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Cultural and pathogenic characterization of *Fusarium* fungi isolated from dieback branches of cacao

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A B S T R A C T

Fusarium species are known as pathogens on cacao causing dieback, but field observation showed apparent variation in suspected Fusarium-caused dieback. We hypothesized that many strains or species infect cacao trees, and, therefore, we evaluated isolates by culturing in semi synthetic PDA medium and infecting cacao seedlings artificially. Of four isolates from cacao branch collected in Luwu, Soppeng, Banyuwangi, and Luwu Timur, one had red maroon mycelia, the second white creamy mycelia, the third pink and white mycelia, and the fourth white mycelia. Symptoms on seedling leaves eight weeks after inoculation of roots with the four isolates were respectively yellowing with green spots, yellowing with green veins, wrinkling, and yellowing with brown spots and green veins. Incidence of disease in seedlings caused by the four isolates was respectively 25.4%, 28.9%, 8.4%, and 29.2%. These data support the occurrence of four Fusarium pathotypes, which are perhaps different species, that infect cacao trees in the field.

Introduction

Fusarium species are a filamentous fungi found in the temperate and tropical areas of the world that enable it to penetrate plant surfaces and cause stem canker,, dieback, Panama disease, malformation, and fruit rots (Waller and Brayford, 1990; Burgess and Bryden, 2012). In Sulawesi, Indonesia, diseases caused by *Fusarium* are important on perennial crops such as mulberry, acacia, silk tree, black pepper, durian, passion fruit, and cacao (Rosmana *et al.*, 2003; Rosmana *et al.*, 2013; Karim, 2014).

On cacao, *Fusarium* disease known as Fusarium vascular dieback (FVD) an important disease with characteristic symptoms of yellowing of leaves and dieback resembling the symptoms of vascular streak dieback (VSD) but without the presence of three darkened vascular traces on leaf scars and petioles. When twigs are split infected xylem is visible as brown coloration within the vascular tissue. Severe infestation with this soil-borne pathogen can cause dieback the tree, with long impacts as

the crop cannot be readily regenerated by planting new seedling or shoot-graft and by side grafting (Rosmana *et al.*, 2013).

On branches affected by dieback of cacao, Adu-Acheampong and Archer (2011) isolated some spesies of *Fusarium* such as *F. chlamydosporum*, *F. solani*, *F. oxysporum* and *F. proliferatum*. In a previous study, the occurrence of other species on cacao was reported, namely *F. decemcellulare* (Leslie and Summerell, 2006). According to field observation, dieback has diverse symptoms varying from light to severe. Therefore, we hypothesize that a number of species or pathotypes of *Fusarium* cause disease in cacao. Characterization of these *Fusarium* types is a prerequisite in disease control studies.

Our aim was to isolate *Fusarium* species from cacao in the field and then characterize them morphologically in medium culture, and, in addition, evaluate symptoms on leaves, incidence, and colonization capability in plant tissues after artificial infection of the roots of cacao seedlings.

Materials and Methods

Isolation and cultural characterization of *Fusarium*

Fusarium species were isolated from cacao branches showing die back symptoms in Luwu regency (South Sulawesi), Soppeng regency (South Sulawesi), Banyuwangi regency (East Java), and Luwu Timur regency (South Sulawesi), Indonesia. These branches were cut into lengths about 10 cm long and then surface sterilized. Surface sterilization was done by sequential immersion in 2% sodium hypochlorite, 70% ethanol and sterilized water (Arnold *et al.*, 2003). To reveal the sapwood, bark of branches was removed using a sterilized knife, and the sapwood of 0.25–0.5 cm was placed in Petri dish containing 20 ml potato dextrose agar (PDA). After three days, *Fusarium* mycelia were selected and purified by moving to new PDA medium. Cultural characterization of this *Fusarium* was done by identification of their morphology, colony pattern, density, color, and zonation of mycelia.

Pathogenic assessment of *Fusarium*

To differentiate pathogenic ability, four isolates (Luwu Timur, Soppeng, Banyuwangi, and Luwu Utara) were inoculated into one-month-old cacao seedlings. The inoculation was performed through roots with approximately 1 x 10^6 spores/ml. These seedlings were planted in poly-bags containing about 1 kg soil and they were placed in a green house. For each isolate, five seedlings were inoculated and raised in poly-bags, therefore in total there were 25 poly-bags including controls.

Observations of symptoms caused by *Fusarium* were conducted on leaves every week until eight weeks after inoculation. The incidence of *Fusarium* was calculated by using the formula of I = a/b x 100%, where I is disease incidence by *Fusarium*, b is the number of leaves with symptoms and b is the total number of leaves observed.

To determine colonization of *Fusarium* in seedling tissues, these four isolates were re-isolated from petiole, leaf, and stem of cacao seedling nine weeks after inoculation. For this purpose, petiole, leaf and stem were surface sterilized by sequential immersion in 2% sodium hypochlorite, 70% ethanol and sterilized water as mentioned above and a small portion of these plant parts were placed on PDA medium in Petri dish. The occurrence of *Fusarium* isolates was then identified and compared with the isolates used for inoculation.

Data analysis

The data of incidence by *Fusarium* were analyzed after transformation to log x + 1. Duncan's multiple range test (DMRT) was then used for evaluating significant differences between the treatment means.

Results and Discussion

Fusarium was found in all cacao sapwood samples collected from Luwu, Soppeng, Banyuwangi, and LuwuTimur. Each isolate was different, notably in cultural aspect and color. The Luwu isolate had a cultural aspect that was thick and velvety with a red maroon color, while the Soppeng isolate was thick and velvety with white creamy color, the Banyuwangi isolate was effuse and powdery with pink and white color, and the Luwu Timur isolate was effuse and powdery with white color (Table 1).

Inoculation of the four *Fusarium* isolates resulted in symptoms on leaves starting from lower leaves and moving to upper leaves. The symptoms produced by Luwu, Soppeng, and Luwu Timur appeared at five weeks after inoculation, while for the Banyuwangi isolate they appeared at seven weeks after inoculation. Infection by Luwu isolate resulted in symptoms firstly as greening of veins on leaves, which gradually became clear, together with yellowing of leaf beginning from the leaf margin. Infection by Soppeng isolate presented also leaf symptoms with green veins, but this greening of veins persisted longer than that produced by the Luwu isolate. In addition, clearing between veins occurred in the Soppeng isolate. Infection by Banyuwangi isolate resulted in a symptom of leaf wrinkling. The symptom expressed by Luwu Timur isolate was similar at first to symptoms of the Soppeng isolate, but the clearing symptom between veins developed to a brown reddish coloration (Figure 1).

After 3-4 days of culture on PDA medium, the Soppeng isolate grew from stem sections, petiole sections and leaf pieces from two test seedlings nine weeks after inoculation, while Banyuwangi and Luwu Timur isolate grew respectively from stem section and leaf pieces. No growth was recorded on any Luwu isolate treated seedling or the control stems, petioles, or leaves.

Applications of four *Fusarium* isolates have been demonstrated to result in disease on cacao seedlings. This suggests these isolates possibly cause dieback on trees in the field. Based on their characteristics in medium culture, expression of symptoms, degree of incidence, and colonization in cacao tissue, we can consider that these four isolates were different species. So far, *Fusarium* species identified as agent of dieback on cacao are *F. chlamydosporum*, *F. solani*, *F. oxysporum F. proliferatum*, and *F. decemcellulare* (Adu-Acheampong and Archer, 2011; Adu-Acheampong *et al.*, 2012). Species identification of our *Fusarium* isolates is presently on the way.

The degree of *Fusarium* colonization on seedling tissues is apparently important for symptom expression and seedling incidence by this fungus. But, our results showed that even the Luwu isolate offered the most visible symptoms on seedling leaves, we could not detect any fungus on tissue of stems, petioles or leaves.

Table.1 Characteristic of four *Fusarium* strains isolated from dieback branches of cacao grew on PDA medium

Fusarium origine	Upper surface				Lower surface
	Culture aspect	Density	Color	Zonation	
Luwu	Thick, velvety	Hight	Red maroon	Present	Red maroon alternating with red in zonation
Soppeng	Thick, velvety	Light	White creamy	Absent	Yellowish
Banyuwangi	effuse, powdery	Mild	Pink and white	Present	Pink alternating with yellow in zonation
Luwu Timur	Effuse, powdery	Mild	White	Absent	White and yellow

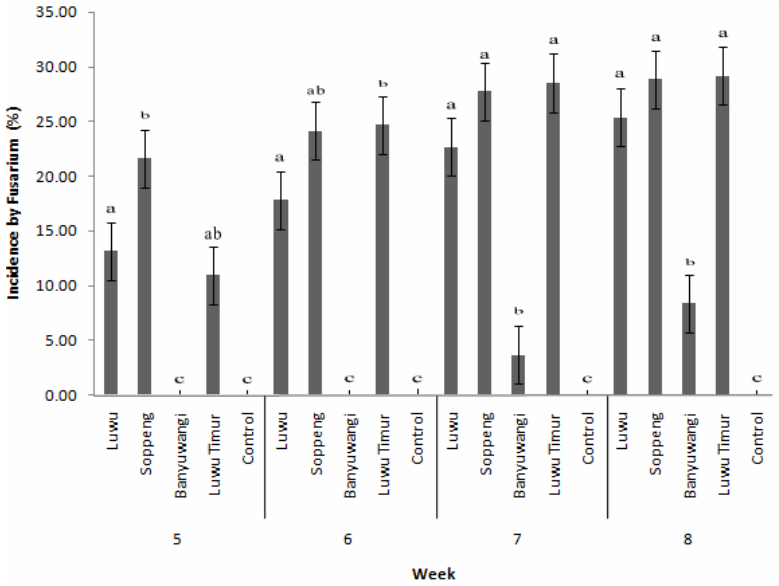

Figure.1 Disease incidence caused by four *Fusarium* isolates (Luwu, Soppeng, Banyuwangi, and Luwu Timur) at five until eight weeks after inoculation. Means in the same week followed by same letter are not significantly different according to DMRT (P = 0.05).

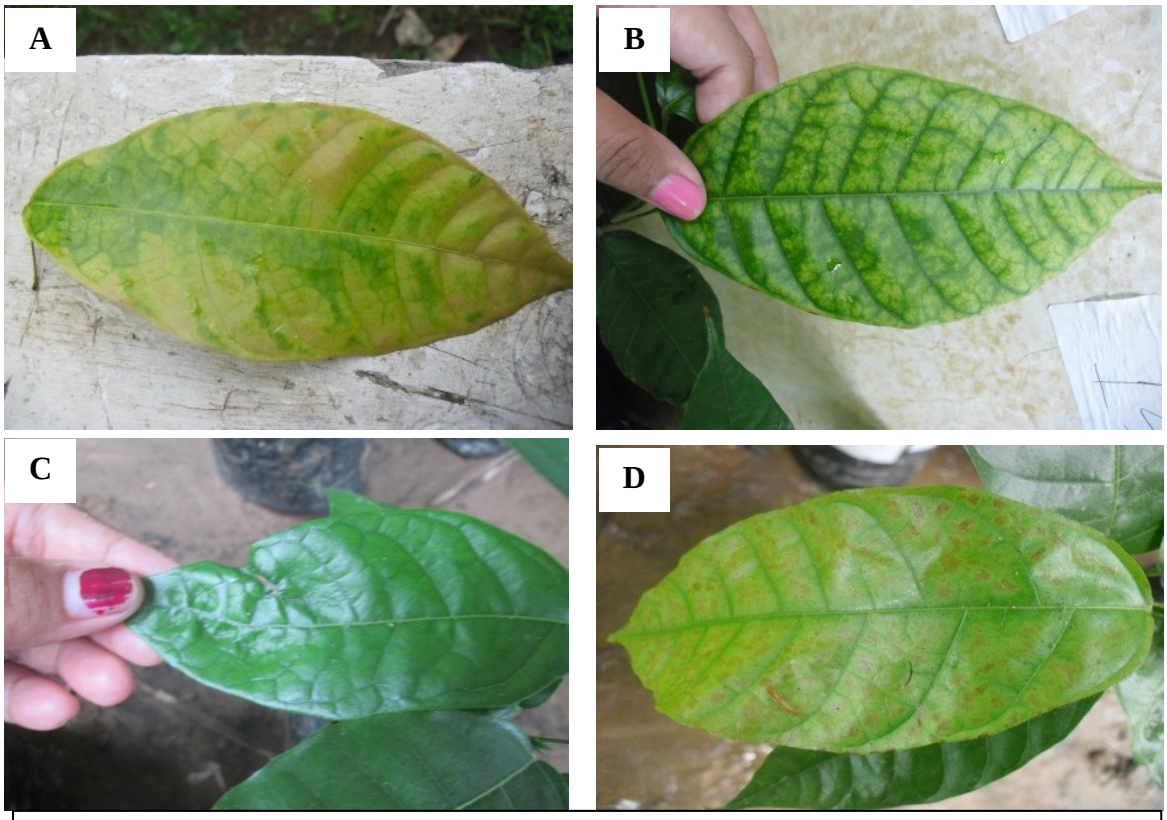

Figure.2 Symptoms expressed on leaf of cacao seedling eight weeks after inoculation by *Fusarim* of Luwu isolate (A), Soppeng isolate (B), Banyuwangi isolate (C), and Luwu Timur isolate (D) through roots.

We will evaluate whether this condition was due to competition with other microorganisms, since the soil used for planting seedlings was not sterile.

Our results provide new insights into the *Fusarium* spp. - cacao interaction and the infection of cacao trees. Many strains or species can infect cacao tree and offer different degrees of dieback. On other hand, in the field dieback can also be caused by other fungi such as *Ceratobasidium theobromae* and *Lasidiplodia theobromae* (Guest and Keane, 2007; Adu Acheampong and Archer, 2011; Adu-Acheampong *et al.*, 2012; Samuels *et al.*, 2012). *Fusarium* could possibly make a complex with two fungi mentioned above and then produce either more severe or decreases in dieback symptoms.

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