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Research Article

Pathogenicity Analysis of Aspergillus Species on Pre-Mature and Mature *Musa Paradisiaca*

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Abstract

Fruit is the primary economic component of the banana plant, and because the fruit is heavily afflicted by several pathogens, primarily fungal infections, producers and retail sellers are suffering significant economic losses as a result of fruit rotting during cultivation and storage. The temperature and relative humidity of the environment have a significant impact on the severity of fruit illnesses. This study aims to compare pathogenicity of pre-mature and mature banana sample and the effect of temperature and Relative Humidity (RH) on the growth of fungal pathogen. Banana fruits were studied over a month period in order to determine the incidence of species of the Aspergillus genus and assess their potential pathogenicity. Pathogenic variability, temperature variability and relative humidity among different isolates of Aspergillus showed that *A.flavus* caused maximum spoilage at 25◦C and *A.fumigatus* caused maximum spoilage at 75% relative humidity in case of mature banana sample. A.niger caused spoilage in case of pre-mature banana.

Keywords: Pathogenicity, Fungal isolates, Relative humidity, Internal transcribed spacer.

INTRODUCTION

The Musaceae family's banana (Musa sp.) is one of the most significant crops in tropical and subtropical nations. Bananas are right up there with rice, wheat, and maize as significant staple foods in many Asian and African nations. The disease-infected ripe fruit causes losses in nearby marketplaces as well. The environmental factors such as temperature and relative humidity have profound influence on the fruit diseases and their severity. Therefore, much more attention has been paid to studies on the economic aspect of fungal disease encountered during storage condition. The effects of temperature and relative humidity on the decay of mango, banana, papaya, guava, citrus, apples, pomegranate and other tropical fruits have been studied by various workers (Edney, 1964; Srivastava et al,1965; Badger, 1965; Bhargava et al, 1966; Kaiser & Lukezic, 1966; Brown, 1975 Narania & Reddy, 1978; Wadia et al, 1986; Singh etal, 1995; Banyal & Tyagi, 1997). In order to understand the nature of diseases, the progression of penetration and disintegration of the fruit tissue by the fungi *A. flavus*, *A. fumigatus*, *A. niger*, it is required to analyse the architecture of healthy and sick banana fruits. Some of the most common fungi isolated from banana samples are *A. niger*, *A. flavus*, and *A. fumigates*. The order of structural and metabolic developments during an infection may depend on how the host and pathogen interact. Cells initially maintain their shape since the majority of their cell walls are still intact, but later on, when the pathogens' enzymes continue to attack on the cell wall, identity is lost as the cell wall begins to break down (Brown, 1936).This study was undertaken to compare the relative pathogenicity of Aspergillus isolates from pre mature and mature banana sample. The prevalence of Aspergillus species and their potential toxicity were examined in banana fruits over the course of a month. When different isolates of Aspergillus were tested on bananas, the pathogenic variability (*A. niger*, *A. flavus* and *A. fumigates*) temperature variability (25◦C

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and 35°C) and relative humidity (i.e., 30%, 50%, & 75%) were studied.

MATERIALS AND METHODS

Isolation of the pathogen: Banana fruits were gathered from Bihar district. Three different isolates of fungus named as *A. niger*, *A. flavus* and *A. fumigatus* were isolated and was subjected to pathogenicity test from the banana samples. The fruits were surface sterilized with 70% ethanol and was rinsed with distilled water and then air dried. A sterile cork borer (5 mm diameter) was used to wound the stem end of each fruit and mycelial discs of equivalent diameter obtained from the edge of actively growing pure cultures were placed on the wound.

Identification of the pathogens: The cultures were identified based on spore morphology and colony characters and further by LPCB staining.

Proving the pathogenicity: Mature bananas were inoculated with different strains of fungus such as *A. niger*, *A. flavus*, and *A. fumigatus* while keeping one as control which was devoid of any fungal inoculation and was inoculated with SDA media. While in case of premature ones, two banana samples were taken in which one was inoculated with *A. niger* while another one was kept as control. All these inculcated fruits were arranged on individual trays and covered with cling film to conserve moisture and avoid contamination. The fruits were incubated in BOD incubator at 24°C for 5 days. The mature fruits were observed to be infected in 5 days of incubation while the premature ones were spoiled after 8 days of incubation respectively. After incubation fungal peel was taken from each infected banana and it was plated on SDA media to confirm that whether the infected fungus is same as the one with which banana samples were treated.

Molecular characterization: The DNA was extracted from the 2 fungal isolates (isolates 1,2) by the kit method [Nucleo-pore Gdna Fungal Mini Kit (Cat. NP- 7006D)] according to manufacturer's instruction. The Internal Transcribed Spacer (ITS) region, was studied to confirm the morphologically identified fungal isolates.

The consensus primers ITS1 (5'TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') were used for the amplification of Internal Transcribed Spacer (ITS) region. In a PTC-100 Peltier Thermal Cycler, PCR amplifications were carried out in a total volume of 25 µL including 0.5 µL of 0.5 µM template DNA, 5 µL of 5 PCR buffer, 4.0 mM MgCl2, 0.8 mM dNTP mix of both forward and reverse primers, and 0.625 U of Taq polymerase (MJ Research Inc., MA, USA). The first 34 PCR cycles consisted of an initial denaturation at 95°C for 1 min, an annealing step at 52°C for 30 s, an extension step at 72°C for 1 min, and a final extension step at 72°C for 10 min.

After being purified using a QIA quick Purification kit from Qiagen (Hilden, Germany) in line with the manufacturer's instructions, the sequencing of the PCR products was performed. The PCR product were further analysed on 1% gel by Agrose Gel Electrophoresis and visulaised by UV-Transilluminator. The sequence obtained was further analysed and identified using Bioinformatics tools viz., BLAST program, Bio Edit tool. The NCBI BLAST (Basic Local Alignment Search Tool) database was used to evaluate the sequences in order to compare them to published sequences from known fungus species. The identified and closely related species were then used to construct phlyogenetic tree for both isolates (isolate 1,2)

Temperature test: Banana in Petri dishes were inoculated with different strains of fungus and incubated in five individual growth chambers at various temperature regimes (25°C or 35°C), with 5 days (mature banana sample) and 8 day (pre mature) incubation.

Relative humidity test: Banana in petri dishes were inoculated with different strains of fungus and incubated in five individual desiccators at various relative humidity regimes (30%, 50% and 75%), with 4-5 days (mature banana sample) and 8 day (pre mature) incubation. Different relative humidity i.e., 30%, 50%, &75% were maintained in desiccators using respective salt solutions and acids according to humidity parameters.

RESULTS

Identification of the pathogen: The morphological study of 3 fungal isolates gave preliminary idea of A.niger, *A.flavus* , A.fumigatus. The A.niger isolates were morphologically identified based on Black powdery, and yellowish colonies on reverse for pre-mature banana samples. A.fumigatus were morphologically identified based on White, brown to black or green colored colonies and *A.flavus* showed Green, powdery and yellowish colonies on reverse, A.niger isolates were morphologically identified based on Black powdery, and yellowish colonies on reverse for mature banana sample as shown in (Figure 1).

The Lacto Phenol Cotton Blue LPCB staining confirm the presence A.niger in pre-mature banana and 3 fungal isolates A.niger, *A.flavus* , A.fumigatus in mature banana as shown in (Figure 2).

Pathogenicity test: The 3 funguses isolated were *A. niger*, *A. flavus*, and *A. fumigates*. The mature fruits were observed to be infected in 5 days of incubation while the premature ones were spoiled after 8 days of incubation respectively. *A. niger* isolates on pre-mature banana causes black discoloration of vascular Bundles shown in (Figure 3). *A. flavus* isolates on mature banana, changes into dark brown spot and yellowish discoloration of the fruit pulp after 5 days

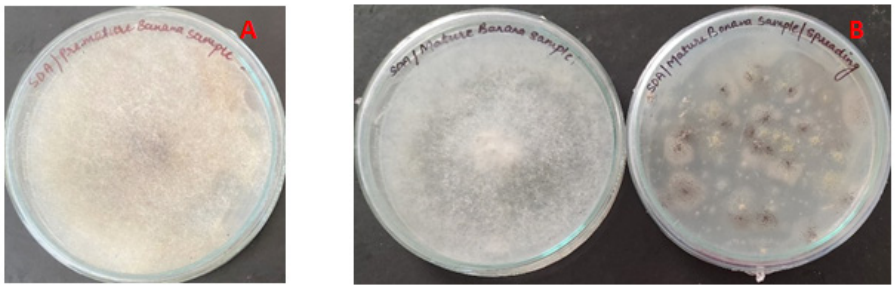

Figure 1: Colony Morphology of isolates [A] *A.niger* (initially white then black powdery) for pre-mature banana [B] *A.niger* (black powdery), *A.flavus* (green powdery), *A.fumigatus* (white,brown to green) for mature banana.

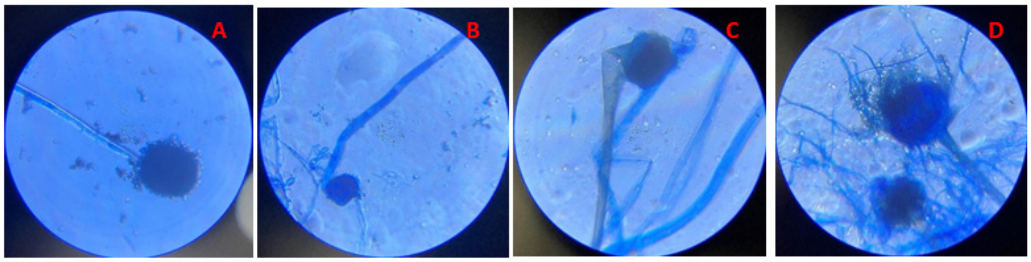

Figure 2: Confirmation of isolates in LPCB mount [A] A.niger Pre-mature banana [B] A.flavus [C] A.niger [D] A.fumigatus for mature banana.

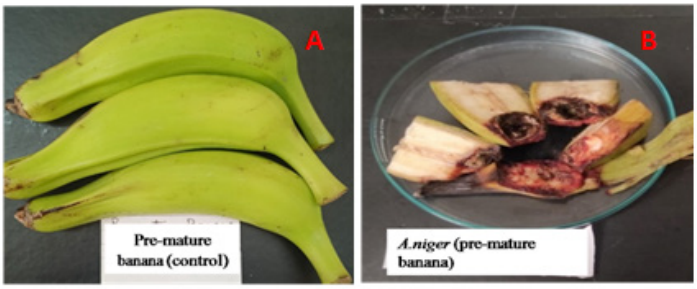

Figure 3: Pathogenecity test of fungal isolate after 8 days of incubation, black discoloration of the vascular bundles[A] Control [B] *A.niger* in pre-mature banana.

of incubation. *A. niger* isolates on mature banana, after 5 days incubation, changes into dark black spot-on banana and brown discoloration of the fruit pulp. *A. fumigatus* isolates on mature banana, after 5 days incubation, where colour changes into black spot and yellowish discoloration of the fruit pulp as shown in (Figure 4).

Further, the pure culture of the isolated fungus confirms that the infected fungus is same as the one with which the banana sample were treated as shown in (Figure 5).

The pathogenic variability among different isolates of Aspergilllus tested on premature and mature banana in incubator is shown in (Table 1) that shows the number of spots increases on increasing the incubation days in both mature and premature banana. Graph 1 shows this data recorded in the form of a bar diagram plotted for 3 fungal isolates.

Molecular characterisation: The query sequence was found closely related to partial sequence of A.niger and *A.flavus* from NCBI database. In order to identify the sequence from its query sequence,phylogentic tree was construced as shown in (Figures 6 & 7) that shows that isolate 1 was A.fumigatus with 98.19% similarity and isolate 2 was A.niger with 99.8% similarity.

Temperature variability: (Table 2) shows temperature variability among different isolates of Aspergillus tested on pre-mature and mature banana at 25°C and 35°C. The mature fruits were observed to be infected in 5 days of incubation and the no. of spots were maximum in the banana sample inoculated by *A.flavus* at 25°C. The premature ones were spoiled after 8 days of incubation respectively. The maximum infection was recorded in fungi that were kept at temperature range of 250C while lowest infection was recorded in temperature range of 350C. This implies that fungus was very well grown in low temperature range while the growth was minimal in high temperature range.

Relative humidity: When the relative humidity increases from 30% to 75% in case of pre-mature banana the spoilage of banana increases as shown in Figure 8. On the other hand, maximum spoilage is seen at 75% humidity in case of mature banana. Among the 3 isolates, most of the infection is seen in *A. fumigates* and *A.flavus* treated mature banana sample as the humidity increased from 30% to 75% as shown in (Figures 9, 10, 11) and (Table 3). Hence, it shows that humidity percentage is directly proportional to the fungal growth. Graph 3 shows the effect of relative humidity on A.niger treated pre-mature banana. Similarly graph 4 and graph 5 shows *A. fumigatus* and A.niger treated mature banana.

This study reveals that the fungus is more commonly responsible for spoilage of fruits as 3 fungal species of Aspergillus were isolated from the banana fruit which were A.niger, *A.flavus* , A.fumigatus. The morphological characteristics of 3 fungal isolates were similar with the descriptions by Zulkifli & Zakaria, 2017 and Oyeleke et al., 2010. The LPCB staining results were similar with the findings of Singh et al., 2015 and Chaurasiya et al., 2021. In the present study, 3 Aspergillus spp. were isolated that shows that it is most commonly responsible for spoilage of fruit that is in line with the work of Rashad et al., 2011. A. aculeatus, A. candidus, *A. clavatus*, *A. flavus*, A. fumigatus, A. japonicus, *A. niger*, A. parasiticus and A. terreus were isolated from Apple, orange by Abdullah et al., 2016. Udoh et al., 2015 isolated different fungus such *F.oxysporum, F.moniliforme, M. indicus* and *R.nigricans* that were associated with the spoilage of Banana. Similar results were recorded while proving pathogenicity by Bhat (1991) & Ekbote (1994) on pomegranate and mango respectively. Kota (2003) proved the pathogenicity of *Colletotrichum gloeosporioides* on mango and banana.

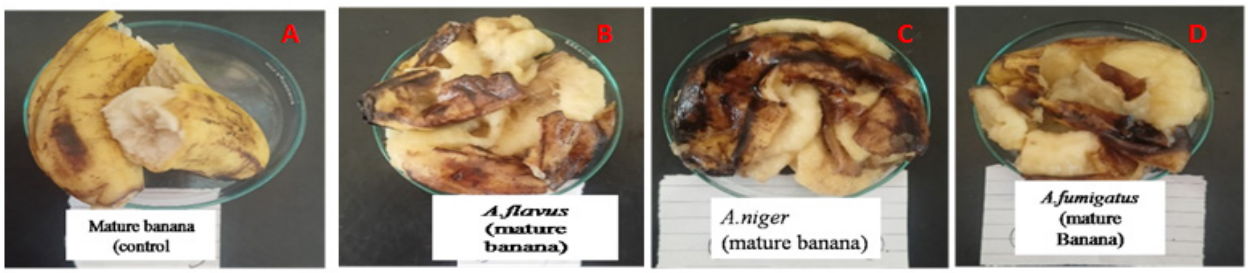

Figure 4: Pathogenecity test of fungal isolates after 5 days of incubation, colour change was observed [A]Control, [B] *A.flavus*, [C] *A.niger*, [D] *A.fumigatus* in mature banana.

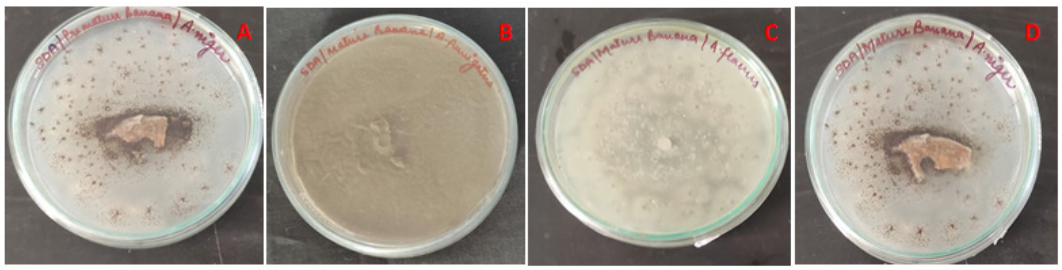

Figure 5: Pure culture of the fungal isolates [A] *A.niger* in pre mature banana [B] *A. fumigatus*, [C] *A. flavus*, [D] *A. niger* in mature banana.

Table1: Pathogenic variability among different isolates of Aspergillus tested on banana in incubator.

S.No	Sample	Isolates	Incubation period(days)	No. of spots on banana
1	Pre mature banana	A. *niger*	2	0
			5	4
			8	6
2	Mature banana	A. *niger*	2	4
			5	8
			8	15
		A. *fumigatus*	2	5
			5	7
			8	11
		A. *flavus*	2	3
			5	9
			8	16

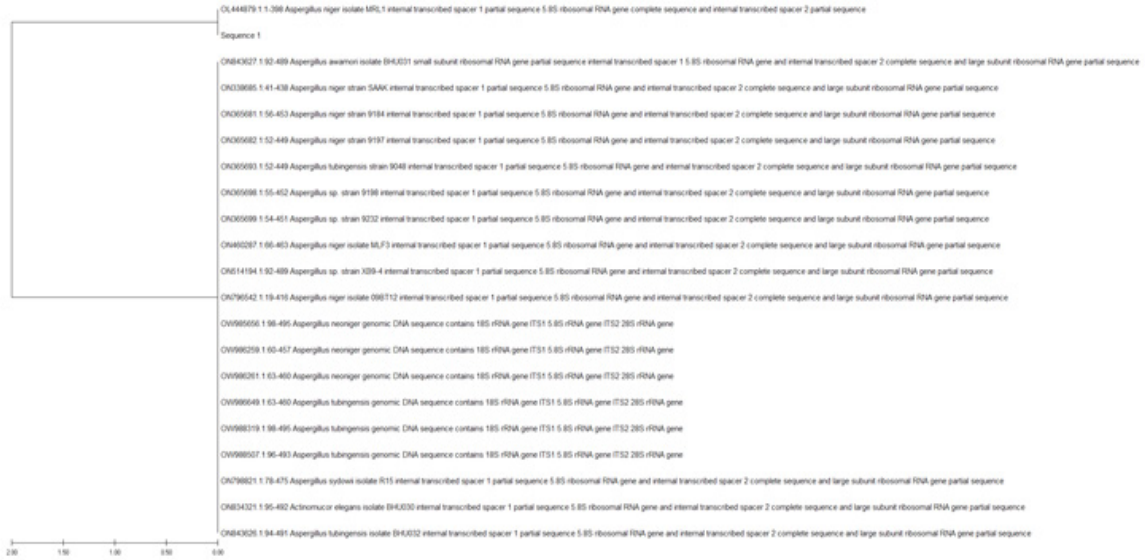

Figure 6: Phylogenetic tree for *A.fumigatus*.

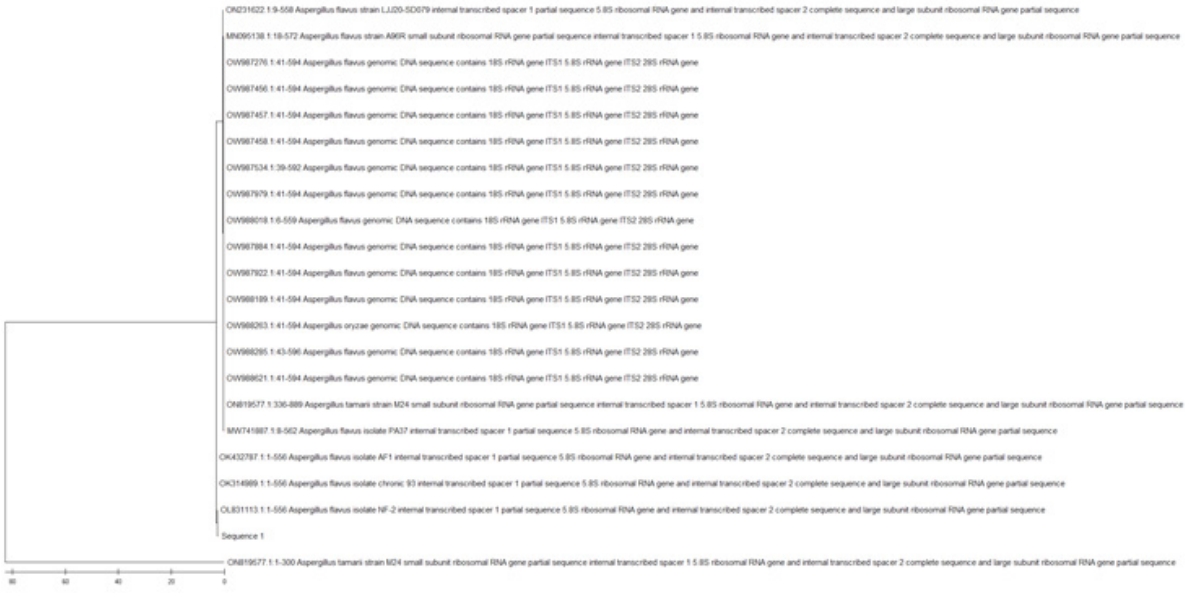

Figure 7: Phylogentic tree for *A.niger*.

Table 2: Temperature variability among different isolates of Aspergillus tested on banana at 35^0C.

S.No	Sample	Isolates	Inoculated culture in (ml)	Incubation period (days)	Incubation temperature (°C)	No. of spots on banana
1.	Pre mature banana	*A.niger*	1ml	2	25	00
				4		02
				6		04
				8		06
2.	Mature banana	*A.niger*	1ml	2		04
				5		08
		A.fumigatus	1ml	2		05
				5		07
		A.flavus	1ml	2		03
				5		09

3.	Pre-mature banana	*A.niger*	1 ml	2	35	00
				4		00
				6		00
				8		00
4.	Mature banana	*A.niger*	1ml	2		00
				5		00
		A.fumigatus	1ml	2		00
				5		00
		A.flavus	1ml	2		00
				5		00

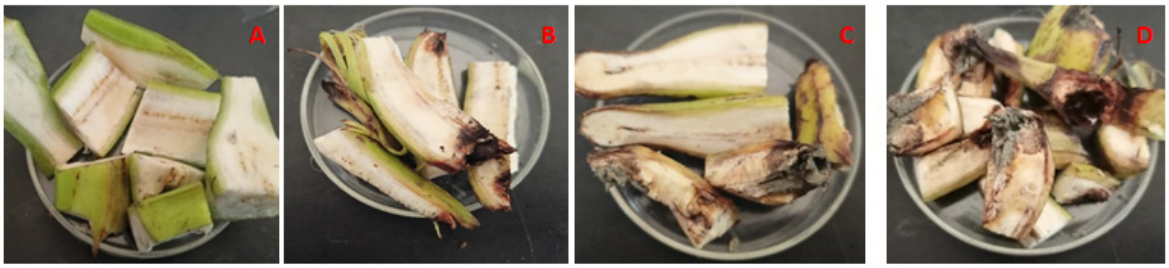

Figure 8: [A] *Control* [B] *A. niger* at R.H. 30%, fungal developing on the surface and black discoloration of the vascular bundles [C] R.H. 50% black discoloration of the vascular bundles, [D] R.H. 75%black discoloration of the vascular stem pre-mature banana sample.

Figure 9: [A] Control [B] *A. niger* at R.H. 30% dark black spot [C]50% black spot developing on the half surface,[D] R.H. 75% black colour developing on the whole mature banana.

Figure 10: [A] Control [B] A. fumigatus at R.H. 30% black spot on the surface brown discoloration of the fruit pulp[C] R.H. 50% dark black patches on the surface, [D]R.H. 75% fungal developing on the whole banana surface, white spot on the surfaces and black discoloration of the fruit pulp of whole mature banana.

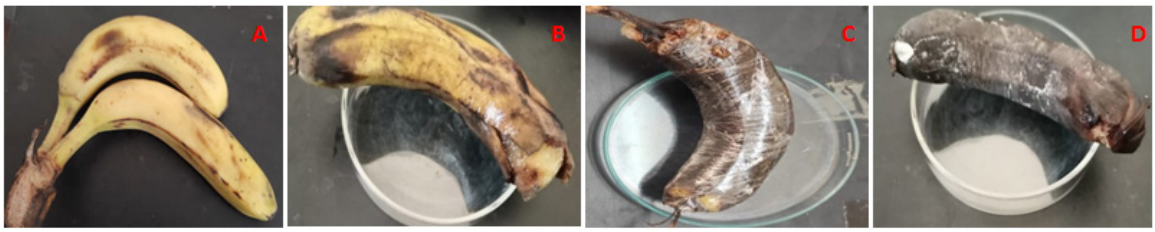

Figure 11: [A] Control [B] A. flavus at R.H. 30% black patches on the surface,[C] R.H 50% fungal developing on the whole surface[D] R.H 75%, color will be change into black colour, white spot on the surfaces of whole mature banana.

Table 3: Different relative humidity i.e., 30%, 50%, & 75% were maintained in desiccators.

S.No	Sample	Isolates	Inoculated culture in (ml)	Incubation period(days)	Relative Humidity (%)	No. of spots on banana
1.	Pre mature banana	*A.niger*	1ml	2	30	01
				4		01
				6		02
				8		03
				2	50	01
				4		01
				6		03
				8		06
				2	75	02
				4		03
				6		06
				8		10
2.	Mature banana	*A.niger*	1ml	2	30	01
				4		02
				6	50	04
				8		03
				2	75	05
				5		08
		A.fumigatus	1ml	2	30	01
				5		01
				2	50	03
				5		04
				2	75	05
				5		06
		A.flavus	1ml	2	30	01
				5		02
				2	50	04
				5		05
				2	75	06
				5		07

DISCUSSION

The molecular characterization of the 2 isolates A.fumigatus and A.niger were in line with the work of Rădulescu et al., 2019. This result align with the findings of Hasan & Zanuddin, 2018 who successfully isolated and identified Aspergillus niger, *Clostradium sp* and *Fusarium oxysporum* from spoilt mango and banana fruits.

Temperature and relative humidity are crucial factors in promoting fungal growth and degradation in the post-harvest environment. According to Wardlaw (1972), temperature has a significant impact on the rate at which fruits deteriorate due to a fungal disease while being stored. The growth of several fungus is typically favoured by extremely high humidity, which reduces market fruit and vegetable losses (Harvey, 1978; Wadia etal., 1986). All of the fungus prefer temperature between 25◦C and 30 °C to flourish, thus an increase in temperature leads to no growth and less infection.

Similar to bacteria, it has been discovered that most fungus thrive at relative humidity levels of 93%, while maximal infections have been linked to relative humidity levels of 100%. As, the humidity increases from 30% to 75% spot formation increases hence, the infectivity of the fungus increases. Most of the infection was seen in *A. fumigates* and *A.flavus* inoculated mature banana as the humidity increased from 30% to 75%. Hence, it shows that humidity percentage is directly proportional to the fungal growth, unlike the work of Ullah et al., 2006 that reported high humidity delay the ripening of banana and increases shelf life that inturn reduces the spoilage time of banana fruit. Snow (1949) has made observations on the impact of humidity on the pace of germ tube elongation, the formation of asexual and sexual fructifications in diverse mould fungus, and spore germination. There is no clear association between temperature and humidity for fungus, according to a broad review of the literature, which also reveals that there is tremendous variation in how different fungi respond to temperature and humidity (Bonner, 1948).

CONCLUSION

This present study revealed that the fungus are most commonly responsible for spoilage of banana fruit. Three spoilage fungi including *A.niger ,A.fumigatus, A,flavus* were isolated and identified that causes spoilage of pre-mature and mature banana. Out of three fungal isolate *A.niger* caused spoilage in pre-mature banana. Among mature banana, *A.flavus* showed high percentage of spoilage in case of temperature variability and *A.fumigatus* showed high rate of spoilage in case of relative humidity variability. Findings in this study suggest that farmers are facing difficulties in ensuring the safety of their food and that they must take suitable management methods against agricultural infections during harvest in order to prevent cuts and minimise losses.

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