Detection of seed-borne pathogens in wheat (Triticum durum)

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Abstract

The research was conducted to investigate the seed borne fungi related to various cultivars of durum wheat (Triticum durum.) grown in Kurdistan region, Iraq. using international rules of seed testing method and used two primer (ITS, LSU) for molecular identification for testing 22 seed samples a total of 15 genera were reported. Their isolation frequency's most prevalent genera were Aurobasidium pullulans , Stemphylium vesicarium, Cladosporium herbarum , Ulocladium sp, Curvularia sp, Fusarium solani , Alternaria alternata, Arthrinium arundinis, Phoma medicaginis Rhizoctonia solani , Nigrospora spaerica, Aspergillus nidulans , Aspergillus fumigatus , Penicillium pinophilum, mucor hiemalis. highest isolation frequency was displayed Aurobasidium pullulans29.19% and Aspergillus fumigatusshowed low isolation 0.83% the disease severity between treatments and using real-time PCR for detection of fungi in wheat tissue treatment with infected fungi they have significant ct valve between the treatments , height ct value of control 38.32 ct value and lowest ct value of Curvularia sp.

Keywords: *Seed borne wheat pathogen, molecular identification, pathogenicity test of seedborne fungi.*

INTRODUCTION

One of the most significan cereal crops in the world is wheat (Triticum aestivum). Worldwide wheat output has grown since 2013, reaching more than 700 million metric tons annually. More over a quarter of the world's wheat output is mostly concentrated in China, India, the United States, and Russia. The first crop ever grown and the most popular grain in Iraq is bread wheat. Iraq produced 2,974,136 million metric tons of wheat in 2017 (Kallio and Solberg, 2018) .

There have been reports of both pathogenic and saprophytic fungus from durum wheat. Fusarium spp., which causes head blight and lowers grain quality, is one of them (Alkadri et al., 2013). Wheat grains were routinely tested for other significant diseases such Alternaria alternata, Bipolaris sorokiniana, Microdochium nivale, and Tilletia spp (Alkadri et al., 2013). Fusarium, Penicillium, and Aspergillus of seed-borne saprophytic species that reproduce by seed sometimes create volatile metabolites such aromatic, ketone, and alcohol chemicals (Laddomada et al., 2014)

Rhizopus sp., Phoma sp., Nigrospora sp., Drechslera tetramera, Fusarium pallidoroseum, Fusarium oxysporum, Fusarium moniliforme, Cladosporium sp., Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, and Alternaria alternata are among the seed-borne mycoflora of sorghum

identified in various parts of the world (El-Nagerabi et al., 2000, Abdullah and Kadhum, 1987) (Ahmed et al., 1991).

The most advantageous and environmentally friendly method of controlling spot blotch disease is seen to be the adoption of resistant cultivars (Gupta et al., 2018). But pathogenic and genetic heterogeneity among pathogen populations made research on resistance breeding more challenging (Ghazvini and Tekauz, 2012); (Al-Sadi, 2016) . Plant pathogens and conserved sequences of glyceraldehyde-3-phosphate dehydrogenase, translation elongation factor 1-alpha (EF1-), RNA polymerase second largest subunit (RPB2), 18S rRNA gene (SSU), -tubulin, 28S rRNA gene (LSU), and internal transcribed spacers (ITS) have been studied phylogenetically using molecular techniques (GAPDH). They offered useful sites for the precise identification of a variety of fungal illnesses (Brunner and Mach, 2010) ,(Raja et al., 2017). Spot blotch disease was routinely detected using several methods according to PCR (Yu, 2018). To increase the effectiveness of plant disease control procedures, new disease detection and evaluation approaches must be developed. Aims of research isolation and identification of wheat seed borne fungi using molecular method in Kurdistan region.

Material and method

Sampling and isolation

Sub-sections A total 26 seed samples of four governorate in Kurdistan/Iraq , related (Erbil , duhok , sulemany , Halabja) Provinces were collected. Based on International rules of seed Testing (ISTA, 2009) the Agar plate method was used for testing of seed health specially for identification of internal seed borne pathogens. Facilities and equipment, sample size, surface sterilization, plating,using detecting standard methods (agar plate) were utilized [Potato dextrose agar (PDA) (Himedia laboratories, India) . according to International rules of seed Testing (Powell, 2009).

Prepare PDA media and sterilized Before pouring, the agar medium cool down to around 50°C.Addtion filter sterilized antibiotic (Striptomycin @ 35 ug/L) solution in the PDA medium to avoid bacteria growth,. Pour the medium in sterile petri dishes (15 ml per8.5 cm dish) in a Laminar Air Flow bench. Plate and incubate surface sterilized seeds ,In such case the seeds should be plated after disinfection of sodium hypochlorite (2%) for 1 minute then wash treated seed three times for 1 minute each by sterilized distilled water. Put seeds in the petri dishes by forceps.10 seeds can be plated for each 8.5 cm petri dishes.. A total of 100 seeds in 10 replication accordint to ISTA standard. The plated grains were incubated for 5-7 days at 25 ºC. Colonies growing out from the grains on each medium were isolated to newly fresh appropriate media for identification (Abdullah and Atroshi, 2016)The percentage frequency of occurrence (FO) of fungi in the seeds of each cultivar and percentage contamination (PC) was calculated.

Number of grains on which :

$$FO\% = \frac{\textit{Fungal species was indentified}}{\textit{Total number of tested grains}} \mathbf{100}$$

Identification of fungi

Based on descriptions and keys, the isolated fungi were provided by (Ellis, 1971),(Klich, 2002) , (Prescott et al., 1986), (Samson et al., 2004), (Asgari and Zare, 2011), (Guarro et al., 2012) Colony morphology and colors were observed by naked eyes and photo was taken. The spores and mycelium of fungal colonies grown on PDA for 5 days were observed by a dissecting microscope (Choi et al., 2019).

DNA extraction genomic DNA was isolated from fungi, samples are extracted by using Beta Bayern tissue DNA preparation Kit (Beta Bayern GmbH .90453 Bayern, Germany) Amplification Ribosomal RNA genes (rRNA) Polymerase Chain Reaction(PCR):PCR amplification for 28S large subunit of rRNA and 5.8S ribosomal RNA partial genes were done in total volume 50 µl of reaction mixture containing; 2x Taq DNA Polymerase Master Mix (AMPLIQON A/S Stenhuggervej 22), 10 Picomol (pmol) of forward primers in 10 pmol concentration and reverse primers (table1), by Bioresearch PTC-200 Gradient thermocycler.

Table 1: The primers are used for identifying of (Coa) and (fnbB)

Gene	Forward primer	Reverse Primer	Band size	Tm
LSU	ACCCGCTGAACTTAAGC	CGCCAGTTCTGCTTACC	1200	56
ITS	TCCGTAGGTGAACCTGCGG	TCCTCCGCTTATTGATATGC	500	55

Sequencing of DNAThe samples of PCR product of 28S and and 5.8S partial genes have sequenced by ABI Prism Terminator Sequencing Kit (Applied Biosystem) at Macrogen Molecular Company of Korea . Chromatograms of both partial gene were modified and using Finch TV program software, base calls examined. Sequence alignmentThe 28S and and 5.8S partial gene of rRNA sequences were utilized to Basic Local Alignment Search Tool (BLAST) is a searching tool that applies the sequence alignment method (https://blast.ncbi.nlm.nih.gov/Blast.cgi) , and is available at the NCBI (National Center for Biotechnology Information) website to comparing and alignment laboratory or query sequence with other biological sequence to find out more similarity with target of genus and species of fungi.

Pathogenicity tests of wheat cultivars isolates

- Tests of pathogenicity on seeds:

Tests of pathogenicity on seeds , soil was autoclaved for 20 minutes at 121 bar .using pots (14 cm in diameter by 12 cm in height), plants were cultivated in a combination of (100) gm peat moss and local Swinton loam soil.

They were used isolated pathogen fungi (Aurobasidium pullulans , Stemphylium vesicarium , Cladosporium herbarum , Ulocladium sp , Curvularia sp , Fusarium solani , Alternaria alternata , Arthrinium arundinis , Phomame dicaginis Rhizoctonia solani , Nigrospora spaerica) 7 days growing , Inoculation soil by cutting culture pathogen fungi mixed with soil 2 days before planting wheat seeds .

Five seeds were then planted at four cm deep in pots , before sowing that had been sterilization seeds by 1 min in an aqueous solution of 0.6% NaOCl and washed twice in sterile distilled water. The control treatment planting wheat seeds in the pots with out fungi pathogen.

The pots were maintained under greenhouse conditions until harvest, receiving regular irrigations of sterile tap water. Used RCBD

experiment with four replicates of all treatments. (Fernandez and Chen, 2005).

- Test of pathogenicity on seedlings

In pots that were made in a way similar to how the pots were made for the seeds' pathogenicity test, The seedling at the four to six leaf stage , perforation tiny hole (1 cm in diameter) was near to the crown of each seedling , and an agar plug containing mycelium from each of the fungal isolates, produced as for the test on seeds, was inserted in the hole next to the crown root. The soil was then placed on top of the agar plug. The control treatment consisted of an agar plug devoid of any fungi. The same growing circumstances and average development stage as in the test on seeds were used to maintain the plants. Each pot served as a duplicate for each testes four times , which were set up in an RCBD experiment . Assessment of plant survival, scale of diseases severity , and chlorophyll content were done as for the pathogenicity test on seeds.

Amplification of Real Time Polymerase Chain Reaction (RT-PCR)

In 20 µl of reaction mixture three biological replicates of PCR amplification wheat genes were done involving DNase free water and template of DNA (Table 2), 10 pmol of ITS4 reverses primer(TCCTCCGCTTATTGATATGC), 10 Picomol (pmol) of ITS1 forward (TCCGTAGGTGAACCTGCGG), 2x SYBER Green Master mix (Addbio company made in Korea) by Applied Biosystem Model 7500 Real Time thermocycler (table 2).

Table 2: Real-time PCR Amplification Reagents

No.	PCR components	Concentration	Volume (µl)
1	SYBER Green Master mix	2x	10
2	Forward Primer	10 Pmol	1
3	Reverse Primer	10 Pmol	1
4	DNase free Water	-	8
5	Template (DNA)	50ng/µl	3
Total			20

Step one of the temperature profile consists of an initial denaturation at 95 C for 5 minutes, followed by 40 cycles of a primer annealing at 55 C for 45 minutes, an extension at 72 C for 1 minute, and a final additional extension at 72 C for 8 minutes.

Statistical analysis

The data were first checked for normality, and then an analysis of variance (ANOVA) was performed. Duncan's multiple range test was used to compare means (Duncan, 1975)at $p \leq 0.01$ by the statistical analysis software IBM SPSS statistics (v28) according to one-way ANOVA (Basto and Pereira, 2012).

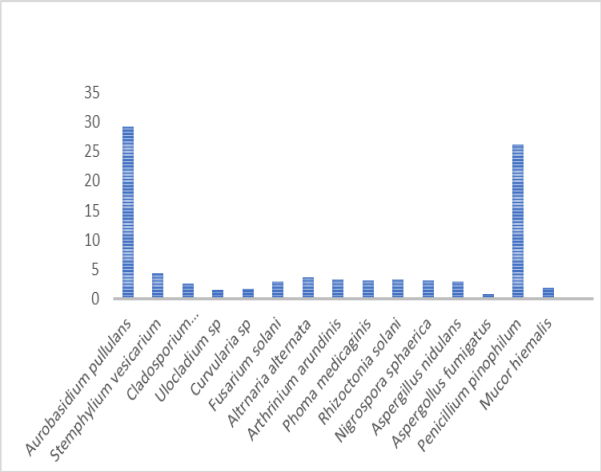
Result and discussion

Isolation fungi of different wheat grains

The proportion frequency of durum wheat grains infected by fungus in various cultivars as determined by international standards for seed testing (ISTA, 2009) synonymy (Powell, 2009). (fig 1)(Abdullah and Atrosh, 2014, Abdullah and Atroshi, 2016) , fifteen fungal genera have been identified from durum wheat grains (figure 2). Highest isolation frequency was reported by the genus Aurobasidium pullulans 29.19% followed by Penicillium pinophilum 26.25% , Stemphylium vesicarium 4.3 % , Alternaria alternata ,Arthrinium arundinis, Phoma medicaginis Rhizoctonia

solani ,Nigrospora spaerica (3.65 ,3.26, 3, 3.20 , 3.14)% respectively . Cladosporium herbarum ,Fusarium solani and Aspergillus nidulans (2.5 , 2.88 , 2.87)% respectively . other fungal genera showed low isolation frequency Ulocladium sp, Curvularia sp , Aspergillus fumigatus and mucor hiemalis (1.51 , 1.67, 0.83, 1.85) %respectively (Abdullah and Atroshi, 2016).

Fig.1. isolation frequency percentage for fungal genera identidied from grains of durum wheat in this study , the results show table (2) that total of 15 fungal species including (Aurobasidium pullulans , Stemphylium vesicarium, Cladosporium herbarum , Ulocladium sp, Curvularia sp, Fusarium solani , Alternaria alternata, Arthrinium arundinis, Phoma medicaginis Rhizoctonia solani , Nigrospora spaerica, Aspergillus nidulans , Aspergillus fumigatus , Penicillium pinophilum, mucor hiemalis) .



According to table (3), Aurobasidium pullulanswas was the genus that was isolated the most often from 22 cultivars, with a percentage frequency ranging from 1% to 68.18%. The prevalence of Stemphylium vesicarium, on the other hand, was observed from 10 cultivars and varied from 1% to 7.5%, the , Cladosporium herbarum , Ulocladiumsp and Penicillium pinophilumwere represented from 4 cultivars at frequency between 1% to 5 , 0.91% to 4 % and 1 % to 99 % respectively . the genus fusarium solani was detected from 6 wheat cultivars the frequency ranged 1% to 5% . , Arthriniumarundinisand mucor hiemaliswere isolated from 7 cultivars frequency between 1% to 8.33% and 1% to 4% respectively . genus Phomamedicaginis , Curvulariasp and Aspergillus fumigatuswere reported from 1 wheat cultivars with low isolation frequency ranged 3% , 1.67% and 0.83 % respectively. Alternaria alternatawas isolated in 17 cultivars ranged between 0.91% to 10.83% frequency , the genus nigrosporaspaerica was reported from 10 wheat cultivars frequency between 1% to 8.33% and Aspergillus fumigatus was isolated from 8 cultivars frequency ranged between 1% to 7 % .(Abdullah and Atroshi, 2016). In contrast, a significant isolation rate of Aspergillus, Fusarium, Penicillium, and Acremonium was found in wheat grains from Turkey (ASKUN, 2007). Alternaria and Fusarium colonized winter durum wheat the most often, while Penicillium and Aspergillus were very sometimes seen in Poland (Pląskowska and Chrzanowska-Drożdż, 2009)). In Tunisia's primary wheat-producing regions, the Microdochium, Fusarium, and genera Alternaria were found to be very prevalent grains of postharvest moulds, while Rhizipous, Mucor, Aspergillus, Penicillium, Fusarium, and Alternaria were the most common postharvest mould genera ((Belkacem-Hanfi et al., 2013).

Table 3: Isolation Frequency percentage for fungal genera detected from durum wheat grains from Kurdistan region

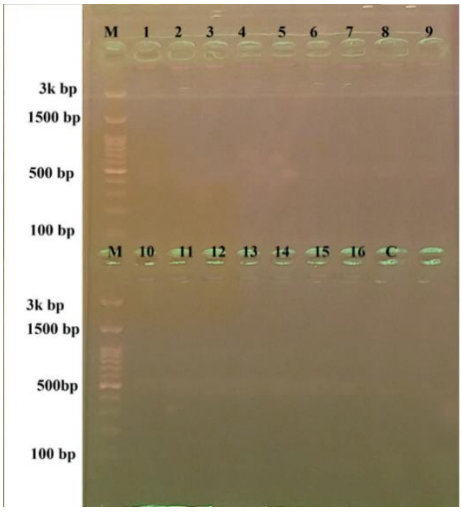
Region	location	cultivars	*Aurobasidium ullulans*	*Stemphylium vesicarium*	*Cladosporium herbarium*	*Ulocladiums p*	*Curvulariasp*	*Fusarium solani*	*Altrnariaalternata*	*Arthriniumarundinis*	*Phomamedicaginis*	*Rhizoctonia solani*	*Nigrosporasphaerica*	*Aspergillus nidulans*	*Aspergollus fumigatus*	*Penicillium sinaehilum*	*Mucor hiemalis*
Hawler	Research center	Hawler 2	8								3			4			4
		Hawler 3	9						3								1
		Hawler 4	14						1			1					2
	Farmer wheat cultivars	jehan	55	7.5			1.67	3.33	8.33			0.83	8.33	1.67			
		adana	68.18	5.45						4.55		0.91	3.64	3.63			
		smnto														99	
		frez	53	11		2			4	2		3	1	1		2	2
Dhok	Research center	Adana 99	18.33		5	1.67		5	3.33	8.33		3.33	6.67	1.67			
		Jehan 99	45		2			3	2	5		3	3			1	2
		Tamuz 2	32	2		2		1	1	1			1	2			
	Farmer wheat cultivars	Adana 99	61	4				3	1	1		6					
		Jehan 99	56	6					5	1		2	4				1
Slemany	Research center	aras	17						2			1	1	2			
		Slemany 2	26.36						3.64			5.45					
		gwaikan	25	0.83					2.5			10.83					
	Farmer wheat cultivars	Aras Gully sur	24		1	1						1					
		Adana	1									1		7		3	1
		Slemany 2	3.33									4.17	0.83		0.83		
Halabja	Research center	Slemany 2	43.63	0.91		0.91			10			0.91					
		Ma3ruf	1		2												
	Farmer wheat cultivars	Slemany 2	25					2	7			6					
		adana	27	1					1			4	2				

Molecular identification of fungi isolated of wheat cultivars

-Genomic DNA isolated

The genomic DNA was isolated by Beta Bayern fungi DNA preparation Kit (Beta Bayern GmbH .90453 Bayern, Germany) spin column filter method. The isolated DNA was electrophorized in 1% Agarose gel (Fig2).

Fig.2. Genomic DNA isolated from fungus samples in which first lane is marker ladder (3k bp-100bp) and second lane to end lanes are DNA extracted bands up of ladder and the last lane(C) is negative control without any band.



-PCR amplification of 28S and 5.8 partial genes

Theprimers of both partial genes were designed for the using the sequences of 28S and 5.8S partial gene available in fungi , Synthesized by Micro-gene Company (South Korea) The primers of LSU could yield a band size ~1200bp fig(3) and the primers of ITS could yield a band size ~500bp fig(4) after PCR product was electrophoresed and visualized by 1.5% Agarose gel.

Fig.3. PCR amplification of partial28S rRNA gene from sixteen fungi samples in which first lane is marker ladder(3k bp-100bp) and second to end are 28S rRNA gene bands with size 1200 bp and the last lane C is negative control without any band.

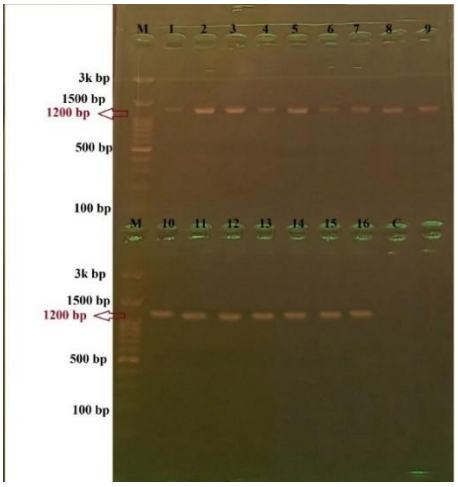
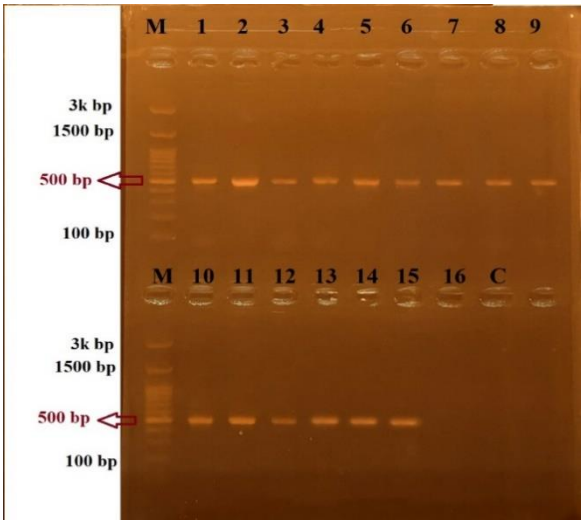


Fig.4. PCR amplification of partial5.8S rRNA gene from sixteen fungi samples in which first lane is marker ladder(3k bp-100bp) and second to end lanes are 5.8rRNA gene bands with size 500 bp and the last lane C is negative control without any band.

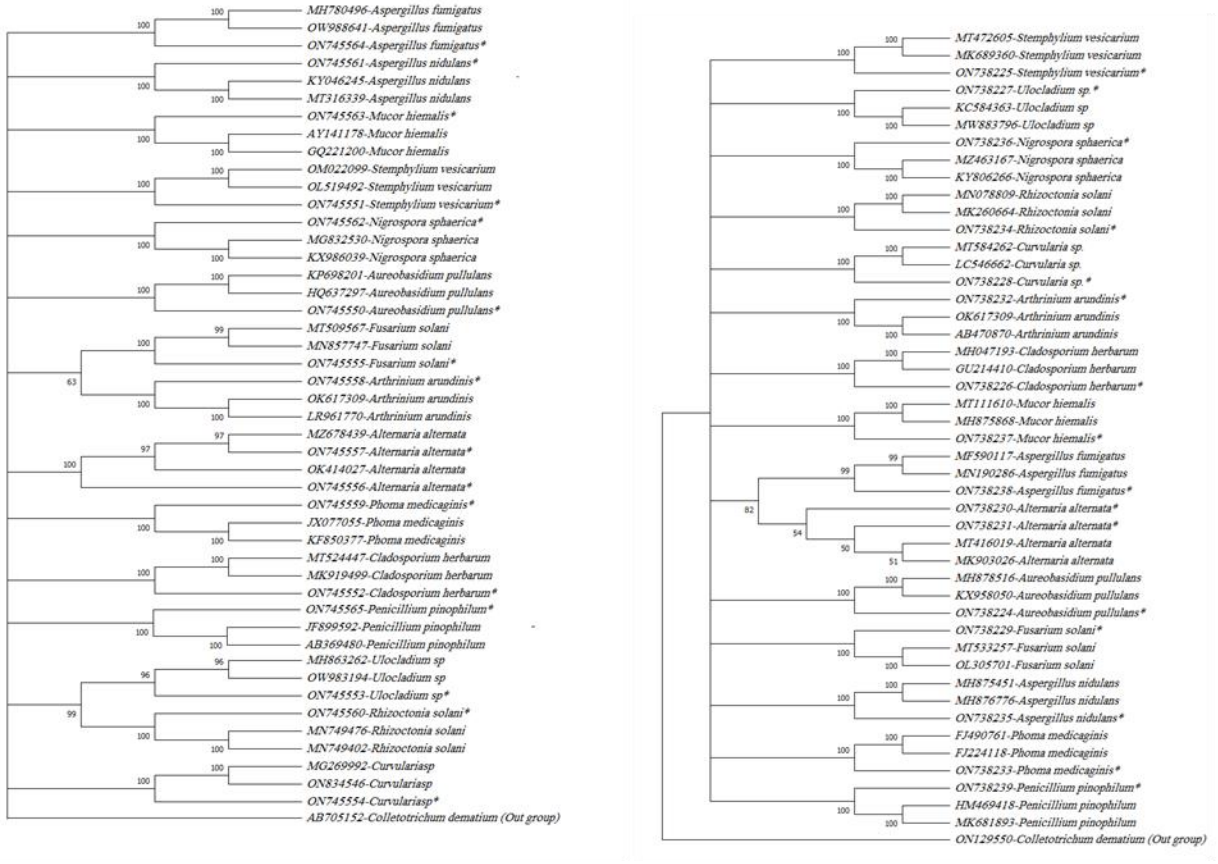


-Phylogenetic inferences

MEGA 11 program of Phylogenetic analysis based on 5.8S and 28S partial gene nucleotide

sequences showed grouping of 16 examined species of fungi on expected lines. Sequence divergence similarity information and a phylogeny were used to determine how closely related species of different genera were to one another. The 16 samples of fungus species grouped in one cluster with high similarity of into genbank fungus species (Fig. 5).

Fig.5. Figure 4 31 Employing Maximum Likelihood with boost strap of Mega 11 program, show phylogenetic positioning of each fungi species of 16 samples with similar Gen Bank sequences of 28S (A) ITS primer and 5.8S(B) LSU primer partial gene that available in Gen Bank. The stars (*) is query samples. Clletotrichum dematium has been selected as an outgroup



pathogenicity of important fungi isolated of wheat cultivars

-Pathogenicity test on seeds

table (4) showed that the wheat seed borne fungi was non significant effect of the foliar and flower length high length (80.50 , 10.25) cm respectively and lower length (64.50 , 8.25)cm respectively , but has significant effect on root length highest length recorded of control (with out any fungi treated) 12.37 cm and lowest length of the wheat plant treated by Curvularia sp 7.25 cm. isolated fungi non significant effect on fresh and dry wheat the highest wheat (7.78, 4.45)gm respectively and lowest weight (3.72 , 1,82)gm

respectively .(Saad et al., 2021) while has significant effect on chlorophyll value (content in plant wheat the highest value content in control treated (33.45) the lowest chlorophyll value content in plant wheat treated by Cladosporium herbarium (10.43).(Aryal et al., 2015) the result showed of diseases severity modified 0-9 scale for yellow spot reaction in wheat plant the diseases expression recorded at the adult plant stage in the control treated have lowest degree of diseases severty (1.00) and highest degree of diseases severity of plant treated recorded by Aureobasidium pullulans , Curvularia sp , Fusarium solani , Alternaria alternata , Arthrinium arundinis Rhizoctonia solani ,and Nigrospora sphaerica (7.25 , 7.25, 7.75,7.50,7.75,7.25,7.75) respectively . (Dinglasan et al., 2016) the isolates fungi had non significant effect on yield seed weight the highest weight of treated was observed by fungi Curvularia sp (4.95)gm and lowest weight of control treated (5.10)gm .(Tian et al., 2020)

Table 4: Effect of elven isolated fungal pathogenic genera on some growth and yield parameters of seed wheat plant in green house

Fungial isolates	Foliar length	Root length	Flower length	Fresh weight	Dry weight	Seed weight	Chlorophyll	Diseases severity
control	72.00^{a}	12.37^{a}	10.25^{a}	4.19^{a}	2.08^{a}	5.10^{a}	33.45^{a}	1.00^{d}
Aureobasidium pullulans	66.00^{a}	9.50^{bcd}	9.50^{a}	3.72^{a}	1.84^{a}	6.20^{a}	19.78^{b}	7.25^{a}
Stemphylium vesicarium	67.50^{a}	9.50^{abc}	8.50^{a}	3.95^{a}	1.82^{a}	6.00^{a}	11.45^{b}	4.50^{bc}
Cladosporium herbarium	70.00^{a}	10.75^{ab}	9.00^{a}	6.51^{a}	4.45^{a}	6.06^{a}	10.43^{b}	6.00^{ab}
Ulocladiumsp	75.25^{a}	9.75^{abc}	9.25^{a}	5.01^{a}	3.07^{a}	5.13^{a}	13.17^{b}	3.75^{c}
Curvulariasp	71.75^{a}	7.25^{C}	8.50^{a}	5.44^{a}	3.32^{a}	5.76^{a}	18.60^{b}	7.25^{a}
Fusarium solani	70.25^{a}	9.00^{bc}	8.50^{a}	4.75^{a}	2.59^{a}	4.95^{a}	18.16^{b}	7.75^{a}
Alternaria alternata	68.00^{a}	8.75^{bc}	8.25^{a}	5.11^{a}	2.66^{a}	5.88^{a}	15.53^{b}	7.50^{a}
Arthrinium arundinis	64.50^{a}	7.75^{bc}	8.50^{a}	4.86^{a}	3.41^{a}	5.35^{a}	13.51^{b}	7.75^{a}
Phomam edicaginis	76.00^{a}	10.25^{abc}	10.00^{a}	6.61^{a}	3.93^{a}	5.71^{a}	17.22^{b}	5.00^{bc}
Rhizoctonia solani	75.75^{a}	9.00^{bc}	9.50^{a}	5.23^{a}	2.67^{a}	5.16^{a}	13.03^{b}	7.25^{a}
Nigrospora sphaerica	80.50^{bc}	9.00^{bc}	9.75^{a}	7.78^{a}	4.13^{a}	5.83^{a}	18.95^{b}	7.75^{a}

Means had a common letter in the columns are non-significant different at p ≤ 0.01 as analyzed by Duncan

Seedling pathogenicity test :

table (5) showed the non significant effect of foliar and flower length, the high length were(80.50 , 9.25)cm and lower length (64.00 , 7.50)cm respectively and they show

significant effect between treatments of root length , highest length of control treatment which (10.50)cm and lowest length of Rhizoctonia solanitreated (7.25) cm obtained . the fresh weight and dry weight was none significant effect between treatments , the high weight of plant fresh and dry weight were (9.90 , 5.41 and lower weight 5.51, 2.53) gm respectively . on the other hand the yield of seed weightshow significant effect between treatments the highest seed weight of treated by Arthrinium arundinis was(6.43) gm and lowest weight of treated by Rhizoctonia solani was (5.05) gm . the chlorophyll content of plant wheat show significant effect between treatments the highest value of chlorophyll content of control treated was (36.33) and lowest chlorophyll value of treated with Phoma medicaginis was (13.03). of the diseases severity has significant effect ,the highest degree of Fusarium solani and lowest degree of control treatment was (1.00) .(Fernandez and Chen, 2005).

Table 5: effect of eleven isolated fungi on seedling wheat plant at some leaf parameters in greenhouse

Fungi	Foliar length	Root length	Flower length	Fresh weight	Dry weight	Seed weight	Chlorophyll	Diseases severity
control	74.75^{a}	10.50^{a}	9.25^{a}	7.08^{a}	3.26^{a}	6.20^{a}	36.33^{a}	1.00^{e}
Aureobasidium pullulans	73.75^{a}	9.00^{a}	8.75^{a}	5.69^{a}	2.86^{a}	6.01^{ab}	15.06^{cd}	8.00^{ab}
Stemphylium vesicarium	78.00^{a}	9.25^{a}	8.75^{a}	7.42^{a}	3.94^{a}	5.96^{abc}	20.76^{bc}	4.75^{d}
Cladosporium herbarium	79.50^{a}	10.00^{a}	8.25^{a}	5.96^{a}	3.05^{a}	5.70^{abc}	22.53^{bc}	4.75^{d}
Ulocladiumsp	69.75^{a}	9.00^{a}	8.75^{a}	5.51^{a}	2.74^{a}	5.93^{abc}	17.60^{bcd}	5.00^{d}
Curvulariasp	75.75^{a}	10.25^{a}	9.00^{a}	9.90^{a}	5.41^{a}	6.13^{ab}	16.22^{bcd}	7.75^{abc}
Fusarium solani	64.00^{a}	9.25^{a}	8.00^{a}	8.02^{a}	4.47^{a}	5.55^{abc}	15.71^{bcd}	8.25^{a}
Alternaria alternata	77.75^{a}	9.50^{a}	9.25^{a}	6.66^{a}	3.88^{a}	5.83^{abc}	22.80^{b}	7.75^{abc}
Arthrinium arundinis	64.50^{a}	8.00^{a}	8.50^{a}	7.46^{a}	2.53^{a}	6.43^{a}	20.13^{bcd}	6.25^{abc}
Phomamedicaginis	76.00^{a}	9.50^{a}	8.75^{a}	9.21^{a}	5.01^{a}	5.56^{abc}	13.05^{d}	6.00^{cd}
Rhizoctonia solani	75.75^{a}	7.25^{b}	7.50^{a}	7.83^{a}	3.84^{a}	5.05^{c}	20.76^{bc}	7.50^{abc}
Nigrospora sphaerica	80.50^{a}	9.00^{a}	8.25^{a}	7.78^{a}	4.61^{a}	5.25^{bc}	19.22^{bcd}	8.00^{ab}

Means had a common letter in the columns are non-significant different at $p \leq 0.01$ as analyzed by Duncan

-Detection of fungi Infection in Wheat Tissues

Table (6) shows they have significant ct valve between the treatments , height ct value of control 38.32 ct value and lowest ct value of Curvularia sp.

The new qPCR technique was very sensitive and allowed for the detection of pathogen DNA down to 0.1 pg. DNA extracting from healthy plant tissue and other fungus species were not amplified by the proposed primer. Matusinsky et al. developed a species-specific PCR (Matusinsky et al., 2010) permitted detecting 0.001 ng of pathogen DNA, whereas a multiplex system of PCR (mPCR) established to detect wheat pathogens detecting Fusarium spp, Bipolaris sorokiniana, and Rhizoctonia cerealis in winter wheat (Sun et al., 2020). Real-time PCR assay discussed by Orina Aleksandra et al. (Yu, 2018) revealed the existence of B. Alternaria tenuissima B.ipolaris sorokiniana , Fusarium graminearum , Fusarium culmorum Fusarium sporotrichioides

Table 6: Real-time PCR Amplification Reagents of detection of fungi Infection in Wheat Tissues

Fungal isolates	Real-time PCR Amplification Reagents
Control	38.32[a]
Aureobasidium pullulans	26.01[b]
Stemphylium vesicarium	24.43[b]
Cladosporium herbarium	26.11[b]
Ulocladium sp	23.02[b]
Curvularia sp	21.46[c]
Fusarium solani	22.55[b]
Alternaria alternata	22.42[b]
Arthriniumarundinis	24.56[b]
Phoma medicaginis	23.02[b]
Rhizoctonia solani	22.88[b]
Nigrospora sphaerica	21.08[b]

Means had a common letter in the columns are non-significant different at $p \leq 0.01$ as analyzed by Duncan

A real-time PCR test detects a positive response by building up a fluorescent signal. The number of cycles needed for the fluorescent signal to reach the threshold is known as the Ct (cycle threshold) (ie exceeds background level). The quantity of target nucleic acid in the sample is negatively correlated with the CT levels (ie the lower the Ct level the greater the amount of target nucleic acid in thesample). WVDL real-time tests run through 40 amplification cycles.

Cts 29 are strong positive responses that show the sample contains a lot of the target nucleic acid. Positive responses with CTs of 30-37 indicate that the target nucleic acid is present in moderate levels. Cts of 38–40 indicate weak responses and low concentrations of the target nucleic acid, which may indicate an infection state (Bathgate, 2014)

Conclusion

Total of 15 genera were isolated Aurobasidium pullulans , Stemphylium vesicarium, Cladosporium herbarum , Ulocladium sp, Curvularia sp, Fusarium solani , Alternaria alternata, Arthrinium arundinis, Phoma medicaginis Rhizoctonia solani , Nigrospora spaerica, Aspergillus nidulans , Aspergillus fumigatus , Penicillium pinophilum, mucor hiemalis by using international ruls of seed testing method and used two primer(ITS, LSU) for molecular identification .

the results of pathogenicity test they effect of fungi used of inoculation seed wheat and seedling plant wheat when 4 to 6 leaves and for detection fungi in wheat tissue used real-time PCR method.

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