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RESEARCH ARTICLE

FUNGAL CONTAMINANTS ON GARLIC SEEDS:CASE STUDY FROM GARLIC STORAGES IN SEMBALUN HIGHLANDS OF EASTERN LOMBOK - INDONESIA

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Abstract

Garlic (*Allium sativum* L.) is one of the horticultural commodities of high economic value in Indonesia where Province of West Nusa Tenggara (NTB) is one of the largest garlic-producing areas. One of the main constraints facing by farmers during postharvest storage is seed-borne fungal contaminants. This study aims to investigate fungal contaminants on garlic seeds in the Sembalun Highlands (1200 m above sea level) of East Lombok Regency, West Nusa Tenggara (NTB) Province of Indonesia. Research was conducted in September–November 2018 using Blotter on Test method. Garlic seed samples (*Sangga Sembalun* variety) were collected from farmers garlic storages in Sembalun Highlands. Preparation of garlic samples, incubation, and observation were conducted at the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Mataram University. Identification was conducted based on Barnett and Hunter (1998) and data percentage of infection were recorded. Results show that there were two main seed-borne fungal contaminants isolated from garlic seed samples, namely *Aspergillus* sp. and *Fusarium oxysporum*. The genus of *Aspergillus* belongs to the *Ascomycetes* class. It can be found everywhere in nature and grows as a saprophyte in decaying plants. While *F. oxysporum* is a plant pathogenic fungus on garlic worldwide that often infects seeds from the nursery period to cause wilting symptoms in plants. This causes crop yields to decrease, resulting in a decrease in production. For further research, the fungi isolated should be identified at the species level using molecular characterization. For farmers, seed treatments are required to anticipate and reduce the impact of these contaminants on garlic production.

Introduction:-

Garlic (*Allium sativum* L.) is known as the 14th most important vegetable crop in the world and the second most important crop of the *Allium* genus worldwide (Bayan *et al*., 2014; FAO, 2018).Garlic production and acreage have increased over the last 50 years by about 200% (FAO, 2018).Commonly, fresh garlic is used for cooking (Kendarini *et al*., 2022). However, garlic has some bioactive compounds(Najda *et al*., 2016)and contains protein, minerals, and

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vitamins A, B1, B6, and C. Therefore, it is used as both traditional and modern medicine, such as for antibacterial, antifungal, antiviral, anti-protozoa, and anti-cancers (Bayan *et al*., 2014), as well as for treating metabolic, cardiovascular, and respiratory system diseases (Najda *et al*., 2016).

In Indonesia, garlic is usually cultivated in irrigated highlands (>1,000 above sea level) due to cooler weather compared to the lowlands (Hidayah *et al*., 2021).The annual demand for garlic in Indonesia is estimated at 500,000 tonnes. However, domestic production is only 88,000 tonnes (Center of Statistical Bureau, 2019),meaning that 82.4% of the need is supplied through importation. However, the cultivation of garlic has been revived by the Indonesian government in the last few years. One of the strategies to increase domestic production is to make more certified seeds available. This is because certified-garlic seeds have not been established in Indonesia, therefore seeds quality is mostly uncontrolled (Hidayah *et al*., 2021).

One of the areas in Indonesia that produces garlic is Sembalun highlands. It is located in the valley of Mount Rinjani in East Lombok Regency, West Nusa Tenggara (NTB) Province of Indonesia. The people of Sembalun recognize garlic as a very important commodity because the history of the area is very closely linked with this commodity. The Sembalun Plateau was the main producer of garlic in Indonesia until the 1990s. With the opening of the imported garlic tap in 1998, Sembalun's garlic immediately slumped, and people switched to planting other commodities such as potatoes, carrots, onions, chillies, and others (Mardiana *et al*., 2018).In the last five years, the government has been trying to revive the heyday of Sembalun garlic. Since the start of garlic cultivation in the 1960s, there have been ups and downs in garlic production. At first, it was grown in the forests of Sembalun with high yields, and then it declined when there was forest cover in 1979. In the 1980s, garlic rose again and reached a period of its glory until 1998, which was marked by very high productivity, reaching 40 tonnes/ha. However, currently, the productivity of garlic in Sembalun highlands is decreasing and ranges from 15–18 tonnes/ha (Mardiana *et al*., 2018). This is probably due to a decrease in soil fertility and an increase in pest and disease infection in the fields. The last would allow it to be carried to the farmers' storages. Therefore, prospective garlic seeds stored cannot be separated from the presence of contaminants in the form of insects, fungi, bacteria, and viruses. Based on the previous studies, fungal pathogens infected garlic dominantly in the Sembalun fields (Hidayah *et al*., 2021). Those pathogenic fungi were very likely to be carried to farmers' storage.

Some seed-borne fungi from the genera of *Aspergillus*, *Botrytis*, *Fusarium* and *Peniccilium* have been reported causing cloves rot of stored garlic bulbs in Egypt (Moharam *et al*., 2013). In addition, their findings indicated that isolates of *F. oxysporum*, *F. proliferatum* and *F. solani* were superior to other tested fungi and induced the highest cloves rot. The isolate of *F. oxysporum* was highly reduced clove germination, produced extensive seedlings damping-off and induced highly disease severity index of rotted roots/cloves followed by *F. Solani* (Moharam *et al*., 2013). Other research from commercially distributed garlic seed cloves from six states of the United States and mainland China were surveyed for the presence of fungi recorded as pathogenic to garlic. They found some fungi such as *Aspergillus niger*, *A. ochraceus*, *Botrytis porri*, *Embellisia allii*, *Fusarium oxysporum* f. sp. cepae, *F.proliferatum* and *Penicillium hirsutum*. In addition, *F. verticillioides*, which was not previously reported as pathogenic to garlic, but here demonstrated to be a pathogen (Dugan *et al*., 2007).

The success of the garlic cultivation business is strongly supported by the seed factor because its production depends on the quality of the seeds used. Bulbs used must be of high quality with plants that grow normally, healthy, free from pests and pathogens. Good quality garlic seeds must meet the requirements such as being free of pests and diseases, the base must be full and firm, the cloves are pithy and the weight of the cloves for seeds is 1.5 to 3 grams (Wibowo, 2007).Therefore, this study aims to investigate fungal contaminants on garlic seeds at farmers' storages in Sembalun highlands of Eastern Lombok, NTB Province of Indonesia.

Materials and Methods:-

This research was conducted from September to November 2018. The garlic seed samples (*Sangga Sembalun* variety) were collected from farmer storages in Sembalun Highlands, East Lombok Regency, West Nusa Tenggara (NTB) Province of Indonesia. Preparation of garlic seed samples, incubation of the samples, observation of the isolates, and identification of the isolates were conducted at the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Mataram University, Indonesia. Tools used were Petri dishes, Erlenmeyer flasks, autoclave, incubator, beaker, hoses, macro pipette, pipette tips, dropper pipette, test tubes, Bunsen burner, sterile cotton sticks, ruler, laminar flow cabinet, stirrer, hot plate, analytical scales, test tube racks, measuring cups, scissors, tweezers, filters, and evaporators. While materials used were garlic seeds, distilled water, cotton, tissue, alcohol 70%, ethanol

90%, paper labels, wrapping paper, filter paper, and aluminium foil. The research equipment was sterilized at a temperature of 121°C for about 15 minutes.

Preparation of the garlic seed samples
Garlic seed samples were soaked in distilled water for two minutes to clean the seeds from dirt, then rinsed with distilled water and drained. Three strands of filter paper were moistened by dipping them in aquadest. Then the filter papers were placed into petri dishes and the testing process was conducted in the Biological Safety Cabinet. Each replication consists of five Petri dishes with 50 cloves of garlic.

Incubation of the garlic seed samples
After the sowing process was completed, it was then incubated for 24 hours with 12 hours of photoperiod. On the second day, the garlic seed samples were put into a freezer at 0-14°C for 24 hours to suppress germination. On the third day, the garlic seed samples were taken out of the freezer and put on a metal rack for 5 days with 12 hours of photoperiod.

Observation of the fungal isolates
The mycelium of fungi which were grew from the garlic seed samples then isolated and placed on the glass objects and dripped with aquadest solution, and closed using cover glass. These isolates then observed under a binocular microscope (400x and 1,000x magnification).

Identification of the fungal isolates
Identification of the isolates was performed by comparing the results of the documentation collected with a publication titled Illustrated Genera of Imperfect Fungi (Barnett and Hunter,1998). The identificationprocess were included morphological properties of colony growth, colony color, insulation on hyphae, and the shape of spores and conidiophores.

Data recorded
The isolates which were found and the number of garlic seeds infected were recorded. The percentage of infection was calculated using the formula:

$$\text{Percentage of Infection (PI)} = \frac{\text{Number of infected seeds}}{\text{Number of incubated seeds}} \times 100\% \quad (1)$$

Results and Discussion: -

Isolation and identification of fungal contaminants on garlic seeds
We found two fungal species on garlic seeds, namely *Fusarium oxysporum* and *Aspergillus* sp. (Table 1). These fungal-infected garlic seeds were seen on the outer surface of the incubated seeds. The fungal colonies were in the form of mycelium and spores.

Table 1:- Percentage of infected garlic seed samples and the present of fungal contaminants (n = 50 cloves).

Replication	Percentage of Infected Seeds (%)	*Fusarium oxysporum*	*Aspergillus* sp.
1	3.5	+	-
2	3.5	-	+
3	5.0	+	+
4	4.0	-	+
5	3.0	-	+
6	3.5	+	-
7	5.0	+	+
8	3.0	-	+
9	5.0	+	+
10	5.0	+	+
11	3.5	-	+
12	3.5	-	+
13	4.0	+	-
14	3.0	-	+

15	5.0	+	+
16	3.0	+	-
17	3.5	+	-
18	3.5	-	+
19	3.0	-	+
20	5.0	+	+

Notes: **+** = present, **-** = not present

Fusarium oxysporum

Most of the tested garlic cloves were infected by *F. oxysporum*, a soil-borne pathogen that belongs to the Hypomycetes (subdivision of Deuteromycotina). Most of these genera are saprophytic fungi that are commonly found in soil, but some are parasitic. Based on the observation of garlic seeds infected by *F. oxysporum* macroscopically, the mycelium was grayish white (Figure 1).

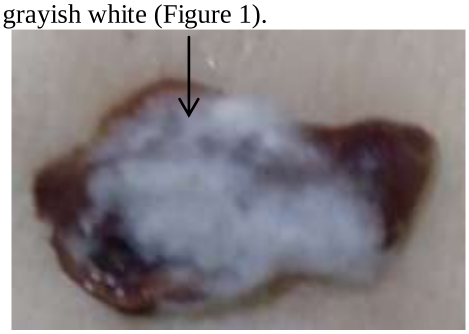

Figure 1:- Grayish white mycelium of *F. oxysporum*grew on the cloves of garlic seed samples (*Sangga Sembalun* variety) after incubation.

The genus of *Fusarium* is from the order of Hypocreales and phylum of Ascomycota. Characterization of this genus is having a body structure in form of branched, hyaline and septa mycelium with 2 microns in diameter. Genus of *Fusarium* also has a phialide structure in form of mono-phialide or poly-phialide as well as solitary or part of a complex branching. Microconidia are asexual reproduction of this fungus and located on the branched and un-branched conidiospores. Phialide formed microconidia with a fine structure and a cylindrical shape and consist of 2 or more cells with thick cell walls. Microconidia usually consist of 1-3 cells, round or cylindrical and arranged into chains and clots (Moretti, 2009). In addition, genus of *Fusarium* consists of large number of species which include plant pathogens causing diseases in some agricultural crops. This genus also produced mycotoxins that can contaminate produced as well as harmful for human and animals (Moretti, 2009). Microconidia in *F. oxysporum* were characterized by oval and non-insulated microconidia, insulated crescent-shaped macroconidia (generally 3 insulated) (Figure 2 and 3).

Figure 2:- Spores of *F. oxysporum*. **a:** microconidia and **b:**macroconidia (observation with an electric light binocular microscope 400x magnification without methylene blue dye).

F. oxysporum has both macroconidia and microconidia. Macroconidia is curved, long with a tapered tip and has one or three septa, while microconidia is smaller than the macroconidia with a non-insulated or single-insulated shape and is produced by sporodocium. Chlamydospores and sclerotia are often formed from their mycelium. Mycelia of *Fusarium* sp. is septate, branched and hyaline, yellowish brown in old age (Rustam *et al*., 2013). *F. oxysporum* is a pathogenic fungus that often attacks seeds from the nursery period to cause wilting symptoms in plants. This causes crop yields to decrease, resulting in a decrease in production. One of the obstacles in the development of garlic plants is the attack of the fungus *F. oxysporum* which causes wilt disease (Rustam *et al*., 2013).

The genus of *Fusarium* is among the most common storage fungi infecting garlic bulbs worldwide (Oh and Kim, 2016), two of them namely *F. proliferatum* and *F. oxysporum* f.sp. cepae have been reported causing garlic seed rot disease (Dugan, 2007). Moreover, *F. oxysporum* f.sp. cepae is one of the *Fusarium* species which was reported on garlic seed in Indonesia and causing basal rot disease (Prihastuti, 2012). *Fusarium* is a soil-borne pathogen, but it can also infect plants through wounds or other organs, including stems, leaves, flowers, and fruit. This infection can also be transmitted by spores carried by wind and water (Adawiyah, 2016).

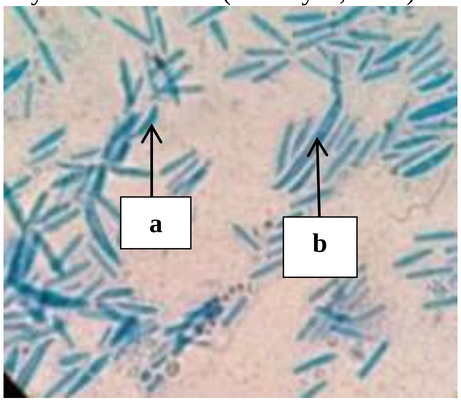

Figure 3:- Spores of *F. oxysporum*. **a:** microconidia,**b:** macroconidia (observation with an electric light binocular microscope at 1000x magnification with methylene blue dye)

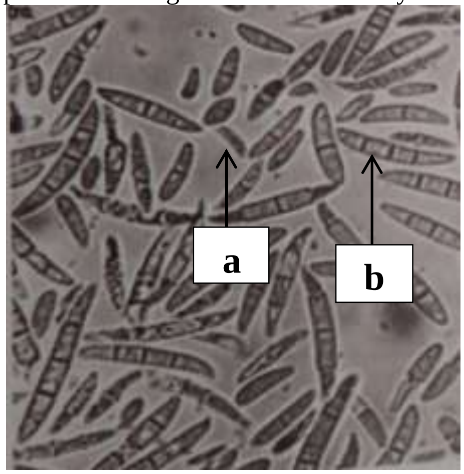

Figure 4:- Comparative images of *F. oxysporum* spores. **a**: microconidia and **b**: macroconidia at 1000x magnification as published by Barnett and Hunter (1998)

There are several factors that can cause bulb rot triggered by *F. oxysporum*, including the possibility of pathogens being carried by garlic seeds because they are not treated prior to planting. Fungal pathogens can also thrive in the soil used to cultivate garlic bulbs. Soil conditions can have an impact on the process of microorganism colonization. The soil's high cation exchange capacity creates a favourable environment for the pathogenic fungus to thrive. Additionally, soil acidity has an impact on disease progression (Koike *et al*., 2008). Furthermore, it was reported that a pH value below 5.5 could intensify the disease (Hadiwiyono and Widono, 2008).

Base rot disease caused by *F. oxysporum* can be managed with non-pathogenic *Fusarium* and also *Trichodermaharzianum* as biological control agents. Non-pathogenic *Fusarium* could minimize plant disease incidence (Kristiana, 2015). *Trichoderma* is a soil fungus that has been commercialized due to its efficacy as a biofertilizer and ability to boost crop yields. *T. harzianum* has been known for a very long time to boost plant growth and development, particularly through the growth of more and stronger roots. In addition to living on the root surface, its colonies can also penetrate the root epidermal layer. Several studies have demonstrated that plants with *T.harzianum* colonies on their root surfaces use 40% less nitrogen fertilizer than plants without colonies. The non-pathogenic fungi *Fusarium* and *Trichoderma* lowered the number of rotting garlic bulbs concurrently. This demonstrated that non-pathogenic *Fusarium* can suppress the incidence of garlic base rot. Non-pathogenic *Fusarium* has been utilized as a microbial antagonist against pathogens found in the soil (Dhingra *et al*., 2006). Non-pathogenic *Fusarium* was not only capable of preventing disease in plants, but it may also stimulate plants growth. Therefore, the combination of *Fusarium* and *Trichoderma*, which are not hazardous, is an effective method for promoting the growth and development of plants (Horinouchi, 2011).

***Aspergillus* sp.**

Another fungus that infected garlic seed samples is *Aspergillus* sp. Morphological observations of *Aspergillus* sp. seen directly, the colonies appear greenish yellow and some are blackish brown (Figure 5). Seeds infected by *Aspergillus* sp. on the outer surface of the seeds there is a fungal mycelium. The mycelium is initially white and then the sporangium becomes yellowish brown, green or blackish (Hafsari and Isma, 2013).The genus of *Aspergillus* belongs to the Ascomycetes class. It can be found everywhere in nature and grows as a saprophyte in decaying plants. In the culture medium, it grew long filaments that branched out into mycelia and conidiospores (Crystovel, 2016).

The sporangium of *Aspergillus* sp. looks like a broom. The important characteristics of *Aspergillus* sp. are a sporangium that resembles a broom, the conidiophores are hyaline, simple or unbranched, sometimes thick-walled as shown in Figure 6. *Aspergillus* sp. is a fungus of the phylum Ascomycota. The morphological characteristics are the same as most of the species in this phylum, such as filamentous, hyphae insulated or having septa, and they can be found abundantly in nature (Watanabe, 2002). Conidiophores that are upright, simple, with rounded edges, and radiating from the tip or the entire surface are some microscopic characteristics of *Aspergillus*. In addition, conidia are round and often colorful (Adawiyah, 2016), while macroscopic morphological characteristics on the PDA media were surface light green to dark green, brown and black, and a round colony with hyphae and septa.

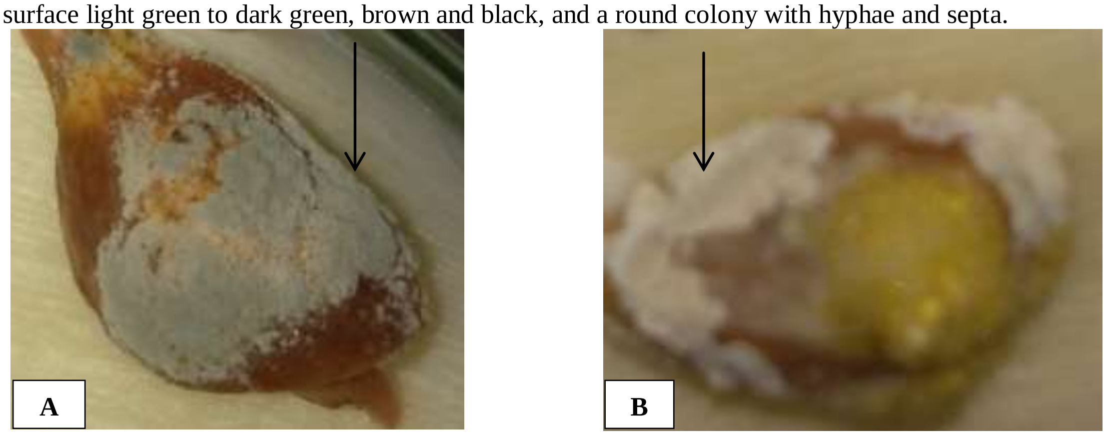

Figure 5:- Mycelium of *Aspergillus* species grew on the cloves of garlic seed samples (*Sangga Sembalun* variety) after incubation.**A:***Aspergillus* sp. 1 and **B:***Aspergillus* sp. 2

The genus of *Aspergillus* has septate hyphae and branched mycelium. The hyphae that appear above the surface are fertile. The colonies are grouped into conidiophores with septa or non-septa that arise from the leg cells. A bubble appears at the end of the hyphae where, out of this bubble, the sterigma appears. Conidiums appear from sterigma, which are arranged sequentially to resemble a string of pearls (Figure 6). The conidiums are colored black, brown, dark yellow, and green, which give the fungus a certain color (Crystovel, 2016). Observation with an electric light binocular microscope with 400x magnification showed the presence of elliptical conidia without septa (Figure 7). The spores of *Aspergillus* sp. are small and light in size, resistant to dry conditions, have leg cells that are not so clearly visible, have non-septal spore conidia and form sterigmata where conidia grow. Conidia of *Aspergillus* sp. have a diameter of 1.5 – 2.4 µm, smooth-walled, long to elliptical, and striate. Microscopically, conidiophores are usually long, columnar, colorless (hyaline) and smooth, giving rise to biseriate round (Crystovel, 2016).

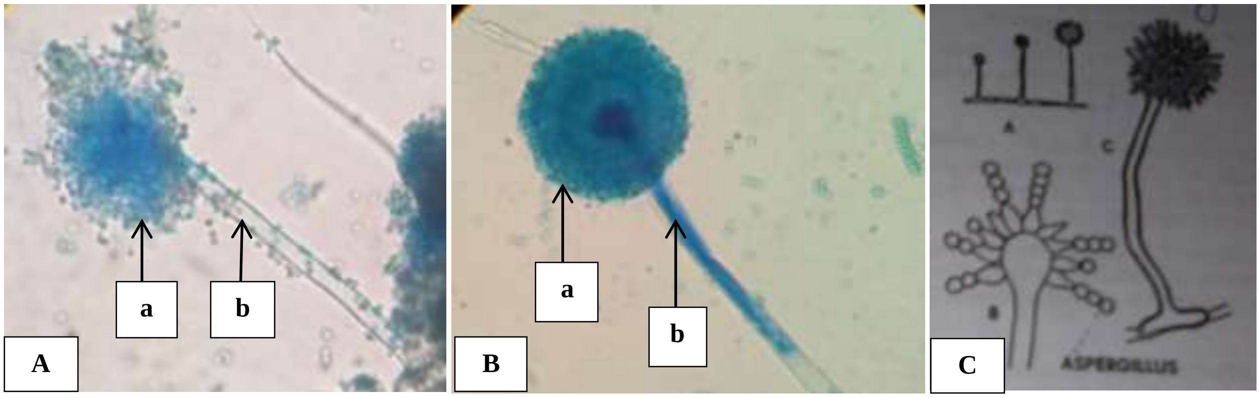

Figure 6:- Sporangium of **A:***Aspergillus* sp. 1, **B:***Aspergillus* sp. 2 on garlic seeds under observation with an electric light binocular microscope with 400x magnification and **C:** Comparative image of *Aspergillus* sp. from Barnett and Hunter (1998).**a:** conidia, **b:** conidiophores.

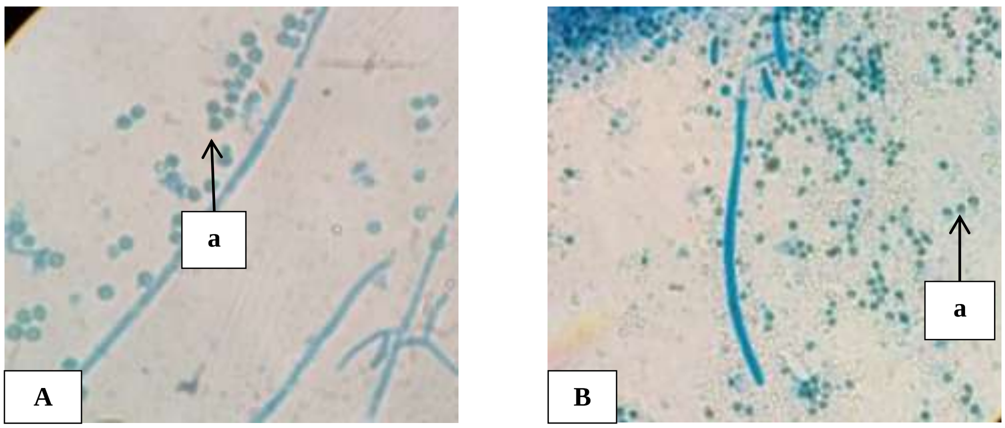

Figure 7:- Spores of *Aspergillus* sp. 1 (**A)** and *Aspergillus* sp. 2 (**B**). **a:** Conidia (observation with an electric light binocular microscope at 400x magnification).

The genus of *Aspergillus* is also among the most common storage fungi infecting garlic bulbs worldwide (Oh and Kim, 2016). Some species have been reported contaminated garlic seeds such as *A. awamori*(Oh *et al*., 2016). Furthermore, it was reported that isolation trails from garlic seed cloves of some cultivars commercially distributed and rotted cloves of stored Baladi cultivar during 2010 and 2011 in different regions of Sohag, Egypt resulted in detection of four fungal genera namely*Aspergillus*, *Botrytis*, *Fusarium* and *Penicillium*(Moharam *et al*., 2013). Other research reported that commercially distributed garlic seed cloves from six states of the United States and mainland China were surveyed for the presence of fungi recorded as pathogenic to garlic and among them were *A. niger* and *A. ochraceus*(Dugan *et al*., 2007).

Aspergillus sp. in its growth is directly related to the nutrients contained in the substrate. Simple molecules around the hyphae can be directly absorbed while more complex molecules must be broken down before being absorbed into the cell by producing several extracellular enzymes such as proteases, amylase, mananase, and glatosidase. *Aspergillus* sp. uses organic matter from the substrate for molecular transport activities, maintenance of cell structure, and cell mobility. *Aspergillus* sp. usually appears during storage. Infection increases when the seed is stored in a room (26–31°C and RH 64–80%) that promotes fungus activity and development in the warehouse. *Aspergillus*sp.grows best in environments with 50.5–100% humidity and temperatures of between 30 and 35°C**.** The genus of*Aspergillus*can produce antimicrobial compounds, namely mevioninand *aspersillin* (Gandjar *et al*., 2006). *Aspergillus* also helps crops in important ways. It ties down free nitrogen from the air and breaks down phosphate in the soil, which plants use as an organic nutrient (Koike *et al*., 2008).

Conclusions:-

Based on the results of this study, it can be conclude that there were two main seed-borne fungal contaminants isolated from garlic seed samples in Sembalun Highlands of Eastern Lombok, Indonesia namely *Aspergillus* sp. and *F. oxysporum*. It is well known that *F. oxysporum* is a plant pathogenic fungus on garlic worldwide that often infect seeds from the nursery period to cause wilting symptoms in plants. This pathogen causes crop yields to decrease, resulting in a decrease in production. Whilst the genus of *Aspergillus* belongs to the Ascomycetes class. This fungus can be found everywhere in nature and grows as a saprophyte in decaying plants. For further research, the fungi isolated should be identified at the species level using molecular characterization. For farmers, seed treatments are required to anticipate and reduce the impact of these fungi on garlic production in the fields.

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Contributorship:-

Baiq Nurul Hidayah is the main contributor of this paper. While Yuli Handayani, Anang Triwiratno, Muhammad Tahir Hamsyah, Mohammad Rani, and Nurhaedah are supporting contributors.

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