ISSN (Print): 2476-8316
ISSN (Online): 2635-3490
Dutse Journal of Pure and Applied Sciences (DUJOPAS), *Vol. 5 No. 1a June 2019*

Preliminary Studies on Fungus Associated with Storage Disease of Garlic (Allium Sativum L.) in Nigeria

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Abstract
Garlic storage fungal diseases causes serious economic losses for farmers and traders with potential risk of health hazards in the populace. The research was conducted at the Department of Biological sciences Nigerian Defence Academy (NDA) to determine the percentage occurrence of disease symptomatic garlic bulbs in local pink and white skinned garlic types Var.Ophioscorodon and the fungus responsible for garlic rot during storage. Garlic were purchased in Kaduna central market. Injured and disease symptoms bulbs were discarded. Fifty healthy bulbs each were selected as a sample, kept separately in different envelopes, labeled and stored for 10 weeks under laboratory condition. They were observed each and recorded at weekly interval for disease symptomatic bulbs in the lots. The significance differences in the two percentages were computed as the percentage occurrence of varietal susceptibility in disease symptomatic bulbs. The pathogen was cultured on potato Dextrose Agar (PDA) medium, incubated and kept at room temperature for 3 days then isolated; and identified. From the result obtained, differences between two percentages, that is, 84% and 72%of varietal susceptibility in pink and white garlic is not significant at .05 and .01 probability levels, since the critical ratio3.45 is greater than 2.58 and 1.96 at both tails. Table 1. The isolate viewed under a microscope was found to be of Penicillium spp. Figure 1. Pink garlic variety had a higher percentage of symptomatic bulb compared to white garlic after days of storage. In all considered cases, bulb decay increased with storage time. From this study it can be recommended that white skinned garlic should be selected for screening of penicillium storage resistance line which can be used in breeding programme and promote safety garlic consumption among the existing local clones of garlic

Keywords: Pathogenicity, Disease susceptibility, Storage rot, *Penicilliumspp,* Garlic

INTRODUCTION
Garlic (*Allium sativum L., Alliaceae*) has been used since as early as 5000 BC as a medicine and food condiment. Beneficial compounds to our health such as *oligosaccharides, steroidal glycosides,* essential oil, *flavonoids, anthocyanins, lectins, prostaglandins, fructan,* pectin, *adenosine*, and vitamins are found in garlic bulbs and leaves, but the organosulfur compound *allicin* is responsible for its medicinal properties (Arzanlou and Bohlooli, 2010).El-Marzoky, *et, al*., (2013) reported that garlic is the most important commercial crops grown all over the world and consumed in various forms. It is also known to lower blood sugar and cholesterol levels. Its many other health-promoting attributes have been resulted in medicinal pills, drinks and powders based on garlic extracts. Rana *et al*., (2011) stated that regular consumption of garlic prevents cardiovascular diseases, diabetes, asthma, and cancer. According to Etoh and Simon

(2002) the centre of origin of garlic has been considered to be Central Asia, with secondary centers of diversification in China and the Mediterranean area and evolved from the wild species *Allium longicuspis*, differing in the exerted anther. In *Allium longicuspis*, as in many onions, possessing bulbils in the umbel; the flowers apparently do not always develop, and the anther not exerted from the perianth; today garlic is known only as a cultivated plant, and its wild relatives are not found. However, recent molecular evidence indicates that *Allium longicuspis* lays within the range of genetic diversity found in *Allium sativum*.Fritsch and Friesen, (2002) reported there are two subspecies of garlic, the hardneck, (*Allium sativum var. ophioscorodon*) and the soft necks, (*Allium sativum var. sativum*). These two subspecies look and grow differently. Interesting fact: "*Allium sativum*'' in the botanical name for garlic, means "pungent cultivated". Garlic has been cultivated for millennia, but the taxonomic origins of this domestication process have not been identified. Modern taxonomy subdivides the world's garlic germplasm into five distinct groups: *Sativum, Ophioscrodon, Longicuspis*, Subtropical and *Pekinense*. According to FAO (2010) the annual world garlic production is 22.2 million tons. Spain has the highest production rate in the European Union and the ninth highest in the world, with approximately 154,000 tons per annum grown on 16,000 ha. Aliyu (2006) reported that in Nigeria, the production of garlic is concentrated in the Northern Guinea and Sudan Savanna ecological zones, where it is mainly grown under irrigation in the dry season. Its diverse distribution can be seen by its diverse common names in different societies. It is called "*Tafarnuwa*" in Hausa, "*Saum*" in Arabic, "*Aglio"* in Italy, "*Chesnoc*" in Russia, "*Lahsan* in Hindi, "*Ail*" in French, "*Ayu*" in Yoruba.El-Marzoky *et, al.,* (2013) reported that numerous *fungi* attack garlic cloves during storage resulting in decay causing considerable losses and decreasing the quality. Ghangaonkar (2013) indicated that *Aspergillus niger*, *Fusarium oxysporum*, *Macrophomina Phaseolina,Botrytis alli,Penicillium corymbiferum, Aspergillus flavus, Rhizopus stolonifer, Chaetomium globosum* are specially found on bulbs from storage.

Steven (2013) reported that blue Mold of garlic may be caused by any of several *Penicillium* species, but most commonly *Penicillium hirsutum*. While the pathogen can cause poor plant stands in the field, *Penicillium* decay of stored bulbs is more common and more economically devastating. Advanced stages of bulb and clove rot have a characteristic mass of blue to blue-gray growth. Susan (2008) also reported that the blue mold disease occurs at harvest and in storage. Characteristic disease symptoms of the infected bulbs in storage are seen as water soaked areas on the outer surfaces of scales. This leads to development of the green-blue, powdery mold on the surface of the lesions. When the bulbs are cut, these lesions are seen as tan or grey colored areas. There may be total deterioration with a secondary watery rot. *Penicillium* survives in infected bulbs and cloves from one season to the next. Spores from infected heads are spread when they are cracked prior to planting. The fungus does not persist in the soil. Air-borne spores spread the disease often invade plants through wounds, bruises or uncured neck tissue. In storage, infection on contact is through surface wounds or through the basal plate; the fungus grows through the fleshy tissue and sporulation occurs on the surface of the lesions. Entire cloves may eventually be filled with spores.

According to Valdez *et al*, (2006) blue mould disease in garlic (*Allium sativum*) is associated worldwide with various *Penicillium species*, and has been attributed to significant annual crop losses in Argentina; the world's second largest exporter of garlic. *Penicillium Viridicatum* was first reported as the causal agent of blue mould of garlic in Argentina (Gatica and Oriolani, 1984) before the characterization of *P. allii* (Vincent and Pitt, 1989). *Penicillium allii* is micromorphologically similar to *P. Viridicatum* and both species produce yellow exudates in pure culture. The *P. Viridicatum* strains were not able to sporulate on the garlic cloves. *Penicillium hirsutum* was also reported as a pathogen on garlic in Argentina (Cavagnaro *et al*.,

2005). However, *P. allii* but not *P. hirsutum* has been reported as an aggressive pathogen of garlic in comparative pathogenicity trials conducted in damp chambers (Overy *et al.,* 2005). Valdez *et al*, (2006.) reported that *P. allii* rather than *P. hirsutum*or P. *Viridicatum*, is the pathogenic species responsible for garlic crop losses due to blue mould rot in Argentina. Prevention and control of some post-harvest fungal diseases of garlic bulbs was reported by Abdel-Al et.al. (1991). Smalley and Hansen, (1962) indicated that *Penicillium* on stored garlic (Blue mold) *Penicillium hirsutum* Dierckx (syn. *P. corymbiferum* Wrestling) seems to be the most common and widespread species occurring in storage and is reported as being one of the primary causes of decay in stored garlic. According to Kirk *et al.,* (2008) the widespread genus *penicillium* contains over 300 species. The genus name is derived from the Latin root *penicillum*, meaning "painter's brush", and refers to the chains of conidia that resemble a broom. Haubrich, (2003) described in the scientific literature by Johann Heinrich Friedrich Link in his 1809 work Observationes in ordines plantarum naturales included three species—*P. candidum, P.expansum, and P. glaucum*—all of which produced a brush-like *conidiophore (*asexual fruiting structure). Pitt (1979) divided *Penicillium* into four subgenera based on *conidiophore* morphology and branching pattern: *Aspergilloides, Biverticillium, Furcatum*, and *Penicillium. Penicillium* is also classified as a genus of anamorphic fungi in the division *Ascomycota* (order *Eurotiales,* class *Eurotiomycetes*, family (*Trichocomaceae*). The *thallus* (mycelium) typically consists of a highly branched network of multinucleate, septate, usually colorless hyphae. Many-branched *conidiophores* sprout on the *mycelia*, bearing individually constricted conidiospores. The *conidiospores* are the main dispersal route of the fungi, and often are green in color. Sexual reproduction involves the production of *ascospores,* commencing with the fusion of an archegonium and an antheridium, with sharing of nuclei. The irregularly distributed asci contain eight unicellular ascospores each.

Samson et al,(2004) also reported that *Penicillium* commonly known as molds, are among the main causes of food spoilage, especially species of subgenus *penicillium*. Pitt et al, (2000) stated that many species produce highly toxic mycotoxins. The ability of these *penicillium* species to grow on seeds and other stored foods depends on their propensity to thrive in low humidity and to colonize rapidly by aerial dispersion while the seeds are sufficiently moist. Cantwell (2013) stated that Seed bulbs (clove to be planted for the following next season) are generally stored at ambient temperature from harvest (June, July, or August depending on location and cultivar) until planting in the fall. These storage practices are similar to those outlined in the revised USDA handbook. The commercial storage of fruits, vegetables, and florist and nursery stock. Recommendation suggest that garlic intended for consumptions be stored at -1℃ to 0℃, 60-70℃ relative humidity (RH), which allowed it to keep for >9 months. Letetia (2015) indicated that four factors affect the storage of garlic;

i) how well it was grown and cured
ii) its group type
iii) temperature and
iv) humidity.

Garlic that was poorly grown and improperly cured will not get any better in storage. The optimum storage temperature for bulbs for replanting is 10°C, with limits of 5°C and 18°C.Garlic stores best long term when it is kept between 12°C and 18°Cand between 40% and 60% humidity. If the humidity stays below 40% for a couple of weeks or more, garlic has a tendency to dry out faster than it otherwise would. If humidity goes higher than 60% for any extended period of time, fungus and molds can set in. If the temperature goes below about 12°C for an extended period of time, garlic tends to want to sprout and grow, even if it is not the right time of year (that's why the refrigerator is not a good place to store garlic). If temperatures stay much over 21°C. For any extended length of time, garlic tends to dry out and deteriorate for storage at room temperature: Before storing, spread garlic on newspapers

out of sunlight in a well-ventilated place to cure for 2 to 3 weeks or until skins are papery. Chelan and Douglas(2015)recommended that before storing, spread garlic on newspapers out of sunlight in a well-ventilated place to cure for 2 to 3 weeks or until skins are papery. Store in a cool, dry, well-ventilated place, such as an attic or unheated room in well-ventilated containers, like mesh bags. Storage life is 5-8months.Identification of *penicillium* is not easy. It is a large genus, and many common species look alike to the uninitiated. At the same time there is a great deal of variability within the species, therefore, unambiguous identification of the species require molecular identification (Guerche *et al*., 2004).Among the molecular tools available tubulin gene has proven useful for identification of closely related *penicillium* species (Kim *et al*.,2006).*Penicillium* isolates were identified with the help of keys developed by Pitt, (1979, 2004) and Frisvad and Samson (2004).
This work aims to estimate the percentage occurrence of disease susceptibility to bulbs colour in local garlic Var.*Ophioscorodon* pink and white skinned types and to determine presence of the pathogen responsible for garlic decay at 10 weeks of storage under laboratory condition.

METHODOLOGY
Local clones of garlic Var.*Ophioscorodon* with pink and white skinned types were purchased in Kaduna central market. The infected bulbs were selected from the bulk. Those that were injured; and had the disease symptoms were discarded. Fifty healthy bulb samples were selected; and stored in different envelopes for ten weeks after which each was examined on weekly basis for disease symptoms. Penicillium isolates were identified in the level of genus on Potato Dextrose Agar (PDA)medium, 200g of healthy Irish potatoes were washed under running water, cut into slices; and boiled in distilled water on a hot plate for 25 minutes to soften. The broth were sieved out into 250ml beaker, 5g of glucose and 30g of agar-agar were added to the broth and made up to 1 liter of distilled water. The solution was heated with constant stirring using sterilized glass rod until the agar was completely dissolved into the solution. The potato Dextrose Agar medium was autoclave at 121℃ for 15 minutes, made in to 12 plates and allowed to solidify under laboratory conditions.To improve the sensitivity and specificity of routine culture approach for identification of Penicillium in the level of species, Malt Extract Agar (MEA) {powdered malt extract 20 g, Peptone 10 g, Glucose 20 g, Agar 20 g, DW 1L were used.

The following materials were used; Petri-dishes, inoculating loop , cotton wool, Beaker, Conical flask , methylated sprit , Microscope slides , Distilled water , Bunsen burner , cover slips, scalpel , photographic microscope , Safety razor blade and potato Dextrose Agar PDA medium. The equipment used was sterilized by washing with detergent solution, rinsed and dried in an oven and autoclaved at 121℃ for 15 minutes. A sample from each lots were sterilized with sodium hypo chloride solution for three times, a piece of disease tissue from each bulb samples were cut using a sterilized razor and transferred to the potato Dextrose Agar medium in the plates. The blade was constantly sterilized by flaming over a Bunsen burner and cooling in methylated sprit to avoid contamination during process. The plates were incubated under laboratory condition for 72 hours. Pure cultures from each plate were made by using sterilized inoculating loop. A minute portion from the media containing the required fungal mycelia and spores from each colony was scooped and transferred into freshly prepared sterile potato Dextrose Agar medium using procedures described by Cruickshank et al. (1975) to avoid contamination by bacteria, a broad-spectrum antibiotic (0.5ml Gentamycin) was added to media in the plates. The pure cultures were incubated under laboratory condition for 72 hours before observation under a photographic microscope. Some little fragments of the fungal mycelium from the medium were scooped with sterilized inoculation loop and placed in a drop of saline solution on clean slide. The slides was covered with cover slips, mounted and observed under low power at first and then at high powered

magnification of microscope. Isolate were identified based on their morphological characteristics, that is , the type of mycelia, fruiting body , septation of hyphae , pigmentation of the culture, characteristic of the spores, somatic structures , reproductive stages and types of spores. As identified in accordance with the description of (Ainsworth 1971; Barnett and Hunter 1999).

RESULTS

Table 1.Percentage occurrence of disease symptomatic bulbs in pink and white skinned Garlic (*Allium sativum* L.).At 10 weeks of storage under laboratory condition.

Number of Days	Pink skinned garlic (P_1)	White skinned garlic (P_2)
7	2	0
14	5	1
21	6	2
28	8	6
35	14	11
42	19	17
49	24	21
56	27	25
63	38	31
70	42	36
	$P_1 = 42$	$P_2 = 36$
	$N_1 = 50$	$N_2 = 50$

From table 1. The significance differences in the two percentages of disease symptomatic bulbs in pink and white skinned garlic P_1 and P_2 was computed as the percentage occurrence of a disease symptomatic bulbs in two independent sample of size N_1 and N_2 by the following formula adopted from Lokesh,(n.d)

$$P = \frac{N_1 P_1 + N_2 P_2}{N_1 + N_2}$$

$q = 1 - p$; N_1 and N_2 = Size of the sample

Test of the significance of differences between the two percentages; P_1= 84% and P_2= 72%
$N_1 = 50$ and $N_2 = 50$

$$P = \frac{N_1 P_1 + N_2 P_2}{N_1 + N_2} = \frac{84 \times 50 + 72 \times 50}{50 + 50} = \frac{4200 + 3600}{2500} = \frac{7800}{2500} = 3.12$$

$q = 100 - 3.12 = 96.88$

The standard error of the difference between P_1 and P_2 is $\sigma_{D\%} = \sigma_{P1-P2} = \sqrt{PQ}\left[\frac{1}{N1} + \frac{1}{N2}\right]$ by Substituting the values = $\sqrt{3.12 \times 96.88}\left[\frac{1}{50} + \frac{1}{50}\right] = \sqrt{302.2656 \times 0.04} = \sqrt{12.090624} = 3.47715 \approx 3.48$

The significance of the difference between percentages P_1 and P_2 was found out by a use of critical ratio (C R) as $C R = [P_1 - P_2] / \sigma p_1 - p_2$

Substituting the values in the above formula = $84 - 72 / 3.48 = 3.45$

The obtained critical ratio (CR) 3.45 is greater than 2.58 and also greater than1.96. Hence, the differences between two percentages 84% and 72% is not significant at .05 level and .01 level. This indicates that there is not true differences in the percentages in varietal susceptibility between the pink and white garlic type. The species of the isolated fungi responsible for the storage disease on garlic clones as observed under the photographic microscope was found to be of *penicillium species* Figure 1.

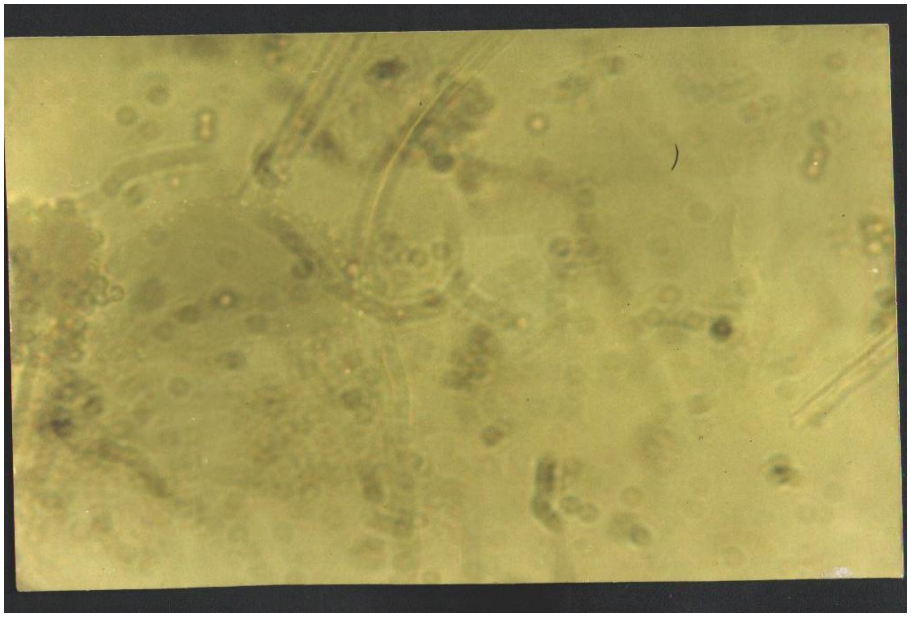

Plate 1.Shows spores and a brush-like conidiophore (asexual fruiting structure) of *Penicillium* species as observed under photographic microscope.

DISCUSSION

Consent over the causal agents of blue mold rot of garlic has yet to be reached, although many *Penicillium spp.* have been reported as pathogens. Smalley and Hansen (1962) first reported the causal agents of the disease as *P.corymbiferum* and *P. cyclopium*. However, since then *P. corymbiferum* has been subdivided into several different species contained within the *Penicillium* series *Corymbiferum*(with synonymy of the original *P.corymbiferum* characterization aligned with that of *P. hirsutum*). (Gatica and Oriolani, 1984; Cavagnaro*et al*., 2005) reported the presence of *P. Viridicatum* and *Penicillium hirsutum* as a pathogen on garlic in Argentina. *Penicillium viridicatum* has also been reported from garlic in Japan (Saito and Tsuruta, 1984). Overy *et al*., (2005) reported that *P. Allii* but not *P. hirsutum*has been reported as an aggressive pathogen of garlic in comparative pathogenicity trials conducted in damp chambers. Valdez *et al*., (2006) first reported the confirmation of *P. allii* as garlic pathogens in Argentina. Onions *et al.,* (1984) reported that species-level identification is often very difficult challenging; in particular, numerous errors related to the identification of *Penicillium* have been reported in the literature. The main difficulties involved in *Penicillium* identification are related to various nomenclature schemes, strain variation and decisions based on minutiae. New varieties and species have been designated, only to be subsequently reclassified as members of existing taxa. Frisvad *et al.* (2000).

CONCLUSION

This study has shown that Pink garlic variety had a higher percentage of symptomatic clove84%compared to white garlic 72% after days of laboratory storage. No significant differences was observed between two percentages at .05 level and .01 level, since the obtained critical ratio (CR) 3.45 is greater than 2.58 and 1.96 and indicated that garlic stored under laboratory condition influenced disease susceptibility in the evaluated garlic clones. In all considered cases, bulb decay increased with storage time. The isolate viewed under a microscope was found to be of *penicillium spp*. It is therefore concluded that the white skinned garlic be encouraged for storage and selected for screening of *penicillium* resistance line which can be used in breeding programme and promote safety garlic consumption among the existing local clones of garlic. Additional studies would be supportive in illuminating an appropriate tools for identification of *Penicillium spp.in* stored garlic.

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