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Molecular identification of *Fusarium* spp causing crown rot and head blight on winter wheat in Iraq

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The study was carried out to characterize Fusarium isolates associated with crown rot and head blight on wheat by morphological and molecular techniques. The molecular characterization was achieved through sequencing translation elongation factor-1 alpha gene (αTEF gene) and RNA polymerase II gene (RPB2) and confirm by GenBank database BLAST. Result showed that among 21 pathogenic isolates obtained, 4 isolate were *Fusarium proliferatum*, 15 of *F. verticelloides*, one of each of *F. solani* and *F. culmorum.* It was found that all the isolates exhibited symptoms of crown rot disease on wheat seedling ranging from faint lesion on sheath outer sheath leaves to severe necrosis on all sheath leaves. The more severe isolate was of *F. culmorum*with severity index 0.7. While the others showed disease index ranging from 0.2 to 0.4. Six of 21 isolates only showed Head blight on spike, 2 of *F. proliferatum*, three of *F. verticelloides* with infection area of 5, 6, 6, 6, 6, 6% and the more severe one of *F. culmorum* with infection area 100% respectively. ELISA test revealed that all 21 isolate of *Fusarium* produced DON toxin on wheat straw at concentration between 0.5-1.9 mg/Kg. The higher DON toxin producer was of *F. culmorum* at 3.8 mg/kg.

Key word: Wheat, *Fusarium* spp., DON, crown rot

Introduction

Crown rot on wheat caused by *Fusarium culmorum* and *F. pseudograminearum*, is of global significance disease and becoming epidemics in recent years in Europe, USA, Canada, China and South America causing heavy losses in yield and grain quality (McMullen *et al*., 1997; Goswami and Kistler, 2004). Crown rot is considered as the second most economically devastating disease on wheat in Australia, where it is chronic, covering most of

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wheat cultivation area and causing annual losses up to $ 56 Australian million dollars (Brennan and Murray, 1998). It was reported that the can reach to 89% in individual crops (Klein *et al*., 1985). Two billion $US in wheat production were lost in the last decade in USA by this disease (Dubin *et al*., 1997), and 20 -100% losses occurred during 1999 and 2000 in New south Wales (Manning *et al.*, 2000).

The *Fusarium spp* predominantly found associated with Fusarium head blight (FHB) in wheat and other small-grain cereals in Europe are *F. graminearum, F. avenaceum* and *F. culmorum* (Bottalico and Giancarlo, 2002). It has been reported also that *F. verticillioides* and *F. proliferatum,* the most common fungi associated with maize had the ability to attack wheat seedling and caused crown and root rot disease with the capacity to produce fumonisins toxin (Chulze *et al*., 1996; Al-Mousa, 2006).

The most characteristic symptoms of crown rot on wheat appeared at the base of plants (crown, lower leaf sheath and tillers) at maturity as brown discoloration and under moist condition sporodochia are formed on infected tissue as pink patches. These symptoms are associated with stunting, reduced tellering and empty heads. Head blight affects flowering spike giving bleached appearance of the spike.

Several previous studies reported that these fungi produce DON toxin in susceptible varieties of wheat (Ansari *et al.*, 2007; Lemmens *et al.,* 2005; Menke-Milczarek and Zimny, 1991). This mycotoxin was found to facilitate the development of Fusarium head blight and crown rot disease (Walter and Doohan, 2011). In addition DON toxin reported to cause damage in cell membrane through released Na and K ions (Cossette and Miller, 1995). DON mycotoxin was found to inhibit seed germination, root and shoot growth, protein and callus formation (Ansari *et al*., 2007; Lemmens *et al*., 2005; Rocha *et al*., 2005; Miller and Ewen, 1997).

Symptoms of stunting, browning at the base of wheat plant, reduction in tillers number associated with spike bleaching suspected to be of crown rot and head blight in wheat fields were observed. The study was conducted to characterizes the causal agents of these symptoms at molecular level and testing their capacity to produce mycotoxin.

Materials and methods

Sample collection and Fusarium spp isolation

Samples of wheat and corn plants showing symptom characteristic of Fusarium infection and of corn grains were collected from 5 different locations in Iraq in 2007-2008 and 2008-2009 seasons. Root and stem pieces (1-2 cm) and corn grains were surface sterilized with 1% sodium hypochloride and placed on potato dextrose agar (PDA) in petri plates of 9 cm dim (4 pieces/plate). The plates were incubated at 25±2º C for 5 day, and the *Fusarium* spp were morphologically identified and purified by single spore technique for molecular identification.

Pathogenicity test

The pathogenicity of *Fusarium* isolates were tested for crown rot (CR) and head blight (HB) disease on wheat seedlings under greenhouse conditions. Disc of 1cm dim from 10 days old Fusarium culture on PDA were mixed with sterile soil and peat moss (1:1 v:v) in pots of 5×10 cm (3 discs/pots). The pots were covered with polyethylene bags for 2 days. The pots were sown with surface sterilized wheat seeds and watering with distilled water. Three seedling were let grown in each pots and crown rot was assessed after 35 days. The proportion of stem discoloration to seedling height and the number of leaf sheath layers showing necrosis were determined and disease severity was calculated according to the equation:

Crown rot severity index = (length of stem discoloration/seedling height)
× (number of leaf sheath layers with necrosis)

For FHB pathogenicity, three wheat seedling in pots, after spike formation, were sprayed with Fusarium spore suspension, (prepared by adding 100 ml distilled water to 7 day old Fusarium culture on PDA) (0.5 ml/spike). The treated spikes were covered with polyethylene bags and the disease symptoms were evaluated after 7-10 days. The disease severity was recorded as percentage of infected spike. The *Fusarium* spp isolates showing high aggressiveness for FHB and CR were maintained on sterilized sand for molecular identification. The molecular identification protocols were carried out in CSIRO, Plant Industry, Queensland –Brisbane.

DNA Extraction

Twenty-one isolates that given high aggressiveness were choosen. The Fusarium isolates were grown in quarantine room on PDA media for 5 days at 25 ± 2ºC. The mycelium from each isolate was collected in eppendrofe tube. The mycelium was lyophilized (freeze-dry) in 2 ml screw cryo-tubes with holes in the caps, then 5mm ball bearing was added to each tube, and the lids were replaced with new one. The tube was shaken in retsch MM300 shaker at frequency of 25 for 3 min, and centrifuged at 3000 rpm in microcentrifuge for 3 min. Two hundred µL of extraction buffer I (20 mL 1M Tris, 5mL NaCl, 5mL 0.5M EDTA, 70mL MilliQ water) was added to each tube, mixed by vortex mixer, and centrifuged at 2000 rpm for 2 min . Ninety µL of supernatant was added to 10 µL of extraction buffer II (5% sodium dodecy sulphate (SDS) in MilliQ water) and mixed by pipetting up and down. The tubes were incubated at 65 °C for 1 hour with tapping to mix after each 30 min of incubation, and centrifuged at 3500 rpm for 10 min. Forty µL of supernatant was added to 100 µL of absolute ethanol, and centrifuged at 2000 rpm for 10 min. The DNA precipitate was air dried, and dissolved with 100 µLMilliQ water at 4 °C overnight. The tubes were centrifuged at 2000 rpm for 5 min and the supernatant was kept at 4 °C for PCR protocol.

The DNA cleaned up using UltraClean™Gelspin™ purification kit from Mo Bio Laboratories Inc. Gel-Bind was added to DNA solution, mix well, and the mixture was passed through spin filter by centrifugation at 10000 xg for 10 seconds. The filtrate discarded, 300 µl of Gel-Wash buffers was added to filter and centrifuged at 10000 xg for 10 sec. The last step was repeated and the filter was carefully transferred to 2 ml clean tube. Fifty µl of elution buffer was added directly onto the center of the filter and centrifuged at 10000 xg for 30 sec. The filtrate containing DNA is ready to use for PCR protocol.

PCR Assays

The concentration of DNA was measured by spectrophotometer model ND-1000 Nano Drop and adjusted to 5 ng/µl. PCR was carried out using translation elongation factor-1 alpha gene (αTEF gene) EF1 $^{5'}$GTGGGGCATTTACCCCGCC$^{3'}$, EF2 $^{5'}$ACGAACCCTTACCCACCTC $^{3'}$ (O'Donnell *et al*., 2000), and RNA polymerase II gene (RPB2) 7cR (5_-CCCATRGCTTGYTTRCCCAT) and the primers 7cF (5_-ATGGG YAARC AAGCYATGGG). PCR reaction mixture for TEF gene consisted of 12.8 µl milliQ H_2O, 2.5 µl 10x buffer, 2.5 µl MgCl2, 0.5 µl dNTP mix(10mM each), 0.8 µl forward primer EF1 (10mM), 0.8 µl reveres primer EF2 (10mM), 0.1 µl Taq (Biotech) and 5 µl DNA sample. All preparation work was in ice bath. Cycling parameter was initial denaturation of 95 °C for 75 s, denaturation 95 °C for 15 s , annealing 53 °C for 30 s, pimer extension 72 °C for 30 s (38 cycles), final extension 72 °C for 30 s and hold at 10 °C.

The mixture for RPB2 reaction consisted of 13.6 µl milliQ H_2O, 2.5 µl 10x buffer, 2.5 µl $MgCl_2$, 0.5 µl dNTP mix(10mM each), 0.4 µl forward primer 5F2 (10mM), 0.4 µl reveres primer 7CR (10mM), 0.1 µl Taq (Biotech) and 5 µl DNA sample. Cycling parameter was initial denaturation 94 °C for 90 sec, denaturation 94 °C for 30 sec, annealing 55 °C for 90 sec, pimer extension = 68 °C for 2 min (40 cycles), final extension= 68 °C for 5 min and hold at 10 °C.

The reaction was carried out by Gene Amp PCR System 9700, AB Biosystems.

Preparation of Agarose gel 1.5 %

The PCR product was analyzed by electrophoresis in 1.5% agrarose gel prepared in ATM buffer 1X which 57.1 ml glacial acetic acid, 242g Tris base, 200 ml of 0.5 M EDTA pH 8.0 in 1L MilliQ water (50X ATE) and placed in microwave in mid-high for 4 min. Ten µl of red gel stain was added to gel at 45 °C and poured in gel container after inserting the comb. The gel was maintained 20 min at room temperature for solidification.

Sequencing of PCR products

The PCR reactions were cleaned up by using UltraClean™ PCR Clean-Up Kit from MO BIO Laboratories, Inc. this kit designed to purify PCR products directly from a PCR reaction. Five volumes of SpinBind were added to the PCR reaction and mix well. The PCR/SpinBind mixture was transferred to a spin filter unit, and centrifuged at 13,000 rpm for 10-30 seconds. The liquid was discarded and spin filter was maintained in the same tube. Three hundred µl of SpinClean buffer was added to the filter and the mixture was centrifuged at 10,000 xg for 10-30 seconds. The process was repeated and the Spin filter was transferred into a clean 2 ml tubes. Fifty µl of Elution Buffer (10 mMTris) solution or sterile water was added directly onto the center of the white spin filter. The mixture was centrifuged at 10,000 xg for 30-60 seconds. The spin filter basket was removed out and DNA in the filtrate was stored at -20°C for sequencing reaction.

Translation elongation factor-1 alpha gene (αTEF gene) and RNA polymerase II gene (RPB2) were sequenced for Iraqi isolates. The DNA concentration was measured by spectrophotometer model ND-1000 Nano Drop. The reaction mix was prepared by adding 13 µl MilliQ H_2O, 3.5 µl 5X buffer, 0.5 µl primer EF1 or EF2 (prepared separately), 1.0 µl BDT and 2 µl DNA samples. Cycling parameter were 96 °C for 2 min 1 cycle, (96 °C for 10 secs, 50°C for 5 sec, 60°C for 4 mins) 30 cycles and final 4°C hold. PCR product was cleaned up using Agencourt CleanSEQ kit, Agencourt Bioscience Corporation. Sixty two µl of 85% ethanol was to PCR product, mix well by pipetting for 7 times in cleaning PCR plate. The pleat was place on SPRIPlate 96R for 3 min to separate beads. The clear phase was discarded and 100 µl of 85% ethanol was added and let for 30 sec at room temperature. The ethanol was aspirated out and the pleat air dried for 10 min at room temperature. Forty µl MilliQ water was added to the pleat and maintained at room temperature for 5 min.

The pleat was placed on SPRIPlate 96R for 3 min to separate beads. The clear phase was transferred into new clean plate for loading on the detector and send to sequencing.

DON toxin production

Ten gram of sterile wheat straw in 9 cm dim Petri dish was inoculated with a disc of 1 cm dim of 7 days *Fusarium* isolate culture on PDA media, 3 Petri dishes for each isolate, and incubated in 25 ±2 ºC for 21 days. The cultures were dried and grounded using coffee grinder. The powder was conserved at 4°C.

DON toxin Extraction and detection

DON toxin was extracted by using Accelerator Solvent Machine ACE200. One gram of each samples powder was extracted with 15 ml of Acetonitril:water 85:15. Five hundred µl of each extract was dried under nitrogen flow in plastic tubes. Fifty five µl of milliQ water was added to each tube and vortex for few seconds each. The toxin was detected in samples by Beacon Deoxynivalenol ELISA kit Analytical Systems Inc.

Results and discussions

Identificatin of Fusarium spp

Twenty one of 93 isolates of *Fusarium* spp isolated from the different locations of infected wheat plants and corn grains were found highly pathogenic. The isolates were firstly identified based on morphological characteristics and then confirmed by PCR analysis using Translation elongation factor-1 alpha gene (αTEF gene) and RNA polymerase II gene (RPB2) and sequencing the reaction product (Fig 1 and 2). The result of morphological examination together with DNA sequencing according to GenBank database BLAST searching using individual sequences confirmed the identity of all isolates and that belong to *Fusarium* spp Table 1 and Fig 1.

Tabl 1. Source and site of isolation for *Fusarium* spp isolates in Iraq

Isolate No.	Species	Source of isolate	Place of isolate
1	*Fusarium proliferatum*	Maize – crown	Baghdad
2	*F. proliferatum*	Maize – crown	Baghdad
3	*F. verticelloides*	Maize – crown	Baghdad
4	*F. proliferatum*	Maize – crown	Baghdad
5	*F. verticelloides*	Maize – crown	Baghdad
6	*F. verticilloides*	Maize – crown	Baghdad
7	*F. verticelloides*	Maize – crown	Baghdad
8	*F. verticelloides*	Maize – crown	Baghdad
9	*F. proliferatum*	Maize – crown	Baghdad
10	*F. verticilloides*	Maize – crown	Al-Anbar
11	*F. verticelloides*	Maize – crown	Al-Anbar
12	*F. verticelloides*	Maize – crown	Al-Anbar
13	*F. verticelloides*	Maize – crown	Al-Anbar
14	*F. verticelloides*	Maize – crown	Al-Anbar
15	*F. verticelloides*	Maize seed	Baghdad
16	*F. verticelloides*	Maize seed	Baghdad
17	*F. verticelloides*	Maize seed	Baghdad
18	*F.solani*	Maize seed	Baghdad
19	*F. verticelloides*	Maize seed	Baghdad
20	*F. verticelloides*	Maize seed	Babylon
21	*F. culmorum*	Wheat – crown	Baghdad

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  1 CCCGCCTACC GCTTTGAGCA AGCTGCTCGC CTCTGGCAGT
 41 CGACCACTGA TGAGTACTAC CCTGGACGAT GAGCTTATCT
 81 GCCATCGTGA TCCTGACCAA GATCTGGCGG GGTACATCTT
121 GGAAGACAAT ATGCTGACAT CGCTTCACAG ACCGGTCACT
161 TGATCTACCA GTGCGGTGGT ATCGACAAGC GAACCATCGA
201 GAAGTTCGAG AAGGTTAGTC ACTTTCCCTT CGATCGCGCG
241 TCCTCTGCCC ACCGATTTCA CTTGCGATTC GAAACGTGCC
281 TGCTACCCCG CTCGAGACCA AAAATTTTGC GATATGACCG
321 TAATTTTTTG GTGGGGCATT TACCCCGCCA CTCGAGCGAT
361 GAGCGCGTTT TTGCCCTTTC CTGTCCACAA CCTCAATGAG
401 CGCATTGTCA CGTGTCAAGC AGCGACTAAC CATTCGACAA
441 TAGGAAGCCG CTGAGCTCGG TAAGGGTTCC TTCAAGTACG
481 CCTGGGTTCT TGACAAGCTC AAGGCCGAGC GTGAGCGTGG
521 TATCACCATC GATATTGCTC TCTGGAAGTT CGAGACTCCT
561 CGCTACTATG TCACCGTCAT TGGTATGTTG TCGCTCATAC
601 CTCATCCTAC TTCCTCATAC TAACACATCA TTCAGACGCT
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681 ACCTCTCCA
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1

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 81 TGCCATCGTG ATCCTGACCA AGATCTGGCG GGGTACATCT
121 TGGAAGACAA TATGCTGACA TCGCTTCACA GACCGGTCAC
161 TTGATCTACC AGTGCGGTGG TATCGACAAG CGAACCATCG
201 AGAAGTTCGA GAAGGTTAGT CACTTTCCCT TCGATCGCGC
241 GTCCTCTGCC CACCGATTTC ACTTGCGATT CGAAACGTGC
281 CTGCTACCCC GCTCGAGACC AAAATTTTTG CGATATGACC
321 GTAATTTTTT TGGTGGGGCA TTTACCCCGC CACTCGAGCG
361 ATGAGCGCGT TTTTGCCCTT TCCTGTCCAC AACCTCAATG
401 AGCGCATTGT CACGTGTCAA GCAGCGACTA ACCATTCGAC
441 AATAGGAAGC CGCTGAGCTC GGTAAGGGTT CCTTCAAGTA
481 CGCCTGGGTT CTTGACAAGC TCAAGGCCGA GCGTGAGCGT
521 GGTATCACCA TCGATATTGC TCTCTGGAAG TTCGAGACTC
561 CTCGCTACTA TGTCACCGTC ATTGGTATGT TGTCGCTCAT
601 ACCTCATCCT ACTTCCTCAT ACTAACACAT CATTCAGACG
641 CTCCCGGTCA CCGTGATTTC ATCAAGAACA TGATCATGGG
681 TACCTCCA
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2

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 81 CCATCGTAAA CCCGGCCAAG ACCTGGCGGG GGATTTCTCA
121 AAGAAAACAT GCTGACATCG CTTCACAGAC CGGTCACTTG
161 ATCTACCAGT GCGGTGGTAT CGACAAGCGA ACCATCGAGA
201 AGTTCGAGAA GGTTAGTCAC TTTTCCTTCT ATCGCGCGTT
241 CTTTGCCCAT CGATTCCCCC CTACGACTCG AAACGTACCC
281 GCTACCCCGC TCGAGCCCAA AAATTTTGCG ATACGACCGT
321 AATTTTTTCT GGTGGGGCAT TTACCCCGCC ACTCGAGCGG
361 CGTGTTTCTG CCCTCTCCCA TTCCACAACC TCACTGAGCT
401 CATCGTCACG TGTCAAGCAG TCACTAACCA TCCGACAATA
441 GGAAGCCGCT GAGCTCGGTA AGGGTTCCTT CAAGTACGCC
481 TGGGTTCTTG ACAAGCTCAA GGCCGAGCGT GAGCGTGGTA
521 TCACCATCGA TATCGCTCTC TGGAAGTTCG AGACTCCTCG
561 CTACTATGTC ACCGTCATTG GTATGTTGTC GCTCTTACTC
601 CGTTCTATAT CTCCTATTAC TAACACATCA CATAGACGCT
641 CCCGGTCACC GTGATTTCAT CAAGAACATG ATCATGGGGT
681 ACCTCTCCA
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3

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 41 ACCACTGTGA GTACTACCCT TGACCATGAG CTTATCGGCC
 81 ATCGTTAACC CGGCCAAAAC CTGGCGGGGG ATGTCTCGGA
121 TAGCTATGCT TTGTTCGTCC TGCAGACCGG TCACTTGATC
161 TACCATTGCG GTGGTATCGA CCAGCGAACC ATCTGGAATT
201 TCGAGAATGT TAGTCAATTT TCCTTCTATC GCGTGTTGCT
241 TGCCCATCGA TTCCCCCTTG TACTCGAAAC GTACCAGGCA
281 CCCCGCTCGA ACCAAAAATT TTGCGATACA ACCGTATTTT
321 TACTGGTGGG GCATTTACCC CGCCACTCGA GGGCGCGTTT
361 CAGCCCTCTC CCATTCCACA ACCTCAGTGA CCTCATCGCC
401 ACGTGTCAAG CAATCACTAA CCATCCAACC ATAGGAAGCC
441 GCTGAGCTCG GTAAGGTTTC CTCATCGTAC GCCTGGGTTC
481 TTGACAAGCT CAAGGCCGAG CGTGAGCGTG GTATCACCAT
521 CGATATCGCT CTCTGGAAGT TCGAGACTCC TCGCTACTAT
561 GTCACCGTCA TTGGTATGTT GTCGCTCTTA CTCCGTTCTA
601 TATCTCCTAT TACTAACACA TCACATAGAC GCTCCCGGTC
641 ACCGTGATTT CATCAAGAAC ATGATCACTG GTTACTCTAC
681 CCA
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4

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 81 AAACCCGGCA AGACCTGGCG GGGGATTTCT CAAAGAAAAC
121 ATGCTGACTT CGCTTTCCAG AACGGTCACT TGATCTACCA
161 GTGGGGGGGT ATCCACCAAC CAAACCTCCA AAAATTTCAG
201 AAGGGTAATC CCTTTTCCTT TCATCCGGCG TTCTTTGGCC
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281 GCTCGAGCCC AAAAATTTTG CGATACAACC GTAATTTTTT
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361 TGCCCTCTCC CATTCCACAA CCTCTGTGAG CTCTGCGTCG
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121 CTCAAAGAAA ACATGCTGAC ATCGCTTCAC AGACCGGTCA
161 CTTGATCTAC CAGTGCGGTG GTATCGACAA GCGAACCATC
201 GAGAAGTTCG AGAAGGTTAG TCACTTTTCC TTCTATCGCG
241 CGTTCTTTGC CCATCGATTC CCCCCTACGA CTCGAAACGT
281 ACCCGCTACC CCGCTCGAGC CCAAAAATTT TGCGATACGA
321 CCGTAATTTT TTCTGGTGGG GCATTTACCC CGCCACTCGA
361 GCGGCGCGTT TCTGCCCTCT CCCATTCCAC AACCTCACTG
401 AGCTCATCGT CACGTGTCAA GCAGTCACTA ACCATCCGAC
441 AATAGGAAGC CGCTGAGCTC GGTAAGGGTT CCTTCAAGTA
481 CGCCTGGGTT CTTGACAAGC TCAAGGCCGA GCGTGAGCGT
521 GGTATCACCA TCGATATCGC TCTCTGGAAG TTCGAGACTC
561 CTCGCTACTA TGTCACCGTC ATTGGTATGT TGTCGCTCTT
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681 GGGTACTTCC CCA
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201 AAGTTCGAGA AGGTTAGTCA CTTTCCCTTC GATCGCGCGT
241 CCTCTGCCCA CCGATTTCAC TTGCGATTCG AAACGTGCCT
281 GCTACCCCGC TCGAGACCAA AAATTTTGCG ATATGACCGT
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7

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561 TATGTCACCG TCATTGGTAT GTTGTCGCTC ATACCTCATC
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601 TCTATCAGAC GCTCCCGGTC ACCGTGATTT CATCAAGAAC
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161 GATCTACCAG TGCGGTGGTA TCGACAAACG AACCATAGAG
201 AAGTTCGAGA AGGTTACTCA CTTTTCCTTC TATAGGGCGT
241 TCTTTGCCCA TCCAGTCCCC CCTACGACTC ATAACGTACC
281 CGCTACCCCG CTCAAGCCCA AAAATTTTGC GATACGACCG
321 TAATTTTTTC TGGTGGGGCA TTTACCCCGC CACTCGAGCG
361 GCGCGTTTCT GCCCTCTCCC ATTCCACAAC CTCACTGAAC
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521 ATCACCATCG ATATCGCTCT CTGGAAGTTC GAGACTCCTC
561 GCTACTATGT CACCGTCATT GGTATGTTGT CGCTCTTACT
601 CCGTTCTATA TCTCCTATTA CTAACACATC ACATAGACGC
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10

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 81 AGTCATCAAC CCCGCCATAC GTGGCGGGGT AATTTTAACT
121 TGAATATCTG CTGATAAGAT TGCATAGACC GGTCACTTGA
161 TCTACCAGTG CGGTGGTATC GACAAGCGAA CCATCGAGAA
201 GTTCGAGAAG GTTGGTTCCC ATTCCCCTCG ATCGCACGCT
241 CTCTACCCTC CGATCTATCA GTCGAATCAG TTTTACGACG
281 ATTGAATATG TGCCTGTTAC CCCGCTCGAG TACAAAATTT
321 TGCGGTTCAA CCGTAATTCT TTTGGTGGGG TTTCAACCCC
361 GCTACTCGGG TGACAGGCGC TTGCCCTTCC CACAAATCCA
401 TGTCTCGCGC ATCACGTGTC AACCAGTCAC TAACCACCCG
441 ACAATAGGAA GCCGCCGAGC TCGGTAAGGG TTCCTTCAAG
481 TACGCCTGGG TTCTTGACAA GCTCAAGGCC GAGCGTGAGC
521 GTGGTATCAC CATCGATATC GCCCTCTGGA AGTTCGAGAC
561 TCCTCGCTAC TATGTCACCG TCATTGGTAT GTTGTCATCA
601 CTTTCACTCA TTATCTTCTC ATTCTAACAT GTGCTTCAGA
641 CGCTCCCGGT CACCGTGATT TCATCAAGAA CATGAGCACG
681 GGGAAACCTC CC
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11

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  1 GGGCTTTTTA GCCGTCTAAT GCACGTCGCT TCTGGCAGTC
 41 GACCACTGTG ACTACTACCT CCTGATGATG AGCTTATAGG
 81 CTATCGTAAA CTGAGCCAGA ACTGGGCGGG GGATTTCTGA
121 TGAAAACTTG CTGACATGGC TTCACAGACC GGTCACTTGA
161 TCTACCAGAG CGGTGGTATC GACAAACGAA CCATAGAGAA
201 GTTCCAGAAG GTTACTCCCT TTTCCTTCAA TAGGGCGTTC
241 TTTGCCCATC CAGTCCCCCC TACGACTCAT AACGTACCCG
281 CTACCCCGCT CAAGCCCAAT AATTTTGCGA TACGACCGTA
321 ATTTTTTCTG GTGGGGCATT TACCCCGCCA CTCGTTCGGC
361 GCGTTTCTGC CCTCTCCCAT TCCACAACCT CACTGAACTC
401 ATCATCACCT GTTAAGGAGT CACTAACCGT CCGACAATAA
441 GAAGCCGCTG ACCTCGGTAA AGGTTCCTTC AAGGACACCT
481 GGGTTCTTGA CAAGCTCAAG GCCGAGCGTG AGCGTGGTAT
521 CACCATCGAT ATCGCTCTCT GGAAGTTCGA GACTCCTCGC
561 TACTATGTCA CCGTCATTGG TATGTTGTCG CTCTTACTCC
601 GTTCTATATC TCCTATTACT AACACATCAC ATAGACGCTC
641 CCGGTCACCG TGATTTCATC AAGAACATGA TCCGG
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12

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  1 GGGGAAGTTG GGGGGTATCC TACCTTATCC TCTGGCCAGT
 41 CGACCACTGT GAGTACTACC TCTTGATCAT GAGGTTATTG
 81 GCCATCGTTC ACCCGGCCAA AACCTGGCGG GGGATTTCTC
121 AAATAAAACA TGCTGACATC GTCCCGCAGA CCGGTCACTT
161 GATCTACCAT TGCGGTGGTA TCGACAAGCG AACCATCGAG
201 AAATTCGAGA AGGTAATGCA CTTTTCCTTC TAGCGCGCGA
241 TCTTTGCCCA TCGATTCCCC CCTACGACTC GAAACGTACC
281 CGCTACCCCG CTCGAGCCCA AAAATTTTGC TATACGACCG
321 TAATTTTTTC TGGTGGGGCA TTTACCCCTC CACTCCAGCG
361 GCGCGTTTCT GCCCTCTCCC ATTCCACAAC CTCACTGAGC
401 TCATCGTCAC GTGTCAAGCA TTCACTAACC ATCCGAGAAT
441 ACGAAGCCGC TGAGCTCGGT AAGGGTTCCT TCAAGTACGC
481 CGGGGTTCTT GACAAGCTCA AGGCCGAGCG TGAGCGTGGT
521 ATCACCATCG ATATCGCTCT CTGGAAGTTC GAGACTCCTC
561 GCTACTATGT CACCGTCATT GGTATGTTGT CGCTCTTACT
601 CCGTTCTATA TCTCCTATTA CTAACACATC ACATAGACGC
641 TCCCGGTCAC CGTGATTTCA TCAAGAACAT GATCATGGGT
681 ACCTTCC
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13

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  1 GGGGAATTCG TTAGTCTAGG TACACGTCGC CTCTGGCCAG
 41 TCGACCACTG TGAGTACTAC CTCCTGATCA TGATGTTGCT
 81 GGCCCTCCTA AACCCGGCAA AAAGCTGGGG GGGGATTTCT
121 CTTGCAAAAC ATATTGATAT CGCTTCACAG ACCGGTCACT
161 TGATCTACCA GTGCGGTGGT ATCGACAAGC GAACCATCGA
201 GAAGTTCCAG AAGGTGATTC CCTTTTCCTT CTATCGCACG
241 TTCTTTGCCC ATCAATTCGC CCCTACTACT CTAAACTTAC
281 CCGCTACCCC GCTGGAGACC AAAAATTTTG GGATACGACC
321 GTAATTTTTT CTGGTGGGGC ATTTACCCCG CCACTCAAGC
361 GGCGCGTTTC TGCCCTCTCC CATTCCACAA CCTCACTGAG
401 CTCATCGTCA CGTGTCATGC AGGCACTAAC CATCGAACAA
441 TAAGAAGCCG CTGAGCTCGG TAAGGGTTCC TTCAAGTAGC
481 TAGGGTTCTT GACAAGCTCA AGGCCGAGCG TGAGCGTGGT
521 ATCACCATCG ATATCGCTCT CTGGAAGTTC GAGACTCCTC
561 GCTACTATGT CACCGTCATT GGTATGTTGT CGCTCTTACT
601 CCGTTCTATA TCTCCTATTA CTAACACATC ACATAGACGC
641 TCCCGGTCAC CGTGATTTCA TCAAGAACAT GATCATGGGA
681 CCTTTCAA
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14

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  1 TGGGGTAGGT CTGTCTAGCA GCAGCTTCCT CTGGCAGTGC
 41 GACCACTGAT GAGTACTACC CTTGACGATG AGCTTATCGG
 81 CCATCGTAAA CCCGGCCAAG ACCTGGCGGG GGATTTCTCA
121 AAGAAAACAT ACTGATATCG CTTCACAGAC CGGTCACTTG
161 ATCTACCAGT GCGGTGGTAT CGACAAGCGA ACCATCGAGA
201 AGTTCGAGAA GGTTAGTCAC TTTTCCTTCT ATCGCGCGTT
241 CTTTGCCCAT CGATTCCCCC CTACGACTCG AAACGTACCC
281 GCTACCCCGC TCGAGCCCAA AAATTTTGCG ATACGACCGT
321 AATTTTTTCT GGTGGGGCAT TTACCCCGCC ACTCGAGCGG
361 CGCGTTTCTG CCCTCTCCCA TTCCACAACC TCACTGAGCT
401 CATCGTCACG TGTCAAGCAG TCACTAACCA TCCGACAATA
441 GGAAGCCGCT GAGCTCGGTA AGGGTTCCTT CAAGTACGCC
481 TGGGTTCTTG ACAAGCTCAA GGCCGAGCGT GAGCGTGGTA
521 TCACCATCGA TATCGCTCTC TGGAAGTTCG AGACTCCTCG
561 CTACTATGTC ACCGTCATTG GTATGTTGTC GCTCTTACTC
601 CGTTCTATAT CTCCTATTAC TAACACATCA CATAGACGCT
641 CCCGGTCACC GTGATTTCAT CAAGAACATG ATCTGGGGAA
681 ACCTTTCAAA
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15

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  1 GGGGGGGCGG GTGGCTGTGG AGAAATCCTC ATACACTAGC
 41 GGAGTAGACC ATTGAGACTC CACCTCTTGA TGATCATGTT
 81 ATGGCGCATA GTACACCCGG CCAAGACCTG GCGGGGGATT
121 TCTCAAAGAA AACGTGCTGT GCTCGTCCAG CAGACGGGTC
161 AGTTGATCTA GGGTTGGGGT GGTATCGACA GGCGCACCAT
201 CTACAATTTG GATAATGGTA GTCACTTTTC CTTCCATCGC
241 GTGTTGTTTG CCCATCCATC CCCCCCTACG ACTCGAAACC
281 GACCCGCTAC CCCGCTCGAG CCCAAAAATT TTGCGATACG
321 ACCGTAATTT TTTGTGGTGG GGCATTTACC CCGCCACTCA
361 AGCGGCGCGT TTCTGCGTTC TCCCATTCCA CACCCTCAGT
401 GATTTTAACG TGTCATGCAC TGACCACCCA ACCCATCCAA
441 CAATACGAAG CCGCTGAGCT CGGTTAGGTT TCCTTAGGGT
481 ACGCCTGGTT TCTTGACAAG CTCGAGCCGA GCGTGATTGT
521 GGTATCAACA TCGATATCTG TATCTGGAAG TTCCAGACTC
561 CTCGCTACCA TGTCACCGTC GTTGGTATGT TGTCCCTCTT
601 ACTCCATTCT ATATCTCCAA TTATCAAAAC CTCACATCCA
641 CGCTCCCTGG TCACCGTGAT ATCATCACGA ACATGATGAC
681 TAGTACCTCC AATG
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16

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  1 TGCCCCTTCC ACTGACGCCT GCATCTCGCC TCTGGCAGTG
 41 CGACCACTGA TGAGTACTAC CCTTGACGAT GAGACTTATC
 81 GGCCATCGTA AACCCGGCCA AGACCTGGCG GGGGATTTCT
121 CAAAGAAAAC ATACTGATAT CGCTTCACAG ACCGGTCACT
161 TGATCTACCA GTGCGGTGGT ATCGACAAGC GAACCATCGA
201 GAAGTTCGAG AAGGTTAGTC ACTTTTCCTT CTATCGCGCG
241 TTCTTTGCCC ATCGATTCCC CCCTACGACT CGAAACGTAC
281 CCGCTACCCC GCTCGAGCCC AAAAATTTTG CGATACGACC
321 GTAATTTTTT CTGGTGGGGC ATTTACCCCG CCACTCGAGC
361 GGCGCGTTTC TGCCCTCTCC CATTCCACAA CCTCACTGAG
401 CTCATCGTCA CGTGTCAAGC AGTCACTAAC CATCCGACAA
441 TAGGAAGCCG CTGAGCTCGG TAAGGGTTCC TTCAAGTACG
481 CCTGGGTTCT TGACAAGCTC AAGGCCGAGC GTGAGCGTGG
521 TATCACCATC GATATCGCTC TCTGGAAGTT CGAGACTCCT
561 CGCTACTATG TCACCGTCAT TGGTATGTTG TCGCTCTTAC
601 TCCGTTCTAT ATCTCCTATT ACTAACACAT CACATAGACG
641 CTCCCGGTCA CCGTGATTTC ATCAAGAACA TGATCCGGGG
681 ACCCTTTCCA
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17

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  1 CCGAATTACA TCTGAATTTC ATCCATCCTC CTCTGGCAGT
 41 CGACCACCGA TAAGTCAAAC CCTCATCGCG ATCTAGCTTA
 81 TCTCGGGTCG TGGAACCCCG CCTGGCATCT CGGGCGGGGT
121 ATTCATCAGT CACTTCATGC TGACAATCAT CTACAGACCG
161 GTCACTTGAT CTACCAGTGC GGTGGTATCG ACAAGCGAAC
201 CATCGAGAAG TTCGAGAAGG TTGGTGACAT CTCCCCCGAT
241 CGCGCCTTGC TATTCCACAA CGAATTCCCT CCCTCGCGAT
281 ACGCTCTGCG CCCGCTTCTC CCGAGTCCCA AATTTTTTGC
321 GGTCCGACCG TAATTTTTTT TGGTGGGGCA TTTACCCCGC
361 CACTCGGGCG ACGTTGGACA AAGCCCTGAT CCCTGCACAC
401 AAAAACACCA AACCCTCTTG GCGCGCATCA TCACGTGGTT
441 CACAACAAAC GCTAACCGGT CCAACAATAG GAAGCCGCTG
481 AGCTCGGTAA GGGTTCCTTC AAGTACGCCT GGGTCCTTGA
521 CAAGCTCAAG GCCGAGCGTG AGCGTGGTAT CACCATCGAC
561 ATTGCCCTCT GGAAGTTCGA GACTCCCCGC TACTATGTCA
601 CCGTCATTGG TATGTTGCTG TCACCTCTGT CACACATGTC
641 TCACCACTAA CAATCAACAG ACGCCCCCGG CCACCGTGAT
681 TTCATCAAGA ACATGATCAT GGG
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18

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  1 TCCCCTCGTC TGCGTACCAC TGCAGCTTCC TCTGGAAGTC
 41 GACCACTGAT GAGTACTACC CTTGACGATG AGCTTATCGG
 81 CCATCGTAAA CCCGGCCAAG ACCTGGCGGG GGATTTCTCA
121 AAGAAAACAT ACTGATATCG CTTCACAGAC CGGTCACTTG
161 ATCTACCAGT GCGGTGGTAT CGACAAGCGA ACCATCGAGA
201 AGTTCGAGAA GGTTAGTCAC TTTTCCTTCT ATCGCGCGTT
241 CTTTGCCCAT CGATTCCCCC CTACGACTCG AAACGTACCC
281 GCTACCCCGC TCGAGCCCAA AAATTTTGCG ATACGACCGT
321 AATTTTTTCT GGTGGGGCAT TTACCCCGCC ACTCGAGCGG
361 CGCGTTTCTG CCCTCTCCCA TTCCACAACC TCACTGAGCT
401 CATCGTCACG TGTCAAGCAG TCACTAACCA TCCGACAATA
441 GGAAGCCGCT GAGCTCGGTA AGGGTTCCTT CAAGTACGCC
481 TGGGTTCTTG ACAAGCTCAA GGCCGAGCGT GAGCGTGGTA
521 TCACCATCGA TATCGCTCTC TGGAAGTTCG AGACTCCTCG
561 CTACTATGTC ACCGTCATTG GTATGTTGTC GCTCTTACTC
601 CGTTCTATAT CTCCTATTAC TAACACATCA CATAGACGCT
641 CCCGGTCACC GTGATTTCAT CAAGAACATG ATGCGGGG
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19

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  1 CAAAAATTAA CTGTGAATCT GCAATCTCTC AATCTGGCAG
 41 TCGACCACTG ATGAGTACTA CCCTTGACGA TGAGCTTATC
 81 GGCCATCGTA AACCCGGCCA AGACCTGGCG GGGGATTTCT
121 CAAAGAAAAC ATGCTGACAT CGCTTCACAG ACCGGTCACT
161 TGATCTACCA GTGCGGTGGT ATCGACAAGC GAACCATCGA
201 GAAGTTCGAG AAGGTTAGTC ACTTTTCCTT CTATCGCGCG
241 TTCTTTGCCC ATCGATTCCC CCCTACGACT CGAAACGTAC
281 CCGCTACCCC GCTCGAGCCC AAAAATTTTG CGATACGACC
321 GTAATTTTTT CTGGTGGGGC ATTTACCCCG CCACTCGAGC
361 GGCGCGTTTC TGCCCTCTCC CATTCCACAA CCTCACTGAG
401 CTCATCGTCA CGTGTCAAGC ACTCACTAAC CATCCGACAA
441 TAGGAAGCCG CTGAGCTCGG TAAGGGTTCC TTCAAGTACG
481 CCTGGGTTCT TGACAAGCTC AAGGCCGAGC GTGAGCGTGG
521 TATCACCATC GATATCGCTC TCTGGAAGTT CGAGACTCCT
561 CGCTACTATG TCACCGTCAT TGGTATGTTG TCGCTCTTAC
601 TCCGTTCTAT ATCTCCTATT ACTAACACAT CACATAGACG
641 CTCCCGGTCA CCGTGATTTC ATCAAGAACA TGATCACTGG
681 GCTACCTCCC
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20

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  1 TGGTCGTTCT TCGTTAGCTG CACTTCATCG GGCCGGCGAC
 41 CACTGATGAG TACCACTGCA TGCCCAACCC CAGCCGATAC
 81 TTGGCGGGGT AGTTTCAAAT TTCCAATGTG CTGACATACT
121 TTGATAGACC GGTCACTTGA TCTACCAGTG CGGTGGTATC
161 GACAAGCGAA CCATCGAGAA GTTCGAGAAG GTTGGTCTCA
201 TTTTCCTCGA TCGCGCGCCC TTTTCCCTTT CGAAACATCA
241 TTCGAATCGC CCTCACACGA CGACTCGATA CGCGCCTGTT
281 ACCCCGCTCG AGGTCAAAAA TTTTGCGGCT TTGTCGTAAT
321 TTTTCTGGTG GGGCTCATAC CCCGCCACTC GAGCGACAGG
361 CGCTTGCCCT CTTCCCACAA ACCATTCCCT AGGCGCGCAC
401 CATCACGTGT CAATCAGTTA CTAACCACCT GTCAATAGGA
441 AGCCGCCGAG CTCGGTAAGG GTTCCTTCAA GTACGCCTGG
481 GTTCTTGACA AGCTCAAAGC CGAGCGTGAG CGTGGTATCA
521 CCATTGATAT CGCTCTCTGG AAGTTCGAGA CTCCTCGCTA
561 CTATGTCACC GTCATTGGTA TGTTGTCACT ACTGCTGTCA
601 TCACATTCTC ATACTAACAC GACTATCAGA CGCTCCCGGT
641 CACCGTGATT TCATCAAGAA CATGATCATG GG
```

21

Fig. 1. DNA sequencing for Fusarium isolates for translation elongation factor-1 alpha gene (αTEF gene) and RNA polymerase II gene (RPB2) regent products. 1,2,3 …..21 the sequencing of Fusarium isolates as Table 1.

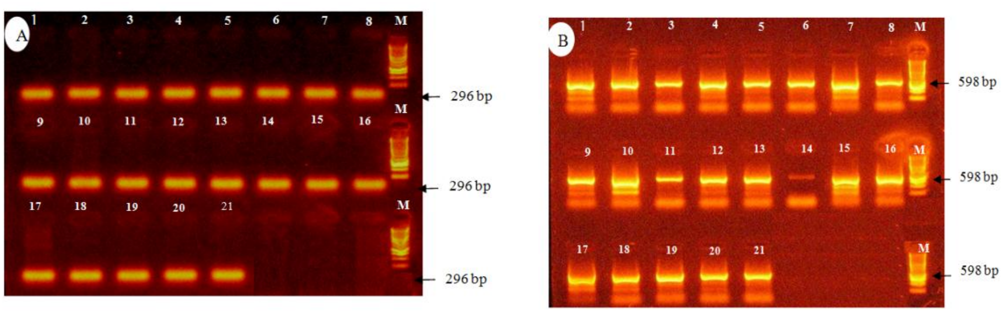

Fig. 2. Agarose gel 1.5% photograph showing PCR amplified products for A- α-TEF gene B- RPB2 for *Fusarium* spp isolates, the product prepared for sequencing test. 1, 2, 3…..21= the *Fusarium* spp isolates , M= marker.

Aggressiveness of Fusarium isolates for CR

The pathogenic isolates showed various symptoms of CR on wheat seedlings ranged from faint lesions on outer sheath to intense brown necrosis on all sheath leaves. The more pathogenic isolate with 0.7 severity index was found belong to *F. culmorum*. Most isolates were belonged to *F. verticelloides*and shown similar symptoms of CR on wheat seedling with severity index ranging from 0.4-0.5. One isolate of *F. verticelloides* No.10 was gave 0.2 disease severity. Four isolates of *F. proliferatum* shown CR symptoms with disease index 0.3-0.4 Table 2.One isolate of *F. solani* shown CR symptoms with disease severity of 0.4. Several previous studies reported that *F. verticelloides, F. solani* and *F. proliferatum* caused CR disease and root rot on wheat seedling (Al-Mousa, 2006; Bottalico and Giancarlo, 2002). Result showed that all the 21 isolates of Fusarium in this study produced DON toxin on wheat straw with concentration ranged between 0.5 - 3.8 mg/kg. A relationship was found between the quantity of toxin produced and the aggressiveness of Fusarium isolates. It was reported that the isolates of high production of DON caused more severe disease symptoms of CR disease (Bottalico and Giancarlo, 2002; Bakan *et al.,* 2012).

Aggressiveness of Fusarium spp for FHB

Two isolates of *F. proliferatum* and four *F. verticelloides* from 21 isolate obtained showed FHB symptoms with disease incidence 33.3, 66.6% for *F. proliferatum* and 66.6, 66.6, 66.6 and 66.6% for *F. verticelloides* respectively. Variation of aggressiveness between isolates was observed. Three isolates of *F. verticelloides* showed FHB symptoms on wheat spike with infection area of 6.6 and 6% respectively .Two isolate of *F. proliferatum* showed symptoms with infected area of 5 and 6%. The most aggressive isolate was of that belong to *F. culmorum* which gave higher percentage of disease area (100%) on spike Table 2. A previous study reported that *F. culmorum* strains is very aggressive in causing CR or FHB disease on wheat through producing high-deoxynivalenol (Bakan *et al*., 2012). This toxin was considered as fungal virulence factor that facilitates the development of Fusarium head blight (FHB) disease (Walter and Doohan, 2011). Another study shown that treated Durum wheat grain with more than 5 µg/ml DON toxin can caused deletion in DNA sequencing and abnormal chromosomal during meiotic division (Mohammad and Fadl-Allah, 2008).

In this study we demonstrated that several species of *Fusarium* were associated with CR anf HB disease on wheat plants. Certain of these species were highly pathogenic and caused severe symptoms on wheat seedlings as well as had capacity to produce high concentration of DON mycotoxin on wheat straw. The capacity of the isolate to produce DON toxin may be responsible of disease symptoms development. These results indicated the necessity of searching of means to protect wheat plants against *Fusarium spp* and reduced the production of DON in the plants.

Table 2. Aggressiveness and disease incidence of Fusarium *spp* testing for CR and FHB assay on seedling wheat

Isolate No.	**Species**	**CR**		**FHB**		**DON toxin µg/g**
		Disease incidence %	Aggressiveness index	Disease incidence %	Infected spike %	
1	*F. proliferatum*	100	0.3	33.3	5	0.81
2	*F. proliferatum*	100	0.4	0	0	1.91
3	*F. verticelloides*	100	0.4	0	0	1.35
4	*F. proliferatum*	100	0.5	0	0	0.89
5	*F. verticelloides*	100	0.4	0	0	0.78
6	*F. verticilloides*	100	0.4	0	0	1.57
7	*F. verticelloides*	100	0.4	66.6	6	0.76
8	*F. verticelloides*	100	0.4	66.6	6	0.83
9	*F. proliferatum*	100	0.5	66.6	6	1.33
10	*F. verticilloides*	33.3	0.2	0	0	0.85
11	*F. verticelloides*	100	0.5	0	0	0.95
12	*F. verticelloides*	100	0.4	0	0	0.70
13	*F. verticelloides*	100	0.5	0	0	1.02
14	*F. verticelloides*	100	0.5	0	0	0.74
15	*F. verticelloides*	100	0.4	0	0	0.59
16	*F. verticelloides*	100	0.5	0	0	1.40
17	*F. verticelloides*	100	0.4	0	0	1.07
18	*F.solani*	100	0.4	0	0	0.62
19	*F. verticelloides*	100	0.5	0	0	0.82
20	*F. verticelloides*	100	0.4	66.6	6	0.55
21	*F. culmorum*	100	0.7	100	100	3.87

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