Varietal variations of nutritional content and analysis of toxigenic *Aspergillus flavus* colonization of artificially infested wheat grains through scanning electron microscopy in relation to aflatoxin elaboration

DEBALIKA DALAL AND NANDLAL MANDAL

J. Mycopathol. Res. 60(1) : *75-80 2022;*
ISSN 0971-3719

Department of Botany,
University of Calcutta,
Kolkata 700 019, India

J. Mycopathol. Res. ***60(1)*** *:* 75-80, 2022*;* (ISSN 0971-3719)

Varietal variations of nutritional content and analysis of toxigenic *Aspergillus flavus* colonization of artificially infested wheat grains through scanning electron microscopy in relation to aflatoxin elaboration

DEBALIKA DALAL AND NANDLAL MANDAL*
Department of Botany, Visva-Bharati, Santiniketan, 731235

Received : 24.11.2021 Accepted : 31.01.2022 Published : 28.03.2022

In the present study, six wheat varieties viz., NW5054, PBW343, K1006, K0307, DBW39, and HD2733 were selected to study nutrient content variations. Maximum P (7678.71), S (3541.36), Cu (18.03) and Mn (111.92) content in ppm was noticed in PBW343 varieties whereas, maximum K (36030), Zn (80.66) and Fe (584.86) content were recorded in K0307, K1006 and HD2733 varieties, respectively. Minimum P (4196.46) and S (1041.6) were recorded in NW5054 variety, whereas the HD2733 variety showed minimum K (21149), Zn (7.86), and Cu (8.03) contents. Similarly, minimum Fe (243.2) and Mn (6.9) were noticed in K1006 and DBW39 variety, respectively. Colonization potentials of *A. flavus* on wheat grain surface were studied through SEM after artificial infestation with aflatoxigenic *A. flavus* isolate (BAF-4), and aflatoxin elaboration potentials was estimated. Maximum *A. flavus* colonization on grain surface was observed in K0307 variety followed by K1006, NW5054, HD2733, PBW343 and minimum was observed in DBW39 variety. Aflatoxin B_1 elaboration potentials in all artificially infested wheat varieties were quantified in µg/kg. Maximum (329) aflatoxin B_1 production was recorded in K0307 variety followed by K1006 (237.66), NW5054 (165.33), HD2733 (160.66), PBW343 (142.66) variety, whereas minimum (56.33) was recorded in the case of DBW39 variety. The variable amount of aflatoxin elaboration in different wheat varieties was also supported by scanning electron microscopy (SEM) of infested grain, inoculated flask as well as in chromatogram. The present study indicates that the highest aflatoxin elaboration was estimated when the surface colonization was observed maximum in SEM. In contrast, if colonization is meager, then the amount of aflatoxin production is reduced proportionally.

Key words: Wheat varieties, nutrient content, *Aspergillus flavus*, aflatoxin, scanning electron microscopy

INTRODUCTION

Almost all foods and feed substrates, including cereals grains, are proven to be a suitable substrate for aflatoxin production in natural as well as in laboratory conditions; however, their amount is always higher in later conditions. Aflatoxins are several structurally related coumarin-derived secondary fungal metabolites and reported as class I carcinogen by International Agency for Research on Cancer (IARC) having hepatotoxicity, teratogenicity, immunotoxicity and carcinogenicity properties in humans and other animals (IARC, 2002). Three well-known aflatoxin-producing fungi, viz., *A. flavus, A. parasiticus*, and *A. nomius* are widely investigated in terms of their occurrence and toxigenicity; however, the former is more ubiquitous than others. The *A. flavus* fungi are common saprobes and are frequently found to contaminate several food crops in the field conditions from early plant growth to till harvest, storage, processing, and elaborate aflatoxin in them. There are four significant types of aflatoxins (AFs) viz., AFB_1, AFB_2, AFG_1, and AFG_2, of which AFB_1 is the most potent carcinogen. Amongst all cereals, wheat is one of the susceptible substrates for *A. flavus* contamination and aflatoxin elaboration in storage as well as field condition; however, in field condition reports on aflatoxin contamination is meager (Giray *et al*., 2007). Aflatoxin elaboration potentials in wheat are also varied in different varieties and are mainly due to physical properties of seed such as the surface structure of seed coat, the thickness of seed coat, presence of cuticle layer and also the nutritional composition of grains (Sharma *et al*. 2018). The present study aimed to determine the nutritional variations in P, K, S, Fe, Mn, Zn and Cu content in selected wheat varieties. The present study also focuses on observing the colonization potential

*Correspondence: nandlal.mandal@visva-bharati.ac.in

of *A. flavus* on a wheat surface through SEM after artificial infestation and determining the varietal variations of aflatoxin production.

MATERIALS AND METHODS

Wheat grain Sample Collection

Six certified wheat grain varieties, viz., NW5054, PBW343, K1006, K0307, DBW39 and HD2733 were procured from Field Crop Research Station (FCRS) Burdwan, West Bengal and were denoted as V-1 to V-6, respectively. All wheat samples were stored in the refrigerator at 4° C for further study.

Determination of wheat nutrient variations

Wheat nutrient variations such as phosphorus (P), potassium (K), sulphur (S), zinc (Zn), iron (Fe), manganese (Mn), and copper (Cu) was determined by the following tri-acid digestion methods of Sahrawat *et al.,* (2002). For this, 0.5 g of each wheat variety was taken separately in 100 ml of a conical flask and added 10ml of the tri-acid mixture ($9HNO_3$:$4HClO_4$:$1H_2SO_4$) and heated on a hot plate till the red NO_2 fumes ceased. After cooling the digested flask, added distilled water slowly to make up the final volume of 100 ml and filtered through Whatman filter paper No.1. Filtered digested solution (aliquots) was used to quantify P, K, S, Fe, Mn, Zn and Cu content by adding specific reagents and optical density was measured through a Spectrophotometer at the desired wavelength or directly analyzed by Atomic Absorption Spectrophotometer.

Determination of P content

For this previously digested 5 ml aliquot was taken into 25ml of volumetric flask and added 10ml of Barton's reagent. After mixing a few minutes, add distilled water to make up the final volume to 25 ml and allow to stand for up to 30 minutes until yellow colour development. The yellow colour intensity was measured in a spectrophotometer at 420 nm. The amount of phosphorus in different wheat grain varieties was determined with the help of the following formula.

P content in ppm = OD × Dilution factor × Correction factor

Determination of K content

For this 5 ml of aliquots were taken into 25 ml volumetric flask, diluted to 25 ml with distilled water and filtered through Whatman No.42. Aspirate the standards and then the sample to the flame and meter reading was recorded. The emission intensity is proportional to the potassium content and their amount is calculated with the help of the following formula.

K content in ppm = (Flame Photometer reading – Blank reading) × Dilution factor

Determination of S content

To determine S contents, 5ml of aliquots was taken into 25 ml volumetric flask, then added 10 ml sodium acetate buffer, 1 g of barium chloride and 1ml of gum acacia (2.5 g in 1liter distilled water) and mixed properly. Finally, by adding distilled water, volume is adjusted to 25 ml after proper mixing reading was measured by Spectrophotometer at 490nm (Thakur, 2012).

S content in ppm = OD × Dilution factor × Correction factor

Determination of Zn, Fe, Mn and Cu content

For this, adequate aliquots were taken directly in the test tube and absorbance was measured through Atomic Absorbance Spectrophotometer (AAS). Their amount is calculated in ppm by applying the following formula.

Zn, Fe, Mn and Cu content in ppm = AAS reading × Dilution factor

Molecular identification of toxigenic A. flavus

Previously morphologically identified, toxigenic *A. flavus* (BAF-4) isolate was taken from the same laboratory (Dalal and Mandal, 2021). For molecular identification, isolated BAF-4 was subjected to PCR amplification at ITS region 1 and 2 of ribosomal RNA gene with the help of universal forward (ITS1) and reverse (ITS4) primer using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The consensus sequence of the PCR amplicon was generated using aligner software. The consensus sequence was used to carry out BLAST with the database of NCBI Gene bank. Based on the maximum identity score first ten sequences were selected and aligned using multiple alignment software programs. Clustal W Distance matrix was generated, and the phylogenetic tree was constructed using MEGA 7 (Kumar *et al.* 2016).

SEM observation of artificially infested wheat varieties

One ml of spore suspensions (2×10^7 spores/mL) of freshly cultured aflatoxigenic BAF-4 isolate was taken and mixed in 50g wheat grain taken in 250 ml flask and kept in an incubator at room temperature. After incubation of 10 days, a few grains of each variety were selected for CPD (critical point drying), and the rest were kept for the study of aflatoxin elaboration potentials. During CPD, selected grains were dehydrated in acetone and transferred to a critical point dryer (Quorum K 850) using liquefied carbon dioxide as a transitional fluid. Finally, dehydrated grains were coated with gold using an ion sputter (Coater IB-2, Gike Engineer, Japan) and were observed under the Scanning Electron Microscope (Gemini SEM 450, Zeiss) at different magnifications.

Aflatoxin extraction from wheat grains

Extraction of aflatoxin from previously infested wheat grains was done by following the method of Thomas *et al*. (1975). For this, 50 g of artificially infested wheat grain was powdered and blended with 250 ml of Methanol: Water (60:40 v/v) for 15 minutes at high speed. The methanolic extract was filtered with Whatman no. 1 filter paper. 125 ml of filtrate was taken in a 500 ml separating funnel and then extracted with 80 ml saturated NaCl and 50 ml of n-Hexane. The upper hexane layer was discarded, and the lower methanol layer was then extracted with 40 ml chloroform and shaken again. The layers were allowed to separate. The lower chloroform layer was drained into a 125 ml flask containing 5 g of cupric carbonate. Cupric carbonate was allowed to settle down, and chloroform was decanted through Whatman No- 42 filter paper passing through the bed of anhydrous sodium sulphate. The chloroform extract was evaporated to dryness, and the residue was dissolved in one ml of chloroform and reserved for Thin Layer Chromatography (TLC). Spotted TLC plates were developed in a TLC tank containing running solvent of Toluene: Isoamyl alcohol: Methanol in a ratio of 90:32:2 as suggested by Reddy *et al.* (1970). The developed plates were air-dried and then observed under ultraviolet light (360nm). Identification of aflatoxins was made visually by comparing the color of fluorescence and Rf value (Stack and Pohland, 1975). For quantitative assay of aflatoxin, spots on chromatograms were scrapped out and subsequently extracted with cold methanol (5ml/spot) and quantified with a Spectrophotometer (Nabney and Nesbitt, 1965).

RESULTS AND DISCUSSION

Molecular identification of BAF-4 Isolates

During molecular identification, the PCR product of the ITS region of isolated BAF-4 shows a single band of ~700 bp DNA on 1.0% Agarose Gel (Fig.1A). Based on forward and reverse DNA sequences, a consensus sequence was generated. The consensus sequence of BAF-4 is as follows.

CAATCAGTTAAAACTTTC AACAATGGATCTCTT
G G T T C C G G C A T C G A T G A A G
AACGCAGCGAAATGCGATAACTAGTGTGAATTG
CAGAATTCCGTGAATCATCGAGTCTTTG
A A C G C A C A T T G C G C C C C C
T G G T A T T C C G G G G G G C A T G C C T G T
CCAAGCGTCATTGCTGCCCATCAAGCACGGCTT
GTGTGTTGGGTCG TCGTCCCCTCTCCG
G G G G G G A C G G G C C C C A A A G G
CAGCGGCGGCACCGCGTCCGATCCTCGAGC
G T A T G G G G C T T T G T C A C C C
G C T C T G T A G G C C C G G C C G C C G G G G G
C T C T C A G C C C C G G G
C C C G C G C C C G C C G G A G A C A C C A
C G A A C T C T G T C T G A T C T A G T G A A
GTCTGAGTTGATTGTATCGCAATCAGTTAAA
A C T T T C A A C A A T G G A T C T C T T G G T
TCCGGCATCGATGAAGAACGCAGCGAA
ATGCGATAACTAGTGTGAATTGCAGAATT
C C G T G A A T C A T C G A G T C
TTTGAACGCACATTGCGCCC

After BLAST of the above sequence with the database of NCBI Gene bank, maximum identity score first ten sequences were selected (Fig. 1B) and aligned using multiple alignment software programs. After Clustal W. Distance matrix and the phylogenetic tree construction using MEGA 7 and based on the maximum likelihood method (Kimura, 1980) of the Kimura 2-parameter model, selected BAF-4 is identified as *Aspergillus flavus* (Fig. 1C). Growth patterns with colony morphology of identified *A. flavus* and SEM micro-photograph are depicted inFig 2A and Fig 2B respectively.

Nutritional variations in wheat varieties

In the present study, selected wheat varieties showed a variable amount of nutritional content

Table 1: Nutrient content variations in different wheat varieties

Wheat varieties	Nutrient content in ppm*						
	P	K	S	Zn	Cu	Fe	Mn
NW5054 (V-1)	4196.46 ± 2.18	23220.67 ±0.57	1041.6 ±0.95	27.06 ±0.30	10.6 ±0.43	403.63 ±1.30	67.66 ±0.65
PBW343 (V-2)	7678.71 ±0.19	27690.67 ±0.57	3541.36 ±0.45	28.96 ±0.45	18.03 ±0.15	445.56 ±0.35	111.92 ±0.91
K1006 (V-3)	6126.19 ±0.40	27190.33 ±1.52	1529.82 ±0.76	80.66 ±0.40	11.1 ±0.3	243.2 ±1.17	82.26 ±2.25
K0307 (V-4)	7144.5 ±1.17	36030 ±2.0	3194.27 ±0.32	33.43 ±0.05	17.5 ±0.36	302.66 ±0.76	51.46 ±2.01
DBW39 (V-5)	5088.83 ±1.54	26761 ±1.0	2013.18 ±1.43	36.1 ±0.7	11.3 ±0.26	342.6 ±0.6	6.9 ±0.9
HD2733 (V-6)	4376.18 ±1.22	21149 ±1.0	1944.29 ±0.50	7.86 ±0.65	8.03 ±0.15	584.86 ±2.11	65.96 ±0.65

*Average of three replicate with standard deviation

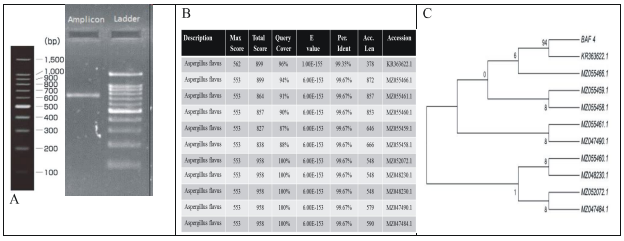

Fig. 1 : (A-C). PCR amplicon of BAF-4 isolate, nucleotide homology and Phylogenetic analysis.
Ladder specification and ITS amplicon (A); Nucleotide homology with *A. flavus*(B); Phylogenetic analysis (C)

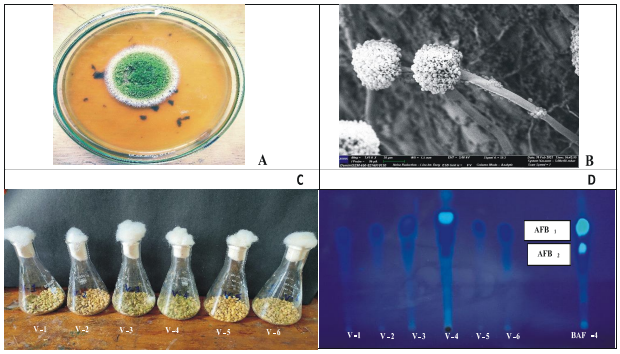

Fig. 2: (A) Colony morphology of Toxigenic *A. flavus* (B) SEM image of BAF-4(C) Artificially infestation of seed and (D) Aflatoxin on TLC plate AFB_1

V-1 (NW505), V-2 (PBW343), V-3 (K1006), V-4(K0307), V-5(DBW39), V-6 (HD2733)

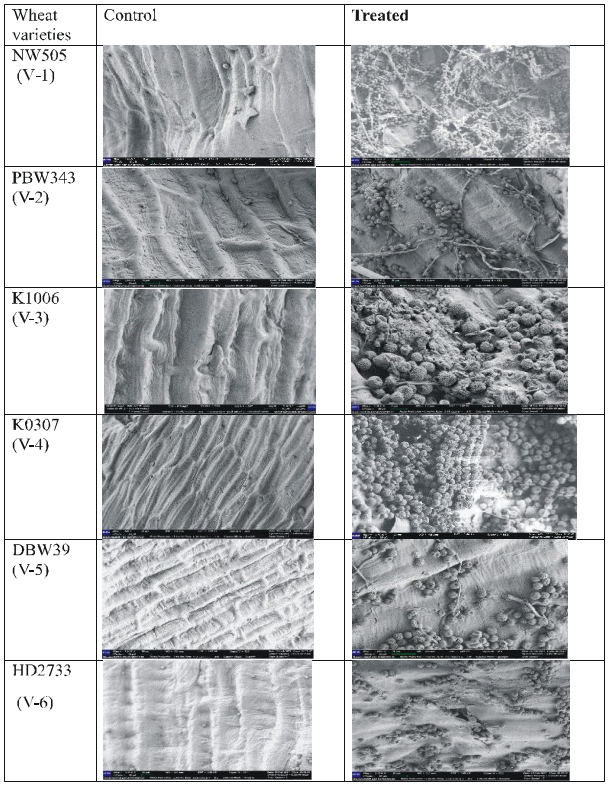

Fig. 3: Colonization potentials of *A. flavus* on infested wheat variety under SEM

and are quantified in ppm. The range of different nutrient content such as P (4196.46-7678.71), K (21149-36030), S (1041.6-3541.36), Zn (7.86-80.66), Cu (8.03-18.03), Fe (243.2-584.86), and Mn (6.9-111.92) was recorded (Table-1). Maximum P (7678.71), S (3541.36), Cu (18.03) and Mn (111.92) content in ppm was noticed in PBW343 (V-2) varieties whereas, maximum K (36030), Zn (80.66) and Fe (584.86) content was recorded in K0307 (V-4), K1006 (V-3) and HD2733 (V-6) varieties, respectively. Minimum P (4196.46) and S (1041.6) were recorded in NW5054 (V-1) variety, whereas HD2733 (V-6) variety showed minimum K (21149), Zn (7.86) and Cu (8.03) contents. Similarly, minimum Fe (243.2) and Mn (6.9) were noticed in K1006 (V-3) and DBW39 (V-5) variety,

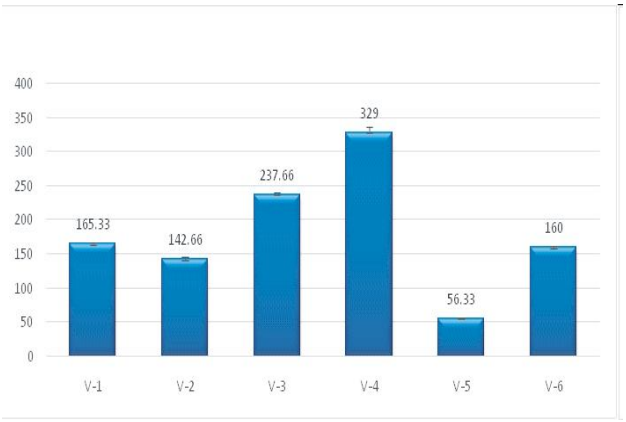

Fig.4: Aflatoxin production potentials (µg/kg) in artificially infested wheat variety
V-1 (NW505), V-2 (PBW343), V-3 (K1006), V-4(K0307), V-5(DBW39),V-6 (HD2733)

respectively. The wheat varieties grown in India are diverse in physicochemical and nutritional properties (Kumar *et al.* 2014; Kundu *et al.* 2016). Kundu *et al.* 2017 also reported a significant variation in the physicochemical and flour characteristics of the same wheat varieties grown at different geographical conditions. During analysis of Fe and Zn content of 265 wheat genotypes by Zhang *et al.* (2010) recorded quite significant variations ranging from 28.0 to 65.4 mg kg^{-1} and 21.4 to 58.2 mg kg^{-1} respectively, with a mean of 39.2 and 32.3 mg kg^{-1}. Similarly, Ikhtiar and Alam (2007) during the study of Pakistani wheat varieties, maximum potassium (84.25×10-6) and phosphorus (0.84×10-6) values are observed in Chudry-97 and Watan, whereas minimum (77.65×10-6) and (0.24×10-6) was noticed in Ghaznawy and Gandam-2002 variety which indicates that different wheat variety contains a variable amount of nutrients.

SEM observation of A. flavus colonization on wheat varieties

Variable *A. flavus* colonization potentials on the surface of wheat variety were observed under SEM study and are compared with the control set (Fig 3). Maximum *A. flavus* colonization on grain surface was observed in K0307 (V-4) variety as their surface showed very fine threadlike venation with multiple dense ridges and furrows. Rest other varieties such as K1006 (V-3), NW5054 (V-1), HD2733 (V-6), PBW343 (V-2) showed grain surface colonization in decreasing order, and the minimum was observed in DBW39 (V-5) variety. The study of wheat grain surface structures through SEM of two wheat cultivars (Eser and Fuatbey-2000) by Kalkan and Palamanit (2017) reported several longitudinal parallel ridges but variable patterns. The variations in grain surface colonization are either due to variations in seed coat structure, the thickness of seed coat, and the presence of cuticle layer or are due to nutrient content variations in different varieties.

Aflatoxin elaboration potentials of wheat varieties

Aflatoxin B_1 elaboration potentials in all six artificially infested wheat varieties were quantified in µg/kg. The maximum (329) aflatoxin B_1 production was recorded in K0307 variety followed by K1006 (237.66), NW5054 (165.33), HD2733 (160.66), PBW343 (142.66) variety, whereas minimum (56.33) was recorded in the case of DBW39 variety (Fig. 4). Maharjan *et al.* (2002) were also studied aflatoxin elaboration by toxigenic *A. flavus* on artificially infested ten common wheat varieties of Nepal, viz. Nepal-297, Kalyansona, Lerma Rojo-64, RR-21, BL-1022, BL-1473, Annapurna-1, Bhrikuti, UP-262, and Annapurna-4 and found that all the varieties are susceptible for aflatoxin B_1 production with a range of 57 (Nepal-297) to 824 (Nepal-297) µg/kg. Similarly, Toteja *et al.* (2006), while working on 1,646 samples of wheat grain collected from different parts of India as a multicenter study and found that 40.3% of the samples were positive for aflatoxin B_1 production in natural conditions. However, in the case of artificially infested wheat grains, the amount of aflatoxin is always higher than natural contamination (Kumar *et al.,* 2013). During working on the effects of different nutrients on aflatoxin production in wheat and other cereals by Liu *et*

al. (2016), Zn concentration showed increasing effects on dry mycelial weight and significantly induced AFB_1 production up to 1.7- to 26.6-fold. Similarly, other nutrients such as glycine and glutamic acid are also essential for aflatoxin production. Varied physical properties of grains and nutritional content variations may help or prevent fungal colonization as well as aflatoxin elaboration (Sharma *et al.* 2018). The variable amount of aflatoxin elaboration in different wheat varieties is also supported by colonization potentials of *A. flavus* on infested wheat variety under SEM (Fig. 3), artificially infestation of seed (Fig 2C) as well as thin layer chromatogram of Aflatoxin (Fig 2D). The present study indicated thatthe highest amount of aflatoxin elaboration is observed when the surface colonization is maximum as revealed in SEM. In contrast, if colonization is meager, then the amount of aflatoxin production is reduced proportionally.

ACKNOWLEDGEMENT

Authors are thankful to the Head, Department of Botany (UGC-DRS-SAP & DST-FIST Sponsored) Visva-Bharati, Santiniketan, for providing necessary laboratory facilities and the University Grant Commission for financial support as UGC-BSR research Start-up grant. The authors are also thankful to the Department of Integrated Science, Visva-Bharati Santiniketan, for providing Scanning Electron Microscope facilities.

REFERENCES

Dalal, D., Mandal, N. 2021. Isolation and screening of aflatoxigenic *Aspergillus flavus* from the wheat rhizosphere of Birbhum, West Bengal and its growth inhibition through solvent extract of medicinal plants. *J. Mycopathol. Res*. **59:** 285-290.

Giray, B., Girgin, G., Engin, A.B., Aydýn, S.,Sahin, G. 2007. Aflatoxin levels in wheat samples consumed in some regions of Turkey. *Food Control*. **18:** 23–29.

Ikhtiar, K.,Alam, Z. 2007. Nutritional composition of Pakistani wheat varieties. *Journal of Zhejiang University Science B*. **8:** 555-559.

International Agency for Research on Cancer (IARC). 2002. Summaries and evaluations: aflatoxins. *Lyon: IARC Press*. **82:** 171

Kalkan, F., Palamanit, A. 2017. Scanning Electron Microscopy Images of Moistened Wheat Grains: Frictional Properties. *Int. J. Adv. Chem. Eng. Biol. Sci*. **4:** 159-161.

Kimura M.1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution***16:**111-120.

Kumar S., Stecher G., Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol.Evol***. 33***:* 1870-1874*.*

Kumar, A., Mandal, N., Roy, S., Roy, A. K. 2013. Deterioration in nutritive value of wheat grains infested with aflatoxigenic strains of *Aspergillus flavus*. *Ind.Phytopathol*. **66:** 311-312.

Kumar, A., Maurya, B. R.,Raghuwanshi, R. 2014. Isolation and characterization of PGPR and their effect on growth, yield and nutrient content in wheat (*Triticum aestivum* L.). *Biocatal. Agricult.Biotechnol*. **3:** 121-128.

Kundu, M., Khatkar, B.S.,Gulia, N. 2017. Assessment of chapatti quality of wheat varieties based on physicochemical, rheological and sensory traits. *Food Chem.***226:** 95–101.

Kundu, M., Gulia, N.,Khatkar, B.S. 2016 Diversity in quality traits of Indian wheat cultivars: grain, whole wheat flour and protein characteristics. *Int. J.Innov. Res. Sci. Eng. Technol.***5:**17590–17594.

Liu, J., Sun, L., Zhang, N., Zhang, J., Guo, J., Li, C., Qi, D. 2016. Effects of nutrients in substrates of different grains on aflatoxin B1 production by *Aspergillusflavus*. *BioMed Res.Inter*. **2016:** Article ID 7232858, 10 pages.

Maharjan, K., Karki, T., Sinha, K. 2002. Screening of some common varieties of wheat from Nepal against Aflatoxin elaboration by *Aspergillus flavus*. *J. Inst. Agri.Animal Sci*. **23:** 85–87.

Nabney, J.,Nesbitt, B. E. 1965. A spectrophotometric method for determining the aflatoxin. *Analyst, Lond*. **90:** 155-160.

Reddy, T. V., Viswanathan, L.,Venkitasubramanian, T. A. 1970. Thin layer chromatography of aflatoxin. *Anal.Biochem*. **38:** 568 – 571.

Sahrawat, K. L., Ravi Kumar, G., Rao, J. K. 2002. Evaluation of triacid and dry ashing procedures for determining potassium, calcium, magnesium, iron, zinc, manganese, and copper in plant materials. *Comm. Soil Sci.Plant Analysis* **33:** 95-102.

Sharma, A.C., Proshad, R., Kormoker, T., Islam, M.S., Chandra, K. 2018. A review on aflatoxins in stored grain food, their sources, mechanisms and possible health hazard. *Arch. Agri. Environ. Sci.* **3:** 416-423.

Stack, M. E.,Pohland, A. E. 1975. Collaborative study of a method for chemical confirmation of identity of aflatoxin. *J. Assoc. Anal. Chem.* **58:** 110- 113.

Thakur, R. K. 2012. Determination of Sulphur in Soil and Plant. *Training Programme on Advance in Agro-technolies for Improving soil, Plant and Atmosphere system.*

Thomas, F. Eppley, R.M., Trucksess, M.W. 1975. Rapid screening methods for aflatoxin and zearalenone. *J.Assoc. Anal.Chem*. **58:** 114-116.

Toteja, G. S., Mukherjee, A., Diwakar, S., Singh, P., Saxena, B. N., Sinha, K. K., Sinha, A. K., Kumar, N., Nagaraja, K. V., Bai, G., Prasad, C. A., Vanchinathan, S., Roy, R.,Parkar, S. 2006. Aflatoxin B1 contamination in wheat grain samples collected from different geographical regions of India: A multicenter study. *J Food Prot*. **69:**1463-1467.

Zhang, Y., Song, Q., Yan, J., Tang, J., Zhao, R., Zhang, Y., He Zhonghu., Zou, Chungin, Ortiz-Monasterio, I. 2010. Mineral element concentrations in grains of Chinese wheat cultivars. *Euphytica*, **174:** 303-31