the ratio of shoot regeneration on the selective medium is important, the number of transgenic plants and their gene expression level are the determining factors. Of fact, *Agrobacterium*-mediated transformation is possible with other types of peanut explants. Iqbal et al (2012) obtained an efficiency of 42% for cotyledonary node explants. Whereas it is around 63 and 62% for embryo cotyledons or around 72 and 77% for mesocotyl-derived explants of two peanut cultivars Huayu 20 and 26 (Chen et al. 2015).

Expression of chitinase 42 kDa

The expression of three genes encoding chitinase 42 kDa in transgenic peanuts was examined using SDS-PAGE and dot blot analysis. Figure 4 illustrates several protein bands with a molecular weight of 42 kDa, as expected for *T. asperellum* SH16 chitinase, from various samples on the polyacrylamide gel. Transgenic peanut lines with putative recombinant chitinase were chosen for dot blot analysis. Dot signals were observed in the majority tested transgenic peanut lines and positive control, except for the non-transgenic negative control and a few others (Fig. 5). In this work, the gene *syncodChi42-2* (line S2A-8) still has the strongest dot signal like our earlier study employing the 35S promoter to drive the expression of the chitinase gene in peanuts (Hoa et al. 2021). These findings imply that chitinase 42 kDa genes were successfully expressed in transgenic peanuts. However, some transgenic individuals were unable to produce or only produced a small amount of chitinase 42 kDa due to the position effect, resulting in no or weak signals on the blots (Betts et al. 2019, Pérez-González and Caro 2019). To our knowledge, until now no studies have employed antigen-antibody interaction, such as dot blot, to determine chitinase gene expression in transgenic peanuts. The majority of studies have focussed on transcriptional expression or chitinase activity in transgenic peanuts, as well as their antifungal effectiveness (Sharma and Anjaiah 2000; Iqbal et al. 2012; Prasad et al. 2013; Chen et al. 2015; ul Hassan et al. 2016).

Chitinase activity assay

Colloidal chitin hydrolysis activity of chitinase 42 kDa from transgenic peanuts was determined using the agar plate assay. The transgenic peanut lines with the highest chitinase expression are shown in Figure 6. The largest *D-d* difference (about 2.5 cm) was found in *syncodChi42-2* transgenic peanuts (line S2A-8), which was 1.3- and 1.7-fold greater than *syncodChi42-1* (line S1A-9) and *Chi42* (line WTA-2) transgenic peanuts, respectively. Where *D* denotes the clear zone diameter and *d* denotes the diameter of the pre-punched hole for loading enzyme. Whereas in the non-transgenic control, hydrolysis was negligible (Fig. 6). These findings suggest that chitinase 42 kDa was present in transgenic peanuts in an active form.

Line S2A-8 also displayed the highest chitinase specific activity of 1124 U/mg among transgenic peanuts regenerated from embryonal cotyledon, 1.25 and 2.21 times higher than line S1A-9 and line WTA-2, respectively. However, their total activity revealed the opposite result, with the line S1A-9 (140 U/mL) being approximately 1.4 and 1.6 times higher than the lines S2A-8 (100 U/mL) and WTA-2 (87 U/mL), respectively. The non-transgenic control had the lowest chitinase activity, only 53.8 U/mg (31.03

U/mL). Overall, the chitinase expression levels in the transgenic peanuts and the tested genes were different. In every case, the synthetic genes *syncodChi42-1* and *syncodChi42-2* were expressed stronger than the wild-type *Chi42* gene. Plants do not or only produce minimal quantities of chitinase unless they are triggered by a fungal disease, according to several studies (Grover 2012; Collinge et al. 1993; Punja and Zhang 1993). In the present study, the root extract from *in vitro* non-transgenic and chitinase transgenic peanuts without fungal disease infection was used to determine chitinolytic activity on the colloidal chitin plate and chitinase activity. Although non-transgenic peanuts also produced chitinase, it was only in negligible amounts, so the chitinase activity was low and the hydrolysis region was small.

Peanuts with the *syncodChi42-2* gene driven by the 35S promoter also exhibited the higher chitinase activity compared to *syncodChi42-1* and *Chi42*, according to a recent study (Hoa et al. 2021). They peaked at 823 U/mg, which is 1.4 times lower than the gene regulated by the Asy promoter in this study. This demonstrates that the methods employed to optimize codon use for plant expression in previous work (Luong et al. 2021), as well as the control of genes by the root-specific promoter in this study, were successful in peanuts.

Prasad et al (2012) and Iqbal et al (2012) improved the chitinase activity of peanuts up to 2-14 times and 1.8-6.5 times higher than non-transgenic controls, respectively, by transferring the *Rchit* and chitinase genes from rice. Recently, Tien et al (2021) also reported that transient expression of *syncodChi42-1* and *syncodChi42-2* synthetic genes in *Nicotiana benthamiana* was 1.7 and 2.6 times greater than wild-type *Chi42* gene, respectively.

In vitro antifungal activity of chitinase

To assess antifungal effectiveness, WTA-2, S1A-9, and S2A-8 transgenic peanut lines with the highest chitinase activity were chosen. After 96 h of treatment, the transgenic roots of all three peanut lines completely inhibited the growth of *S. rolfsii*, which causes white mold wilt disease. The positive control also showed similar findings. While *S. rolfsii* grew strongly in treatments for negative controls (Fig. 8). This investigation revealed that chitinase had significant antifungal activity against *S. rolfsii* in all three transgenic peanut lines examined, with no differences found. Our results also demonstrated that the signal peptide of the rice amylase 3D gene was effectively active in chitinase transgenic peanut roots. Peanut recombinant chitinase was extracellularly secreted against *S. rolfsii*. Comparing Figures 8B, it seems that non-transgenic peanut chitinase (Fig. 8C) was also produced but not enough to completely inhibit the fungus growth.

According to studies conducted by Rohini et al (2001) and Iqbal et al (2012), chitinase transgenic peanuts outperformed non-transgenic controls in terms of resistance to *Cercospora arachidicola*, an ascomycete that causes early leaf spots in peanuts. Prasad et al (2012) discovered that only 0-10% of *Ritch* transgenic peanut seeds were infected with *Aspergillus flavus* under *in vitro* seed inoculation tests.

Conclusion

The chitinase 42 kDa genes from *T. asperellum* SH16, which are driven by the root-specific Asy promoter, were successfully heterologously expressed in peanut cultivar L14. All three genes, one wild-type (*Chi42*) and two synthetic genes (*syncodChi42-1* and *syncodChi42-2*) were secreted extracellularly by the guidance of a signal peptide of the rice amylase 3D gene. Chitinase activity of two synthetic genes outperformed the wild-type gene but all of them exhibited a strong antifungal activity for *S. rolfsii*. These findings suggest that the *Chi42*-derived optimized chitinase genes might aid peanuts to fight the phytopathogenic fungus *S. rolfsii*.

Abbreviations

2,4-D: 2,4-dichlorophenoxyacetic acid

ANOVA: analysis of variance

BAP: benzylaminopurine

BCIP: 5-bromo, 4-chloro, 3-indolyl phosphate

CTAB: cetyltrimethylammonium bromide

EtOH: ethanol

IAA: indoleacetic acid

NAA: naphthaleneacetic acid

NaClO: s*odium hypochlorite*

NBT: nitro-blue tetrazolium

PDA: potato dextrose agar

pNpGlcNAc: 4-nitrophenyl-N-acetyl-β-D-glucosaminide

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TBST: Tris-buffered saline with Tween 20

TSP: total soluble protein

Declarations

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Conflict of interest

The authors declare that they have no conflicts of interest.

Compliance with ethical standards

Authors' contributions

NHL and NQDT designed this study. NQDT, NXH and NHT designed plant expression vector. NHT performed the triparental mating experiment. PTBH, NHT, HTQT, HAT and LNHN performed *in vitro* culture and transformation experiments. PTBH and NNL performed gene expression analyses. NHL prepared the manuscript. All authors have read and approved the manuscript.

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Tables

Table 1
Oligonucleotide sequence of specific primers for indicators of chitinase genes.

Genes	Directions	Sequences (5'-3')	PCR products (bp)
Chi42	Forward	TGGTACTATGCAGCTTGACCT	505
	Reverse	GTACTCCCAGTCGACGTCAA	
SyncodChi42-1	Forward	TGGTACTATGCAGCTTGACCT	689
	Reverse	CGTAATCCAAGACTTGACCCAG	
SyncodChi42-2	Forward	TGGTACTATGCAGCTTGACCT	931
	Reverse	AGAACCGGAACCAATACCATT	

Note: TGGTACTATGCAGCTTGACCT sequence of all three forward primers was located in signal peptide segment of rice amylase 3D gene (Jung et al. 2016) that flanked 5' end of chitinase genes (data not shown). The signal peptide was used for the extracellular expression of the enzyme chitinase.

Table 2
Chitinase transgenic efficiency in peanuts through embryonal and de-embryonal cotyledon.

Explant type	Gene	Number of samples	Number of shoot regeneration samples	Number of shoot/sample	Number of surviving shoots	Number of shoots with positive PCR
Embryonal cotyledon	*Chi42*	200	200	4.26	68	33
	SyncodChi42-1	200	200	4.07	74	39
	SyncodChi42-2	200	200	4.01	65	26
De-embryonal cotyledon	*Chi42*	200	35	7.29	33	18
	SyncodChi42-1	200	36	7.05	36	22
	SyncodChi42-2	200	30	6.23	27	15

Figures

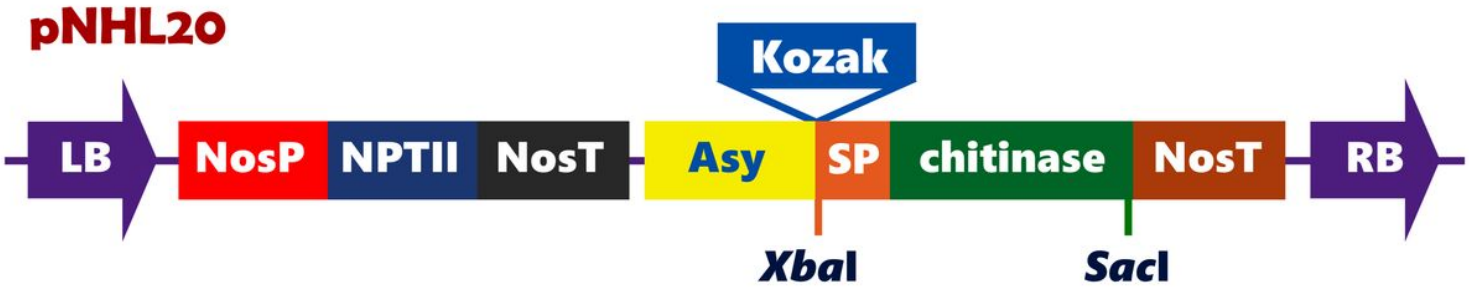

Figure 1

A signal peptide of amylase 3D gene from rice (Jung et al. 2016) was situated at the 5’ end of the genes guides newly synthesized chitinase to the periplasm for extracellular secretion (Fig 1).

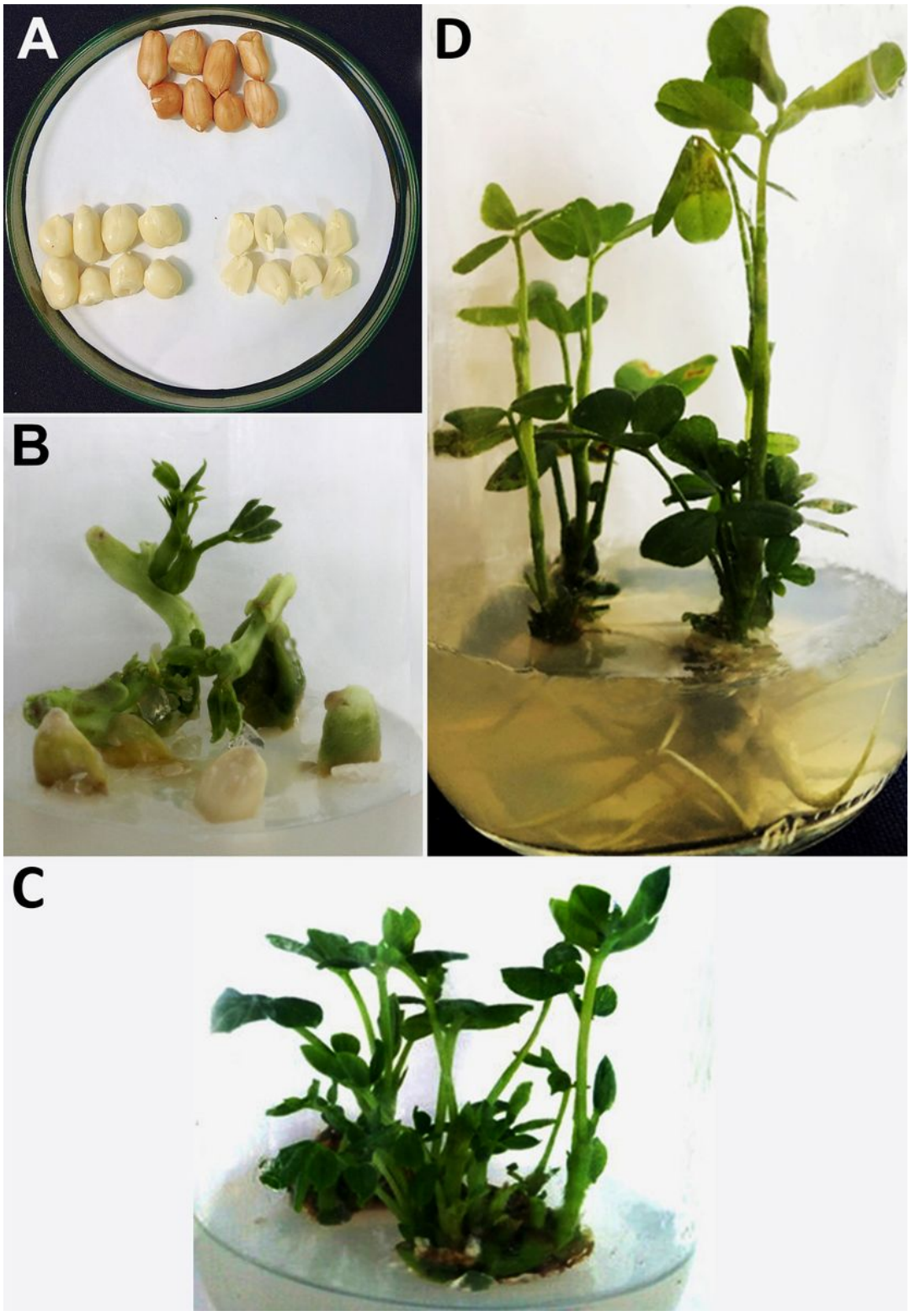

Figure 2

Table 2 shows that after 4 weeks of culture, 303 surviving shoots were obtained from two types of transformed explants on a selective medium containing kanamycin and cefotaxime (Fig. 2).

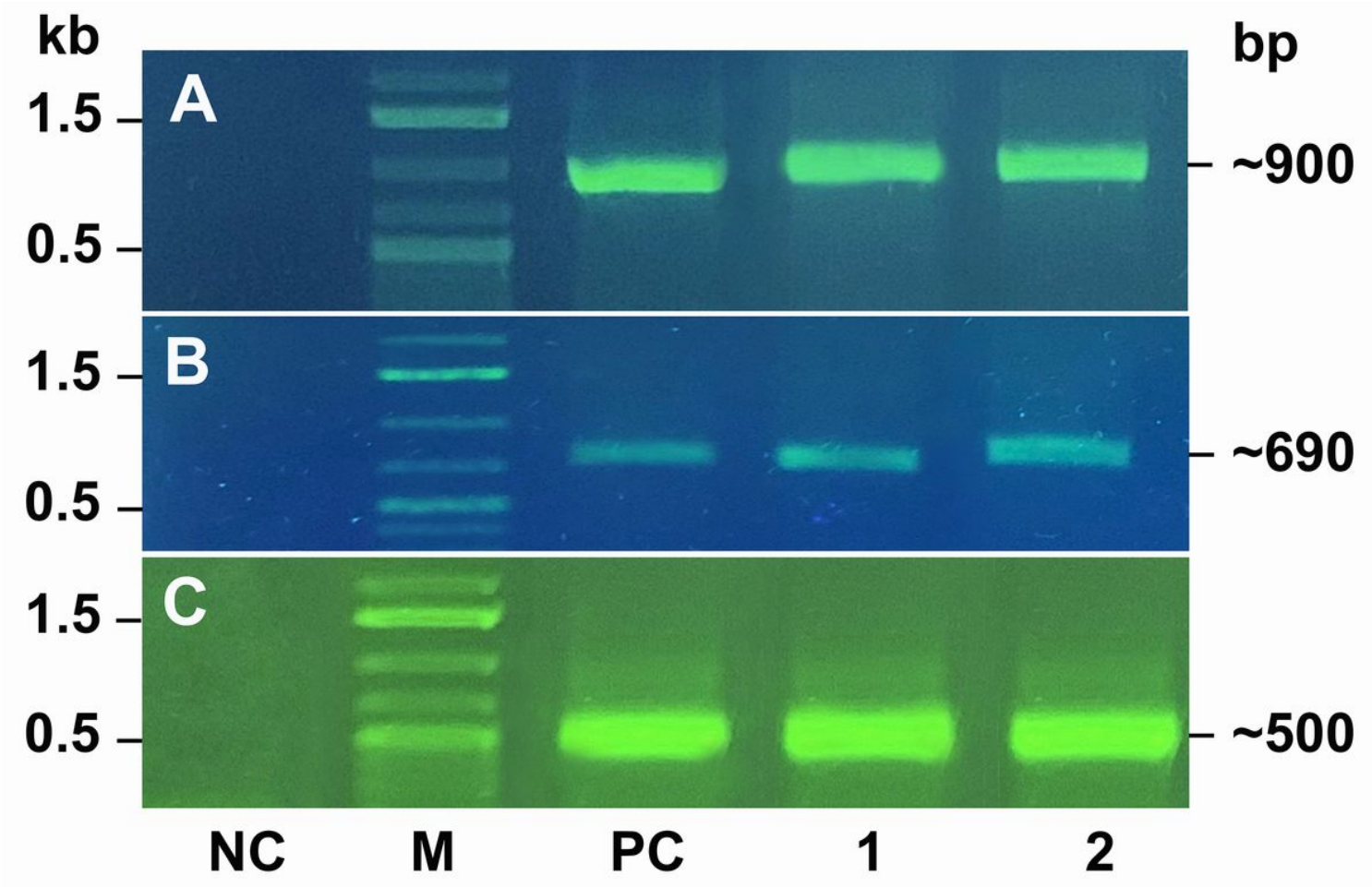

Figure 3

In embryonal cotyledons, it was just 48.5% for Chi42, 52.7% for syncodChi42-1, and 40% for syncodChi42-2, whereas in de-embryonal cotyledons, it was 54.5% for Chi42, 61.1% for syncodChi42-1, and 55.6% for syncodChi42-2 (Fig. 3).

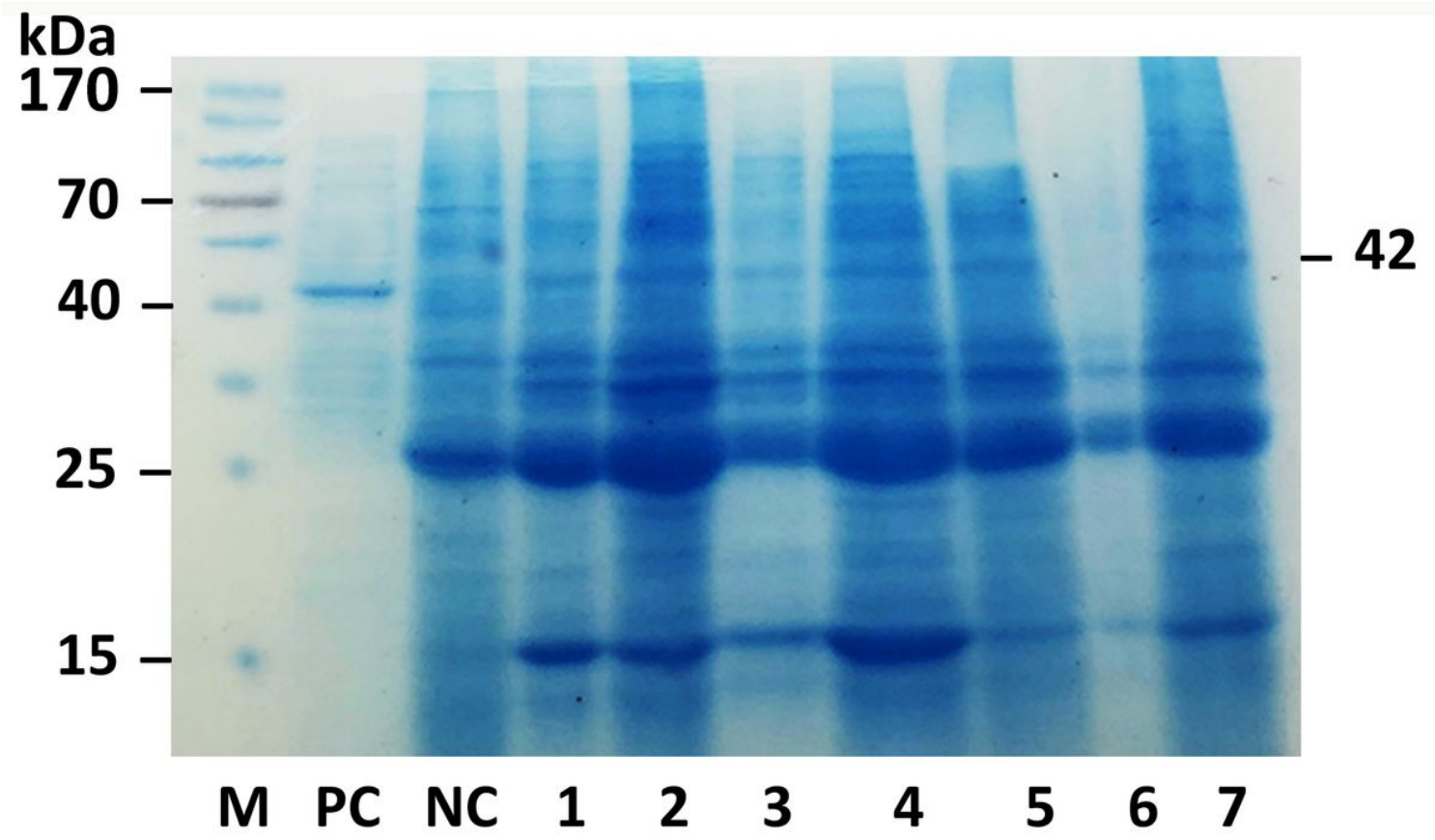

Figure 4

Figure 4 illustrates several protein bands with a molecular weight of 42 kDa, as expected for T. asperellum SH16 chitinase, from various samples on the polyacrylamide gel.

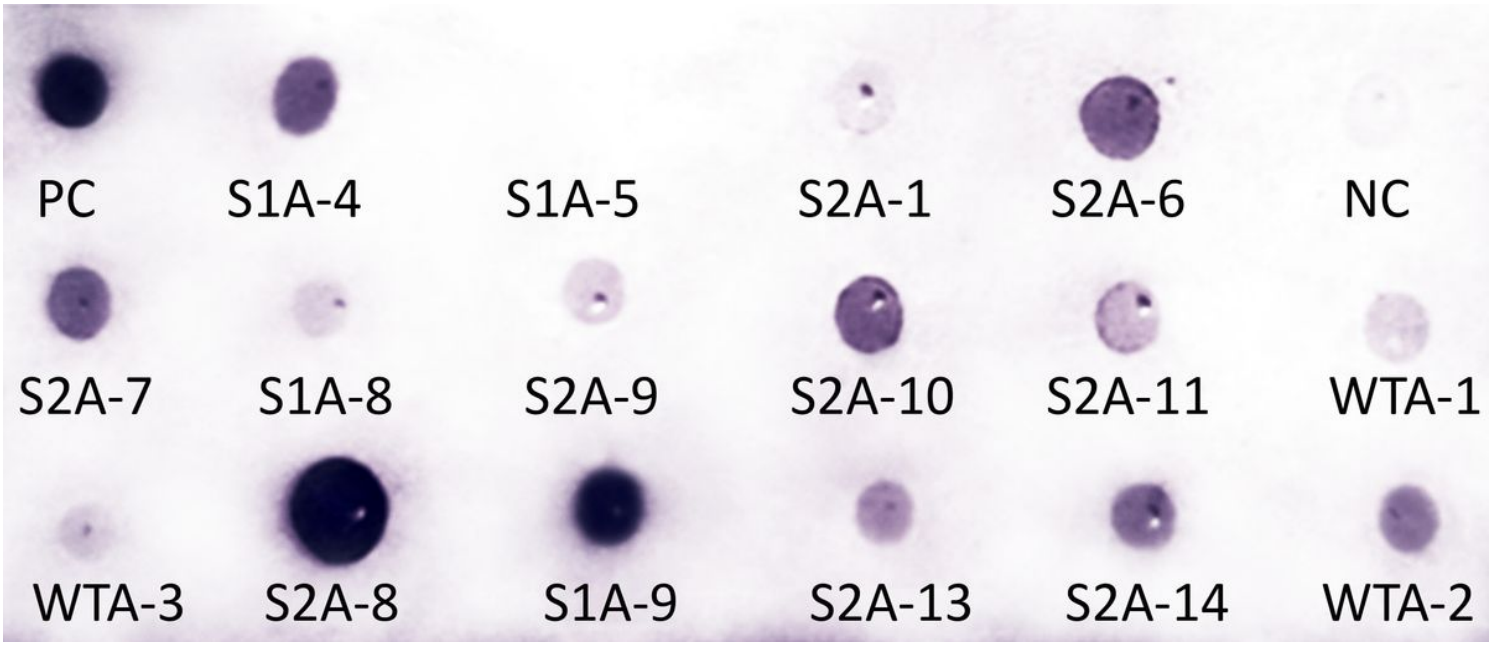

Figure 5

Transgenic peanut lines with putative recombinant chitinase were chosen for dot blot analysis. Dot signals were observed in the majority tested transgenic peanut lines and positive control, except for the non-transgenic negative control and a few others (Fig. 5).

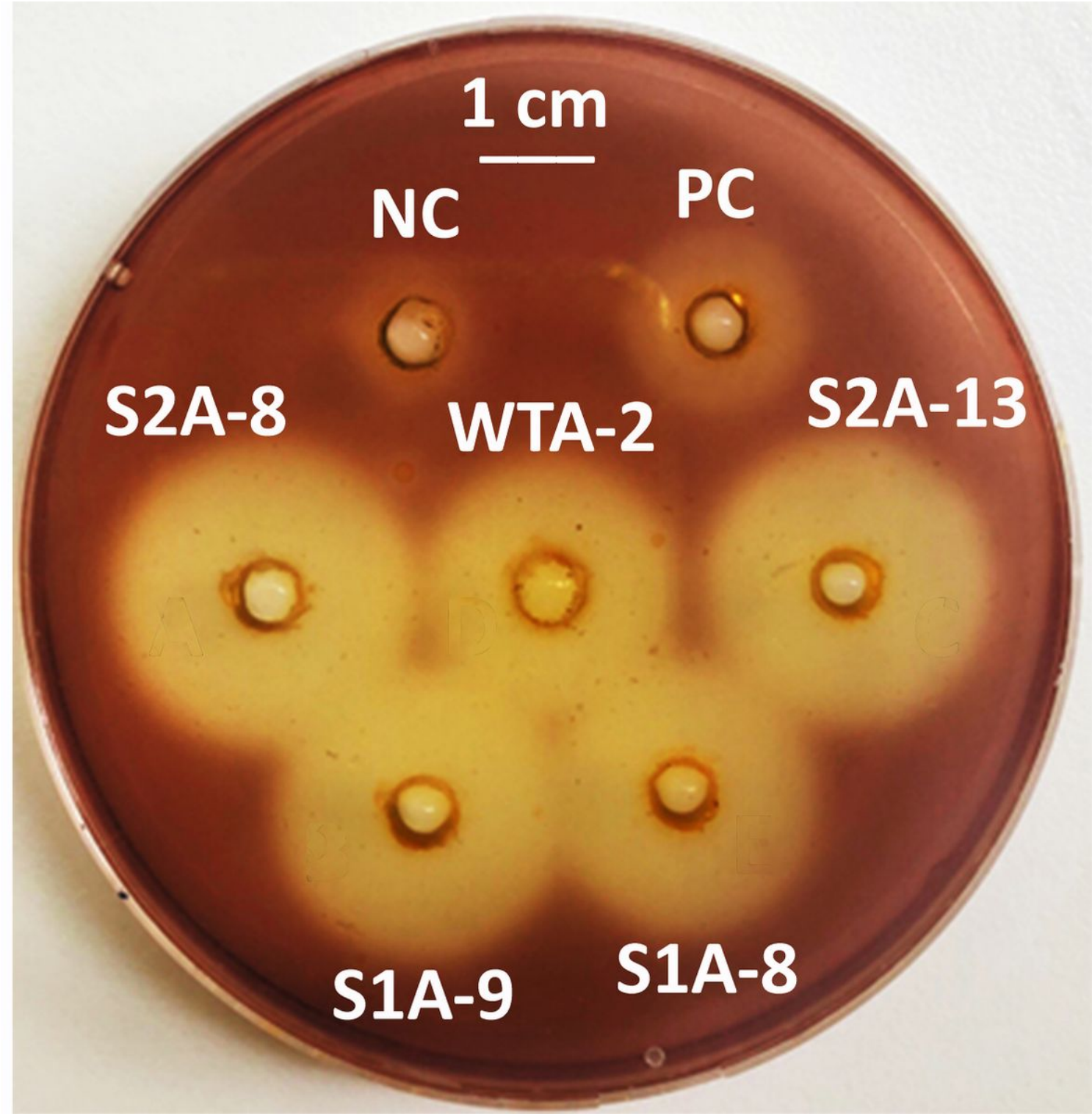

Figure 6

Colloidal chitin hydrolysis activity of chitinase 42 kDa from transgenic peanuts was determined using the agar plate assay. The transgenic peanut lines with the highest chitinase expression are shown in Figure 6.

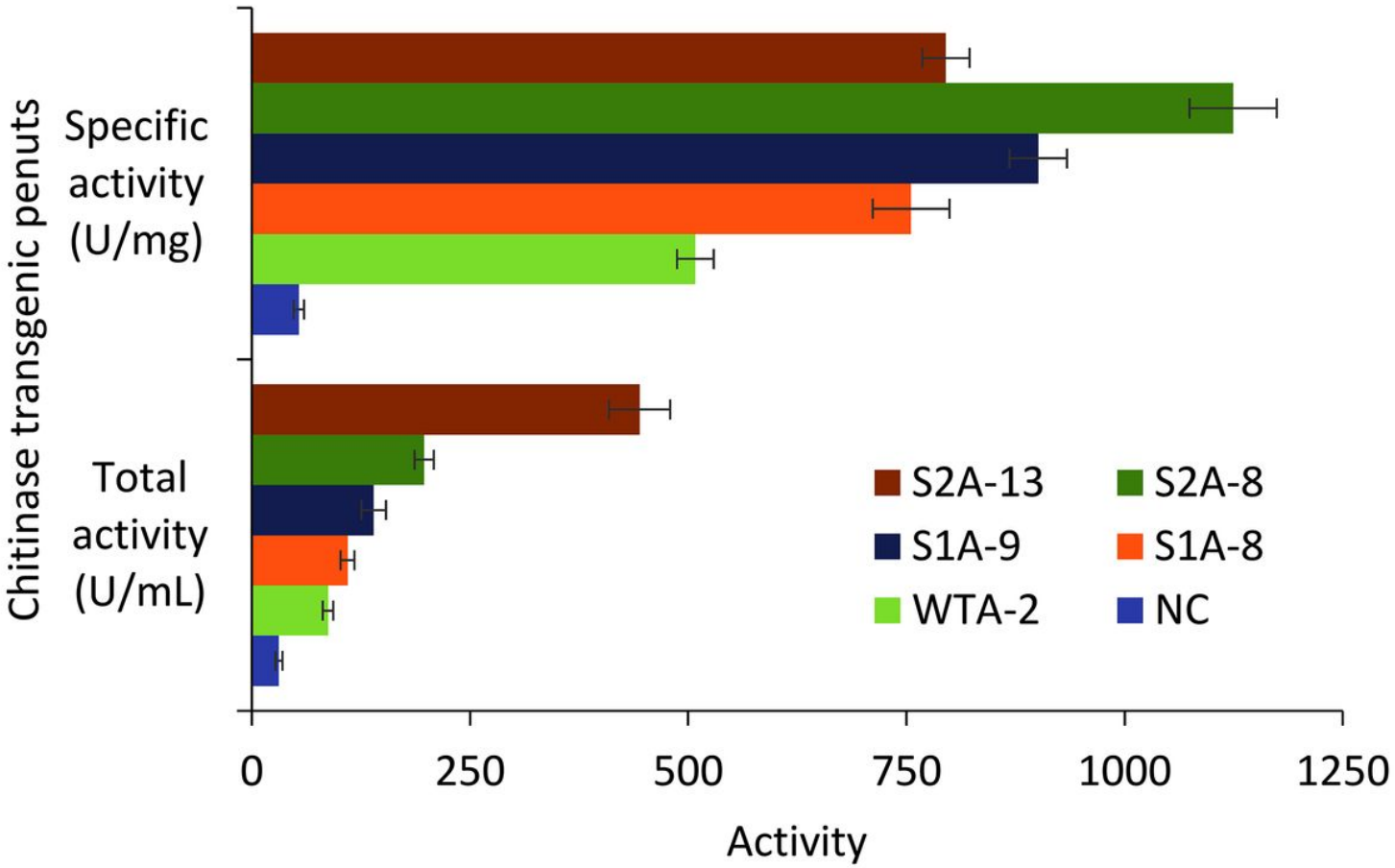

Figure 7

The largest D-d difference (about 2.5 cm) was found in syncodChi42-2 transgenic peanuts (line S2A-8), which was 1.3- and 1.7-fold greater than syncodChi42-1 (line S1A-9) and Chi42 (line WTA-2) transgenic peanuts, respectively. Where D denotes the clear zone diameter and d denotes the diameter of the pre-punched hole for loading enzyme. Whereas in the non-transgenic control, hydrolysis was negligible (Fig. 7).

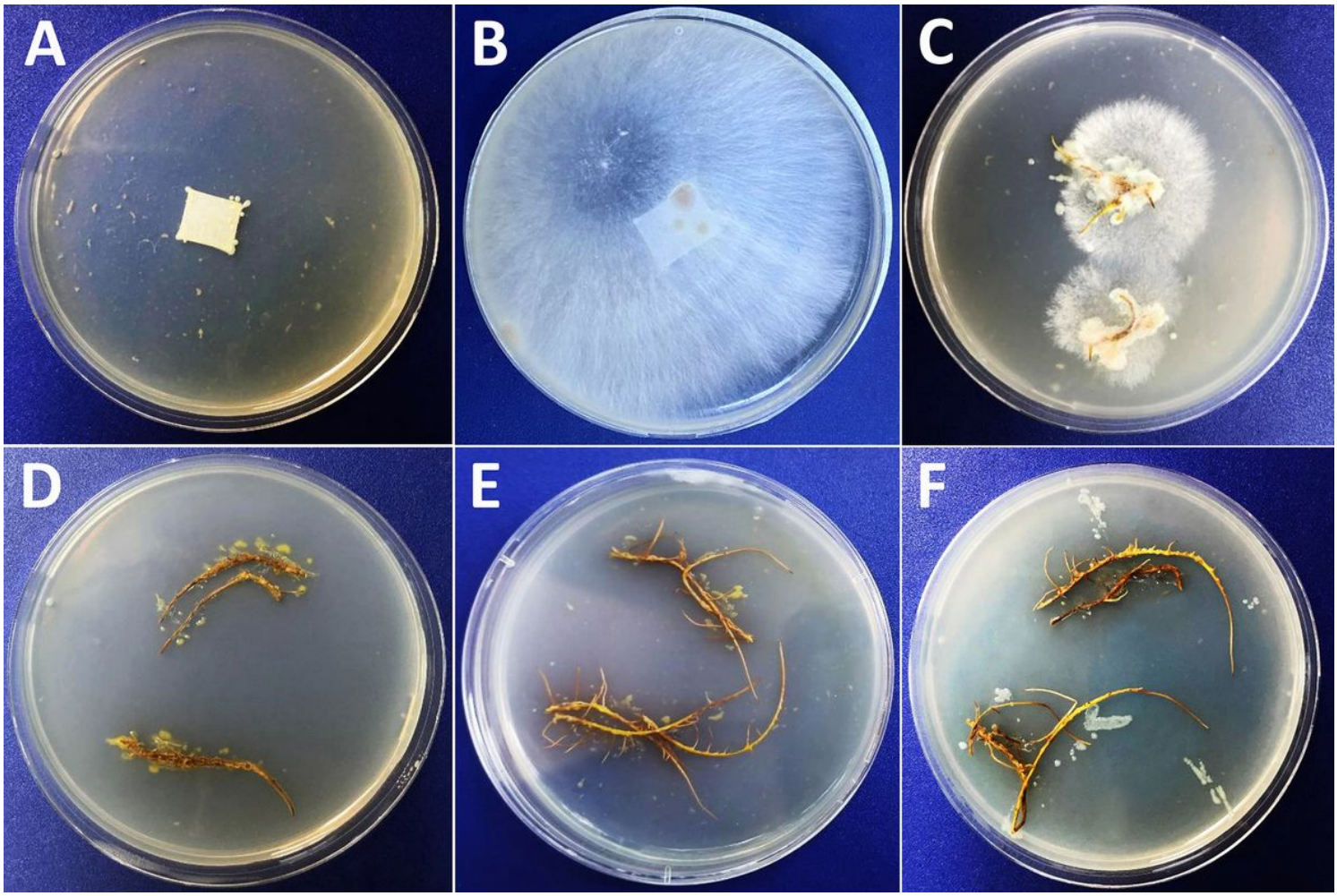

Figure 8

The positive control also showed similar findings. While S. rolfsii grew strongly in treatments for negative controls (Fig. 8). This investigation revealed that chitinase had significant antifungal activity against S. rolfsii in all three transgenic peanut lines examined, with no differences found. Our results also demonstrated that the signal peptide of the rice amylase 3D gene was effectively active in chitinase transgenic peanut roots. Peanut recombinant chitinase was extracellularly secreted against S. rolfsii. Comparing Figures 8B, it seems that non-transgenic peanut chitinase (Fig. 8C) was also produced but not enough to completely inhibit the fungus growth.