



Armillaria root rot threatens Cameroon's Penja pepper (*Piper nigrum* L.)

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Received: 15 November 2019 / Accepted: 27 April 2020 / Published online: 27 May 2020

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Abstract

Penja pepper (*Piper nigrum*) produced in Cameroon has long been recognized for its exceptional organoleptic quality. The pepper vine is grown using a support tree (*Spondias mombin*) in Cameroon. A root disease is associated with plant deaths in both plants. The disease symptoms are characterized by collar cracking and gummosis and the disease was tentatively identified as *Armillaria* root rot. In this work the extent of the problem was characterized by surveying 35 farms in Cameroon. Samples were taken from diseased support trees and pepper vines. Support trees exhibiting typical symptoms were found in approximately one third of the surveyed farms. In these farms, disease incidence and Pepper vine mortality ranged from 1.3 to 50% and 1.2 to 87.7%, respectively. Analysis of the ribosomal internal transcribed spacer region (ITS) locus suggested that the isolates collected from *S. mombin* and *P. nigrum* are most likely, which is a first for both hosts, represented by *Armillaria camerunensis* (Henn.) Volk & Burdsall, a fungus previously associated with declining cacao trees in Cameroon. Given the increasing demand for Penja pepper and the great damaging potential of this root rot, research on management strategies should be prioritized.

Keywords *Armillaria camerunensis* · Disease incidence · Molecular identification · Slow decline · Support tree

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Introduction

The pepper vine (*Piper nigrum*) is a tropical perennial crop, native to India, which belongs to the Piperaceae family (Ravindran 2000). It is cultivated in several countries including Brazil, Indonesia and Vietnam which are the world's largest producers of pepper (Faostat 2017). According to the International Pepper Community (IPC 2017) pepper is the “King of spices” since it is the most consumed and marketed spice in the world. Besides its popular use in cuisine and seasoning, the spice has shown medicinal attributes. Pepper can help relieve viral respiratory ailments such as flu and colds, as well as digestive problems such as constipation, flatulence and colic (Meghwal and Goswami 2012; Ahmad et al. 2012).

Black pepper was introduced to Cameroon in the 1930s and although the exact origin of first introduction is unknown, the first vines were introduced into Cameroon by the Germans, and later by French Planters and the Cameroon Development Corporation (CDC). They brought in cultivars from Cambodia, the Democratic Republic of Congo (DRC) (at the time the Belgian Congo) and India. It is the descendants

of this miscellany of cultivars that are now grown in Cameroon.

The pepper produced in Cameroon has long been recognized by connoisseurs to be among the world's best, owing to its outstanding organoleptic attributes. This exceptional quality has earned Penja black pepper the first sub-Saharan protected geographical indication (GI) label in September 2013 by the African Intellectual Property Organization (OAPI), further boosting its reputation. Although Cameroonian production remains marginal (<0.02% of global production) (Faostat 2017), it has increased over the past few years by over a factor of 10, as pepper attracts increasing interest from local farmers, stimulated by the GI label for Penja pepper and a concomitant price increase from \$4.2 before the GI to \$23.6 USD per kg in 2015. Prices oscillated between \$20.20 and \$23.6 US dollars between 2016 and 2018.

As is the case for any intensively propagated crop, diseases and pests are a major constraint limiting black pepper productivity. Numerous pests and diseases have been reported to affect pepper vines; some of which are well known to cause severe losses, e.g. *Phytophthora* foot rot caused by *Phytophthora capsici* Leonian, (Ravindran 2000; Truong et al. 2008), blackberry disease caused by *Colletotrichum* species (Keuh 1990), and slow decline (or yellow disease) which can result from various pathogens and complexes of biotic and abiotic stressors (Ramana and Santhosh 2000). In Cameroon, as cultivation of pepper is intensifying so is the pressure of phytosanitary problems. In recent years especially, there has been a growing concern among farmers about the losses associated with diseases, which in some cases have resulted in up to 50% mortality in certain pepper plantations. The “Plantations du Haut Penja” (PHP) organization, the biggest pepper producer in Cameroon, saw its production area reduced from 40 to 21 ha because of disease pressure (Mbodiam 2019). According to preliminary assessments, these losses are most likely associated with a range of biotic and abiotic stressors that remain to be resolved (Petchayo et al. 2015).

In Cameroon, a disease affects the pepper's support tree, *Spondias mombin* L (hog plum), a native of the Americas and West-Indies. The support tree is critical for pepper cultivation as it provides a structure for optimum growth and productivity of pepper plants (Ravindran 2000). The disease affecting *S. mombin* is characterized by collar cracking, gummosis and the presence of white fungal mycelial fans beneath the bark, which are all typical symptoms of *Armillaria* root rot (Fig. 1c, d). A preliminary investigation indicated that like the support tree, the pepper vine was also susceptible to this disease (Petchayo et al. 2015). However, further studies are needed to support the development of effective management strategies. The aim of this study was therefore two-fold: 1) to elucidate the cause of the disease by means of DNA sequence analyses and; 2) to assess the incidence of root rot in Penja Pepper farms across the certified geographic area using field surveys.

Materials and methods

Field observations and sample collection

The Penja pepper production basin lies within the fourth agro-ecological zone, as defined by the Institute of Agricultural Research for Development (IRAD) of Cameroon. This region straddles the Littoral and South-West administrative regions of the country. It features a tropical rain forest as native vegetation cover and a subequatorial climate type, characterized by 2500–4000 mm annual rainfall, 22–29 °C mean temperatures and 61–90% relative humidity.

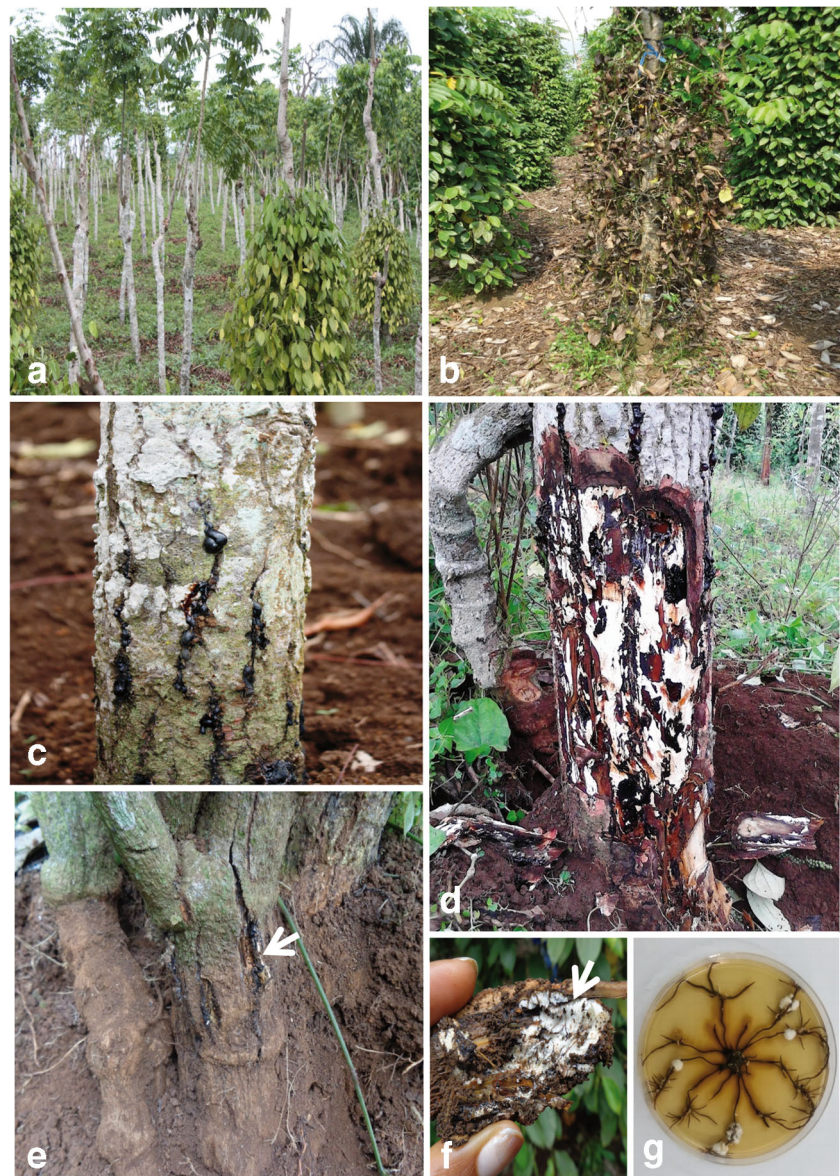
Field observations for root rot and sample collection were carried out in May–June 2016, on a total of 35 farms, selected from the database of the association of Penja pepper producers (Penja Pepper Geographical Indication Group, IGPP for its abbreviation in French) and based on farmer's indications whether the disease was present or not. Farms were distributed over the districts of Penja-Njombe ($n = 14$; N4° 38. 799' E9° 40. 331'), Loum ($n = 7$; N4° 42. 140' E9° 46. 318') in the Littoral region, and Tombel ($n = 13$; N4° 39. 685' E9° 36. 853') in the South-West region. Each plantation was surveyed to determine the prevalence of the disease. Two transects, perpendicular to each other, were laid out in the center of each pepper field. Each transect was 28 m long and included three rows of pepper plants planted together with support trees (*S. mombin*) at 2 × 2 m spacing. Thus, a maximum of 81 pepper vines and support trees were examined and a sanitary status was assigned to vines and support trees separately, either healthy, diseased, or dead/absent (absence of a support tree or vine was assumed to be due to the death of the plant). Potential differences per district and plantation age group (3–6, 7–10 and > 10 yrs. of age) were analyzed using arcsin-transformed percentage disease incidence data for support trees and mortality incidence for vine trees with the appropriate generalized linear model in XLSTAT v. 2019.4.1.

Eleven symptomatic vine/support tree combinations were randomly sampled in one farm per district. Infected samples were collected from roots, collar and trunk of pepper and support trees (Fig. 1a–f) following a purposive stratified random sampling technique. All samples were placed in paper bags, duly labeled and brought back to the laboratory for further analyses. Additional information regarding age of the plantation, associated crops and previous land use was also collected.

Isolation of pathogen

Isolation of suspected *Armillaria* cultures from infected plant materials was performed as described by Tchotet Tchoumi et al. (2017). Small pieces (2–3 mm³) of woody tissue were taken from the advancing edge of lesions and were surface sterilized in 7% sodium hypochlorite for 90 s, then rinsed twice in sterile distilled water and dried using sterile tissue paper. These

Fig. 1 Disease symptoms in pepper plantations in the Penja region of Cameroon, (a) support trees (*S. mombin*) most of which are devoid of pepper vines; (b) a dead pepper vine, reminiscent of slow decline; (c) gum exuding from the base of a dying support tree; (d) white mycelial fans underneath the bark of support tree; (e) basal stem cracking of black pepper; (f) white mycelium underneath the root bark of *P. nigrum*; (g) Morphological characteristics *in vitro* of *Armillaria* isolate



sterilized plant materials were plated onto Basidiomycete selective medium, made up of 2% Malt Extract Agar (MEA) supplemented with 2 mg/L benomyl, 2 mg/L dichloran and 0.01 g/L streptomycin sulphate (Worrall 1991). Plates were incubated at ambient temperature (25 ± 2 °C) in the dark for two weeks, and pure cultures were obtained from these by transferring rhizomorph tips onto new MEA plates, which were also maintained at ambient temperature.

DNA extraction, amplifications and phylogenetic analysis

Mycelium from pure cultures was harvested, freeze-dried and ground to a fine powder using a cell disruptor machine (Retsch GmbH, Germany). Genomic DNA was extracted using the CTAB protocol as described by Möller et al. (1992). Prior to

amplifications, the initial DNA concentrations were adjusted to 100 ng/μL using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). PCR amplifications targeted the ITS regions (ITS1, ITS2), including the 5.8S subunit of the ribosomal DNA and was done using the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). PCR and sequencing reaction mixtures and reaction conditions were the same as outlined in Tchotet Tchoumi et al. (2017). DNA sequencing was done at the sequencing facility of the University of Pretoria. The CLC Main Workbench v.7.6.1 package was used to edit bidirectional-sequencing reads and to assemble contigs. The ITS sequences were subsequently queried against the global fungal DNA sequence database in the GenBank nucleotide database using the NCBI BLASTn search tool (<https://blast.ncbi.nlm.nih.gov>). A

dataset of *Armillaria* ITS sequences was assembled, comprising representatives from the different localities and hosts (*P. nigrum* and *S. mombin*) targeted by this study, the sequences obtained by Roux et al. (2012) on cacao (*Theobroma cacao*) in Cameroon, as well as all BLASTn hits in GenBank with $\geq 97\%$ sequence identity. Sequences representing *A. hinnulea* were included in the dataset as outgroups (Table 1). The dataset was submitted to multiple sequence alignment with the online program MAFFT (<http://mafft.cbrc.jp/alignment/server/>) prior to phylogenetic analysis based on Maximum likelihood (ML) criterion using RaxML (Stamatakis 2006) in raxmlGUI 1.3 (Silvestro and Michalak 2012). The analysis used 10 parallel runs and 1000 bootstrap replications and applied the GTR + G model of sequence evolution determined as best fit under the Akaike Information Criterion (AIC) using JModeltest v2.1.6 (Darriba et al. 2012; Miller et al. 2010).

Pathogenicity test

A pathogenicity test was conducted using six-month old *Piper nigrum* plants obtained from cuttings and kept under natural conditions, and two *Armillaria* isolates (CMW44956 and CMW45278). Two inoculation methods were used: soil inoculation and artificial wounding modified from Ford et al. (2017).

For the soil inoculation technique four mycelial agar plugs of the same size, overgrown with the respective *Armillaria* isolates, were mixed with the soil of each pot containing pepper plants. In the wound inoculation study 5×5 mm agar plugs containing three-week-old mycelium from each isolate were inserted into a wound made on the collar of each plant. A total of seven pepper seedlings per fungal isolate were inoculated for each inoculation technique. Seven plants were inoculated with sterile agar plugs to represent the controls. Plants were maintained for four months, with daily watering. At the beginning and over the course of the experiment, several growth parameters were evaluated, such as number of leaves, max plant length and mortality. At the end of the experiment, plants were collected and leaf and root dry weight data were collected. Changes in plant height and leaf area were determined, as well as lesion length, measured from the center of the inoculation point, between the beginning and end of the experiment. The general health status of the roots was also determined. Treatment effects were evaluated by fitting a one-way Anova model in XLSTAT v.2019.4.1.

Results

Field observations

The average plantation age of the 35 surveyed farms was around 8 years (3 to 15 years) (Table 2). From a total of

2832 inspected vine/support tree combinations (three support trees were absent), 157 (5.5%) of the support trees were visibly affected and showed symptoms of collar cracking and gummosis. The percentage of affected support trees ranged from zero to more than 50% between farms (Table 2). There was no significant effect of districts ($P = 0.501$). In the Tombel district, 7 out of 13 farms (53.8%) were affected and disease incidence in affected farms ranged from 1.2 to 22.2%. In the Njombe-Penja district 3 out of 13 farms (23.1%) were affected and disease incidence in affected farms ranged from 1.2% to 50.6%. Finally, in the district of Loum 2 out of 9 farms (22.2%) showed symptoms with disease incidences of 3.7% and 45.7%. Some surveyed farms ($n = 13$) had no support trees affected by the disease even though the owners indicated that the disease was present (Table 2). On the surveyed farms for which the farmers had indicated the absence of the disease, the disease was indeed absent. This discrepancy between farmer indications and transect data is partially due to the fact that the transect was laid out in the middle of the plot, where in certain cases indeed no diseased trees were present, although diseased trees were sometimes present outside of the transect area. The transect area was put in the middle of the plot to avoid edge effects for certain other parameters measured.

With regard to the pepper vines, a total of 515 out of 2832 support trees (18.2%) (Table 2) were devoid of vines. In other words, on average a fifth of any plantation was unproductive. Contrary to “*Armillaria*” incidence, all farms had a certain level of pepper vine mortality, however, once again there were no marked differences between the three districts ($P = 0.123$). Pepper vine mortality was often associated with yellowing of the vine before subsequent desiccation. Unfortunately, the etiology of yellowing disease in Penja pepper remains to be resolved. Biotic stressors such as nematodes, *Phytophthora* and *Pythium* species as well as *Fusarium solani* (f. sp. *piperis*) might be involved (Cardoso and Albuquerque 1979; Ramana et al. 1987; Anandaraj et al. 1996; Shahnazi et al. 2012). Although nematodes, *Pythium/Phytophythium* spp. and *Fusarium*, have been found in these pepper plantations (data not shown), it has not been possible to unequivocally link these to the slow decline of pepper vines. Correct identification of these organisms, like for *Armillaria* here, will be a first step. Recent advances allowing the correct determination of e.g. the presence of *Fusarium solani* f. sp. *piperis* (Costa et al. 2017) will be useful in this respect.

For both support tree and pepper vine, disease was not related to plantation age ($P = 0.208$, $P = 0.958$, respectively). This might indicate that the problems/pathogen(s) afflicting support trees and/or pepper vines were already present at the time of plantation establishment or arrived very shortly after.

Pathogen identification

All sampled diseased plants presented similar symptoms, such as cracking of the bark and wood (Fig. 1c, e) and presence of

Table 1 Isolates used in the phylogenetic analysis

Species	Strain*	Host	Geographical origin	GenBank accession numbers (ITS)	Reference
<i>Armillaria camerunensis</i>	TH 9926	Dead wood	Cameroon	KU170951	Koch et al. 2017
<i>A. camerunensis</i>	TH DJA 91	<i>Gilbertiodendron dewevrei</i>	Cameroon	KU170952	Koch et al. 2017
<i>Armillaria</i> sp.	CMW34125	<i>T. cacao</i>	Cameroon (B)	MG254549	Roux et al. 2012
<i>Armillaria</i> sp.	CMW36819	<i>T. cacao</i>	Cameroon (N)	MG254551	Roux et al. 2012
<i>Armillaria</i> sp.	CMW36820	<i>T. cacao</i>	Cameroon (N)	MG254552	Roux et al. 2012
<i>Armillaria</i> sp.	CMW36821	<i>T. cacao</i>	Cameroon (K)	MG254553	Roux et al. 2012
<i>Armillaria</i> sp.	CMW43766	<i>Spondias mombin</i>	Cameroon (P)	MG182003	This study
<i>Armillaria</i> sp.	CMW44921	<i>S. mombin</i>	Cameroon (P)	MG182004	This study
<i>Armillaria</i> sp.	CMW44922	<i>S. mombin</i>	Cameroon (P)	MG182005	This study
<i>Armillaria</i> sp.	CMW44923	<i>S. mombin</i>	Cameroon (P)	MG182006	This study
<i>Armillaria</i> sp.	CMW44924	<i>S. mombin</i>	Cameroon (P)	MG182007	This study
<i>Armillaria</i> sp.	CMW44927	<i>S. mombin</i>	Cameroon (L)	MG182008	This study
<i>Armillaria</i> sp.	CMW44956	<i>Piper nigrum</i>	Cameroon (Njombe)	MG182009	This study
<i>Armillaria</i> sp.	CMW45278	<i>Piper nigrum</i>	Cameroon (P)	MG182010	This study
<i>Armillaria</i> sp.	CMW45281	<i>S. mombin</i>	Cameroon (Njombe)	MG182011	This study
<i>A. fuscipes</i>	CMW10165	<i>Prunus persica</i>	Zimbabwe	AY882966	Coetzee et al. 2005
<i>A. fuscipes</i>	CMW2717	<i>Pinus elliottii</i>	South Africa	AY882971	Coetzee et al. 2005
<i>A. fuscipes</i>	CMW2740	<i>Pinus patula</i>	South Africa	AY882970	Coetzee et al. 2005
<i>A. fuscipes</i>	CMW3164	<i>Pelargonium asperum</i>	La Reunion	AY882975	Coetzee et al. 2005
<i>A. fuscipes</i>	CMW4871	<i>Widdringtonia whytei</i>	Malawi	AY882976	Coetzee et al. 2005
<i>A. fuscipes</i>	CMW4873	<i>Indigenous shrub</i>	Malawi	AY882977	Coetzee et al. 2005
<i>A. fuscipes</i>	CMW4874	<i>Araucaria cunninghamii</i>	Zimbabwe	AY882967	Coetzee et al. 2005
<i>A. fuscipes</i>	CMW4949	<i>Pinus elliottii</i>	Tanzania	AY882978	Coetzee et al. 2005
<i>A. fuscipes</i>	CMW4950	<i>Pinus strobes</i>	Tanzania	AY882979	Coetzee et al. 2005
<i>A. fuscipes</i>	CMW4953	<i>Pelargonium asperum</i>	La reunion	AY882974	Coetzee et al. 2005
<i>A. fuscipes</i>	CMW5844	<i>Pinus patula</i>	Ethiopia	AY882969	Coetzee et al. 2005
<i>A. fuscipes</i>	CMW5846	<i>Pinus patula</i>	Ethiopia	AY882968	Coetzee et al. 2005
<i>A. fuscipes</i>	CMW7184	<i>Cypress</i> sp.	Kenya	AY882973	Coetzee et al. 2005
<i>A. fuscipes</i>	CMW7187	<i>Camellia sinensis</i>	Kenya	AY882972	Coetzee et al. 2005
<i>Armillaria</i> sp.	CMW3173	<i>Tectona grandis</i>	Zambia	AY882981	Coetzee et al. 2005
<i>Armillaria</i> sp.	CMW4455	<i>Camellia sinensis</i>	Zimbabwe	AY882985	Coetzee et al. 2005
<i>Armillaria</i> sp.	CMW4456	<i>Brachystegia utilis</i>	Zimbabwe	AY882984	Coetzee et al. 2005
<i>Armillaria</i> sp.	CMW3152	Unknown	Cameroon	AY882980	Coetzee et al. 2005
<i>Armillaria</i> sp.	CMW10115	<i>Acacia albida</i>	Zimbabwe	AY882983	Coetzee et al. 2005
<i>Armillaria</i> sp.	CMW10116	<i>Newtonia buchananii</i>	Zimbabwe	AY882982	Coetzee et al. 2005
<i>A. hinnulea</i>	NZFS22376	Unkown	New Zealand	EU734746	Unpublished
<i>A. hinnulea</i>	NZFS2394	Unkown	New Zealand	EU734745	Unpublished

B: Bokito; L: Loum; N: Ngat; P: Penja

*Strain in bold were collected during this study

white fungal mycelial mats beneath the bark (Fig. 1d, e, f). Gum exudation was only observed on support trees (Fig 1c). All isolates collected had the typical culture morphology of *Armillaria* species, such as the presence of abundant rhizomorph growth (Fig. 1g).

PCR amplification and sequencing of isolates yielded ITS amplicons ranging in size from 616 to 618 bp, and showing high (99%) sequence identity with *Armillaria* isolates obtained previously from declining *Theobroma cacao* trees in Cameroon (Roux et al. 2012) and *Tectona grandis* in

Table 2 Armillaria root rot incidence at three localities in Cameroon

District	Plantation	Farmer obs. ¹	Armillaria Incidence (%) ²	Pepper vine Mortality (%) ³	Age (yrs.)	Associated crops	Previous land use
Tombel	T1	+	3.4	27.2	9	<i>Prunus africana</i> , <i>Persea americana</i> , <i>Cola acuminata</i>	<i>Coffea</i> sp.
	T2	+	30.0	87.7	3	None	<i>Coffea</i> sp., <i>T. cacao</i>
	T3	+	4.4	16	3	<i>P. africana</i> , <i>P. americana</i> , <i>C. acuminata</i>	<i>Coffea</i> sp.
	T4	+	17.2	21	15	<i>T. cacao</i> , <i>Dioscorea alata</i>	<i>Coffea</i> sp., <i>A. comosus</i>
	T5	+	1.3	1.2	7	<i>T. cacao</i>	Native forest
	T6	+	33.3	33.3	15	<i>Musa</i> sp.	<i>T. cacao</i>
	T7	+	28.6	56.8	7	<i>T. cacao</i> , <i>Musa</i> sp.	<i>T. cacao</i>
	T8	–	–	21	10	<i>T. cacao</i> , <i>D. alata</i>	None
	T9	+	–	22.2	5	<i>Ananas comosus</i> , <i>Musa</i> sp.	<i>Coffea</i> sp.
	T10	–	–	3.7	5	<i>Musa</i> sp.	None
	T11	+	–	67.6	10	<i>T. cacao</i> , <i>D. alata</i>	<i>T. cacao</i>
	T12	–	–	19.8	4	<i>T. cacao</i> , <i>Musa</i> sp.	<i>T. cacao</i> , <i>Coffea</i> sp.
	T13	+	–	4.9	8	<i>T. cacao</i> , <i>Musa</i> sp.	<i>Coffea</i> sp.
Njombe- Penja	N1	+	1.3	3.7	10	<i>Citrus sinensis</i> , <i>C. acuminata</i>	<i>T. cacao</i>
	N2	+	5.5	32.1	11	None	<i>T. cacao</i>
	N3	+	51.9	3.7	12	None	Native forest, <i>Musa</i> sp.
	N4	+	–	6.2	7	None	<i>T. cacao</i>
	N5	+	–	38.3	8	Fruit trees	<i>T. cacao</i>
	N6	+	–	6.2	25	<i>Musa</i> sp., <i>D. alata</i>	<i>C. acuminata</i> , <i>T. cacao</i>
	N7	+	–	9.9	10	None	<i>T. cacao</i>
	N8	+	–	18.5	n.d.	<i>Musa</i> sp.	Forest trees
	N9	–	–	8.6	8	<i>Musa</i> sp.	<i>C. papaya</i> , <i>A. comosus</i>
	N10	+	–	32.1	7	<i>C. acuminata</i> , <i>Musa</i> sp.	<i>T. cacao</i> , <i>C. acuminata</i>
	N11	–	–	6.2	3	<i>Carica papaya</i> , <i>A. comosus</i>	<i>Carica papaya</i>
	N12	+	–	2.5	6	None	<i>A. comosus</i>
	N13	+	–	8.6	6	<i>Musa</i> sp., <i>C. papaya</i>	<i>Musa</i> sp.
Loum	L1	+	4.5	18.5	4	None	<i>T. cacao</i>
	L2	+	64.9	28.4	6	None	<i>T. cacao</i>
	L3	–	–	7.4	16	<i>C. papaya</i>	<i>T. cacao</i>
	L4	+	–	4.9	5	None	<i>Coffea</i> sp., <i>Manihot esculenta</i>
	L5	–	–	9.8	10	<i>Musa</i> sp.	<i>Zea mays</i> , <i>Musa</i> sp.
	L6	–	–	39.5	17	<i>Musa</i> sp., <i>D. alata</i>	<i>Musa</i> sp.
	L7	–	–	2.5	8	None	<i>T. cacao</i>
	L8	+	–	18.5	8	None	<i>Coffea</i> sp.
	L9	–	–	29.6	11	None	<i>Musa</i> sp.

¹ ± presence or absence of the disease in the plantation according to the farmer

² Armillaria incidence based on transect data, – = absence of Armillaria incidence within the transect

³ Pepper vine mortality based on transect data

n.d. = not determined

Zambia (Coetzee et al. 2005). In the ML based phylogram, the *Armillaria* isolates from Penja pepper fields grouped together, with strong bootstrap support (86%), into a single phylogenetic clade, alongside the type *A. camerunensis*, as

well as isolates associated with cacao root rot in Cameroon. This clade also included isolates from diverse host plants in Zambia and Zimbabwe (Fig. 2). The *A. camerunensis* clade is sister to another clade

representing *A. fuscipes*, which is well supported by a strong bootstrap value of 99%.

Pathogenicity test

The pathogenicity test showed no significant differences between treatments with regard to changes in plant height or leaf surface area nor for root and leaf dry weight at the end of the assay (P -values >0.2), although on some uprooted pepper plants what seemed like necrotic root lesions were observed. However, a significant difference ($P = 0.018$, $df = 2$, $F = 4.97$) was observed with regard to lesion length from the point of collar inoculation. Control plants had no lesion development, yet pepper seedlings infected by the pepper isolate had an average lesion size of 4.5 mm and those infected by the *S. mombin* isolate on average of 7.1 mm which was significantly different from the control. Certain plants presented yellowing, unfortunately, re-isolation efforts from wounds and necrotic roots were unsuccessful. Care has to be taken when interpreting these results. Pepper cuttings take several months before developing a root system and initially grow very slow. This, combined with the difficulty of *Armillaria* pathogenicity assays and the relatively short duration of the trial (4 months) may be the reason that no effects, other than the presence of lesions were observed.

Discussion

The ITS based phylogenetic tree clearly shows how the *Armillaria* isolates from the Penja pepper fields grouped together into a single phylogenetic clade alongside the type *A. camerunensis* and isolates from Cameroon associated with cacao root rot. This clade is sister to another clade representing *A. fuscipes* (Fig. 2). This result is similar to those of Coetzee et al. (2005) who, relying on ITS and IGS1 based phylogenies, split *A. fuscipes sensu lato* (s. l.), at the time considered a single specific entity, into two distinctive sister species. One species represented *A. fuscipes sensu stricto* (s. s.), and included isolates from South Africa, Zimbabwe, Kenya, and La Reunion. The second species, to which the Penja isolates appear to belong to, represented an undescribed species and included isolates from Cameroon, Zambia, and Zimbabwe. When Roux et al. (2012) used IGS1 phylogeny to identify *Armillaria* isolates associated with cacao root rot in Cameroon; they concluded that these isolates were conspecific with the unnamed second clade of *A. fuscipes* s. l. This clade was later described as *A. camerunensis* by Koch et al. (2017). Unlike with the ITS as seen in this study, the phylogenetic tree based on IGS1 shows substantial sub-structuring in the *A. camerunensis* clade in Cameroon (Roux et al. 2012), which could be indicative of a high genetic diversity and possibly cryptic speciation in this species. Although two other

Armillaria species have in the past been reported from Cameroon, including *A. elegans* and *A. heimii* (*A. fuscipes*) (Hennings 1895; Roux et al. 2012; Koch et al. 2017), DNA sequence studies have to date only recovered *A. camerunensis* in the country.

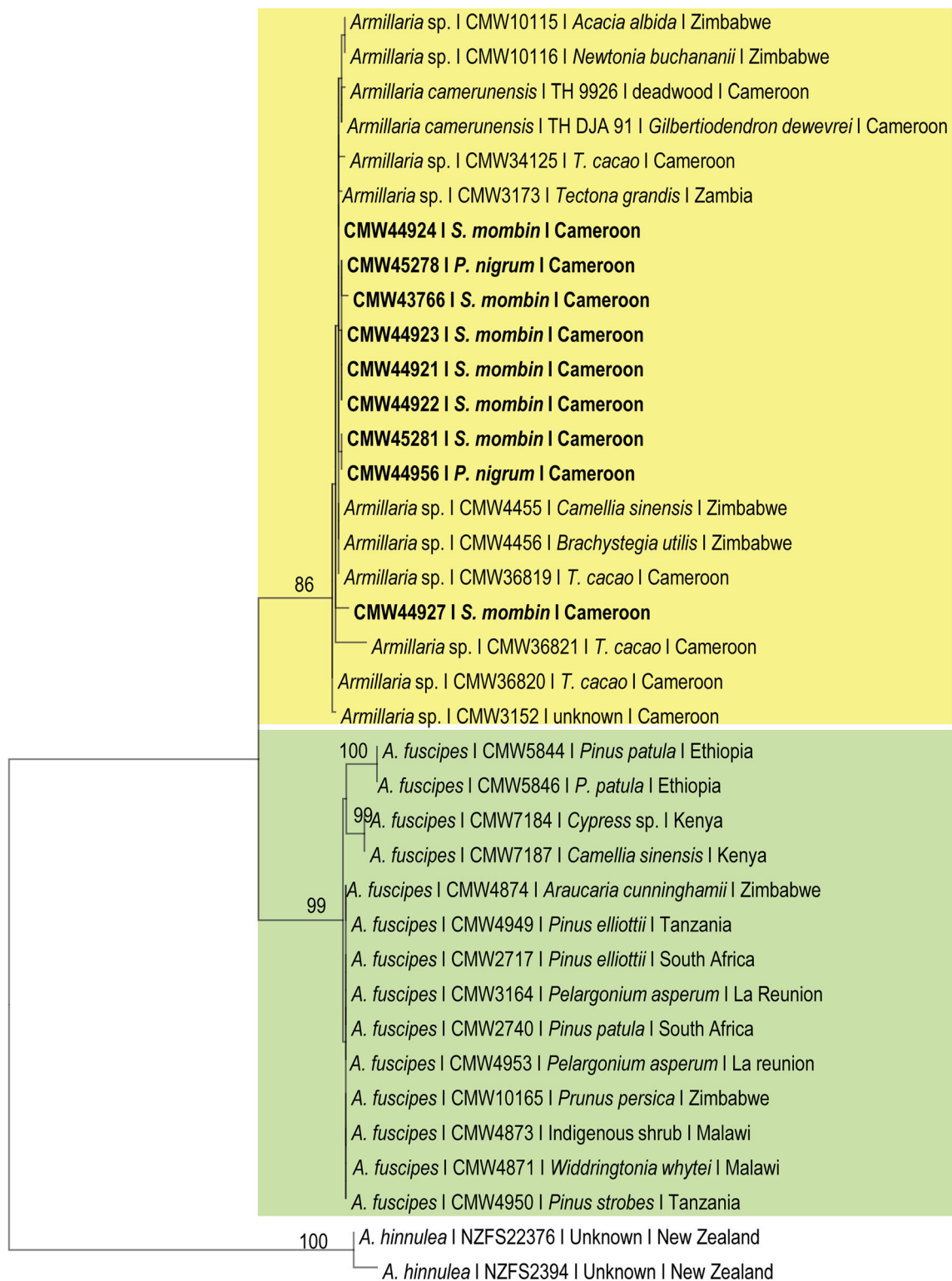
Many of the farms had previously grown, or were still growing, crops like cacao, *Coffea* spp. or fruit trees such as *Citrus* species, avocado (*Persea americana*), African plum (*Dacryodes edulis*) or cola nut (*Cola nitida*), next to or associated with the pepper (Table 2). Certain plots contained forest trees or remnants (stumps) thereof. Many of these are potential hosts for *Armillaria* (Roux and Coetzee 2016), which would explain the presence of the pathogen. *Armillaria* has been reported in Cameroon as early as 1892 (Hennings 1895) and has been found on cocoa in Cameroon, apparently as early as 1907 (Roux and Coetzee 2016), as well as in several other West African countries such as Sao Tomé, Togo and Ghana (Dade 1927; Rishbeth 1980; Roux and Coetzee 2016).

Since *Armillaria* spp. may survive decades in stumps and roots in the soils (Roux and Coetzee 2016), preparation of the terrain where a pepper plantation is to be established is key. Generally speaking, this disease spreads *via* root to root contact between infected and healthy plants, or through rhizomorphs or by mycelium present in the soil (Denman et al. 2000). Thus, manual removal of potentially infected plant material such as stumps and their root systems before establishment of the pepper plantation is recommended. Soil fumigation and soil inoculation with antagonistic fungi (*e.g.* Otieno et al. 2003) could further help to prepare a terrain. However, the efficacy of these methods is certainly not a hundred percent.

Once the disease is present, trenching is often used to prevent spread (Worrall 2004). This is only successful if the trench is dug well beyond the last affected trees. In one farmer's field in the Njombe-Penja district they tried this approach, which unfortunately did not seem to be very successful. This was either because the trench was dug too close to the area that was visibly affected or, since the affected area was on a slope, there was a downward movement of infectious propagules that was not stopped by the trench.

Many Penja pepper farmers use fungicides to control the disease cause by *A. camerunensis*, as this is the principal means they are comfortable with and knowledgeable about when it comes to pest and disease control. Unfortunately, chemical control is not very efficient in controlling *Armillaria* root rot (Thomidis and Exadaktylou 2012; Roux and Coetzee 2016).

Since the disease was more prevalent and more clearly visible on *S. mombin* than on pepper vines, we hypothesize that generally speaking the disease spreads through direct root contact from *S. mombin* to *P. nigrum*. If so, potentially using a different support tree not affected by *Armillaria* could solve the problem. Otherwise, support systems such as metal or



H
0.005

Fig. 2 Maximum Likelihood (ML) tree inferred from the ITS region showing the phylogenetic position of isolates obtained from *Piper nigrum* and *Spondias mombin* (in bold) from Cameroon. The tree was

rooted with sequences of *Armillaria hinnulea* (KX618553 and KX618555). Bootstrap values $\geq 70\%$ from 1000 replicates are displayed at the nodes

concrete poles could be considered, although this would require an investment out of reach of most farmers. However, since a few pepper vines with clear *Armillaria* disease symptoms were observed attached to seemingly healthy support trees, this raises the question whether the disease can spread from *P. nigrum* to *S. mombin*. Ultimately, it seems that both species are potential hosts of this *Armillaria* sp.

Based on the results presented here, it is clear that *Armillaria* root rot, caused by *A. camerunensis*, is one of the main phytosanitary problems of Penja pepper cultivation, causing considerable economic impact. Familiarization of farmers with the causal agent and appropriate control methods are urgently needed to ensure sustained production of highly valued Penja Pepper. This is the first report of *Armillaria* root rot of *S. mombin* and *P. nigrum* globally.

Acknowledgements The authors would like to thank the Penja Pepper Geographical Indication Group (IGPP) producers, in particular René Claude Metomo Elogo (President of Association) and Emmanuel Nzenowo (Executive Secretary) for allowing database and field access. We also thank Fokou Rodrigue, Diazem Clovis, Tocké, Ngonga Florian, and Njonkam Ferdinand, agricultural technicians who assisted in the surveys. Funding and resources were provided by CIRAD, IRAD, the University of Pretoria and through the “Debt reduction-development contract (C2D) France Cameroon” Poivre de Penja project. The authors were free to publish any or all results originating from their work.

Author contributions Conceptualization: Petchayo TS, M Ndoumbé Nkeng, GM ten Hoopen; Methodology: Petchayo TS, JM Tchotet Tchoumi, M Ndoumbé Nkeng, GM ten Hoopen; Investigation: Petchayo TS, JM Tchotet Tchoumi, M. Mbenoun, GM ten Hoopen; Formal analysis: Petchayo TS, JM Tchotet Tchoumi, J Roux, M Mbenoun, M Ndoumbé Nkeng, GM ten Hoopen; Resources: J Roux, GM ten Hoopen; Funding acquisition: CV Mfegue, GM ten Hoopen; Project administration: S Nyassé; Supervision: J Nguefack, BAD Begoude, GM ten Hoopen; Writing original draft: Petchayo TS, GM ten Hoopen; Manuscript revision: Petchayo TS, GM ten Hoopen, M Mbenoun, JM Tchotet Tchoumi, J Roux. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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