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Article

Antimicrobial Effects of Nitric Oxide against Plant Pathogens

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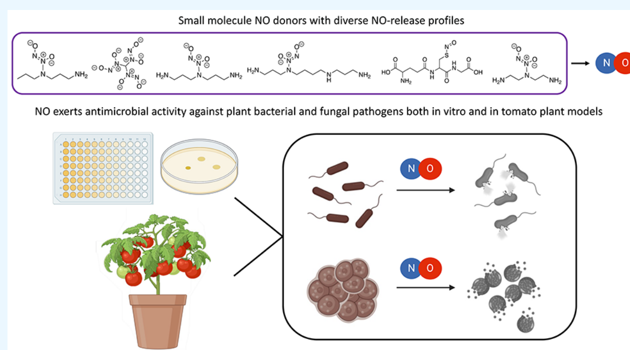
ABSTRACT: Pathogen infection represents the greatest challenge to agricultural crop production, resulting in significant economic loss. Conventional pesticides are used to control such infection but can result in antimicrobial resistance and detrimental effects on the plant, environment, and human health. Due to nitric oxide's (NO) endogenous roles in plant immune responses, treatment with exogenous NO represents an attractive nonpesticide approach for eradicating plant pathogens. In this work, the antimicrobial activity of small-molecule NO donors of varying NO-release kinetics was evaluated against *Pseudomonas syringae* and *Botrytis cinerea*, two prevalent plant pathogens. Intermediate NO-release kinetics proved to be most effective at eradicating these pathogens in vitro. A selected NO donor (methyl tris diazeniumdiolate; MD3) was capable of treating both bacterial infection of plant leaves and fungal infection of tomato fruit without exerting toxicity to earthworms. Taken together, these results demonstrate the potential for utilizing NO as a broad-spectrum, environmentally safe pesticide and may guide development of other NO donors for such application.



INTRODUCTION

Tomato (*Solanum lycopersicum*) is the highest-value horticultural crop, with over 256 million tons of tomatoes harvested from 6.3 million hectares worldwide (2021).[1−3] The importance of tomatoes is largely due to their nutritional value, as they are a good source of phytochemicals and nutrients (i.e., lycopene, phytoene, β-carotene, Vitamin A, ascorbic acid, potassium, and folate).[4,5] Consumption of tomatoes has been associated with reduced risk of cancer, inflammatory processes, and cardiovascular diseases due to the interaction of phytochemicals with metabolic pathways that are related to oxidative stress and the inflammatory response.[6] The greatest challenge in tomato production is infection caused by pathogens in the field and during postharvest processing, resulting in huge economic loss.[7,8] More than 60 pathogens including fungi, bacteria, oomycetes, and viruses can cause disease in tomatoes and account for 70−95% of annual losses in global tomato production.[9,10] *Botrytis cinerea* (*B. cinerea*) is the most extensively studied necrotrophic fungal pathogen. *B. cinerea* can cause gray mold disease both pre- and postharvest in over 200 crops worldwide, with serious losses in postharvest tomato fruits.[11,12] Similarly, *Pseudomonas syringae* (*P. syringae*) causes economically significant bacterial infections in a wide range of plant species and is a source of bacterial speck disease on tomatoes.[13−15]

The application of chemical pesticides is the primary method for disease management in crop and vegetable production.[7] While conventional pesticides have beneficial effects on crop yield and productivity, less than 10% of the applied pesticides reach their target.[7] Furthermore, the extensive use of pesticides results in detrimental effects on the plant, environment, and human health.[16,17] Pesticides negatively impact a plant's photosynthetic ability, seed production, and nutritional content.[7,18] Continuous use of pesticides can alter soil texture, reduce soil respiration, and inhibit the activities of commensal organisms in the soil.[19,20] Additionally, the overuse of pesticides leads to the development of antimicrobial resistance. For example, resistance in *B. cinerea* has been reported against almost all of the target-site-specific fungicides used for its control across several crops. To date, four types of multidrug-resistant strains have been identified.[21] The chemical control of bacterial speck disease caused by *P. syringae* is primarily dependent on copper compounds.[22] Bacterial strains that are resistant to these compounds have been identified globally.[22,23] The need for the development of broad-spectrum, efficacious, and environmentally safe substitutes for conventional pesticides is significant.

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Local delivery of nitric oxide (NO), an endogenously produced free radical, represents a promising alternative to traditional pesticides due to its inherent antimicrobial activity and native functions in the plant immune response. The first report on the biological activity of NO was actually in a plant.[24] More recently, NO was reported to be involved in multiple processes integral to plant growth and development.[25,26] Nitric oxide plays key roles in plant defense against pathogens, inducing the expression of defense genes (i.e., phenylalanine ammonia lyase (PAL) and pathogenesis-related protein-1 (PR-1)) after pathogen attack. Nitric oxide also produces reactive byproducts such as hydroxyl radicals and peroxynitrites that can directly exert toxicity against the pathogen and lead to localized plant cell death, known as the hypersensitive response (HR).[27,28] The HR, in turn, determines the activation of systemic acquired resistance (SAR), a salicylic acid-dependent response in unaffected tissue that limits subsequent infection by a broad range of pathogens.[29] Nitric oxide has been shown to increase the levels of salicylic acid (SA), further enhancing SAR.[26]

Exogenous NO has been utilized to promote plant defense against pathogens. Fumigation with NO gas was shown to control insects in fresh produce, including the codling moth in apples, the spotted wing drosophila in strawberries, and aphids in lettuce.[30−32] Due to the high reactivity and short half-life (3−5 s) of NO gas, chemical NO donors have been developed to store and controllably release NO for both biomedical and agricultural applications.[33−36] Postharvest treatment of tomato fruits with the NO donor sodium nitroprusside (SNP) and preharvest treatment of tomato plants with L-arginine, a precursor of NO, were shown to both enhance the resistance of the fruits against *B. cinerea* invasion and promote the activity of enzymes involved in plant defense.[37,38] Similarly, immersion of pitaya fruit and apples in SNP solutions reduced infection by the fungi *Colletotrichum gloeosporioides* and *Alternaria alternata*, respectively.[39,40] Sodium nitroprusside also inhibited the germination of *Penicillium expansum* spores in vitro, decreasing their virulence to apple fruit.[41] Likewise, *S*-nitrosoglutathione (GSNO) induced the activation of pathogenesis-related (PR) genes in *Arabidopsis thaliana*, resulting in resistance to virulent *P. syringae* pathovar (pv) tomato and promoted the expression of SAR-related genes in harvested peach fruit, the combination of which prevented infection by the fungus *Monilinia fructicola*.[42,43]

While these results demonstrate the potential for NO to be used as a broad-spectrum pesticide, one of the most common classes of NO donors, diazeniumdiolates, have not been explored for use as pesticides in plants. Additionally, utilizing the same NO donor to treat both bacterial and fungal plant pathogens without ecological toxicity has yet to be evaluated or demonstrated. Herein, we determined the in vitro antimicrobial effects of six small-molecule NO donors with high NO payloads and diverse NO-release kinetics against both *B. cinerea* and *P. syringae*. The antimicrobial effects of the most promising NO donor were evaluated against *P. syringae* infection of tomato plants and postharvest *B. cinerea* infection of tomato fruit. An earthworm toxicity assay was utilized to evaluate the ecological toxicity of the NO donor to confirm preliminary environmental safety of utilizing NO as a broad-spectrum pesticide.

■ MATERIALS AND METHODS

Materials. Glutathione, sodium nitrite, diethylenetriamine (DETA), bis(3-aminopropyl)amine (DPTA), spermine (SPER), and *N*-propyl-1,3-propanediamine (PAPA) were purchased from MilliporeSigma (Burlington, MA). Glycerol, glucose, Proteose Peptone No. 3, agar, Silwet L-77, magnesium chloride ($MgCl_2$), and common laboratory salts and solvents were obtained from Fisher Scientific (Fair Lawn, NJ). Methyl tris diazeniumdiolate (MD3) aqueous solution was a gift from Vast Therapeutics (Durham, NC). Sabouraud dextrose agar (SDA) and broth (SDB) were obtained from Becton, Dickinson, and Company (Franklin Lakes, NJ). Rifampicin was purchased from Research Products International (Mt. Prospect, IL). Nitrogen (N_2), oxygen (O_2), pure nitric oxide (NO; 99.5%), and NO calibration gas (25.87 ppm balance N_2) were obtained from Airgas National Welders (Raleigh, NC). All chemicals were used as received without further purification unless otherwise specified. *Botrytis cinerea* Persoon (*B. cinerea*) was purchased from the American Type Culture Collection (ATCC; Manassas, VA). *Pseudomonas syringae* pv tomato DC3000 (*P. syringae*) was a gift from Professor Jeff Dangl from the Department of Biology at the University of North Carolina at Chapel Hill (Chapel Hill, NC). King's Medium B (KMB; ATCC Medium 2441) was prepared with 20 g of Proteose Peptone No. 3, 10 mL of glycerol, 1.5 g of potassium phosphate dibasic, 1.5 g of magnesium sulfate heptahydrate, and 50 mg of rifampicin per liter and adjusted to pH 7.2 with 5 M hydrochloric acid (HCl). King's Medium B agar was prepared as described above with the addition of 15 g L^{-1} agar. *Solanum lycopersicum* (Roma tomato) seeds were purchased from Southern States Cooperative (Richmond, VA). Earthworms (*Eisenia fetida andrei*) and alfalfa pellets were purchased from Amazon. Distilled water was purified to a resistivity of 18.2 MΩ·cm, and a total organic content of ≤6 ppb using a Millipore Milli-Q UV Gradient A10 system (Bedford, MA).

Synthesis of NO Donors. Small-molecule *N*-diazeniumdiolate NO donors were synthesized as described previously.[44] Briefly, a 50 mg mL^{-1} solution of SPER, PAPA, DPTA, or DETA in anhydrous acetonitrile was added to Teflon cups within Parr hydrogenation reaction vessels. The vessels were purged with argon (7 bar) a total of six times (three short 10 s purges followed by three 10 min purges) and then pressurized with NO (15 bar). After 72 h, the same argon purging protocol was repeated to remove unreacted NO. The resulting white precipitate (SPER/NO, PAPA/NO, DPTA/NO, or DETA/NO) was collected via vacuum filtration, washed with cold diethyl ether, dried in vacuo, and then stored at −20 °C.

The NO donor *S*-nitrosoglutathione (GSNO) was synthesized following a previously published protocol.[45] Briefly, glutathione (1.53 g; 5 mmol) was dissolved in 5.5 mL of Milli-Q water with 5 mL of 1 M HCl and stirred on ice. Sodium nitrite (0.345 g; 5 mmol) was added to the solution and stirred on ice in the dark. After 40 min, 10 mL of cold acetone was added to the reaction flask and stirred for an additional 10 min. The resulting dark pink precipitate was collected via vacuum filtration, washed with 15 mL of cold Milli-Q water, 9 mL of cold acetone, and 9 mL of cold diethyl ether, dried in vacuo, and stored in the dark at −20 °C in a vacuum-sealed bag.

Characterization of NO Release. Real-time NO release was evaluated using a Sievers 280i nitric oxide analyzer (NOA; Boulder, CO). Prior to analysis, the NOA was calibrated with air passed through a zero NO filter and 25.87 ppm of NO

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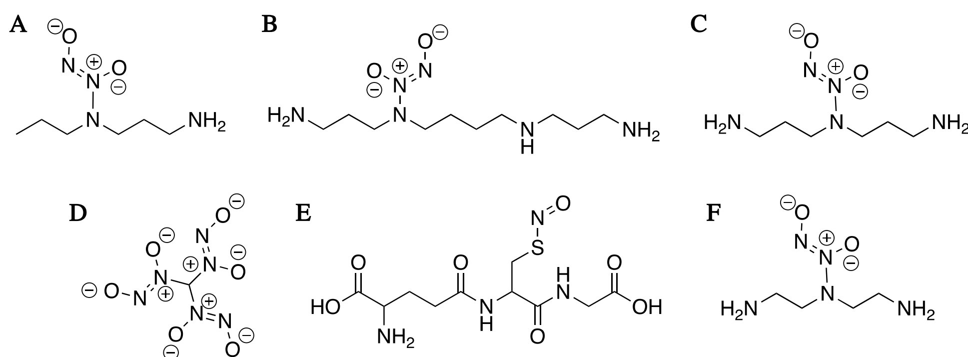

Figure 1. Structures of (A) PAPA/NO, (B) SPER/NO, (C) DPTA/NO, (D) MD3, (E) GSNO, and (F) DETA/NO.

calibration gas (balance N_2). The NO donor (approximately 1 mg) was added to a round-bottom flask containing 30 mL of deoxygenated phosphate-buffered saline (PBS; 10 mM; pH 7.4) at 37 °C. The solution was purged with N_2 gas (200 mL min^{-1}) to carry liberated NO to the instrument. Analysis was terminated when NO levels fell below 10 ppb.

Evaluation of Antibacterial Activity against *Pseudomonas syringae*. The minimum inhibitory and bactericidal concentrations (MIC and MBC, respectively) of the NO donors were evaluated against *P. syringae* using the broth microdilution method.[46] Bacteria cultures of *P. syringae* were grown from frozen (−80 °C) stocks on KMB agar plates. Colonies were isolated from the plate, resuspended in KMB, and incubated at 28 °C overnight. The overnight solution was diluted to a final concentration of 5×10^6 CFU mL^{-1} in fresh KMB. Solutions of the NO donors (4 mg mL^{-1}) were prepared in KMB, titrated to pH 7.4 with 1 M HCl, and 2-fold serially diluted in a 96-well plate to give a final volume of 100 μL per well. After 10 μL of the bacteria solution was added to each well, resulting in a final bacteria concentration of 5×10^5 CFU mL^{-1}, the 96-well plates were incubated at 28 °C. The MIC was determined as the lowest concentration of NO donor that resulted in no visible bacteria growth after 24 h. To determine the MBC, 5 μL from each well was spot-plated on a rectangular KMB agar plate and incubated at 28 °C overnight. The MBC is defined as the lowest concentration of material that resulted in no bacterial growth.

Evaluation of Antifungal Activity against *Botrytis cinerea*. The MIC and minimum fungicidal concentration (MFC) of the NO donors against *B. cinerea* were determined using the broth microdilution method adapted from the Clinical and Laboratory Standards Institute (CLSI) M27 Reference Method.[47] *Botrytis cinerea* was incubated on SDA at 25 °C for 5−7 days. Spore suspensions of approximately 5×10^6 conidia mL^{-1} were prepared by brushing the surface of the SDA plates with a sterile cotton swab to collect the spores and suspending them in sterile PBS (10 mM; pH 7.4). Solutions of the NO donors (8 mg mL^{-1}) were prepared in SDB, titrated to pH 7.4 with 1 M HCl, and 2-fold serially diluted in a 96-well plate to give a final volume of 100 μL per well. The spore suspension was then added to each well (10 μL), and the plates were kept at 25 °C. After 48 and 72 h, the MIC and MFC, respectively, were determined as the lowest concentration with no visible fungal growth.

Bacterial Infection and Treatment of Tomato Plants. Tomato plants were grown in a greenhouse at 25 °C supplemented with high-intensity sodium lamps for 14 h each day. To evaluate the ability of NO donors to treat bacterial infection in tomato plants, 2-month-old tomato plants were syringe-infiltrated with 1×10^6 CFU mL^{-1} *P. syringae* in 10 mM $MgCl_2$ by adapting a previously published protocol.[48] To prepare this solution, colonies of *P. syringae* were isolated from a KMB agar culture plate and incubated in KMB at 28 °C overnight. The overnight solution was centrifuged at 6000*g* for 2 min at room temperature and washed with 10 mM $MgCl_2$ three times. The bacterial cells were then resuspended in 1 mL of 10 mM $MgCl_2$ and diluted to a concentration of 1×10^8 CFU mL^{-1}. This suspension was further diluted in 10 mM $MgCl_2$ to a final bacterial concentration of 1×10^6 CFU mL^{-1}. After infection, the plants were treated daily with either a spray treatment of 50, 100, or 500 mg MD3 in 200 mL of water with 0.02% Silwett L-77 or through the addition of 100 or 500 mg MD3 to the plants' watering trays and stored at room temperature. To determine the bacterial burden, two leaf disks were collected per plant using an 8 mm biopsy punch on days 0, 1, 3, 5, and 7 after infection. The leaf disks were ground with 1 mL of 10 mM $MgCl_2$ using a mortar and pestle and 10-fold serially diluted in 10 mM $MgCl_2$. The dilutions were drip-plated on KMB agar plates, incubated at 28 °C for 2 days, and enumerated to determine viable CFU mL^{-1}. At least three biological replicates were included in each treatment and control group.

Fungi Inoculation of Fruit and Disease Symptom Measurement. Tomato plants were grown in a greenhouse as described above, and Roma tomatoes were harvested at the red stage and immediately transported to the laboratory. Fruits were surface disinfected with 2% (v/v) sodium hypochlorite for 2 min, washed with distilled water, and dried at room temperature. Tomatoes were wounded with a 2 mm biopsy punch and inoculated with a 10 μL suspension of *B. cinerea* spores (2×10^6 conidia mL^{-1}) using a pipet.[37,49] Tomatoes were randomly divided into treatment and control groups, with 15 fruits per group, and stored at 25 °C and 90−95% relative humidity. Tomatoes were monitored for decay, photographed, and treated either by pipetting 10 μL of MD3 solution into the wound daily over the course of a week or soaking the tomatoes for 24 h in an MD3 solution. Untreated tomatoes and tomatoes soaked in sterile Milli-Q water were used as controls. Disease incidence was expressed as the percentage of fruits in each group displaying visible fungal growth.

Evaluation of Ecological Effects via Earthworm Toxicity Tests. An earthworm toxicity test was performed by adapting the United States Environmental Protection Agency (EPA) OCSPP 850.3100: Earthworm Subchronic Toxicity Test guidelines.[50] Earthworms were added to soil and acclimated for 14 days. Twice a week during this period, the

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Table 1. Nitric Oxide-Release Properties of Small-Molecule NO Donors in PBS (10 mM; pH 7.4; 37 °C)[a]

donor	$[NO]_t$ (μmol mg^{-1})[b]	$[NO]_{max}$ (ppb mg^{-1})[c]	t_d (h)[d]	$t_{1/2}$ (h)[e]
PAPA/NO	10.3 ± 1.2	36,395 ± 10,293	6 ± 1	0.5 ± 0.1
SPER/NO	5.9 ± 0.5	4185 ± 569	34 ± 5	3.3 ± 1.0
DPTA/NO	8.4 ± 1.2	3374 ± 1253	36 ± 4	4.0 ± 0.9
MD3	7.0 ± 0.3	2733 ± 1012	56 ± 1	4.4 ± 2.0
GSNO	2.7 ± 0.1	214 ± 66	51 ± 15	24.8 ± 8.0
DETA/NO	11.6 ± 1.2	608 ± 153	143 ± 16	25.1 ± 5.4

[a]Error represents the standard deviation for $n \geq 3$ separate syntheses. [b]Total NO released over full duration. [c]Maximum instantaneous NO concentration. [d]Duration of NO release. [e]Half-life of NO release.

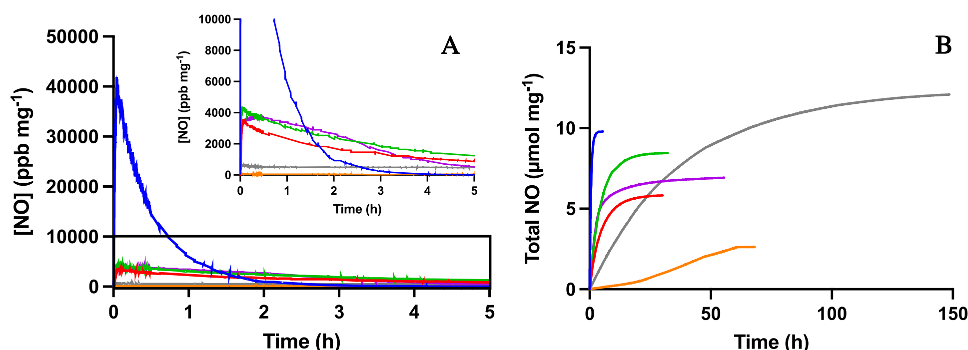

Figure 2. (A) Representative NO-release profiles for the first 5 h of release with inset of boxed region from 0 to 10,000 ppb mg^{-1} and (B) cumulative NO release over time for PAPA/NO (blue), SPER/NO (red), DPTA/NO (green), MD3 (purple), GNSO (orange), and DETA/NO (gray) in PBS (pH 7.4; 10 mM; 37 °C).

earthworms were fed alfalfa pellets that were previously saturated with Milli-Q water at a ratio of approximately 1 g of dry pellets per 2 mL of water and aged for 2 weeks in a covered container. Soil (100 g) was added to test containers (glass canning jars with 1-pint capacity) along with the treatment material (MD3 dissolved in Milli-Q water to give a final volume of 1.23 mL) at concentrations of 250, 500, and 1000 mg MD3 per kg soil. Treatment of soil with an equivalent volume of Milli-Q water was used as a control. The total biomass of test organisms placed in each test container was measured at the beginning and end of the test. Before measuring the biomass, worms were rinsed with water to remove soil, placed in Petri dishes with wet filter paper to allow purging of their gut contents overnight, and then weighed. Worms were added to the top of the soil in the test containers and their burrowing behavior was observed. The soil temperature, soil pH, worm viability, and worm behavior were monitored weekly for a period of 21 days.

RESULTS AND DISCUSSION

Nitric oxide-releasing small molecules from different classes of NO donors including *N*-diazeniumdiolates (i.e., PAPA/NO, SPER/NO, DPTA/NO, and DETA/NO), *C*-diazeniumdiolates (i.e., MD3), and *S*-nitrosothiols (i.e., GSNO) were selected to comprehensively evaluate the effects of NO on the eradication of bacterial and fungal plant pathogens (Figure 1). These NO donors are characterized as having large NO payloads (2.7−11.6 μmol mg^{-1}) and varying NO-release kinetics (Table 1 and Figure 2). The fastest-releasing NO donor, PAPA/NO, had the largest burst of NO released ($t_{1/2}$ of 0.5 h and release duration of 6 h). An inverse relationship was observed between maximum instantaneous NO concentration and half-life of NO release, with NO-release systems of longest NO-release kinetics (i.e., GSNO and DETA/NO) having lowest overall initial NO burst but half-lives up to 24 h. While GSNO and DETA/NO have similar NO-release half-lives, the release duration for DETA/NO (143 h) is significantly longer than GSNO (51 h). Typically, *N*-diazeniumdiolates, including DETA/NO, follow pseudo-first-order decompositions to NO with the rate constant being dependent on the pH of the solution, whereas the release of NO from *S*-nitrosothiols is impacted by several factors (i.e., temperature, light, metal ions) which plays a more significant role on their release kinetics.[51,52] Selecting NO donors with diverse NO-release profiles allowed for the evaluation of the effects of NO-release kinetics on antimicrobial activity.

In Vitro Antibacterial Activity against *Pseudomonas syringae*. A broad-spectrum pesticide should effectively eradicate both bacterial and fungal pathogens. In this study, the antibacterial activity of the NO donors was thus evaluated against *P. syringae*, a common plant pathogen often utilized as a model organism.[53] Over 60 pathovars of *P. syringae* have been identified, with each pathovar infecting characteristic groups of host plants, many of which are economically important crop species.[53] For example, *Pseudomonas syringae* pv tomato causes bacterial speck disease, one of the most pervasive biological adversities in tomato cultivation.[54] The in vitro antibacterial activity of the NO donors against *P. syringae* pv tomato was evaluated using a broth microdilution assay.

All six NO donors that were evaluated effectively eradicated *P. syringae* (Table 2). The NO donors with the shortest half-lives (i.e., PAPA/NO and SPER/NO) required the greatest concentrations (i.e., larger MICs) to achieve inhibition of bacterial growth; however, similar MBCs (500−1000 μg mL^{-1}) were observed for the NO donors with both the shortest and longest (i.e., GSNO and DETA/NO) NO-release half-lives. The higher concentrations of NO needed from the faster NO-releasing systems suggest a more rapid burst release of NO may not effectively inhibit bacterial growth or lead to eradication, likely due to NO release occurring prior to the NO

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Table 2. In Vitro Antibacterial Activity of NO Donors against Planktonic *Pseudomonas syringae*[a]

	MIC (μg mL^{-1})		MBC (μg mL^{-1})	
donor	donor concentration	NO dose	donor concentration	NO dose
PAPA/NO	500	155	1000	309
SPER/NO	500	89	500	89
DPTA/NO	31.25	8	125	32
MD3	125	26	125	26
GSNO	125	10	1000	81
DETA/NO	62.5	22	500	176

[a]MIC and MBC determined from $n \geq 3$ biological replicates.

donor reaching the bacteria and the short diffusion distance of NO.[33] While the NO donors with longer half-lives of NO release effectively inhibited bacterial growth at low concentrations, complete eradication was not achieved and the bacteria were able to grow back, as the lower amounts of NO released toward the end of the release profile are likely unable to exert substantial oxidative and nitrosative stress needed to eradicate the bacteria. Complete eradication is preferred over inhibition to limit the potential for bacterial regrowth. The most effective NO donors at bacterial eradication were DPTA/NO and MD3, which had intermediate NO-release kinetics with half-lives around 4 h and maximum instantaneous NO concentrations around 3000 ppb mg^{-1}, suggesting that this NO-release exposure is optimal for *P. syringae* eradication.

In Vitro Antifungal Activity against *Botrytis cinerea*. To further demonstrate the potential of NO as a broad-spectrum pesticide, the antifungal activity of the small-molecule NO donors was evaluated against *B. cinerea*, one of the most destructive and extensively studied fungal pathogens which affects over 200 crops and causes gray mold disease in tomatoes and other important agricultural crops.[12,55] *Botrytis cinerea* has high agricultural and scientific importance as it often acquires resistance to commonly employed fungicides used to control it.[55] As observed for *P. syringae*, the NO donors with the shortest (i.e., PAPA/NO) and longest (i.e., GSNO and DETA/NO) half-lives of NO release required the highest NO payloads to achieve fungal eradication (Table 3). Overall,

Table 3. In Vitro Antifungal Activity of NO Donors against *Botrytis cinerea* Spores[a]

	MIC (μg mL^{-1})		MFC (μg mL^{-1})	
donor	donor concentration	NO dose	donor concentration	NO dose
PAPA/NO	4000	1236	8000	2472
SPER/NO	<15	<3	<15	<3
DPTA/NO	62.5	16	62.5	16
MD3	125	26	125	26
GSNO	2000	163	4000	326
DETA/NO	1000	351	1000	351

[a]MIC and MFC determined from $n \geq 3$ biological replicates.

larger concentrations of NO donor (1000−8000 μg mL^{-1}) were needed to eradicate *B. cinerea* relative to *P. syringae* (500−1000 μg mL^{-1}). *Botrytis cinerea* is notoriously difficult to control due to its sexual and asexual stages that allow it to survive as spores in unfavorable conditions.[56] The NO donors with intermediate NO-release half-lives (i.e., DPTA/NO, and MD3) proved ideal for eradicating *B. cinerea*, requiring similar concentrations of NO (16−26 μg mL^{-1}) to those needed to eradicate *P. syringae*, demonstrating the ability of NO to effectively treat this notoriously robust pathogen. Of note, SPER/NO had the greatest antifungal activity against *B. cinerea*, with significant efficacy observed at doses less than 15 μg mL^{-1}, suggesting that the NO-release profile from SPER/NO is most suited for eradication of *B. cinerea*.

Taken together, the results from the in vitro evaluation of antimicrobial activity against *P. syringae* and *B. cinerea* suggest that DPTA/NO and MD3 have the most optimal NO-release profiles for the broad-spectrum eradication of these plant pathogens. Unfortunately, small-molecule polyamine NO donors, such as DPTA/NO, have been associated with the formation of nitrosamines that are potent human carcinogens and highlighted as environmental contaminants of increasing health concern.[57,58] The carbon-bound MD3 NO donor was thus selected for further studies evaluating the antimicrobial activity in plant systems and corresponding ecological toxicity.

Treatment of *Pseudomonas syringae*-Infected Tomato Plants. As *P. syringae* commonly causes black speck disease in tomato plants, the ability of MD3 to eradicate the pathogen after infection of tomato plants was evaluated to appropriately assess the utility of NO treatment. The leaves of tomato plants were inoculated with *P. syringae* pv tomato via syringe infiltration. Two routes of administration were explored to deliver MD3 and facilitate local NO release. In the first treatment route, 50, 100, or 500 mg MD3 was diluted in 200 mL water and then sprayed onto the leaves of the plants daily. For the second treatment route, 100 or 500 mg MD3 was added directly to the watering tray of the plants each day. Spray treatment proved to be more effective, as the plants treated with MD3 in their watering trays had similar bacterial burdens to untreated controls (Figure 3), likely due to the

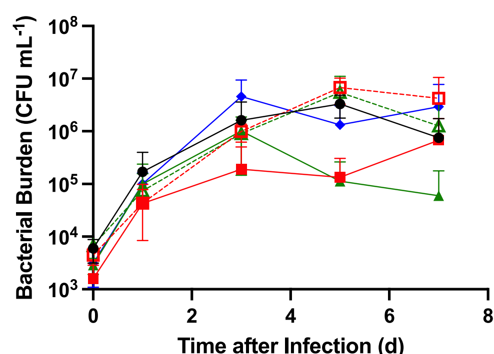

Figure 3. *Pseudomonas syringae* burden in infected tomato plant leaves after no treatment (black circles) or treatment with 50 mg MD3 (blue diamonds), 100 mg MD3 (green triangles), or 500 mg MD3 (red squares), administered by spray treating the leaves of the plants (solid lines and shapes) or adding treatment to the water tray of the plants (dotted lines and hollow shapes). Three tomato plants were included in each treatment condition.

more localized application of NO at the site of infection. While the same mass of MD3 was used in each treatment condition, the effective concentration of spray treatment was higher due to the smaller volume of water used compared to the volume of water in the tray. Though spray treatment with 500 mg of MD3 initially proved to be most effective in eradicating *P. syringae* leaf infection, after 7 days of infection, treatment with 100 mg of MD3 led to the greatest reduction in bacterial burden. This treatment led to a 92% decrease in bacterial

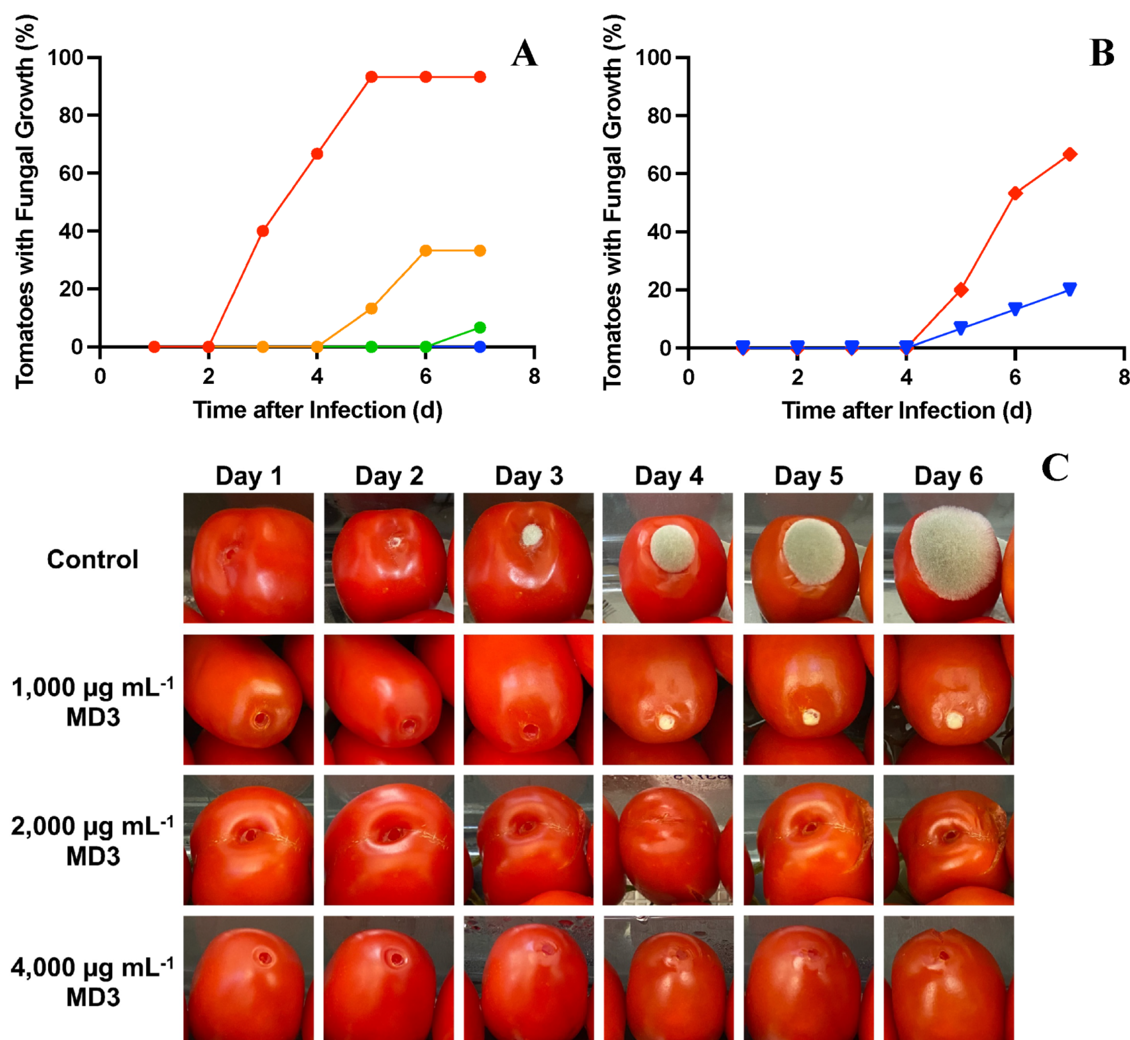

Figure 4. Percent of tomatoes with visible *Botrytis cinerea* growth after (A) daily treatment with 1000 (orange), 2000 (green), and 4000 (blue) μg mL^{-1} MD3 compared to untreated tomatoes (red) or (B) a 24-h soak on day 0 in either water (red) or a 1000 μg mL^{-1} solution of MD3 (blue). (C) Representative images of *B. cinerea*-inoculated untreated tomatoes and those with daily treatment with MD3. Fifteen tomatoes were included in each treatment condition.

viability compared to the control, suggesting the promise of utilizing MD3 as a treatment for black speck disease and controlling bacterial infection of tomato plants. The use of NO-releasing nanomaterials has the potential to increase their internalization into plant tissue and further enhance NO's antimicrobial efficacy, and represents a promising direction of future work.[33]

Treatment of *Botrytis cinerea*-Infected Tomato Fruit. *Botrytis cinerea* has a wide range of infection sites, including the leaves, stems, flowers, and fruits of plants, and can infect plants throughout their development process.[59] Gray mold disease caused by *B. cinerea* causes substantial postharvest losses of fruit and vegetables worldwide. To further demonstrate the potential use of NO as a broad-spectrum pesticide, the antifungal activity of MD3 was evaluated against *B. cinerea*-infected tomato fruit. Again, two treatment methods were compared. In the first method, aqueous solutions of MD3 ranging from 1000 to 4000 μg mL^{-1} were directly pipetted into the wound created for inoculation with *B. cinerea*. After 5 days, over 90% of the untreated controls showed visible growth of *B. cinerea* (Figure 4A) with fungal growth appearing as early as 3 days after inoculation. Treatment with MD3 was efficacious in preventing the growth of *B. cinerea*, even at the lowest treatment concentrations. At 1000 μg mL^{-1}, MD3 was able to prevent visible fungal growth until day 5 and only 33% of treated tomatoes had any growth on day 7. This growth was substantially less than the fungal growth observed in the untreated tomato fruit (Figure 4C). Only 7% of the tomatoes treated with 2000 μg mL^{-1} MD3 had visible fungal growth on day 7, and treatment with 4000 μg mL^{-1} MD3 completely prevented visible fungal growth. Visually, treatment with MD3 at these concentrations did not have any negative effects on the appearance of the tomato fruit (Figure 4C).

As daily treatment of postharvest fruit may be too labor intensive, a one-time soak in an aqueous MD3 solution immediately after fungal inoculation was also evaluated with tomatoes soaked in water as controls. Soaking tomatoes in water did reduce the occurrence of fungal infection compared to untreated tomatoes, as visible fungal growth was delayed until day 5 and 67% of tomatoes displayed visible fungal growth on day 7 (Figure 4B). This decrease in fungal growth is likely due to the water washing away some of the inoculated fungi rather than exerting an antifungal effect. Soaking tomatoes in MD3 further decreased the occurrence of fungal

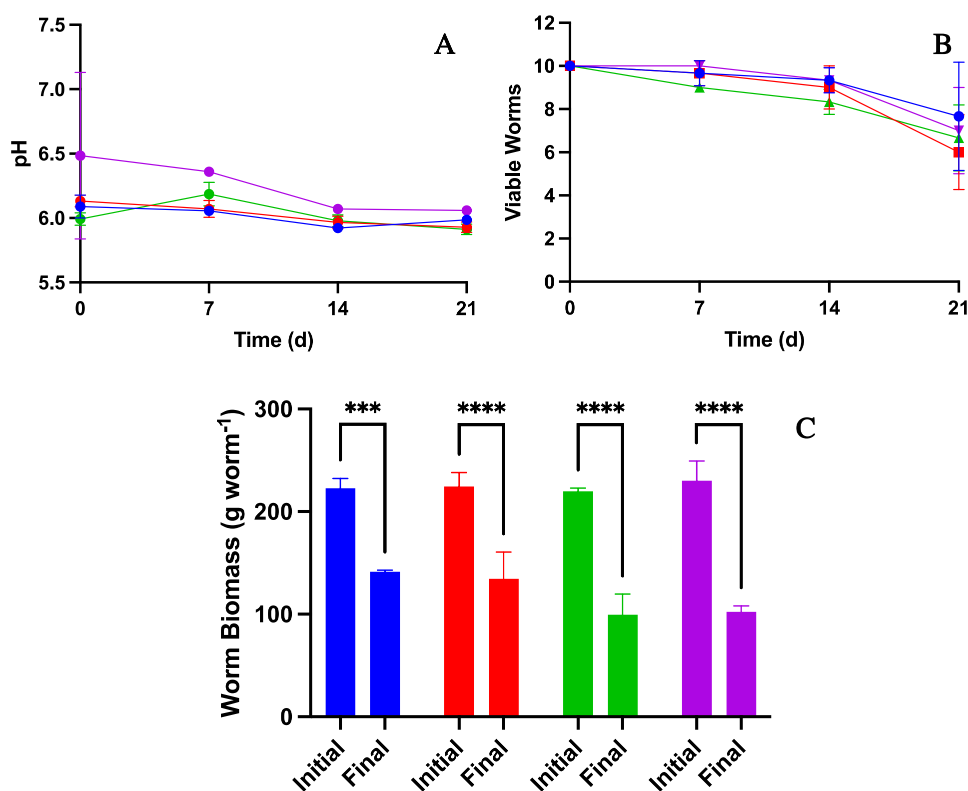

Figure 5. (A) Soil pH, (B) earthworm viability, and (C) normalized biomass of worms for earthworm toxicity test after treatment with PBS (blue), 250 (red), 500 (green), or 1000 (purple) mg MD3 kg^{-1} soil. Three groups with 10 worms each were evaluated for each treatment condition. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

infection, as only 20% of tomatoes had visible growth of *B. cinerea* on day 7, suggesting that a one-time treatment with MD3 is effective at preventing *B. cinerea* growth. Utilizing a higher concentration of MD3 may potentially further decrease the occurrence of fungal growth and should be explored in future work.

Evaluation of Ecological Toxicity via an Earthworm Toxicity Assay. Nitric oxide has the potential to be an environmentally safe substitute for conventional pesticides due to its endogenous roles in both plant and human biology.[33] Additionally, the short lifetime of released NO precludes the accumulation of NO in the environment. As NO donor molecules are used to deliver NO, an evaluation of the environmental toxicity of the NO donor and its resulting decomposition products is still needed. To assess the ecological toxicity of MD3, an earthworm toxicity test was conducted following the Earthworm Subchronic Toxicity Test guidelines from the EPA, which were established for use in testing pesticides.[50] Earthworms (*Eisenia fetida andrei*) are often utilized as a model organism for toxicity screening, as they are sensitive to toxicants and can be cultured under laboratory conditions.[20,60] Higher concentrations of MD3 (250−1000 mg MD3 kg^{-1} soil) than were needed to exert antimicrobial activity were evaluated to ensure that no adverse environmental effects would be observed from the use of MD3 as a broad-spectrum pesticide. The temperature and pH of the soil, as well as the viability and behavior of the earthworms were evaluated weekly after treating the soil with MD3. The temperature of the soil remained constant at 22−23 °C over the course of the experiment for each treatment group. The pH of the soil was not significantly altered upon treatment with MD3 and remained constant around pH 6 over the duration of the study (Figure 5A). No significant changes in earthworm viability were observed between the treatment and control groups at any time points (Figure 5B), with all worms showing normal behavior (i.e., burrowing and response to stimuli), indicating that treatment with MD3 does not have adverse environmental consequences. The biomass of the worms (normalized to number of worms) was also compared at the beginning and end of the study (Figure 5C). While the average biomass of the worms significantly decreased over the duration of the experiment, no significant changes in biomass were observed between the control and treatment groups, further confirming that the NO is unlikely to negatively influence the environment.

Taken together, these results demonstrate the potential use of NO as a broad-spectrum, efficacious, and environmentally safe substitute for conventional pesticides. Intermediate NO-release kinetics (i.e., half-lives of release around 4 h) proved most effective at eradicating both *P. syringae* and *B. cinerea* in vitro at NO doses as low as 16−32 $\mu g\ mL^{-1}$. Treatment with a *C*-diazeniumdiolate, MD3, demonstrated the ability to treat *P. syringae* infection of tomato plants and prevent *B. cinerea* growth in postharvest tomato fruits. The environmental safety of MD3 treatment was determined in an established earthworm toxicity assay in which no adverse effects were observed resulting from treatment with the NO donor. Likewise, NO treatment did not influence growth or other plant biology at the macroscale. Future studies should explore this promising NO donor for the treatment of pathogen infections of additional plant species and incorporate the NO donor into a delivery system for targeted delivery and enhanced uptake by plant tissue.

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Author Contributions

The manuscript was written through contributions of all authors. Q.E.G. and M.H.S. conceived and designed the experiments which were carried out by Q.E.G. and M.E.P. Data interpretation was performed by Q.E.G. The manuscript was primarily written by Q.E.G. and M.H.S. All authors provided critical feedback and have given approval to the final version of the manuscript.

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Notes

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