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MANAGEMENT OF LOOSE SMUT DISEASE (*USTILAGO TRITICI*) AND DETERMINATION OF FUNGICIDES RESIDUES IN WHEAT MATRICES USING QuEChERS METHODOLOGY

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ABSTRACT: *Loose smut of wheat caused by Ustilago tritici is a disease of economic important in Egypt. It is an exclusively seedborne disease and can be controlled by applying pre-sowing dry seed treatment with any of the nature products as black seed oil and cumin oil or gamma rays@ 150, 200, 250 Gy or four systemic fungicides as Premis® 25% FS, Sumi-8® 2%WP, Dividend Extreme® 11.5% FS and Raxil® 2% DS. The present study was carried out during 2016/2017 and 2017/2018 growing seasons and aimed to evolve an efficient method of application of fungicides, nature products and radiation for an economic control of loose smut of wheat and also evaluated for the determination of four azole fungicides residues in wheat and soil samples. The tested fungicides were highly effective in controlling the disease and gave more than 98% disease control with high grain yield while nature products as black seed oil and cumin oil gave 61.2% and 56.6% control, respectively. For determining the fungicides residues, the fungicides were extracted and cleaned using the 'Quick Easy Cheap Effective Rugged and Safe' (QuEChERS) method and followed by gas chromatographs coupled to electron capture (μECD) detector analysis. The analytical method was evaluated in terms of recoveries, repeatability, limit of quantitation (LOQ) and matrix effects. The recoveries were between 80.12 to 97.85% with relative standard deviation (RSDs) ranged between 6.12-10.65%. Limit of detections ranged from 0.002 to 0.01mg/kg. LOQ for tested fungicides less than or met the maximum residue limits (MRL).*

Key words: *Wheat, Loose smut, Ustilago tritici, Disease control, Fungicides residues.*

INTRODUCTION

Wheat crop is attacked by a large number of diseases among which rusts and smut are highly destructive and cause enormous damage. Loose smut of wheat caused by *Ustilago segetum* var. *tritici* is one of the most important diseases of wheat. Loose smut is a cereal disease present around the world (Agrios, 2005). It was known by man already during the historic time. Thus, Roman people recognized the disease and designated it *Ustilago*, meaning *burn* in Latin (Wilcoxson and Saari, 1996). The disease can cause both yield and quality reduction of wheat. Smuts along with the rusts were the main concern of farmers until the 20th century in most of the wheat growing areas (Agrios, 2005). Loose smut is mainly spread by infected seed; it can also be spread by air, but not for long distance (Nielsen and Thomas, 1996). Reduction in yield is approximately equivalent to the percentage of smutted heads because a smutted spike generally results in the loss of all grains from that spike (Morton, 1961, Green *et al*., 1968). The loose smut disease is internally seed-borne, i.e. the spores remain in the seed embryo, and

thereafter in a presence of favourable conditions it affects the new plant from the same infected seed. The disease is usually seen at higher elevations, and wet and cool weather is favourable for the disease (Saari *et al.*, 1996). Contaminated seeds are the only source of perpetuation and loose smut causes yield losses up to 5-7 % where farmers recycle their own seed (Anon., 1992, Ramdani *et al.*, 2004). Presence of loose smut infection can't be predicted until plant impregnated with the inoculum produces a spike characteristic symptoms i.e. early emergence and blackening of emerging spike. In a diseased plant spikelet and kernels of each spike there is smut infected mass instead of grains enveloped in greyish membrane which later on ruptures and black powdery mass i.e. teliospores emerge out (Agrios, 2005). When these teliospores land on healthy flowers germinate and establish in pericarp and tissue of embryo before kernel matures. The mycelium then becomes inactive and remains dormant until the infected kernel germinates next year. The disease is spread by windblown teliospores. Loose smut-infected wheat plants produce fewer tillers and reduced tiller height (Agarwal and Gupta, 1989). Infection with certain pathogens causes discoloration and shriveling of seeds, reducing grain quality (Agarwal, 1986, Mathur and Jørgensen, 1992). None of the wheat varieties under cultivation in Egypt is resistant against this disease. Looking at these facts, the use of pre-sowing fungicidal seed treatment therefore, is only viable and a popular method for its effective management. The systemic fungicides like Raxil (tebuconazole), Premis, Sumi-8, were recommended in past decades (Goel *et al.*, 2001). New molecules and formulations are however, required to bring down the cost of treatment and the harmful effects as well as manage buildup of resistance against these, in pathogen. The use of nature products as black seed oil and cumin oil are safer on consumers. Gamma rays is well established for inducing useful mutants has been used in many crops such as wheat, rice, barley, maize, etc. (Njau *et al.*, 2006, Abdel-Hady *et al.*, 2008, Sharma *et al.*, 2011, Marcu *et al.*, 2013). These induced mutation help breeders to develop many agronomical important traits such as shorter growing period, increased tolerance or resistance to biotic stresses (Maluszynski and Kasha, 2002, Kenzhebayeva *et al.*, 2013). But nowadays problem is that most of farmers prefer using chemical control, because it gives immediately results and highly effective in controlling the disease. Using pesticides to protect crops production may cause impacts on the environmental and on consumers as chemical residues in wheat products. The 'Quick Easy Cheap Effective Rugged and Safe' (QuEChERS) sample preparation method has been used for preparation wheat and soil samples for determination of fungicides residues. This method has many advantages over traditional techniques, such as high recovery for wide polarity and volatility range of fungicides; high sample throughput; the use of smaller amounts of organic solvent; and the use of no chlorinated solvents (Nguyen *et al.,* 2007, Jallow *et al.,* 2017). To maintain plant food safety, regulation (EC) No39/2005 on maximum residue levels of fungicides in or on food and feed of plant and animal origin require the member states of the European union to monitor pesticide residue levels in food commodities and submit the monitoring results to EFSA and the European commission (Regulation, 2005, Lawon food safety and nutrition, 2006). After many years of research, it's possible now to assess health hazards caused by plant protection products (Lozowicka *et al.,* 2012). Calculation of dissipation rate of a

pesticide after its treatment is a key process for determining the residual behavior of pesticides in agricultural crops and for detecting pre-harvest interval (PHI). Additionally, residues dissipation curves can be used to estimate the time required for decreasing the residues below MRLs (Fenoll *et al.,* 2009).

Azole group is the largest group of antifungal agents and extensively used in a wide range of crops in many countries for its good control of fungi diseases like powdery mildew, rusts, *Septoria* leaf blotch. Azoles work by targeting the sterol 14α-demethylase CYP51 (a member of the cytochrome P450 family), which is an important regulatory enzyme in the ergosterol biosynthetic pathway. Azole fungicides bind through direct coordination of the triazole N-4 or the imidazole N-3 nitrogen as the sixth ligand of the haem iron (Price *et al.,* 2015)

Gas chromatographs coupled to electron capture (μECD) detector are also widely used, mainly for the detection of different compounds as (azole compounds) (Gowda and Somashekar, 2012, Hunter *et al.,* 2010). μECD is cheaper and have lower maintenance costs, hence are more readily available in some countries than mass spectrometers. Most importantly, they are very sensitive for those groups of fungicides, which are essential if the data are used for dietary exposure assessment (Jardim *et al.,* 2014). Therefore, the main objectives of this study were conducted to evaluate the efficacy of nature products, radiation and systemic fungicides to control loose smut disease of wheat and to assess occurrence of fungicides residues of wheat and soil samples.

MATERIAL AND METHODS

1. Experimental design, disease and grain yield assessment

An experiment was conducted in research area of El-Gemmeiza Agricultural Research Station, Agricultural Research Center (ARC), El-Gharbiya, Egypt during 2016/2017 and 2017/2018 growing seasons. Seeds of wheat cultivar Sakha 61 were provided from Field Crop Research Institute, ARC, Egypt. The lines of wheat cultivar Sakha 61were sown in 2 m long row and spikes were inoculated at growth stage' 59' of Zadoks' scale (Zadoks *et al*., 1974) with loose smut teliospores using modified 'go-go' method (Joshi *et al*., 1988*),* during 2016/2017 growing season. The seeds obtained during previous year were treated 24 h before sowing in the next year using slurry treatment method with four fungicides viz. Premis® 25% FS (Triticonazole), Sumi-8® 2%WP (Diniconazole), Dividend Extreme® 11.5% FS (Difenoconazole + Mefenoxam), Raxil® 2% DS (Tebuconazole) and other treated with nature products as black seed oil, cumin oil and other treated seeds were exposed to 150 Gy, 200 Gy and 250 Gy of gamma rays. Irradiation was achieved at the National Center for Research and Radiation Technology, Atomic Energy Authority, Nasr City, Egypt. The untreated seeds served as check. The experiments were laid out in randomized block design, with three replications during 2017/2018 growing season. Each wheat line was sown in 2 m long row with 20 infected seed, spaced 10 cm apart. Normal agronomic practices were followed for wheat cultivation. The observations on total number of smutted tillers were recorded after 75 and 90 days of sowing. Percent disease control over check was calculated based on infected tillers. The statistical analysis of smutted tillers and grain yield per replicate was done.

2. Residues analysis of fungicides:

2.1. Chemicals and Reagents

The references standard materials of fungicides were purchased from Dr.

Ehrenstorfer (Ausburg, Germany). The purities of the standard fungicides were (>98%). Acetonitrile and methanol (HPLC-grade) were supplied by SDS (France). Primary, secondary amine (PSA, 40 µm Bondesil) and C18 were purchased from Supelco (Supelco, Bellefonte, USA). Anhydrous magnesium sulfate and Sodium chloride were of analytical grade, purchased from Merck and were activated by heating at 135ºC overnight in the oven, then cold and kept in desiccators before usage.

2.2. Sample preparation

The QuEChERS (quick, easy, cheap, effective, rugged, and safe) sample preparation method was modified according to (Mastovska *et al.,* 2010) to accommodate wheat and provide good analytical results for the majority of the target fungicides; Difenoconazole, Diniconazole, Tebuconazole and Triticonazole.

Five grams of milled wheat samples were shaking for 1 h in 20 ml of 1:1 (v/v) water/acetonitrile to provide simultaneous matrix swelling and analyte extraction. Then, a mixture salt of $MgSO_4$/NaCl (4:1, w/w) is added to the extract to induce phase separation and force the fungicides into the upper acetonitrile layer, a 1 ml aliquot of which is subsequently cleaned up using dispersive solid phase extraction with 150 mg of PSA and 150 mg of $MgSO_4$.

The analytical procedure for soil according to (Brondi *et al.,* 2011) consisted of the following steps: (1) adding a 10 g of sample into a centrifuge tube; (2) adding 10 mL of methanol (MeCN), QuEChERS extraction salts in each tube, and centrifuging it at 3,000 rpm for 1 min; (3) transferring 5 mL of MeCN extract to a solid phase extraction (SPE) cartridge containing 330 mg PSA, 330 mg C18 and a 1 cm layer of $MgSO_4$ activated with 3 mL of MeCN. Then, (4) the extract was passed in the column SPE and collected; (5) transfer 1.0 mL of the collected extract to a vial for analysis by GC-µECD.

2.3. Instrumentation

The HP6890 gas chromatograph equipped with an HP7673 auto-sampler, an electron-capture detector. A 30 m x 0.32 mm capillary column coated with a 0.25 µm thick film of 5% phenyl methylpolysiloxane (HP-5) from Hewlett and Packard was used in combination with the following oven temperature program:

Initial temperature 180 ºC for 5 min., 5 ºC / min. up to 220 ºC and held for 5 min., 5 ºC/min. up to 240 ºC and held for 5 min. The carrier gas (N_2) flow rate was 3 ml/min., splitless injection of a 1µl volume was carried out, detector and injector temperatures were 300 ºC and 280 ºC, respectively.

2.4. Method performance

Selectivity was checked by analyzing the GC-µECD chromatogram profiles of a blank and a fortified sample, verifying for interferences at the same retention time of the fungicides. The trueness (recovery) and precision as relative standard deviation (RSD %) were determined by analyzing replicate samples fortified at three levels (n = 5 at each level) for wheat and soil samples.

Linearity was accessed by injecting external matrix-matched calibration standards curves, each with 6 points. The calibration standard curves ranged from 0.01 to 2µg/ml. The linear regression was evaluated by checking the residues variance and the correlation coefficient (r^2). The matrix effect was investigated by comparing the slopes of calibration curves at (0.01 to 2 µg/ml) of 4 azole fungicides in wheat and soil. The % matrix effect (ME) could be negative or positive and would be classified in three

categories: no matrix effect (between -20% and 20%), medium matrix effect (between -50% to -20% or 20 to 50%) and strong matrix effect (below -50% or above 50%) (Ferrer *et al.,* 2011, Saber *et al.,* 2016).

The method limit of quantification (LOQ) was defined as the lowest concentration that could be quantified with acceptable recovery (70-120%) and precision (RSD ≥20%) (SANCO, 2013). The method limit of detection (LOD) was set at 1/3 LOQ.

3. Data Analysis

Data was subjected to analysis of variance (ANOVA) and Least Significant Difference (LSD 5%) used to compare the means for all the variables within the experiment (Gomez and Gomez, 1984)

RESULTS AND DISCUSSION

1. Assessment of disease and yield components

The data in (Table 1) revealed that no wheat replica/lines was found immune towards disease; four advance lines treated with Triticonazole, Diniconazole, Difenoconazole + Mefenoxam and Tebuconazole were exhibited efficiency more than 98% disease control, while five lines treated with black seed oil, cumin oil and gamma rays @ 150, 200, 250 Gy were found to be moderately resistant with 61.2, 56.6, 39.6, 43.2 and 49.3% disease control, respectively comparing to untreated control that was exhibited high susceptible to loose smut disease. In case of untreated seeds the numbers of infected tillers were also significantly higher than the other treatments. Loose smut of wheat can be controlled by the use of clean seed, seed treatment with systemic fungicides or hot water, and host resistance (Bailey *et al*., 2003). Husnain *et al.* (2017) reported that the six fungicides; Dividend Star, Raxil Ultra, Score, Crest, Topsin-M and Hombre were found equally effective as seed treatment for loose smut disease control. The yield data revealed that the fungicides and nature products; black seed oil and cumin oil out yielded all other treatments (Table 1). The wheat seed yield per replica during year of trial was also significantly high in almost all agent treatments. For number of grains/ spike in the treatments; Triticonazole, Diniconazole, Difenoconazole + Mefenoxam, Tebuconazole, black seed oil, cumin oil and gamma rays @ 150, 200, 250 Gy was 55.00, 51.33, 54.00, 53.00, 38.33, 37.00, 26.33, 27.00 and 29.67, respectively while untreated control was 13.00 and for grain weight / spike (gm) was 2.62, 2.48, 2.53, 2.58, 1.54, 1.50, 1.38, 1.43 and 1.47, respectively while untreated control was 0.76 gm. It is also evident from the yield data (Table 1) that the highest yield was not dependent on seedling emergence in the field, in other words, the replicates having highest seedling emergence did not yield significantly very high.

2. Method validation

GC-μECD chromatograms showed no interfering peaks present in the blank samples of wheat and soil at the same retention time for all fungicides, indicating that the extraction, clean-up and instrument conditions were satisfactory and the method was selective. Fig. (1) Shows the chromatograms of fortified control wheat samples and treated samples which analyzed by GC-μECD.

The linearity study of the matrix-match calibration curve showed that for most compounds, in the matrices, the standard deviations of the calibration curve residues increased with fungicides concentrations. The correlation coefficients (R^2) were 0.95 to 0.98. The matrix effects (ME) were investigated for wheat and soil samples by comparison of the slopes of the matrix-matched and

solvent-based calibration curves. The results showed a remarkable %ME for tested fungicides in all matrices in a range from -15.7% to +18.4%. Therefore, matrix-matched calibration curves were used for accurate quantitation for tested fungicides. %ME was indicated that no interfering endogenous peak appeared and did not significantly suppress or enhance the response of the instrument.

The results of the trueness or the mean recoveries found during the method validation showed in Table (2). Overall, the compounds combinations had recoveries between 80.12 to 97.85% with relative standard deviation (% RSD) between 6.12-10.65 %, at all tested levels (0.01, 0.1 and 0.5 mg/kg), five replicates per level used to check the recovery at acceptable mean recoveries (70-120%), according to (SANCO, 2013). So, the value indicating that the method was sensitive and able to detect and quantify the analyte at low levels, and it is suitable for the determination of Diniconazole, Difenoconazle, Tebuconazole and Triticonazole in wheat and soil samples. The (%RSDr) value ranged from 1.63 to 3.18% According to (SANCO, 2013) the obtained (% RSDr) value was within the acceptable range ≤20%. LOD ranged from 0.002 to 0.01mg/kg. LOQ for tested fungicides ranged from 0.01 to 0.03mg/kg.

3. Fungicide residues in wheat and soil samples

The four azole compounds were not found in any sample of wheat and soil samples or were present at levels below the LODs. Table (3) showed the LOD, LOQ, MRL and residues in wheat and soil samples of (Fig 1).

Table (1): Effect of different agents on loose smut and grain yield of the tested wheat cultivar during 2017/2018 growing season.

Sr. No.	Treatments	Dosage per kg of seed (Rate of application)	No. of grains / spike	Grain weight / spike (gm)	Total number of infected tillers	Percent disease control over check
1	Triticonazole	2 cm^3	55.00	2.62	0.0	100.0
2	Diniconazole	2 g	51.33	2.48	2.0	98.9
3	Difenoconazole + Mefenoxam	1.2 cm^3	54.00	2.53	1.0	99.3
4	Tebuconazole	1.2 cm^3	53.00	2.58	1.0	99.3
5	Black seed oil	8 h soaking (15 cm^3)	38.33	1.54	59.0	61.2
6	Cumin oil	8 h soaking (15 cm^3)	37.00	1.50	66.0	56.6
7	Gamma Rays	150 Gy	26.33	1.38	91.0	39.6
8	Gamma Rays	200 Gy	27.00	1.43	86.0	43.2
9	Gamma Rays	250 Gy	29.67	1.47	77.0	49.3
10	Control (untreated)	-	13.00	0.76	151.0	-
LSD 5%		-	1.10	0.09	3.86	-

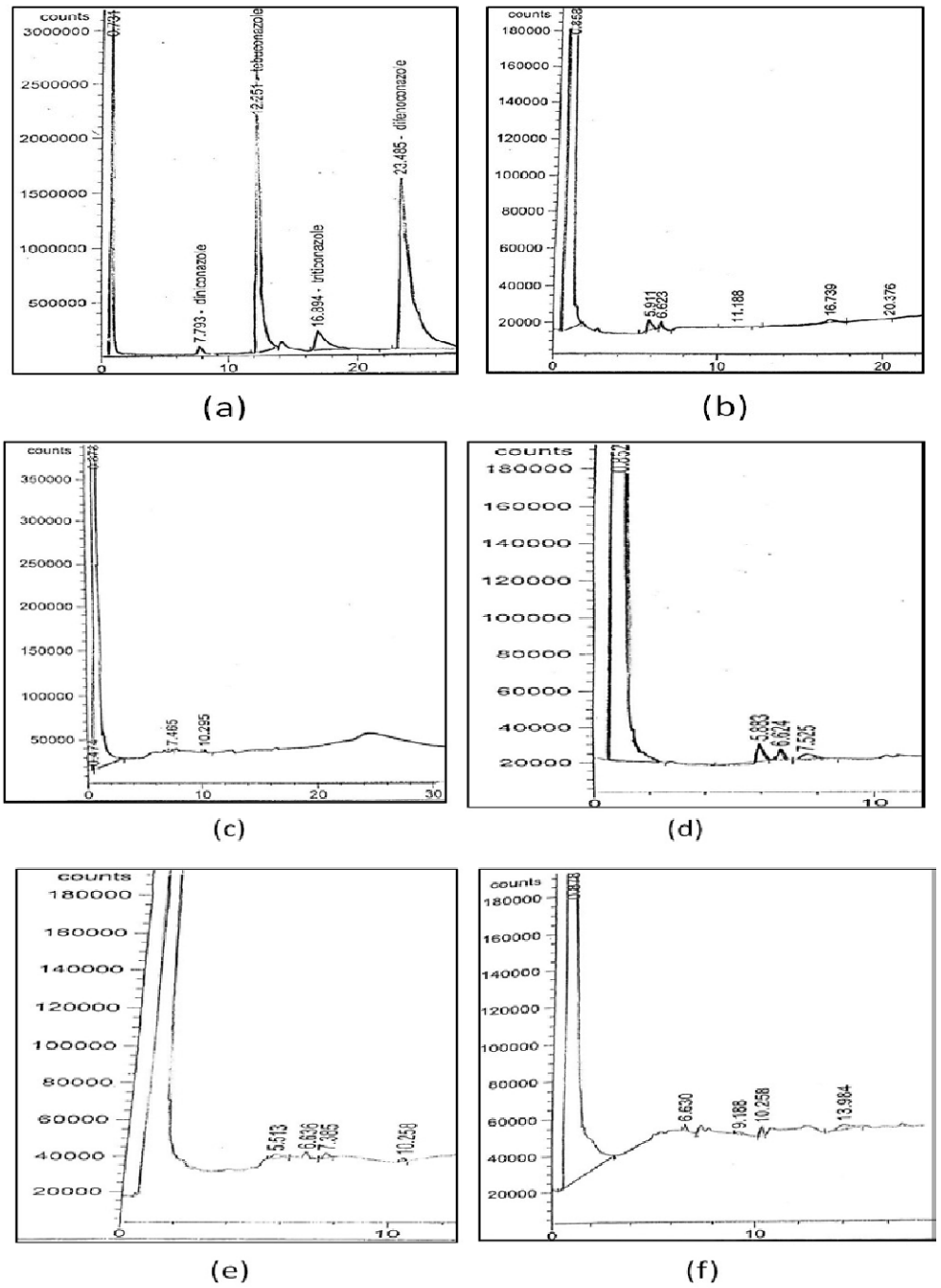

Fig. (1): Shows the chromatograms of

- **(a) Fortified control wheat samples with tested fungicides.**
- **(b) Soil sample.**
- **(c) Wheat sample treated with difenoconazole.**
- **(d) Wheat sample treated with dinioconazole.**
- **(e) Wheat sample treated with tebuconazole.**
- **(f) Wheat sample treated with triticonazole.**

Table (2): Average recoveries and repeatability of the tested fungicides for wheat and soil samples

Fungicides	Recovery (%) ± RSD (%) (*n* = 5) at spiked level (mg/kg)					
	Wheat			Soil		
	0.01	0.1	0.5	0.01	0.1	0.5
Difenoconazole	90.98±6.28	92.45±10.52	97.85±10.65	91.36±6.58	93.08±9.71	92.78±7.54
Diniconazole	88.61±9.02	86.15±9.36	95.01±10.39	90.97±7.53	97.91±10.37	95.24±10.02
Tebuconazole	82.55±10.23	86.59±8.64	96.89±6.91	80.12±6.12	92.34±10.55	96.15±9.43
Triticonazole	92.30±7.52	94.02±6.58	92.49±10.12	85.62±8.09	89.27±6.38	92.19±8.97

Table (3): LOD, LOQ, MRL and residues in wheat and soil samples.

Fungicides	LOD mg/kg	LOQ	MRL mg/kg	Residues mg/kg	
				Wheat	Soil
Difenoconazole	0.002	0.01	0.1	ND	ND
Diniconazole	0.007	0.01	0.01	ND	ND
Tebuconazole	0.01	0.03	0.3	ND	ND
Triticonazole	0.005	0.01	0.01	ND	ND

ND: Non detectable

The loss of fungicide residues in crops depends on the climatic conditions, type of application, dosage, the interval between application and harvest. In addition, the rapid dissipation of originally applied pesticide is dependent on a variety of environmental factors such as sunlight and temperature (Waghulde *et al.*, 2011). However, the major factor in reducing the pesticides from the plant surface is high temperature. Light plays an essential role in the behavior of pesticide in the environment. Also, the decline of pesticides may due to biological, chemical or physical processes, or if still in the field, due to dilution by the growth of the crop. Plant growth, particularly for fruits are also responsible to a great extent for decreasing the pesticide residue concentrations due to growth dilution effects (Nasr *et al.,* 2014).

CONCLUSIONS

It is concluded that the four fungicides, natural products and gamma rays were able to suppressed loose smut fungal pathogen at different levels also the four azole compounds were not found in any sample of wheat and soil samples. LOQ for tested fungicides was

less than or met the maximum residue limits (MRL).

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مقاومة مرض التفحم السائب في القمح وتقدير متبقيات المبيدات الفطريه باستخدام طريقة كيتشيرز

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الملخص العربي

مرض التفحم السائب في القمح من الامراض الاقتصاديه الخطيره التي تؤثر علي محصول القمح في مصر ويسببه فطر يستلاجوا تريتساي. يمكن مقاومة المرض عن طريق معاملة التقاوي قبل الزراعه باحد المبيدات الفطريه او باستخدام بعض المنتجات الطبيعية او عن طريق تشعيع التقاوي قبل الزراعه باشعة جاما. واستخدم في هذه الدراسه زيت الحبه السوداء وزيت الكمون وثلاث جرعات مختلفة من اشعة جاما بمعدل ١٥٠و ٢٠٠ و٢٥٠ جريي وكذلك اربعة مبيدات فطريه جهازية هم بريمس (٢٥%) و سومي–٨ (٢%) وديفيديند اكستريم (١١,٥%) و راكسيل (٢%). هذه الدراسة قد نفذت خلال موسمي الزراعة ٢٠١٧/٢٠١٦ و ٢٠١٨/٢٠١٧ وذلك لتقييم فاعلية بعض المركبات الطبيعيه واشعة جاما وبعض المبيدات الفطريه لمقاومة مرض التفحم السائب في القمح وكذلك لتقدير المتبقي من هذه المبيدات في كل من عينات القمح والتربه للتاكد من خلوها من الاثار المتبقيه الضارة. واوضحت النتائج الكفاءه العاليه للمبيدات لمقاومة المرض يليها المنتجات الطبيعيه. حيث اعطت المبيدات مقاومة لمرض التفحم السائب وصلت لاكثر من ٩٨% مع ناتج محصول عالي بينما اعطي كل من زيت الحبه السوداء وزيت الكمون ٦١,٢ و ٥٦,٦% مقاومة علي التوالي. وباستخدام طريقة كيتشيرز لتقدير نسبة المتبقي من المبيدات في كل من عينات القمح والتربه اوضحت النتائج خلو كل من عينات القمح والتربه المختبره من اي من الاثار المتبقية للمبيدات وان النسبه الناتجه اقل من النسبه العالميه السموح بها.

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