



Screening of Plant Endophytes as Biological Control Agents against Root Rot Pathogens of Pepper (*Capsicum annum* L.)

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Abstract

In this study, 16 endophytes previously isolated from flowering dogwood (*Cornus florida*) stem tissue without causing any external symptoms, were screened for bioactivity against different root rot pathogens, *Fusarium solani*, *Fusarium oxysporum*, *Macrophomina phaseolina* and three *Phytophthora* species. While most of the endophytes suppressed pathogen growth in dual culture and exhibited potential as biological control agents, one fungal endophyte A22F1, (*Nigrospora sphaerica*) was evaluated further in growth chamber and greenhouse experiments in which it markedly reduced *Phytophthora capsici* root rot on three pepper cultivars 'California Wonder', 'Pepper Cayenne' and 'Numex Primavera'. Thus, endophyte A22F1 has high potential as a biological control agent for *P. capsici* in pepper. Its *in vitro* bioactivity displayed against *F. solani*, *F. oxysporum*, and *M. phaseolina* require additional *in vivo* studies on susceptible hosts.

Keywords: Soil-borne pathogens; Fungicide resistance; Biological-based IPM; Vegetable diseases

Introduction

Diseases are the most important limiting factor affecting bell pepper production in the United States, severely reducing productivity when environmental conditions are favorable [1,2]. Destructive fungal pathogens that cause root rot diseases include *Phytophthora capsici*, *P. nicotiana* var *parasitica*, *Fusarium solani*, *F. oxysporum*, *Verticillium* spp, *Rhizoctonia solani*, *Pythium aphanidermatum* and *Macrophomina phaseolina* [1,2]. Of these pathogens, *P. capsici*, *F. oxysporum*, *M. phaseolina* and *R. solani* are among the most important and widely distributed root rot pathogens [3-6]. In addition to the root rot problems, these pathogens affect all parts of the plant including stem, leaves, and fruits. The most obvious symptom of root rots is wilting and death of plants even when soil has enough moisture. However, in disease progression, the stem dries up and withers, die back occurs, leaves defoliate and the whole plant finally die. Root rot caused by *P. capsici* has become a serious threat to pepper production; up to 100% yield loss has been reported when environmental conditions are favorable for disease development [7-9]. The *P. capsici* pathogen affects 45 species of cultivated plants and weeds belonging to 14 families of flowering plants, out of which 19 species belonging to 8 families are highly susceptible [10]. Cultural strategies such as crop rotation have been insufficient in controlling *P. capsici* because oospores acting as resting structures survive in the soil for a long time even in the absence of a host plant [11-12]. The use of chemical fungicides such as mefenoxam (Ridomil Gold®) has been effective and a common practice in controlling *P. capsici*, but the rise of resistant populations has been reported throughout the United States [8]. Furthermore, there is an increasing concern over toxicity hazards from accidental exposures to the users, non-target organisms and potential environmental contaminations [13-17]. Thus, there has been an increasing demand for chemical-free food produce and the organic production system has become the fastest growing sector of crop production. Disease constraints significantly reduce crop productivity in organic production systems. There is need to develop eco-friendly products that can be incorporated in integrated disease management programs [18-21]. Biological control is a viable strategy for disease management and sustainable pepper production [21,22].

Biological control agents (BCAs) antagonize pathogens directly by hyperparasitism, production of antibiotics and lytic enzymes, indirectly by competing for space and nutrients, inducing systemic resistance, and

promoting plant growth [18,23-26]. Several fungal and bacterial BCAs have shown potential as effective antagonists against plant pathogens [27-33]. However, attempts to introduce eco-friendly microbial pesticides as biological agents for combating plant pathogens have been hampered by the lack of consistent field results. This problem has been attributed to environmental fluctuations including temperature, moisture, and nutrient availability as well as harmful effects of UV-light [34,35].

Microbial strains that colonize plants internally as endophytes without causing harm to their host plants are naturally abundant and less vulnerable to external environmental fluctuations. Such organisms may serve as BCAs that are effective in field environments. Endophytic microorganisms are mostly fungi and bacteria [31,33]. In our previous research, several bacteria that colonized flowering dogwood (*Cornus florida*) endophytically, displayed great potential in controlling powdery mildew and macrophomina root rot in greenhouse conditions [30-32]; some showed potential in controlling *Phytophthora* root rot in greenhouse conditions [36]. This study was initiated to screen additional endophytes for bioactivity against diverse root rot pathogens and identify those with potential as biological control agents for pepper (*Capsicum annum*) root rot pathogens.

Materials and Methods

Endophyte cultures and root rot pathogens

Endophytes evaluated in this study (Table 1) were previously isolated from stem tissue of *C. florida* that looked healthy with no apparent external symptoms [33]. Initial screening of 16 endophytes for biological activity against root rot pathogens was carried out *in vitro* using dual cultures. The root rot pathogens used in this study were

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previously isolated from roots of symptomatic bell pepper (*C. annum*) plants that exhibited wilting symptoms at Tennessee State University's (TSU) research field and TSU extension service community gardens in Nashville, TN (Table 2).

In vitro screening of endophytes for bioactivity against selected pathogens

A total of 15 fungal endophytes and one bacterium presented in Table 1 were screened for potential as biological control agents (BCAs) against seven root rot pathogens presented in Table 2. The *in vitro* screening was conducted in a growth chamber set at 27 ± 2°C. A dual culture technique was used in which an endophyte and a pathogen were grown opposite each other in the same Petri plate using 5 mm diam. plugs collected from 7-10 day-old fungal cultures grown in potato dextrose agar (PDA) and 24 h-old bacterial cultures grown in Nutrient agar (NA). Control treatments consisted of plugs of plain media placed opposite each pathogen. Each treatment was replicated with three plates arranged in a randomized complete block design. Pathogen growth was measured after 7-10 days and the endophyte effects on pathogen growth were assessed using the formula:

$$\% \text{ Growth Inhibition} = \frac{R2 - R1}{R2} \times 100$$

in which R2 is pathogen mycelia radius in the control plate (cm), and R1 is pathogen mycelia radius in pathogen-endophyte dual culture (cm). Three endophytes, A22F (*Nigrospora sphaerica*), E (*Enterobacter* sp.) and A27F3 (*Dothideales* sp) were evaluated further against three isolates of *Phytophthora capsici* (OP97, Lt263, Lt6745), and single isolates of *P. irrigata*, *P. cryptogea* and *P. nicotianae*.

In vivo pathogenicity tests of endophytes and selected pathogens

Transparent plastic lunch boxes bedded with damp sterilized paper towels were used as a growth chambers to screen the selected fungal pathogens and endophytes for pathogenicity on bell pepper seedlings. Three-week-old seedlings of 'California Wonder' grown in growth chambers were inoculated with different pathogens including three isolates of *Phytophthora capsici* (OP97, Lt263 Lt6745), and single isolates of *Phytophthora nicotianae*, *Phytophthora irrigata*, *Phytophthora*

cryptogea, *Fusarium solani*, *Fusarium oxysporum*, *Macrophomina phaseolina*, and *Cercospora nicotiana*. Seedlings for this study were surface sterilized by dipping in 2% NaOCl for 2 min and rinsed in sterile water 3-4 times. Five mm culture plugs of selected endophytes and pathogens listed in Tables 1 and 2 were used to inoculate the pepper seedlings; fungal cultures were 7 day-old and bacterial cultures were 24 h-old. Six inoculum plugs were placed around the root of each seedling; roots were then covered with sterilized paper towels and kept moist with sterile water. Control treatments consisted of PDA agar plugs. Each treatment was replicated with three seedlings and arranged in a randomized complete block design at 27°C. The seedlings were monitored for disease development.

Plant inoculation with P. capsici isolate Lt6745 and its pathogenicity in greenhouse conditions

Seeds of 'Pepper Cayenne', 'Numex Primarvera' and 'California Wonder', were sown in 64 plastic cells plug trays (Morton's Horticultural Supplies, McMinnville, TN) filled with heat sterilized commercial grade bedding soil. At three weeks after sowing, seedlings were transplanted to 10 cm² plastic pots filled with heat sterilized commercial potting mixture (Morton's grow mix mixed with Miracle Gro potting mix (1:3)). At 10 days after transplanting, the seedlings were inoculated with *P. capsici* isolate Lt6745 using inoculum concentration of 2 × 10⁶ zoospores/ml and three methods: (a) lower stem (crown region) application in which the cotton wool was soaked in inoculum and placed directly on the stem crown region and taped onto the stem; (b) soil drenching with inoculum using 25 ml of inoculum per plant with inoculum applied around the plant base; and (c) root dipping in inoculum of 500 ml in which plants were gently uprooted and washed, blotted dry with paper towels and then dipped in the inoculum for 30 min before re-planting in 10cm² pots using fresh heat sterilized Grow Mix. Control treatments used sterile distilled water. Each treatment was replicated with three seedlings and arranged in a randomized complete block design at 27°C. The seedlings were monitored for disease development.

Plant inoculation with endophyte A22F1 and its effect on P. capsici root rot in greenhouse conditions

Spores of endophyte A22F1 were harvested from 7-10-day old cultures grown in PDA, flushed with sterile water and the suspension of mycelia and spores filtered through two layers of cheese cloth. Spores were counted using a haemocytometer and inoculum concentration was adjusted to 2 × 10⁶ spores/ml. Plant inoculation with endophyte A22F1 was by root dipping in the inoculum in which plants were gently uprooted and washed, blotted dry with paper towels, and then dipped for 30 min in 500 ml inoculum before re-planting in 10 cm² pots using fresh heat sterilized Grow Mix. The effect of A22F1 on *P. capsici* root rot was assed as follows: (i) plant inoculation on *P. capsici* inoculated plants

SN ¹	Endophytes codes	Taxonomic group of the endophyte	Source
1	A11F1	<i>Dothideomycetes</i> sp.	<i>Cornus florida</i> stem
2	A20F2	<i>Hypoxylon howeanum</i>	<i>Cornus florida</i> stem
3	A22F2	<i>Hypoxylon</i> sp.	<i>Cornus florida</i> stem
4	A22F3	Unidentified fungus clone	<i>Cornus florida</i> stem
5	A22F1	<i>Nigrospora sphaerica</i>	<i>Cornus florida</i> stem
6	A23F1	<i>Entonaema</i> sp.	<i>Cornus florida</i> stem
7	A23F3	<i>Whalleya microplaca</i>	<i>Cornus florida</i> stem
8	A26F3	<i>Hypoxylon submonticulosum</i>	<i>Cornus florida</i> stem
9	A26F5	<i>Hypoxylon rubiginosum</i>	<i>Cornus florida</i> stem
10	A27F1	<i>Hypoxylon</i> sp.	<i>Cornus florida</i> stem
11	A27F2	Unidentified fungus endophyte	<i>Cornus florida</i> stem
12	A27F3	<i>Dothideales</i> sp	<i>Cornus florida</i> stem
13	A32F2	Unidentified	<i>Cornus florida</i> stem
14	A40F2	<i>Hypoxylon submonticulosum</i>	<i>Cornus florida</i> stem
15	A8F1	<i>Botryosphaeria dothidea</i>	<i>Cornus florida</i> stem
16	Bacteria E	<i>Enterobacter</i>	<i>Cornus florida</i> stem

¹Strain number

Table 1: Fungal endophytes isolates evaluated for biological control of root rot pathogens.

SN ¹	Pathogens	Source
1	<i>Fusarium solani</i>	Tennessee State University (TSU) ² , Nashville
2	<i>Fusarium oxysporum</i>	TSU, Nashville
3	<i>Macrophomina phaseolina</i>	TSU, Nashville
4	<i>Phytophthora nicotianae</i>	TSU Nursery Research Centre, McMinnville
5	<i>Phytophthora irrigata</i>	TSU Nursery Research Centre, McMinnville
6	<i>Phytophthora cryptogea</i>	TSU Nursery Research Centre, McMinnville
7	<i>Phytophthora capsici</i> isolates Lt6745, OP97 and Lt263	University of Tennessee, Knoxville (Courtesy of Dr. Lamour)
8	<i>Cercospora nicotianae</i>	Tennessee State University (TSU), Nashville

¹Strain number, ²Tennessee State University (TSU), Community gardens.

Table 2: Fungal pathogens evaluated in biological control using endophytes.



Figure 1: Disease rating scale used to quantify *Phytophthora capsici* infection in 'California Wonder' pepper plants in which 0=asymptomatic plant, 1=plant with yellow leaves, 2=wilted plant with leaves drooping, 3=defoliated plant; 4=plant with crown rot (arrow), and 5=dead plant.

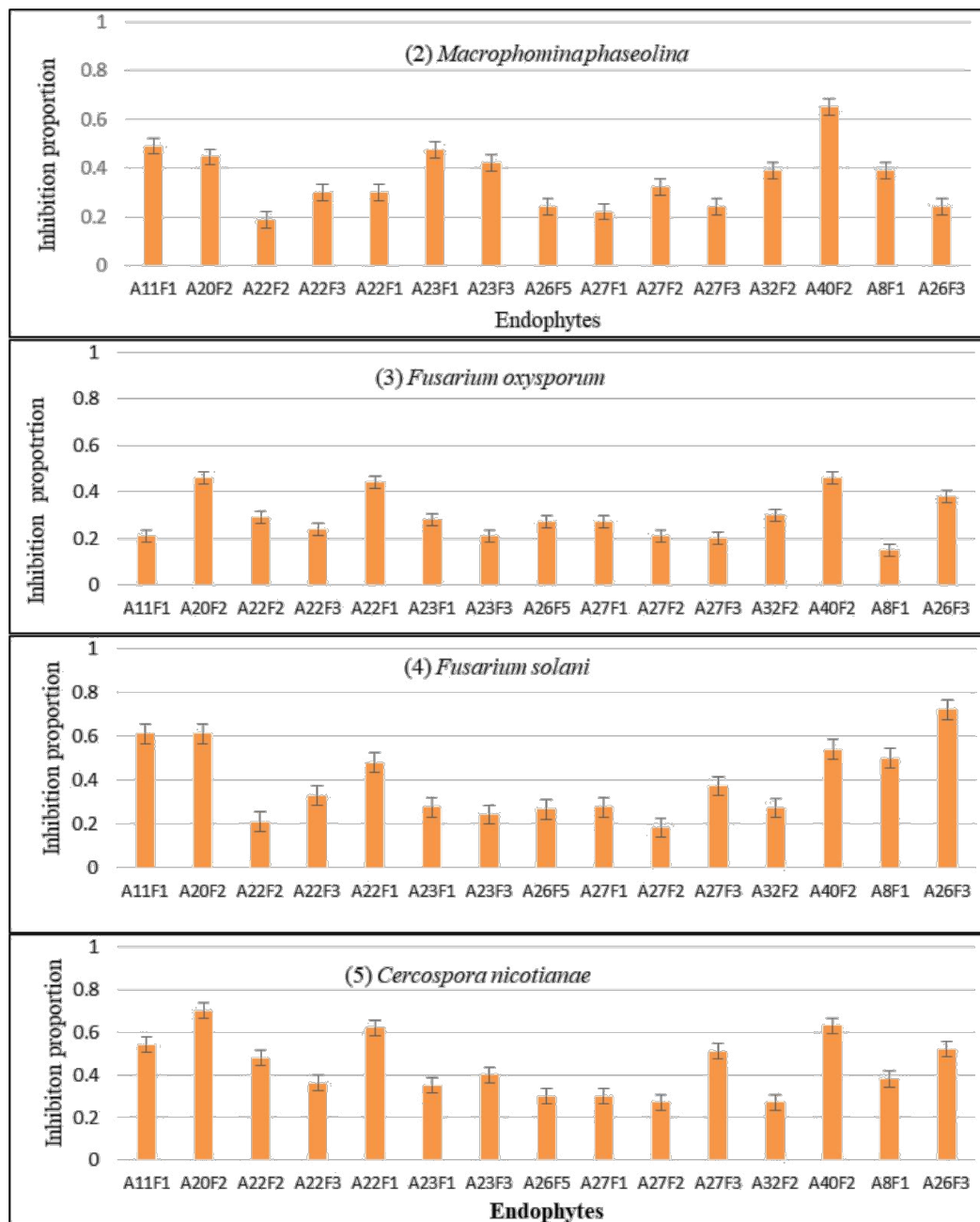


Figure 2: Inhibition of mycelial growth of root rot pathogens (4) *Macrophomina phaseolina*, (5) *Fusarium solani*, (6) *F. oxysporum*, and (7) *Cercospora nicotiana* by 15 fungal endophytes isolated from flowering dogwood.

(pathogen before endophyte), (ii) plant inoculation with A22F1 before the pathogen (endophyte before pathogen), (iii) pathogen only, (iv) endophyte only, and (v) water control with no pathogen or endophyte. In treatments where plants were inoculated with both pathogen and endophyte either as pathogen before endophyte, or pathogen after endophyte, plant roots were dipped in one inoculum for 30 minutes; blotted dry and then dipped in the second inoculum for the next 30 min and then blotted dry before replanting in heat sterilized soil in fresh pots.

Evaluation of disease development was initiated three days after inoculation, using a disease rating scale of 0-5 according to Abdou et al. [37] with minor modifications in which 0=healthy, 1= chlorotic leaves, 2= wilted leaves, 3=wilted and defoliated leaves, 4=crown and stem rot, and 5=dead plants observed (Figure 1).

Statistical data analysis

The data obtained were subjected to analysis of variance using SAS 9.4 software [38]. Mean values among treatments were compared by using least significant difference (LSD) test at the 5% level ($p=0.05$) of significance and presented as the mean values \pm standard deviation (SD).

Results

In vitro screening of endophytes for bioactivity against selected pathogens

Different endophytes exhibited different levels of bioactivity against different root rot pathogens in dual cultures as presented in Figures 2. Endophyte A40F2 was most effective in suppressing *Macrophomina phaseolina* growth in culture by up to 65% (Figure 2). The highest inhibition of *Fusarium oxysporum* mycelia growth in culture was by endophyte A20F2 (46%) and A40F2 (46%) followed by A22F1 (44%) as shown in Figure 2. Endophyte A26F3 caused 72% growth inhibition of *Fusarium solani* in culture followed by A11F1 and A20F2 with growth inhibition of 61% (Figure 2). Highest inhibition of *Cercospora nicotianae* mycelia growth was by 20F2 (70%) followed by A40F2 and A22F1 with 63% and 62% inhibition respectively (Figure 2).

Of the three endophytes evaluated against *P. capsici* isolates Lt6745, Lt263 and OP97, *P. nicotianae*, *P. irrigata*, and *P. cryptogea*, A22F1 caused 55%, 50% and 52% mycelial growth inhibition against Lt6745, Lt263 and OP97 respectively and also inhibited mycelial growth of *P. nicotianae*, *P. irrigata*, and *P. cryptogea* by 52%, 58% and 48% respectively (Figure 3). *Enterobacter* sp. caused varying degrees of growth inhibition with 66%, 40%, 40%, inhibitions against Lt6745, Lt263, and OP97, respectively while A27F3 caused the least inhibition (Figure 3). Overall, the antagonistic activity against root rot pathogens was indicated by a clear zone of inhibition in dual cultures as exemplified by A22F1 in Figures 4 and 5. Endophyte A22F1 was fast growing and out-competed the pathogens, overgrowing *C. nicotianae* that was quite slow growing (Figure 4).

In vivo pathogenicity tests of selected pathogens and endophytes

All three isolates of *P. capsici* (Lt6745, Lt263 and OP97) and *P. nicotianae* were virulent on pepper and infected all inoculated pepper seedlings. However, Lt6745 was the most virulent and all three seedlings were dead within seven days after inoculation. Although Lt263 and OP97, and *P. nicotianae* caused disease on pepper, they took more time to infect and kill the seedlings. Similarly, *P. cryptogea* was not aggressive on pepper and produced brown lesions on two out of three pepper

seedlings in 12-16 days. Other species, namely *P. irrigata*, *Fusarium solani*, *F. oxysporum*, *Macrophomina phaseolina* and all endophytes did not cause any symptoms on inoculated pepper seedlings; plants remained green and healthy up to the end of the experiment at 16 days after inoculation.

Plant inoculation with *P. capsici* isolate Lt6745 and its pathogenicity in greenhouse conditions

Root inoculation with Lt6745 by root drenching resulted in disease development that was evident at seven days after inoculation with browning of the stem clearly visible in the crown region. As the disease progressed, leaf yellowing, drooping of lower leaves and wilting followed, roots and rootlets revealed dark, brown, or blackened and decayed tissue. Inoculated seedlings were dead at 24 days after inoculation. Plants inoculated on the lower stem or crown region by using a cotton plug soaked in inoculum displayed disease symptoms at 4 days after inoculation and symptom development commenced with browning of the collar regions followed by wilting; pepper plants were dead in 11 days after inoculation (Figure 5). Disease development on plants inoculated by root dipping was similar to when stem crown region was inoculated using cotton wool, with initial symptoms evident in 3-4 days after inoculation and plants dead at 11 days after inoculation.

Plant inoculation with endophyte A22F1 and its effect on *P. capsici* Lt6745 in greenhouse conditions

Overall, all three cultivars exhibited maximum disease severity at 15-17 days after inoculation when all plants were dead (Figure 6). The three pepper cultivars were susceptible to *P. capsici* with 'California Wonder' being most susceptible and displayed faster disease development than the other two cultivars. Highest disease severity was observed on control plants inoculated with Lt6745 alone and not treated with endophyte A22F1 (Figure 6). Treatments with Lt6745 and endophyte A22F1 resulted in significantly lower disease severity in all three pepper varieties compared to pathogen only (Figure 5).

Plants inoculated with A22F1 before the pathogen Lt6745, developed significantly lower disease severity than plants inoculated with A22F1 after Lt6745 was applied. In one experiment, 'Numex Primarvera' plants did not develop disease symptoms throughout the experiment when A22F1 was applied before the pathogen was introduced (Figures 7a-7f). However, when the experiment was repeated, plants inoculated with the endophyte remained disease-free for 13 days and then developed some disease symptoms that were significantly less than in other treatments (Figure 7d). All plants of 'Pepper Cayenne' inoculated with A22F1 before the pathogen developed significantly lower disease severity than 'California Wonder' in repeated experiments (Figures 7c and 7e). Initially, 'California Wonder' plants treated with A22F1 displayed lower disease severity, but the disease increased steadily, and plants died at day 15-17 after inoculation similar to the non-treated plants. When the study was repeated, no significant differences were observed between the two experiments (Figures 7b and 7f).

Discussion

Almost all plant species examined to date (over 400,000) have been found to host endophytic microorganisms that live within intra- and intercellular spaces of plant tissues interacting with their host plants in symbiotic, mutualistic, and other types of relationships without showing symptoms or causing harm to their host plants [39-48]. Endophytes colonize the same ecological niche as plant pathogens and share an intimate relationship with their host plants [43,49]. Thus, endophytes present a valuable natural resource for potential utilization

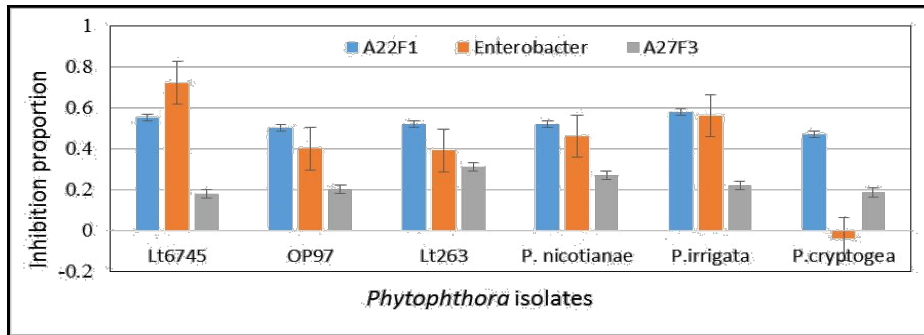


Figure 3: *In vitro* growth inhibition of root rot pathogens *Phytophthora capsici* isolates Lt6745, OP97, Lt263) *P. nicotiana*, *P. irrigata*, *P. cryptogea*, and by two fungal endophytes (A22F1 and A27F3) and one bacterial endophyte *Enterobacter* sp.

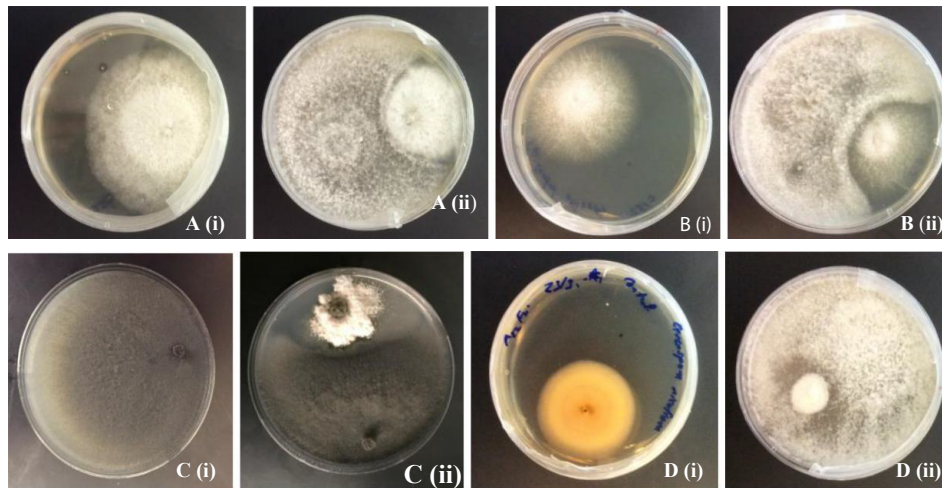


Figure 4: Inhibition of pathogen growth by biological control agent A22F1 (*Nigrospora sphaerica*) in dual cultures with [A] *Fusarium solani*, [B] *F. oxysporum*, [C] *Macrophomina phaseolina*, and [D] *Cercospora nicotiana* in which (i) is pathogen alone and (ii) is pathogen and A22F1.

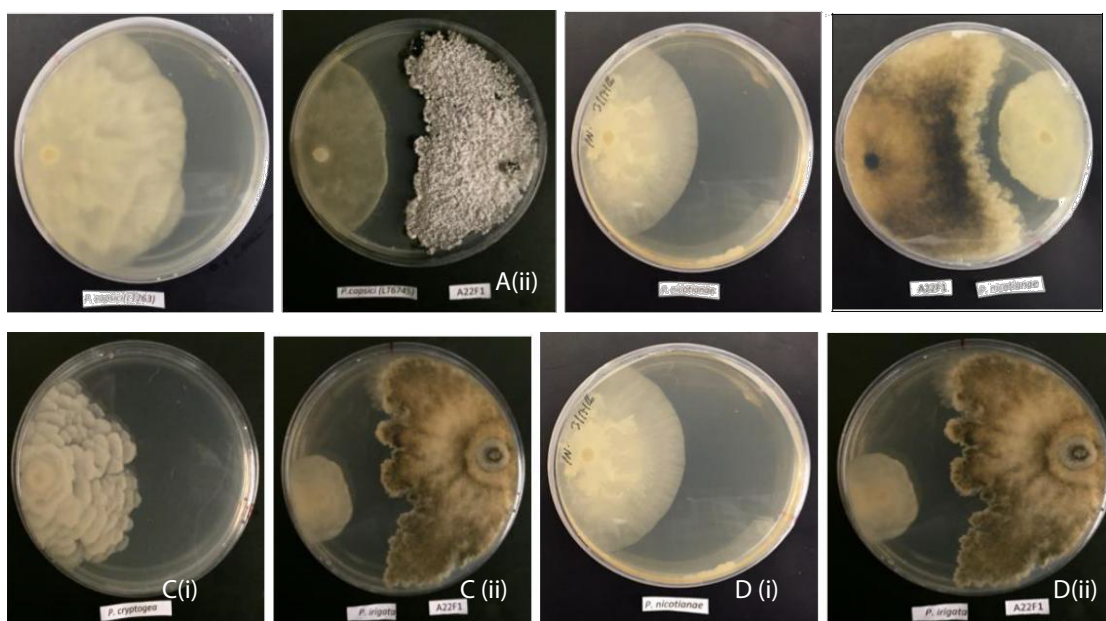


Figure 5: Inhibition of pathogen growth by biological control agent A22F1 (*Nigrospora sphaerica*) in dual cultures with [A] *Phytophthora capsici*, [B] *P. nicotiana* [C] *P. cryptogea*, and [D] *P. irrigata* in which (i) is pathogen alone and (ii) is pathogen and A22F1.



Figure 6: Disease progression in pepper plants inoculated with *Phytophthora capsici* isolate Lt6745 by (a) lower stem inoculation method in a greenhouse using a cotton plug soaked in inoculum (arrow); (b) taped onto the stem (arrow); (c) 4 d after inoculation (DAI), (d) 6 DAI (e) 8 DAI.

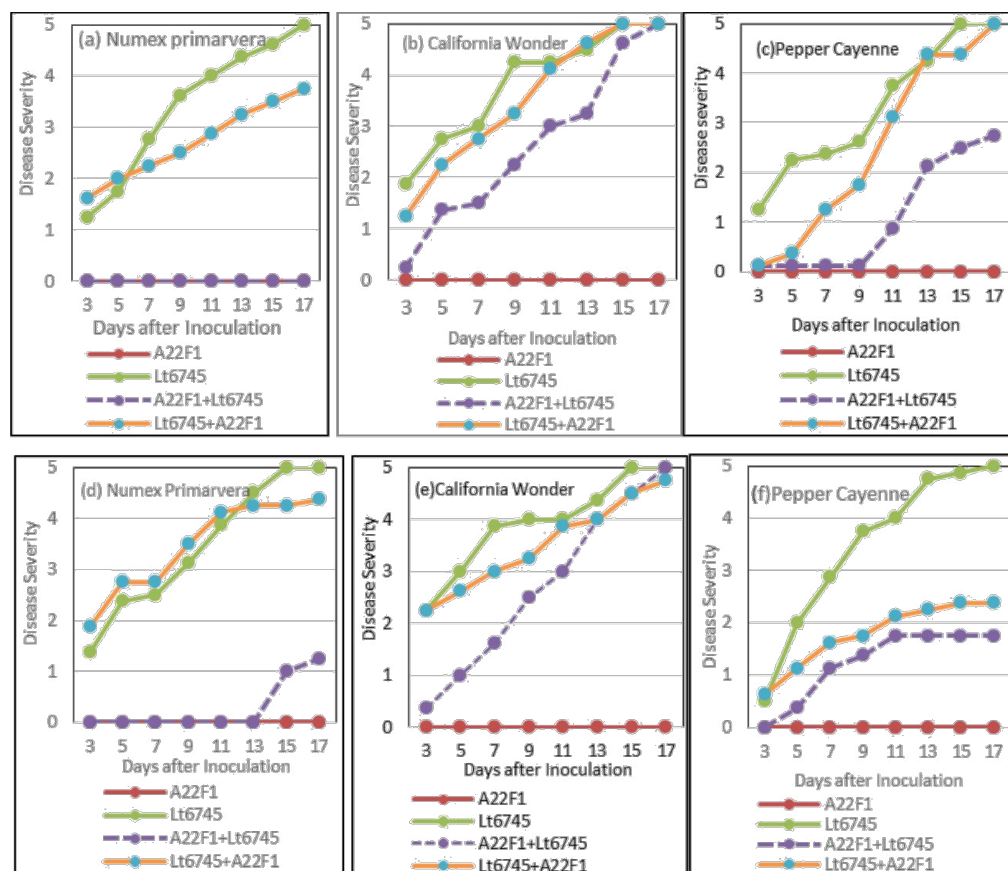


Figure 7: *Phytophthora capsici* disease severity in three pepper cultivars inoculated with endophyte A22F1 and *P. capsici* isolate Lt6745 in two experiments in greenhouse conditions in which plants were inoculated with pathogen before the endophyte (Lt6745 +A22F1) and endophyte before the pathogen (A22F1 + Lt6745), endophyte alone (A22F1) and pathogen alone (Lt6745) were the controls. Disease severity assessed on a 0-5 scale in which 0 was free of infection and 5 was an either dead or nearly dead plant.

as biological control agents. In our bioassays of endophytes (shown to be none-pathogenic on pepper), different fungal endophytes exhibited potential as biological control agents for different pathogens in dual cultures.

The endophyte that was most effective in suppressing *Macrophomina phaseolina* growth by 65% was A40F2, while A20F2 was most suppressive on *Fusarium oxysporum* with 46%; A26F3 was most effective against *Fusarium solani* causing 72% growth inhibition and 20F2 was most suppressive to *Cercospora nicotianae* by 70%. However, *in vitro* results do not always translate to disease suppression on plants.

Results by Rajkumar et al. [47] suggested that *in vitro* seedling assays can be used as a rapid and more accurate technique for the selection of promising biocontrol agents against *P. capsici*. In our studies, endophyte A22F1 was not the most suppressive organism to any of the pathogens evaluated in dual cultures, but it was suppressive with a clear zone of pathogen inhibition in dual cultures causing 55%, 50% and 52% inhibition against three *P. capsici* isolates Lt6745, Lt263 and OP97. This suggested that A22F1 may produce chemical compounds that are inhibitory to the pathogen. Furthermore, A22F1 was very fast growing and out-competed most pathogens in agar culture and could also function by competition for space and nutrients. Low inhibition

in growth of pathogen colonies in dual cultures has sometimes been associated with significant reduction of disease incidence in greenhouse and field trials [46]. For example, reports on *Bacillus cereus* isolate BT8 showed a lack of antagonism to *P. capsici* in *in vitro* studies, but the same organism suppressed lesion development from *P. capsici* on cocoa (*Theobroma cacao*) leaves [43]. Strong antagonism of some endophytes with organisms such as *Phytophthora capsici*, *Fusarium solani*, *F. oxysporum*, *Cercospora nicotianae* and *Macrophomina phaseolina* suggests a need to evaluate the effective endophytes *in vivo* using susceptible hosts.

Of the organisms evaluated for pathogenicity on pepper, *P. capsici* isolate Lt6745 was the most pathogenic on pepper while *Fusarium solani*, *F. oxysporum*, *Cercospora nicotianae* and *Macrophomina phaseolina* did not produce disease symptoms on pepper plants. *P. capsici* is economically very important in causing damping off, blight, root, crown and fruit rots in diverse plants including Solanaceae, Cucurbitaceae and Fabaceae [6,8,47,50]. According to Latha et al. [18] and Pal and Gardener [26], the most effective biological control agents inhibit plant pathogens by multiple mechanisms of action including the production of antimicrobial compounds, competing for space and nutrients, plant growth promotion and induced systemic resistance. Our dual culture results suggest that A22F1 may have multiple modes of action in pathogen suppression that may include biochemical compounds, and competition; more studies are needed on its mechanism of action. Previous reports have provided evidence that endophytes synthesize myriads of secondary metabolites, which are not directly involved in the metabolism of the microorganisms but play a role in the fitness and survival of the endophytes and their hosts [39,44]. These functional metabolites include alkaloids, terpenoids, steroids, quinines, isocoumarin derivatives, flavonoids, phenols/phenolic acids, and peptides [39,44]. Thus, endophytes can be an important source of bio-compounds for combating fungal pathogens, reducing crop losses, and improving agricultural productivity with reduced agricultural chemical pesticide inputs.

Of the endophytes evaluated in this study, A22F1 (*Nigrospora sphaerica*) has been reported to be a plant endophyte in medicinal plants, sea grass, palm trees, tomato, and pepper [48]. Various studies have identified novel metabolites from *N. sphaerica*, some of which have antifungal properties [51]. Although the production of many of these metabolites by the endophytic fungi are not fully understood or known, a compound, phomalactone (5, 6-dihydro-5-hydroxy-6-prop-2-enyl-2H-pyran-2-one) produced by *N. sphaerica* has been reported to inhibit mycelial growth of *Phytophthora infestans* and sporangia and zoospore germination of both *P. infestans* and *P. capsici* [12]. The clear inhibition zone formed between A22F1 and *P. capsici* isolates and other fungal pathogens (Figures 4 and 5) suggest the production of some antifungal chemical metabolites and merits further studies. Although *in vitro* tests do not always translate to *in vivo* activity, results from A22F1 in greenhouse conditions provide evidence on the potential of this endophyte as a biological control agent for *Phytophthora* pathogens. Our results indicated that inoculation of the crown region and root dipping method positioned the inoculum directly on plant tissue for faster infection establishment and could be regarded as maximum challenge methods. Thus, root dipping was used for both endophyte and pathogen inoculation.

Plant inoculation with the endophyte prior to the pathogen was more effective than introducing the pathogen before the endophyte. This was more clearly expressed on 'Numex Primaver' plants which did not develop disease symptoms throughout the experiment when endophyte A22F1 was applied before the pathogen (Figure 6a). Results

from repeated experiments showed that plants inoculated with the endophyte remained disease-free for 13 days and then developed significantly less disease than in other treatments (Figure 6d). Similarly, 'Pepper Cayenne' displayed significantly lower disease severity when inoculation with A22F1 occurred before the pathogen (Figures 6c and 6e). The observation is supported by other reports on *Nigrospora sphaerica* that a high level of host root colonization prior to pathogen infection is effective in reducing damage from root rots and other diseases in most crops studied [12]. However, *N. sphaerica* was reported to cause leaf blight on blueberry in Argentina [52] and on tea [53] and Chinese fir (*Cunninghamia lanceolata* Lamb.) in China [54], it was also reported to cause shot holes in mulberry in China [55]. Although results from this study clearly show that A22F1 was not pathogenic on dogwood from where it was isolated or on bell pepper where it exhibited effective biological control of *P. capsici*, it is important to test A22F1 for pathogenicity on blueberry, mulberry and other crops that have been reported as hosts to *N. sphaerica*.

Conclusion

Results on the three pepper cultivars also suggest that the integration of the BCA A22F1 and moderate resistance would likely provide complete control of the disease. In this study, the time of application of A22F1 before the pathogen was only 30-35 min and it is unlikely that the endophytic fungus would have been well established in that short time, yet disease was significantly reduced. Furthermore, plant inoculation with the pathogen may have provided an unusually high inoculum level. In another study in which pepper seed was soaked in bacterial BCA inoculum for 1 h before planting, root rot disease severity from *P. capsici* was reduced and pepper yield increased [36]. Previous analysis of mechanisms of some bacterial strains that protect their hosts from root diseases have shown that the bacteria produce antifungal antibiotics, elicit induced systemic resistance in the host plant or interfere with fungal pathogenicity factors [24]. More studies are needed to understand how A22F1 protects bell pepper plants and the timing and method of BCA applications need to be refined to optimize efficacy.

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