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Cumin Transformation for Resistance to Fungal Diseases

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ABSTRACT

Cumin (*Cuminum cyminum* L.) is an economically valuable medicinal and spice plant that is highly susceptible to fungal pathogens, including *Fusarium oxysporum f. sp. cumini*, which can cause yield losses of up to 80%. In this study, a multi-gene construct (pBI121) carrying *chitinase, β-1,3-glucanase*, and *PRP1* genes, each driven by an independent *CaMV 35S* promoter and accompanied by a *nptII* selectable marker, was introduced into cumin through *Agrobacterium tumefaciens*–mediated transformation. Cotyledon explants were co-cultivated with *Agrobacterium* strain *EHA105* at OD_{600} = 0.5, followed by selection on MS medium containing 50 mg L^{-1} kanamycin. A total of nine transgenic lines were confirmed by PCR amplification of specific bands at approximately 870 bp (*glucanase*), 680 bp (*chitinase*), and 580 bp (*PRP1*), corresponding to a transformation efficiency of about 1.5%. Protein extracts from transgenic plants exhibited clear antifungal activity against *Fusarium oxysporum* in vitro, with inhibition zones averaging 6.3 ± 0.5 mm and 9.8 ± 0.7 mm for 50 µg and 100 µg protein concentrations, respectively. No inhibition was observed in extracts from non-transgenic plants or buffer controls. The antifungal effect is attributed to synergistic action among *chitinase*, *glucanase*, and *PRP1* proteins, which degrade fungal cell wall components and activate defense signaling pathways. These results demonstrate the feasibility of *Agrobacterium*-mediated transformation in cumin and highlight the potential of multi-gene stacking strategies for enhancing fungal resistance in medicinal plants. This work represents an important step toward developing disease-resistant cumin genotypes and reducing reliance on chemical fungicides in sustainable agriculture.

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1. Introduction

Medicinal plants have received worldwide attention because of their healing properties and the side effects of synthetic drugs. This interest has led to an increase in the growing and trading of these species. Cumin (*Cuminum cyminum* L.) grows well in hot, dry areas and holds great economic value, especially in Iran. It is the second most important plant exported from the country, after saffron, and is considered a vital domesticated medicinal plant (Bahmankar *et al*., 2017; Bahmankar *et al*., 2019). Cumin is commonly used to treat digestive issues, gas, and pain, and it also serves as an antiseptic in certain traditional uses (Pandey *et al*., 2015). Fungal diseases like powdery mildew (*Erysiphe polygoni*), leaf blight (*Alternaria burnii*), and fusarium wilt (*Fusarium oxysporum f.sp. cumini*) have caused yield losses between 50-80% in cumin production. These diseases have greatly lowered cumin farming in Iran. Since 2010, the area for cumin cultivation has dropped from 108,000 hectares to less than half (Esfandiari *et al*., 2009; Patel *et al*., 2012; Özer and Bayraktar, 2018).

Conventional control methods, such as chemical fungicides, are often limited in effectiveness, environmentally detrimental, and unsustainable for long-term cumin cultivation. Consequently, alternative

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strategies have been sought, with genetic engineering emerging as a promising approach to enhance disease resistance. Among these, *Agrobacterium*-mediated transfer of pathogenesis-related (PR) proteins has been widely employed. PR proteins are innate plant defense molecules that are activated in response to pathogen attack or environmental stress, contributing to the inhibition of pathogen growth and the activation of plant defense mechanisms (van Loon *et al*., 2006). By introducing these proteins into cumin through genetic transformation, it is possible to strengthen the plant's innate defense system and reduce dependence on chemical pesticides (van Loon *et al*., 2006). These proteins can kill pathogens by breaking down their cell walls and playing a part in defense signaling. When PR proteins are expressed in transgenic plants, they can boost resistance. However, their effectiveness varies based on the type of protein, the plant species, and the specific pathogen.

Agrobacterium-mediated transformation allows stable integration of multiple antifungal genes (*chitinase*, *glucanase*, *PRP1*) into cumin. Conventional breeding or chemical treatments are slower, less precise, and often ineffective against fungal diseases. The various functions and evolutionary consistency of PR proteins support their use in creating fungal resistance, including *chitinase*, *glucanase*, and *PRP1* (van Loon *et al*., 2006). The initial report of *Agrobacterium*-mediated transformation of cumin employing the EHA105 strain was made by Indian scientists (Pandey *et al*., 2013). They utilized a *pCAMBIA1301* construct featuring a *GUS* reporter and an *hptII* selectable marker, rather than genes that confer resistance to fungi (Pandey *et al*., 2013). In 2010, other researchers demonstrated direct gene transfer into cumin embryos by employing a gene gun for temporary *GUS* expression (Singh *et al*., 2010). The level of tolerance to salt stress in cumin has been enhanced by transfection of the *SbNHX1* gene, indicating the potential for genetic engineering of this species (Pandey *et al*., 2016).

Current methods build on early transformation studies. They focus on expressing anti-pathogenic proteins to provide disease resistance. Currently, most methods for creating fungal-resistant transgenic plants focus on adding genes that code for anti-pathogenic proteins. However, research on combining multiple *PR* genes in cumin for better resistance is limited. Therefore, this study aimed to create transgenic cumin plants using *Agrobacterium tumefaciens*-mediated transformation with a three-gene construct (*chitinase*, *glucanase*, and *PRP1*) controlled by separate *35S* promoters. We assessed the antifungal activity of transgenic cumin genotypes through in vitro bioassays against *Fusarium oxysporum f.sp. cumini*, the pathogen responsible for cumin wilt. This disease is one of the most harmful threats to cumin production in dry and semi-dry areas. We demonstrate the successful transformation and in vitro antifungal effectiveness of transgenic cumin.

2. Materials and methods

For the transformation of cumin, cotyledon explants of the Golestan ecotype were used in an optimized medium containing 0.1 mg L^{-1} of kinetin, which was optimized in another study (Bahmankar *et al*., 2017). It is worth noting that before starting the transfer, in order to increase the cell uptake ability, the explants were cultured on embryogenesis medium for 48 hours (Fig. 1). A single colony of bacteria grown on solid medium was removed and cultured into a Falcon containing 5 ml of liquid LB medium containing appropriate concentrations of kanamycin (50 mg L^{-1}) and rifampicin (75 mg L^{-1}). The bacterial growth steps to reach the appropriate concentration were similar to the seed embryo transfer experiment in the previous step. After reading the OD using a spectrophotometer, appropriate concentrations of 0.6 and 0.5 were determined. Then, the falcons containing the bacterial solution were centrifuged for 15 minutes, the supernatant was removed, and an equal amount of optimized somatic embryogenesis liquid culture medium containing 300 µM astosyringone was added to the bacterial sediment in the falcon.

In the next step, this solution was transferred into a sterile Petri dish and cotyledon explants were transferred and immersed in it (Fig. 1). After about 15-20 minutes, the explants were transferred onto co-culture medium (MS medium containing 0.1 kinetin mg L^{-1}) and kept at 24°C for 48 hours. After the co-culture period, the explants were removed from the co-culture medium, washed with sterile distilled water, and then transferred to sterile filter paper to remove excess moisture. The explants were transferred to MS medium (containing 0.1 kinetin mg L^{-1}) containing 50 mg L^{-1} kanamycin and 500 mg L^{-1} cefotaxime. Explants

were subcultured every 2–3 weeks under controlled growth chamber conditions until embryogenic callus formed and regeneration occurred, with full seedling regeneration achieved in about 10–12 weeks. MS medium containing 0.1 mg L^{-1} kinetin and 50 mg L^{-1} kanamycin antibiotic was used as the initial transgenic selection medium. Although newer vector systems exist, *pBI121* was selected for its proven reliability and ability to carry multiple genes under separate promoters. This choice allowed us to efficiently evaluate the combined antifungal effects of *chitinase*, *glucanase*, and *PRP1* in transgenic cumin.

Figure 1. Cotyledon explants immersed in bacterial solution and co-culture medium

2.1. Confirmation of cumin transformation using PCR

After regeneration of cumin seedlings in a medium containing kanamycin, leaf sampling was performed, and DNA extraction was performed to extract DNA from the regenerated, putatively transgenic seedlings (Bahmankar *et al*., 2018). PCR reaction contained 1× buffer, 200 µM dNTPs, 0.5 µM primers, 1 U Taq polymerase, and 50 ng template DNA. A Bio-Rad T100™ Thermal Cycler was used to perform the polymerase chain reaction (PCR), and Table 1 lists the reaction components. The amplified products were then separated using a 1 kb DNA ladder as a molecular size indicator on a 2% agarose gel.

Table 1. PCR program used to amplify transferred genes to cumin

Step	Temperature	Time	Cycle number
1	95°C	5 min	
2	95°C	45 sec	
3	58°C	45 sec	30 cycles
4	72°C	1 min	
5	72°C	7 min	

2.2. Protein extraction and quantification

Protein extraction steps were carried out separately for each sample at 4°C. The total soluble protein content was determined using the Bradford assay (Bradford, 1976), with bovine serum albumin (BSA) as the standard. Absorbance was measured at 595 nm, and protein concentrations were adjusted to 1 mg mL^{-1} before use in the antifungal bioassay.

2.3. Antifungal bioassay against Fusarium oxysporum f. sp. cumini

For each treatment, 50 µL of protein extract, which is equal to 50 µg or 100 µg of total protein, was placed into wells that were 6 mm in diameter on PDA plates. These plates were pre-inoculated with a 0.5 cm mycelial plug from the actively growing edge of a Fusarium colony. The plates were incubated at 25 ± 1 °C for 7 days. After incubation, the inhibition zone, defined as the distance between the edge of the fungal colony and the well margin, was measured in millimeters (Dolatabadi *et al*., 2014; Loebenstein *et al*., 2010).

To investigate the effect of protein, 50 and 100 µg protein were loaded per well, with total protein concentration quantified by Bradford assay. The treatments applied were: Well 1: 50 micrograms of transfected cumin protein extract, Well 2: 100 micrograms of transfected cumin protein extract, Well 3: 50 microliters of extraction buffer, Well 4: 100 microliters of extraction buffer, Well 5: 50 micrograms of untransfected cumin protein extract, and Well 6: 100 micrograms of untransfected cumin protein extract. After treatment, Petri dishes were incubated at 25°C. After 7 days, the morphology of the fungal mycelium and the antifungal activity of the treatments were evaluated by measuring the distance from the edge of the well to the advancing front of fungal growth (inhibition zone).

3. Results and discussion

3.1. Plant regeneration

A total of 1,800 explants were subjected to treatment with bacterial suspensions at optical density (OD_{600}) levels of 0.5, 0.6, and 0.8. Only the suspension at OD 0.5 promoted regeneration; the higher optical densities caused necrosis (Fig. 2).

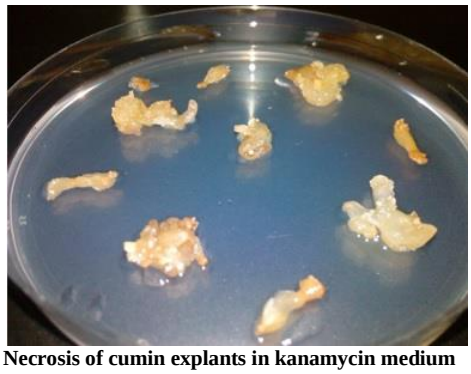

Figure 2. Necrosis of cumin explants in kanamycin medium

Researchers also observed in previous studies that higher densities of *Agrobacterium* can cause necrosis due to bacterial overgrowth (Tohidfar *et al*., 2005). The small size of the explants might also influence their sensitivity. Out of the 600 explants treated at OD 0.5, 11 showed regeneration, with 9 being PCR-positive, leading to a transformation efficiency of 1.5% (Table 2 and Fig. 3).

Table 2. Regeneration characteristics at different OD_{600}

Bacterial OD	Explants inoculated	Explants regenerated	PCR-positive	Transformation efficiency (%)
0.5	600	11	9	0.015
0.6	600	0	0	0
0.8	600	0	0	0

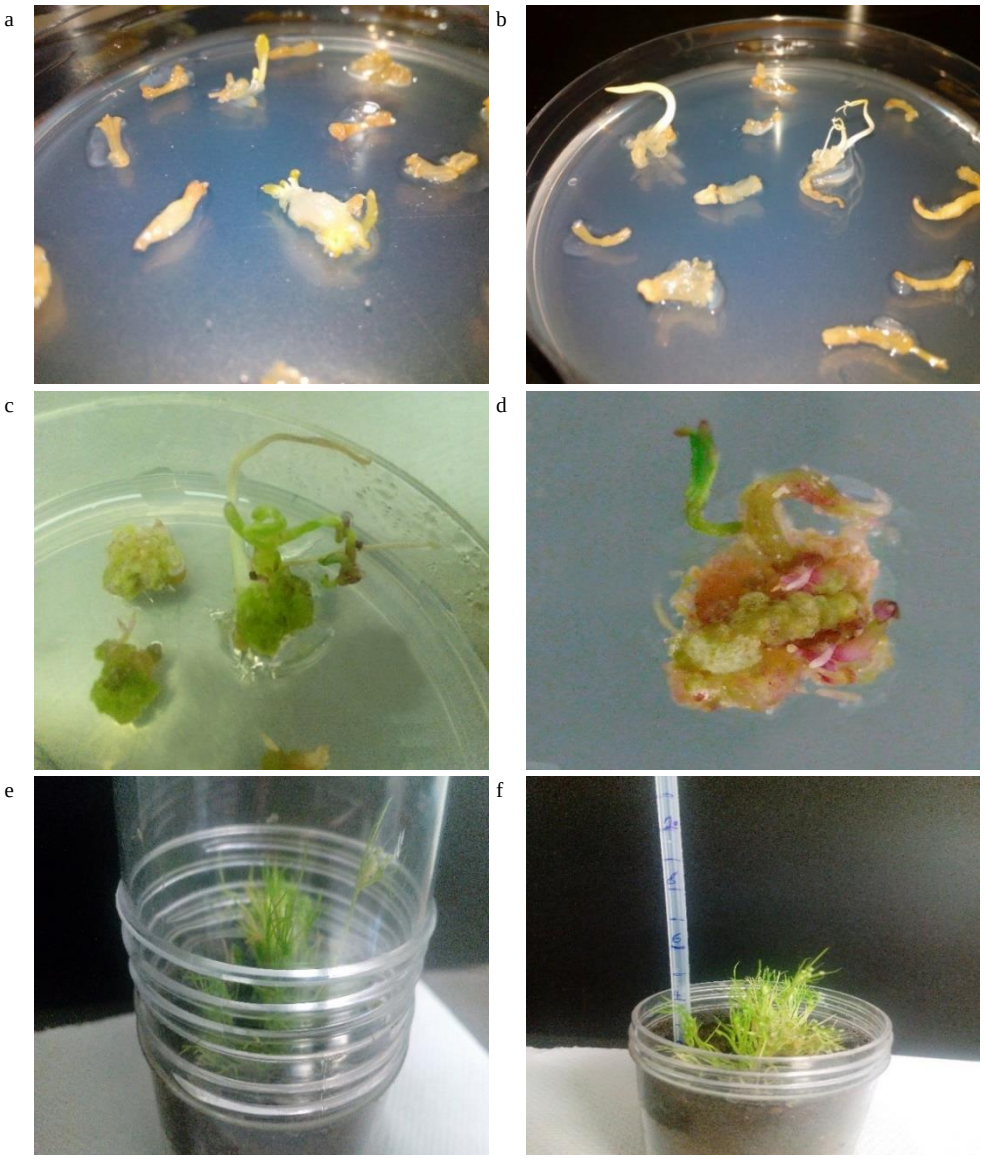

Figure 3. Stages of regeneration and acclimatization of putative transgenic cumin (*Cuminum cyminum*) derived from cotyledon explants following *Agrobacterium tumefaciens*-mediated transformation. (a-b) Initiation of regeneration: embryogenic callus and early shoot primordia formed on MS medium with 0.1 mg L^{-1} kinetin under selection (50 mg L^{-1} kanamycin). This was observed about 3-4 weeks after transferring to the selection medium. (c-d) Shoot emergence: actively growing putative transgenic shoots were seen 6-8 weeks after selection. (e-f) Acclimatization: regenerated plantlets were rooted and gradually adapted to ex vitro conditions by moving them from tissue culture vessels to soil or peat substrate before greenhouse growth. Cefotaxime (500 mg L^{-1}) was added to early subcultures to get rid of *Agrobacterium*. Photographs were taken at the indicated stages; scale bars = 1 cm.

The relatively low transformation efficiency may have resulted from the high sensitivity of cumin explants to *Agrobacterium* infection, kanamycin selection pressure, and bacterial overgrowth during co-culture, as previously observed in similar transformation systems (Liu *et al*., 2016). In the present study, two PCR-negative regenerants may represent escapes from kanamycin selection. Pandey et al. (2013) reported ~1.5% efficiency with similar explants. The transformation efficiency of 1.5% matches the 1.5% reported by Pandey et al. (2013). The low efficiency might result from explant sensitivity and selection pressure.

3.2. Molecular confirmation of transgenic cumin by PCR

The presence of transferred genes in the genome of putative transgenic cumin plants was initially verified using the polymerase chain reaction (PCR), a widely adopted method for preliminary screening of transgenic events due to its simplicity, sensitivity, and cost-effectiveness (Raji *et al*., 2022). Gene-specific primers were designed for the chitinase, glucanase, and PRP1 transgenes, and amplification was performed using genomic DNA extracted from regenerated cumin seedlings. The resulting PCR amplification profiles are presented in Fig. 4.

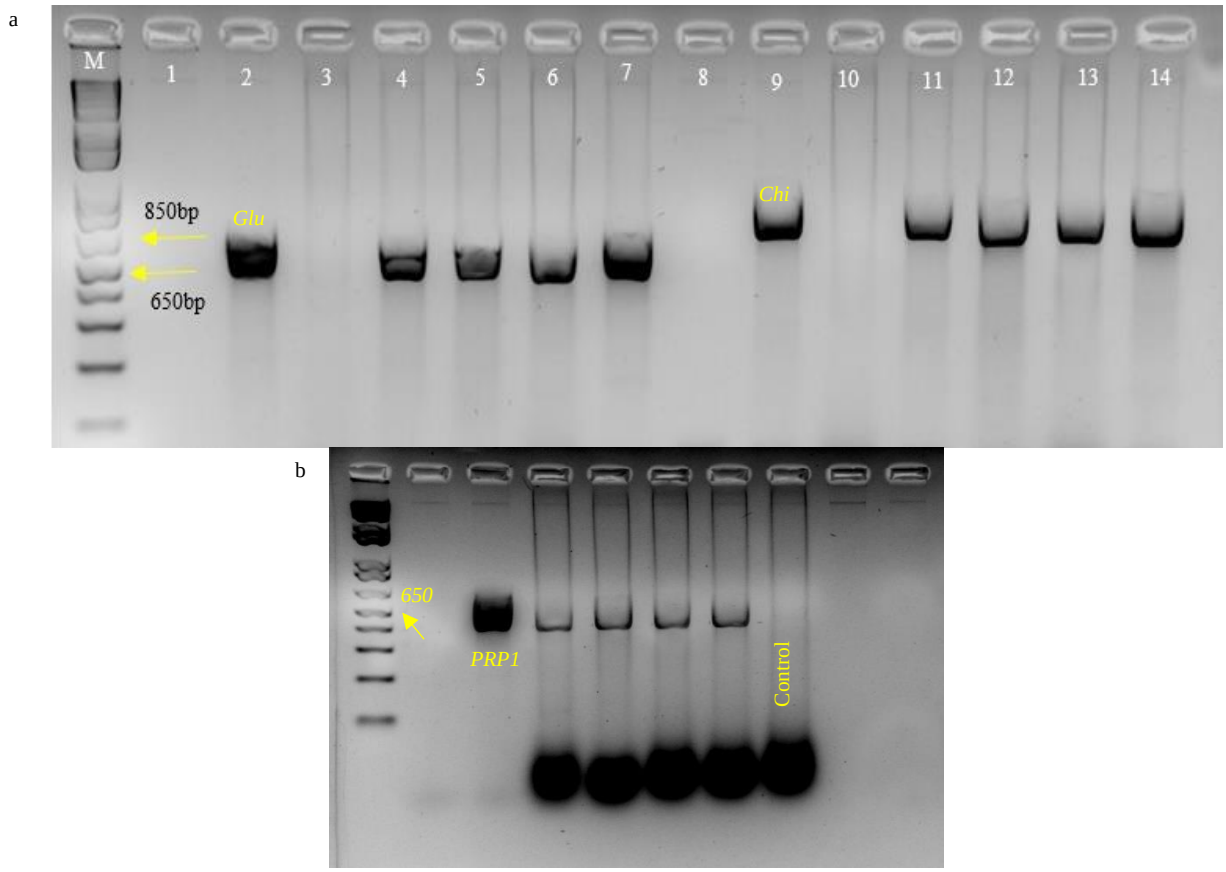

Figure 4. Banding pattern of *glucanase*, *chitinase* (A), and *PRP1* (B) genes in transgenic cumin (*Cuminum cyminum* L.). Figure description for (A) gel: Lane M: 1 kb DNA ladder. Lane 1: negative control plasmid for glucanase. Lane 2: positive control plasmid for β-*glucanase*. Lanes 4-7: PCR-amplified bands corresponding to the *glucanase* gene in transgenic cumin plants. Lane 8: negative control plasmid for *chitinase*. Lane 9: positive control plasmid for *chitinase*. Lanes 11-14: PCR-amplified bands corresponding to the *chitinase* gene in transgenic cumin plants. Lanes 3 and 10: non-transgenic cumin controls. Figure description for (B) gel: Lane M: 1 kb DNA ladder. Lane 1: negative control plasmid for *PRP1*. Lane 2: positive control plasmid for *PRP1*. Lanes 3-6: PCR-amplified bands corresponding to the *PRP1*gene in transgenic cumin plants. Lanes 7: non-transgenic cumin controls.

The PCR amplification products were separated by agarose gel electrophoresis and visualized under UV light following ethidium bromide staining. As expected, distinct bands of approximately 870, 680, and 580 bp corresponding to the glucanase, chitinase, and PRP1 genes, respectively, were detected in the putative transgenic cumin plants (Fig. 4). No amplification was observed in DNA samples from non-transgenic control plants or in negative plasmid controls, confirming the specificity of the PCR reactions. The findings indicated that of the 600 cotyledon explants exposed to *Agrobacterium*, 11 regenerated successfully. From this group, 9 were verified as transgenic via PCR analysis. This indicates

that the transformation efficiency was approximately 1.5% (9/600). PCR confirmation may detect residual plasmid DNA; future work should include Southern blotting to confirm stable integration. Studies indicated that only a few reports exist on cumin transgenic plants, mainly by Indian researchers (Singh *et al*., 2010; Pandey *et al*., 2013). Detailed investigations revealed that the first successful *Agrobacterium*-mediated genetic transformation of cumin was reported by Pandey et al. (2013) using the *Agrobacterium tumefaciens* strain *EHA105*. The researchers employed the binary vector *pCAMBIA1301*, which carried the *β-glucuronidase* (*GUS*) reporter gene and the *hygromycin phosphotransferase II* (*hptII*) selectable marker, both driven by the *CaMV 35S* promoter. Cumin embryos were used as explants for transformation, and transient expression of the *GUS* gene was successfully demonstrated, indicating that *Agrobacterium*-mediated gene delivery is feasible in cumin. The transformation efficiency was reported to be approximately 1.5%. These findings provided an important foundation for subsequent studies aiming to introduce functional genes into cumin through *Agrobacterium*-mediated transformation.

Singh et al. (2010) reported the first attempt to genetically transform cumin using the particle bombardment (gene gun) method for direct DNA delivery, employing embryonic axes as explants. The transformation vector pCAMBIA1301 contained the *β-glucuronidase* (*GUS*) reporter gene and the hygromycin phosphotransferase II (*hptII*) selectable marker, both driven by the constitutive *CaMV35S* promoter. Transient *GUS* expression was observed in transformed tissues, demonstrating the feasibility of direct gene transfer in cumin and providing an initial framework for subsequent studies on stable transformation approaches. According to their findings, approximately 91% of embryos exhibited transient expression of the *GUS* gene, proving that cumin cells can temporarily express foreign genes. This study offered the first experimental proof that *C. cyminum* is genetically manipulable, despite the fact that stable integration was not attained. Building on this basis, the current study successfully transformed cumin by transferring several defense-related genes (*chitinase*, *glucanase*, and *PRP1*) via *Agrobacterium*, which is a major step in creating fungal-resistant genotypes. Recent studies have demonstrated that *Agrobacterium*-mediated transformation can enhance disease resistance in medicinal plants via PR protein expression (Perrot *et al*., 2022; Han and Schneiter, 2024). These approaches are more efficient and precise than conventional breeding. They offer targeted strategies to develop fungal-resistant plant varieties."

3.3. Antifungal efficacy of transgenic cumin protein extracts against Fusarium *wilt pathogen*

According to the results of the bioassay, the protein extracts from transgenic cumin plants successfully stopped *Fusarium oxysporum* mycelia from growing toward the wells that contained them (Fig. 5). After seven days of incubation, the distance between the fungal colony margin and the well edge was measured to measure the inhibitory effect. Wells containing 50 µg and 100 µg of transgenic protein extract had mean inhibition zone diameters of 6.3 ± 0.5 mm and 9.8 ± 0.7 mm, respectively.

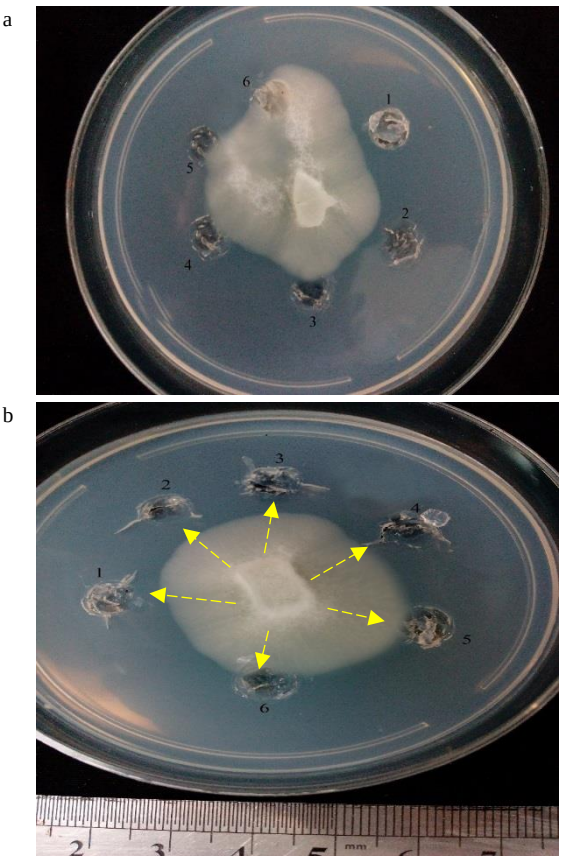

Figure 5. Inhibitory effect of transgenic cumin (*Cuminum cyminum* L.) protein extracts on the growth of *Fusarium oxysporum f. sp. cumini*. (a) Wells 1 and 2 contain 50 and 100 µg of transgenic cumin protein extract, respectively; Wells 3 and 4 contain 50 and 100 µL of extraction buffer, respectively; Wells 5 and 6 contain 50 and 100 µg of non-transgenic cumin protein extract, respectively. (b) Wells 1–4 contain 100 µg of transgenic cumin protein extract; Wells 5 and 6 contain 100 µL of extraction buffer and 100 µg of non-transgenic cumin protein extract, respectively.

However, there was no detectable inhibition zone in wells that contained the extraction buffer or protein extracts from non-transgenic cumin. The antifungal effect of transgene products is confirmed by the absence of inhibition in non-transgenic and buffer controls. It is worth noting that no abnormal hyphal morphology, such as lysis or swelling, was observed in vitro near the wells. According to these results, the antifungal activity was concentration-dependent and mostly linked to the expression of the enzymes chitinase and glucanase, which most likely broke down the fungal cell wall by breaking down chitin and β-1,3-glucan polymers. Transgene copy number and expression levels may affect antifungal efficacy and will be assessed in future studies. The transformation construct's incorporation of the *PRP1* (*Pathogenesis-Related Protein 1*) gene, in addition to *chitinase* and *glucanase*, might have played a role in the transgenic cumin lines' overall antifungal response. Among the most conserved defense-related proteins in higher plants, PRP1 proteins are part of the *PR-1* family. They are strongly induced during pathogen attack, especially through the salicylic acid (SA)-dependent signaling pathway (Bhardwaj *et al*., 2021; Almeida-Silva and Venancio, 2022; Wangorsch *et al*., 2022).

PR-1 proteins are known to have antimicrobial and antifungal properties, although their exact biochemical mechanism is still partially unknown. This may be due to the proteins' disruption of pathogen membrane integrity or interference with sterol-binding processes that are necessary for fungal growth (Gamir *et al*., 2017). It has been demonstrated that co-expressing *PRP1* with hydrolytic enzymes like *chitinase* and *β-1,3-glucanase* in transgenic systems has a synergistic effect that improves the plant's durability and resistance spectrum (Raji *et al*., 2022; Datta *et al*., 1999).

Thus, the strong inhibition zones seen in our bioassay may be explained by the combined action of *PRP1*-mediated defense signaling and the direct cell wall degradation brought on by chitinase and glucanase. *PRP1's* functional role in fungal resistance may be further elucidated by future research concentrating on *PRP1* transcript accumulation and protein localization in transgenic cumin. Other transgenic systems expressing pathogenesis-related proteins, like transgenic cotton (*Gossypium hirsutum*) expressing a bean *chitinase* gene, have shown similar inhibition patterns (Tohidfar *et al*., 2005). Overall, the results of this bioassay indicate that simultaneous expression of *chitinase, glucanase,* and *PRP1* genes in cumin leads to measurable *in vitro* inhibition of *Fusarium* growth. This combined defense strategy represents a promising step toward developing genetically enhanced cumin lines with improved resistance to fungal pathogens.

4. Conclusion

This study successfully demonstrated the genetic transformation of *C. cyminum* through Agrobacterium tumefaciens-mediated delivery of a multi-gene construct carrying *chitinase, β-1,3-glucanase*, and *PRP1* genes, each under independent 35S promoters. The regenerated cumin plants were molecularly confirmed as transgenic by PCR amplification of the target genes, and protein extracts from these plants exhibited significant in vitro antifungal activity against *Fusarium oxysporum f. sp. cumini*. The observed inhibition zones (6–10 mm) highlight the combined role of hydrolytic enzymes and *PRP1*-mediated defense signaling in suppressing fungal growth. These results provide the first experimental evidence that stacking multiple pathogenesis-related (PR) genes can effectively enhance the antifungal defense potential of cumin. This approach may serve as a foundation for developing disease-resistant genotypes in other medicinal and spice crops, reducing dependence on chemical fungicides and contributing to sustainable agriculture. However, the transformation efficiency (≈1.5%) remained low, likely due to explant sensitivity, stringent antibiotic selection, and bacterial overgrowth during co-culture. Moreover, the antifungal resistance was assessed only in vitro; therefore, the stability of gene expression, transgene copy number, and resistance performance under greenhouse or field conditions remains to be evaluated. Future research should focus on molecular characterization of transgene integration, quantitative expression analysis (RT-qPCR, Southern blot), and in planta infection assays to validate durable resistance. Expanding this approach to include additional PR or stress-responsive genes may further improve the spectrum and durability of fungal resistance in cumin and related medicinal plants.

Conflict of interests

All authors declare no conflict of interest.

Ethics approval and consent to participate

No humans or animals were used in the present research. The authors have adhered to ethical standards, including avoiding plagiarism, data fabrication, and double publication.

Consent for publications

All authors read and approved the final manuscript for publication.

Availability of data and material

The authors embedded all data in the manuscript.

Authors' contributions

All authors had an equal role in study design, work, statistical analysis and manuscript writing.

Informed consent

The authors declare not to use any patients in this research.

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