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Root and collar rot disease: a new threat to young cacao (*Theobroma cacao* L.) plants in Sulawesi, Indonesia

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Abstract

A root and collar rot disease caused by a fungal complex has been identified for the first time in orchards of cacao on Sulawesi Island, Indonesia. The disease affects young plants, from eight to eighteen months old. The present work describes the disease and the isolation of potential pathogens associated with it. The symptoms in the field are rotting of the collar and tap root, reduced growth of deep and lateral roots, and leaf chlorosis and fall. Thirteen fungal isolates were recovered from diseased roots, 62% were *Lasiodiplodia theobromae* and *L. parva*, 23% *Diaporthe eucalyptorum, and* 15% *Neocosmospora rubicola*. All isolates caused chlorosis, drying and abscission of leaves, and reduced growth of lateral roots and reduced root weight when inoculated into wounded young cocoa plants. By nine weeks after inoculation more than 75% of leaves were affected. There was no significant difference between the isolates in symptom development. Root weight was reduced by from 25 to 73%. All isolates except one (ARL-.08) caused a significant reduction in root weight compared to the control at nine weeks after inoculation. It is likely that these well-known endophytic fungi became pathogenic following drought and nutrient induced stress known to have occurred in the dry season of 2019 in the particular districts affected.

Keyword *Diaporthe* · *Lasiodiplodia* · Leaves chlorosis · *Neocosmospora* · Root weight · First report

Introduction

Cacao is a major cash crop in Indonesia, cultivated mainly by around one million smallholders. It is an important economic driver on the island of Sulawesi, which produces more than 60% of the country's total cacao crop. Currently, after a rapid increase in cacao production beginning in the early 1980s, production is waning due to factors such as poor management and pests and diseases, negatively impacting household incomes (Moriarty et al. 2014). The main diseases have been Phytophthora Pod Rot (PPR) and Vascular Streak Dieback (VSD) caused by *Phytophthora palmivora* and *Ceratobasidium theobromae*, respectively (Ruf 2007; Rosmana et al. 2010, 2015). The main insect pest has been Cocoa Pod Borer, *Conopomorpha cramerella.* Lately, *Lasiodiplodia* has been causing significant pod rot (Ali et al. 2019; Asman et al. 2020). In the last five years, *Lasiodiplodia* was found to cause a dieback and canker diseases in some regencies. Dieback disease is evident as sudden yellowing and browning of leaves, and eventually dieback. Dark brown streaks within vascular tissue are conspicuous upon splitting the infected stems (Asman et al. 2020). Stem canker is characterized by blackish and sunken regions and the presence of boreholes with powdery frass. In this disease, the fungi, including *Lasiodiplodia, Fusarium*, and *Ceratocystis, are* associated with stem borer *Xylosandrus compactus* as the causal agents (Asman et al. 2020, 2021). The canker causes an estimated 10%-15% of tree death on some farms, especially in wet regions (Rosmana, unpublished data). More recently, a disease that is also associated with *Lasiodiplodia* has been discovered on young cacao plants, eight to eighteen months old. This disease typically manifests as root rot. Lower leaves yellow first, and yellowing may extend to the whole plant, eventually resulting in leaves drying and plant death. The extent and number of deep and lateral roots of these seedlings are greatly reduced. Eventually, these roots disappear, leaving only a taproot on the surface of which fungal mycelium may be evident. In addition to root rot, the collar region of the stem presents blackening and decaying symptom.

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Lasiodiplodia and *Fusarium* species have been known to asymptomatically colonize root, stem, branch, and leaf tissues of both cacao seedlings in the nursery and mature cacao plants (Rosmana et al. 2018, 2019). Botryosphaeriaceae, including *Lasiodiplodia,* are a common component of endophytic communities. The ecological and pathological role of endophytic fungi is poorly understood (Slippers and Wingfield 2007). Some populations of *L. theobromae* found in cocoa tissues are pathogenic, causing cushion gall disease, while others are non-pathogenic (del Castillo et al. 2016). Genes involved in carbohydrate, pectin, and lignin catabolism, cytochrome P450, and necrosis-inducing proteins are expressed during infection (Ali et al. 2019). This disease expression is enhanced in general by plant stress conditions including drought stress, extreme heat or cold, high rainfall, physical damage, damage by other pests and diseases, competition with other plants, and unsuitable sites (Slippers and Wingfield 2007; Songy et al. 2019).

On plants other than cacao, *Lasiodiplodia* species have been reported as causing collar rot and or root rot on peanut (*Arachis hypogaea* L.) (Phipps and Porter 1998), rice (*Oryza sativa* L.) (Claudius-Cole 2018), cassava (*Manihot esculenta* Crantz) (Machado et al. 2014), mulberry (*Morus alba* L.) (Xie et al. 2014), physic nut (*Jatropha curcas* L.) (Latha et al. 2009; Prajapati et al. 2014) and Australian bottle plant (*Brachychiton populneus* Schott & Endl.) (Sandlin and Ferrin 1992). Symptoms on these plants are blackening and decaying of stem collar and roots, the eventual yellowing, drooping, shedding of leaves, and plant death (Sandlin and Ferrin 1992; Latha et al. 2009; Pappachan et al. 2020). While on cacao, the root diseases called black root rot, Armillaria root rot, brown root, and white rot have been known for a long time (Ploetz 2007; Delgado-Ospina et al. 2021). These diseases caused by *Rosellinia pepo* and *Rosellinia bunodes*, *Armillaria mellea*, *Phellinus noxius*, and *Rigidoporus lignosus,* and their infection offer in general similar symptoms including rapid wilting of the leaves and the plant's immediate death (Delgado-Ospina et al. 2021).

The above fungi are considered a rhizomorphic pathogen with an ectotrophic growth habit, while *Lasiodiplodia* on cacao is an endophytic fungus. The present work focused on two aspects of the cacao root rot and collar rot disease observed in Malili, Gantarangkeke, and Ngapa districts in Sulawesi. Firstly, the characterization of symptoms, isolation and identification of *Lasiodiplodia* and other fungi from infected young cacao roots. secondly, the pathogenicity of the isolated fungi when inoculated into young cacao plants.

Materials and methods

Sample collection and isolation of pathogenic fungi

Young cacao plants showing yellowing leaves and root damage were collected from Malili, Gantarangkeke and Ngapa Districts, Sulawesi, Indonesia. The symptoms were observed and described before the plants were transported to the laboratory. The taproot was separated from the above-ground part, and after removing the bark, the remaining root was cut into 1 cm long segments. Then these segments were surface sterilized by dipping in 70% alcohol and 2% NaOCl for three minutes each, followed by three rinses in sterile distilled water. Five root segments were placed in 9-cm-diameter Petri dishes containing 20 ml water agar (WA) and incubated at room temperature (27 °C – 29 °C). After 3-4 days, fungal mycelia growing from the root segment in contact with agar were transferred into PDA medium.

Identification of isolated fungi

Fungi growing on PDA medium in Petri dishes were incubated at 27 °C –29 °C and observed every day for further purification and identification. These Isolated were identified firstly based on their colony characteristics and morphology under the microscope. Secondly, they were identified through their DNA sequences. The mycelium from six-day-old cultures was scraped off, washed with sterile distilled water, dried on sterile absorbent paper towels, and transferred to a 1.5 mL Eppendorf tube for immediate DNA extraction. DNA was extracted as previously reported by Dodd et al. (2002). Polymerase chain reaction for amplification of the internal transcribed spacer (ITS) region was performed using the primers ITS4 and ITS5 (White et al. 1990) with one cycle of pre-denaturation for 120 s at 95 °C followed by 35 cycles of denaturation for 60 s at 94 °C, annealing for 30 s at 50 °C, elongation for 90 s at 72 °C, and post-elongation for 5 min at 72 °C. An amplicon of 600 bp and 900 bp were obtained and sequenced. Sequencing and assembly were done at Axil Scientific, Singapore.

Assessment of disease incidence

The pathogenicity of 13 fungal isolates was evaluated on six-week-old plants (having 8-12 leaves) of the cacao clone MCC 01. After wounding their collar using a needle, fungal inoculation was done by putting a 5 mm diameter agar plug from a five-day-old culture on the surface of this injured collar. The agar plug was then covered with parafilm to avoid contamination by other microorganisms. Five plants were inoculated with each fungus isolate. Leaves were observed for nine weeks for evidence of yellowing and drying symptoms. From these leaf symptoms, the disease incidence was calculated using the formula of Inc = N/Tn x 100%, where Inc is the disease incidence, N is the number of leaves with

yellowing and drying symptoms, and Tn is the total number of leaves observed. The incidence of disease was also observed on below-ground parts by weighing fresh root weight. Roots were cut vertically for observation of internal symptoms. From these infected roots and collar, the inoculated fungus was re-isolated and cultured in PDA medium for proving the present of the same fungal pathogen.

Statistical analysis

Thet disease incidence on leaves and below-ground parts of cacao was analyzed using ANOVA. MS Excel 2013 was used for this analysis, and the data were not transformed after testing normality using skewness and kurtosis. The least significant difference (LSD) was then used in evaluating significant differences ($p \leq 0.05$) among the treatment means.

Results

Disease observation in the field

In the field, this disease mainly affects young cacao plants, from eight to eighteen months old. The first symptom is evident as chlorosis of leaves. The lower leaves become yellow first and then yellowing often extends to the entire plant, and leaves become dry and abscise. Lateral and deep roots are seen to be brown, rotted and reduced in number and extent, the taproot is brown and reduced in size, and white hyphae arise from the surface of the taproot (Fig. 1).

Isolation and identification of fungal species

Fungi were isolated from the roots of affected cacao in three districts of Sulawesi: Malili (ten cultures), Gantarangkeke (one culture), and Ngapa (two cultures) districts. Species identified from Malili included *Lasiodiplodia theobromae* (two isolates), *L. parva* (four isolates), *Diaporthe eucalyptorum* (two isolates), and *Neocosmospora rubicola* (two isolates). *Lasiodiplodia parva* was recovered from Gantarangkeke, and *L. theobromae* and *D. eucalyptorum* were recovered from Ngapa (Table 1). *Lasiodiplodia* species (eight isolates) made up 62% of isolates, *Diaporthe* species (three isolates) made up 23% of isolates and *Neocosmospora* species made up 15%. Base on observation of colony characteristics and morphology under the microscope. *L. theobromae* and *L. parva* colonies grown on PDA 27 °C for five days had white-greyish color on upside and same color for the first and concolorous for the second on reverse side. Their conidia with septate were found in *L. theobromae*, but not in *L. parva*. The *N. rubicola* colony was white on the upside and brown-yellow in the reverse side center. Its microconidium was with one-septum (Fig. 2). The colony of *D. eucalyptorum* was white on the upside and yellowish on the reverse side, and alpha conidia were just observed.

Damage to cacao seedling infected by fungal species

All strains of fungi isolated, including *Lasiodiplodia, Diaporthe,* and *Neocosmospora* were capable of inducing root damage on cacao seedling when inoculated into the stem

Table 1 Fungi isolated from rotted tap roots of cacao plants in three districts in Sulawesi

No	District origin	Isolate number*)	Identified as	Genbank accession number
1	Malili	ARL-01	*Lasiodiplodia parva*	CBS 456.78
2	Malili	ARL-02	*L. theobromae*	CBS 164.96
3	Malili	ARL-03	*L. parva*	CBS 456.78
4	Malili	ARL-04	*L. parva*	CBS 456.78
5	Malili	ARL-05	*Diaporthe eucalyptorum*	CBS 132525
6	Malili	ARL-06	*D. eucalyptorum*	CBS 132525
7	Malili	ARL-07	*Neocosmospora rubicola*	CBS 101018
8	Malili	ARL-08	*L. parva*	CBS 456.78
9	Malili	ARL-09	*L. theobromae*	CBS 164.96
10	Malili	ARL-10	*N. rubicola*	CBS 101018
11	Gantarangkeke	ARB-01	*L. parva*	CBS 456.78
12	Ngapa	ARK-01	*D. eucalyptorum*	CBS 132525
13	Ngapa	ARK-02	*L. theobromae*	CBS 164.96

*) The isolate collection is owned by Ade Rosmana (AR), Plant Disease Laboratory, Hasanuddin University

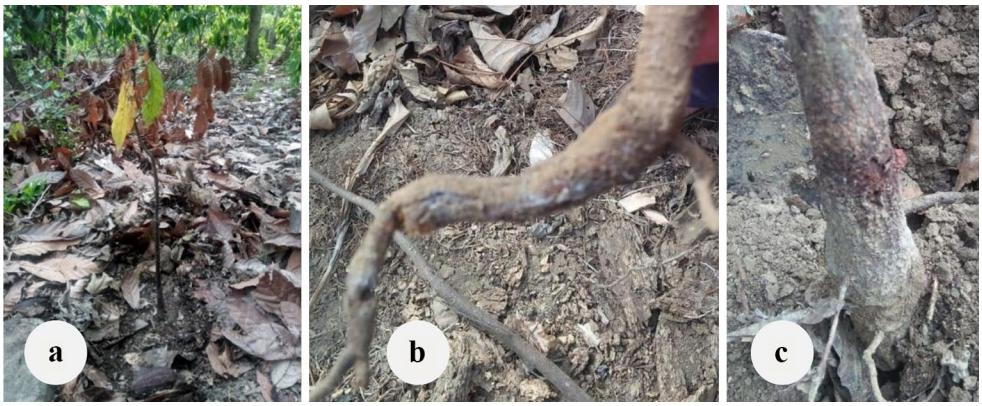

Fig. 1 Symptoms of root and collar rot disease on cacao on a farm in Gantarangkeke district, Sulawesi; **a** eleven-month-old grafted plant with chlorotic and drying leaves; **b** rotten taproot showing emergence of white mycelium and absence of lateral roots; **c** rotting brown collar

under-ground. This damage was expressed externally by reduced lateral root number, taproot size and root fresh weight and internally by vascular tissue browning (Fig. 3). At nine weeks after inoculation, root fresh weight reduction was about 24.6% to 72.7%. The lowest was found in seedlings treated with ARB-01 isolate (*L. parva*), and this root weight was significantly different ($P \leq 0.05$) from those untreated and treated by ARL-05 (*D. eucalyptorum*), ARL-08 (*L. parva*), and ARL-09 (*L. theobromae*) isolates (Fig. 4). In addition to root damage, plants exhibited yellowing and drying of leaves. Isolates ARL-03 (*L. parva*), ARL-08 (*L. parva*), and ARL-10 (*N. rubicola*) were the fastest to show leaf symptoms, at one to three weeks post-inoculation. However, at the last observation, nine weeks

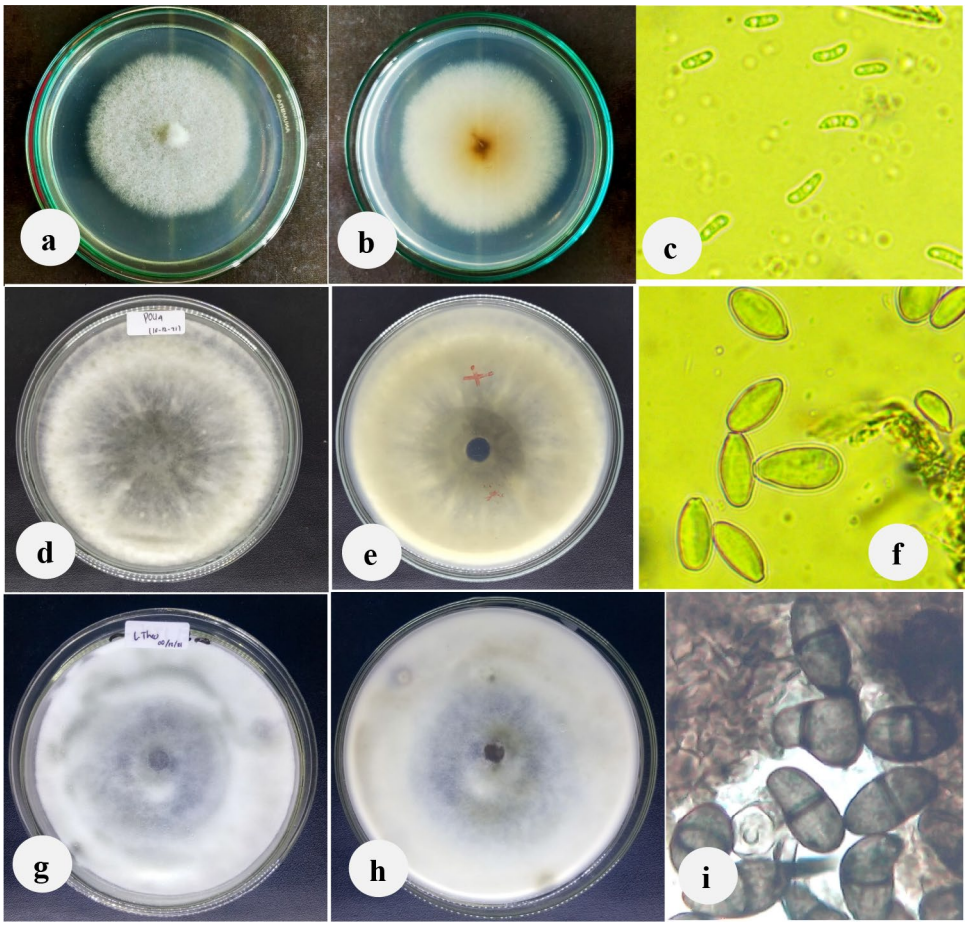

Fig. 2 Colony of *Neocosmospora rubicola* ARL-10 (**a**, front; **b**, reverse), *Lasiodiplodia parva* ARB-01 (**d**, front; **e**, reverse), and *Lasiodiplodia theobromae* ARL-02 (**g**, front; **h**. reverse) grown on potato dextrose agar (PDA) for seven, five, and five days, respectively, at 27 °C. Microconidia of *N. rubicola* with one-septum (**c**), conidia of *L. parva* without septum (**f**), and conidia of *L. theobromae* with septum (**i**)

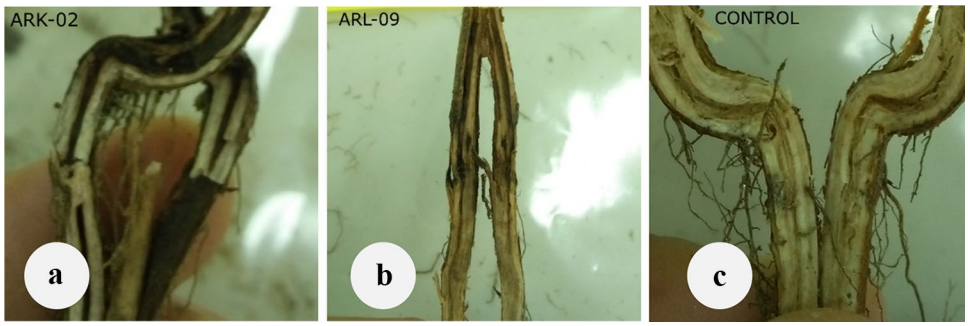

Fig. 3 The taproots of cacao plants split in half longitudinally showing reduced lateral roots and vascular tissue rot nine weeks after inoculation in the laboratory with *Lasiodiplodia theobromae*; **a** isolate ARK-02; **b** isolate ARL-09; **c** uninfected tap root with lateral roots

post-inoculation, all isolates caused 75%-100% of leaves to show yellowing and drying and there was no significant different ($P \leq 0.05$) between isolates in inducing leaves symptoms (Fig. 5). All fungi inoculated can be reisolated from root infected at nine weeks post inoculation.

Discussion

The work showed that in addition to *Lasiodiplodia* (*L. theobromae* and *L. parva*), *Diaporthe eucalyptorum,* and *Neocosmospora rubicola* species were also isolated from diseased roots of cacao seedlings showing collar and root rot symptoms in three districts in Sulawesi. All isolates were capable of inducing collar and root rot symptoms after being artificially inoculated into cacao seedlings. As mentioned previously, *Lasiodiplodia* has been reported in causing root and or collar rots both on annual and perennial plants and offer symptoms including root and collar blackening, the eventual leaves yellowing, drooping, shedding, and plant death**.** *Diaporthe* has been known to cause root rot of perennial herb coptis (*Coptis chinensis* Franchet) (Mei et al. 2020). *Neocosmospora* causes root rot on chickpea (*Cicer arietinum* L.), peanut (*Arachis hypogaea* L.) and muskmelon (*Cucumis melo* L.), with symptoms including yellowing and wilting of leaves, rotting at the stem base and upper root and collapse of the entire plant (Dau et al. 2010; Ali et al. 2011; González et al. 2020). These symptoms resemble those found on young cacao plants in Sulawesi. After inoculation, the rots were only found internally in vascular tissues. It is

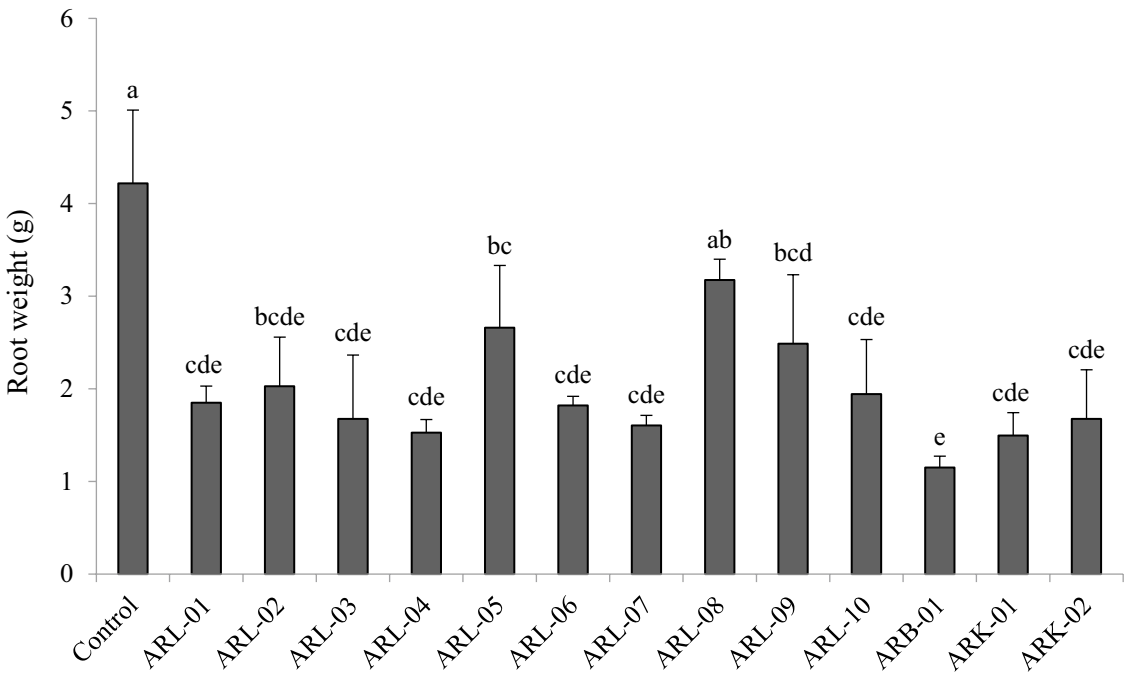

Fig. 4 Mean fresh root weight of young cacao plants at nine weeks after inoculation with 13 fungal isolates including *Lasiodiplodia theobromae* (ARL-02, ARL-09, ARK-02), *L. parva* (ARL-01, ARL-03, ARL-04, ARL-08, ARB-01), *Diaporthe eucalyptorum* (ARL-05, ARL-06, ARK-01), and *Neocosmospora rubicola* (ARL-07, ARL-10). Means with the same letter are not significantly different according to the LSD test ($p \leq 0.05$). Vertical bars show standard errors of means

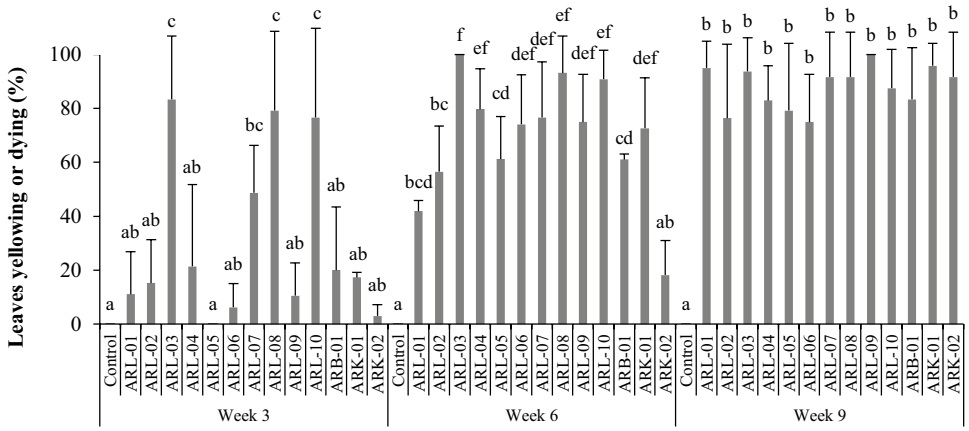

Fig. 5 Leaf damage (% of all leaves either chlorotic or dying) on young cacao plants at three, six, and nine-weeks after inoculation with 13 fungal isolates including *Lasiodiplodia theobromae* (ARL-02, ARL-09, ARK-02), *L. parva* (ARL-01, ARL-03, ARL-04, ARL-08, ARB-01), *Diaporthe eucalyptorum* (ARL-05, ARL-06, ARK-01) and *Neocosmospora rubicola* (ARL-07, ARL-10). Means of incidence at the same time, followed by the same letter, are not significantly different according to the LSD test ($p \leq 0.05$). Vertical bars show standard errors of means

likely that the fungi, known to have endophytic capacity, develop systemically in the vascular tissue, and eventually damage the tissues they pass through

Lasiodiplodia species are known to be endophytes found frequently in cacao both in the nursery and field (Rosmana et al. 2018, 2019). It has been suggested that they may play a role in reducing the infestation by primary pathogens like *Ceratobasidium theobromae* (vascular streak dieback, VSD) and *Phytophthora palmivora* (pod rot, PPR) (Rosmana et al. 2018, 2019; Risda 2020). This *Lasiodiplodia* produces secondary metabolites, such as diketopiperazines, indoles, jasmonates, melleins, lactones and phenols (Salvator et al. 2020a). Jasmonic acid is one of the most critical signal molecules involved in defense, including plant defence against pathogens. *Diaporthe* spp. have also been identified as endophytes in cacao stems together with *Lasiodiplodia* spp. (Ali et al. 2019). The role of *Diaporthe* in protecting plants from fungal diseases has been explored for Dutch elm disease (Brayford 1990). *D. endophytica* and *D. terebinthifoli* can infect and colonize citrus plants and control citrus black spot disease caused by *Phyllosticta citricarpa* (Santos et al. 2016). *Neocosmospora* species occur as fungal endophytes in roots of licorice (*Glycyrrhiza uralensis* Fisch.) and olive (*Olea europaea*) and are considered as having a fundamental role in plant growth and protection (Kim et al. 2017; Nicoletti et al. 2020).

An endophyte is defined as the ability of microorganisms to colonize plant tissues at least part of their life cycle without inducing disease symptoms (Azevedo and Araújo 2007, Salvatore et al. 2020b). The duration of this stage depends on changes in the host susceptibility induced by several kinds of stress, which may reduce their tolerance or trigger a more aggressive and pathogenic behavior by the endophyte (Sakaladis et al. 2011, Salvatore et al. 2020a). Plant stress can be caused by extreme weather conditions such as drought, physical damage, other pathogens and insects, and interplant competition (Slippers and Wingfield 2007).

The current study found that root rot and collar rot disease were widely distributed in Gantarangkeke district in the south of South Sulawesi Province, and in Malili and Ngapa districts in the north of South Sulawesi. It is likely that stress factors in these districts caused the emergence of the disease. Long dry periods during the dry season in 2019 are likely to have stressed young cacao plants, especially those growing without shade. This stress may have been exacerbated by a lack of nutrient supply from the soil, especially in new plantings on land previously planted with cacao. Studies at several locations in Sulawesi showed that the average soil C content was 1.54% and N content was 0.14% (Agoume and Birang 2009; McMahon et al. 2015), while the content recommended for cacao production is > 3.5% for C and > 0.2%, for N (Murray 1967).

The development of Lasiodiplodia--related diseases depends on the interaction between extreme weather conditions and biological pressure from pathogens and pests expanding their geographic ranges (Desprez-Loustau et al. 2006). The same phenomenon, some species of *Diaporthe* can be either pathogenic or harmless endophytes depending on the host and its health (Gomes et al. 2013). Several studies indicate that many different genus or species of endophytes can co-occur in the same host or plant part infected (Gomes et al. 2013; del Castillo et al. 2016; Ali et al. 2019). Flowery cushion gall of cacao is associated with *Fusarium decemcellulare, Lasiodiplodia theobromae*, *F. equiseti*,

Fusarium spp., *F. solani, F. incarnatum, Rhizocthonia solani* and *Penicillium* sp., of which some isolates are pathogenic, and others non-pathogenic (del Castillo et al. 2016). Fungi associated with cacao stem canker include *Fusarium, Lasiodiplodia*, and *Ceratocystis* (Asman et al. 2021, Rosmana, unpublished data). In this research, some strains consisting *Lasiodiplodia, Diaporthe*, and *Neocosmospora* from the Malili district can also co-occur within a single root, and each can cause collar and root rot disease of cacao seedling. The association of several species with the root and collar rot observed in this study suggests that they are likely to be common endophytes of the cacao plants that have become pathogenic following stressing of the plants.

The work concludes that a new disease on cacao called root rot and collar rot was discovered on Sulawesi Island, Indonesia. This disease is associated with two species of *Lasiodiplodia* (*L. theobromae* and *L. parva*), one species of *Diaporthe* (*D. eucalyptorum*) and one species of *Neocosmospora* (*N. rubicola*). It is hypothesised that these well-known endophytic fungi became pathogenic following drought and nutrient induced stress known to have occurred in the dry season of 2019 in the particular districts affected.

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Declarations

Conflicts of interest No potential conflict of interest relevant to this article was reported.

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