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INOCULATION TECHNIQUE OF *COLLETOTRICHUM GLOEOSPORIOIDES* AFFECTS *Phyllanthus niruri* L. GROWTH AND BIOACTIVE COMPOUND SYNTHESIS

Irna Surya Bidara[1], Siti Himawati[1], Eka Nurhangga[1], Yomi Guno[2], Winda Nawfetrias[1]*

[1] Research center for Horticulture, Research Organization for Agriculture and Food, National Research and Innovation Agency (BRIN), KST Soekarno, Jl. Raya Jakarta Bogor KM 46, Cibinong, Bogor, Indonesia 16915

[2] Research center for Aeronautics Technology, Research Organization for Aeronautics and Space, National Research and Innovation Agency (BRIN), KS Rumpin (Jacob Salatun), Jl. Raya Lapan Sukamulya, Rumpin, Bogor, Indonesia 16350

*Correspondence: wind005@brin.go.id

ABSTRACT

Colletotrichum gloeosporioides, an endophytic fungus, significantly enhances growth, defense against pathogens, and remediation of environmental stress in medicinal plants through induced resistance and phytohormone synthesis. The application technique affects the fungi's effectiveness in stimulating growth and inducing bioactive compounds in plants. *Phyllanthus niruri* is an Indonesian medicinal plant with bioactive compounds like flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannins, and saponins. They offer health benefits like antioxidant, anti-inflammatory, analgesic, and antiviral effects. However, research on *Phyllanthus*-endophytic fungi from the *Colletotrichum* has not been reported. This study proposes that the application technique used for *C. gloeosporioides* affects the fungi's effectiveness in stimulating plant growth and inducing beneficial bioactive compounds. We experimented during 2022 and 2023 at an experimental greenhouse with two different methods of fungi inoculation: seed and planting medium. The study reveals that *C. gloeosporioides* inoculation impacts *P. niruri* growth differently, with seed inoculation promoting growth and planting medium inoculation potentially hindering it. Metabolic profiling through clustering analysis indicates that fungal inoculation significantly alters the plant's chemical composition, particularly increasing the abundance of specific alkenes, suggesting activation of defense mechanisms involving very long-chain fatty acids. It highlights the critical role of the inoculation method in shaping plant-microbe interactions and influencing the production of pharmacologically relevant compounds like steroids, diketones, alkanes, and alkenes, which may serve in the plant's defense responses and have beneficial impacts on human health. Therefore, optimizing inoculation strategies and understanding the underlying biological mechanisms are crucial for harnessing the potential benefits of microbial inoculants in agriculture and biotechnology.

Key words: Endophytic fungi, Inoculation, *Phyllanthus niruri*, Planting medium, Seed

INTRODUCTION

Endophytic fungi inhabit plant tissues and play important roles in promoting plant growth, enhancing nutrient uptake, increasing resistance to pathogens and environmental stress, and influencing the synthesis of bioactive compounds in medical plants. Our previous research identified *Colletotrichum gloeosporioides*, an endophytic fungus from *Phyllanthus* sp. stem, as having the potency to produce Indole Acetic Acid (IAA). *Colletotrichum gloeosporioides,* a fungal pathogen, causes anthracnose disease in plants, causing significant crop yield losses (Sharma & Kulshrestha, 2015). However, it can also benefit plant growth by enhancing iron nutrition through coumarin-mediated changes, providing protective benefits to host plants potentially acting as biocontrol agents (Rojas *et al*., 2010). *Colletotrichum gloeosporioides* from *Dillenia indica* L. showed high phosphate solubilization activity and increased IAA production in Czapek medium, boosting seed germination and growth of *Vigna radiata* and *Cicer arietinum*(Kumar & Prasher, 2023).

Phyllanthus niruri, a traditional Indonesian medicinal plant rich in bioactive compounds such as alkaloids, flavonoids, lignans, polyphenols, terpenoids, tannins, and saponins. These compounds offer pharmacological benefits like antioxidant, anti-inflammatory, analgesic, and antiviral effects (Rhetso *et al*., 2020). Endophytic fungi are extensively applied to isolate new bioactive compounds that offer various pharmacological advantages. The extract from endophytic fungi identified in *P. niruri* has been reported to possess antioxidant and hepatoprotective potential (Kalimuthu *et al*., 2022). However, there is no available research on *Phyllanthus* endophytic fungi that is associated with the genus *Colletotrichum*.

Endophytic fungi have attracted considerable attention in agricultural biotechnology because of their ability to promote plant growth, enhance stress tolerance, improve seed performance, and stimulate the production of valuable secondary metabolites (Li *et al*., 2017). The effectiveness of these beneficial interactions depends on several factors, including fungal species, host genotype, environmental conditions, and inoculation methods (Aleynova & Kiselev, 2023). Among the available approaches, seed inoculation and soil inoculation are the most widely applied. Seed inoculation facilitates early fungal colonization during germination and seedling establishment, thereby enhancing nutrient acquisition and stress tolerance (Dudeja *et al*., 2012). In contrast, soil inoculation promotes interactions within the rhizosphere and root system, potentially improving nutrient uptake and plant development through different physiological mechanisms (Kuźniar *et al*., 2019). Since inoculation method is a critical determinant of successful colonization and plant response, optimizing inoculation strategies is essential to maximize the beneficial effects of endophytic fungi.

Despite the growing interest in endophyte-assisted crop improvement, information regarding the effects of different inoculation methods on *Phyllanthus niruri* remains limited. In particular, the influence of *Colletotrichum gloeosporioides* inoculation on plant growth and metabolite production has not been thoroughly investigated. Therefore, this study aimed to evaluate the effects of seed inoculation and growing-medium inoculation with *C. gloeosporioides* on the growth performance and metabolite profile of *P. niruri*.

MATERIALS AND METHODS

The present study was carried out at experimental green house of National Research and Innovation Agency (6°21'27"S 106°39'51"E and elevation of 57 m asl), Banten, Indonesia. The experiments used the seeds of *P. niruri* collected from Jember, East Java, Indonesia. Isolate *C. gloeosporioides* was provided by isolation from healthy stem tissues of *Phyllanthus* sp and has been analyzed molecularly using ITS 1. Fungus cultures were maintained at Potato Dextrose Agar (PDA) from Himedia Laboratories (India) incubated at 27±2°C in dark conditions for seven days. The spore suspension was prepared in Potato Dextrose Broth (PDB) from Himedia Laboratories (India) medium and incubated on a shaker 120 rpm at 27±2°C for seven days. Spore concentration was determined using a hemocytometer under a light microscope (100×) and adjusted to 1×10^{10} CFU mL^{-1}.

The experiment employed two inoculation methods: seed inoculation and growing-medium inoculation. For seed inoculation, 3 g of seeds were soaked in 100 mL of spore suspension for 24 h under shaking conditions (120 rpm, 27 ± 2°C), while control seeds were soaked in sterile water. Treated seeds were then sown in pots containing sterile growing medium. For planting-medium inoculation, 100 mL of spore suspension was applied directly to the medium surface prior to sowing sterilized seeds. Control treatments received sterile water only. Plants were maintained under greenhouse conditions and watered as needed. The experiment was arranged in a completely randomized design with five replicates per treatment.

Germination rate, vigor index, and root-to-shoot ratio were recorded at 7 days after planting (DAP). Plant height, leaf number, leaflet number, fresh weight, dry weight, and chlorophyll content (SPAD-502 Plus, Konica Minolta, Japan) were measured at 49 DAP. Plant height was measured from the base of the stem to the apical bud. Fresh biomass was determined using an analytical balance, while dry weight was measured after oven-drying at 40°C for seven days.

For metabolite analysis, plant extracts were prepared following a modified method (Nawfetrias *et al*., 2023). Chemical profiling was performed using Gas Chromatography–Mass Spectrometry (GC–MS; Agilent 7890B) equipped with helium as the carrier gas. Relative compound abundances were calculated based on peak area normalization.

Statistical analysis was conducted using R software. Multivariate analysis included principal component analysis (PCA) and Pearson correlation. Hierarchical clustering was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm, and results were visualized accordingly.

RESULTS AND DISCUSSION

This study evaluated the effects of *Colletotrichum gloeosporioides* inoculation on the growth parameters of *Phyllanthus niruri*, using two inoculation technique: seed inoculation and planting medium inoculation. The principal component analysis (PCA) explained 93.8 % of the total variation, with Dim1 and Dim2 accounting for 71.5 % and 23.3 %, respectively (Figure A). The PCA clearly distinguished the effects of the two inoculation methods. Plant derived from *C. gloeosporioides*-inoculated seeds were positively associated with most growth parameters, including shoot-root fresh and dry weights, total biomass, germination rate, vigor, chlorophyll content, and vegetative growth (number of leaves, leaflets, and plant height). İn contras, plant grown in inoculated planting media were associated with lower biomass and growth performance, although they exhibited a slightly higher root-shoot ratio.

These findings indicate that the effects of *C. gloeosporioides* on *P. niruri* are strongly dependent on the inoculation method. Seed inoculation enhances plant growth, vigor, chlorophyll content, and biomass accumulation, likely throught improved nutrient availability and phytohormones regulation (Dudeja *et al*., 2012; Lopes *et al*., 2021). Increased chlorophyll content in seed- inoculated plants reflects higher photosynthetic efficiency, enhancing growth rates and taller plant heights. In contrast, planting medium inoculation reduced growth, possibly due to competition for nutrients and space between the fungus and the host plant.

The contrasting responses highlight the importance of inoculation strategy in determining plant microbe interactions. In addition, environmental conditions, soil characteristic, and interactions with indigenous rhizosphere microorganisms may influence inoculation outcomes (Lopes *et al*., 2021). Another study suggests that endophytic fungi application on planting medium by soil drenching has been successful in inoculating crops like cassava, common bean, sorghum, wheat, coffee, opium poppy, and sweet pepper, improving plant growth, controlling wireworms, and improving yield (Tefera & Vidal, 2009). While the positive effects of seed inoculation with *C. gloeosporioides* on *P. niruri* are evident, the negative impacts observed with planting medium inoculation highlight the complexity of plant-microbe interactions. These findings underscore the need for careful consideration of inoculation methods and environmental conditions to optimize the benefits of microbial inoculants in agriculture. Further research into the mechanisms of interaction and the role of environmental factors could provide deeper insights into maximizing the potential of such biotechnological applications.

Figure 1B displays the Pearson correlation coefficients between each pair of growth parameters of P. niruri. Strong positive correlation was observed between shoot fresh weight with shoot dry weight (r = 0.99), shoot dry weight with total dry weight (r = 0.98) and root dry weight with total dry weight (r = 0.86). these relationships indicate that increases in shoot and root biomass contribute substantially to overall plant biomass accumulation. In contrast, the root-shoot ratio showed weak correlation with most growth parameters, suggesting that biomass allocation patterns are regulated independently of absolute growth and may be influenced by environmental conditions and inoculation treatments. A strong positive correlation was also observed between germination rate and vigor index, indicating that improved seed vigor contributes to more efficient germination and seedling establishment (Basu & Groot, 2023). Overall, the correlation analysis suggests that successful plant growth is associated with coordinated development of both aboveground and belowground organs, highlighting the importance of balanced resource allocation in *P. niruri* development.

Clustering analysis showed that control grouped in the same group. In contrast, seed and planting medium inoculation of *C. gloeosporioides* were grouped in the other group (Figure 2). These results showed that *P. niruri* gave the same response to *C. gloeosporioides*, which applied both seeds and a planting medium. The clustering of control planting medium and seed inoculation indicates similar baseline levels of measured compounds in uninoculated planting medium and seed, establishing a baseline metabolic state. In other clusters, the alkene groups such as octacosane 2-methyl- (C66), nonacosane 2-methyl (C63), tetracosane (C80), octacosane (C65), and docosane (C40) showed higher abundance in fungi treatment. The *C. gloeosporioides*-inoculated group exhibits significant changes in compound abundance, indicating the endophytic fungi's ability to disrupt the existing metabolic equilibrium. *Colletotrichum gloeosporioides* inoculation significantly drives metabolic change in *P. niruri*, altering their chemical fingerprints and making them more similar to control in both planting medium and seed inoculation.

The increased abundance of alkene groups such as octacosane 2-methyl, nonacosane 2-methyl, tetracosane, octacosane, and docosane in *P. niruri* inoculated by *C. gloeosporioides* can be attributed to the complex interactions between plant lipids and pathogen-induced stress responses. These alkenes, which are very-long-chain fatty acids (VLCFAs), play significant roles in plant defense mechanisms, particularly in response to biotic stress. The biosynthesis and accumulation of these compounds are influenced by various lipid-mediated signalling pathways and enzymatic activities that are activated during endophyte treatment. Lipids play a crucial role in plant defense, acting as structural components and signalling molecules. VLCFAs, found in alkenes, are essential for plant cuticular waxes, providing a protective barrier against pathogens. Their biosynthesis is often upregulated in response to pathogen attacks (Seth *et al*., 2024)

The contrasting patterns observed between seed and planting medium inoculation highlight that the plant's defense response can be influenced by the initial point of contact and the systemic nature of the infection. Seed inoculation might lead to an earlier activation of certain defense mechanisms as the plant develops in the presence of the pathogen. This could result in a different profile of compound accumulation compared to when the fungus interacts with roots in planting medium inoculation. Some of these compounds might be being broken down or metabolized by the plant as part of its defense response. The plant might be converting them into other substances that are more effective response to the fungus. Alternatively, the fungus itself might be metabolizing or absorbing these compounds from the plant tissues. For compounds like Phytol and Stigmasterol, which are involved in various plant metabolic pathways and membrane structure, a decrease (green bar) could indicate stress or a reallocation of resources towards defense-related compounds (Figure 3). γ-Sitosterol, another sterol, might be similarly affected by the plant's response or fungal activity. Increased compounds (yellow bar) are likely being produced or accumulated by the plant as part of its defense response to the fungal infection. They might have antifungal properties or play a role in signaling defense pathways within the plant. Our study showed that the abundance of tetracosane in planting medium treatment is increase than in seed treatment is decreased.

The fungus establishes a relationship with the plant when *C. gloeosporioides* is inoculated into *P. niruri* seeds or planting medium. This relationship can be endophytic, where the fungus influences the plant's metabolism and produces compounds, or elicitation, where the plant perceives the fungus as a threat, activates defense-related genes, leading to the production of protective secondary metabolites. The methanolic extract of *P. niruri* treated with *C. gloeosporioides* revealed 11 compounds, including steroid, flavonoid, alkane, and alkene (Table 1). These compounds have pharmacological potential that is beneficial for human health, such as anti-inflammatory, anticancer, antidiabetic, antimicrobial, and antioxidant.

CONCLUSION

1. *C. gloeosporioides* inoculation significantly impacts *P. niruri* growth, with outcomes tied to the application method. Seed inoculation boosts growth, while planting medium inoculation

may impede it due to resource rivalry.
2. Fungal presence drastically alters the plant's chemical makeup, notably increasing specific alkenes that likely trigger very long-chain fatty acid-mediated defense mechanisms.
3. This underscores the criticality of inoculation strategy in plant-microbe interactions, influencing pharmacologically important compounds linked to both plant defense and human health benefits. Optimizing these strategies and understanding their biological underpinnings is vital to harnessing microbial inoculants' full potential.

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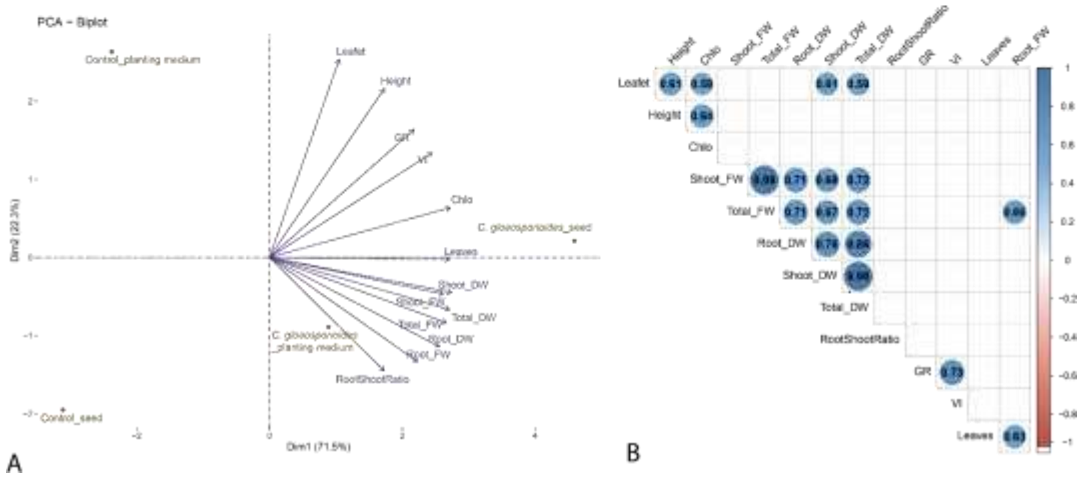
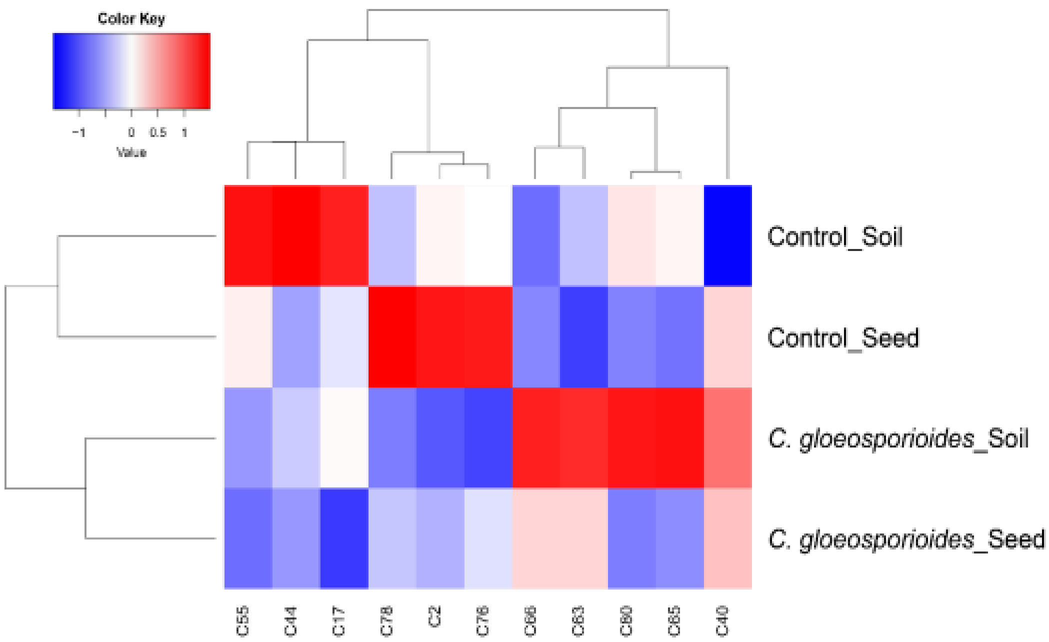
Table 1. Identified compounds in *P. niruri* under seed and planting medium inoculation treatments with *C. Gloeosporioides.*

Compound	Retention Time	Molecule Formula	Class	Activity	Reference
Ɣ-Sitosterol	31.861	$C_{29}H_{50}O$	Steroid	anti-inflammatory anticancer antidiabetic anti-angiogenic antimicrobial	((Tripathiathi et al., 2013)
7,9-Di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione	12.678	$C_{17}H_{24}O_3$	diketone	unidentified	-
Docosane	28.054	$C_{22}H_{46}$	alkane	antimicrobial	(Zouaghi *et al.*, 2021)
Eicosane	24.96	$C_{20}H_{42}$	alkane	antimicrobial	(Suyambumani *et al.*, 2024)
Hexadecane, 2,6,10,14-tetramethyl-	23.019	$C_{20}H_{42}$	alkane	antioxidant	(Indira *et al.*, 2024)
Nonacosane, 2-methyl-	27.661	$C_{30}H_{62}$	alkane	antibacterial	(Wei *et al.*, 2024)
Octacosane	26.297	$C_{28}H_{58}$	alkane	antimicrobial antioxidant anticancer	(Ochieng Nyalo *et al.*, 2022)
Octacosane, 2-methyl-	26.845	$C_{29}H_{60}$	alkane	antimicrobial antioxidant anticancer anti-inflammatory diuretic antimalarial	(Rhetso *et al.*, 2020)
Phytol	16.425	$C_{20}H_{40}O$	alkene	antioxidant antinociceptive anti-allergy anti-inflammatory	(Patil & Singh, 2022; Rhetso et al., 2020)
Stigmasterol	30.984	$C_{29}H_{48}O$	Steroid	anticancer antimicrobial antibacterial antioxidant	(Patil & Singh, 2022)
Tetracosane	21.503	$C_{24}H_{50}$	alkene	anticorrosive antioxidant antimicrobial	(Joseph *et al.*, 2016)



Leaflet: number of leaflets, Height: plant height, GR: germination rate, VI: vigor index, Chlo: chlorophyll content index, Leaves: number of leaves, Shoot_DW: shoot dry weight, Shoot_FW: shoot fresh weight, Total_DW: total dry weight, Root_DW: root dry weight, Root_FW: root fresh weight, RootShootRatio: root shoot ratio.

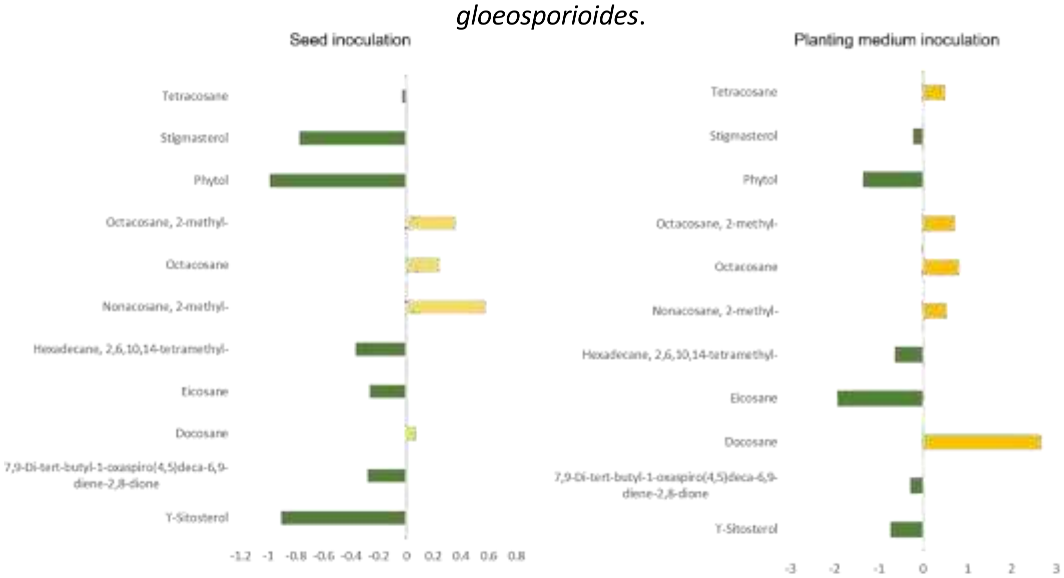
Figure 1. Principle component analysis (A) and correlation analysis (B) between growth parameters of *P. niruri* on seed and planting medium inoculation of *C. gloeosporioides*.



C55: Hexadecane, 2,6,10,14-tetramethyl-, C44: Eicosane, C17: 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione, C78: Stigmasterol, C2: ϒ-Sitosterol, C76: Phytol, C66: Octacosane, 2-methyl-, C63: Nonacosane, 2-methyl-, C80: Tetracosane, C65: Octacosane, C40: Docosane.

Figure 2. Heatmap clustering of eleven compounds observed in Gas Chromatography Mass Spectrophotometry analysis of *P. niruri* on seed and planting medium inoculation of *C.*

gloeosporioides.



Green bars pointing to the left indicate a decrease in the amount of that compound in the *P. niruri* plant after inoculation compared to a control group. Yellow bars pointing to the right indicate an increase in the amount of that compound after inoculation. The length of the bar shows the magnitude of the change – longer bars mean a bigger change.

Figure 3. Comparison of eleven compounds determined by Gas Chromatography Mass Spectrophotometry of *P. niruri* between seed and planting medium inoculation of *C. gloeosporioides*.