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Growth and development of transgenic peanut (*Arachis hypogaea*) lines containing chitinase 42 kDa gene from *Trichoderma asperellum* SH16

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Transgenic peanut lines

Yield

ABSTRACT

Peanut (*Arachis hypogaea* L.) is vulnerable to many diseases. Vietnam and other regions where peanut is widely cultivated have a high threat of fungal and other plant diseases. Various fungicides are available to control the fungal disease but these have various harmful effects on the natural flora, fauna, and environment. Transgenic peanut lines which possess antifungal activity provide a possible solution in managing fungal diseases apart from the traditional resistance and fungicide usage. Therefore, this study evaluated the probable growth and development of chitinase transgenic peanut lines against *Sclerotium rolfsii*, a pathogen that causes "southern blight" in plants, under greenhouse conditions. This study provided evidence that through *Agrobacterium itumefaciens* mediated transformation, 42 kDa chitinase genes from *Trichoderma asperellum*, which is under the regulation of 35S promoter, were successfully incorporated into the peanut's (*A. hypogaea* L.) genome and expressed in their plants. This evidence also demonstrated that transgenic peanut lines were suitable for growing and developing in the greenhouse. Further, it was reported that transgenic peanut lines took approximately 133 to 145 days from planting to maturity. These results also revealed that various growth characteristics of transgenic peanut lines having two synthetic genes (*syncod Chi42-2* i.e. S2-2, S2-4, S2-6, and *syncod Chi42-1* i.e. S1-1, S1-2, S1-3) were greater than that from the wild-type *Chi42* (WT-1, WT-2, and WT-3). In addition, yield-related parameters including the number of mature pods, 100 pods weight and 100 seeds weight for

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all the transgenic peanut lines were higher than that of the non-transformed plant. Among the transgenic lines, line S2-4 exhibited significantly higher growth and yield than the other transgenic lines. These results demonstrated that 42 kDa chitinase genes overexpressing peanut lines could be a candidate for improvement against plants to phytopathogenic fungus *S. rolfsii* and high yield.

1 Introduction

Peanut (*Arachis hypogaea* L.) is an annual leguminous plant that is cultivated in many countries around the world. In Vietnam, it is one of the most crucial oil seed crops with a total cultivating area of 177.043 hectares and a productivity of 0.44 million tons in 2019 (FAO 2020). Despite its values, peanut cultivation is hampered by many pathogens. The most harmful soil-borne pathogen of groundnut is root-and stem-rot caused by *Sclerotium rolfsii*. Further, S. *rolfsii* is difficult to control as a result of its wide variety of hosts (Javaid et al. 2021; Sharf et al. 2021) and persistent sclerotia (Kumar et al. 2012). This fungus is mostly associated with the stem and pod rot of peanuts and might cause 10 – 25% pod yield losses which sometimes reached up to 80% (Mehan et al. 1994). Currently, only a few resistant cultivars are commercially available (Branch and Brenneman 1999; 2009; Woodward et al. 2008). Control of stem rot disease mostly relies on cultural practices and fungicide treatment. However, cultural practices are not always effective due to the wide range of pathogens. Besides, fungicides are often too expensive for local groundnut farmers in Vietnam.

Nowadays, with the advancement of agricultural biotechnology, scientists are developing more and more new transgenic crop plants having desired qualities such as higher yield, resistance to insects, phytopathogenic fungi, and diseases. By using these technologies, the different origins derived chitinase genes have been successfully transformed into different types of plants such as rice (Lin et al. 1995), tobacco (Zhu et al. 1994), cucumber (Kishimotoiet al. 2002), Italian ryegrass I (Takahashi et al. 2005), banana (Sreeramanan et al. 2009), cotton (Ganesan et al. 2009), and peanut I (Chu et al. 2008, 2013) and developed the ability of fungal resistance in these crops. Though various attempts were made to enhance the fungal resistance in groundnut by utilizing tobacco chitinase (Rohini and Rao 2001), barley oxalate oxidase (Livingstone et al. 2005), mustard of defensin (Anuradha et al. 2008) and β-1,3-glucanase from tobacco (Sundaresha et al. 2009), there are currently no reports regarding the usage of 42 kDa chitinase genes from *Trichoderma asperellum* SH16, except for those published by Loc et al. (2022), Hoa et al. (2022a) and Tue et al. (2022). The antifungal activity of peanut-containing 42 kDa chitinase genes (Hoa et al. 2022b) and two genes *(syncod Chi42-1 and syncod Chi42-2)* were codons optimized for expression in the plant from the *Chi42* gene (Luong et al. 2021) were reported in these two types of research. Therefore, this study aimed to evaluate the growth and development rate under greenhouse conditions of the three previously mentioned transgenic peanut lines. The transgenic plants with an increase in chitinase activity could become a valuable source of biocontrol genes against plant-pathogenic fungi.

2 Materials and Methods

2.1 Plant Materials

Nine chitinase transgenic peanut lines were used as test materials in the present study. Peanut varieties having the plant expression vector pMYV719 harboring three genes (*Chi42*, *syncodChi42-1*, and *syncodChi42-2*) expressing i42 kDa chitinase were used in this study I (Loc et al. 2022).

2.2 Greenhouse experiments

Nine transgenic peanut lines containing *Chi42* (WT-1, WT-2, WT-3), *syncodChi42-1* (S1-1, S1-2, S1-3), and *syncodChi42-2* (S2-2, S2-4, S2-6) transgenic gene and one non-transgenic control (NC) were planted in greenhouse conditions at Institute of Bioactive Compounds and Department of Biotechnology, University of Sciences, Hue University, Hue, Vietnam. Tissue-cultured peanuts have enough stems, leaves, roots, and height (6 – 8 cm) and are grown under greenhouse conditions. Cultured plants were gently removed from the culture tubes and carefully washed in the medium using sterile distilled water. Transferred the rooted shoots to pots filled with a mixture of antisepticized - soil: sand: and vermiculite (1:1:1) and immediately enclosed in polythene bags to retain a high moisture content (85%) at 25°C in a growth cabinet with a 16-h photoperiod and 60 µE/m^2/s light intensity. Small holes were made in the plastic bags and left for 7 to 8 days for plant acclimatization. After 2 weeks, these plants were transferred to the 20 cm diameter pots containing autoclaved field soil (Better, HIEUGIANG Co., Ho Chi Minh, Vietnam) and shifted to the greenhouse with 26 to 30°C/20 to 25°C day/night temperatures and about a 10 to 12 h photoperiod for flowering and seed set. The plants were irrigated with nutrient solution (TANNONGPHAT Co., Ha Noi, Vietnam) once a month and gradually with fresh water whenever required.

2.3 Data collection growth, development, and productivity

2.3.1 Time of growth and development of transgenic peanut

Tissue-cultured peanuts have enough stems, leaves, and roots, plants with 6 – 8 cm stem height were used to grow under

greenhouse conditions. Peanut line growth was calculated from the planting in the greenhouse to the stage when 50% of the total plants have 5 mature leave, level 1 branches, and appear the first flower per stem. Further, the total duration of growth was calculated from planting to the stage when 80 – 85% of peanuts are ripe, the veins of the hull are prominent, the inside of the hull has turned dark and the leaves turn yellow.

Plant height was determined by a ruler from the plant's base to the top of the highest point. It was collected in 10 randomly chosen plants. Further, the number of leaves and number of branches were calculated at the end of the growth period.

2.3.2 Factors that constitute yield and yield components

The number of mature pods/plants was calculated by counting pods of ten sample plants of each plot (with three replications). Further, the weight (g) of one hundred pods and one hundred seeds was obtained by weighing a random sample of 100 pods and 100 seeds, respectively.

2.4 Statistical Analysis

All numerical data accumulated from this study were subjected to statistical analysis and significance tests. All data were subjected to statistical analysis using Duncan's test with SPSS (ver. 20.0) (IBM, Armonk, NY, USA). Differences reported as significant are at $p < 0.05$.

3 Results and Discussion

3.1 Time of growth and development of transgenic peanut

Transgenic peanuts after 18 to 20 days of planting in the greenhouse began to form real leaves (Table 1) while in the case of seed-grown plants, real leaves start appearing only after 15 days of seed sown. Transgenic peanut lines grow and develop well under greenhouse conditions. The time from planting to the first branch appearing is 27 to 30 days with chitinase transgenic peanut lines (Table 1) while in the case of non-transgenic peanuts growing from seed (NC-1), the first branch appeared 20 days after planting. Chitinase transgenic peanut lines were harvested in 140 – 144 days after planting in the greenhouse while this period was reported as only 122 days for NC-1. These results have shown that the transgenic peanut lines had a longer growth period compared to the peanuts grown from seed. This is comprehensive because changing from *in vitro* to *in-vivo* conditions requires more time to adapt plants in the soil. These results are in agreement with the findings of Minh and Hieu (2012) who reported a 125 to 140 days period between planting and harvesting in peanut cultivar L14.

3.2 Plant height

The results presented in Table 2 showed that plant height ranges from 9.3 to 10.6 cm when the plant reached to 5 leave stage after planting. In general, plant height among transgenic and non-transgenic plants did not show any statistical deviation at this stage. At a full-bloom stage, the plant height of *syncodChi42-2* transgenic peanut lines varied from 17.6 to 17.8 cm while in the case of *syncodChi42-1* and *Chi42* transgenic peanut lines, it varied from 16.8 to 17.4 cm, 15.8 to 15.9 cm, respectively. Plant height continuously increased until the plant reaches the end of flowering. At this stage, plant height ranges from 20.8 to 23.0 cm for *syncodChi42-2* transgenic peanut lines and 20.5 to 21.8 cm, 20.4 to 20.8 cm, and 17.9 cm for *syncodChi42-1*, *Chi42*, NC, respectively. Among the tested transgenic peanut lines, the highest plant height of 30.0 cm was reported for the S2-4 line (Table 2). From the results of the current study, it can be concluded that the plant

Table 1 Time of growth and development of chitinase transgenic peanut lines

Gene	Transgenic peanut lines	Growth and development (days)				
		5 leaves	Level 1 branches	Beginning of Flowering	End of flowers	Harvest
syncodChi42-2	S2-2	18	27	57	77	142
	S2-4	18	27	59	77	140
	S2-6	19	27	59	75	140
syncodChi42-1	S1-1	19	30	59	75	144
	S1-2	20	29	57	77	142
	S1-3	20	30	57	77	142
Chi42	WT-1	18	30	59	77	144
	WT-2	19	30	59	77	142
	WT-3	18	30	58	77	144
NC	NC-1	15	20	43	64	122
	NC-2	20	30	58	77	145

Note: NC: non-transgenic peanut from *in vitro*.

Table 2 Plant height of transgenic peanut lines (cm) during the study period

Gene	Transgenic peanut lines	Plant height (cm) at different time after planting			
		5 leaves	Full-bloom	End of flowers	Harvest
syncodChi42-2	S2-2	$10.6^{a} \pm 0.42$	$17.8^{a} \pm 1.30$	$20.8^{b} \pm 1.92$	$29.3^{ab} \pm 0.97$
	S2-4	$9.8^{ab} \pm 0.84$	$17.8^{a} \pm 1.30$	$23.0^{a} \pm 1.87$	$30.0^{a} \pm 1.60$
	S2-6	$10.6^{a} \pm 0.89$	$17.6^{a} \pm 1.52$	$23.0^{a} \pm 1.73$	$29.8^{a} \pm 1.27$
syncodChi42-1	S1-1	$10.6^{a} \pm 0.55$	$17.4^{ab} \pm 1.34$	$20.6^{b} \pm 0.82$	$28.1b^{c} \pm 0.74$
	S1-2	$9.9^{ab} \pm 0.65$	$16.8^{ab} \pm 0.84$	$21.8^{ab} \pm 1.15$	$27.9^{bc} \pm 1.78$
	S1-3	$9.3^{b} \pm 0.57$	$17.4^{ab} \pm 1.52$	$20.5^{b} \pm 0.87$	$29.0^{ab} \pm 0.94$
Chi42	WT-1	$9.7^{ab} \pm 0.67$	$15.9^{b} \pm 1.02$	$20.6^{b} \pm 1.82$	$26.1^{d} \pm 1.34$
	WT-2	$10.0^{ab} \pm 1.22$	$15.9^{b} \pm 1.24$	$20.4^{b} \pm 1.52$	$26.0^{d} \pm 0.71$
	WT-3	$9.6^{ab} \pm 0.89$	$15.8^{b} \pm 1.10$	$20.8^{b} \pm 1.92$	$26.8^{cd} \pm 1.15$
NC		$10.1^{ab} \pm 0.55$	$14.2^{c} \pm 0.45$	$17.9^{c} \pm 1.24$	$23.3^{e} \pm 1.48$

Here a-e Means with different superscripts in the same column that followed the mean and standard deviation are significantly different ($p < 0.05$), NC: non-transgenic peanut from *in vitro*.

Table 3 Number of leaves per plant during the study period

Gene	Transgenic peanut lines	Number of leaves per plant at different times after planting				
		Flowering	Full-bloom	End of flowering	Harvest	Number of green leaves at harvest
syncodChi42-2	S2-2	$12.4^{a} \pm 0.55$	$15.2^{ab} \pm 0.45$	$17.2^{bc} \pm 0.45$	$21.2^{a} \pm 0.45$	$4.4^{a} \pm 0.55$
	S2-4	$12.8^{a} \pm 0.45$	$16.0^{a} \pm 0.45$	$18.4^{a} \pm 0.45$	$21.8^{a} \pm 0.45$	$4.4^{a} \pm 0.55$
	S2-6	$12.6^{a} \pm 0.55$	$15.6^{a} \pm 0.55$	$17.6^{b} \pm 0.55$	$21.6^{a} \pm 0.55$	$4.4^{a} \pm 0.55$
syncodChi42-1	S1-1	$12.4^{a} \pm 0.55$	$15.4^{a} \pm 0.55$	$17.4^{b} \pm 0.55$	$21.4^{a} \pm 0.55$	$4.2^{a} \pm 0.45$
	S1-2	$12.4^{a} \pm 0.55$	$15.4^{a} \pm 0.55$	$17.4^{b} \pm 0.55$	$21.4^{a} \pm 0.55$	$4.2^{a} \pm 0.45$
	S1-3	$12.4^{a} \pm 0.55$	$15.6^{a} \pm 0.55$	$17.6^{ab} \pm 0.55$	$21.6^{a} \pm 0.55$	$4.2^{a} \pm 0.45$
Chi42	WT-1	$11.0^{b} \pm 1.00$	$14.4^{b} \pm 0.89$	$16.4^{d} \pm 0.55$	$20.0^{ab} \pm 1.00$	$4.2^{a} \pm 0.45$
	WT-2	$10.8^{b} \pm 0.84$	$14.6^{b} \pm 0.89$	$16.4^{d} \pm 0.55$	$20.0^{ab} \pm 0.71$	$4.2^{a} \pm 0.45$
	WT-3	$11.2^{b} \pm 0.84$	$14.6^{b} \pm 0.55$	$16.4^{d} \pm 0.55$	$20.2^{b} \pm 0.84$	$4.2^{a} \pm 0.45$
NC		$9.8^{c} \pm 0.84$	$13.0^{c} \pm 1.00$	$15.6^{d} \pm 1.14$	$19.2^{c} \pm 0.84$	$4.0^{a} \pm 0.55$

Here a-d Means with different superscripts in the same column that followed the mean and standard deviation are different ($p < 0.05$), NC: non-transgenic peanut from *in vitro.*

height of chitinase transgenic peanut lines was higher than the non-transgenic peanut at different times after planting, and these differences were statistically significant ($p<0.05$). L14 is a peanut with a balanced shape, strong growth, and larger plant height, which will affect flowering and pod formation. Therefore, the plant height of peanuts will create a premise for flowering, better pod formation, and higher peanut yield corresponding to transgenic peanut lines. Improved plant height of transgenic cotton plants were also reported by Bashir et al. (2022), who used the barley chitinase I and chitinase II gene to create resistance against fungi.

3.3 Number of leaves per plant

A perusal of the data presented in Table 3 revealed that different transgenic peanut lines (*Chi42, syncodChi42-1*, and *syncodChi42-2)* showed a significant effect on the number of leaves per plant. Overall, the experimental result showed that the number of leaves was found higher in the S2A-12 line at all growth stages starting from beginning to the flowers production (12.8 leaves) and harvesting (21.8 leaves), and these differences are statistically significant ($p < 0.05$) as compared to the non-transgenic peanut lines. The number of leaves increased during the pod development phase and decreased during harvest. Leaves are the essential source from which the photosynthates are channeled to the sink. During the pod development phase, leaves provide nutrition to pods and a higher number of leaves contribute to higher pod yield. According to Yang et al. (2020) research, transgenic soybean plants weren't witnessing any detrimental impacts on growth and development by the overexpression of the chitinase gene *CmCH1*. Similarly, Zaynab et al. (2017) informed that transgenic potatoes expressing the rice chitinase gene had a higher number of leaves per plant than non-transformed plants.

Table 4 Number of branches per plant during the study period

Gene	Transgenic peanut lines	Number of level 1 branches/ plant		Total number of branches/plant	
		Full-bloom	Harvest	Full- bloom	Harvest
syncodChi42-2	S2-2	$3.4^{ab} \pm 0.55$	$4.6^{a} \pm 0.55$	$4.8^{ab} \pm 0.45$	$6.4^{a} \pm 0.55$
	S2-4	$3.6^{a} \pm 0.55$	$4.8^{a} \pm 0.45$	$5.0^{a} \pm 0.00$	$6.4^{a} \pm 0.55$
	S2-6	$3.4^{ab} \pm 0.55$	$4.8^{a} \pm 0.45$	$4.8^{ab} \pm 0.45$	$6.4^{a} \pm 0.55$
syncodChi42-1	S1-1	$3.4^{ab} \pm 0.55$	$4.6^{a} \pm 0.55$	$4.8^{ab} \pm 0.45$	$6.0^{ab} \pm 0.00$
	S1-2	$3.6^{a} \pm 0.55$	$4.4^{a} \pm 0.55$	$5.0^{a} \pm 0.00$	$6.2^{ab} \pm 0.45$
	S1-3	$3.2^{ab} \pm 0.45$	$4.4^{a} \pm 0.55$	$4.8^{ab} \pm 0.45$	$6.4^{a} \pm 0.55$
Chi42	WT-1	$3.2^{ab} \pm 0.45$	$4.4^{a} \pm 0.55$	$4.8^{ab} \pm 0.45$	$6.0^{ab} \pm 0.45$
	WT-2	$3.2^{ab} \pm 0.45$	$4.4^{a} \pm 0.55$	$5.0^{a} \pm 0.45$	$6.2^{ab} \pm 0.00$
	WT-3	$3.6^{a} \pm 0.55$	$4.6^{a} \pm 0.55$	$4.8^{ab} \pm 0.00$	$6.2^{ab} \pm 0.45$
NC		$2.8^{b} \pm 0.45$	$3.4^{b} \pm 0.55$	$4.4^{b} \pm 0.55$	$5.6^{b} \pm 0.55$

Here a-b Means with different superscripts in the same column that followed the mean and standard deviation are different ($p < 0.05$), NC: non-transgenic peanut from *in vitro.*

Table 5 Yield parameters of transgenic peanut lines

Gene	Transgenic peanut lines	Yield parameters of transgenic peanut lines		
		Number of mature pods/plant	100 pods weight i(g)	100 seeds weight (g)
syncodChi42-2	S2-2	$8.6^{a} \pm 0.55$	$114.84^{ab} \pm 1.21$	$35.22^{ab} \pm 0.61$
	S2-4	$9.0^{a} \pm 0.71$	$115.48^{a} \pm 0.58$	$36.22^{ab} \pm 0.93$
	S2-6	$8.4^{ab} \pm 0.55$	$115.32^{a} \pm 0.58$	$35.62^{ab} \pm 1.32$
syncodChi42-1	S1-1	$8.4^{ab} \pm 0.55$	$112.58^{ab} \pm 2.38$	$33.66^{c} \pm 0.88$
	S1-2	$8.4^{ab} \pm 0.55$	$114.88^{ab} \pm 1.29$	$34.54^{bc} \pm 1.05$
	S1-3	$8.6^{a} \pm 0.55$	$114.70^{ab} \pm 0.78$	$34.54^{bc} \pm 0.77$
Chi42	WT-1	$8.6^{a} \pm 0.89$	$111.86^{b} \pm 2.64$	$33.06^{d} \pm 0.96$
	WT-2	$8.2^{ab} \pm 0.84$	$111.86^{b} \pm 2.42$	$33.10^{d} \pm 0.51$
	WT-3	$8.4^{ab} \pm 0.55$	$111.68^{b} \pm 2.07$	$32.28^{d} \pm 0.44$
NC		$7.6^{b} \pm 0.55$	$107.26^{c} \pm 4.81$	$31.76^{e} \pm 1.01$

Here a-e Means with different superscripts in the same column that followed the mean and standard deviation are different ($p < 0.05$). NC: non-transgenic peanut from *in vitro.*

3.4 Number of branches per plant

The number of level 1 branches and the total number of branches per plant at different chitinase transgenic peanut lines varied from 3.2 to 3.6 and 4.8 to 5.0, respectively for the full-bloom stage (Table 4). There weren't any visible changes in the total number of branches per plant in transgenic peanut lines compared to the untransformed control at full bloom and harvest, except for line S2-4 (Table 4). At the maturity stage, in comparison with the control, the number of level 1 branches and the total number of branches/plant of all transgenic lines were remarkably increased, particularly for *syncodChi42-2* genes associated with S2-4 (Table 4). According to Dapaah et al. (2014) number of branching in peanuts may positively impact the final yield. Similarly, Cuong et al. (2019) studied the impact of level 1 branches in cultivar L14 and found a significant association with the final yield, and in this manner findings of this study are in agreement with the findings of these studies.

3.5 Yield parameters of transgenic peanut lines

Differences in yield parameters among transgenic and non-transgenic test lines are shown in Table 5. Regarding the number of mature pods per plant, all transgenic peanut lines had a significantly higher number of pods as compared to the non-transgenic plants (Table 5). In the case of 100 pods and 100 seeds weight, all chitinase transgene peanut lines have significantly higher averaged pod and seed mass as compared to the non-transgenic. Among the various tested lines, *syncodChi42*-2 (S2-2, S2-4, and S2-6) transgenic peanut lines have a higher number of

mature pods per plant (10.5 – 18.4%) as compared to the non-transgenic cultivar (Table 5). Further, *syncodChi42-1* and *Chi42* transgenic peanut lines showed an enhanced number of mature pods per plant (7.9 – 13.2%) compared with non-transgenic cultivars. Further, the S2-4 line showed significantly higher yield parameters as compared to the other transgenic lines (Table 5).

According to Nagpure et al. (2014), a crop's productivity is diminished by the effects of chitinase in improving the crop's defense mechanisms against several types of stresses. Jeong et al. (2013) created OsNACS transgenic rice plants with the control of RCc3 and GOS2 promoters. Crop yields under normal conditions increased from 9% to 26%. In addition, the investigation also mentioned that RCc3:OsNAC5 plants had a larger grain yield of 22 – 63% under water deficiency conditions.

Growth and yield characteristics including plant height, number of leaves/plant, number of branches/plant, number of mature pods/plant, pod and seed weights of the peanut lines were evaluated under greenhouse conditions and found statistically comparable between the transformed and non-transformed plant lines. Similar types of results had been previously recorded under both greenhouse and field conditions with other transgenic crops (Arnoldo et al. 1992; Chenault et al. 2006).

Conclusion

In this study, the total of nine transgenic peanut lines including three wild-type (*Chi42* i.e. WT-1, WT-2, WTA-3) and six synthetic gene lines (*syncodChi42-1* i.e. S1-1, S1-2, S1-3 and *syncodChi42-2* i.e. S2-2, S2-4, S2-6) were tested. The results of the study suggested that all transgenic lines did not show any major changes in the growth and development characteristics in greenhouse conditions. Peanut lines expressing chitinase 42 kDa showed significantly increased various yield parameters. This study provides a reasonable approach for the genetic improvement of peanuts to enhance resistance to the pathogen fungus *S. rolfsii*. The promising transgenic peanut lines identified in this study can be exploited as stable fungal disease-resistant peanut lines in the future for other plant breeding programs.

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Conflicts of interest

All authors declare that they have no conflicts of interest.

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