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Clay-based azoxystrobin formulation enhances cotton protection against charcoal rot disease

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Abstract

Background Cotton charcoal rot disease, caused by *Macrophomina phaseolina*, is a major threat to cotton production in Israel and globally, leading to severe yield loss due to post-flowering plant collapse. Current management relies heavily on chemical fungicides. However, rising environmental concerns and fungicide resistance underscore the need for sustainable alternatives. This study evaluated novel clay-based formulations for the controlled release of azoxystrobin (AS). The approach aims to protect cotton during the sensitive early growth stages when pathogen penetration and colonization occur. Two types of clay carriers—bentonite (montmorillonite) and sepiolite—were tested for their ability to deliver AS effectively.

Results While seedlings grown in the greenhouse showed minimal visible symptoms at the disease latent stage, quantitative real-time PCR analysis indicated that both formulations effectively suppressed root infection, reducing *M. phaseolina* DNA levels by 81%. In a full-season open-enclosure trial, bentonite-AS treatment exhibited reduced efficacy, possibly due to phytotoxicity at the tested concentration. In contrast, the sepiolite-AS treatment increased flower bud number by 87% and reduced pathogen infection by up to 92% at 68 days post-sowing, although these effects were not statistically significant due to high variability. At harvest, sepiolite-AS and bentonite alone increased shoot dry weight per plant by 129% and 128%, respectively, and reduced pathogen DNA levels by 63% and 69%, respectively.

Conclusion Overall, although environmental variability led to statistical insignificance, the findings support the clay-AS approach, especially sepiolite, as an effective means to prevent early infection and reduce late-season disease outbreaks. This formulation holds promise for seed coating or sowing strip applications, offering a practical, eco-friendly approach that can be integrated with biological control to reduce chemical inputs across diverse cultivation systems.

Keywords Azoxystrobin, Bentonite, Crop protection, Disease control, *Macrophomina phaseolina*, Sepiolite, Quantitative real-time PCR

Introduction

Cotton (*Gossypium* spp.) is the world's primary natural fiber crop and a crucial income source for farmers globally. In 2023, cotton was cultivated on approximately 32 million hectares (FAOSTAT 2023), yielding over 24 million metric tons (U.S. Department of Agriculture, Foreign Agricultural Service, Production – Cotton 2025). According to a recent report (Ashraf et al. 2024; Saleem et al. 2024), cotton has an annual economic impact of

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approximately 600 billion US dollars, and remains the leading natural fiber produced and traded worldwide. The cotton industry supports nearly one billion individuals, including 250 million laborers, spans about 150 countries, and provides livelihoods for around 100 million families. While cotton is adapted to a wide range of climates—including temperate, subtropical, and tropical regions—its future cultivation may be hindered by climate change (Bange et al. 2016), which can exacerbate the prevalence and severity of soil-borne diseases such as cotton charcoal rot disease (CRD) (Cohen et al. 2022). Biotic stresses, particularly from pathogens and pests, substantially reduce cotton yield and drive up production cost due to necessary control interventions. *Macrophomina phaseolina* (Tassi) Goid., the causal agent of CRD, is among the most damaging pathogens. The fungus is a broad-host-range pathogen capable of infecting over 500 plant species across about 75 families (Marquez et al. 2021; Banaras et al. 2021). Disease severity in cotton is influenced by the level of soil infestation, pathogen virulence variability, and host susceptibility (Su et al. 2001). Infected cotton plants typically exhibit symptoms during the flowering and fruiting stages, including wilting, leaf and stem desiccation, and eventual plant death (Degani et al. 2020). Yield loss might be severe and localized, with complete plant mortality observed in affected field patches (Yadav et al. 2021). The fungus usually penetrates the roots through cracks in the phelloderm, often formed in dry, compacted soils during hot weather conditions (Cohen et al. 2022). Timely irrigation, particularly before the onset of heat stress, can enhance plant performance and disease tolerance (Degani et al. 2023b). *M. phaseolina* employs cell wall-degrading enzymes and mycotoxins to invade and kill host cells (Islam et al. 2012). Early symptoms, appearing in young plants in early summer, include foliar yellowing—presumably due to toxin translocation from the roots to above-ground tissues—often leading to plant dehydration despite adequate irrigation (Abbas et al. 2019). A characteristic symptom of CRD is vascular discoloration, likely resulting from fungal toxins interacting with host-derived compounds such as gossypol, a phytoalexin central to cotton defense mechanisms (Cohen et al. 2022). In Israel, the incidence of CRD has increased, potentially due to the adoption of more susceptible *Gossypium barbadense* cultivars and rising temperature associated with climate change (Cohen et al. 2022).

The persistence of *M. phaseolina* in soil and plant residues is primarily due to its production of melanized microsclerotia and pycnidia (Marquez et al. 2021), which are multicellular structures that enable long-term survival and complicate disease management (Kumar et al. 2023; Lodha et al. 2020; Alizadeh et al. 2025). Control strategies to reduce disease pressure include crop

rotation, resistant cultivars, cultural practices, soil solarization, and optimized irrigation (Lodha et al. 2020). However, these methods often require long implementation periods and may be undermined by the pathogen's strong saprophytic capacity (Kumar et al. 2023; Ghosh et al. 2018). In the absence of highly resistant cotton varieties, systemic fungicides have become the principal tool for disease suppression (Bashir et al. 2017). Various fungicides, such as carbendazim, difenoconazole, benomyl, and azoxystrobin (AS), have shown differing levels of efficacy against *M. phaseolina*. Bashir et al. (2017) identified carbendazim and thiophanate-methyl as effective treatments. In Israel, Cohen et al. (2022) evaluated chemical control strategies and reported that seed coating with AS or Signum (a combination of boscalid and pyraclostrobin) prevented root infection for up to 12 days, after which root elongation exposed tissues to infection. The results also showed that AS applications in soil through the drip irrigation system reduced the disease incidence to 15%, whereas untreated controls and treatments with prochloraz or prothioconazole resulted in about 30% incidence. Given increasing concerns about the overuse of synthetic fungicides and the emergence of fungicide-resistant strains (Shcherbakova 2019), alternative, environmentally sustainable strategies for managing CRD are urgently needed (Uroos et al. 2022; Javed et al. 2021; Banaras et al. 2021). The utilization of agriculturally beneficial microorganisms for disease management represents an environmentally sustainable and safer alternative to conventional methods that may negatively impact ecosystems and human health. Notably, plants associated with *Trichoderma* spp. demonstrate increased growth and a reduced incidence of multiple diseases compared with those of plants cultivated without these fungal biocontrol agents (Nakkeeran et al. 2016; de Oliveira et al. 2021; Elshahawy et al. 2018; Degani et al. 2021a, 2021b; Gordani et al. 2023). Specific *Trichoderma* species have demonstrated efficacy against CRD across a range of experimental systems, including in vitro dual-culture assays, growth room seedling trials, and full-scale commercial field evaluations (Degani et al. 2023a, 2024; Iqbal et al. 2020a; Waheed et al. 2024).

In recent decades, clay-based substrates have been investigated to minimize the ecological damage caused by pesticides by preventing their rinsing, decomposition, or loss of activity (Aranda et al. 2018; Rytwo et al. 2012, 2015; Shuali et al. 2011; Undabeytia et al. 2011; Mishael et al. 2003; Sheng et al. 2001). Clay is inexpensive (affordable), simple, and readily available, and there is no fear of using it for environmental hazards. Various clays have been proven as potential materials suitable for slow-release protection against leaching, evaporation, or photochemical decomposition (Nir et al. 2013).

They can be applied in their natural form or as an "engineered" form by organic cations while optimizing the adsorption process, release, and activity. An example of such use was recently demonstrated (Shaltiel-Harpaz et al. 2023). In this study, clay was shown to be effective for the slow release of essential oils to control thrips in chives in a greenhouse experiment without harming the chives. Beyond the limitations discussed above, chemical CRD management is costly and dependent on drip irrigation and specific cultivation practices. In contrast, the proposed clay-based formulation—applicable by sowing strip placement or seed dressing—offers a cost-effective, environmentally friendly alternative that significantly reduces chemical inputs, and can be efficiently deployed under diverse field conditions without the need for specialized infrastructure. The slow release of fungicide from a clay dispersed in the sowing strip allows continuous protection of the seedlings over time while preventing the loss of activity due to movement of the fungicide in soil. This spot application will also enable a significant reduction in pesticide use and its combination with biological control solutions. Indeed, AS fungicide has already been proven effective in combination with *Trichoderma* species for CRD protection (Degani et al. 2024, 2023b). Finally, the azoxystrobin-clay (AS-clay) formulation may also protect plants against other soil diseases, as shown for *Magnaportheopsis maydis* (Gordani et al. 2023). The two pathogens, *M. phaseolina* and *M. maydis*, can be found abundantly in Israeli commercial fields that share two summer crop hosts, cotton and maize, cultivated alternately in two-year rotations (Degani et al. 2022, 2020). The goal of the present study was to develop clay-based substrates for the binding and slow release of AS and test their efficiency against the CRD pathogen *M. phaseolina* in a greenhouse and over an entire growth period under semi-field conditions. The efficacy of this substrate against *M. phaseolina* infections was evaluated by tracking the plants' growth and health. Sensitive pathogen infection assessment was performed via a molecular method in which the DNA abundance of *M. phaseolina* in the plant roots was detected via quantitative real-time PCR (qPCR).

Materials and methods

Rationale and research design

The study consisted of two parallel experiments with a similar design. The first experiment focused on cotton sprouts grown for up to 33 days in greenhouse conditions. The second experiment was performed in pots within an open enclosure (semi-field conditions) throughout a full growth season of up to 106 days. Conducting experiments in pots offers advantages over open-field trials, including greater uniformity of environmental

conditions and more precise control of inoculum pressure, irrigation, soil composition, and fertilization. Additionally, this setup prevents cross-contamination among treatments through a randomized distribution and allows the inclusion of mock controls—uninfected, healthy plants.

AS clay-based formulation preparation

In this study, two types of clays, bentonite (purchased from Sigma-Aldrich, Rehovot, Israel, CAS No: 1302–78-9), a platelet-structured clay mineral primarily composed of montmorillonite, and sepiolite (supplied by Tolsa, Spain, CAS No: 63800–37-3), a naturally occurring fibrous structured clay mineral, were used. Both minerals are known for their capacity to adsorb organic molecules (Shuali et al. 2011). To prepare the formulation, a commercial AS pesticide (Amistar S.C.; Syngenta, Basel, Switzerland, supplied by Adama Makhteshim, Ashdod, Israel, 30.76 mL of a tenfold diluted commercial solution) was thoroughly mixed with a clay sample (9.24 g) on baking paper to form a uniform slurry. Deionized distilled water (DDW) at the same volume was used as a control instead of AS. The mixture was then dried in a room environment until complete water evaporation occurred. The resulting dry material was ground into fine powder using a mortar and pestle.

Fungal origin and growth conditions

The phytopathogenic fungus *M. phaseolina* (isolate Mp-1) was isolated in 2017 from infected cotton plants at the laboratory of Roni Cohen, Newe Ya'ar Research Center, Israel. Identification was based on physiological profiling, colony morphology, microscopic characteristics, and molecular analysis (Degani et al. 2023a, 2020). Several studies previously demonstrated its pathogenicity toward cotton (Degani et al. 2022, 2024, 2023b). The fungus was maintained on potato dextrose agar (PDA; Sisco Research Laboratories Pvt. Ltd., Maharashtra, India) in 90-mm Petri dishes incubated at (28 ± 1) °C, in darkness for 4–5 days, using MaxQ™ 6000 Incubated/Refrigerated Stackable Shakers (Thermo Fisher Scientific, Waltham, MA, USA). Subculturing was performed by transferring a 6-mm-diameter mycelial plug excised from the edge of a 5–7-day-old colony onto a fresh PDA plate, followed by incubation under identical conditions.

Greenhouse sprouts' experiment

Experimental setup

The seedling assay employed seven independent biological replicates (pots) per treatment. The following treatments were evaluated: each AS-clay-based formulation (bentonite or sepiolite) was applied to protect *M. phaseolina*-stressed plants compared with a DDW-clay control

(clay treated with DDW instead of pesticide). These treatments were compared with the corresponding treatments without *M. phaseolina* soil infection. Two additional controls included infected, unprotected plants and mock healthy plants. In total, the experiment comprised 10 treatments and 70 pots. The trial was conducted in 2.5 L pots placed within a greenhouse at the Avni Etan Experimental Farm, located in Golan Heights (32°49′03.3″ N, 35°45′46.4″ E). The local peat soil used for the experiment was sourced from a field with no recorded history of *M. phaseolina* infection. Any potential presence of the pathogen was presumed negligible and unlikely to affect the results. The soil was amended with 33% (v/v) Perlite No. 4 to improve soil aeration. Seven seeds of the Goliath V-6 cotton cultivar (*G. barbadense*, extra-long staple Pima-type, from Israel Seeds) were sown per pot at a depth of approximately 3 cm. The sprouts were subsequently diluted to 5 per pot 13 days after seeding. The plants were grown in a greenhouse under an 11 h light/13 h dark photoperiod, with relative humidity ranging between 32% and 100% (average: 66% ± 19%) and temperature ranging from 9.8 °C (min) to 32.1 °C (max), with an average of (21.1 ± 4.3) °C. Pots were irrigated daily via a computerized drip system, delivering tap water at a flow rate of 2 L·h⁻¹ for 3 min per pot (total of 100 mL daily). The experiment was terminated on day 33 post-sowing.

AS-clay-based protective treatment and soil inoculation

To protect each seed, the AS-clay-based formulation (bentonite or sepiolite) was applied at sowing by placing 55–65 mg of the prepared powder (see Section “AS-clay-based formulation preparation”) directly into the seed pit. Soil inoculation was carried out at sowing and approximately two weeks later by embedding 25 fungal culture discs (6 mm in diameter, excised from the margins of 5–7-day-old PDA colonies; see Section “Fungal origin and growth conditions”) per pot. The discs were evenly distributed across the soil surface and buried at a depth of approximately 2.5 cm. The specific dates and detailed procedures for the greenhouse trial are provided in Table 1.

Measurements of traits

Seedling emergence rate was evaluated on day 6 post-sowing, and the plant canopy cover percentage was assessed on day 13. Plant survival rate and growth parameters, including aboveground fresh weight per plant (shoot weight), shoot length, leaf number, and canopy cover percentage, were assessed at the conclusion of the experiment on day 33. Canopy cover percentage was quantified via Canopeo software (version 2.0; <https://canopeoapp.com>, accessed on 27 April 2025), using a Samsung Galaxy S24 Ultra smartphone (Patrignani

Table 1 Experimental arrangement of the greenhouse pot trial

Date	Experimental timeline and sampling scheme	Days from sowing
08 October 2024	Sowing, AS-clay application, 1 st inoculation	0
12 October 2024	Above-ground emergence	4
14 October 2024	Sprouting assessment	6
21 October 2024	2 nd inoculation and thinning	13
10 November 2024	Final sampling	33

et al. 2015). The mobile application measured the green canopy cover of cotton sprouts from a height of 0.5 m above the pot base. Pathogen colonization levels were determined by quantifying *M. phaseolina* DNA level in cotton roots via qPCR, as detailed in Section “Analysis of the *M. phaseolina* DNA level”. Minimal qPCR signals detected in the roots of uninoculated control plants, likely reflecting the trace natural presence of *M. phaseolina* in the soil, were used to establish baseline values. These average background values were subtracted from all treatment groups.

Virulence trials in semi-field, open enclosure, full-season pots

Experimental setup

Plastic pots (10 L) were filled with heavy soil obtained from the Northern R&D experimental farm in the Hula Valley, Upper Galilee, northern Israel (33°09′08.2″ N, 35°37′21.6″ E), which was selected to simulate local field conditions closely. The soil had no prior history of CRD, and any indigenous presence of *M. phaseolina* was presumed to be negligible. Soil aeration was enhanced by incorporating 25% (v/v) Perlite No. 4. Fertilizers and pest control measures were applied throughout the season to manage diseases other than those caused by *M. phaseolina*. Each pot was sown with seven seeds of the cotton cultivar Goliath V-6, placed at a depth of approximately 3 cm. Each clay–AS formulation (bentonite or sepiolite) was applied to protect plants stressed by *M. phaseolina*. These treatments were compared with bentonite or sepiolite treated with DDW instead of pesticide (Bent-DDW and Sep-DDW). In addition, two controls were included: infected, untreated plants, and mock healthy plants. Each treatment was set up with eight biological replicates (pots).

Key dates and meteorological conditions

The semi-field experiment was carried out during the summer of 2024, with key dates provided in Table 2. The temperature and humidity conditions throughout the

Table 2 Arrangement of the semi-field open enclosure pot experiment

Date	Experimental timeline and sampling scheme	Days from sowing
15 July 2024	1 st inoculation (sterilized millet grains)	−9
24 July 2024	Sowing, AS-clay application	0
30 July 2024	2 nd inoculation, above-ground emergence assessment	6
06 August 2024	3 rd inoculation, sprouting assessment	13
08 September 2024	Thinning to 2 plants per pot	46
27 September 2024	Beginning of the flowering	65
30 September 2024	Mid-sampling (one plant per pot), and thinning	68
07 November 2024	Final sampling	106

Table 3 Meteorological data for the semi-field experiment (15 July–7 November in 2024)

Parameters	Value
Temperature/°C	26.3
Humidity/%	55.5
Soil temperature top 5 cm/°C	31.4
Radiation/(W·m ^{−2})	227.6
Precipitation/mm	5.2
Evaporation/mm	741.3

Data according to the Israel Northern Research and Development, Dafna Meteorological Station

cotton growing season were typical and favorable for disease development, as described previously (Degani et al. 2023b). The experiment was terminated on 7 November, 2024, owing to the end of the autumn season and the approaching winter. The detailed meteorological data are presented in Table 3.

Preparation of infected grains

Sterilized millet grains (*Panicum miliaceum*) were used to inoculate the soil with *M. phaseolina*, following a previously described protocol (Gordani et al. 2023) with modifications. Briefly, 3.8 kg of dry millet kernels were boiled in 6.35 L of tap water for 100 min. Next, 49.4 g of gypsum (CaSO₄·2H₂O) was added to the mixture. The grains were then distributed into four 3.5 L glass jars, each containing 1.8 kg of wet kernels, and subjected to autoclaving at 121 °C for 20 min, followed by 134 °C for 40 min. After sterilization, 90 mL of sterile DDW and two 7-day-old *M. phaseolina* cultures grown on PDA—including the entire agar surface from 9-cm Petri dishes—were added to each jar. The contents were mixed with a sterile spatula. Each jar was loosely sealed to allow gas exchange, covered with aluminum foil,

and incubated in the dark at (28 ± 1) °C for 11 d until the grains were fully colonized by the fungal mycelium, which was visible as dark-pigmented hyphae.

Soil inoculation and AS-clay-based protective treatment

The soil was initially infected nine days prior to the experiment by incorporating 100 g of sterilized millet grains colonized by *M. phaseolina* into the upper 5 cm soil layer of each pot, following the protocol described by Gordani et al. (2023). Complementary soil inoculations were conducted across all treatment pots at 6 days (seedling emergence) and 13 days after sowing. For each supplementation infection event, three fungal culture discs (6 mm in diameter) obtained from *M. phaseolina* grown as described in Section “Fungal origin and growth conditions” were inserted into the soil around each sprout (totaling 15 discs per pot). The AS-Clay-based protective treatment was applied following the same procedure as in the greenhouse seedling trial (Section “AS-clay-based protective treatment and soil inoculation”).

Measurements of indices

The treatment impact on plant development and health was studied similarly to the growth room evaluation (Section “Measurements of traits”). The percentage of seedling emergence was assessed 6 days after sowing for each treatment. Canopy cover percentage, shoot dry weight per plant, and number of flower buds per plant, as well as the fungal DNA level in plant roots, were assessed at 68 days post-sowing. At 106 days post sowing, shoot dry weight per plant and fungal DNA level in plant roots were investigated. Yield traits were not investigated, as plant development was disrupted and delayed, possibly due to CRD pressure or growth conditions that exposed the plants to wind and other environmental stresses.

Analysis of the *M. phaseolina* DNA level

DNA was extracted from cotton roots pooled from each pot. The roots were thoroughly rinsed with running tap water, then cut into sections about 2 cm in length, and 0.7 g of tissue was used per replicate. Fungal DNA extraction was performed according to a previously described protocol (Shofman et al. 2025). Briefly, the plant tissue was homogenized in 4 mL of CTAB buffer. An aliquot of 1.2 mL was transferred to microcentrifuge tubes and incubated at 65 °C for 20 min. After centrifugation at 18 000 ×g for 5 min at 24 °C, 700 µL of the supernatant was mixed with an equal volume of chloroform: isoamyl alcohol (24:1). This purification step was repeated twice, with 500 µL of each phase used in the second repetition (1:1, v/v). The aqueous phase (300 µL) was then combined with 200 µL of pre-chilled isopropanol and incubated at -20 °C for 20–60 min to precipitate DNA. The DNA was pelleted by centrifugation (12 000 r·min⁻¹, 4 °C, 20 min), then washed with 0.5 mL of ethanol and dried overnight in a sterile environment. Finally, the DNA was resuspended in 100 µL of ultra-pure water and stored at -20 °C until qPCR analysis.

A standard qPCR protocol was optimized for *M. phaseolina* identification (Degani et al. 2023a) via species-specific primers (Table 4). To normalize the relative DNA level across samples, amplification of the cytochrome C oxidase (*COX*) gene, a mitochondrial housekeeping gene involved in the final step of the respiratory electron transport chain, was conducted (Weller et al. 2000). Relative fungal DNA abundance was calculated using the ΔCt (Delta cycle threshold) method, assuming equal amplification efficiency across all samples. All qPCR reactions were conducted in four technical replicates via the CFX384 qPCR Detection System (Bio-Rad, Hercules, CA, USA) in 384-well plates. Each 5 µL reaction contained 0.25 µL of forward and reverse primers (5 µmol·L⁻¹) each, 2.5 µL of iTaq™ Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), and 2 µL of diluted DNA template. The cycling conditions were as follows: an initial activation step at 95 °C for 60 s, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 30 s, and a final melting curve analysis (Degani et al. 2023a).

Statistical analysis

The greenhouse sprout and semi-field pot experiments were conducted in accordance with a completely randomized statistical design. No random effects with statistical significance were found in the pots' setting. Analysis and statistical processing were performed using GraphPad Prism software [version 10.4.2 (633), GraphPad Software Inc., San Diego, CA, USA]. For both experiments, the data were first tested for normality using the Shapiro–Wilk test. When the normality assumption was met, a one-way analysis of variance (ANOVA) was applied, followed by Fisher's least significant difference (LSD) test at a significance level of *P* < 0.05. In cases where normality was not confirmed (*P* < 0.05), the nonparametric Kruskal–Wallis test was used, followed by an uncorrected Dunnett's post hoc test. It should be noted that, despite efforts to standardize experimental conditions in both greenhouse and open-enclosure pot trials, environmental fluctuations could not be fully controlled. Such variability may have contributed to differences in plant growth and disease severity among replicates. Additionally, minor heterogeneity in soil nutrient composition, native microbial communities, and seed genetic background could have influenced pathogen activity and plant susceptibility. These inherent sources of biological variation were reflected by high standard error values.

Results

In this study, the protective potential of clay-AS-based powder to prevent or mitigate the early and late stages of cotton charcoal rot was evaluated. Both a greenhouse sprout experiment and a full-season open-enclosure pot trial demonstrated the effectiveness of certain treatments, as detailed below.

Greenhouse sprouts experiment

Under greenhouse conditions, cotton sprouting above the soil surface progressed uniformly across treatments (Fig. 1). Six days post-sowing, the aboveground emergence rate reached 100% in all experimental groups, except for the Sep–AS treatments with or without pathogen stress, which recorded 94% and 97% emergence rates, respectively. These differences were not statistically significant (data not shown). However, the

Table 4 Primers for qPCR detection of *M. phaseolina* DNA levels

Primer name	Sequence	Uses	Amplification	References
MpKFI MpKRI	5'-CCGCCAGAGGACTATCAAAC-3' 5'-CGTCCGAAGCGAGGTGATT-3'	Target gene	300–400 bp <i>M. phaseolina</i> species-specific fragment	(Babu et al. 2007)
COX-F	5'-GTATGCCACGTGCGATTCCAGA-3'	Control	COX gene	(Li et al. 2006; Weller et al. 2000)
COX-R	5'-CAACTACGGATATATAAGRCCRRAACTG-3'			

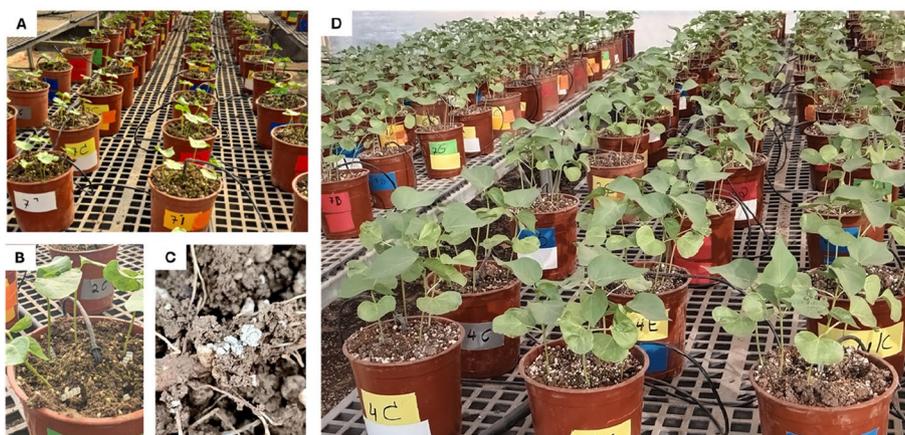


Fig. 1 Greenhouse sprout experiment. **A** Sprouts on day 8. **B** Sprouts' second inoculation on day 13. **C** Bentonite adheres to sprout roots. **D** Sprouts on day 23

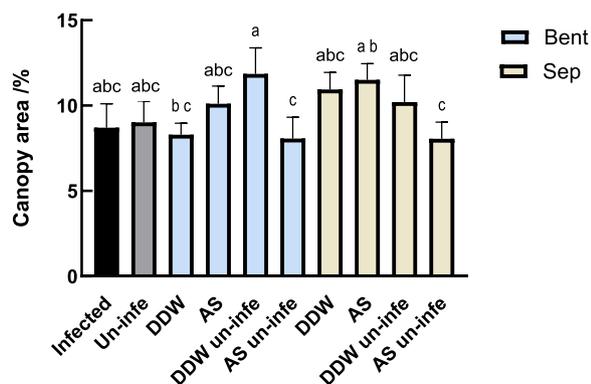


Fig. 2 The canopy cover percentage 13 days post-sowing. Treatments included bentonite (Bent) and sepiolite (Sep) clays, each combined with azoxystrobin (AS) or deionized distilled water (DDW, as the control). "Infected" refers to inoculated, untreated plants, while "Un-infe" represents uninoculated healthy controls. Bars represent 7 biological replicates, and error bars indicate standard errors. Different letters above the bars denote statistically significant differences (ANOVA, $P < 0.05$)

canopy cover percentage evaluation performed 13 days after sowing revealed a contrasting trend (Fig. 2). At this stage of plant development, none of the treatments differed significantly from the infected control. However, several treatments differed significantly from one another ($P < 0.05$). Specifically, both clay-AS treatments (sepiolite-AS and bentonite-AS) applied in uninfected soil exhibited significantly lower growth. In contrast, bentonite alone, applied in uninfected soil, and the Sep-AS treatment increased vegetative growth (canopy cover percentage) by 36% and 32%, respectively, compared with the infected control.

During the 33-day experimental period, the growth parameters (survival rate, canopy cover percentage, shoot weight per plant, leaf number per plant, and shoot length) of the sprouts were minimally impacted by pathogen inoculation or protective treatments (Table 5), which aligns with expectations for this latent disease stage (Fig. 1). Plant survival rate remained

Table 5 Growth parameters in the greenhouse sprouts experiment (33 d post-sowing)

Clay	Treatment	Survival rate/%	Canopy cover percentage/%	Shoot weight per plant/g	Leaf number per plant	Shoot length/cm
Control	Infected	100 ± 0.0 ^a	36 ± 2.3 ^a	4.9 ± 0.09 ^{ab}	4.0 ± 0.00 ^{abc}	17 ± 0.24 ^a
	Uninfected	98 ± 2.8 ^a	30 ± 5.8 ^a	4.9 ± 0.24 ^{ab}	4.0 ± 0.00 ^{abc}	17 ± 0.52 ^a
Bentonite	DDW	100 ± 0.0 ^a	32 ± 4.6 ^a	4.9 ± 0.15 ^{ab}	4.1 ± 0.04 ^a	17 ± 0.75 ^a
	AS	100 ± 0.0 ^a	33 ± 5.4 ^a	4.9 ± 0.35 ^{ab}	4.0 ± 0.08 ^{abc}	17 ± 0.81 ^a
	DDW uninfected	100 ± 0.0 ^a	32 ± 3.8 ^a	5.3 ± 0.12 ^a	4.0 ± 0.00 ^{abc}	18 ± 0.56 ^a
	AS uninfected	100 ± 0.0 ^a	32 ± 5.0 ^a	4.8 ± 0.10 ^{ab}	3.8 ± 0.10 ^c	17 ± 0.96 ^a
Sepiolite	DDW	100 ± 0.0 ^a	36 ± 5.1 ^a	5.0 ± 0.21 ^{ab}	4.0 ± 0.03 ^{ab}	18 ± 0.77 ^a
	AS	100 ± 0.0 ^a	29 ± 4.3 ^a	4.8 ± 0.37 ^{ab}	3.9 ± 0.07 ^{bc}	18 ± 0.82 ^a
	DDW uninfected	100 ± 0.0 ^a	33 ± 3.2 ^a	4.7 ± 0.18 ^b	4.0 ± 0.00 ^{abc}	17 ± 0.45 ^a
	AS uninfected	92 ± 4.0 ^b	28 ± 4.4 ^a	4.7 ± 0.18 ^b	3.9 ± 0.09 ^{abc}	17 ± 0.46 ^a

Data is mean ± standard error. Different lowercase letters in the same column indicate significant differences among treatments

uniformly high across all treatments, with only a significant reduction observed in the Sep-AS uninoculated group ($P < 0.05$). Compared with the two control treatments, the vegetative growth—expressed as the leaf number per plant—did not differ significantly across the remaining eight treatments. However, a small but statistically significant reduction was observed in the Bent-AS treatment under non-infected conditions compared with the bentonite-alone treatment under pathogen stress ($P < 0.05$).

Based on Table 5, shoot weight per plant remained statistically unchanged across all eight treatments relative to the two controls. However, within the infected treatments, both Sep-DDW and Sep-AS resulted in higher shoot weight per plant compared with their corresponding non-infected treatments, implying a treatment-specific positive effect under pathogen pressure. Interestingly, the bentonite-alone uninfected treatment exhibited a significantly higher shoot weight per plant than the two uninfected sepiolite treatments ($P < 0.05$), highlighting a differential response between the clay types under non-infected conditions. The shoot length and canopy cover percentage showed no significant differences among the 10 treatments. Importantly, compared with the infected control, both bentonite and sepiolite clay-based AS formulations reduced the DNA level of *M. phaseolina* in cotton roots by 81%; however, this difference did not reach statistical significance (Fig. 3).

Virulence trials in semi-field, open enclosure, and full-season pots

The open-enclosure semi-field trial enabled the evaluation of treatment potential throughout an entire growth

season (Fig. 4). The above-soil-surface emergence was assessed on day 6 post-sowing. Nearly all the treatments resulted in proper germination, with an average of 5.0 ± 0.34 sprouts per pot, except for the bentonite-DDW treatment (clay without fungicide), which resulted in a reduced average of 4.0 sprouts per pot. By mid-season sampling (day 68), obvious differences were observed between treatments in both plant performance and pathogen presence (Fig. 5). Infected control plants exhibited an evident reduction in the number of flower buds per plant (50% decrease relative to healthy, uninoculated plants), yet unexpectedly developed higher shoot dry weight per plant—43% above that of the healthy control—although this difference was not statistically significant for either parameter. The sepiolite-based formulation (Sep-AS) induced pronounced recovery, improving the number of flower buds per plant by 87% relative to the infected control (not statistically significant) and restoring development to levels comparable with or exceeding those of the healthy plants.

In contrast, the Bent-AS treatment achieved the lowest growth parameters across all indices, yielding values comparable to those of the infected control, and, for canopy cover percentage and shoot dry weight per plant, even lower (39% and 47% reductions relative to the infected control, respectively). This outcome is likely due to an excessively toxic dosage. However, at this stage (day 68), the bentonite-alone treatment outperformed all other treatments under *M. phaseolina* stress, exhibiting increases of 34% in canopy cover percentage, 129% in flower bud number, and 35% in shoot dry weight per plant (Fig. 5A-C; without statistical significance). Both canopy cover percentage and flower bud number in the Bent-DDW treatment were significantly higher than those recorded for Bent-AS ($P < 0.05$).

Quantitative PCR analysis (Fig. 5D) supported these observations by showing that the relative abundance of *M. phaseolina* DNA in the root tissue was drastically reduced following the clay-AS treatments. The Sep-AS formulation decreased the pathogen DNA level by 92%, whereas Bent-AS achieved a 59% reduction compared with the infected untreated control. Although the statistical analysis revealed no significant differences among the treatments ($P > 0.05$), the consistent trend of pathogen suppression and growth improvement demonstrates the biological efficacy of the clay-based AS carriers in mitigating the impact of CRD.

At the end of the season (day 106), clear disease symptoms were evident (Fig. 4E, F). The shoot dry weight per plant increased by 129% and 128% in the plants treated with sepiolite-AS and bentonite alone ($p < 0.05$), respectively, compared with the infected untreated control (Fig. 6A). Additionally, these two treatments were

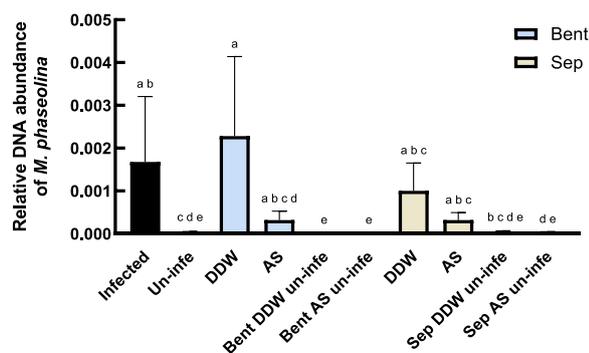


Fig. 3 Effects of AS-clay formulations on the DNA level of *M. phaseolina* in cotton roots (33 d post-sowing) under greenhouse conditions. Pathogen load was quantified using qPCR. Treatments included bentonite (Bent) and sepiolite (Sep) clays, each combined with AS or with DDW as a control. “Infected” refers to inoculated, untreated plants, while “Un-infe” represents uninoculated healthy controls. The bars represent the means of 5–7 biological replicates; the error bars indicate standard errors. Different letters above the bars denote statistically significant differences (Kruskal–Wallis test, $P < 0.05$)

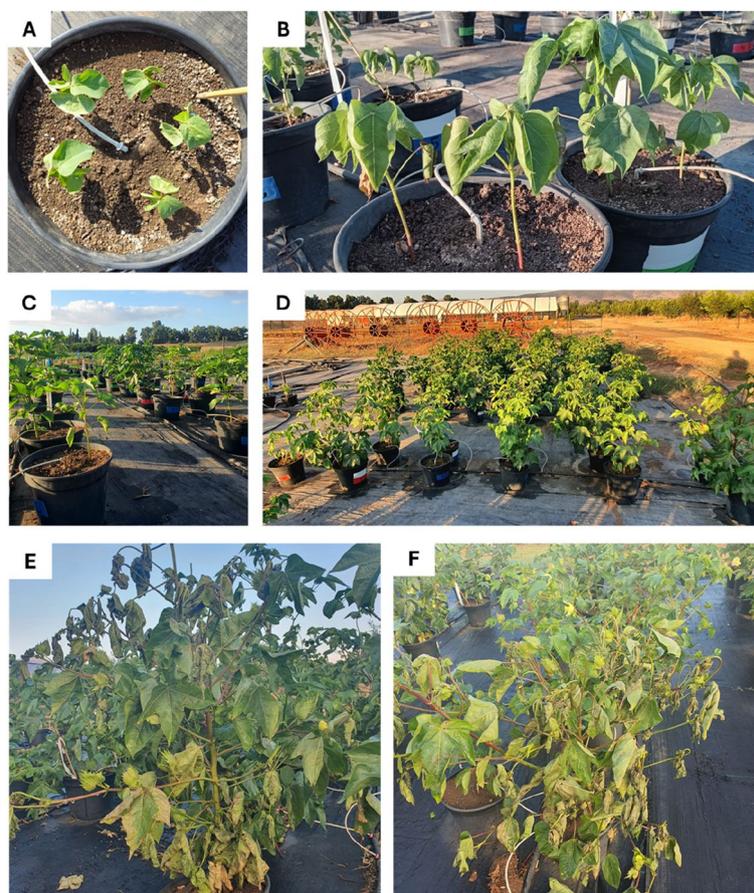


Fig. 4 The semi-field, open enclosure, full-season pots' trial. **A** Sprouts on day 16. **B** Sprouts on day 49. **C** Plants on day 70. **D** Plants on day 96. **E, F** Diseased plants on day 103

most effective at reducing pathogen abundance in cotton roots by 63% and 69%, respectively, although the differences were not statistically significant (Fig. 6B).

Discussion

The current study tested an innovative clay-based method that enables the controlled release of AS, providing targeted protection for cotton during its vulnerable early growth stages, when CRD pathogen penetration and colonization are most likely to occur. The substrates used for this purpose are based on clay minerals, which can be applied in their natural form or engineered using organic cations to optimize adsorption, release, and bioactivity (Masini et al. 2021; Nomicisio et al. 2023; Degani et al. 2025). The importance of the results aligns with earlier studies driven by comparable goals. Previously, to protect crops against fungal pathogens, kaolin and bentonite were incorporated into starch–alginate beads to control the release of the fungicide thiram and reduce environmental pollution (Singh et al. 2009). Beads with varying clay contents were characterized using Fourier

transform infrared spectroscopy, scanning electron microscopy, energy-dispersive X-ray analysis, thermogravimetry, and swelling studies. Compared with kaolin, the formulations showed high entrapment efficiency and modified thiram release, with bentonite providing a slower, non-Fickian diffusion profile. Another study that exemplifies such a methodology investigated modified bentonite–alginate nanocomposites that release pesticides—including fungicides—in a controlled, pH-responsive manner (Yang et al. 2024). This approach enhances the precision of pesticide delivery, potentially reducing environmental exposure and improving efficacy.

The design of clay-based controlled-release systems has been used in other agricultural management challenges, such as improving the effectiveness of pesticides and herbicides. For example, in a recent greenhouse study, clay-based biopesticide effectively controlled pests in chives without damaging the foliage (Shaltiel-Harpaz et al. 2023). This environmentally friendly method involves essential rosemary oil absorbed onto sepiolite, a mineral known for its ability to bind uncharged molecules

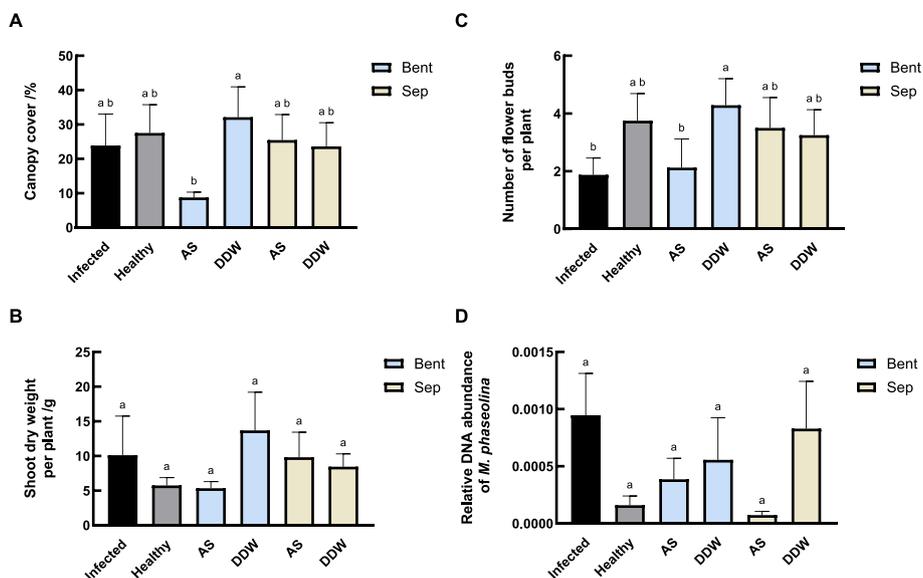


Fig. 5 Effects of AS-clay formulations on plant performance and fungal abundance in flowering cotton at 68 days post-sowing. **A** Canopy cover percentage, **B** shoot dry weight per plant, **C** number of flower buds per plant, and **D** relative DNA level of *M. phaseolina* in roots via qPCR. The treatments included bentonite (Bent) and sepiolite (Sep) clays, which were combined with either AS or DDW as a control. "Infected" represents inoculated, untreated plants; "Healthy" denotes uninoculated controls. The values represent the means of 7–8 biological replicates, with each replicate consisting of one plant per pot. The error bars indicate the standard errors. Different letters above the bars indicate statistically significant differences (ANOVA or Kruskal–Wallis test, $P < 0.05$)

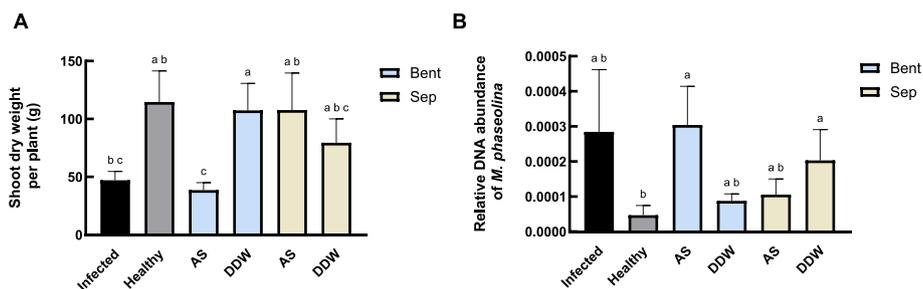


Fig. 6 Effects of AS-clay formulations on biomass accumulation and fungal abundance in cotton at harvest (106 days post-sowing). **A** shoot dry weight per plant; **B** relative amount of *M. phaseolina* DNA in roots via qPCR. The treatments included bentonite (Bent) and sepiolite (Sep) clays, each combined with AS or with DDW as a control. "Healthy" represents uninoculated mock controls, whereas "Infected" refers to inoculated, untreated plants. The data represent the means of 3–8 biological replicates; the error bars indicate standard errors. Different letters above the bars denote statistically significant differences according to the Kruskal–Wallis test ($P < 0.05$)

(Shuali et al. 2011; Aranda et al. 2018). Another work demonstrated that clay–surfactant formulations can be developed to enable the slow release of herbicides (Galán-Jiménez et al. 2013). In this former study, in vitro and soil tests confirmed delayed release and improved bioactivity compared with those of commercial formulations. The results from the growth room and semi-field trials were consistent during the early growth phase (days 33 and 68, respectively), particularly regarding pathogen DNA levels in plant roots.

While the slow-release properties of the bentonite–AS and sepiolite–AS formulations have recently been demonstrated in vitro (Degani et al. 2025), the present work constitutes the first report of their in-plant application for protection against cotton CRD. Interestingly, under non-infected conditions, the plant growth response to the clay–AS formulations exhibited a distinct pattern compared with pathogen-stressed plants. Bentonite applied alone in healthy soil improved growth, whereas both clay–AS treatments (sepiolite–AS and bentonite–AS) resulted in reduced biomass. This suggests that, in

the absence of pathogen pressure, localized or sustained AS release from the clay matrices may induce mild phytotoxicity. Similar effects have been documented for strobilurin fungicides, where excessive or prolonged exposure can impair physiological processes such as chlorophyll stability or mitochondrial electron transport (Dias 2012; Amaro et al. 2020). Phytotoxic outcomes have also been reported when fungicides interact with soil physico-chemical properties or disrupt root-associated microbial communities (Chou et al. 2025).

In contrast, under *M. phaseolina* infection, the same clay-AS formulations mitigated disease severity and partially restored growth, demonstrating a net beneficial effect despite their potential phytotoxicity in healthy plants. This contrasting response highlights the strong context-dependence of fungicide performance in planta: while slow-release AS may transiently reduce growth under non-infected conditions, its protective effect under pathogen pressure clearly outweighs such impacts. Overall, these findings underscore the importance of testing controlled-release formulations under both healthy and infected conditions to accurately assess their agronomic relevance and avoid underestimating their disease-suppressive potential.

Thus, both the growth room and semi-field trials experiments demonstrated the superiority of the AS-clay formulation over clay alone in this research. This trend continued in the AS-sepiolite treatment throughout the season; however, it did not hold for the Bent-AS combination. By harvest, this treatment failed to protect plant growth under CRD stress or reduce the pathogen infection level. Surprisingly, bentonite alone (Bent-DDW) had a beneficial effect on both parameters (growth promotion and inhibition of *M. phaseolina* root colonization). Previous studies have shown that bentonite may have antifungal activity. For example, bentonite loaded with zinc oxide nanoparticles exhibited antifungal activity in laboratory tests against *Aspergillus niger* (de Lucas-Gil et al. 2020). Additionally, this clay might have provided a physical barrier limiting fungal penetration, as shown for kaolin in previous studies (Lamb et al. 2002). Alternatively, bentonite may improve soil moisture retention and structure, promoting beneficial microbial communities known to suppress pathogens such as *Alternaria*, *Bipolaris*, *Fusarium*, *Leptosphaeria*, and *Microdochium* (Chen et al. 2023). Its use in conjunction with copper compounds has also proven effective against fungal diseases such as downy mildew and *Monilia* (Al-Taey et al. 2023). Furthermore, clays such as bentonite are known to bind fungal toxins (e.g., aflatoxins), reducing their harmful impact (Nadziakiewicz et al. 2019).

It is important to recognize the limitations of pot trials in replicating field conditions. AS-based treatments may have stronger effects under greater disease pressure,

which is often difficult to replicate in pot experiments, even more so in open environments. Currently, AS-based treatments for CRD control in cotton (Degani et al. 2024, 2023b) are relatively expensive and less practical in fields irrigated by methods such as pivot irrigation. Moreover, pesticide exposure poses significant human health concerns (El Afandi et al. 2024), and the extensive use of pesticides in agriculture may affect a diverse range of non-target species, which is linked to global biodiversity loss (Wan et al. 2025). To mitigate the environmental impact of pesticides, clay-based substrates are being explored as carriers that can prevent pesticide leaching, degradation, or loss of efficacy (Nir et al. 2013; Rytwo et al. 2005, 2008, 2012). By utilizing adsorption processes and specific molecular interactions, these substrates could enable cost-effective, long-lasting applications across diverse field conditions while minimizing activity loss due to fungicide movement in the soil. Moreover, this formulation could significantly reduce overall pesticide usage through localized (spot) application and seed coating. It also offers compatibility with biological control strategies.

A combined AS-*Trichoderma* strategy for managing CRD was recently validated. Previous studies identified high-potential *Trichoderma* species for *M. phaseolina* biocontrol applications (Degani et al. 2023a). Across a series of trials—ranging from growth room sprouts to field conditions—seed coating with *Trichoderma* mixtures, either alone or in combination with low-dose AS irrigation, achieved disease suppression and plant recovery comparable to chemical-only treatments. In an open-air pot trial, mixed management achieved up to 91% pathogen suppression. Under commercial field conditions, the sole biological management or the mixed approach reduced infection by 28% and 37%, respectively, decreased the plant dehydration and mortality by 77% and 27%, and increased yield by 9% and 17%, respectively (Degani et al. 2024, 2023b). Following this two-year field evaluation, it was concluded that the biological and integrated treatments significantly outperformed chemical control, supporting the integrated approach as a robust and sustainable solution for CRD control, potentially mitigating the risk of resistance development.

Indeed, combining biological and chemical management practices to protect plants against phytopathogens should be considered, particularly when single active molecule-based fungicides are used (Gordani et al. 2023). There is a significant risk of evolving AS-resistant pathogen strains, particularly when this fungicide is used extensively over consecutive growing seasons (Leadbeater 2014; Castroagudín et al. 2015; Fernández-Ortuño et al. 2010a). Fungicides with a single-site mode of action, such as AS, are especially prone to resistance development (Massi et al. 2021). AS, a member of

the strobilurin class, acts as a quinone outside inhibitor (QoI) that disrupts mitochondrial respiration by binding to the quinol oxidation (Qo) site of the cytochrome bcl complex in the fungal electron transport chain, thereby blocking ATP synthesis (Fernández-Ortuño et al. 2010b). Resistance to QoI fungicides has already been reported in more than 20 fungal genera, including *Rhizoctonia solani*, *Alternaria alternata*, *Botrytis cinerea*, *Venturia inaequalis*, and *Mycosphaerella graminicola* (Koehler et al. 2019). Although, to our knowledge, there is no data on AS resistance mutations in *M. phaseolina*, the potential for resistance development is evident. Therefore, it is essential to implement fungicide resistance management strategies (Koehler et al. 2019). Exploring alternative chemical agents for integration into *M. phaseolina* management program may expand our options for preventing the emergence of resistance (Bashir et al. 2017; Iqbal et al. 2020b). In addition, the long-term effectiveness of fungicides can be enhanced by combining active ingredients with different modes of action (Brent et al. 2007; Davies et al. 2021).

Bridging the gap between this clay-based experimental stage and its application in agriculture will require substantial further work. The following efforts should focus on field-scale validation of the new AS-clay preparation, formulation refinement, combination with biological control agents, and optimization of its application method. A key step will be optimizing how the AS-clay formulation is applied to the seeds—evaluating its adhesion, potential powder release during seed coating, handling, and sowing operations. These aspects are critical for ensuring uniform coverage, user safety, and environmental protection. In conclusion, the AS-clay technology provides a promising platform for developing next-generation controlled-release fungicide systems that can be tailored for diverse crops and soil-borne pathogens, offering a sustainable path forward for modern plant protection. By using the methodology demonstrated here, similar clay–fungicide combinations could be developed and adjusted to protect crop plants from additional soil–borne pathogens.

Conclusion

This study demonstrated the potential of clay-based AS formulations as an innovative and sustainable approach for controlling *M. phaseolina*, the causal agent of cotton CRD. The use of clay-AS formulations may reduce chemical loss under adverse environmental conditions, and maintain effective disease control with lower fungicide input. As demonstrated in the present study, the slow release of fungicides such as AS during the critical early stages of pathogen penetration and establishment in seedlings is particularly important. Moreover, the

beneficial effect of bentonite alone on plant performance suggests that certain clays may also contribute directly to improved soil structure, water retention, or microbial balance, further supporting plant resilience. Future efforts should focus on translating this concept into practical agricultural applications.

Abbreviations

AS	Azoxystrobin
AS-clay	Azoxystrobin–clay formulation
Bent	Bentonite
CAS No.	Chemical Abstracts Service number
COX	Cytochrome c oxidase (housekeeping gene, qPCR reference)
CRD	Charcoal rot disease
DDW	Deionized distilled water
PDA	Potato dextrose agar
qPCR	Quantitative real-time polymerase chain reaction
QoI	Quinone-outside inhibitor (fungicide mode of action)
Sep	Sepiolite

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Authors' contributions

Conceptualization: Degani O, Hadad A, Etedgi E, Dimant E, Levi-Lion A, Hadari P, Rabinovitz O, and Rytwo G. Data curation: Degani O, Hadad A, Etedgi E, Dimant E, Levi-Lion A, Hadari P, Rabinovitz O, and Rytwo G. Formal analysis: Degani O, Hadad A, Etedgi E, Dimant E, Levi-Lion A, Hadari P, Rabinovitz O, and Rytwo G. Funding acquisition: Degani O and Rytwo G. Investigation: Degani O, Hadad A, Etedgi E, Dimant E, Levi-Lion A, Hadari P, Rabinovitz O, and Rytwo G. Methodology: Degani O, Hadad A, Etedgi E, Dimant E, Levi-Lion A, Hadari P, Rabinovitz O, and Rytwo G. Project administration: Degani O, Dimant E, and Rytwo G. Resources: Degani O and Rytwo G. Supervision: Degani O, Rabinovitz O, and Rytwo G. Validation: Degani O, Hadad A, Etedgi E, Dimant E, Rabinovitz O, and Rytwo G. Visualization: Degani O, Hadad A, Etedgi E, Dimant E, and Rytwo G. Writing (original draft): Degani O. Writing (review and editing): Degani O, Hadad A, Etedgi E, Dimant E, Levi-Lion A, Hadari P, Rabinovitz O, and Rytwo G. All authors have read and approved the final version of the manuscript.

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Data availability

All the data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

All methods were performed according to the Ministry of Agriculture and Rural Development, Consultation Service (Shaham, Beit-Dagan, Israel), and Israel Northern Research and Development (Northern R&D, Kiryat Shmona, Israel) guidelines and regulations. These authorities authorized and supervised the experimental research and semi-field study of the plants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicts of interest.

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