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DETECTION OF TRICHOTHECENE TOXIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY(HPLC) ANDTHIN LAYER CHROMATOGRAPHY TEST (TLC) TECHNOLOGY OF GRAIN CONTAMINATED WITH *FUSARIUMGRAMINEARUM* FUNGI

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ABCTRACT : The current study aimed using several techniques in detecting some isolates of the *Fusariumgraminearum*fungi, testing their ability to produce or not producing tricothecencs toxin, and the techniques used in this study like the technique of High-performance liquid chromatography (HPLC) and *Thin Layer Chromatography test* (TLC) technology. Isolates eight isolates of different origin and source, isolating one clinical isolation (cow's lung) and seven environmental isolates (wheat and barley),These fungi were cultured on Potato dextrose agar (PDA) and then sub cultured on Sabouraud dextrose agar (SDA). The isolates were identified depending on their morphological characteristics (cultural and microscopical). The results showed that the color of the *Fusarium* colony was initially cotton us but soon became white in the anterior and zebra direction in the opposite direction, while its shape was similar to the large banana in microscopic tests. The study showed no differences in the results of the technique of liquid chromatography and chromatography test. This is a confirmation of the findings of the present study with other studies. If isolates were found to have the ability to produce poison and the highest percentage of semolina in barley samples was (18.72 - 18.75) compared to wheat samples Which was observed to be unable to produce the poison and this indicates the existence of significant differences between the isolates of wheat and barley in terms of their origins, while no significant differences between the isolates in terms of origin (clinical, environmental). As for the test of accurate color separation was observed discoloration of the same samples producing the poison Boas Of technical liquid high chromatography test performance by comparing it with standard, and the reason is due to the ability of some isolates the toxin production and other do not have the ability to produce poison for the absence of the gene responsible for the production of this poison or mutation genetic led to his absence in the other isolates.

Key words : *Fusarium graminearum,* tricothecencs*,* HPLC, TLC.

INTRODUCTION

Mycotoxin produced by the genus Fusarium trichothecenes - B were monitored in different samples of wheat grain (Jesus *et al* 2014)Human food can be contaminated with mycotoxins at various stages in the food chain (Bennett and Klich, 2003) and the most important generas of mycotoxigenic fungi are Aspergillus, Alternaria, Claviceps, Fusarium, Penicillium and Stachybotrys,Mycotoxins occur more frequently in areas with a hot and humid climate, favourable for the growth of molds, they can also be found in temperate zones (Zain,2011). Some Fusarium species produce trichothecene mycotoxins under certain growth conditions (Kimura *et al*, 2007; Desjardins,2009) is an important pathogen of wheat and barley can accumulate type B trichothecenes, such as deoxynivalenol (DON)and nivalenol (NIV), in infected grains (Marin*et al*, 2013). TrichothecenesNumerous kinds of trichothecenes are producedby taxonomically unrelated fungal genera, including*Fusarium, Trichothecium, Myrothecium, Stachybotrys*, andother saprophytic fungi. Among the trichothecene producers,*Fusarium culmorum* and *Fusarium graminearum*aremajor hemibiotrophic pathogens that cause a devastatingdisease in cereal crops, collectively known as *Fusarium* headblight (FHB) (Goswami&Kistler, 2004). In addition tosignificant losses of yield, these *Fusarium* species causemycotoxin contamination in infected grains,Thereforemost of the concerns about trichothecenes are related to *Fusarium* trichothecenes such as deoxynivalenol, nivalenol,and acetylated derivatives thereof, which actually poseserious

health threats to humans and animals (Pestka&Smolinski, 2005).The T-2 toxin is well-known to cause acute and chronic tox-icity, as well as alimentary toxic aleukia (ATA) and Kashin- Beck Disease (KBD) in humans and animals. It has been shown to cause alteration of blood–brain barrier (BBB)(Chen *et al,* 2012).

Methodologically, T-2 analysis methods are divided intotwo classes; screening methods such as Thin Layer Chromatography (TLC) and high-performanceliquid chromatography (HPLC) methods. Ler *et al,* 2006) In this study, HPLC and TLC detection of T-2 toxin from*Fusarium* strains is studied.

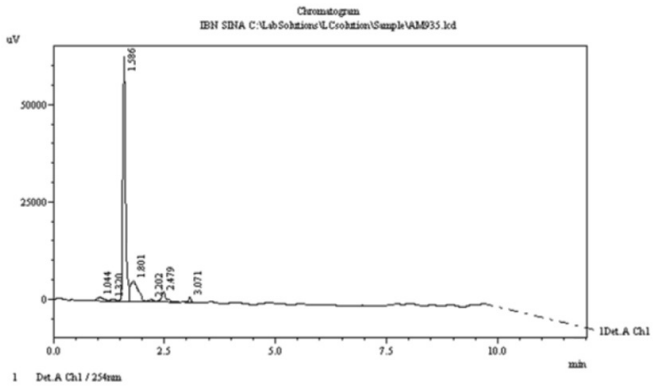
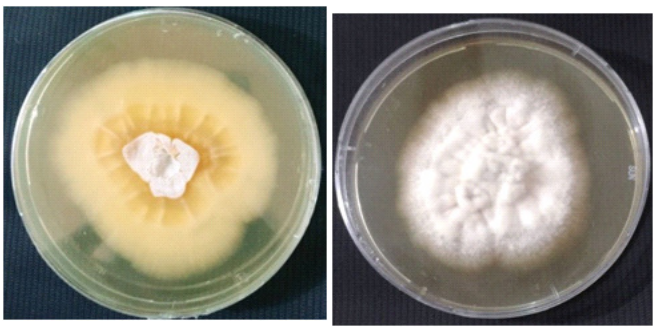


Figure 1 : The standard curve of Tri concentrations using HPLC technique.



B=Reversed A= Front

Figure 2 : *Fusariumgraminearum* growth 28 °C after 7 day.

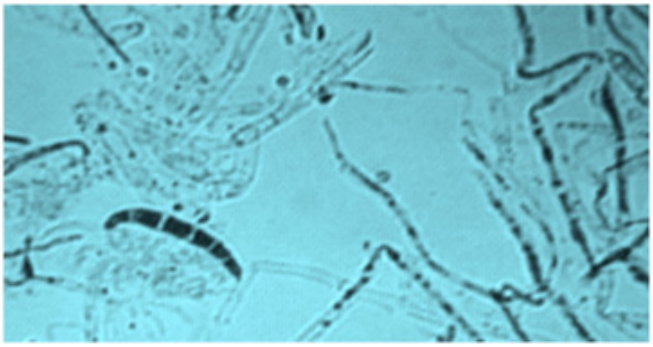


Figure 3 : Microscopic features of *Fusarium graminearum*

MATERIALS AND METHODS

Fungi and Culture preparation

Eight samples of wheat and barley were collected, (Table 1). A section of pure and proliferated colony onPDA medium were added to sterile rice substrate (50 mg)of rice in 50 mL sterile distilled water) and was incubated (27±1 °C for 5 days). Then 3 weeks was incubated at 10±2°C according to literature (Joffe,1986) Then growth medium wastransferred onto a sterile aluminum foil and then was oven dried(45°C for 24-48 hours). The dry weight was powderedby electronic grinder. Afterward the extract was prepared and used for further analysis.

Trichothecene extract

The Trichothecene toxin was extracted according to Ehrlich &Lillehoj(1984). Taken 25 g of representative sample of soybeans were placed into an 100ml Erlenmeyer flask.,Add 20 ml of a acetonitrile –water solvent mixture (86:14, vol/ vol),The flasks was shaken for 1 h.,Then the suspension was filtered through apaper filter with 5 g of sodium sulphate, After filtration and evaporation of the solvent on a rotary evaporator, the residue from the solvent extraction was dissolved (1 g/10 ml) in pyridine-acetic anhydride (1:1,vollvol). After 24 h at room temperature, the solvent was removed in vacuo with a mechanical pump (40°C, 2.5 torr and the residue was dissolved in diethyl

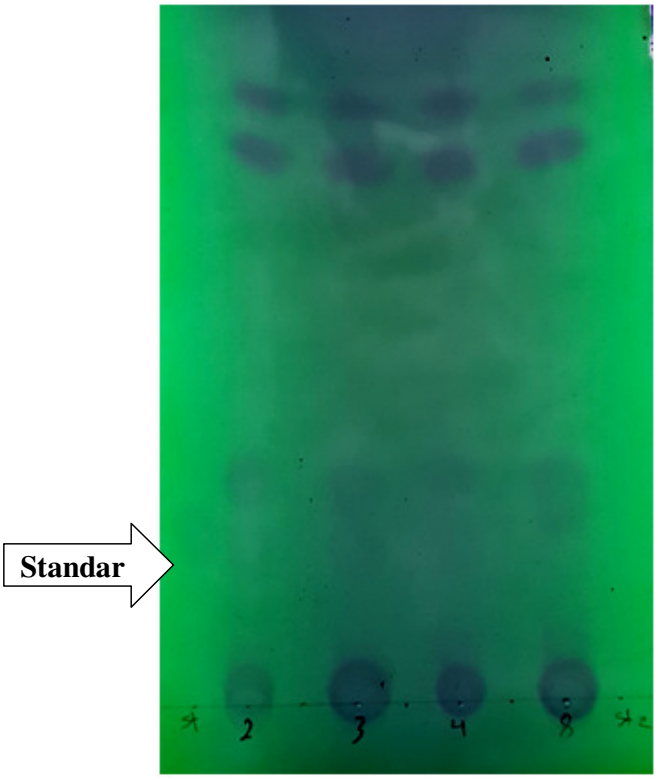


Figure 4 : Thin Layer chromatography (Blue colour spot showing Standard T-2 Toxin), acetone-chloroform –isopropanol (1: 8: 1,vol/vol/vol) as mobile phase.

ether (1 g/5 ml) and filtered, The ether solution was placed on a column (6.0 by 30 cm) of Silica Gel 60 (Brinkmann Co.) and the triacetyldeoxynivalenol was eluted with ethyl ether. (Approximately 10 200-ml fractions were collected). Since triacetyldeoxynivalenol does not stain with AlCl3 spray, portions of the fractions were treated with 0.5 M NaOH in 90% aqueous ethanol to regenerate DON, The DON was identified by its mobility (Rf = 0.6) and characteristic bluefluorescence on Brinkmann GF254 silica thin-layer plates (5by 10 cm) (developed with acetone-chloroform-isopropanol [1:8:1, vol/vol/vol], sprayed with 20% aqueous aluminum chloride, and heated at 110°C for 10 min). After Fractions containing triacetyldeoxynivalenol were pooled and evaporated to dryness on a steam bath. The resulting residue was dissolved in 5 ml of 0.5 M sodium hydroxide in 90% aqueous methanol and, after 10 min, was neutralized with 1 M citric acid and evaporated to dryness at reduced pressure.

The hydrolysis product was dissolved in 20 ml of acetonitrile-water (86:14) and filtered under vacuum through a column containing a mixture of 1.4 g of charcoal (Darco G60; J. T. Baker Chemical Co.), 1.0 g of neutral alumina (Matheson, Coleman, Bell Co.), and 0.6 g Celite (HyfloSupercel; Johns Manville Corp.).

The column was then washed with 100 ml of acetonitrile-water (86:14). The solution after silica gel chromatography was pale yellow, and after passage through the charcoal-alumina column, it was essentially colorless,Although DON could not be crystallized at this stage, the purity as determined by quantitative HPLC and comparison to standard.

Table 1 : *Fusarium spp.* Isolates from different source and origin

Isolate No.	Isolate origin	Source of isolate
Tri1	Lung cow	Clinical
Tri2	Barley	Environmental
Tri3	Wheat	Environmental
Tri4	Barley	Environmental
Tri5	Wheat	Environmental
Tri6	Wheat	Environmental
Tri7	Wheat	Environmental
Tri8	Barley	Environmental

Table 2 : Comparison between different isolates in Co.TRI.in HPLC

Isolate No.	Isolate origin	Source of isolate	Co. toxin. in HPLC (ppm) (Soybean)
Tri1	Clinical	Lung cow	0.00±0.00
Tri.2	Barley	Environmental	18.75±3.23
Tri.3	Wheat	Environmental	0.028±3.07
Tri.4	Barley	Environmental	18.72±2.7
Tri.5	Wheat	Environmental	0.00±0.00
Tri.6	Wheat	Environmental	0.00±0.00
Tri.7	Wheat	Environmental	0.00±0.00
Tri.8	Barley	Environmental	18.72±2.7

HPLC determination

The quantity and quality of Tri were detected according to Hyun *et al*, (2018) The Tri has dissolved in acetonitrile 2 ml and filtered through a Millipore filter (0.45µm) then 100 µl was drawn to inject into the HPLC.

The requirements for HPLC experiment

* Column type: 250x4.6 mm 5 micron C18.
* Mobile phase: Acetonitrile: water (85% -15%).
* Flow rate: 1.5 ml / min.
* Detector UV: 254 mm
* Temperature: 40C
* Pressure: 12 psi
* Injection volume: 20 µl

The concentration of Tri for each sample could be measured from the area under the peak, relative to those of Tri standard peak.

Tri Standard Curve

The standard Tri solution was prepared in acetonitrile at a concentration of 25 µg/ml to prepare a stock solution and kept at (-20) °C. Tri standard curve was drawn with concentrations (1.25, 2, 5 and 10) µg/ ml for 1an apposite area of Tri by using HPLC technique (Figure 1)

Thin-layer Chromatography (TLC)

T-2 toxin was extracted and detected by thin layer chromatographic method,The plates were prepared by

Table 3 : Mean concentration of toxin concentrations by origin

Isolate origin	Cow lung	Barely	Wheat
Cow lung	-	-18.75*±6.52	-0.02±6.18
Barely	18.75*±6.52	-	18.72*±5.45
Wheat	0.028±6.18	-18.72*±5.45	-

Table 4 : Mean by concentration of toxin concentration source.

Source of isolate	Number	Co.meantoxin. in HPLC (ppm) (Soybean)
Clinical	1	0.00± 0.00
Environmental	7	8.055±7.799

adding 50 ml of distilled water to 25g silica gel powder and mixed uniformly to get homogenous mixture (slurry) and then drawn on glass plates of 20 x 20cm using TLC applicator with thickness of 2mm and allowed to dry. The coated plates were activated by drying in forced draft hot air oven at 1100 C for one to two hours. The activated plates were then cooled to room temperature.

After that, 10 microliters of the solution added to the soybean samples, Taken 25 g of representative sample of soybeans were placed into an 100ml Erlenmeyer flask and added acetonitrile - water (86:14,vol/vol). It is then placed in a TLC basin containing acetone-chloroform – isopropanol (1: 8: 1,vol/vol/vol) Extracted toxin (crude) was spotted on TLC plate with capillary tube and allowed to run As Plates were developed for 15 to 18 minutes, dried, and viewed under ultraviolet light at 254 nm wavelength(Omurtag and Yazicioðlu,2000)(Rachitha *et al*,2018)

RESULTS AND DISCUSSION

Fusaruim spp. cultural features and microscopic examination

The isolates were cultured on SDA medium and supplied with 0.05 g/l chloramphenicol to prevent the bacterial growth, then incubated at 28±1 ºC. The growth reached to the edge of the petri dishes after a period of time estimated until one week. Colonies of *Fusarium spp.* on SDA showed a rapid growth compared with colonies that cultured on PDA. The diameter of the colony reached to 9 cm within a week when grows on SDA and 7 cm when grows on PDA at 28°C, the colonies are like powdery. The color of this colony appeared as cottony aerial mycelium when young but soon became white in the front direction and their color was floral in the opposite direction (Fig. 2-1). As for the study of optical microscopy, it was a large banana-like form (Fig. 2-2) This is consistent with what (Bryan *et al,* 2013).

Thin Layer Chromatography test (TLC)

Quantitative thin Layer Chromatography requires the application of several standards to each plate,reducing the number of actual samples that can be analyzed in a single run. Using pure standard compounds and a selective detection method,As a limitation, changes to the distribution of samples and standards within the plate as well as interferences from matrix compounds must be observed,this approach could also be employed to quantify highly retained substances, which are usually inaccessible for quantitative analysis.

For this purpose, this test was used to confirm the presence of trichothecens toxin in soybean samplesafter adding acetonitrile - water (86:14,vol/vol) to samples andThe TLC plates were allowed to dry for one hour before development in 95:5 chloroform:acetone within TLC tanks that had equilibrated for one hour. Plates were developed for 15 to 18 minutes, dried, and viewed under ultraviolet light at 254 nm wavelength.

The drawing also shows Four of the samples gave positive results for the TLC test when compared with the nivalenolstandard(NIV) *(*standard 1) while the negative results were given when compared with the deoxynivalenol standard (DON) *(*standard 2), The samples showed sky blue color bands under UV. comparable to standard T-2 toxin these results came in line with the results(Rachitha *et al*,2018) Figure(4)Thin Layer chromatography (Blue color spot showing Standard T-2 Toxin), acetone-chloroform –isopropanol (1: 8: 1,vol/vol/vol) as mobile phase.

The HPLC technique for detection the potentiality of *Fusarium graminearum* to produce Trichothecenes

The results HPLC of different isolates were observed in terms of their production of Trichothecenctoxin. These results complement the results of TLC or more specifically to confirm the production of isolates for their production of Trichothecenctoxin. Table(2).

The difference in the percentage of toxins produced by the *Fusarium* fungi may have a significant impact on the health of both humans and animals if a study was done in North Carolina found a significant decrease in the production of milk at cows that consumed concentrated fodder contaminated with 0.8 mg DON/kg, Such a result can be explained through the synergic effect of mycotoxins associated with DON even though these were not identified and the presence of DON residue in the animal tissue ordue to the multiple interactions between mycotoxins in fodder(Whitlow *et al*,1994),and among the general effects of DON on the human, we mention: inhibitor of protein synthesis, affliction of the gastrointestinal tract, immune system depression. (Violeta, 2018). The results of the current study yielded four samples on the production of toxin compared to the other four samples. If the barley samples (Tri2,Tri4,Tri8) were superior to their theory, the samples of the wheat (Tri3,Tri5,Tri6, Tri7) and the sample taken from the cow's lung (Tri1) were (18.75) and the sample of the wheat was (0.288).

The conclusive sample of the cow 's lungs has lost its ability to produce poison, possibly due to its lack of the gene responsible for the production of toxins or has been exposed to mutation genetic to lose its ability to produce.Table (2).

This result was within the range that he had achieved

(Desjardins *et al*, 2009) It was observed that the ratio of toxins to the fungus, including those of trichothecenctoxin isolated from wheat and barley grains, was (2.5-25) ng / g.,we infer the possibility of this toxin entering the food chain (grains, flour, bread) and lead to a toxic effect for both humans and animals,While the results of the current study, when using the three origin (cows lung, Barley, wheat) Significant (P>0.05) differences in the concentration of the origin of the value of F about 27.9, The comparative comparisons between the assets to the superiority of barley on the rest of the assets and showed the differences between barley and the rest of the assets a very significant(P>0.05) difference, but the difference between the wheat andbarley can make a significant difference.

Table (3) shows the differences and their significance T,this result was similar to the study of Korea, which observed the percentage of trichothecenectype B (NIV) when examined by a HPLC ranged between (5.7- 2791.4) µg kg, and this result was much less than daily due to the exposure of the Korean population to fungal toxins (Ok. *et al*, 2018). Thus confirming the danger these toxins have on human health in different countries.As for the source of these isolates was isolated one clinical and other isolates were taken from an environmental source, the results of the statistical analysis showed no significant (P>0.05) difference between the exporters as shown in the table (4). The identity of the host and the evolutionary relationship between the plant and the fungus has a significant role in the ability of fungi to produce toxin, as well as finding future studies to improve agricultural crops (Lofgren *et al*, 2018).

The results of the study indicate that the risk of trichothecenectype B if given a high percentage in the grains of both wheat and barley and thus prepare to be absorbed by the body when eating quantities even if the simple grain of wheat and barley (Arrache *et al*, 2018).

CONCLUSIONS

Samples of wheat and barley have become contaminated with fungi, including *Fusariumgraminearum* which is a danger to the health of humans and animals. Detection of toxic fungus may be studied after methods including (HPLC and TLC) technology. The absence of trichothecens toxin was observed in some isolates and this may be due to several reasons, including the absence of the gene responsible for producing the toxin.

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