Biochem. Cell. Arch. Vol. 21, No. 2, pp. 3971-3977, 2021	www.connectjournals.com/bca	*ISSN 0972-5075*
DocID: https://connectjournals.com/03896.2021.21.3971		*eISSN 0976-1772*

DETECTION AND IDENTIFICATION OF A LOCAL FUNGAL ISOLATES *FUSARIUM* SPP. PRODUCING FOR VOMITOXIN (DEOXYNIVALENOL) FROM WHEAT AND BARLEY USING MORPHOLOGICAL AND MOLECULAR METHODS

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(Received 28 March 2021, Revised 3 June 2021, Accepted 12 June 2021)

ABSTRACT : The current study was aimed to identification a local isolates of *Fusarium* spp. associated with wheat and barely grains that producing Deoxynivalenol, using traditional and molecular methods. The results revealed that five *Fusarium* spp. were found in wheat and barley seed of present study cultured on different media (Potato-Dextrose Agar, Potato-Dextrose Agar + malachite green oxalate, Malachite Green Agar 2.5) and Occurrence of fusarium spp. 32%. In both samples as: *F. oxysporum, F. lateritium*, *F.solani*, *F. sporotrichioides*, *F. tricinctum.* The most productive isolates of Fusarium species was *F. oxysporum* with highest productivity (2175.8975 ± 6.71681) ppb according to Quantitative detection by ELISA of DON production in thirteen isolates, where all isolates of *Fusarium* spp. have the ability to produce Deoxynivalenol in different concentrations. The Identification of this isolate *Fusarium oxysporum* were carried out by culturing them on PDA plates at 25^{0}C for 7 days, PCR analysis confirmed our morphological identifications by used ITS primer pairs : (ITS1 and ITS4) for *ITS* gene. Contamination of wheat and barley samples (different places) with toxigenic *Fusarium* spp. The identification of *Fusarium oxysporum* by PCR analysis are a practical, short, reliable and more accurate method when compared to the traditional methods.

Key words : Wheat, barley, *Fusarium oxysporum*, ITS1.ITS4, deoxynivalenol.

How to cite : Nada Fazei AL-Hadeethi, Nada Sabah Razoqi, Khalid A. Habib and Warqaa Y. Salih (2021) Detection and identification of a local fungal isolates *Fusarium* spp. producing for vomitoxin (Deoxynivalenol) from wheat and barley using morphological and molecular methods. *Biochem. Cell. Arch.* **21**, 3971-3977. DocID: https://connectjournals.com/03896.2021.21.3971

INTRODUCTION

Wheat (*Triticum aestivum* L) is one of the oldest known and most important grain crops cultivated and used as daily food and a major source of energy (Pontonio and Rizzello, 2019). Wheat contains high amounts of carbohydrates that are necessary to provide food calories in addition to its good source of protein, vitamins, and minerals (Lamothe *et al*, 2019). Wheat, which belongs to the family Poaceae, is one of the most essential staple crops for human food and livestock feed. In 2018, 1180443 ha of wheat were harvested in different provinces of Iraq, producing 3211382 tonnes (Jaber and Lahuf, 2020). Wheat flour contains starch, protein, fiber, minerals, fat and low levels of pentosans that make wheat of great economic and nutritional importance. Wheat is prone to infection with many plant pathogens causing seed rot and damping off diseases at different stages of plant growth (Ishtiaq *et al*, 2019; Al-Moussawi, 2010). Barley (*Hordeum vulgare* L.) is one of the most important crop plants worldwide, representing the fourth most broadly cultivated cereal; after wheat, maize and rice (Verstegen *et al*, 2014). The global production reached 156.80 million metric tons in 51.85 million hectares in 2019 (Tomek and Kaiser, 2014). Barley is an ancient cereal which has been traditionally used for animal feeding and as raw material in malting industry (Madakemohekar *et al*, 2018). Barley has a high level (up to 6% average) of β-glucan, which is water-soluble polysaccharide considered as souluble dietary fiber. β-Glucan has been shown beneficial effects for heart patients by reducing blood pressure (Behall *et al*, 2014) lower serum cholesterol and visceral fats. Also, the positive effect of β-glucan on management of diabetes by reducing postprandial blood glucose levels is well established (Cavallero *et al*, 2002).

Barley, along with other small grain cereals can be affected by fungi, resulting in economic losses due to disease, low quality, and mycotoxin contamination (Almeida *et al*, 2018). Deoxynivalenol (DON) is 3α,7α,15-trihydroxy-12,13-epoxy-trichothec-9-en-8-one. DON, one of about 150 related compounds known as the trichothecenes is one of the toxic secondary metabolites produced by a number of species of *Fusarium* fungi and some other fungi. DON can be predominantly found in agricultural crops like barley, rice, maize, and wheat, deoxynivalenol-contaminated grain can produce vomiting, feed refusal, weight loss, and diarrhea in livestock (Valera *et al*, 2019). Human and animal exposure to DON via ingestion of contaminated food can induce acute and chronic effects, such as immunosuppression, neurotoxicity, embryo toxicity, and teratogenicity (Pestka, 2007). The International Agency for Research on Cancer categorized DON as aGroup3 carcinogen (World Health Organization, International Agency for Research, 1993).

Fusarium is belong to the Ascomycota and its species can be supposed as fungi with high impact on life and health of human, directly or indirectly (Karim *et al*, 2016).

Fusarium spp. are classified as very important fungal pathogens due to their association with extremely important crops in almost all steps of the agriculture industry, both pre- and postharvesting, throughout the world (Marin *et al*, 2013). Some members of *Fusarium* produce a multitude of secondary metabolites, considered "mycotoxins" due to their toxic, mutagenic and/or carcinogenic effects on humans and animals once the harvested product is processed into food and consumed (Escrivá *et al*, 2015). Identification of *Fusarium* genus is based on the formation of multicellular and banana-shaped macroconidia with basal foot cell but identification of species is difficult and not sufficient; therefore molecular methods are required to confirm identification (Healy *et al*, 2005).

The current study was aimed to Identification a local isolates of *Fusarium* spp. associated with wheat and barely grains that producing Deoxynivalenol, using traditional and molecular methods.

MATERIALS AND METHODS

Samples collection

Ninety three Wheat and Barley samples were collected between (July 2019 and January 2020) from local markets and from silos (AL-Dura, Al-Tagi, AL-Sulaymaniyah), Quality Control Department and Ministry of Agriculture, (2-4) kilogram of each sample was placed in a plastic bag and stored at 4°C until analysis.

Isolation of fungi

Two hundred grains from each Wheat or Barley sample were surface-sterilized by immersion in 2% sodium hypochlorite (NaOCl) solution in 250 ml conical flask for one minute, and then washed three times using sterilized D.W. The grains were dried by using sterilized filter paper and placed on MGA 2.5 and PDA medium containing chloramphenicol (50 mg/L) using twenty Petri plates for each sample (10 grains/each plate) (Barnett *et al*, 1998; Domsch *et al*, 2007).

Identification of *Fusarium* species

All fungal isolates were identified by traditional methods according to the macro & microscopic characteristics using different culture media as PDA, MGA, MGA2.5+ carnation leaf pieces, PDA+ malachite, PSA and KCLA and Lactophenol cotton blue (Booth *et al*, 1977; Leslie and B A, 2007).

Detection and quantification of the deoxynivalenol

Fungal inoculum preparation : *Fusarimm* spp. isolates were incubated for 4–5 days at 25^0C on potato dextrose agar. Colonies were subcultured on new PDA plates and stored on PDA slants at 4^0C for further use. Plugs (3 mm diameter) were collected with a cork borer from the margin of colonies grown on PDA for seven days at 25^0C. All the plugs were prepared and incubated in the fluid nutrient medium of potato dextrose broth (PDB) that used for fungal growth and mycotoxin production. Then The fungi was incubated in 200 mL of PDB for each run with different environmental factors. Medium pH (4), incubation temperature (25^0C) and time (28 days) were evaluated to find the most suitable culture condition for mycotoxin accumulation (Wu *et al*, 2017).

Deoxynivalenol extraction from growth medium for ELISA analysis

The extraction of DON was carried out after 28 days of incubation in PDB based on the optimized culture conditions. The cultures were filtered with filter paper, and the mycelia were extracted with methanol, then the filtrate and extract were combined and concentrated under reduced pressure using a rotary evaporator at 65^0C. Subsequently, methanol/water (80/20, v/v) was added and the extracts were washed with hexane (60 mL) to defat. The aqueous and methanol layers were collected, concentrated and filtered through a 0.45 μm nylon filter, and stored in brown glass at -20^0C until further purification and isolation (Nilanonta *et al*, 2002; Meca *et al*, 2009).

Enzyme Linked Immune Sorbent Assay (ELISA) technique

Detection of Deoxynivalenol by ELISA technique

Table 1 : Primers.

Primer Name	Seq.	Annealing temp. (°C)	Product size (bp)
ITS1	F 5'-TCCGTAGGTGAACCTGCGG-3'	55	558 bp
ITS4	R 5'-TCCTCCGCTTATTGATATGC-3'		

was performed using eurofins ELISA kit company (Germany) according to the instructions of manufacturer.

Extraction of DNA and PCR assay

Fusarium isolates were grown on PDA plates for seven days by single spore isolation, then mycelia and conidia were harvested and ground (Rahjoo *et al*, 2008). The DNA was extracted from ground mycelium of each isolate by Promega, (USA) DNA kit according to the instructions of manufacturer Primers were selected according to previously published studies (ITS1, ITS4) (White *et al*, 1990) these primers used for diagnosis of Fusarium species as shown in Table 1. Their primer was supplied by Macrogen Company, Korea. PCR Master Mix (Promega company, USA) ready to load (Green) had been used in this work. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel electrophoresis stained with Eth.Br. PCR conditions was set as the following : 1 Cycle at 95^{0}C for 5 min then 30 Cycle of 95^{0}C for 30s, 55^{0}C for 30 s, 72^{0}C for 30s and Final cycle at 72^{0}C for 30 s. PCR product were sent for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation, Korea. The results then analyzed by using geneious software.

Statistical analysis

All analysis was performed using the statistical package (SPSS) version thirteen; the data were expressed as mean, standard deviation SD, percentage. ANOVA was used to analyze repeated measurement and t-test. Results were determined as non-significant at ($P > 0.05$).

RESULTS AND DISCUSSION

Isolation and identification of *Fusarium* spp. in wheat and barley

The results revealed that five *Fusarium* spp. were found in Wheat and Barley seed of present study cultured on different media (Potato-Dextrose Agar, Potato-Dextrose Agar + malachite green oxalate, Malachite Green Agar 2.5) as seen in Table 2.

The occurrence of *Fusarium* spp. 32%. The species of *Fusarium* that appeared in both samples : *F. oxysporum*, *F. lateritium*, *F. solani, F. sporotrichioides, F. tricinctum*.

General characteristics of *Fusarium* species

Fusarium species may produce three types of spores called macroconidia, microconidia, and chlamydospores (Nelson *et al*, 1983). Some species produce all three types of spores, while other species do not. The macroconidia are produced in a specialized structure called a sporodochium in which the spore mass is supported by a superficial cushionlike mass of short monophialides bearing the macroconidia (Hawksworth, 1995). Macroconidia may also be produced on monophialides and polyphialides in the aerial mycelium.

Microconidia are of various shapes and sizes, and those produced in chains have a truncate base. The third type of spore formed by *Fusarium* species is a chlamydospore. The most characteristic of chlamydospores is the formation thick walled conidia filled with lipid material that help the fungus to survive under hard conditions like absence of suitable host. The chlamydospores are arranged in different ways: singly, in pairs, in clumps or in chains. The outer wall of chlamydospore may be smooth or rough (Booth, 1977).

Morphological identification of *Fusarium oxysporum*

As results shown in Table 2 from the 39 *Fusarium* isolates were 17 identified as *Fusarium oxysporium* , when culturing them on PDA plates at (25^{0}C) for 7 days as Colony colour ranged from white to pale violate, *F. oxysporum* usually produce pigmentation with a pale violet as shown in Fig. 1. Also, Macroconidia: Straight to slightly curved, thin walled, usually 3-5 septate, with tapering and curved apical cell and foot shaped basal cell. Macroconidia sparse I: oval, elliptical, straight to curved. Produced in false heads on short monophialidic conidiogenous cells. Chlamydospores: singly, in pairs, in clusters or in short chains. Chlamydospores terminal or intercalary with smooth or rough walled (Ismail *et al*, 2013) as shown in Fig. 2.

Screening of *Fusarium* spp. isolates for production of Deoxynivalenol using ELISA

DON Quantitative detection was done by ELISA Technique in thirteen isolates as results shown in Table 3 that the most productive *Fusarium* species was *F. oxysporum* as highest productivity (2175.8975 ± 6.71681 ppb), while the other isolate ranged between (1874.6880-2153.8573ppb).

When ELISA technique was used to measure the concentration of DON. All the thirteen isolates of *Fusarium* species have the ability to produce DON in

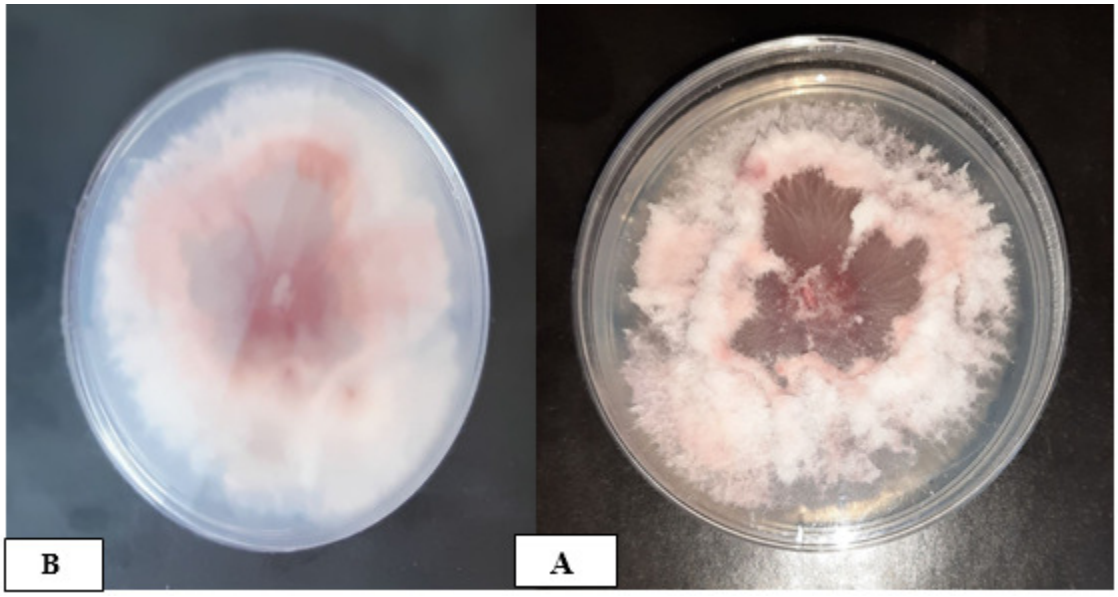

Fig. 1 : Colony Morphology of *Fusarium oxysporum on* PDA at 25ºC for seven days (A) up side, (B) reverse side.

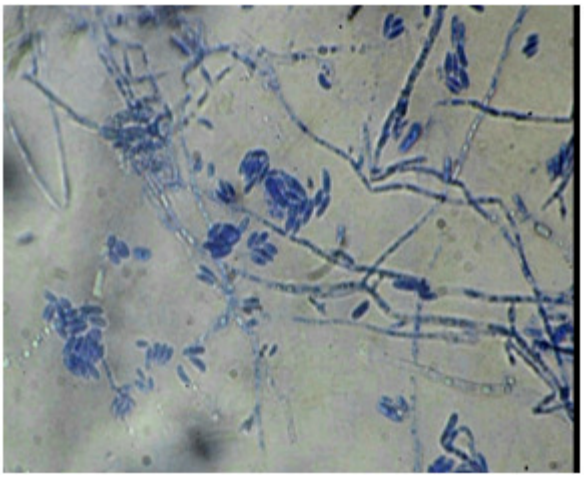

Fig. 2 : Microscopic observation of *Fusarium oxysporum* by lacto phenol cotton blue staining (40X).

Table 2 : Isolation and identification *Fusarium* spp. in wheat and barley.

Isolation number	*Fusarium* spp.	Sample
1	*F.oxysporum*	Barley
2	*F.oxysporum*	Barley
3	*F.oxysporum*	Wheat
4	*F.oxysporum*	Wheat
5	*F.lateritium*	Barley
6	*F. solani*	Wheat
7	*F. sporotrichioides*	Wheat
8	*F.tricinctum*	Wheat
9	*F. solani*	Wheat
10	*F. sporotrichioides*	Barley
11	*F.tricinctum*	Barley
12	*F. solani*	Barley
13	*F. solani*	Barley

different concentrations.

The enzyme-linked immunoassay (ELISA) is probably the most commonly used antibody-based assay for mycotoxin detection. The principle of the ELISA method is based on the specific antigen-antibody chemical reaction; it can be direct, competitive, indirect, Indirect competitive and sandwich ELISA (Suzuki *et al*, 2007). The advantages of this technique are included rapid speed screening, high specificity, simple operation without using any instrument or readers and eco-friendly analysis because radioactive labels and large volumes of organic solvents are not required (Krska and Molinelli, 2009; Sakamoto *et al*, 2018). Quantification and detection of common mycotoxins, including AFM1, AFs, OTA, DON, fumonisins, ZEA and T-2 toxin are possible with commercial ELISA kits (Chun *et al*, 2007; Li *et al*, 2009). This technique is used with many studies in Carolina (Yu *et al*, 2019), in USA (Sanders *et al*, 2016) and Hungary (Tima *et al*, 2016).

Molecular identification of *Fusarium oxysporum*

Locally, for the first time, molecular methods as pcr using for diagnostic a mycotoxin producing fungal isolate.

We used PCR analysis to confirm our morphological identifications by used ITS primer pairs ITS1 (F 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 (R 5'-TCCTCCGCTTATTGATATGC-3') (White *et al*, 1990) for analysis on ITS region and ITS gene to identify unknown fungal species that originally designed for fungi as shown in Fig. 3. This isolate was identified with a BLAST search for similarities present in the NCBI database as well as by comparing their sequences against the *Fusarium oxysporum* database.

PCR assay is precise and complementary techniques, which allow a rapid, sensitive and reliable specific

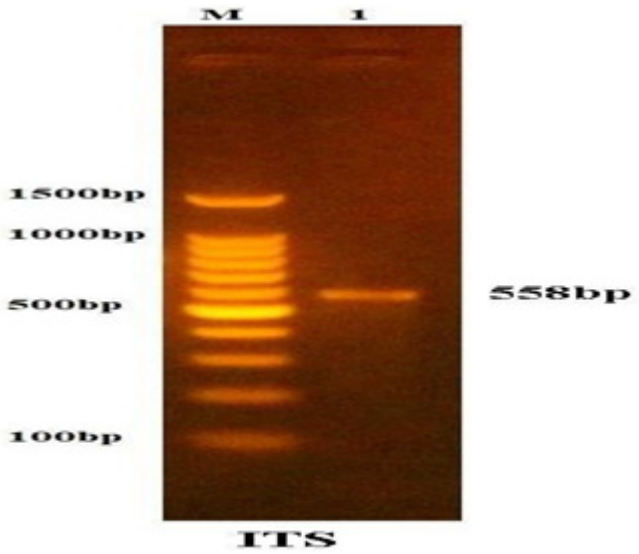

Fig. 3 : Results of the amplification of *ITS* gene of *Fusarium oxysporum* species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1 resemble 558bp PCR products.

Table 3 : Deoxynivalenol production by *Fusarium* species isolates on PDA media at 28ºC for 28 days.

Isolates	*Fusarium* spp.	DON ppb Mean ± Std. Deviation
1	*F. oxysporum*	2175.8975 ± 6.71681 [A]
2	*F. oxysporum*	2153.8573 ± 42.81975 [A]
3	*F. oxysporum*	2082.6728 ± 29.05140 [A]
4	*F. oxysporum*	2066.1375 ± 113.09290 [A]
5	*F. lateritium*	2047.3200 ± 202.29946 [A]
6	*F. solani*	2032.7833 ± 114.43183 [A]
7	*F. sporotrichioides*	2016.7145 ± 157.93060 [A]
8	*F. tricinctum*	1993.0700 ± 32.82457 [A]
9	*F. solani*	1980.6750 ± 139.10761 [A]
10	*F. sporotrichioides*	1958.6568 ± 122.01565 [A]
11	*F. tricinctum*	1939.3058 ± 254.28731 [A]
12	*F. solani*	1909.9675 ± 169.62860 [A]
13	*F. solani*	1874.6880 ± 431.00917 [A]
	Total	2011.5038 ± 178.11658

• A letter within the same column are non-significantly different ($p \leq 0.05$).

TTCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATCCGAG
GTCAACATTCAGAAGTTGGGGGTTTAACGGCTTGGCCGCGCCGCGTACCAGTTGCGA
GGGTTTTACTACTACGCAATGGAAGCTGCAGCGAGACCGCCACTAGATTTCGGGGCC
GGCTTGCCGCAAGGGCTCGCCGATCCCCAACACCAAACCCGGGGGCTTGAGGGTTGA
AATGACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGTTCA
AAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTTGCTGCGT
TCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTTATTTATGGT
TTTACTCAGAAGTTACATATAGAAACAGAGTTTAGGGGTCCTCTGGCGGGCCGTCCC
GTTTTACCGGGAGCGGGCTGATCCGCCGAGGCAACAATTGGTATGTTCACAGGGGTT
TGGGAGTTGTAAACTCGGTAATGATCCCTCCGCAGGTTCACCTACGGA

Fig. 4 : FASTA Sequence of *Fusarium oxysporum* (carried out with the support of Macrogen Company, Korea).

diagnosis of *Fusarium* species (Petrovic *et al*, 2009).

The use of PCR technique was beneficial in epidemiological analyses (Geiser *et al*, 2004; Sreenivasa *et al,* 2008) and used widely in the taxonomy of fungi (Mule *et al*, 2004a; Nayaka *et al*, 2010). Some researchers used PCR for the detection of pathogenic fungi in infected plant tissues (Nayaka *et al*, 2008). The major benefit of PCR technique is that the researcher need only small quantities of genomic DNA to confirm the presence of pathogen in host tissue that might not be detected in culture (Nayaka *et al*, 2008).

CONCLUSION

Contamination of wheat and barley samples (different places) with toxigenic *Fusarium* spp. The identification of *Fusarium oxysporum* by the species specific PCR are a practical, short, reliable and more accurate method when compared to the traditional methods. All isolates of *Fusarium* spp. have the ability to produce Deoxynivalenol in different concentrations.

ACKNOWLEDGMENTS

All Experiments of Pcr and Sequence for detection of *Fusarium oxysporum* was carried out with the support of laboratories of Macrogen Company, Korea.

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