

Article

Comparative Evaluation of Inoculation Methods to Screen *Aspergillus niger*-Induced Crown Rot Disease Resistance in Peanut

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Abstract

Peanut crown rot, caused by *Aspergillus niger* (*A. niger*), can cause significant yield loss ranging from 10 to 50%, mainly as a result of stand loss due to seedling death. Symptoms include damping-off of seedlings; dark grey vascular tissue; and the development of a sooty, black spore mass on the crown region beneath the soil. Currently, there is no commercial peanut cultivar resistant to crown rot, and no inoculation methods have been tested to effectively screen for *A. niger* resistance. In this study, we tested toothpick poking, seed inoculation, and soil inoculation to establish an effective protocol for screening peanut plants for resistance to crown rot under greenhouse conditions. Results showed that toothpick inoculation with *A. niger* spores caused 100% seedling mortality, whereas non-inoculated poked seedlings and controls showed 100% survival. For the seed inoculation method, the seedling stand percentage was significantly higher in the control (>90%) than in spore treatments across eight weeks ($p < 0.001$). Spore concentrations (10^6 , 10^7 , and 10^8 spores mL^{-1}) did not differ significantly, although 10^8 spores mL^{-1} was most effective, reducing the seedling stand percentage to 1% by week 8. Soil inoculation caused seedling mortality from 3 weeks after sowing and significantly reduced the seedling stand percentage from weeks 2 to 8 ($p < 0.01$ to $p < 0.001$). The 10^8 spores per gram of soil treatment was the most effective, reducing the seedling survival rate to 20% by week 8. To conclude, all three tested inoculation methods were effective. However, toothpick inoculation ensured 100% infection by bypassing all the natural barriers to infect the plant. Seed inoculation was more effective than soil inoculation, which takes longer to cause crown rot infection. These results will enable plant breeders to select the appropriate inoculation method to screen breeding materials and germplasm for potential *A. niger* resistance and, ultimately, to develop crown-rot-resistant cultivars.



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1. Introduction

Peanut crown rot is an important disease caused by *Aspergillus niger* (*A. niger*) in cultivated peanut (*Arachis hypogaea*) worldwide. It is more prevalent in regions with high temperatures and low soil moisture content. The yield losses range from 10% to 50%,

depending on the severity of the attack, and may lead to complete destruction of the crop [1,2]. Peanut crown rot can be either seedborne or soilborne, and *A. niger* in soils often serves as the primary inoculum in fields where peanuts have previously been harvested. Peanut seedlings and young plants are more susceptible to crown rot, which can kill seedlings [3] and plants about 4–5 weeks old under severe conditions. The infected stem just above the soil may turn dark brown, and the crown region under the soil may turn black due to the development of a black mass of conidia and mycelia. As the infection progresses, peanut plants can die within 20–30 days, depending on the severity of the infection. Warm drought conditions, poor seed quality, and insect-induced seedling damage may cause outbreaks of crown rot [4].

Aspergillus species are commonly opportunistic pathogens that colonize plants more effectively when host tissues are weakened by wounds, insect injury, drought, heat stress, or other forms of damage [5]. Black aspergillus produces black conidia, which are easy to recognize on infected plants. The cell walls of these black masses of conidia contain melanin, which confers resistance to high salinity, drought, and UV light [6]. In favorable conditions, *Aspergillus* conidia germinate, form hyphae, and penetrate host plant tissues [5]. *Aspergillus* relies on host cell fatty acid metabolism to meet its energy requirements. The glyoxylate cycle plays a major role in providing nutrients to fungi, enabling asexual reproduction and the production of massive numbers of conidia [7]. During infection, *A. niger* can cause tissue necrosis, vascular tissue degradation, and ultimately the rotting of plant tissues. This leads to adversely affected seed germination, seedling establishment, shoot growth, and root development, which can cause serious yield loss [8,9].

Several inoculation methods have been used to study the various seed- and soil-borne pathogens. Previously, several inoculation methods, including toothpick and clothespin inoculation, slurry inoculation, colonized grain/oat inoculum and agar disk assays, have been compared to study the infection severity of *Sclerotium rolfsii* [10]. Recent studies have used soil infestation, seed inoculation, or combined seed–soil inoculation methods to evaluate the pathogenicity of *A. niger* and establish disease pressure in peanut collar rot assays [11,12]. Similar side-needle, spray [13] and toothpick wounding [14,15] have been used in maize, soybean, and sorghum to achieve uniform disease pressure. These studies showed that inoculation methods can be crucial for evaluating disease severity and infection uniformity.

Currently, no peanut variety that is disease-resistant against crown rot has been reported. One of the constraints in managing crown rot is the lack of a genetic resistance source and effective methods for screening current germplasm to breed disease-resistant peanut cultivars. Developing fast, reliable screening methods is necessary to evaluate the available germplasm and develop resistant lines against peanut crown rot. In this study, we aimed to develop robust methods to screen peanut lines for resistance to *A. niger*. The methods established here can be used to screen peanut lines at either large or small scales, thereby helping breeders develop crown-rot-resistant peanut cultivars.

2. Materials and Methods

2.1. Plant Material Preparation

Peanut cultivar FloRunTM ‘331’ [16] was used for *A. niger* inoculation. FloRunTM ‘331’ was developed by the University of Florida (UF) as a runner-type cultivar with medium-sized seeds; a higher oleic acid composition; and tolerance to fungal diseases, including white mold and late leaf spot [16], but with no resistance to peanut crown rot. For the experiments, peanut seeds of FloRunTM ‘331’ were sterilized by treating them with 0.1% mercuric chloride (HgCl₂) solution for 5 min with continuous stirring, followed by 3–4 washes with distilled water to reduce surface microbial contamination. After that, seeds

were also treated with a 0.6% Abound® Flowable Fungicide (22.9% azoxystrobin, Syngenta, US) solution (resulting in 0.14% azoxystrobin) for 2 min with gentle stirring, then rinsed with distilled water three times to remove any residues of HgCl₂ and Abound® before inoculation or planting. Plants were grown in Miracle-Gro Moisture Control All-Purpose Potting Mix, 50 qt, under greenhouse conditions.

2.2. *A. niger* Inoculum Preparation and Inoculation

The *A. niger* isolated from a peanut field, Suwannee Co., FL, USA, was cultured on full-strength 30 g/L potato dextrose agar (PDA) Petri dishes supplemented with 10 g/L agar at room temperature to achieve maximum growth (black spores) within 7–10 days.

2.2.1. Toothpick Inoculation

For toothpick inoculation, a sterile toothpick was rotated on the surface of a fresh fungal culture plate for several rotations. To quantify the approximate *A. niger* spore load per toothpick, sterile toothpicks were rotated across the surface of a pure fungal culture plate for 3, 5, or 10 rotations. Each rotation treatment was performed in duplicate. The inoculated toothpicks were then immersed in 1 mL of sterile distilled water and mixed thoroughly to prepare a spore suspension. The spore concentration was determined by counting the W squares of a hemocytometer and using the formula below:

$$\text{Cells per mL} = (\text{total cells counted}) / (\text{number of squares counted}) \times 10^4$$

For the experiment, sterilized seeds were grown in pots for three weeks, with three replicates for each of the three treatments: (1) Control, where no inoculation or toothpick poking was conducted. (2) Toothpick poking without *A. niger* inoculum, where seedlings were mechanically wounded by poking at the crown area just underneath the cotyledons (Figure 1) with sterile toothpicks without *A. niger* spores to observe any physical damage or death of the seedlings due to injury. (3) Toothpick poking inoculation with *A. niger* inoculum (10 rotations), where the toothpick inoculated with *A. niger* spore was used to poke the crown area of the seedlings and was left in place to ensure sustained contact between the fungal inoculum and the plant tissue.



Figure 1. Toothpick poking site around the crown area underneath the cotyledon of peanut seedlings.

2.2.2. Seed Inoculation

Three concentrations of *A. niger* suspension— 10^6 , 10^7 , and 10^8 spores mL⁻¹—were prepared from the freshly grown *A. niger* PDA culture plate. Spore suspensions were prepared by adding 5–10 mL of 0.005% sterile Tween solution to a fresh fungal culture plate, scraping the hyphal mat with a sterile cell spreader to release the fungal spores, and diluting

with distilled water to achieve the required spore concentrations. The concentrations of 10^6 , 10^7 , and 10^8 spores mL^{-1} were determined by using a hemocytometer.

Sterilized seeds were soaked in spore suspensions at three concentrations separately for 16 h at room temperature, then sown into the soil. For the control, seeds were soaked in distilled water without any fungal spores for 16 h. Each treatment included three replicate trays, with each tray containing 50 seeds.

2.2.3. Soil Inoculation

For soil inoculation, approximately 200 g of soil was first inoculated with 200 mL of inoculum, then mixed well into 1.13 kg of soil in each tray to evenly distribute *A. niger* spores. Four spore concentrations—0 (control), 10^6 , 10^7 , and 10^8 spores per gram of soil—were achieved. As a control, the same volume of distilled water was added to the soil without any fungal spores. For each treatment, three trays were prepared as replicates. Thus, a total of 12 trays were prepared accordingly, and 50 seeds were sown in each tray.

2.3. Greenhouse Condition

All experiments were conducted in a greenhouse at the University of Florida (UF), Gainesville, FL, USA, during October–December 2024. The greenhouse environmental control system was set to maintain a relative humidity of 75% and temperature of approximately 27 °C to favor *A. niger* infection and disease development. Temperature was regulated using automated heating and cooling systems. However, as is typical for greenhouse environments, the temperature fluctuated throughout the day. During the experimental period, daytime temperatures ranged from 23 to 27 °C, while nighttime temperatures ranged from 16 to 18 °C. Plants were grown under natural daylight and managed using standard greenhouse cultural practices. All three inoculation experiments were carried out in a completely randomized block design.

2.4. Data Collection and Analysis

For the toothpick inoculation, the seedling survival percentage for each tray was recorded at 2 weeks post-inoculation. The infected seedlings were confirmed by observing black sporulation and spore accumulation in vascular tissues under the microscope. For seed and soil inoculations, the seedling stand percentage was recorded weekly in each tray for up to 8 weeks.

The data were subjected to one-way analysis of variance (ANOVA) to compare differences between treatment groups, and significance levels were set at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). When ANOVA showed a significant spore concentration effect ($p < 0.05$), Fisher's Least Significant Difference (LSD) test at $\alpha = 0.05$ was applied to compare the treatment means. Data are displayed as mean $\pm$ standard error (SE). Pearson correlation ($p < 0.05$) between log₁₀ spore concentration and seedling stand percentage was performed. All statistical analyses were performed using the Python 3.12.4 statistical software package SciPy 1.18.0 and statsmodels 0.14.6 [17].

3. Results

3.1. Toothpick Inoculation

To standardize the toothpick inoculation method, spore concentration was measured after rotating the toothpick 3, 5, and 10 times on the culture plate. The spore number increased with the rotation number, where 10 rotations gave a mean of 9.15×10^5 spores (Table 1), which were used for the toothpick inoculation method in this study.

Table 1. Effect of toothpick rotation number on *A. niger* spores gathered by a toothpick.

Rotations	R1	R2	Mean
3	1.1×10^5	8.7×10^4	9.85×10^4
5	5.0×10^5	4.8×10^5	4.90×10^5
10	8.4×10^5	9.9×10^5	9.15×10^5

Following toothpick inoculation, crown rot symptoms became progressively more pronounced, resulting in seedling mortality within 2 weeks (Figure 2A–F). As the infection advanced, dense black sporulation developed around the crown region (Figure 2I,J), and infected plants started to show sudden wilting (Figure 2H) and ultimately died approximately two weeks after inoculation (Figure 2A,D). In contrast, seedlings without toothpick poking showed no signs of disease development and demonstrated normal growth, indicating a healthy environment during the experiment (Figure 2C,F). The seedlings poked with a sterile toothpick, without fungal inoculum, showed no crown rot symptoms (Figure 2B,E) and grew normally, confirming that mechanical injury alone did not induce disease and that crown rot symptoms and mortality in seedlings inoculated with *A. niger* were caused by infection. Overall, *A. niger* inoculation caused 100% seedling mortality, whereas sterile-toothpick-poked seedlings and controls showed 100% survival (Figure 2G). Microscopic examination of the cross-section of the infected plant stem revealed dark coloration of the vascular bundle, indicating necrosis and tissue damage (Figure 2J).

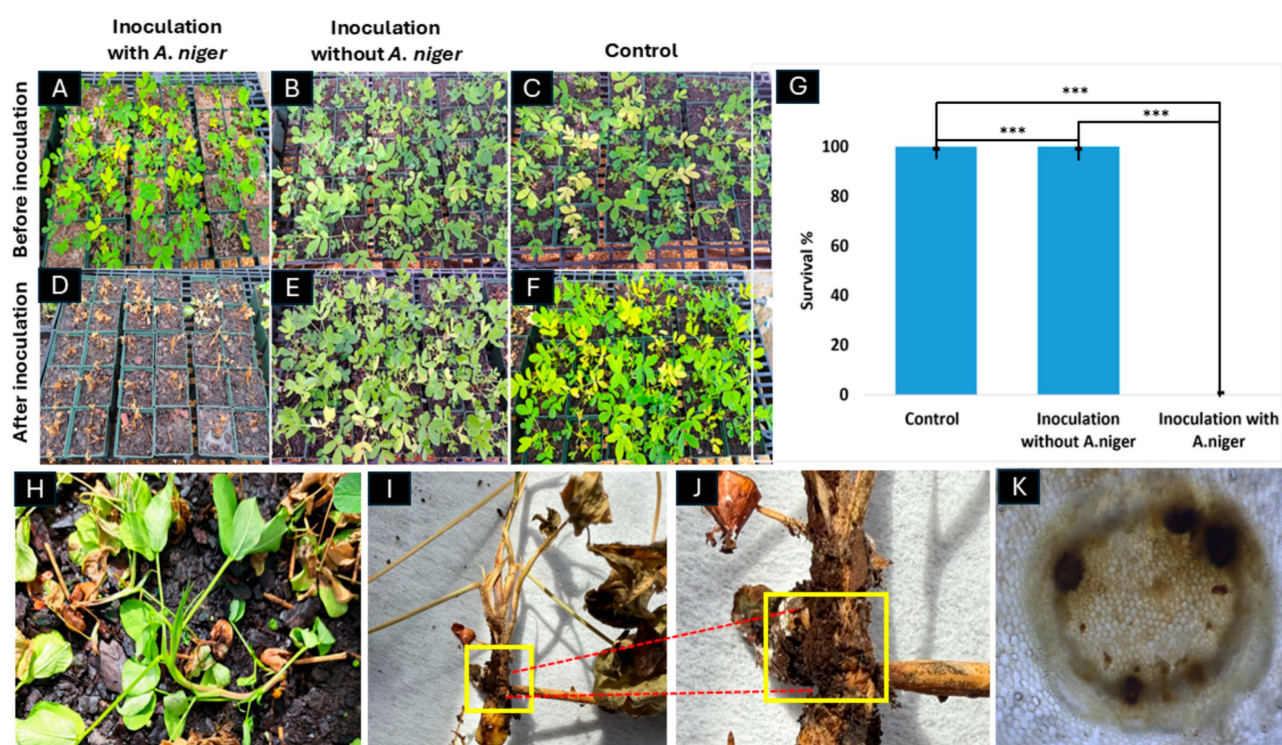


Figure 2. Illustrating toothpick poking inoculation. (A–F) Toothpick inoculation results before and after 2 weeks of *A. niger* infection. From left to right: Control (uninoculated), toothpick inoculation without *A. niger* and toothpick inoculation with *A. niger* spores. (G) Seedling survival percentage at 2 weeks after inoculation. (H) Sudden seedling wilting after 10 days of post-*A. niger* inoculation. The results showed a 100 (%) infection rate for toothpick poking with *A. niger* spores, whereas the control (uninoculated) and toothpick poking without *A. niger* seedlings showing 100 (%) survival. (I,J) *A. niger* black spores around the crown region of the peanut stem. (K) Symptomatic cross-section of infected cells under a microscope showing darkened vascular tissues including xylem and phloem cells, indicating severe tissue necrosis post-*A. niger* infection. Significance levels: $p < 0.001$ ***.

3.2. Seed Inoculation

The control peanut seeds germinated progressively from week 2 to week 5, achieving a final germination rate of 87.33%. This reflects natural variability in the timing of seedling emergence under greenhouse conditions. However, pre-soaking peanut seeds in *A. niger* suspension inhibited germination significantly ($p < 0.001$). By week 8, germination rates were reduced to 6.00%, 4.67%, and 1.33% in the 10^6 , 10^7 , and 10^8 spores mL^{-1} treatments (Figure 3A–H), respectively, corresponding to 93–98.5% reductions compared with the control (Figure 3I). Although, the inoculated treatments with 10^6 , 10^7 , and 10^8 spores mL^{-1} were generally not significantly different from each other, the 10^8 spores mL^{-1} treatment resulted in the greatest reduction in seedling stand percentage.

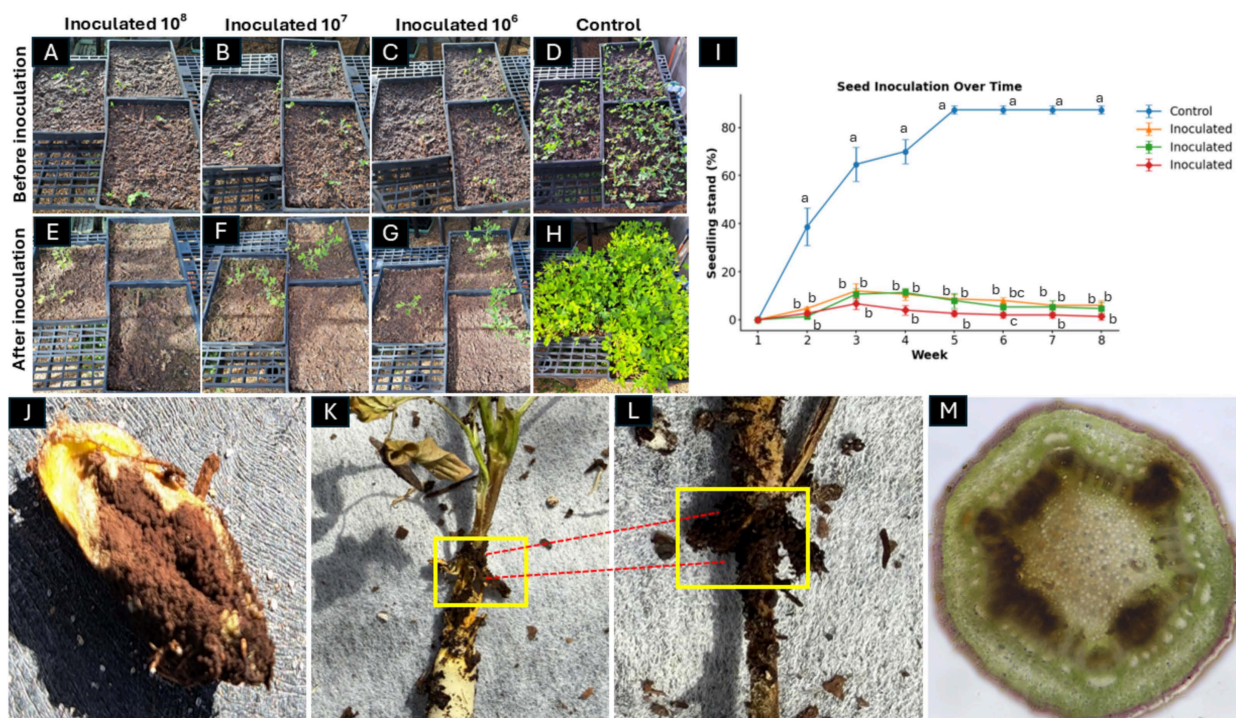


Figure 3. Demonstrating the effect of seed inoculation on peanut seedlings. (A–H) Seed inoculation results after 3 weeks and 8 weeks of *A. niger* infection. From left to right: Control (uninoculated) and 10^6 , 10^7 , and 10^8 spores mL^{-1} treatment groups, respectively. (I) The germination percentage was recorded from week 2 to week 8. Data represent mean $\pm$ standard error (SE) of three biological replicates. Different letters above data points indicate significant differences between treatments within each week, as determined by Fisher's least significant difference (LSD) test at $\alpha = 0.05$. (J–L) *A. niger* black spores around the cotyledons and crown region of the infected peanut seedling. (M) Cross-section of an infected stem under a microscope, showing dead vascular tissue due to infection.

For the seedlings germinated from seeds pre-soaked in *A. niger* suspension, rapid crown rot developed with visible mycelial growth around the crown area that extended to the cotyledons within 2 weeks after planting (Figure 3J–M). The presence of a black mass of spores indicated the presence of *A. niger*, which was re-isolated and re-cultured on PDA to confirm the presence of the same fungal spores. Overall, seed inoculation imposed strong disease pressure, reduced seed germination, and enabled rapid screening of cultivars for crown rot resistance at the germination stage.

3.3. Soil Inoculation Developed Concentration-Dependent Disease Infection

The soil inoculation resulted in seedling mortality 3 weeks after sowing and significantly impacted seedling stand rates ($p < 0.01$ for 2–4 weeks; $p < 0.001$ for 5–8 weeks).

Comparison of seedlings during the second week (Figure 4A–D) and eighth week after planting (Figure 4E–H) showed that all three spore concentrations reduced the seedling stand percentage compared with the uninoculated control. At week 2 after sowing, the seedling stand percentage was higher and not reduced by the lower soil inoculum levels (10^6 and 10^7 spores per gram soil) compared with the non-inoculated control. Also, during the third week, the seedling stand percentages in the 10^6 and 10^7 spores per gram treatments were generally comparable with the control, whereas the 10^8 treatment demonstrated a reduction in seedling stand percentage. After three weeks of sowing, germinated seedlings started to show crown rot symptoms (Figure 4J,K), followed by seedling death in all *A. niger* spore treatment groups, particularly the 10^8 spores per gram treated group, and a progressive decline in seedling stand percentage. In contrast, the seedling stand percentage remained high, above 80% in the non-inoculated control. The infected seedlings were dissected and observed under a microscope, revealing abundant black spores within the vascular tissues (Figure 4L). This revealed that *A. niger* overwhelmed the vascular tissue, leading to damping-off and the ultimate death of peanut plants. The fungal concentration of 10^8 spores per gram in soil was the most effective at impacting the seedling stand percentage, with a 20% survival compared with the other two treatment levels (~40%) by the end of eight weeks after planting (Figure 4I). There was no significant difference in seedling stand percentage between the 10^6 and 10^7 spore per gram treatments.

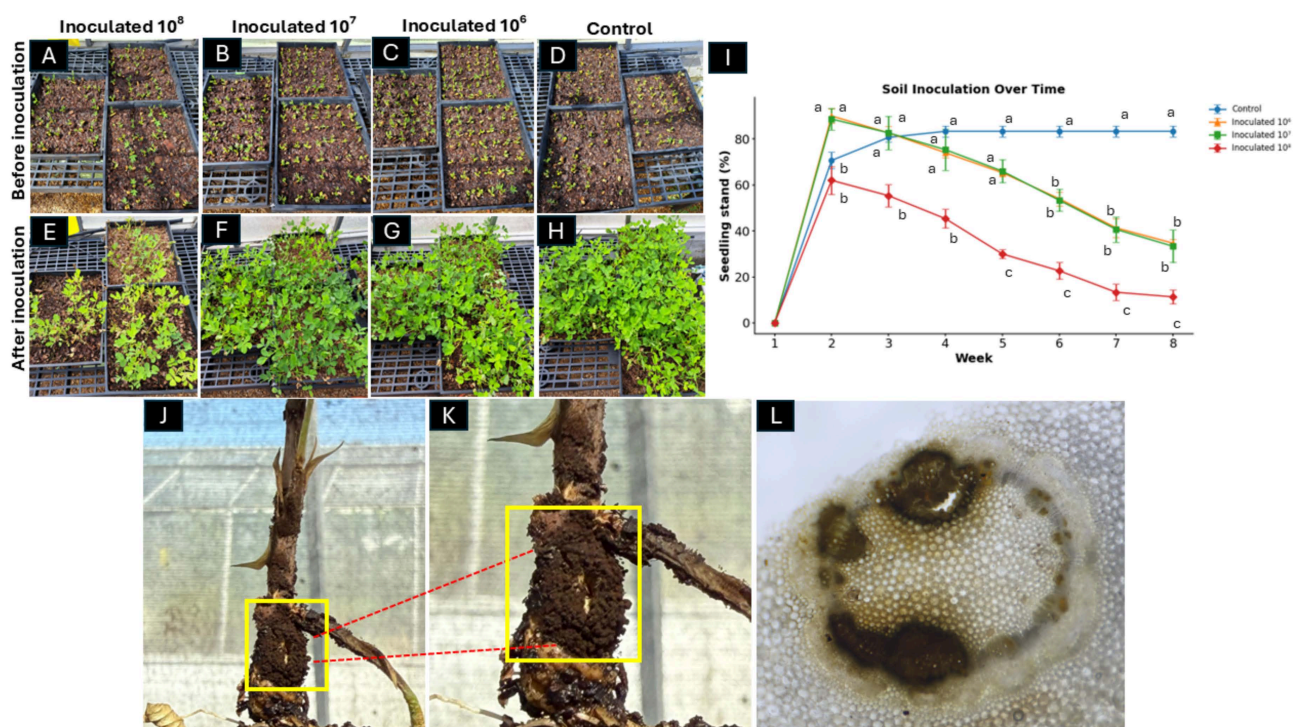


Figure 4. Illustrating the effect of soil inoculation on the germination percentage over time. (A–H) Different spore concentrations (10^6 , 10^7 , and 10^8) were used to inoculate the soil before seed planting. Upper and lower panels showing soil inoculation results in the 2nd week and after the 8th week of *A. niger* infection, respectively. From left to right: Control (uninoculated) and 10^6 , 10^7 , and 10^8 spores per gram treatment groups, respectively. (I) The seedling stand percentage was recorded from week 2 to week 8. Data represent mean $\pm$ standard error (SE) of three biological replicates. Different letters above the data points indicate significant differences between the control and treatments after 3 weeks, as determined by Fisher's least significant difference (LSD) test at $\alpha = 0.05$. (J,K) *A. niger* black spores around the cotyledons and crown region of the peanut. (L) Cross-section of infected cells under a microscope showing dead vascular tissues due to spore accumulation.

4. Discussion

In crop breeding programs, disease screening is typically performed in the field, under controlled greenhouse conditions, or using in vitro methods to evaluate, select, or develop potential pathogen-resistant genotypes. The current study aims to develop efficient inoculation methods to screen the peanut germplasm for peanut crown rot caused by *A. niger*. Three methods, namely, toothpick poking, seed inoculation, and soil inoculation, were tested to induce *A. niger* symptoms in greenhouse conditions. Variations in infection severity and mortality rates between these inoculation methods revealed that the choice of inoculation approach affects the severity and efficacy of *A. niger* infection. These findings are crucial for understanding the relative estimated effectiveness of these inoculation techniques used in the present study and will help scientists develop more effective laboratory and greenhouse methods for screening peanut crown rot resistance.

4.1. Toothpick Inoculation Offers an Extreme Pressure Screening Assay

The toothpick inoculation technique was highly effective and reliable for delivering *A. niger* spores directly to the wounded tissue, bypassing initial physical barriers such as the epidermis, cuticle and cell wall, thereby increasing the likelihood of rapid infection before full activation of host defense responses [18]. It ensured 100% infection and mortality of all inoculated peanut seedlings within 2 weeks, consistent with previous pathogenicity studies, which required mechanical injury to facilitate infection and confirm pathogen aggressiveness [19]. The toothpick inoculation method has also been tested to evaluate the disease resistance against several host–pathogen systems, including soybean—*Diaporthe aspalathi* [14,20], sorghum—*Erwinia chrysanthemi* [21], corn—*Pythium arrhenomanes* [22], maize—*Fusarium graminearum* [23] and cowpea—*Macrophomina phaseolina* [24]. While the toothpick method guarantees high infection rates, it differs from natural infection processes. *A. niger* is typically seed- or soil-borne and infects plants without physical wounds. Mechanical wounding may intensify infection severity compared with natural field conditions. Therefore, this approach is most appropriate when a synchronized and uniform infection is required. This method is particularly beneficial for confirming host resistance, evaluating host resistance under disease pressure, testing fungicide efficacy, or studying host–pathogen interactions after infection has been established. In our study, the toothpick method produced consistent, uniform infections. This contrasts with results reported for *S. rolfii* using the same technique [10], in which toothpick–stem inoculation was less consistent in both greenhouse and field experiments. This may reflect differences in both pathogens' virulence and/or growth characteristics. The aggressive nature of *A. niger*, which rapidly produces abundant conidiospores under favorable conditions and colonizes vascular tissues quickly, led to seedling death within 2–3 weeks post-infection. This reliable, synchronized infection makes the toothpick method valuable for accurate resistance screening for crown rot resistance. However, other inoculation methods (discussed below) provide easy, large-scale operation, and additional insights into infection timing and disease development under more natural environmental conditions.

4.2. Seed Inoculation Strongly Suppressed Seed Germination

For applying the seed inoculation method, peanut seeds were immersed in a suspension of *A. niger* spores at varying concentrations. This approach mimics a common route of natural infection, as contaminated seeds often result in poor germination and damping-off in field conditions [25]. Our results quantitatively demonstrate this: seeds soaked in spore suspensions (10^6 , 10^7 , 10^8 spores mL^{−1}) exhibited poor germination or post-emergence mortality regardless of concentration, indicating that 10^6 spores mL^{−1} is sufficient for infection. The increase in the germination rate in the non-inoculated control

progressed from week 1 to week 5, indicating seed germination variability under greenhouse conditions. The seed inoculation method achieved nearly 100% infection rates at higher spore concentrations compared with ~85% for soil inoculation. This higher efficiency results from direct pathogen–seed contact during the 16 h soaking period. *A. niger* initiates colonization during soaking, and favorable soil conditions (humidity and temperature) after sowing enable rapid spore reproduction. By the time radicles and shoots emerge, black spores have already colonized the seed coat and invaded the cotyledons, resulting in rapid infection of seedlings in the soil before they sprout. This method provides valuable insight into infection processes during germination and early seedling emergence, when seeds and young seedlings are most vulnerable to pathogen attack under natural conditions. Seed inoculation is particularly valuable for evaluating genetic resistance during early developmental stages and for large-scale screening of resistant genotypes. The uniform, high-infection-pressure stringently tests seedling vigor and early defense responses to determine field establishment success.

4.3. Soil Inoculation Induced a Gradual but Progressive Development of Crown Rot

The soil inoculation method resulted in ~85% mortality at 10^8 spores mL^{-1} and required a longer time to produce disease symptoms than seed inoculation. This delay occurs because spores are distributed throughout the soil rather than concentrated on surfaces of seeds. Not every seed immediately contacted spores during germination, and pathogen spread requires time, allowing some seedlings to grow and potentially escape or survive infection, particularly at lower spore densities. This method most closely replicates natural field conditions, where *A. niger* acts as a soilborne pathogen. Soil microbial communities and environmental factors influence pathogen establishment, providing a more realistic assessment of disease development in agricultural settings. Our results demonstrate that infection consistency is influenced by both inoculum density and the duration of host–pathogen contact, which may help explain the variability in crown rot development observed under field conditions. Achieving uniform disease incidence with soil inoculation requires an even pathogen distribution and optimal environmental conditions, soil moisture, and temperatures of 28–30 °C, with drought conditions favoring *A. niger* growth [19]. This method is ideal when the research objective is to evaluate how genotypes perform under field-like conditions with variable infection timing and environmental pressures.

4.4. Variations Between Inoculation Methods

The toothpick inoculation method proved to be the most lethal and uniform for pathogen pressure application of the three methods, achieving the most severe and rapid response and resulting in a 100% infection rate. The variation between seed and soil inoculation methods largely depends on whether the fungal spores achieve direct and immediate contact or not with seeds. Direct contact of fungal spores with the seed resulted in rapid, severe infection, leading to strong suppression of emerged seed growth. In contrast, soil inoculation with similar spore densities retained a higher seedling stand percentage, and the infection spread progressively as compared with the toothpick poking and seed inoculation methods. Recent work suggests that combining seed and soil inoculation can achieve intermediate infection rates (76%) [26], potentially offering insights into multiple infection routes simultaneously. The choice of method should align with research objectives: stringent resistance screening favors seed or toothpick methods, while field-representative evaluations favor soil inoculation or combined approaches.

As a summary, toothpick inoculation can provide a 100% infection rate, making it suitable to select resistant germplasms quickly and accurately and suitable for host

resistance mechanistic studies. Seed inoculation can provide a nearly 100% infection rate, which is optimal for large-scale screening of germplasm or breeding materials at the early emergence stage. Soil inoculation provides an approximately 85% infection rate, which can mimic field conditions in evaluating disease development across plant growth stages and assessing genotype performance under realistic, variable disease pressure.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

<i>A. niger</i>	<i>Aspergillus niger</i>
HgCl ₂	Mercuric chloride
PDA	Potato dextrose agar
LSD	Fisher's least significant difference
ANOVA	Analysis of variance

References

1. Damicone, J. Soilborne Diseases of Peanuts. Available online: <https://extension.okstate.edu/fact-sheets/print-publications/epp-entomology-and-plant-pathology/soilborne-diseases-of-peanut-epp-7664.pdf> (accessed on 6 April 2026).
2. Pande, S.; Rao, J.N. Changing scenario of groundnut diseases in Andhra Pradesh, Karnataka, and Tamil Nadu states of India. *Int. Arachis Newsl.* **2000**, *20*, 42–44. [\[CrossRef\]](#)
3. Carter, E. Crown Rot Creeps In. Available online: <https://nwdistrict.ifas.ufl.edu/phag/2016/07/08/crown-rot-creeps-in/> (accessed on 6 April 2026).
4. Melouk, H.; Damicone, J. *Aspergillus* crown rot. In *Compendium of Peanut Disease*; Kokalis-Burelle, N., Porter, D.M., Rodríguez-Kábana, R., Smith, D.H., Subrahmanyam, P., Eds.; USDA: St. Paul, MN, USA, 1997; pp. 429–494.
5. Baltussen, T.J.; Zoll, J.; Verweij, P.E.; Melchers, W.J. Molecular mechanisms of conidial germination in *Aspergillus* spp. *Microbiol. Mol. Biol. Rev.* **2020**, *84*, e00049–19. [\[CrossRef\]](#) [\[PubMed\]](#)
6. Bennett, S.E.B.J.W. An overview of the genus *Aspergillus*. In *The Aspergilli*; CRC Press: Boca Raton, FL, USA, 2007; pp. 23–34.
7. Ebel, F.; Schwienbacher, M.; Beyer, J.; Heesemann, J.; Brakhage, A.A.; Brock, M. Analysis of the regulation, expression, and localisation of the isocitrate lyase from *Aspergillus fumigatus*, a potential target for antifungal drug development. *Fungal Genet. Biol.* **2006**, *43*, 476–489. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Shankar, J. An overview of toxins in *Aspergillus* associated with pathogenesis. *Int. J. Life Sci. Biotechnol. Pharma Res.* **2013**, *2*, 16–31.
9. Tawfik, E.; Alqurashi, M.; Aloufi, S.; Alyamani, A.; Baz, L.; Fayad, E. Characterization of mutant *Aspergillus niger* and the impact on certain plants. *Sustainability* **2022**, *14*, 1936. [\[CrossRef\]](#)
10. Shokes, F.; Róžalski, K.; Gorbet, D.; Brennen, T.; Berger, D. Techniques for inoculation of peanut with *Sclerotium rolfsii* in the greenhouse and field. *Peanut Sci.* **1996**, *23*, 124–128. [\[CrossRef\]](#)
11. Bajaya, T.; Ghasolia, R.; Bajya, M.; Choudhary, M.; Shivran, M.; Kumari, P.; Sharma, J. Isolation, identification, pathogenicity and host range of *Aspergillus niger* causing collar rot of groundnut (*Arachis hypogaea*). *Pharma Innov. J.* **2022**, *11*, 1441–1445.
12. Kumari, M.; Singh, M. Survey, collection, isolation and pathogenicity test of *Aspergillus niger* van. Tieghem causing collar rot of groundnut in Sikar and Jaipur district of Rajasthan. *J. Food Legumes* **2016**, *29*, 237–240.

13. Windham, G.; Williams, W. Comparison of different inoculating methods to evaluate the pathogenicity and virulence of *Aspergillus niger* on two maize hybrids. *Phytoparasitica* **2012**, *40*, 305–310. [[CrossRef](#)]
14. Campbell, M.A.; Li, Z.; Buck, J.W. Development of southern stem canker disease on soybean seedlings in the greenhouse using a modified toothpick inoculation assay. *Crop Prot.* **2017**, *100*, 57–64. [[CrossRef](#)]
15. Tesso, T.; Ochanda, N.; Claflin, L.; Tuinstra, M. An improved method for screening *Fusarium* stalk rot resistance in grain sorghum (*Sorghum bicolor* [L.] Moench.). *Afr. J. Plant Sci.* **2009**, *3*, 254–262.
16. Tillman, B. Registration of ‘FloRun ‘331’ peanut. *J. Plant Regist.* **2021**, *15*, 294–299. [[CrossRef](#)]
17. Virtanen, P.; Gommers, R.; Oliphant, T.E.; Haberland, M.; Reddy, T.; Cournapeau, D.; Burovski, E.; Peterson, P.; Weckesser, W.; Bright, J. SciPy 1.0: Fundamental algorithms for scientific computing in Python. *Nat. Methods* **2020**, *17*, 261–272. [[CrossRef](#)] [[PubMed](#)]
18. Pryor, B.; Davis, R.; Gilbertson, R. A toothpick inoculation method for evaluating carrot cultivars for resistance to *Alternaria radicina*. *HortScience* **2000**, *35*, 1099–1102. [[CrossRef](#)]
19. Xu, M.; Yang, J.; Wu, J.; Chi, Y.; Xie, L. First report of *Aspergillus niger* causing root rot of peanut in China. *Plant Dis.* **2015**, *99*, 284. [[CrossRef](#)] [[PubMed](#)]
20. Ghimire, K.; Petrović, K.; Kontz, B.J.; Bradley, C.A.; Chilvers, M.I.; Mueller, D.S.; Smith, D.L.; Wise, K.A.; Mathew, F.M. Inoculation method impacts symptom development associated with *Diaporthe aspalathi*, *D. caulivora*, and *D. longicolla* on soybean (*Glycine max*). *Plant Dis.* **2019**, *103*, 677–684. [[CrossRef](#)] [[PubMed](#)]
21. Singh, P.; Singh, Y. Evaluation of inoculation methods and standardization of *Erwinia chrysanthemi* inoculum concentration for germplasm screening against stalk rot in sorghum. *J. Pure Appl. Microbiol.* **2016**, *10*, 2747–2752. [[CrossRef](#)]
22. Mitsuhashi, S.; Masunaka, A.; Kikawada, T.; Sugawara, K.; Tsukiboshi, T.; Tamaki, H.; Sato, H. Evaluation of resistance to *Pythium* root rot by *Pythium arrhenomanes* in corn by using a toothpick inoculation method. *Grassl. Sci.* **2015**, *61*, 181–184. [[CrossRef](#)]
23. Mesterhazy, A.; Toldine Toth, E.; Szel, S.; Varga, M.; Toth, B. Resistance of maize hybrids to *Fusarium graminearum*, *F. culmorum*, and *F. verticillioides* ear rots with toothpick and silk channel inoculation, as well as their toxin production. *Agronomy* **2020**, *10*, 1283. [[CrossRef](#)]
24. Souza, E.M.d.S.; Silva, L.A.B.d.; Pinto, F.Á.C.; Borel, J.C.; Capucho, A.S.; Ishikawa, F.H. Inoculation methods and aggressiveness of *Macrophomina phaseolina* isolates in cowpea. *Ciência Rural* **2021**, *52*, e20210072. [[CrossRef](#)]
25. Morwood, R. Peanut pre-emergence and crown rot investigations. *Qld. J. Agric. Sci.* **2024**, *10*, 222–236.
26. Govind Junjadia, J.R.V.; Dama Ram, M.M.; Kumawat, M.M.; Kumar, M.; Kumar, S. Isolation, Identification, and Pathogenicity of Collar Rot of Groundnut (*Arachis hypogaea*) Caused by *Aspergillus niger* van Tieghem. *Biol. Forum.* **2025**, *17*, 04–08. [[CrossRef](#)]

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