Journal of Microbiological Methods 215 (2023) 106849

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Agrobacterium tumefaciens-mediated transformation of *Nigrospora* sp. isolated from switchgrass leaves and antagonistic toward plant pathogens

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ARTICLE INFO

Keywords:
Agrobacterium tumefaciens
Bipolaris maydis
Endophytic fungi
Fungal transformation
Nigrospora sp.
Parastagonospora nodorum

ABSTRACT

Nigrospora is a diverse genus of fungi colonizing plants through endophytic, pathogenic, or saprobic interactions. Endophytic isolates can improve growth and development of host plants, as well as their resistance to microbial pathogens, but exactly how they do so remains poorly understood. Developing a reliable transformation method is crucial to investigate these mechanisms, in particular to identify pivotal genes for specific functions that correlate with specific traits. In this study, we identified eight isolates of *Nigrospora* sp. internally colonizing the leaves of switchgrass plants cultivated in North Carolina. Using an *Agrobacterium tumefaciens*-mediated transformation approach with control and GFP-expressing vectors, we report the first successful transformation of two *Nigrospora* isolates. Finally, we demonstrate that wild-type and transgenic isolates both negatively impact the growth of two plant pathogens in co-culture conditions, *Bipolaris maydis* and *Parastagonospora nodorum*, responsible for the Southern Leaf Blight and Septoria Nodorum Blotch diseases, respectively. The GFP-transformed strains developed here can therefore serve as accurate reporters of spatial interactions in future studies of *Nigrospora* and pathogens in the plant. Finally, the transformation method we describe lays the foundation for further genetic research on the *Nigrospora* genus to expand our mechanistic understanding of plant-endophyte interactions.

1. Introduction

Nigrospora represents a diverse genus of fungal ascomycetes that can colonize many species of land and marine plants (Xu et al., 2022). Species in the *Nigrospora* genus are defined as endophytic, phytopathogenic, and even saprobic fungi (Hao et al., 2020). These different lifestyles depend on the colonized host plant and environmental contexts. Some examples of plant pathogenic fungi are *Nigrospora osmanthi* that causes leaf blight in *Stenotaphrum secundatum* and *Ficus pandurata*, *Nigrospora sphaerica* that causes leaf blight in *Mentha canadensis*, and *Nigrospora oryzae* that causes leaf spots in *Aloe vera* and *Poa pratensis* (Liu et al., 2019; Mei et al., 2019; Sun et al., 2020; Zhai et al., 2013; Zheng et al., 2012).

The first endophytic *Nigrospora* species was isolated from the leaves of a grass, *Panicum amphibium*, by Zimmerman, 1902 and identified as *Nigrospora panici*. Endophytic *Nigrospora* species produce a large number of secondary metabolites, including polyketides, pyrans, quinones and even some compounds originally considered as plant-specific products like quercetin (Ebada et al., 2016). Particularly, some species of *Nigrospora*, like *N. oryzae*, produce ergoline alkaloids and abscisic acid, sharing a highly similar structure with plant alkaloids and phytohormones (Steiner and Leistner, 2012; Uzor et al., 2015). These metabolites exhibit various beneficial effects on plant growth, such as seed germination and plant cell division, but also antifungal or antibacterial properties. Therefore, it is reasonable to think that some endophytic *Nigrospora* species can be used to protect crop plants against microbial pathogens. Indeed, *Nigrospora* sp. LLGLM003 releases griseofulvin that inhibits the growth of two plant pathogenic fungi, *Botrytis cinerea* and *Colletotrichum orbiculare* (Zhao et al., 2012). Antimicrobial activity of an endophytic *N. sphaerica* strain isolated from the medicinal plant *Dinellia indica* L. was also assessed against several fungal pathogens like *Fusarium oxysporum* and *Aspergillus niger*, resulting in 72% and 55% growth

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https://doi.org/10.1016/j.mimet.2023.106849
Received 11 August 2023; Received in revised form 25 October 2023; Accepted 25 October 2023
Available online 30 October 2023

decrease, respectively (Kumar and Prasher, 2023). However, further progress in understanding the mechanisms underlying endophyte-pathogen interactions requires development of tools that allow us to dissect their molecular biology and genetics.

Developing a reliable genetic transformation method for endophytic fungi, and particularly *Nigrospora* species, is crucial to investigate the molecular players involved in growth, plant tissue colonization, and inhibition of pathogenic fungi. Genetically transforming filamentous fungi can be more challenging than for yeasts, probably due to their more complex cell wall composition. Consequently, multiple methods have been developed with various success, including electroporation, biolistics, protoplast formation, and shock waves (Case et al., 1979; Li et al., 2017; Rivera et al., 2014). Because these transformation methods can have a high risk of random mutations, *Agrobacterium tumefaciens*-mediated transformations (Agrotransformations) have also been recently developed for plant-associating filamentous fungi, including endophytic and ectomycorrhizal fungi (Combier et al., 2003; Felber et al., 2019). For example, the genetic transformation of the plant endophytic fungus *Diaporthe phaseolorum*, isolated from mangroves, was performed with *A. tumefaciens* strain EHA105 to express a green fluorescent protein (GFP) in the fungus, and proved to be quite stable (Sebastianes et al., 2012). To our knowledge, no Agrotransformation strategy has been developed for any *Nigrospora* species so far.

Here, we report the first successful Agrotransformation of two *Nigrospora* strains, *Nigrospora* sp. NC02751 and *Nigrospora* sp. NC02761 isolated from switchgrass leaves in North Carolina. Additionally, we demonstrated that both isolates reduce by 40–60% the growth of two plant pathogenic fungi, *Bipolaris maydis* and *Parastagonospora nodorum*, responsible for the Southern Leaf Blight (SLB) and Septoria Nodorum Blotch (SNB) diseases, respectively, and that the genetic transformation does not affect this ability.

2. Materials and methods

2.1. Focal Nigrospora strains

We randomly selected eight endophytic strains of *Nigrospora* sp. that were isolated from surface-sterilized switchgrass (*Panicum virgatum* L.) leaves collected during a sampling campaign in August 2019 (Whitaker et al., 2023; Table 1). We focused on *Nigrospora* because related strains previously isolated from switchgrass were beneficial to the host in drought conditions (Giauque et al., 2019; Giauque and Hawkes, 2013). Fungal 28S rRNA was sequenced using NL1 and NL4 primers (O'Donnell, 1993) and sequences are available on NCBI Genbank (Accession numbers OR246895-OR246902). The ITS regions were amplified with ITS1-ITS4 primers (Gardes and Bruns, 1993; White et al., 1990) and sequences are available on GenBank (Accession numbers OR724861-OR724868). We report the current best BLAST match for each strain and each primer set (Table 1), but the species level for each fungus cannot be confirmed due to discrepancies for some isolates between 28S rRNA and ITS BLAST matches. Consequently, we will hereafter refer to them as "*Nigrospora* sp." followed by the strain ID as appropriate.

2.2. Vector sources and A. tumefaciens preparation

Agrotransformations were performed using the AGL1 strain of *A. tumefaciens* carrying the pHg and pBGgHg vectors that harbor the control and GFP constructs, respectively (Chen et al., 2000; Kemppainen and Pardo, 2010). To express these vectors, the bacterial strain was grown in 50 ml of liquid LB medium for three days, then centrifuged at 3000 rpm for 15 min. The bacterial pellet was resuspended in 1 ml of cold $CaCl_2$ (20 mM), and 100 μl were mixed with 1 μl of each purified vector. After an incubation on ice for 30 min followed by a flash freeze in liquid nitrogen, tubes were kept on ice for 5 min. A total of 1 ml of LB medium was added before incubation for 2 h at 30 °C under shaking. Finally, 100 μl of culture was spread on a solid LB plate supplemented with rifampicin (100 μg ml^{-1}) and kanamycin (50 μg ml^{-1}). Plates were incubated for three days at 30 °C until transformed colonies appeared.

2.3. Hygromycin sensitivity test

T-DNA of vectors used to transform *Nigrospora* harbored the *hph* gene of *Escherisha coli* coding for an aminocyclitol phosphotransferase that confers resistance to hygromycin. Thus, to evaluate the hygromycin sensitivity of the eight identified *Nigrospora* species, cultures were made on potato dextrose agar (PDA) media with 0, 50, 75 and 100 μg·ml^{-1} of hygromycin (Thermo scientific). Growth was observed on each media for seven days to determine the correct concentration to use and pictures were taken.

2.4. Fungal preparation for Agrotransformations

Only hygromycin-sensitive fungal strains were selected for subsequent Agrotransformations. Therefore, fungal cultures were prepared in 60 ml of potato dextrose (PD) broth for seven days. Thalli formed in liquid media were transferred in fresh PD solution and grinded for approximately 15 s with an immersion homogenizer (Fisherbrand™ 150 Homogenizer). The lysates were poured into two Petri dishes filled with PD broth, and placed at 26 °C until the formation of a new thallus. Each time a thallus was formed, it was transferred into fresh PD medium, homogenized, and incubated again at 26 °C. The process continued until a new thallus was able to form overnight.

2.5. Agrotransformation of hygromycin-sensitive strains of Nigrospora

Agrotransformations were carried out using the method developed for the ectomycorrhizal fungus *Hebeloma cylindrosporum* (Combier et al., 2003; Garcia et al., 2013). *A. tumefaciens* AGL1 strains expressing the pBGgHg and pHg vectors were inoculated in 5 ml of LB supplemented with rifampicin (100 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) at 26 °C under shaking conditions. After three days, 200 μl of the culture were inoculated in 5 ml of sterile induction medium (minimal medium

Table 1
Identification of the focal *Nigrospora* isolates based on ITS and 28S rDNA comparisons to the NCBI Genbank nucleotide database. Best matches are reported as the first named match in the BLAST results. *E*-values of matches ranged from 0 to 7e-160, query coverage from 99 to 100%, and percent identity from 99.4 to 100.0%. Note that, for the 28S, all matches had alternative identifications with the same scores.

Isolate	Isolate ITS Accession	Best ITS BLAST Match Accession	Best ITS BLAST Match ID	Isolate 28S Accession	Best 28S BLAST Match Accession	Best 28S BLAST Match ID
NC02705	OR724861	EU272486.1	*N. oryzae*	OR246895	NG_069399.1	*N. lacticolonia*
NC02749	OR724862	MG098254.1	*N. oryzae*	OR246896	MG888641.1	*N. oryzae*
NC02751	OR724863	MN341509.1	*N. musae*	OR246897	MH867051.1	*N. musae*
NC02757	OR724864	MK247346.1	*N. oryzae*	OR246898	MG888641.1	*N. oryzae*
NC02761	OR724865	KT898587.1	*N. oryzae*	OR246899	NG_069402.1	*N. hainanensis*
NC02769	OR724866	MG098254.1	*N. oryzae*	OR246900	MG888641.1	*N. oryzae*
NC02788	OR724867	KT898587.1	*N. oryzae*	OR246901	KX958066.1	*N. oryzae*
NC02793	OR724868	KT898587.1	*N. oryzae*	OR246902	KX958066.1	*N. oryzae*

supplemented with 40 mM of MES and 0.2 mM of acetosyringone) and incubated under shaking conditions until the OD600 reached at least 0.4. For the co-cultures, mycelia were grinded as described above and 50 μl of lysate were placed onto sterilized circular glass microfiber filter discs (Cytiva's Whatman Grade GF/D Glass Microfiber Prefilter). Discs were transferred into Petri dishes filled with induction medium and 50 μl of bacterial culture were placed on each one of them. In total, 100 discs were prepared as described above for each fungus-vector combination. Plates containing the discs were incubated at 26 °C for three days.

After three days, discs were transferred into Petri dishes filled with the following selection medium: PDA supplemented with 100 μg ml^{-1} of hygromycin and 200 μg ml^{-1} of cefotaxime. Growing mycelia were collected between 10 and 15 days after the discs were placed on the selection medium, and transferred on fresh PDA plates supplemented with 100 μg ml^{-1} of hygromycin to confirm the transformants. Confirmed transformants were successively sub-cultured on hygromycin-free PDA plates for five successive generations, and then finally transferred to PDA medium supplemented with 100 μg ml^{-1} of hygromycin to check their mitotic stability of the integrated T-DNA. This step ensured that transformants maintained their hygromycin resistance phenotype in the absence of antibiotic, indicating that the selective marker was mitotically stable.

2.6. PCR-based validation of fungal transformants

Each transformant was grown on a PDA medium supplemented with hygromycin (100 μg ml^{-1}) at 26 °C for 10 days. The hyphae were scraped from these plates and genomic DNA was isolated using the cetyltrimethylammonium bromide method. All the transformants were first analyzed for their ability to express *hph,* the hygromycin-resistance gene of *E. coli*, using HygR-F and Hyg-R primers (5′- CTCGGAGGGCGAAGAATCTC-3′ and 5'-CAATGACCGCTGTTATGCGG-3′, respectively). Fungi transformed with the pBGgHg vector were also verified with the GFP-specific primers GFP-F and GFP-R (5'-ATGGTGAGCAAGGGCGAGGAGCT-3′ and 5′- GTCGCGGCCGCTTTACTTGTACA-3′, respectively). Purified PCR products were also sequenced using the same primers to confirm the sequences.

2.7. GFP observation by confocal microscopy

Analysis of GFP expression in transformants was done using a Zeiss LSM 710 confocal microscope on fungi growing in PDA supplemented with hygromycin (100 μg ml^{-1}) for seven days. Image acquisition was performed using the following configuration: AF488 filter for chs1 detector with range 494–582 nm, Track1-LSM Laser 488 nm with master gain for chs1:500; chsD:240, pinhole: 32 μm. Control transformants were also analyzed as non-fluorescent controls. GFP fluorescence was quantified and converted into pseudocolor images using the ImageJ software program. Background noise was subtracted from the fluorescence intensity value for quantification. Data are presented from four to six representative images from each fungal transformants, and differences between averages were analyzed by one-way ANOVA followed by LSD post hoc tests.

2.8. Pathogen-Nigrospora interactions

We assayed potential antagonism of *Nigrospora* sp. NC02751 and NC02761 endophytic strains with plant pathogenic fungi of maize (SLB; *B. maydis* Nisikado & Miyake) and wheat (SNB; *P. nodorum* (Berk.) Quaedvlieg, Verkley & Crous). We included both wild-type (Nsp51WT and Nsp61WT) and two transformed strains (Nsp51GFP-1 and Nsp61GFP-1) for each isolate. Each *Nigrospora* strain was grown paired with each pathogen on PDA plates in three replicate 10-cm petri dishes for a total of 8 treatments. Controls consisted of pairs of the same fungus; *Nigrospora* controls were repeated for each pathogen. Plates were scanned at seven and fourteen days and areas of each fungus were calculated in ImageJ (Schneider et al., 2012). Because plate areas were largely full after the first week, growth rates (cm^2/d) were calculated at seven days. We arbitrarily selected the left-hand fungus for growth rate calculations in control plates. We used one-way ANOVA (R package car v.3.1.2, Fox and Weisberg, 2019) to address how fungal growth rates were affected by the fungal pair. We used planned contrasts (multcomp v. 1.4.25; Hothorn et al., 2008) to address whether pathogen growth was affected by being paired with each *Nigrospora* strain relative to pathogen-only controls and whether growth rates differed between transformed and wild-type strains of *Nigrospora*. A Bonferroni correction was used for multiple comparisons. All statistics were performed in R v.4.2.2 (R Core Team, 2022).

3. Results

3.1. Establishment of Agrobacterium mediated transformation protocol for endophytic Nigrospora

To perform Agrotransformations on various *Nigrospora* species, we used a control vector, pHg (Kemppainen and Pardo, 2010), and a GFP-expressing vector, pBGgHg (Chen et al., 2000). Both vectors confer resistance to hygromycin. Therefore, only hygromycin-sensitive fungi can be transformed with these vectors. The eight *Nigrospora* strains isolated from switchgrass plants were placed on PDA media containing various levels of hygromycin (0, 50, 75, and 100 μg l^{-1}) for seven days. Among these strains, seven showed limited growth on media supplemented with 50 and 75 μg l^{-1} of hygromycin, and only five did not show any growth at all on the highest tested hygromycin concentrations: *Nigrospora* sp. NC02705, NC02749, NC02751, NC02761, and NC02793 (Fig. 1). It was important that no growth was recorded at 100 μg l^{-1} of hygromycin since it is the concentration typically used for Agrotransformation with these vectors. Only *Nigrospora* sp. NC02769 did not show significant growth reduction at any hygromycin concentrations (Fig. 1).

Agrotransformations were performed for the five *Nigrospora* strains that showed resistance to 100 μg l^{-1} of hygromycin following the protocol described in (Combier et al., 2003), but no transformants were obtained for *Nigrospora* sp. NC02705, NC02749, and NC02793 (data not shown). Concerning the other two selected strains, *Nigrospora* sp. NC02751 and NC02761, individual transformations were performed on 100 replicates per strain with pBGgHg and pHg vectors. Transformations of *Nigrospora* sp. NC02761 resulted in a total of 30 colonies growing on selection media, but only six of them could successfully grow again on PDA supplemented with hygromycin after five generations without antibiotics. Thus, the individual number of confirmed transformants was three for each vector for *Nigrospora* sp. NC02761. Concerning *Nigrospora* sp. NC02751, a total five colonies were growing on the selection medium (2 and 3 for pBGgHg and pHg vectors, respectively) and all of them survived the mitotic stability test. Consequently, the transformation efficiency was on average 2.5% for *Nigrospora* sp. NC02751 and 3% for *Nigrospora* sp. NC02761, depending on the vector used. The GFP-expressing transformants were named Nsp51GFP-1/2 and Nsp61GFP-1/2/3 for *Nigrospora* sp. NC02751 and NC02761, respectively. Transformations with the control vector pHg were named Nsp51EV-1/2/3 and Nsp61EV-1/2/3. Consequently, a total of five transformants for *Nigrospora* sp. NC02751 and six for *Nigrospora* sp. NC02761 were selected for PCR-based validation.

3.2. Verification of transformed isolates by PCR analysis

Genomic DNA was isolated from Nsp51EV-1/2/3, Nsp61EV-1/2/3, Nsp51GFP-1/2 and Nsp61GFP-1/2/3 transformants growing in PDA supplemented with hygromycin, and PCRs were performed to check the presence of the hygromycin-resistance and GFP genes using the primers HygR-F/R and GFP-F/R, respectively (Fig. 2). Purified pHg and pBGgHg

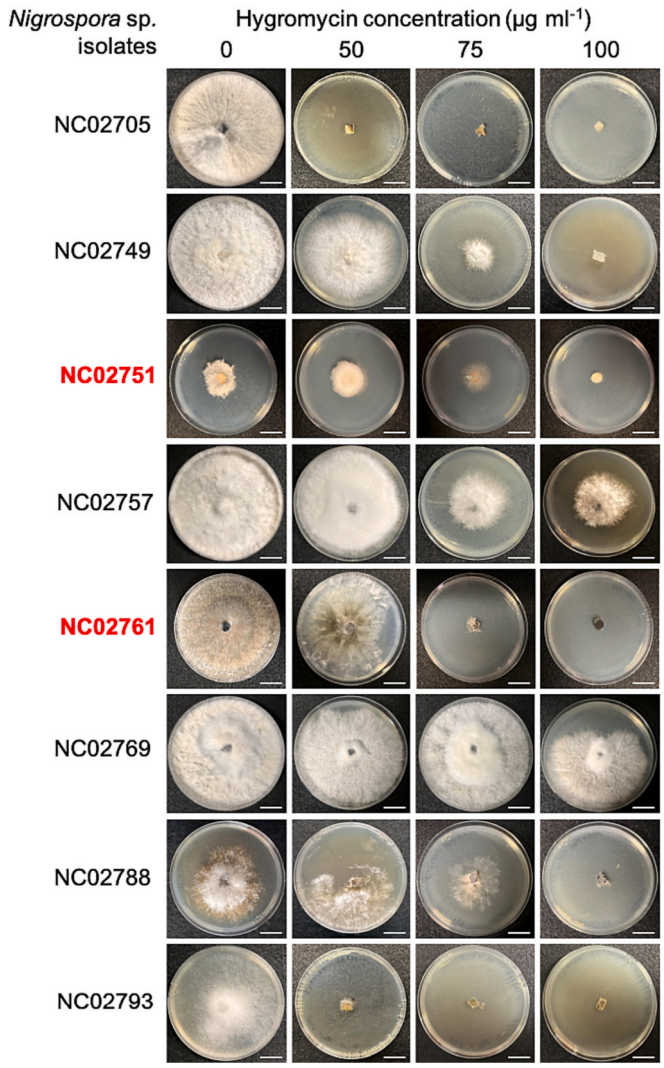

Fig. 1. Hygromycin-resistance test on various *Nigrospora* species isolated from switchgrass leaves in North Carolina. Culture plugs of wild-type *Nigrospora* sp. NC02705, NC02749, NC02751, NC02757, NC02761, NC02769, NC02788, and NC02793 were placed on PDA media supplemented with 0, 50, 75, and 100 μg ml^{-1} of hygromycin for seven days. Isolates NC02751 and NC02761 are written in red font because there are the ones we successfully transformed. Scale bar = 2 cm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vectors were used as templates for the positive control lines. Using HygR-F/R primers, an amplification at the expected size was observed in all five *Nigrospora* sp. NC02751 (Fig. 2a) and six *Nigrospora* sp. NC02761 transformants (Fig. 2), confirming the T-DNA integration of the corresponding vectors in these fungi. Surprisingly, two amplifications were obtained in Nsp51GFP-1/2 with the GFP-F/R primers (Fig. 2b). The ~750 bp band was at the expected size as confirmed by the positive control amplification, while the ~500 bp band was due to a random amplification in the genome of *Nigrospora* sp. NC02751, as also observed in the wild-type strain (Fig. 2b). Regarding Nsp61GFP-1/2/3, an amplification was obtained at the expected size, similar to the positive control vector pBGgHg, while no band was detected in the wild-type *Nigrospora* sp. NC02761 strain (Fig. 2d). Altogether, these results validate the T-DNA integration from pHg and pBGgHg vectors in the corresponding transformants and the efficiency of the Agrotransformations in *Nigrospora* sp. NC02751 and NC02761.

3.3. Verification of GFP-expressing transformants by confocal microscopy

The fluorescence of the GFP-expressing transformants Nsp51GFP-1/2 and Nsp61GFP-1/2/3 was performed after culture on solid PDA for seven days using a Zeiss LSM 710 confocal microscope (Figs. 3 and 4). Nsp51EV-1 and Nsp61EV-2 were used as negative controls to ensure that the genetic transformation approach did not confer any autofluorescence to *Nigrospora* sp. NC02751, and NC02761, respectively. Both Nsp51GFP-1/2 transformants displayed similar significant fluorescence in fungal hyphae compared to the Nsp51EV-1 control line (Fig. 3). Regarding the *Nigrospora* sp. NC02761 transgenic lines transformed with the pBGgHg vector, Nsp61GFP-1 displayed higher levels of fluorescence than Nsp61GFP-2 and Nsp61GFP-3 (Fig. 4). However, all fungal lines showed greater fluorescence than the Nsp61EV-1 control line. Altogether, these results indicate that all *Nigrospora* sp. NC02751 and NC02761 transgenic lines constitutively expressing the GFP construct express a functional GFP protein.

3.4. Wild-type and transformants of Nigrospora sp. NC02751 and NC02761 consistently reduce growth of the pathogenic fungi Bipolaris maydis and Parastagonospora nodorum

We used culture assays to test the ability of *Nigrospora* sp. NC02751 and NC02761 to influence the growth of the pathogenic fungi *B. maydis* and *P. nodorum* (Fig. 5). For this experiment, we focused on wild-type (Nsp51WT and Nsp61WT) and selected transformants (Nsp51GFP-1 and Nsp61GFP-1). The combination of pathogen and any *Nigrospora* isolates significantly affected fungal growth rates ($F_{13,40} = 83.403$, $P < 0.001$; Fig. 5). In planned contrasts, all *Nigrospora* strains reduced growth rates of both pathogens by 40–60% ($P < 0.001$ for all). Growth rates were the same for wild-type and transformed isolates of both *Nigrospora* sp. NC02751 ($P = 0.595$) and NC02761 ($P = 1.000$) and they did not differ in their effects on growth of either pathogen ($P > 0.373$).

4. Discussion

Endophytic fungi have demonstrated enormous potential to benefit their plant hosts by increasing abiotic stress tolerance and enhancing defense (Carrión et al., 2019; Rodriguez et al., 2008). Yet that potential is tempered by the knowledge that their benefits can be context dependent (Giauque and Hawkes, 2013). Moreover, recent work shows that gene expression pathways of host plants can specifically depend on colonization by endophytic fungi (Aimone et al., 2023), making identification of genetic mechanisms challenging. Therefore, genetic transformation of strains will be key to expanding our mechanistic understanding of plant-endophyte interactions. Here, we demonstrated that two strains of endophytic *Nigrospora* can be transformed via *A. tumefaciens*, and that this transformation does not alter their inhibitory effects on two plant pathogens.

Although *A. tumefaciens*-mediated transformation successes have been reported for a number of filamentous fungi (Li et al., 2017), this is the first time to our knowledge that two strains of the *Nigrospora* genus have been genetically transformed using this approach. Various vectors were constructed using the *hph* gene for selection, such as pUR5750, pCAMBIA0380, pC-Hyg-GFP, pC-g-418-GFP, pBHt-2, pAN7–1, pBI-hph, pBGgHg, and pHg (Chen et al., 2000; de Groot et al., 1998; Sayari et al., 2019). In this study we used the pHg and pBGgHg vectors in which a glyceraldehyde-3-phosphate dehydrogenase (gpd) promoter from *Agaricus bisporus* and Cauliflower Mosaic Virus 35S (CaMV 35S) terminator were driving the various cassettes in the T-DNA region. pHg was selected as a control vector since it can only confer the resistance to hygromycin and nothing else, as already reported with the ectomycorrhizal fungus *Laccaria bicolor* (Kemppainen and Pardo, 2010). The vector pBGgHg is also able to confer the hygromycin resistance to the transformed fungi, and also possess a GFP cassette allowing further analyses and verification of transformants. It has been used for a variety of fungi,

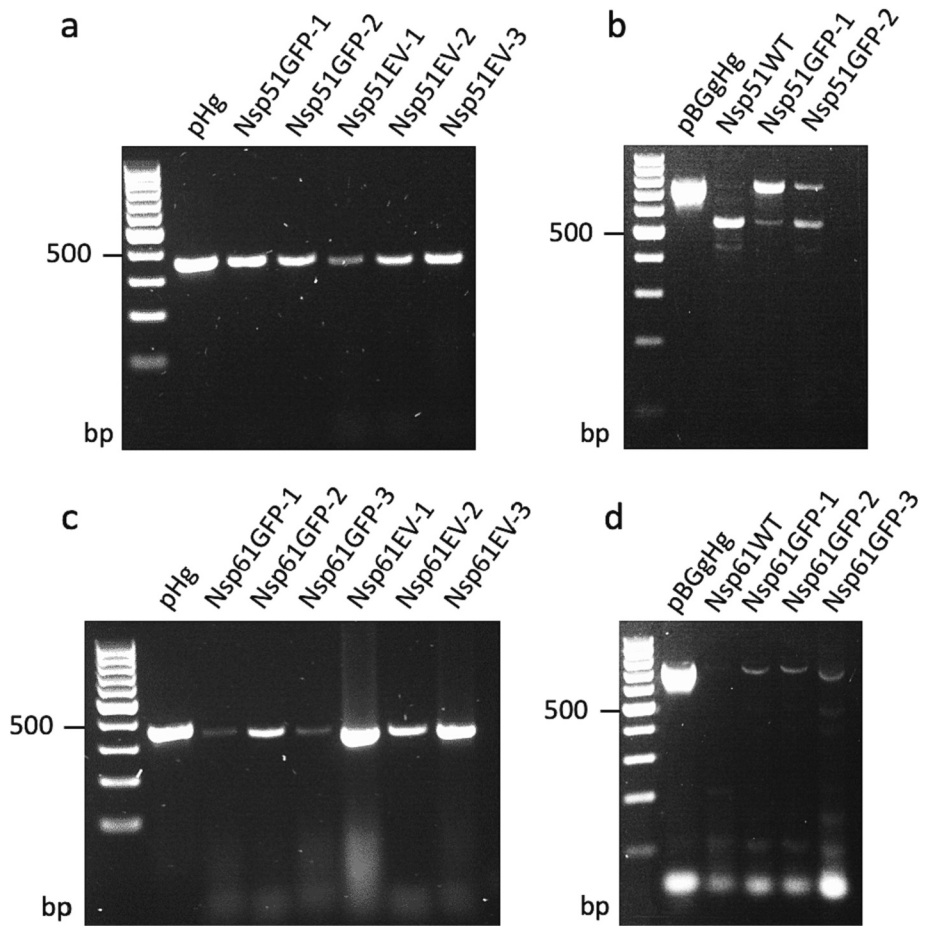

Fig. 2. PCR-based validation for *Nigrospora* sp. NC02751 and NC02761 transformants. PCRs were performed using HygR-F/R (a,c) and GFP-F/R (b,d) primers on pHg or pBGgHg purified vectors, as well as gDNA from *Nigrospora* sp. NC02751 and NC02761 wild-type strains (Nsp51WT and Nsp61WT, respectively), isolates transformed with the control vector pHg (Nsp51EV-1/2/3 and Nsp61EV-1/2/3, respectively), and isolates transformed with the GFP-expressing vector pBGgHg (Nsp51GFP-1/2 and Nsp61GFP-1/2/3, respectively).

including *Beauveria bassiana*, *Tuber borchii*, *H. cylindrosporum*, and *Ganoderma boninense* (dos Reis et al., 2004; Grimaldi et al., 2005; Lim et al., 2021; Müller et al., 2006).

The first criterion to use vector with *hph* gene as selection marker is to check the hygromycin sensitivity of the desired fungal strain. Out of the eight strains tested here, only five were inhibited by hygromycin concentration of 100 μg l^{-1}. We used this threshold for our transformation approach, although a wide variation in tolerance can be observed among different fungal strains. For example, *Ceratocystis albifundus* is sensitive at 10 μg l^{-1} only, while 200 μg l^{-1} is needed for *Aspergillus fumigatus* (Sayari et al., 2019; Sugui et al., 2005). Therefore, it is possible that a higher hygromycin concentration might be needed to inhibit the growth of the other three *Nigrospora* strains that showed tolerance at 100 μg l^{-1} (Fig. 2), but we did not test it. Alternatively, vectors conferring resistance to different antibiotics could be tested to transform these hygromycin-resistant *Nigrospora* strains. Indeed, other studies used zeocin, geneticin, or carboxin as selection markers to transform various filamentous fungi (Becquer et al., 2018; Garcia et al., 2020; Han et al., 2018; Takeno et al., 2005).

Multiple reports also suggest that not only vectors but co-culture conditions could impact transformation efficiency. The transformation efficiency we report here is quite low compared to other fungi, indicating the need for further improvement, which could be achieved by optimizing co-culture conditions. We used a co-culture period of three days on the induction medium for all tested *Nigrospora* strains. In other work, two-days of co-culture was optimal for both *Colletotrichum trifolii* (Takahara et al., 2004) and *A. niger* (Li et al., 2017), with decreased transformation efficiency in the latter fungus when co-culture was extended to three or four days. On the other hand, *L. bicolor* transformation required a co-culture incubation of five days for optimal transformation efficiency (Kemppainen et al., 2005), and three days works best for *H. cylindrosporum* (Garcia et al., 2013). Further tests are needed to determine if changing the length of the co-culture period will improve transformation efficiency for the various *Nigrospora* isolates reported here.

Even with an optimal transformation efficiency, detection of true positive transformants can sometimes be difficult while using an antibiotic-resistance gene as the sole selection marker since spontaneous resistance may appear. Therefore, the use of a reporter gene coding for a fluorescent protein can be another method to assess genetic transformation success. Particularly, GFP has been used in many studies for fluorescence-based detection of fungal transformants (Abello et al., 2008), including in numerous endophytic fungi such as *Acremonium implicatum, Muscodor albus, Acremonium implicatum*, and *Diaporthe schini,* (Abello et al., 2008; Ezra et al., 2010; Yao et al., 2015). The accumulation of GFP could be detected by microscopy mostly in the actively growing region of transformant hyphae, which primarily lies in the outer growth region (Sebastianes et al., 2012). To make sure the fluorescence emitted by the transformed fungal isolate is not due to the transformation technique or T-DNA integration itself as a stress-response, it is usually crucial to check any auto-fluorescence in wild-type or transgenic isolates expressing a control vector. Here, we used a transformant expressing the pHg vector as negative control while GFP-expressing lines were tested for each fungus. Both the detection of *gfp*

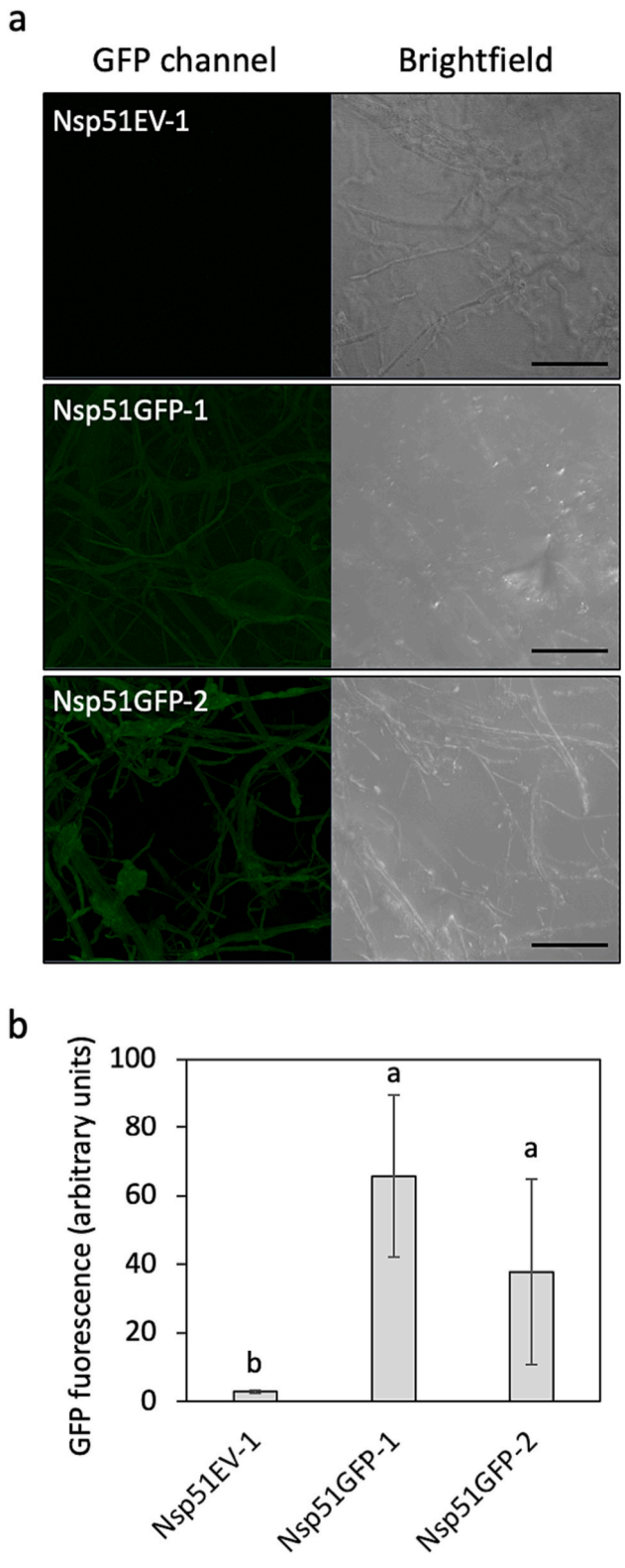

Fig. 3. GFP fluorescence in *Nigrospora* sp. NC02751 isolates in axenic culture. Fluorescence was checked on one transgenic isolate of *Nigrospora* sp. NC02751 transformed with the control vector pHg (Nsp51EV-1), and three transformed with the GFP-expressing vector pBGgHg (Nsp51GFP-1/2) grown on PDA (a). Images were obtained using a Zeiss LSM 710 confocal microscope. Scale bar = 150 μm. Quantified data from four to six representative images taken from each transformants (b). Different letters indicate significant differences between treatments according to one-way ANOVA followed by LSD post hoc tests ($P < 0.05$). $n = 4–6$.

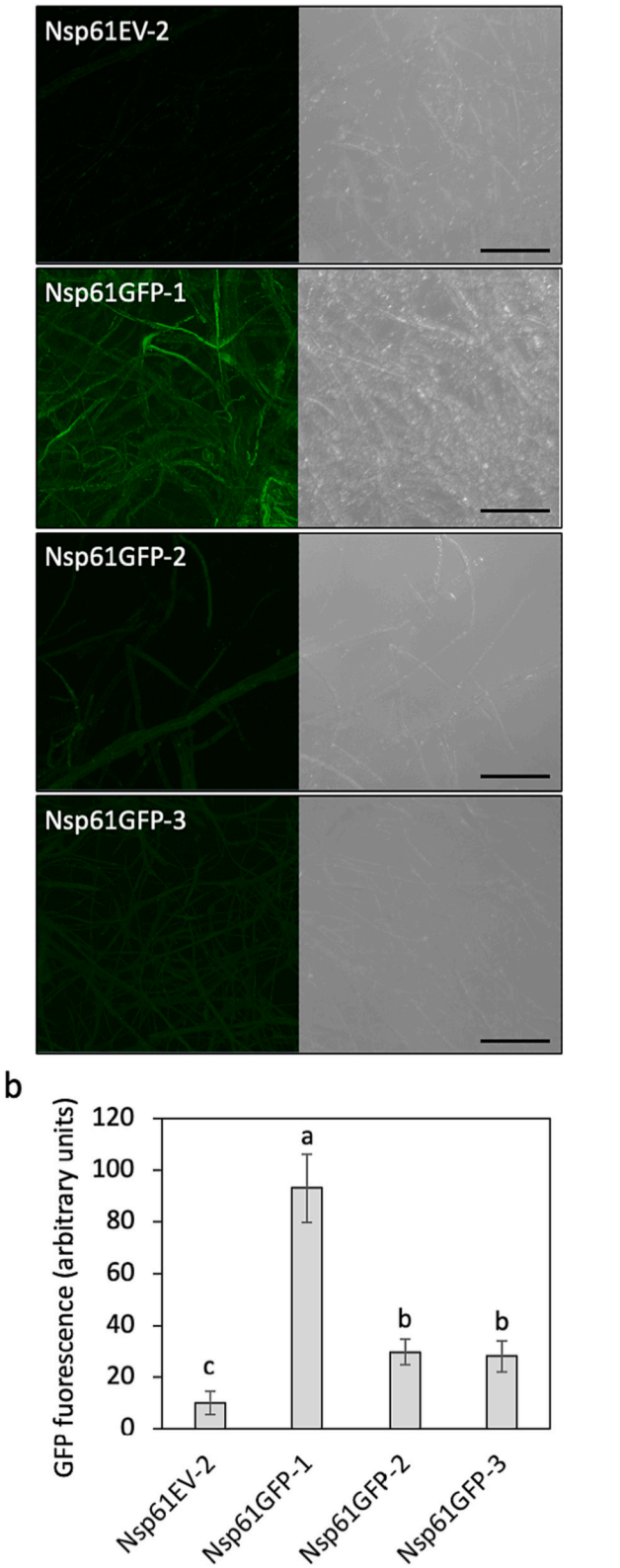

Fig. 4. GFP fluorescence in *Nigrospora* sp. NC02761 isolates in axenic culture. Fluorescence was checked on one transgenic isolate of *Nigrospora* sp. NC02761 transformed with the control vector pHg (Nsp61EV-1), and three transformed with the GFP-expressing vector pBGgHg (Nsp61GFP-1/2/3) grown on PDA (a). Images were obtained using a Zeiss LSM 710 confocal microscope. Scale bar = 150 μm. Quantified data from four to six representative images taken from each transformants (b). Different letters indicate significant differences between treatments according to one-way ANOVA followed by LSD post hoc tests ($P < 0.05$). $n = 4–6$.

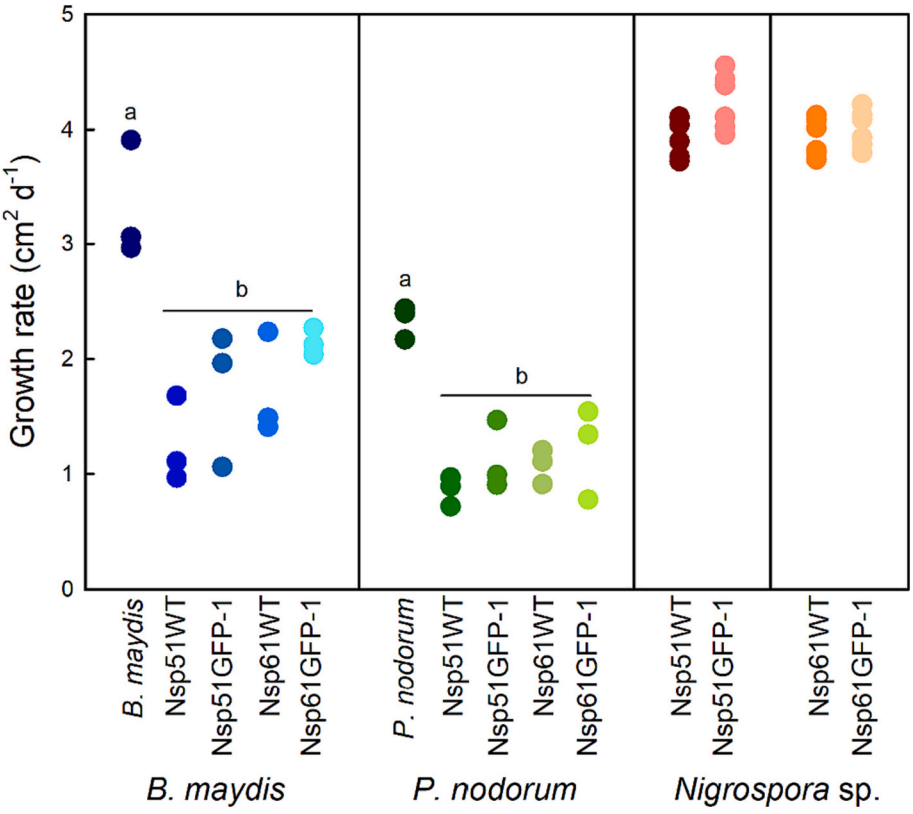

Fig. 5. Growth rate of fungi in paired culture assays. *Nigrospora* sp. consistently reduced growth rates of the pathogens, *Bipolaris maydis* and *Parastagonospora nodorum*, including both wild-type (Nsp51WT and Nsp61WT) and GFP-expressing (Nsp51GFP-1 and Nsp61GFP-1) isolates. Controls consisted of each fungus grown with itself. Pairs are indicated on the x-axis. Letters indicate significant differences in planned contrasts. $n = 3$.

gene and the fluorescence of pBGgHg-expressing transformants only validated the transformation approach and the use of GFP as a reported protein in *Nigrospora* sp. NC02751 and NC02761.

Finally, both *Nigrospora* endophytes tested here reduced the growth of plant pathogens in culture, and this effect was maintained in transformed isolates. The lack of observed side effects on a key interaction is promising for the use of GFP reporter to study endophyte-pathogen interactions. In the future, additional testing for inadvertent effects of transformation would be needed when targeting genes affecting specific functions such as leaf colonization or reproduction. Of course, culture-based assays do not necessarily translate to the same benefits in planta (Whitaker and Bakker, 2019), but some fungal traits are predictive of plant-fungal interaction outcomes (Giauque et al., 2019). Ultimately, one goal of genetic transformation of filamentous fungi is to identify key genes for specific functions that correlate with specific behaviors. Our success here is the first step in that pathway.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the North Carolina State University Game-Changing Research Incentive Program for Plant Sciences Initiative (GRIP4PSI), through the FUN-CROPS project. We thank Briana Whitaker for leaf collection and fungal isolations, as well as Shailesh Karre and Harry Ervin for fungal DNA sequencing.

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