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Assessment of the efficacy of garlic (*Allium sativum* L.) extract for the control of A*spergillus flavus* and other fungi on maize seeds in North central, Nigeria

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Abstract: This study was carried out to evaluate the antifungal activities of aqueous extracts derived from garlic (*Allium sativum* L.) aqueous extract on maize seed-borne fungi with emphasis on *Aspergillus flavus.* A total of 114 samples of rot-infected unshelled maize cobs were collected from three States and the Federal Capital Territory (FCT), Abuja in the North Central zones of Nigeria. After shelling and bulking, the fungal load and molecular identification of *Aspergillus flavus* isolated from samples were determined. Thereafter, one set of 200 g seed portions from the samples were treated with aqueous garlic extract while the other set were left untreated before being stored in propylene bags for four months. Then, using agar plate method, fungal load and radial growth inhibition of the *A. flavus* and other fungi by garlic extract were determined. The study revealed that the incidence of *A. flavus* isolated from the maize grains samples from the FCT, Abuja and Benue State were significantly higher ($p \leq 0.05$) than those from other two states. Out of the five suspected pure *A. flavus* isolates sent for molecular identification, four of them were duly identified as *A. flavus*. It was confirmed from the treated stored maize seeds that garlic extract significantly inhibited ($p \leq 0.05$) *Aspergillusflavus*, with radial growth reduced by 59.05%. The findings can serve as basis for developing an organic agricultural compatible garlic-based management strategy for maize rot fungi complex.

Keywords: *Aspergillus spp,* fungi species, garlic extract, incidence,maize seed, molecular identification.

INTRODUCTION

Maize (*Zea mays* L.) is a cereal crop belonging to the family Poaceae. It is reported as the most important cereal in the world after wheat and rice (Hussain et al., 2013). Maize is used as fodder, feed, vegetable, and industrial raw material. It is a staple for 1.2 million people in Africa south of the Sahara and the Americas (IITA, 2009; USDA, 2016). Maize is rich in vitamins A, C and E, carbohydrate, essential minerals, protein, fibre and calories. Annually, 8.63 million MT are produced in Nigeria (Suleet al., 2014; Ammani et al., 2010). Most of maize production is carried out in the middle and northern belt States(Adamawa, Bauchi, Borno, Yobe, Jigawa, Gombe, Taraba, Plateau, Sokoto, Kebbi, Katsina, Nasarawa, Niger and Zamfara) (Foramfera, 2015) of the country where sunshine is adequate and rainfall moderate (Iken and Amusa, 2004). Grain yields of 4 - 6 tonnes/ha is reported in northern Nigeria whereas 2-4 tonnes/ha is typical of southern part of the country (ICS-Nigeria, 2016).

However, several factors have been reported to limit the quality of maize seeds. These include biotic pressures from stem and ear borers, parasitic weeds (*Striga hermontica*) and microbial diseases in both field and storage (IITA, 2009; Ammani et al., 2012). Fungi ranked second after insects as the cause of deterioration and loss of maize (Debnath et al., 2012). Twelve fungi genera are among the principal causes of deterioration and loss of maize grain (Bhattacharya and Raha (2002). Seed-borne fungi of the genera *Aspergillus, Fusarium, Curvularia, Bipolaris, and Penicillium* have been reported to be associated with maize seeds (Hussain *et al*., 2013). These fungal pathogens affect maize systemically reducing its nutrient quality and quantity, causing seed rots, seedling blight, germination failure, depressed seedling vigour and poor crop performance (Enyiukwu and Ononuju, 2016). In addition, seed-borne mycobiota have been reported also to contaminate maize grains with hazardous metabolites implicated in several forms of allergies, birth defects, cancers and even death in livestock and humans (Enyiukwu et al., 2014). *Aspergillus flavus* is one of the causal organisms of rot infection and aflatoxins in maize seed (Calderari et al*.,* 2013). This fungus is important in producing secondary metabolites which are carcinogenic to both humans and animals.

Natural plant products are important sources of new agro-chemicals for the control of plant diseases (Hassan, 2014). It is known that various natural plant products can reduce populations of foliar pathogens and control diseases development, and then these plant extracts have potentials as environmentally safe alternatives and as components in integrated pest management programs (Browers and Locke, 2004). A number of plant species have been reported to possess natural substances that are toxic to several plant pathogenic fungi (Goussous et al., 2010). Plant extracts have played a significant role in reducing the incidence of seed-borne pathogens and in the improvement of seed quality and germination of plant seeds in the field (Arya and Perelló, 2010; Rukhsana et al., 2010). *Allium sativum* juice extract belongs to such non-traditional treatments and among the natural fungicide substances. Despite the prevalence and seriousness of these storage fungi causing losses to the seed of maize crop in the store in Nigeria, adequate studies have not been undertaken on sustainable means of controlling them. Information on the effective control of these fungi with eco-friendly botanicals is limitedly available.

Therefore, this research will help ascertain the incidences of *Aspergillus flavus* from maize seeds in the middle belt zone, precisely Benue, Nasarawa, Niger States and the FCT Abuja, Nigeria. The study also intended to identify morphologically and molecularly the *Aspergillus flavus* isolated from the maize seeds and determine the effect of garlic extracts on the control of stored maize seed-borne fungi. This is with the long term aim of developing a new alternative methods for managing maize fungal pathogens as a substitute for chemical fungicides.

MATERIALS AND METHODS

Sample collection

A total of one hundred and fourteen rot-infected unshelled maize grains samples were collected from the North Central, Nigeria involving Niger, Benue, Nasarawa States and the FCT Abuja. The sampling was based on purposive random selection. One kilogram of samples from 10 locations from each of the States and the FCT were used for laboratory analysis, using purposive random selection.

The three maize samples collected within each location were bulked, thus amounting to 10 bulked samples from Benue, Niger and Nasarawa States respectively, and nine bulked samples the FCT Abuja, respectively. Each sample was carefully packaged and labelled by location. This resulted in a total of 39 bulked samples which were used for laboratory analysis.

Isolation of fungi before storage with garlic

The isolation of fungi was carried out by the agar plate method as previously described by Klich (2000) and Samson (1991). Sabouraud Dextrose Agar (SDA) medium was prepared for fungal growth and sterilized at 121°C and cooled until mild hot and poured 15 ml in each Petri dish having 90 cm diameter. Seeds were surface sterilized with 1% NaOCl solution for one and half minute and given three washes in sterilized double distilled water. After surface sterilization seeds were placed in Petri dishes containing media and incubate at 22 °C ± 1 °C for 5 to 7 days in an incubator. All processes were carried out under laminar flow chamber to maintain hygienic condition. Further, the morphological and growth characteristics of all the isolates were analysed on solid SDA medium. Slides of each of the organisms were prepared, fixed, mounted and examined under a low-high power compound microscope. Identification of the species was done on the basis of the colour of the colonies and by viewing under the microscope with reference to fungi identification manual (Klich, 2000).

Collection of samples and preparation of garlic bulbs

Allium sativum (garlic) were obtained from Gwagwalada market in Gwagwalada Area Council, Abuja, Nigeria and transported to the Crop protection laboratory of University of Abuja for preparation and studies. For the preparation of the garlic extracts, 200g fresh bulbs were rinsed with sterilized distilled water and cut into small pieces. These chopped pieces were subsequently milled using a blender using 200 ml of sterilized distilled water in line with the description of Goussous et al. 2010.The extracts were filtered into separate labeled containers by muslin cloth and centrifuged at 4000g for 30 mins then filtered through Whatmann's No. 1 filter papers in funnels. A concentration of 1 g/ml was termed as stock solution i.e. 100%, in line with Deshmukh and Borie (1987).

Seed treatments with garlic extract before storage

The maize seed samples were soaked in the garlic extract for 2 h. After soaking, the seeds were surface-dried in an incubator with forced air circulation for 48h on filter paper at 25^{o}C to return to their original moisture of 12-14 % (on dry wet basis). The dried seeds were then stored in propylene bags for four months, before subjecting them to microbial load analysis

Experimental design and layout

The experimental design was complete randomized design involving 2 treatments i.e. (garlic treated and untreated i.e. controls), 39 locations and 3 replicates.

Preparation and sterilization of media

Sabouraud Dextrose Agar was used in this study and prepared according to the manufacturer's instructions thus, 65g of SDA is dissolved in 1000 ml of sterile water and then sterilized (autoclaved) at 121^{o}C and pressure of 15pa for 15 minutes.

Preparation of pure culture of fungal isolate

The young fungal colony was aseptically picked up and transferred to fresh sterile SDA plates to obtain pure culture. The pure cultures on SDA plates was grown at 25 ± 2^{0}C for 7 days and stored in the refrigerator at 4^{o}C until required for further use. The isolate was sub-cultured to obtain young cultures for further studies (Klich, 2000).

Identification of the fungal isolate

Cultural identification

Isolates obtained are characterized and identified on the basis of their colonial and morphological characteristics which include macroscopic and microscopic examinations. Among the characteristics used were colonial characteristics such as size, surface, appearance, texture, and reversed pigmentation of the colonies of sporing structures. Appropriate references were done by using mycological identification keys and taxonomic description (Harrigan and McCance, 2006).

Molecular identification of *Aspergillus flavus*

Following the initial isolation, the *Aspergillus flavus* isolates from the seed samples were purified for molecular identification, characterization. The morphology of colonies and sporulating material were initially confirmed to match species descriptions provided in published taxonomic keys (Klich, 2000). The pure culture of five samples of suspected *A. flavus* was prepared in medical bottles and sent for microbial identification service of CABI where unique reference number were assigned. The International Mycological Institute (IMI) numbers assigned to each of the samples were SA1506178, SA2506179, SA3506180, SA4506180 and SA506180.

The genomic DNA was extracted from mycelia of fungal isolates obtained from 7 days old cultures grown in Yes extract sucrose agar (YES) liquid media. The mycelia was frozen and grounded into a fine powder in liquid nitrogen and DNA was extracted by the Qiagen DNeasy Plant Mini-Kit. The concentration and purity of extracted DNA was determined according to Proirier et al., (2009). Primers and their sequences were used in the PCR amplification experiments (Diba et al., 2014). The primers were obtained from Alpha DNA/Canada.

The reported PCR amplification conditions for amplification of DNA fragments specified by OmtBII and Nor1 primer pairs was conducted according to (Rahimi, 2016) and (Criseo et al., 2001) respectively. The amplified DNA fragments and DNA marker ladder of 100bp (Qiagen) was separated using 1.5% agarose gel and visualized under UV light after staining with ethidium bromide for molecular size determinations in base pair (bp) of DNA fragments (Sambrook et al., 2001)

Effect of garlic products on radial growth of colonies

A plug (10 mm) of mycelium cut from the freshly growing edge of plate cultures of *A. flavus* with a cork borer is inoculated into the centre of a SDA plate. There were eight fungal cultures, two each obtained from the maize samples from Benue, Nasarawa, Niger and the FCT, Abuja. The plates were arranged in Complete Randomized Design involving four replicates. A stock solution of garlic extract was prepared and pipetted onto 5 discs of filter paper distributed around each plug of the fungal inoculum. The resultant colony diameter after 7 days was measured along two axes and the mean diameter of replicate colonies was calculated. The percentage of mycelial growth inhibition (Pi) is calculated using the formula: $Pi = (C - T)/C \times 100$, where C is the diameter of the control colony and T is the diameter of the treated colony.

Data analysis

All data collected from this study were analyzed by simple percentages and Analysis of Variance (ANOVA) using SPSS, version 16.0, at 5% level of significance. Duncan Multiple Range Test (DMRT) at $p<0.05$ was applied to assess the differences amongst the means.

RESULTS

Incidence of fungi load on the rot-infected maize seeds from North Central Nigeria before storage

The mean incidence of fungi species isolated from collected maize seed samples from the three States and the FCT, Abuja is a shown in Table 1. The occurrence of *A. flavus* isolated from the maize grains samples from the FCT and Benue State were significantly higher ($p\leq 0.05$) than those isolated from maize grains from other two States. The incidence of *A. niger* colonies in maize grains from Benue and Nasarawa States and the FCT were not significantly different ($p > 0.05$) from each other, though Benue State had the highest incidence. Generally, the FCT had the highest incidence of *A. flavus*, Benue had the highest incidence of *A. niger* and *Penicillium* spp while *Nasarawa* State had the highest incidence of *Fusarium* spp. Some isolated fungi colonies from Nasarawa and Benue States are shown in Plates 1a-c.

Mean incidence of fungi species isolated from infected maize grains from North Central Nigeria

The occurrence of *A. flavus* isolated from the maize grains samples from the FCT and Benue State was significantly higher ($p< 0.05$) than other two states, while the least was from Nasarawa State. *A. niger* colonies in maize grains from the FCT, Benue and Nasarawa were not significantly different ($p \geq 0.05$) from each other though Benue had the highest incidence. Niger State had the significantly lower ($p\leq 0.05$) colonies of *A. niger* than from other states. The highest incidence of *Fusarium* spp and on the samples was from Niger State (39.00%) while that of *Penicillium* spp was Benue State (17.33%). *Rhizopus* spp had relatively lower incidence than other fungi spp. The colonies of *Rhizopus* spp isolated from maize from the FCT were significantly higher than in the three States

Table 1. Mean incidence (%) of fungal colony isolated from infected maize grains from North Central Nigeria

State	*A. flavus*	*A. niger*	*Fusarium* spp	*Penicillium* spp	*Rhizopus* spp
Benue	50.33[a]	21.00[a]	21.67[b]	10.00[b]	2.33[b]
Nasarawa	14.33[c]	10.33[ab]	14.33[c]	1.67[c]	2.33[b]
Niger	38.67[b]	1.33[c]	39.00[a]	17.33[a]	3.33[b]
FCT	53.00[a]	16.33[a]	19.00[b]	7.10[b]	9.00[a]

Plate A : *A. niger* colony on seeds from Akunza, Nasarawa State

Plate B: *Penicillium* spp colony from seeds from Mbaka, Benue State

Plate C: *A. flavus* (top) and *Penicillium* spp colonies on seeds from Bussan, Nasarawa

Plates 1A-C showing some fungi isolates from the seed samples

Molecular identification of *Aspergillus flavus*

The IMI assigned number and result is as shown in Table 2. Out of the five suspected *A. flavus* samples sent out for molecular identification, four of them were duly identified as *A. flavus* while the fifth one was said to be contaminated with bacteria.

Table 2. Molecular identification of *Aspergillus flavus* samplesinfected maize samples from the FCT Abuja

	Assigned IMI number to each of the five suspected samples	**Result on the isolates**
1	SA1506178	Identified as *A. flavus*
2	SA2506179	Identified as *A. flavus*
3	SA3506180	Identified as *A. flavus*
4	SA4506180	Identified as *A. flavus*
5	SA5506180	No identification was possible because they were contaminated with bacteria

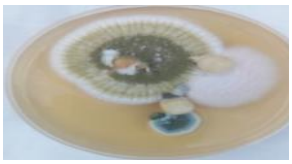
From CABI Identification Report, 2019

Mean incidence of fungi species from garlic-treated and untreated maize seeds at 7 days after inoculation (DAI) in three North Central States and FCT Abuja.

In Benue State, 48.44% of the fungi associated with maize was *Fusarium* spp at 7DAI while *Penillium* spp and *Rhizopus* spp had least incidence of 7.81% respectively (Table 3) . From the treated maize seeds, the growth of the *Fusarium* spp was most inhibited by 66.67%). In the FCT Abuja, *A. flavus* had the highest incidence in the untreated seeds (26.15%) out of the five major fungi isolated at 7DAI while the least was *A. niger* (15.63%). In Niger State, 42.50 % of the fungi observed were *Fusarium* spp while the incidence of this fungus was only 11.11% under garlic treated maize samples. While the incidence of *Penicillium* spp was 25.00 % in untreated maize samples, it was reduced to 22. 22 % in garlic treated maize. The only *Cladosporium* spp colony observed in the stored untreated maize was not inhibited in the garlic-treated maize seeds. The mean incidence of fungi on treated and untreated maize seeds is pictorially shown in Fig. 1.

Table 7: Incidence of fungi species at 7DAI in untreated and garlic-treated maize seeds from North Central Nigeria

State	Fungi genera/spp	No of fungi colony on untreated maize seeds at 7DAI	Incidence of Fungi load on untreated maize seeds at 7DAI	No of fungi colony on treated maize seeds at 7DAI	Incidence of Fungi load on treated maize seeds at 7DAI
Benue	*Fusarium* spp	31	48.44	3	33.33
	Penicillium spp	5	7.81	1	11.11
	A.flavus	17	26.56	2	22.22
	Rhizopus spp	5	7.81	2	22.22
	A. niger	6	9.38	1	11.11
FCT Abuja	*Fusarium* spp	16	25	0	0
	Penicillium spp	13	20.31	3	33.33
	A.flavus	17	26.15	1	11.11
	Rhizopus spp	7	10.93	1	11.11
	A. niger	10	15.63	4	44.44
Niger	*Fusarium* spp	17	42.50	1	11.11
	Penicillium spp	10	25.00	2	22.22
	A.flavus	4	10.00	2	22.22
	Rhizopus spp	4	10.00	2	22.22
	A. niger	4	10.00	1	1.11
	Cladosporium spp	1	2.5	1	1.11
Nasarawa	*Fusarium* spp	18	38.30	1	10.00
	Penicillium spp	17	36.17	3	30.00
	A.flavus	6	12.77	3	30.00
	Rhizopus spp	3	6.38	2	20.00
	A. niger	2	4.25	1	10.00
	Cladosporiumssp	1	2.13	0	0



Untreated seed at 4DAI showing *A. flavus* (top), Penicillium spp (below) ; Fusarium spp (Right)

Garlic-treated seeds at 4DAI (No fungal colony observed)

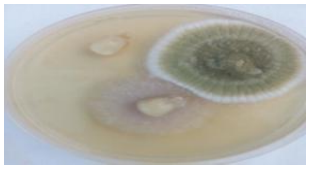
Fungal colony on seed samples from Gwagwalada Abuja

Untreated seed at 4DAI showing *A. flavus* (on 2 top seeds); Fusarium spp (left)

Garlic-treated seeds at 4DAI(No fungal colony observed)

Fungal colony on seed samples from Lambata, Kwali Abuja

Untreated seed at 7DAI showing *A.* flavus colony on seed samples from Lambata, Kwali Abuja



Garlic-treated seeds at 7DAI showing *A. flavus* colony only on a seed sample from Lambata Kwali)

Plate 2: Comparative incidence of fungi colonies at 7DAI in plated untreated and garlic-treated maize seeds from North Central Nigeria

After the maize samples were treated with garlic and stored, the combined incidence of major fungi colonies are as shown in Fig. 1.

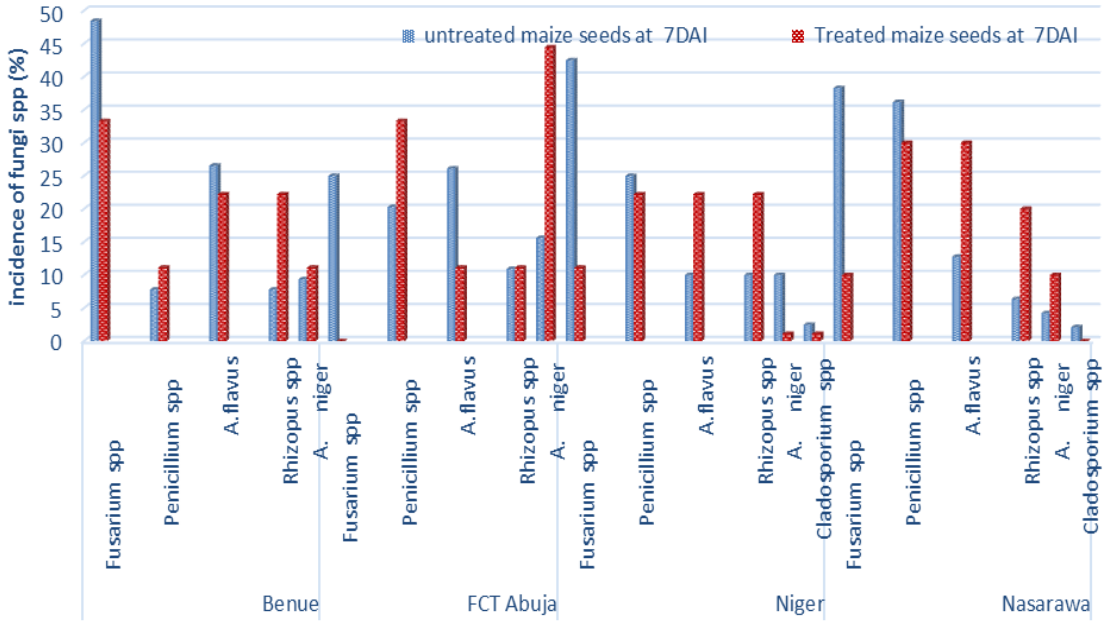


Fig. 1: Incidence of fungi species associated with maize at 7 DAI

Effect of garlic products on mycelium radial growth of *A. flavus*

At 7DAI, the radial growth of *A. flavus* was inhibited in the garlic treated culture medium as shown in the Table 4. Out of the samples, the *A. flavus* mycelium growth in sample five was the most inhibited (63.53%), while that in plate two was the least inhited by 46.84%.

Table 8. Inhibitory effect of garlic extracts on radial growth of *A.flavus* at 7DAI

No of maize seed samples	Diameter of the fungal colony in the control-untreated (mean *cm)	Diameter of garlic-treated colony (mean* cm)	% of mycelial growth inhibition
1	8.8	3.6	59.09
2	7.9	4.2	46.84
3	8.1	3.1	61.72
4	8.6	3.8	55.81
5	8.5	3.1	63.53
6	8.9	3.3	62.92
7	7.8	3.0	61.54
8	8.7	3.4	60.92
Mean	8.41	3.44	59.05

*mean of four replicates

DISCUSSION

From this study *A. flavus* , *Fusarium* spp*, Aspergillus niger,* and *Rhizopus stolonifer* were found associated with the seeds of maize seeds from North central zone of Nigeria. This result agreed with the findings of Kiran et al., 2010; Debnath et al., 2012; Awurum and Enyiukwu, 2014; Hussain, 2013 who reported that the genera *Penicillium, Diplodia, Botrytis, Fusarium*, *Aspergillus, Rhizopus, Curvularia* and *Botrydiplodia* are among the seed-borne fungi associated with seeds in the tropics. For molecular identification of *A. flavus*, one out of the five pure isolates was confirmed to be contaminated. This implied that initial caution should be taken in order to obtain pure, uncontaminated culture. Selective culturing medium could aid getting a pure isolates. Identification to genera level is often based on cultural characteristics such as nature of growth, spore colour, and pigment production and morphological of mycelia and fruiting bodies (Domsch et al*.,* 1980). A lot of difficulties often arose for phenotypical identification of *Aspergillus* spp due to its unstable characteristics. After DNA isolation, comparatively DNA sequence-based identification format appeared to be the most promising in terms of its speed, ease, objectivity and reliability for species identification (Shalini and Amutha, 2015).

During storage, several kinds of fungi can remain associated with maize seeds either causing their deterioration or simply remain viable to infect germinating seedling. The fungi genera typically found in stored grains in most part of Nigeria are *Aspergillus, Penicillium, Fusarium* (Orsi et al., 2000) and some xerophytic species, several of them with capabilities of producing toxins (Castlellarie et al,. 2010). There is a general increase in consumption of contaminated grain with mycotoxins which causes different health problems including death (Adetunji et al, 2014; Voss et al*.,*2007*)A. flavus* becomes systemic and produces aflatoxin and vivesces in seedling of maize and damage stored corn. *Fusarium* invade more than 50% of maize grain before harvest and produce mycotoxin (Uzma and Shahida, 2007), while *Aspergillus flavus* is a food contaminant and capable of producing aflatoxin (Charity et al., 2010)

Chemical control of phytopathogens preventing rot development results in environmental pollution, health hazard and affects the natural ecological balance. Thus, early detection of grain rotting fungi and nonchemical fungicides application would result in more efficient control and may lead to improved storage (Yassin et al., 2012). It was confirmed from this study that the treated stored maize seeds with garlic extract significantly had lower ($p \leq 0.05$) incidence of *Aspergillusflavus*, *Fusarium* spp, *Penicillium* spp and *Rhizopus* spp.

Among many plant substances generally used against seed-borne mycoflora; garlic is reported to be very promising and safer, particularly for food preservation (Haciseferogullari et al., 2005; Aqil et al., 2010). Garlic has broad antimicrobial properties (Irkin and Korukluoglu, 2007) and its activity against *Aspergillus* and mycotoxins production had frequently been documented (Ismaiel, 2008; Salim, 2011).

Garlic extract treatment of wheat seeds significantly reduced the incidence of seed-borne fungi, increased seed germination, the number of healthy seedlings and vigour index (Grozav and Foarce, 2005; Khalaf *et al*., 2011). Josling (2003) reported that allicin is the most important biologically active substance of *A. sativum* crude extract; it is formed from its precursor, alliin, by the action of allinase enzyme (Poonam et al., 2015; Masum et al., 2009).

It was noticed that the mechanism of action of essential oil obtained from the garlic bulbs can act as a an antifungal agent in different ways such as damaging to the enzymatic cell system, including those associated with energy production and synthesis of structural compounds (Vasileet al., 2012); denaturation of the enzymes responsible for spore germination or interference with the amino acid involved in germination (Ruhul et al., 2009); irreversible damage in cell wall, cell membrane and cellular organelles when *A. parasiticus* and *A. flavus* were exposed to different essential oils (Rasoli and Owlia, 2005; Helal et al., 2007; Okigbo et al., 2009).

CONCLUSION

In the North central States of Nigeria, *Aspergillus* spp was the most dominant storage fungi of maize and followed by *Fusarium* spp. Accurate detections and identifications of these fungi are necessary in order to develop appropriate management strategies. This study has provided information on the efficacy of garlic extract in the control of important seed-borne fungi of maize, especially during storage. Confirmation of the effect of garlic in the control of aflatoxins in maize seeds and on germination is necessary.

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