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Original Article

Abundance of arbuscular mycorrhizal fungi in the rhizosphere of healthy and declining citrus in East Nusa Tenggara, Indonesia

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Abstract

Citrus is an important commercial crop in Timor Tengah Selatan, East Nusa Tenggara, Indonesia, but many trees are suffering a decline in health. As citrus is heavily dependent on arbuscular mycorrhizal fungi (AMF), this study investigated the abundance of AMF in the rhizosphere of healthy and declining trees in citrus orchards at 12 geographical locations. In each orchard, 6 soil and 6 root samples representing 3 healthy and 3 declining trees were collected. The soil was analyzed for AMF spore abundance as well as physical (texture) and chemical properties (organic carbon, nitrogen, phosphorus, potassium, pH, and soil exchangeable capacity), while the fine roots of citrus were assessed for colonization. The data were analyzed using analysis of variance (ANOVA) where the health condition of the trees was under the geographical location/site factor. The results showed that the abundance of AMF spores was significantly affected by the geographical location from where the soils were collected, but the health condition of the trees had no effect. However, AMF colonization was significantly affected by both site and tree health. The number of AMF morphotypes tended to be higher under healthy trees than under declining trees. Soil analysis indicated that soil fertility (N and organic C) may be important for tree health. These results provided a new perspective on the possible involvement of AMF and soil nutrients in citrus decline. Further studies are required to define the interactions between AMF, soil fertility, and disease incidence to identify strategies for managing citrus decline in the region.

Keywords: Arbuscular mycorrhizal fungi, Citrus decline, Orchard management, Orchard location, Southeast Asia

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Introduction

Citrus is well known for its nutritional value and health benefits to humans and is one of the most traded horticultural products in the world. The tangerine (*Citrus reticulata*), in particular, is an important citrus

species where Indonesia ranks as the 2nd producing country in the world after China (FAO, 2021). Furthermore, Timor Tengah Selatan (TTS) is one of the 2 districts with the largest orchards of tangerine in East Nusa Tenggara Province, Indonesia. Locally known as *Jeruk Keprok Soe*, this citrus is a local genetic type that has become an important horticultural commodity in the district for many years (Namah and Sinlae, 2012). Despite the importance of citrus to the local economy, a decline in tree health has been affecting production for more than a decade. Several studies reported that the decline is associated with plant pathogens including Huanglongbing bacterial disease (Mudita, 2011), *Botryodiplodia theobromae* basal stem rot (Retnosari et al., 2014), and *Phytophthora* root rot (Retnosari et al., 2014; Simamora et al., 2017).

Arbuscular mycorrhizal fungi (AMF) are well known for their ability to form associations with more than 80% of terrestrial plant species. In this relationship, AMF provide mineral nutrients and water to the host plants in exchange for carbohydrates (Smith and Read, 2008). AMF can also improve plants resistance to abiotic stress, such as drought (Wu et al. 2019; Cheng et al., 2022) and salinity (Zhang et al., 2017; Evelin et al., 2019). In addition, AMF provide other ecosystem services, including soil structure improvement (Leifheit et al., 2014; Wu et al., 2019). Previous studies revealed that citrus forms an association with AMF (Ortas, 2012; Srivastava, 2014; Zou et al., 2020). Furthermore, most varieties of citrus have short or sparse root hairs, which makes them heavily dependent on mycorrhizal colonization for sufficient mineral nutrients and water absorption. Plants with limited root hairs are categorized as being obligatory mycorrhizal dependent (Ortas, 2012; Ortas and Ustuner, 2014). Previous studies have reviewed the importance of AMF for the growth, nutrition, and production of plants (Ortas, 2012; Zou et al., 2020).

Despite the dependence of citrus on AMF for the acquisition of essential nutrients and water, there is a limited knowledge about these fungi in citrus orchards in TTS. Considering the presence of potentially devastating pathogens in citrus orchards in the region (Mudita, 2011; Retnosari et al., 2014; Simamora et al., 2017) and the important role of AMF in supporting growth, more attention is required to investigate the involvement of these beneficial symbionts in citrus decline. Several studies demonstrated the importance of AMF in improving the fitness of plants at the seedling stage (Wu et al., 2012) or in the field (Cao et al., 2021). These fungi have also been reported to enhance resistance to diseases, such as those caused by *Phytophthora* spp. (Watanarojanaporn et al., 2011; Youpensuk et al., 2012).

A preliminary study showed that AMF spore density and colonization were low under declining trees compared to healthy trees (Ishaq et al., 2020). This study was limited to a few locations and no soil analysis was carried out, hence a more detailed study needs to be conducted. Therefore, this study aims to determine the abundance of AMF under healthy and declining trees in a broader range of citrus orchards in TTS. Soil analysis was carried out to evaluate the soil properties of the study sites, and to relate this to the AMF population and citrus health.

Material and Methods

Study site and sampling

Citrus orchards located in TTS District, East Nusa Tenggara Province, Indonesia were surveyed for tree health (Figure 1) and 12 orchards were randomly selected based on the occurrence of healthy and declining trees. Within each orchard, 3 healthy and 3 declining trees were randomly selected for sampling. A healthy tree was defined as a tree without any symptoms of nutrient deficiency or disease. A declining tree had symptoms of disease and/or nutrient deficiency. The 12 selected citrus orchards included Ajoubaki 1 ($9^0$41'38.19" S; $124^0$14'51.7" E), Ajoubaki 2 ($9^0$41'55.7" S; $124^0$15'3.77" E), Ajoubaki 3 ($9^0$42'31.46" S; $124^0$15'41.84" E), Tunua 1 ($9^0$41'12.12" S; $124^0$14'23.58" E), Tunua 2 ($9^0$41'9.94" S; $124^0$14'24.56" E), Tunua 3 ($9^0$41'10.71" S; $124^0$14'26.01" E), Oenali ($9^0$52'43.86" S; $124^0$19'14.41" E), Tubuhue ($9^0$53'8.97 S; $124^0$14'13.21" E), Buat ($9^0$50'44.77" S; $124^0$15'37.5" E), Kesetnana ($9^0$50'56.64" S; $124^0$14'45.40" E), Nusa 1 ($9^0$51'2.79" S; $124^0$23'57.41" E), and Nusa 2 ($9^0$50'50.63" S; $124^0$23'53.93" E).

One soil sample was collected about 15 cm from the trunk to a depth of 0 - 30 cm using a trowel. Fine roots, diameter of 0.5 - 1.0 mm, were collected adjacent to where the soil samples were taken. The soil and root samples were enclosed in sealed plastic bags and taken to the laboratory in Kupang (Indonesia). A total of 72 soil and root samples were obtained from the 12 orchards. A portion of soil from each orchard was bulked into a composite sample for soil analysis. The remaining 72

samples were used for AMF spore assessment.

The annual temperature in the region ranges from 18 - 31°C, the annual rainfall is 1000 – 1700 mm per year (Meteorology, Climatology and Geophysical Agency, 2022) and the altitude ranges from 0 - 2477 m above sea level (asl). Orchard management practices were obtained from the orchardists. A detailed description of the study sites is presented in Table 1. The 12 sites differed in management (presence/absence of intercropping between the trees and fertilizer application), tree age, and presence of nearby vegetation. Some orchards were located on flat topography land, while others were on slopes, and the altitude range was 673 – 1211 m asl.

Soil analysis

Twelve soil samples (one composite sample from each tree) from each study site were analyzed for soil chemical and physical properties, including pH (H_2O; ratio soil to water 1 : 2.5), total nitrogen (Kjeldahl method; digestion, distillation and titration), organic Carbon (C; loss on ignition method), total and available phosphorus (P, Olsen method), exchangeable potassium (K), cation exchange capacity (CEC) (saturation of 1 N NH_4 CH_3CO_2 pH 7), and texture (pipette method) (Black, 1965).

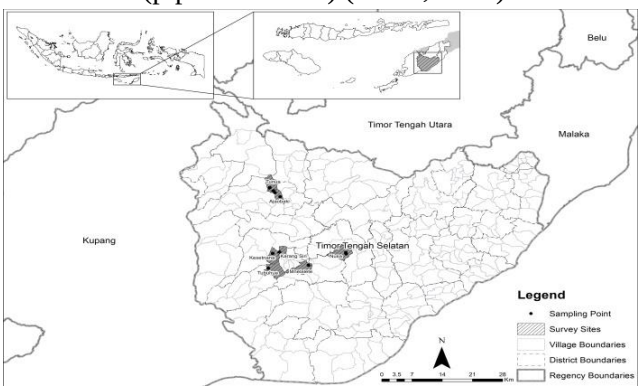

Figure-1. Study sites at 12 locations in TTS District, East Nusa Tenggara, Indonesia

Table-1. Description of the citrus orchards that were sampled

Site	Description
Ajoubaki 1 (A1)	The citrus trees are around 15-20 years old. The land is relatively flat, 1209-1211 m asl, and the soil is Inceptisol. Pumpkin, and corn are planted between the citrus trees during the rainy season without any fertilizer application. Nearby vegetation is mostly perennial native grass with scattered guava and *Sesbania grandiflora*.
Ajoubaki 2 (A2)	The citrus trees are around 10-15 years old. The land is slightly sloped, 1146-1148 m asl, and the soil is Inceptisol. Cow manure is frequently applied to the tree around the trunk. The nearby vegetation is mostly perennial native grass with scattered bananas and the legumes *S. grandiflora* and *Leucaena leucocephala*.
Ajoubaki 3 (A3)	The citrus trees are around 8-10 years old. The land is slightly sloped, 924-926 m asl, and the soil is Inceptisol. Cow manure is frequently applied to the trees around the trunk. The surrounding vegetation is perennial native grass with scattered citronella (*Cymbopogon nardus*), banana, and areca nut (*Arecha catechu*).
Tunua 1 (Tn1)	The citrus trees are around 15 years old. The land is relatively flat, 1284 m asl, and the soil is Inceptisol. Chili is planted between the trees without any fertilizer application. The vegetation nearby is perennial native grass with shrubs such as guava and coffee.
Tunua 2 (Tn2)	The citrus trees are around 15-20 years old. The land is slightly sloped, 1284-1287 m asl, and the soil is Inceptisol. Corn is planted between the trees during the rainy season without fertilizer application. Nearby vegetation is mostly perennial native grass.
Tunua 3 (Tn2)	The citrus trees are around 10 years old. The land is flat, 1285 m asl, and the soil is Inceptisol. Corn is planted between the trees during the rainy season without fertilizer application. Nearby vegetation is mostly perennial native grass, and some bananas and guava.
Oenali (O)	The orchard belongs to the Agriculture Department of TTS district. The trees are around 15-20 years old, the land is flat, 772 m asl, and the soil is Alfisol. Nearby vegetation is mostly perennial native grass.
Tubuhue (Tb)	The citrus trees are around 10 years old, the land is sloped, 654-673 m asl with terrace farming, and the soil is Alfisol. Vegetables are planted between the trees with cow manure application.
Buat (B)	The orchard belongs to the Agriculture Department of TTS district. The trees are around 15-20 years old, the land is flat, 932 m asl, and the soil is Alfisol. The surrounding vegetation is mostly dense perennial native grass.
Kesetnana (Kn)	The orchard belongs to the Agriculture Department of TTS district. The trees are around 15-20 years old, the land is sloped, 834-838 m asl, and the soil is Alfisol. Corn and chili are planted between the citrus trees and NPK inorganic fertilizer is applied. The nearby vegetation is perennial native grass and shrubs.
Nusa 1 (N1)	The citrus trees are around 8-10 years old, the land is sloped, 807-810 m asl, and the soil is Inceptisol. Chili and taro (*Colocasia esculenta*) are planted between the trees without fertilizer application. The nearby vegetation is perennial native grass and shrubs.
Nusa 2 (N2)	The citrus trees are around 15-20 years old. The land is slightly sloped, 793-794 m asl, and the soil is Inceptisol. Corn and vegetables are planted between some citrus trees with cow manure application. Most citrus trees are surrounded by dense shrubs.

Soil analysis was carried out at the Laboratory of Soil Chemistry of the Agricultural Faculty of Nusa Cendana University, Kupang, East Nusa Tenggara, Indonesia.

Isolation and characterization of AMF spores

Soil samples were wet-sieved for spores and this was based on protocols in Brundrett et al. (1996) with minor changes. Air-dried soil (100 g) was suspended in 600 ml of tap water, stirred for 5 minutes, and the suspension was passed through 40, 125, 250, and 400 µm sieves, respectively The process was repeated 4 to 5 times until the suspension was relatively clear. The suspensions were transferred to 50 ml centrifuge tubes and centrifuged for 3 minutes at 2500 rpm. The supernatant was removed, 60% sucrose was added and then centrifuged again at 2500 rpm for 1 minute. The supernatant obtained was poured into a 40 µm sieve, rinsed with water, and then placed in a Buchner funnel laid with Whatman 41 paper. The AMF spores were removed from the Whatman paper and examined under a compound microscope (Olympus CX 24). The spores were sorted based on their size and color, counted, and mounted onto slides using PVLG (polyvinyl lacto-glycerol) with and without Melzer's reagent. The broken spores were discarded and spore density was expressed as the number of spores found per 100 g of soil.

The final characterization of the AMF spores into morphotypes was based on color, size, the surface of the spore, and subtending hyphae using a compound microscope at 100-400 magnification or higher. The descriptions and identifications were based on Schenck and Perez (1990), and the INVAM website (http://invam.caf.wvu.edu). Morphological characterization of the spores was carried out at the Microbiology Laboratory of Nusa Cendana University and the Laboratory of Forest Biotechnology, IPB University Bogor (Indonesia).

Arbuscular mycorrhizal colonization

Citrus fine roots were rinsed in water, cut into 1- 2 cm-long segments, and preserved in 50% ethanol before being processed. The presence of AMF structures was undertaken based on the modified protocols of Brundrett et al. (1996) and Moukarzel et al. (2020). Briefly, approximately 1-2 g sub-samples were rinsed with water and soaked in 10% (w/v) KOH overnight. The roots were removed from KOH, strained over 160 µm nylon mesh, rinsed with running water, and observed under the microscope. If the roots still contained pigment, they were placed again in 10% KOH for a half day to reduce the pigment content in the root. When the roots were almost clear, they were dipped in 10% KOH and heated for 15 minutes at 95 °C. Afterwards, the roots were transferred into a sieve, rinsed thoroughly under running water and soaked in 2% hydrogen peroxide for 20 minutes. The roots were then rinsed under running water, soaked in 1% HCl for 3 minutes, washed under running water and placed in 0.05% (w/v) Trypan blue (CI 23850) in lactoglycerol (1:1:1 lactic acid, glycerol, and water) overnight. The roots were de-stained in 50% glycerol for 30 minutes to remove excess dye. Fifteen to twenty pieces of roots were randomly taken and placed in a gridded petri dish and observed under a compound microscope (Olympus CX 24) for the occurrence of vesicles and arbuscules. The AMF colonization rate was calculated as the percentage of the colonized root versus the total observed segment (Brundrett et al., 1996). The measurement was repeated three times for each sample, and the average was then obtained.

Statistical analysis

The data were analyzed using Nested (Hierarchical) analysis of variance (ANOVA) with a 95% degree of significance where the health condition of the trees was nested within the geographical locations of the study (site) factor. When the main factor had a significant effect, the analysis was followed by Duncan's Multiple Range Test. (DMRT). These analyses were carried out using Microsoft Excel.

Results

Soil analysis

Soil characteristics of the study orchards are presented in Table 2.

Table-2. Soil physical and chemical properties of the 12 study sites

No	Site	Org-C (%)	Total-N (%)	Available P (mg.kg^{-1})	Total-P (mg/100)	K (cmol(+) .kg^{-1})	CEC (cmol(+) .kg^{-1})	pH	Texture
1	A1	0.92 (VL)*	0.15 (L)	41.76 (M)	70.60 (VH)	0.63 (VH)	35.29 (H)	7.07	Sandy loam
2	A2	1.08 (L)	0.09 (VL)	51.07 (H)	85.63 (VH)	0.84 (VH)	39.97 (H)	6.96	Sandy loam
3	A3	2.24 (M)	0.15 (L)	114.69 (VH)	127.72 (VH)	1.38 (VH)	40.90 (VH)	6.31	Sandy loam
4	Tn1	0.45 (VL)	0.05 (VL)	19.36 (L)	43.15 (H)	0.92 (VH)	27.34 (H)	7.10	Sandy loam
5	Tn2	1.27 (L)	0.09 (VL)	62.81 (VH)	104.81 (VH)	1.14 (VH)	49.77 (VH)	7.22	Sandy loam
6	Tn3	0.41 (VL)	0.09 (VL)	21.11 (M)	74.68 (VH)	1.93 (VH)	23.75 (M)	6.11	Sandy loam
7	N1	1.04 (L)	0.14 (L)	50.90 (H)	90.25 (VH)	1.12 (VH)	41.82 (VH)	7.39	Sandy loam
8	N2	1.24 (L)	0.08 (VL)	63.41 (VH)	108.45 (VH)	1.38 (VH)	40.37 (VH)	7.45	Sandy loam
9	O	1.93 (L)	0.12 (L)	94.16 (VH)	127.22 (VH)	0.96 (VH)	40.41 (VH)	7.25	Sandy loam
10	B	0.93 (VL)	0.12 (L)	43.91 (M)	77.44 (VH)	0.90 (VH)	29.14 (H)	5.75	Sandy loam
11	Tb	0.70 (VL)	0.12 (L)	33.20 (M)	65.10 (VH)	1.05 (VH)	27.01 (H)	7.04	Sandy clay loam
12	Kn	2.88 (M)	0.13 (L)	146.70 (VH)	130.43 (VH)	1.38 (VH)	40.97 (VH)	7.0	Sandy clay loam

*)VL (very low), L (low), M (medium), H (high), VH (very high)
Note: *): Indonesian Center for Agricultural Land Resources Research and Development (ICALRRD) (2009)

The criteria used to interpret the results of soil analyses was based on ICALRRD (2009). The 12 study sites had very low to low levels of organic-C, except for Ajoubaki 3 (A3) and Kesetnana (Kn) sites that had medium soil organic-C. In addition, the soil total-N was very low to low in all study sites. Total-P of soil for all sites was high to very high level. However, available P varied from a low level at Tunua 1 (Tn 1; 19.36 mg.kg^{-1}**)** to medium at Tunua 3 (TN3; 21.11 mg.kg^{-1}), Tubuhue (Tb; 33.2 mg.kg^{-1}), Ajoubaki 1 (A1; 41.76 mg.kg^{-1}), and Buat (B; 43.91 mg.kg^{-1}) sites, to high and very high for the remaining sites. Potassium in all orchards was very high, and the CEC ranged from high to very high, except for Tunua 3 (Tn 3) which was in the medium category. The soil had neutral to slightly alkaline pH except at Buat (B), Ajoubaki 3 (A3), and Tunua 3 (Tn3) sites that were slightly acid pH with values of 5.75, 6.11, and 6.31, respectively. The soil texture of ten study sites were sandy loams, but the Tubuhue (Tb) and Kesetnana (Kn) sites had sandy clay loams (Table 2).

AMF spore density

Nested analyses showed that the health condition of the citrus trees had no significant effect on the spore density of AMF. However, Nested analysis indicated that the geographical location/site where the soil samples were taken significantly affected the spore density of AMF. The highest spore density of AMF was in the rhizosphere of citrus in Buat with an average of 892.8 spores/100 g soil, while the lowest of 241.2 spores/100 g soil was in the rhizosphere of Citrus from Nusa 2 site (Figure 2).

Characterization and occurrence of AMF spores

Fourteen (14) AMF spore morphotypes were found in this study (Figure 3) belonging to three genera, *Glomus*, *Acaulospora*, and *Gigaspora*. The most common morphotypes were *Glomus* spp. followed by *Acaulospora* spp. and *Gigaspora* spp. with 7, 6 and 2 morphotypes, respectively.

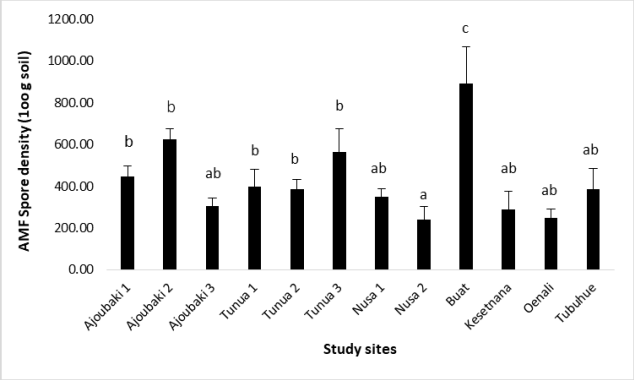

Figure-2. Spore density of AMF in the rhizosphere of citrus at 12 study locations. Value of each bar is an average of six replicates with ± standard error (SE). Bars with the same letter are not different at P = 0.05 using DMRT

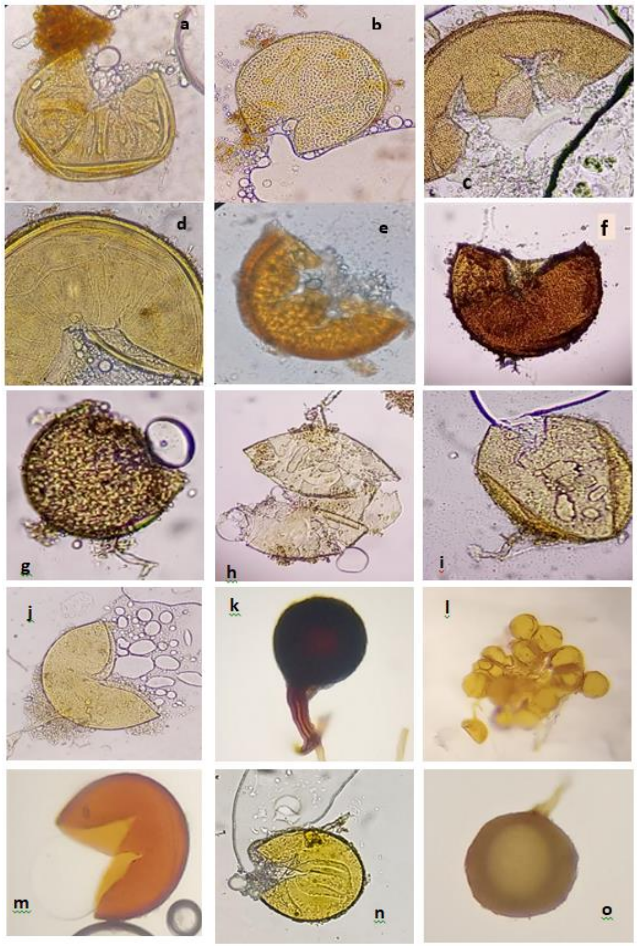

Figure-3. Morphotypes of AM fungal spores: a-f are morphotypes of *Acaulospora* , g-m are morphotypes of *Glomus*, and n, o are morphotypes of *Gigaspora*. The magnification for a-j and n is 1600x, and for k, l, m and g is 400x. The size of spores are 100 -150 µm, except for n (around 200 µm), h (around 80 µm), and l (around 40 µm)

Based on the morphological characteristic of the spores, there was a greater diversity of AMF in the rhizosphere of healthy citrus trees than in the declining trees (Table 3). In nine out of 12 sites, the number of AM morphotypes were higher in the rhizosphere of healthy citrus trees than in the declining citrus trees, excluding A2, N1 and Tn1 sites where the number of AM morphotypes were the same between the healthy and declining trees. Most notably, at A1 and O sites the number of morphotypes in the declining trees were very low compared to the number of morphotypes observed in rhizosphere of healthy trees. Some morphotypes of *Acaulospora* and *Glomus* were generalists as they were found in almost all the citrus orchards, but some morphotypes were restricted to particular sites. By contrast, morphotypes of *Gigaspora* were restricted to a few sites, and were found only in the rhizosphere of healthy citrus trees.

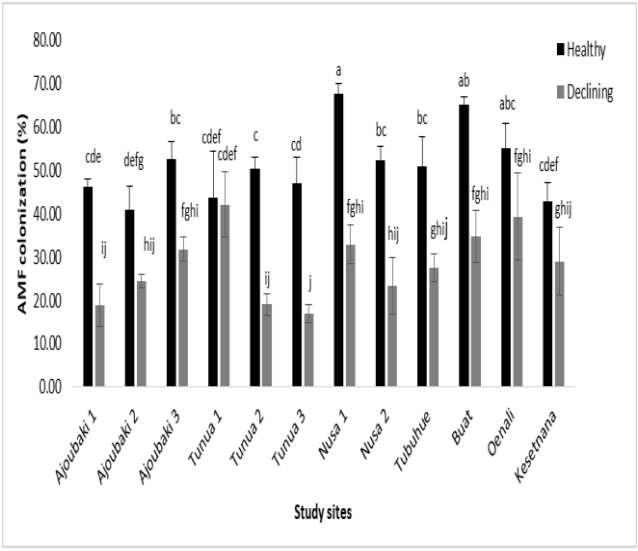

Figure-4. Arbuscular mycorrhizal colonization of fine roots in healthy and declining citrus at 12 sites. Each value is an average of three replicates with ± SE. Bars with the same letter are not different at P = 0.05 using DMRT.

Table-3. Occurrence of AMF spore morphotypes in the rhizosphere of healthy (H) and declining trees (S) in the 12 study sites

Site	*Glomus*							*Acaulospore*						*Gigaspore*		H	S	%H	%S
	1	2	3	4	5	6	7	1	2	3	4	5	6	1	2				
A1H	+	+	+		+	+	+		+		+	+	+			10		**83.3**	
A1S	+	+				+			+								4		**33.3**
A2H	+	+		+		+	+		+	+			+			8		**66.7**	
A2S	+	+	+	+		+	+		+	+							8		**66.7**
A3H		+	+	+		+	+		+				+	+		8		**66.7**	
A3S		+		+		+	+			+	+						6		**50**
KnH	+	+		+		+	+		+		+			+	+	9		**75**	
KnS		+	+	+		+	+		+			+					7		**58.3**
N1H	+		+	+		+	+				+	+	+			8		**66.7**	
N1S	+		+	+		+	+	+	+		+						8		**66.7**
N2H	+	+	+	+		+	+		+	+	+					9		**75**	
N2S				+			+		+		+	+					5		**41.7**
OH	+	+	+	+			+		+		+	+	+			9		**75**	
OS	+	+					+										3		**25**
TbH	+	+	+		+		+		+	+	+	+		+		10		**83.3**	
TbS		+	+				+	+	+			+					6		**50**
BH		+	+	+	+		+	+			+	+	+	+		10		**83.3**	
BS		+	+	+			+		+								5		**41.7**
Tn1H		+		+	+						+			+	+	6		**50**	
Tn1S	+	+	+						+		+	+					6		**50**
Tn2H	+	+	+	+			+	+	+							7		**58.3**	
Tn2S		+		+					+		+		+				5		**41.7**
Tn3H	+	+		+			+	+	+				+	+		8		**66.7**	
Tn3S	+	+	+	+		+	+		+								7		**58.3**

Nested analysis also revealed that the geographical location (site) factor had a significant influence on root colonization by AMF (Figure 5). Among the 12 sites, Nusa 1 and Buat exhibited the highest value of 50.3% and 50%, respectively. However, this was not significantly different from the colonization observed at Tunua 1 and Oenali. The lowest of 32% was observed in citrus grown at Tunua 3, but it was not significantly different from others, except for Tunua 1, Oenali, Buat, and Nusa 1, as shown in Figure 5.

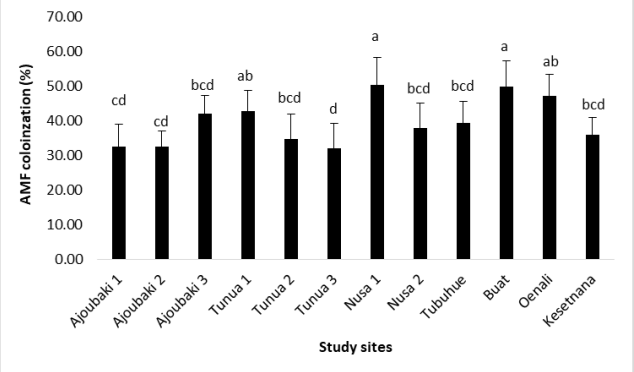

Figure-5. Arbuscular mycorrhizal colonization in citrus grown at 12 sites. Each value of AM fungal colonization is an average of six replicates with ± standard error (SE). Bars with the same letter are not different at P = 0.05 using DMRT.

Discussion

It is well known that citrus is heavily dependent on AMF for mineral and water absorption due to its limited root hairs. However, there are several complex factors that can affect the development of these fungi in the rhizosphere including host, soil physicochemical properties, and soil management (Wang et al., 2012; Wang and Wu, 2016). Furthermore, the composition of the AMF communities may be related to the health condition of the host plants (Yu et al., 2012; Binet et al., 2020).

This study showed that the health condition of the citrus trees and the sites where the soil samples were taken had different effects on the abundance of AMF in terms of spore density and root colonization. The spore density in the rhizosphere was influenced by the sampling region, while the health condition of the citrus trees had no effect. However, AMF colonization was affected by both factors. This raises the question as to whether the capacity of these fungi to colonize the citrus roots is more sensitive to the health condition of the trees than their ability to sporulate, or whether sporulation was responding to factors other than the fitness of the host. In addition, the diversity of AMF morphotypes tended to be higher in the rhizosphere of healthy trees than in declining trees.

In this study, the spore density was only affected by the sites where the soil samples were collected. Since this study was carried out in 12 different sites, where the conditions are highly variable in terms of nearby vegetation, age of citrus trees, altitude, soil, and management of the orchards, it is not surprising that the site factor strongly affected the number of spores. Although AMF have low host specificity (Smith and Read, 2008), host specificity may also occur (Torrecillas et al., 2012), thus it is likely that the presence of other host species in or near citrus orchards may influence the abundance and diversity of AMF. For example, the grass or Gramineae family are alternative hosts for AMF (Wang and Wu, 2016), and were shown to be beneficial in citrus orchards (Xiao et al., 2022). However, some weeds may not favor the development of AMF in the desired crop, and weed control can facilitate more efficient colonization of the crop species (Brito et al., 2013). In this study, grass was the common natural vegetation for land cover in some sites, while citrus trees were surrounded by dense shrubs in others. Furthermore, in some sites, maize and other crops, which can be potential hosts for AMF, were intercropped between the citrus trees. These practices may contribute to the site effect on AMF spore density. Further research is required to identify the relative contribution of different orchard management practices on AMF population in citrus orchards.

Based on the result of soil analysis, it was surprising that organic C was low to very low in all study sites, except at Kesetnana and A1 sites with moderate level of organic C. Similarly, total-N was in the low to very low categories in all sites. This is the first study to report the possible involvement of soil nutrition in the declining citrus in TTS. Therefore, more efforts are needed to deeply investigate the relationship between soil fertility and citrus health. For the development of AMF in the soil, it has been reported that organic C and total-N had a positive correlation to spore number and root colonization (Wang et al., 2015). In this study, organic C and total-N were low in all sites, but the number of spores and root colonization were high and low in some of them. This condition reflects the genetic variation of the fungi in response to the soil mineral condition (Wang and Wu, 2016). Some species are insensitive to low or high soil mineral content, while other species are highly sensitive, and can be severely impacted by this condition.

Apart from soil organic C and total-N, it has also been reported that the AMF communities in citrus orchards are strongly affected by pH, soil management, and available phosphorus (Wang et al., 2012). In this study, soil management, such as the application of fertilizer as well as types of crops for intercropping between the plants was different between orchards. This indicates that it may affect the sporulation and colonization of AMF between the sites. The available P is an important soil mineral that mainly favors the development of AMF when it has a low availability (Smith and Read, 2008). The sporulation and colonization of these symbionts were inconsistent with soil P level. They were also high at some sites where the available P was in the low to medium level as well as high. AMF species/isolates can respond differently to available soil P. Some species/isolates may be impacted by the high P availability depending on the genetic variation of the fungi (Wang and Wu, 2016; Ishaq et al., 2021).

In this study, 14 morphotypes of AMF belonging to *Glomus*, *Acaulospora*, and *Gigaspora* were observed in the rhizosphere of citrus trees. Previously, a similar diversity of AMF morphotypes were observed in the rhizosphere of *Citrus* sp. in Gianyar district, Bali province, Indonesia (Suamba et al., 2014). This

diversity is slightly lower than in citrus orchards in mountainous areas of Southern China (Wang et al., 2013). It has been suggested that elevation can influence the abundance of AMF (Wang et al., 2013). In this study, the citrus orchards were located in hilly areas that were above 600 m asl. Sampling in this study was carried out once, and as sporulation of AMF is likely to be seasonal, repeated sampling across seasons is desirable in the future. Also, in the future, the use of molecular techniques should be used to determine the AMF taxa in citrus orchards in the region.

Arbuscular mycorrhiza fungi have been reported to play an important role in plant health and can potentially be used for biological control of some pests (Song et al., 2015; Khairani et al., 2017; Fiorilli et al., 2018). Although the number of AMF spores was not impacted by the health condition of the trees, which was possibly due to the presence of other nearby potential host plants and site condition, the root colonization by AMF was influenced by the fitness of the host. This indicates that AMF colonization is sensitive to the health status of the host. These results are consistent with previous studies reporting that the fitness level of the host plants can affect the development of the fungi (Yu et al., 2012; Corredor et al., 2014; Binet et al., 2020). AMF are obligate biotrophic symbionts, which cannot live without photosynthates from plants (Smith and Read, 2008). It has also been estimated that plants contribute 10 to 30% of the total photoassimilate carbon to the fungal symbiont in exchange for up to 90% of their nutritional requirement (Walder and van der Heijden, 2015). Since AMF organic carbon supply is dependent on the host plants, any changes in net primary production inevitably affect their development. Furthermore, it has been suggested that in the symbiosis, resource exchange occurs between AMF and the host plants. The cost and benefit between these two symbionts also determine the net outcome of the interaction (Salmeron-Santiago et al., 2022). When the benefits for the host exceed the nutrient consumption of AMF, the fungi can provide symbiosis for the plants. However, when the benefits are less, the AMF takes advantage of organic carbon from the host with less contribution, and this depresses plants growth. Walder and van der Heijden (2015) proposed a hypothesis that in symbiosis, conditional mutualism occurs at a certain condition. Accordingly, AMF are not beneficial in every situation because the resource exchange could be regulated by other factors, such as competition for surplus resources, functional diversity, and sink strength.

The results of this study showed that soil chemical properties, orchard management, and AMF are involved in the health condition of citrus trees in the field. Organic C and N are important soil factors that strongly affect plant health. Furthermore, since citrus is dependent on AMF for their nutrient and water absorption due to the scarcity of root hairs, more attention must be paid to investigating fungi that are beneficial in the field. Diseases are also related to the declining citrus population in the region. This indicates that it is important to investigate the type of AMF that have a role as a biological fertilizer as well as a biological agent for *Jeruk Keprok Soe*. Research will now focus on identifying compatible orchard management practices in combination with effective strains of AMF and their introduction into orchards to improve tree health.

Conclusions

Arbuscular mycorrhizal fungi and soil factor are related to declining citrus in East Nusa Tenggara, Indonesia. Number of AMF spore was affected by geographical location/site factor, and the colonization of AMF was affected by geographical location and the health condition of citrus trees. AMF colonization and diversity of was low under the declining citrus. Soil nitrogen and organic-C are low in almost all study sites and may be involved declining citrus.

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Ishaq L: Planned and managed the study, performed experiment, data collection, data analysis and wrote the manuscript.
Simamora AV & Benggu YI: Performed experiment, data collection and wrote the manuscript.
Bako PO & Roefaida E: Performed experiment, interpreted data and wrote manuscript.
Airthur MM & Nguru ESO: Designed research methodology, performed data analysis and interpretation.