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Short Communication: First report of *Nectria haematococca* causing a moler disease on shallots in West Nusa Tenggara, Indonesia

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Abstract. *Mariani, Suprapta DN, Sudana IM, Temaja IGRM, Sudantha IM. 2022. Short Communication: First report of* Nectria haematococca *causing a moler disease on shallots in West Nusa Tenggara, Indonesia. Biodiversitas 23: 2768-2774.* Moler disease caused by *Nectria haematococca* is the main disease of shallots in West Nusa Tenggara, Indonesia. Moler disease caused significant yield loss and even crop failure. The purpose of this study was to isolate and identify the pathogen causing moler disease on shallots. This research was conducted from February 2018 to November 2019. Survey was conducted at 13 shallot planting locations in West Nusa Tenggara, namely: Ngali, Ncera, Cenggu, Renda, Sajang, Rensing, Dasan Baru, Batu Layar, Anyar, Sembalun, Jerowaru, Batujai and Sakuru Village. Ten infected plants were taken, at each observation point, then put in a labeled plastic bag and brought to the laboratory for isolation purposes. The results showed that pathogen causing moler disease on shallot was isolated NTB2018. On PDA isolate NTB2018 produced white color colony, the mycelium growth direction was sideways with smooth mycelium structure, sideways growth direction of mycelium and fine structure mycelium. The fungus produced insulated mycelium consisting of macroconidia and microconidia as asexual spores and ascospores as sexual spores. Based on analysis of 18S rRNA gene, isolate NTB2018 DNA fragment of ±569 bp.

Keywords: *Fusarium* sp., Indonesia, moler- disease, *Nectria haematococca*, shallots

INTRODUCTION

Shallots (*Allium ascalonicum* L.) are vegetables that have high economic value (Saidah et al. 2020) and nutritional content. In addition, shallots can be used as a traditional medicine to reduce high fever in infants and children and can also increase body's endurance (Mariani and Sugiarta 2016; Tripati and Lawande 2016; Romadhon and Mudji 2018). In Indonesia, there is great potential for shallot cultivation. Throughout the year, shallots are planted on agricultural land from Aceh to Merauke, including the West Nusa Tenggara region. In 2015, West Nusa Tenggara was designated as one of the national shallot centers to meet domestic and international demand, with annual exports ranging from 18,000 to 30,000 tons. Shallots from West Nusa Tenggara are exported to a number of countries, including Malaysia, Singapore, Vietnam, and several others (Maharani 2015).

The Provincial Government of West Nusa Tenggara (2019) reported that shallot production decreased from 2016 to 2017 in West Nusa Tenggara, from 2,118,037 tons/ha in 2016 to 1,954,577 tons/ha in 2017. The decrease in shallot production in West Nusa Tenggara was 19275 ha in 2016 to 17904 ha in 2017. The productivity of shallots in West Nusa Tenggara decreased from 2015 to 2017 from 110.30 tons/ha to 109.17 tons/ha.

Moler disease is the main disease of shallots, which causes significant reduction in productivity (Ilhe et al. 2013; Umiyati 2017; Futane et al. 2018; Safitri et al. 2019). Moler disease of shallots is caused by fungus *Nectria haematococca.* Moler control efforts that are mostly carried out in West Nusa Tenggara are chemical control, technical culture, and the use of resistant varieties, but have not been able to control the disease. This is indicated by the incidence of disease which continues to increase until it reaches 100% (Direct survey in Batu Jai Village and Jerowaru Village, 2019). This is because the use of synthetic pesticides is ineffective in controlling moler disease. Sudantha (2015), reported that the symptoms shown by diseased shallots were sudden wilt in shallots, twisted and shriveled, finally leaves became drooped and dry and eventually shallot bulbs became rot. Therefore, it is necessary to isolate and identify the pathogens that cause moler disease in shallots in West Nusa Tenggara.

Moler disease control on shallots in West Nusa Tenggara is known to be ineffective either on different shallot varieties or at different altitudes. This can be seen from the high incidence of moler disease in shallot plantation areas in West Nusa Tenggara which reached 100% in some shallot plantation areas. Ineffective control is caused by the habit of red onion farmers who are not wise in applying environmentally friendly controls, namely

the application of synthetic pesticides to control moler disease is carried out excessively, with very high doses, resulting in resistance to pathogens. In addition, this can cause the shallot plantation area to become poor in antagonists to inhibit the development of pathogenic spores that cause moler disease in shallot plants. Therefore, it is necessary to isolate and identify the pathogens that cause moler disease on shallots in West Nusa Tenggara as an effective control effort against moler disease in shallots.

Based on the aforementioned information, the objectives of this study were as follows: (i) to determine the symptoms of moler disease in shallots, (ii) to determine the incidence of moler disease in shallots, and (iii) to identify the pathogenic species causing moler disease on shallots.

MATERIALS AND METHODS

Instruments and materials

The glassware and instruments used were petri dishes, erlenmeyer, test tubes, beakers, measuring cups, microscopic, ent needles, spirit lamps, cork borer, analytical balances, laminar airflow, and office stationery. Whereas materials used were samples of sick shallots, soil samples from the rhizosphere of sick shallots, PDA media, WA media, aquadest, alcohol, cling wrap, aluminum foil, spirit solution, water, methylene blue, antibiotics, PCR primers, and pathogenic fungal isolate.

Survey for moler disease incidence

In West Nusa Tenggara, thirteen villages where shallots were grown were surveyed for the incidence of moler disease. The disease incidence was observed on 100 shallot plants at five sites per location. To determine disease prevalence, the number of plants with moler symptoms was tallied. Disease incidence was determined by observing 10 plants per sampling location.

Sampling for moler disease in shallots

Shallots samples were taken from the thirteen villages in districts and cities of West Nusa Tenggara, namely Ngali, Ncera, Cenggu, Renda, Sajang, Rensing, Dasan Baru, Batu Layar, Anyar, Sembalun, Jerowaru, Batujai, and Sakuru village. Observations were made on the symptoms of moler disease that occurred in each village. Observation was carried out on diseased shallot leaves and bulbs. Thirteen sampling points for sick shallots were determined based on observations in each village. Different varieties of shallots were grown at all 13 sampling sites. Sampling of diseased shallots plants was carried out by 20% of plants out of total population. Ten infected plants were taken, at each observation point, then put in a labeled plastic bag and brought to the laboratory for isolation purposes.

Isolation and identification of pathogen causing moler disease in shallots

Total of thirteen samples of symptomatic shallots was taken for the isolation of pathogen. The pathogenic fungus was isolated according to Li-Wang et al. (2019). Symptomatic shallots were cut and then inoculated in water agar (WA) media. Then the fungal spores were isolated on potato dextrose agar (PDA) media and incubated at 25°C for 7 days. One of 13 fastest growing isolates was inoculated into shallots. The development of symptom was observed 7 days after incubation. The fungus re-isolated from infected shallots thus pathogenicity of respective pathogen was confirmed. The fungus was then maintained on PDA slant medium for further analysis.

The pathogen was identified at the genus level by observing the characteristics of mycelium, colonies, conidia, conidiophores, and hyphae (Klaubauf et al. 2014). Morphological identification was done using the key of Alexopoulus and Mims (1979). Molecular identification was carried out according to Khalimi et al. (2019). Further observations were made on the hyphal structure of pathogenic fungus using scanning electron microscopic (SEM) according to Kawuri (2012).

Molecular identification

Fungal pathogen was cultured on PDA media for three days at room temperature (28±2°C). The genomic DNA was extracted by taking hyphae from the edge of colony. The sample was then vortexed for 30 seconds and kept on hot plate at a temperature of 95 to 100°C for 10 minutes and allowed to stand at room temperature for 2 minutes. The sample was then centrifuged at 10,000 rpm for 2 minutes and pellets containing DNA were taken (Kawuri 2012). Genomic amplification of fungal DNA was amplified using universal primers ITS1 and ITS4. The PCR cycles included 3 minutes of pre-denaturation at 95°C, 30 seconds of denaturation at 95°C, 30 seconds of annealing at 50°C, 1 minute of extension at 72°C, 1 minute of final extension at 72°C. PCR products were analyzed by electrophoresis in 1% agarose gel and treated with 0.75 l DNA dye (Nandika et al. 2021; Putri et al. 2022). DNA double helix sequences were assembled and analyzed using Genetyx (version 11.0) and Genetyx-ATSQ (version 4.0) software (Genetyx, Tokyo, Japan) sequentially and compared with same DNA sequences accessed from DDBJ/ EMBL/ GenBank via NCBI BLAST program (Parwanayoni 2018). The phylogenetic analysis was carried out using MEGA 4.0 program. Phylogenetic tree was created based on the method of Khalimi et al. (2019).

RESULTS AND DISCUSSION

Disease incidence of moler on shallots

Moler disease was observed in all thirteen shallot-growing villages of West Nusa Tenggara. The disease incidence ranged from 24.40 % to 100% , with an average of 64.74%. It was noticed that in West Nusa Tenggara, four varieties of shallots were commonly cultivated, namely Bima Brebes, Bali Karet, Philifine, and Cultivar Ampenan. All of these cultivars were found susceptible to moler disease (Table 1).

Table 1. Disease incidence of moler on shallots in 13 villages of West Nusa Tenggara, Indonesia

Survey site	Disease incidence (%)	Varieties of shallots
Village of Ngali	61.20	Bima Brebes
Village of Ncera	61.20	Bima Brebes
Village of Cenggu	62.00	Bima Brebes
Village of Renda	59.65	Bima Brebes
Village of Sajang	52.80	Bali Karet
Village of Rensing	47.20	Philifine
Village of Dasan Baru	83.20	Cultivar Ampenan
Village of Batu Layar	24.40	Cultivar Ampenan
Village of Anyar	67.60	Cultivar Ampenan
Village of Jerowaru	100.00	Philifine
Village of Sembalun	72.40	Bali Karet
Village of Sakuru	49.60	Bima Brebes
Village of Batujai	100.00	Bima Brebes
Average disease incidence (%)	64.71	

Symptoms of moler disease on shallots

Symptoms shown by diseased shallots were sudden wilt in shallots, twisted and shriveled, finally leaves became drooped and dry and eventually shallot bulbs became rot as presented in Figure 1A, while healthy shallots showed no symptoms (Figure 1B). The results of this study also showed that the disease symptoms were similar in different shallow varieties in all samples from 13 villages (Figure 2).

Morphology of *Nectria haematococca*

On PDA media *N. haematococca* produced white thick cottony mycelium expanded like a cloud, periphery of the colony was uneven, front and reverse surface of the colony was white, the mycelium growth direction was sideways with smooth mycelium structure, and the diameter of colony was 9 cm (Figure 3A), have microconidia (Figure 3B1), macroconidia (Figure 3B2), chlamydospores (Figure 3C), and have ascospore in ascus as a sexual spores.

Molecular identification of *Nectria haematococca*

The molecular identification result showed that isolate NTB2018 produced DNA fragment was then purified and subjected to sequencing to determine the species of the fungus.

Based on sequence comparison with database of GenBank, isolate NTB2018 showed homology with several isolates of *Fusarium* spp. such as: *Fusarium solani* strain FsDAG11 (KX583231.1), *Fusarium phaseoli* isolate PR1 (KF717534.1), *N. haematococca* (AY310442.1), *Fusarium solani* isolate TN252D16 (MH329792.1), *Fusarium* sp. isolate X122 (KX008640.1), *N. haematococca* strain NKCM3301 (LC097319.1), *Fusarium solani* strain Cc-149 (JQ910159.1), *Fusarium* sp. BAB-4825 (KU571534.1), *N. haematococca* isolate 164AS/R (GU066713.1), and *Fusarium solani* strain GFW-1 (MK418649.1) (Table 2, Figure 5). The result of phylogenetic tree analysis showed that the fungus had a percentage similarity of 99.82% with *N. haematococca* (AY310442.1). Based on this percentage of similarity can be concluded that species of isolate NTB2018 is *N. haematococca*.

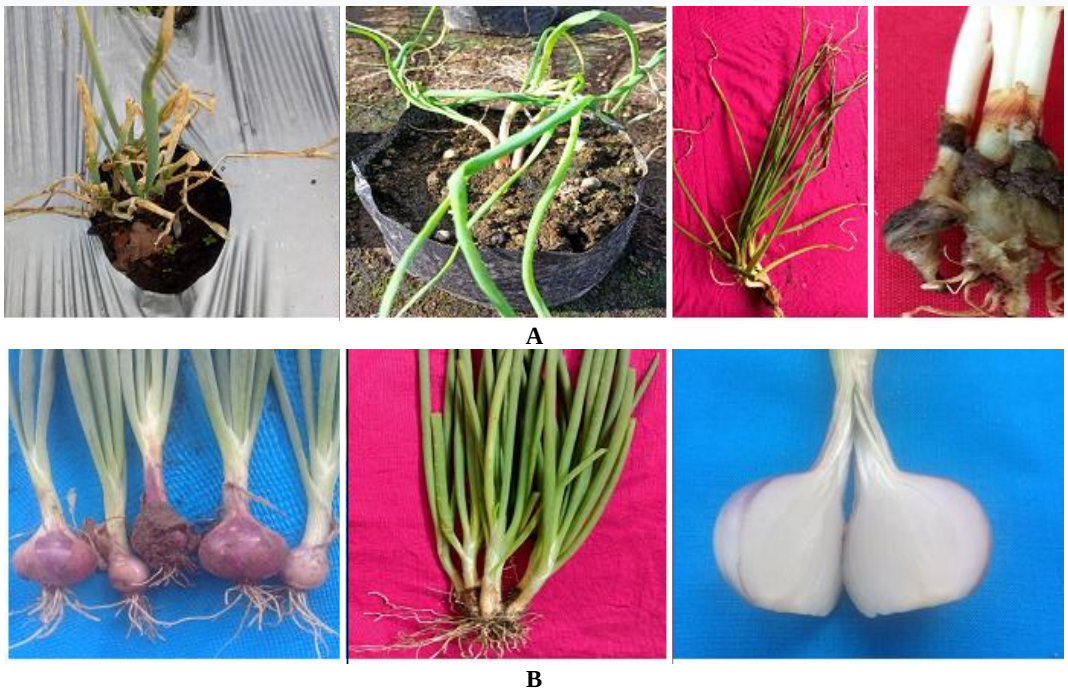

Figure 1. A. Symptoms of moler disease, B. Healthy shallots

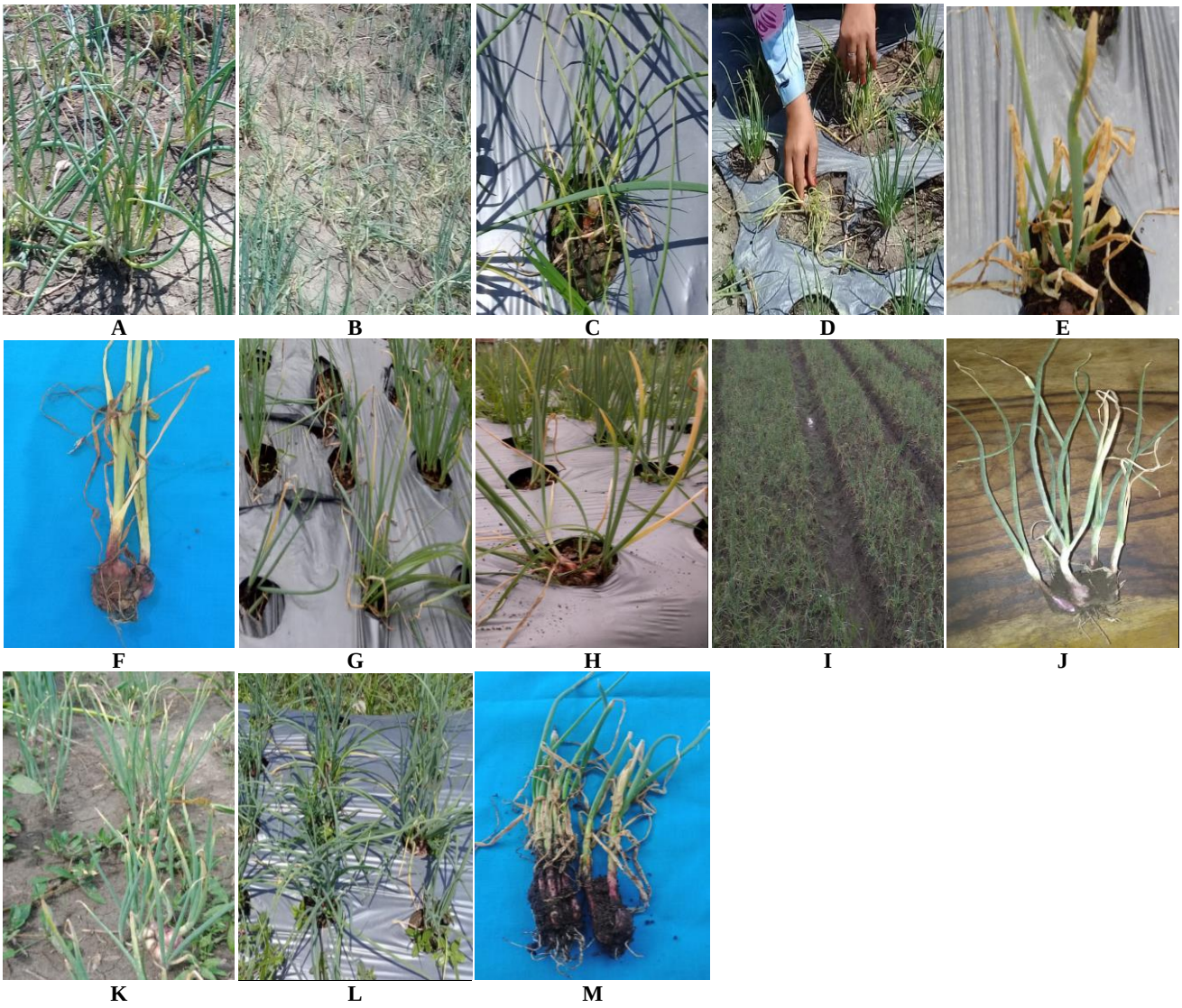

Figure 2. Symptoms of moler disease in different shallot varieties: A. Desa Ngali, var. Bima Brebes; B. Desa Ncera, var. Bima Brebes; C. Desa Cenggu, var. Bima Brebes; D. Desa Renda, var. Bima Brebes; E. Desa Sajang, var. Bali Karet; F. Desa Rensing, var. Philifine; G. Kelurahan Dasan Baru, Kultivar Ampenan; H. Desa Batu Layar, Kultivar Ampenan; I. Desa Anyar, Kultivar Ampenan; J. Desa Batujai, var. Bima Brebes; K. Desa Jerowaru, var. Philifine; L. Desa Sakuru, var. Bima Brebes; M. Desa Sembalun, var. Bali Karet

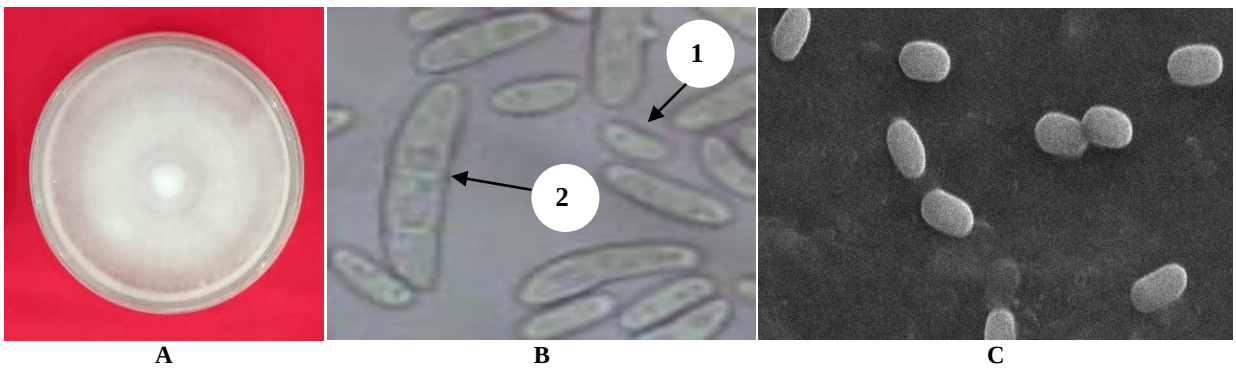

Figure 3. Morphology of isolate NTB2018 causing shallots moler disease in West Nusa Tenggara: A. Colony, B.1. Microconidia, B.2. macroconidia, C. Chlamydospores

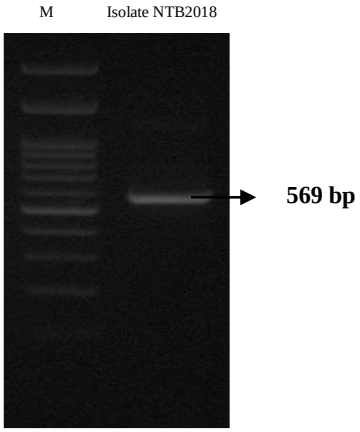

Figure 4. PCR amplification of 18S rRNA gene of isolate NTB2018 (arrow), and M. 1 kb DNA marker

Table 2. Percentage of similarity of isolate NTB2018 causing shallots moler disease in West Nusa Tenggara, Indonesia, with several homologous sequences in the gene bank

Isolates	Percentage similarity (%)
Fusarium solani Strain FsDAG11 (KX583231.1)	99.82
Fusarium phaseoli Isolate PR1 (KF717534.1)	99.82
Nectria haematococca (AY310442.1)	99.82
Fusarium solani Isolate TN252D16 (MH329792.1)	99.82
Fusarium sp. Isolate X122 (KX008640.1)	99.82
Nectria haematococca Strain NKCM3301 (LC097319.1)	99.82
Fusarium solani Strain Cc-149 (JQ910159.1)	99.82
Fusarium sp. BAB-4825 (KU571534.1)	99.65
Nectria haematococca Isolate 164AS/R (GU066713.1)	99.82
Fusarium solani Strain GFW-1 (MK418649.1)	99.65

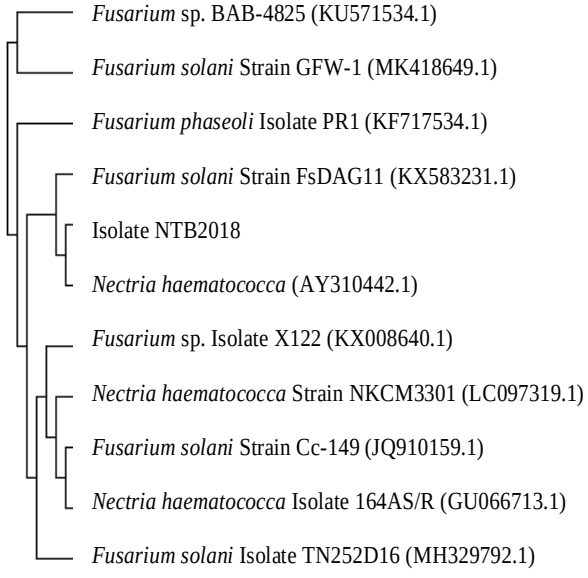

Figure 5. Phylogenetic relationship tree of isolate NTB2018 with several isolates of fungi based on 18S rRNA gene using maximum parsimony method

Discussion

The result of present study showed that moler disease caused by *Nectria haematococca* (*Fusarium solani)* occurred in West Nusa Tenggara. The colony of *N. haematococca* was white in color from reverse front side, mycelium growth direction was sideways and its structure was smooth. On PDA medium an insulated mycelium was found, consisting of macroconidia and microconidia asexual spores and ascospore as sexual spores. *N. haematococca* is a sexual reproduction of *Fusarium solani* (Wu et al. 2011; Yang et al. 2018). Jee et al. (2005), also reported that asexual characteristic of *N. haematococca* is similar to that of *F. solani* having macroconidia and microconidia, while sexual characteristics are similar to that of ascospore contained in ascus. *Nectria haematococca* produced monophialides, microconidia, macroconidia and chlamydospores (Garibaldi et al. 2016).

According to Soleha et al. (2021) *Fusarium oxysporum* causes vascular wilt disease in *A. mangium*. Fusarium survive by forming chlamydospores so that they can survive saprophytically and for a long time (Koyyappurath et al. 2016; Rana et al. 2017; Muslim et al. 2019). In addition, it attacks almost all types of plants, even weeds (Joshi 2018), such as water hyacinth weeds (Alwiguna 2010). *Fusarium* sp. can live as saprophytes or parasites. At the time *Fusarium* sp. live in plant tissue, it will be parasitic, after the shallots die and there is no host, then *Fusarium* sp. will live as saprophytes in the soil by forming a defensive structure in the form of chlamydospores. Chlamydospores from *Fusarium* sp. can survive in the soil and will germinate when environmental factors support their growth. *Fusarium* sp. can survive as saprophytes for about 3 years (Mariani 2009).

Molecular identification results revealed that DNA fragments of *N. haematococca* produced ±569 bp. The phylogenetic tree analysis showed that the pathogenic fungus had a close relationship with *N. haematococca* (AY310442.1) with a percentage similarity of 99.82%. According Garibaldi et al. (2016), DNA of *N. haematococca* produced 413 bp. Taieb and Triki (2016) reported that revealed 99% homology of the amplified product with a reference sequence of *N. haematococca*. Yang et al. (2018) also reported that *N. haematococca* has chromosome length of 454 found on rhizosphere *Notoginseng* in China.

The results showed that disease symptoms were similar in all diseased shallots of different shallot varieties collected from 13 villages. The presence of the same molar disease symptoms in several different varieties in each village was caused by the fungus *N. haematococca*. *N. haematococca* is first reported from shallots in West Nusa Tenggara, Indonesia. Other researchers reported that *N. haematococca* is associated with *Cucurbita* (Hawthorne et al. 2001), piperis (Duarte and Simon 2003), paprika (Jee et al. 2005), *Jatropha curcas* in China (Wu et al. 2011), notoginseng in China (Yang et al. 2018), *Annona muricata* L. at postharvest storage in Mexico (Rubio-Melgarejo et al. 2021), *Mammillaria zeilmanniana* in Italy (Garibaldi et al. 2016), and dieback of olive tres in Tunisia (Taieb and Triki 2016). *N. haematococca* is also found to be associated with

various cultivated plants (O'Donnel 2000). Based on the results of this study, it can be suggested that the effective control of shallot moler disease should be applied in West Nusa Tenggara, Indonesia.

Several researchers of Indonesia reported that moler disease is caused by *Fusarium oxysporum* (Suganda dan Satryo 2017) and can cause a decrease in shallots yield between 10-40% (Lutfia 2018). Juwanda et al. (2016), reported that moler disease is the main disease in shallots caused by *F. oxysporum* f. sp. *cepae* (Foc). Losses due to moler disease can reach up to 50%, even causing crop failure. As a result, the quality and quantity of crop yield decrease. According to Umiyati (2017), one of the destroyers of shallot cultivation is the attack of moler disease caused by *Fusarium* sp. In addition, Hidayati et al. (2019) reported that the disease that attacks shallots is moler disease, caused by *F. oxysporum*. Sari and Siti (2020) observed that moler disease is caused by *F. oxysporum* f.sp. *cepae* is occurred in the Trisula variety of shallots, with the symptoms of necrosis and chlorosis, and almost all parts of the plant became infected. Hikmahwati et al. (2020) reported that efforts to increase shallot production are often constrained by the presence of *F. oxysporum* f.sp. *cepae* which can reduce shallots production. Wibowo et al. (2016) also reported that moler disease on shallots is also found in wetlands.

The results of the present study concluded that (i) Diseased shallots exhibited sudden wilt, twisting, and shriveling, followed by leaves that drooped and became dry and shallot bulbs that rotted, (ii) the incidence of moler on shallots in West Nusa Tenggara was 100 percent with an average of 64.71% and (iii) the pathogen responsible for moler disease on shallots was isolated NTB2018. On PDA isolate NTB2018, the colony color was white, and the mycelium growth direction was lateral with a smooth mycelium structure, lateral mycelium growth direction, and smooth mycelium structure. The fungus produced insulated mycelium composed of asexual spores macroconidia, microconidia and sexual spores ascospores. Based on the analysis of the 18S rRNA gene, isolate NTB2018 had a 569-bp DNA fragment that was 99.82% identical to *N. haematococca* strain NKCM3001 and *N. haematococca* isolate 154AS/R.

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