Antagonistic activities of needle-leaf fungal endophytes against *Fusarium* spp.

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Abstract
The use of living beneficial microbes and/ or their products to control plant pathogens can offer a safer alternative to chemical treatments. In this study, needle-leaf fungal endophytes (NLE) were isolated from symptoms-free needle-leaves of two host plants collected in Cavite and Batangas, Philippines. A total of 73 NLEs were observed from three tree samples for each of the angiosperm *Casuarina equisetifolia* Engl. and the gymnosperm *Pinus kesiya* Royle ex Gordon. These were identified as belonging to 17 morphospecies. Of these, seven NLEs, mainly isolated from *C. equisetifolia,* were tested for their antagonistic activities against three potential plant pathogens, *Fusarium oxysporum* s.l. Smith & Swingle, *F. solani* s.l. (Mart.) Sacc., and *F. moniliforme* s.l. J. Sheld. using the dual-culture method at three strategies. Our results showed NLEs inhibited *F. oxysporum* on contact via the preventive, eradicative, and simultaneous approaches indicating that fungal endophytes may be used as potential biocontrol agents against *F. oxysporum* s.l..

Keywords: agriculture, biocontrol, dual-culture method, fungal pathogens, pathogen

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Introduction
In the Philippines and many Southeast Asian countries, *Musa acuminata* Colla (banana), *Oryza sativa* L. (rice), and *Zea mays* L. (corn) are among the region's top agricultural crops. However, production of these crops can be severely limited by diseases such as those caused by soil fungi of the genus *Fusarium*. For example, *Bakanae* or the "foolish seedling" in Japanese is a common disease in rice caused by *Fusarium fujikuroi* Nirenberg (Cruz et al. 2013). *Fusarium verticillioides* (Sacc.) Nirenberg is considered as a leading pathogen of corn worldwide. Thirty-five *Fusarium* isolates were identified from corn grown in Laguna and Isabela provinces in the Philippines (Cumagun et al. 2009). Recent reports also showed that *F. oxysporum* wilt disease or the Panama disease of banana was previously restricted only to East and parts of Southeast Asia, but has spread even to South Asia, the Middle East and Africa (Dita et al. 2018). The *Fusarium* wilt

caused symptoms such as yellowing and stunted growth of older plants and seedlings, respectively, which eventually results in the death of the banana plant.

The use of agrichemicals can mitigate these fungal-initiated diseases, albeit pesticides can persist in soil, groundwater, and surface water and accumulate in the food chain. Because of the various health hazards associated with the use of pesticides, researches were focused on developing alternative approaches to control plant diseases (Gardener and Fravel 2002, Tjamos et al. 2010). The potential use of endophytic fungi, i.e. fungal strains residing within the tissues of living plants without causing any disease or harm to host plant has been explored as biocontrol agents (Dagamac et al. 2008, Song et al. 2015). Fungal endophytes isolated from *Panax notoginseng* F.H. Chen roots have shown inhibitory activity against the root pathogens *F. oxysporum, F. solani, Alternaria panax* Whetzel*, Phoma herbarum* Westend.*,* and *Mycocentrospora acerina* (R. Hartig) Deighton (Chen et al. 2015, Zheng et al. 2016). In this study, fungal endophytes associated with needle leaves of *Pinus kesiya* (khasi or khasya pine) and *Casuarina equisetifolia* (Australian pine or casuarina) were isolated and tested for their antagonistic activity against the three potential fungal pathogens – *F. moniliforme, F. oxysporum,* and *F. solani*, thereby providing baseline information on their potential use as biocontrol agents against these plant pathogens. We hypothesized that the fungal endophytes from needle leaves of the host plants can reduce growth of the target test fungi.

Materials and methods

Isolation and identification of needle-leaf fungal endophytes

Needle leaves from apparently healthy *C. equisetifolia* (an angiosperm) and *P. kesiya* (a gymnosperm) were collected from three trees along the national road in Tagaytay City (14°05'60.00"N, 120°55'59.99"E) in Cavite and within the Caleruega Retreat Compound (14°04'19.01"N, 120°50'07.67"E) in Nasugbu, Batangas, respectively. The leaves were placed in paper bags and stored in a cool box for transport to the laboratory. Herbarium specimens were also prepared from the collected host plants and sent for species identification to the Botany Division, National Museum in Taft Ave., Manila. To isolate the fungal endophytes**,** needle leaf samples were cut into approximately 10 mm length, soaked in 70 % ethanol for one minute and in 2 % NaOCl solution for five minutes following the protocol of Zamora et al. (2008). The leaf explants were then rinsed thrice using sterile distilled water. To ensure the efficacy of the surface-sterilization, leaf samples were imprinted on freshly prepared culture medium. Five leaf explants (30 per host tree) were then placed at equidistant points on a culture plate containing Malt Extract Agar (MEA) supplemented with 0.5 gL^{-1} Streptomycin (Sigma) and 0.5 gL^{-1} Tetracycline (Sigma). All culture plates (6 per host tree) were incubated at room temperature (26–28 ^{o}C) in the dark. Fungi growing out of the explants were isolated and purified by subsequent subculture on freshly prepared MEA. For fungal identification of test NLEs, the isolates were initially grown on MEA plates and incubated at room temperature for seven days. Morphological characteristics such as description of colony and spores for each fungal isolate were noted and compared with literature (Barnett and Hunter 1998). Percent colonization rate was calculated as the number of explants with fungal growth over the total number of explants multiplied by 100.

Screening for antagonistic activities against Fusarium *species*

Dual-culture method. The test fungi, *F. moniliforme*, *F. oxysporum,* and *F. solani,* were obtained from the Philippine National Collection of Microorganisms (PNCM) at the University of the

Philippines – Los Baños in Laguna, Philippines. Seventeen representative NLEs including the test fungal pathogens were initially grown on MEA plates at room temperature for seven days. Following incubation, mycelial agar blocks, about 6 mm in diameter, were cut off from the actively growing part (colony edges) of the 17 NLE cultures and the three test fungal pathogens using a flame-sterilized cork borer and placed at opposite sides on challenge plates, i.e. 90-mm diameter Petri plates pre-filled with 2 % MEA. All culture plates (in duplicates) were incubated at room temperature (26–28 ºC) for up to eight days or until contact between the opposing fungi was observed. As control, unchallenged test fungal pathogens and NLE were prepared. Antagonistic activities of the NLE isolates against each of the three test *Fusarium* species were determined based on the type of interactions as described by Wicklow et al. (1980): Type A - mutual intermingling of the two fungi, Type B - mutual inhibition at a distance, Type C - inhibition of one fungus on contact, and Type D - inhibition of one fungus at a distance. Needle-leaf fungal endophytes exhibiting B, C, or D type of interactions were considered positive for antagonistic activities and thus, were tested again against the susceptible *Fusarium* species based on three approaches: preventive, simultaneous, and eradicative.

Preventive Approach. The preventive approach was done to investigate whether the promising NLE can protect the host plant before colonization of plant pathogens. NLE mycelial agar blocks were prepared and placed on one side of MEA plates. The fungal endophytes were then incubated for four days, after which, mycelial agar blocks of each of the susceptible fungal pathogen were inoculated on the opposite side of the NLE.

Simultaneous Approach. The simultaneous approach was used to test whether the NLE can inhibit the pathogen when grown together. The mycelial agar blocks of the NLE and the susceptible test fungi were placed simultaneously at opposite sides of the MEA plates.

Eradicative Approach. The eradicative approach was performed to determine the ability of NLE to eliminate plant pathogens after infection. Mycelial agar blocks of the test fungal pathogens were placed on one side of the MEA plates. The plant pathogens were incubated for four days, after which, NLE mycelial agar blocks were inoculated on the opposite side of the test pathogens.

All culture plates (in duplicates) were incubated at room temperature (26–28 ºC) for up to eight days or until contact between the opposing fungi was observed. As described above, unchallenged test fungal pathogens and NLEs were prepared. Colony radial growth (three readings per colony) was measured after day 2, day 4, day 6, and day 8 of incubation. Exact point of measurement was noted on the petri plates. The mean radial colony growth of the test fungi exhibiting inhibited growth by selected NLE was then plotted against the control (unchallenged test fungi). One-way Analysis of Variance (ANOVA) was done to test for the significant differences between the mean radial growth of the test pathogens in dual culture with NLE (test treatment) and the unchallenged test pathogens (control treatment).

Results

A total of 73 fungal endophytes were observed from surface-sterilized and cultured needle leaves collected from three tree samples for each of the host plant, the angiosperm *C. equisetifolia* and the gymnosperm *P. kesiya*. These were characterized morphoculturally and categorized as belonging to 17 morphospecies (Table 1). Interestingly, a higher number of NLEs and a higher colonization rate

were observed from the needle-leaves of the angiosperm *C. equisetifolia* as opposed to the gymnosperm *P. kesiya*.

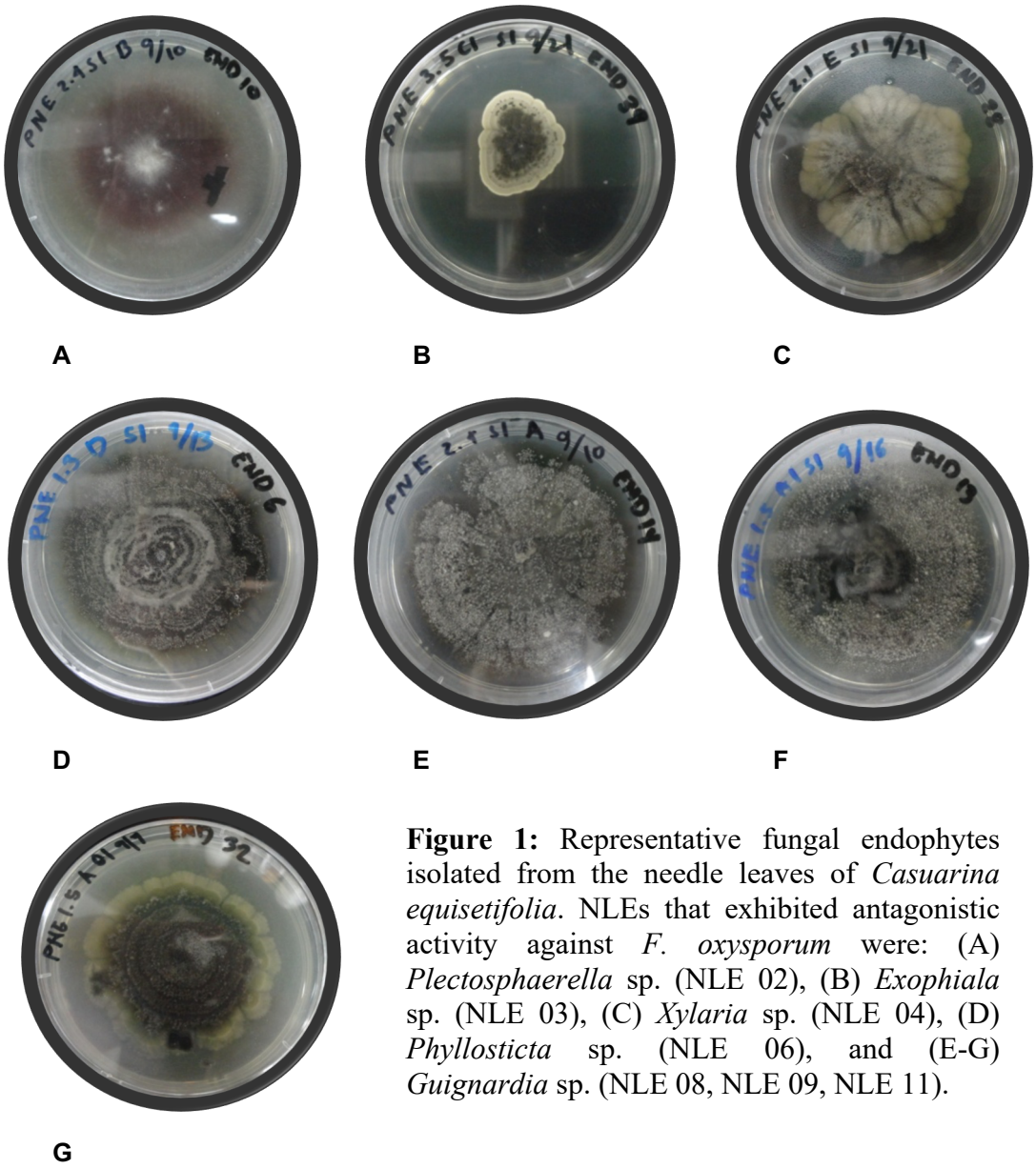

Figure 1: Representative fungal endophytes isolated from the needle leaves of *Casuarina equisetifolia*. NLEs that exhibited antagonistic activity against *F. oxysporum* were: (A) *Plectosphaerella* sp. (NLE 02), (B) *Exophiala* sp. (NLE 03), (C) *Xylaria* sp. (NLE 04), (D) *Phyllosticta* sp. (NLE 06), and (E-G) *Guignardia* sp. (NLE 08, NLE 09, NLE 11).

In this study, we tested 17 NLEs representing each of the 17 morphospecies for their antagonistic activities against the three Fusaria. Of these, only seven NLEs exhibited antagonistic activities against *F. oxysporum* while none exhibited antagonistic activities against *F. moniliforme* and *F. solani*. This led us to focus our efforts on these seven promising fungal endophytes. The seven NLEs were identified based on morphocultural characteristics as *Plectosphaerella* sp. (NLE 02), *Exophiala* sp. (NLE 03), *Xylaria* sp. (NLE 04), *Phyllosticta* sp. (NLE 06), and *Guignardia* sp. (NLE 08, NLE 09, NLE 11) (Fig. 1). All of these NLEs inhibited the growth of *Fusarium oxysporum* upon contact of its mycelia with the mycelia of the NLEs, i.e. interaction type C (Fig. 2). Interestingly, all antagonistic NLEs were isolated from *C. equisetifolia.*

To further evaluate the antagonistic activity of the seven NLEs against *F. oxysporum*, we designed three approaches and measured their radial colony growth over time.

Preventive approach. The preventive approach was done to assess whether NLE can protect its host plant before any infection. After incubation for 8 days with the pre-grown individual NLE (4-day old), the mycelial radial growth of *F. oxysporum* was significantly reduced in contrast to that of the control ($p < 0.05$), indicating antagonistic activity of the fungal endophytes against the plant pathogen (Fig. 3A).

Table 1: The needle-leaf fungal endophytes recorded from *C. equisetifolia* and *P. kesiya*.

Collection Site	Host Tree	Tree Sample	No. of explants with NLE (n = 30)	No. of morphospecies	Colonization rate
Cavite	*Casuarina equisetifolia*	A	18	10	71%
		B	16	7	
		C	19	9	
Batangas	*Pinus kesiya*	A	2	2	27%
		B	8	6	
		C	10	5	

Simultaneous approach. To test whether the NLE can inhibit the pathogen during infection, the simultaneous approach was performed. As also observed in the preventive approach, the seven NLEs significantly reduced the colony radial growth of *F. oxysporum* in contrast with the unchallenged control ($p < 0.05$) (Fig. 3B).

Eradicative approach. The eradicative approach was performed to test the fungal endophytes' capability to suppress the growth of the pathogen after infection. Only two of the seven NLEs, i.e. *Plectosphaerella* sp. (NLE 02) and *Exophiala* sp. (NLE 03), were found to significantly reduce the colony radial growth of *F. oxysporum* in contrast to the unchallenged control ($p < 0.05$) (Fig. 3C).

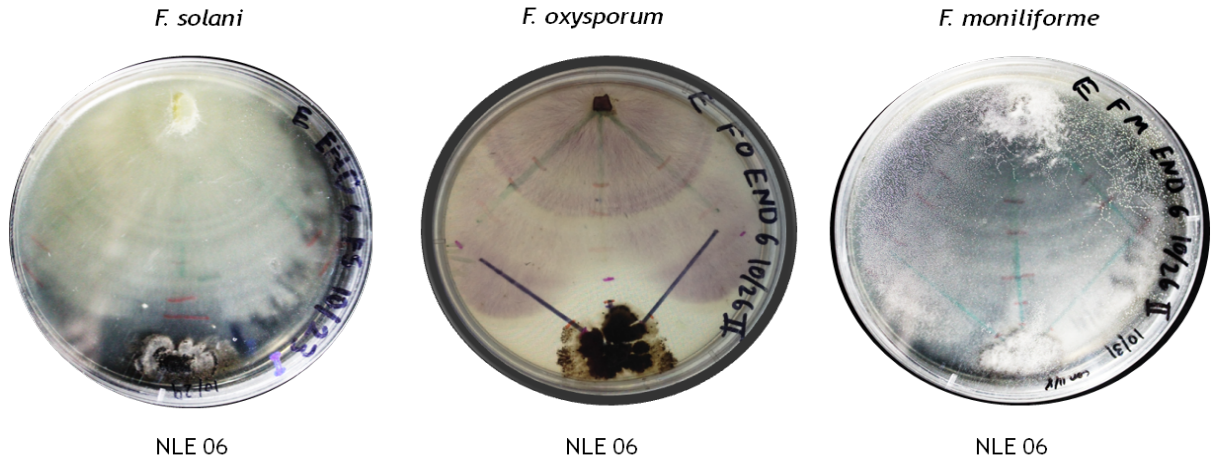

Figure 2: *Phyllosticta* sp. (NLE 06) showing positive antagonism against *F. oxysporum* and the absence of antagonism against *F. solani* and *F. moniliforme.*

Discussion

Fungal endophytes as earlier defined by Petrini (1999) reside within tissues of living plants but do not cause any harm to its host. This unique group of fungi is widely distributed among tropical plants as special hotspots of fungal diversity (Arnold and Lutzoni 2007). Therefore, fungal endophytes can easily be isolated from varied leaf types including needle leaves of gymnosperms and other seed-bearing plants. In this study, we identified 17 morphospecies from the 73 recorded NLEs. Paul and Yu (2008) reported two species of *Cladosporium* from the needles of *Pinus* spp. collected in Korea while Yuan and Chen (2014) isolated the endophytic *Lophodermium* sp. from decomposed needle leaves of *Pinus massiona* Lamb. These studies just exemplified some taxa of fungi that can be isolated from needle leaves as also shown here. Between the two host plants, we also observed a higher colonization rate of *C. equisetifolia* as compared to *P. kesiya* (Table 1). Sieber (2007) noted that density of colonization of conifer needles by endophytes increased with needle age. Needles collected from *C. equisetifolia* were older as compared to those collected from *P. kesiya.* This might explain the disparity in the colonization rates observed in this study.

Fungal endophytes are known for their ecological role and for their potential to control the growth of insects and pathogens (Mishra et al. 2014). Aly et al. (2011) reviewed the promising properties of fungal endophytes and noted their potential for drug discovery. They further listed studies that showed fungal endophytes could produce metabolites attributed to their host plants. In our other studies, we showed fungal endophytes isolated from terrestrial plants, e.g. *Musa* spp. (Dagamac et al. 2008), *Pandanus amaryllifolius* Roxb. (Bungihan et al. 2011, 2013a), *Canarium ovatum* Engl. (Torres and dela Cruz 2015), and from medicinal plants (Eskandarighadikolaii et al. 2015), that exhibited antimicrobial activities including antibacterial and antitubercular, antioxidant properties, and biocontrol of *F. oxysporum*. Some species of these plant-associated fungi produced novel metabolites (Bungihan et al. 2010, 2013b) or exhibited xylanolytic enzymes (Torres and dela Cruz 2013). Even host plants at the interface of aquatic and terrestrial ecosystems such as mangroves can be hosts to fungal endophytes with antimicrobial and cytotoxic activities (Tan et al. 2015, Moron et al. 2018, Apurillo et al. 2019). In this present study, we tested the needle-associated fungal endophytes for antagonistic activities against *Fusarium*. Interestingly, only seven NLEs which were all isolated from *C. equisetifolia* exhibited antagonistic activities against *Fusarium oxysporum.* The NLEs failed to inhibit *F. moniliforme* and *F. solani*. In the findings of Liu et al. (2001), fungal endophytes isolated from evergreen plants showed broad inhibition against various phytopathogens, e.g. *Rhizoctonia cerealis* E.P. Hoeven, *Helminthosporium sativum* Pammel, C.M. King & Bakke, and *F. oxysporum*, in contrast to the findings in this study.

Fungal endophytes also protect their host plants, directly and/or indirectly, through a variety of ways. As stated by Fadiji and Babalola (2020), direct mechanism includes production of antibiotics, phytohormones, and siderophores, secretion of lytic enzymes, and phosphate solubilization. Fungal endophytes also compete with pathogens, thereby helping to suppress them. Fadiji and Babalola (2020) further stated the indirect mechanism, that is, through stimulation of plant secondary metabolites. The antagonistic action against the pathogen can also be through induction of systemic resistance, mycelial inhibition, and expression of defence genes at the onset of certain pathogens in their hosts (Omar and Ahmed 2014). In this study, the antagonistic interactions could be due to the mycelial inhibition of the fungal pathogen upon contact with the NLE. In order to determine the strategies by which the needle-leaf fungal endophytes inhibited *F. oxysporum*, we conducted dual-culture methods through different approaches. First, the ***preventive***

approach (Fig. 3A) tested if NLEs protect its host plant before any infection. Our results showed a significant reduction of the mycelial radial growth of *F. oxysporum* grown with NLE as opposed to the unchallenged *F. oxysporum* (control). Among the isolated fungal endophytes, *Guignardia* sp. (NLE 09) showed the highest inhibitory activity, i.e. with the greatest colony growth reduction, albeit inhibitory activities between the seven NLEs did not differ significantly. Secondly, the ***simultaneous approach*** (Fig. 3B) tested NLE and *F. oxysporum* simultaneously. *Exophiala* sp. (NLE 03) showed the highest inhibitory effect, albeit no significant difference was again noted between the seven NLEs. However, it is clearly shown from the two approaches the reduced colonial growth of *F. oxysporum* when grown in the presence of the NLEs. Redmond et al. (1987) noted the effectiveness of *Exophiala jeanselmei* (Langeron) McGinnis & A.A. Padhye as a biological control agent against *Botrytis* sp. Though the earlier study of Redmond et al. (1987) was done *in planta* as opposed to the *in vitro* approach used in the present study, it was noted that nutrient competition may be a possible mechanism for biological control by *E. jeanselmei*. This may also explain the inhibitory activity exhibited by the *Exophiala* strain isolated from needle leaves against *F. oxysporum*, though further studies are needed to prove this. Lastly, the ***eradicative approach*** (Fig. 3C) tested the fungal endophytes' capability to stunt the growth of the pathogen after infection of its host plant. Here, only two of the seven NLEs, i.e. *Plectosphaerella* sp. (NLE 02) and *Exophiala* sp. (NLE 03), were found to significantly reduce the colony radial growth of *F. oxysporum* in contrast to the unchallenged *F. oxysporum*. In the study of Zheng et al. (2016), the culture extracts of the two fungal isolates of *Plectosphaerella* from the host plant *P. notoginseng* exhibited no to weak inihibitory activity against *F. oxysporum* and other test fungal root-rot pathogens contrary to the significant reduction of radial colony growth observed in this study.

Comparing the three approaches it was evident that the *simultaneous approach* showed a greater growth reduction of *Fusarium oxysporum* as compared to the *preventive* and even to the *eradicative approaches*. This study also supports the general idea of the "protective role" of fungal endophytes in their host plants (Bayman 2007). Barka et al. (2002) supported this observation. In their study, the simultaneous co-culture of the fungal pathogen, *Botrytis cinerea* Pers. and the endophytic bacteria did not stop the spread of the fungus, but a clear inhibition was observed when the fungus was introduced two days after bacterial inoculation. However, it is still unclear how the needle-leaf fungal endophytes isolated in this study could inhibit *F. oxysporum*. Taechowisan et al. (2009) noted that the various modes of protection to host plants by the fungal endophytes which included nutrient competition, direct parasitism, and production of hydrolytic enzymes that destroy cell walls of the fungal pathogens. Tripathi et al. (2008) also stated that fungal endophytes can directly attack pathogens through penetration of the pathogen hyphae or secretion of degradative enzymes. Grosch et al. (2006) likewise observed this penetration of *Rhizoctonia solani* by *Trichoderma* sp. Further studies are still needed to ascertain the mechanism of action by the isolated needle-leaf fungal endophytes.

In conclusion, the findings of this paper suggested that the needle-leaf fungal endophytes exhibited antagonistic activity when co-cultured with the fungal pathogen *Fusarium oxysporum*, and thus can be potential biocontrol agents against this plant pathogen. Additional studies are still required to demonstrate the practical utility of the isolated NLEs for the biocontrol of pathogens. It is necessary to test the isolated fungal endophytes for antagonistic activity *in planta* and for the ability to colonize any intended host plants. Alternatively, the fungal endophytes can be cultured and extracted for bioactive secondary metabolites which can be tapped as agrochemicals.

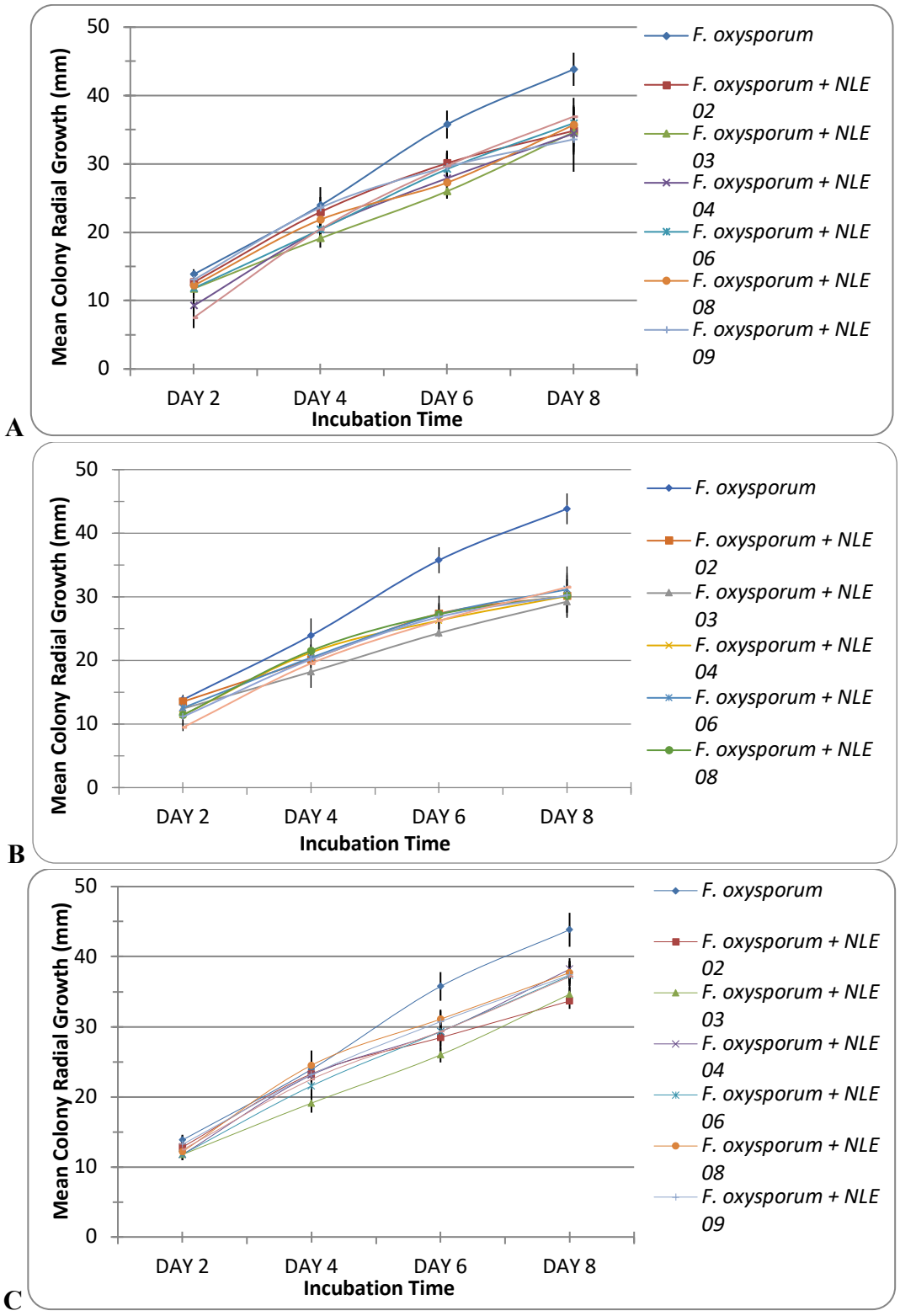

Figure 3: Mean colony radial growth of *F. oxysporum* challenged with NLEs using the (A) preventive, (B) simultaneous, and (C) eradicative approaches.

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Statement on conflict of interest
The authors declare no conflict of interest.

Authors contribution
RBCDM, IRE, MCRRA, MATC, MVBP, JCDP and TEEDC designed the research study, conducted the field collection, analyze the data, and/or wrote the manuscript. The authors contributed equally to this work.

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