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GARLIC AND BLACK PEPPER EXTRACTS AS RESISTANCE INDUCERS AGAINST TOMATO FUSARIUM WILT DISEASE IN KAZAKHSTAN

El-Fiki, I.A.; A.O. Sagitov* and G. M. El-Habbaa
Botany Department, Plant Pathology Branch, Fac. Agric., Benha Univ. – Egypt.
*Academician of the Kazakh National Academy, Kazakh Scientific Research Institute for Plant Protection and Quarantine – Kazakhstan.
The corresponding author, E. mail:ibrahimelfiki@yahoo.com

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ABSTRACT

This study as a part of Ph.D. Thesis accomplished at the National Academy, Kazakh Scientific Research Institute for Plant Protection and Quarantine – Kazakhstan. Tomato Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL) was reported herein for the first time on tomatoes grown under glasshouse conditions in Kazakhstan. Treatment of tomato (cv. Carolina Gold) at the seedling stage with 4% garlic extract (Immerging roots or spraying shoots) or 4% black pepper extract (spraying shoots) prevented the wilt disease symptoms expression on plants even after two months from inoculation by FOL. Most tested treatments induced a marked increase in activities of polyphenoloxidase (PPO) and peroxidase (POD) enzymes in the leaves of treated plants comparing to the untreated control plants. Anatomy of the fifth leaf petioles of tomato plants treated with garlic or black pepper extract (4% conc.) at seedling stage showed induced positive changes in the water conductive elements particularly xylem vessels and width of the vascular bundles in compared with untreated plants (check). These positive changes due to tested treatments, might be involve also in the induced systemic resistance which lead to resist or delay development of the Fusarium wilt disease in tomato plants.

INTRODUCTION

Tomato Fusarium wilt (*Fusarium oxysporum* f.sp. *lycopersici*) become one of a limiting factor in the production of tomato and accounts for yield losses annually. It has become one of the most prevalent and damaging diseases wherever tomatoes are grown intensively because the pathogen persists indefinitely in infested soils. Controlling of plant diseases mainly depend on fungicides treatments (**El-Mougy, *et al.* 2004**). However, fungicidal applications cause hazards to human health and increase environmental pollution. Therefore, alternatives, eco-friendly approach treatments for control of plant diseases are needed (**Abd-El-Kareem, 2007**, **Mandal, *et al.* 2009**). Induction of resistance to pathogen is a promising approach for controlling plant diseases. Exogenous or endogenous factors could substantially affect host physiology, leading to rapid and coordinated defense-gene activation in plants normally expressing susceptibility to pathogen infection (**Mandal, *et al.* 2009**). Resistance of plant to pathogens can be enhanced by application of various biotic and abiotic agents, called induce systemic resistance in plants (**Sarwar, *et al.* 2005**, **Abd-El-Kareem, 2007**). Some plant extracts could be used to control the soil borne diseases including tomato Fusarium wilt (**Aba AlKhail, 2005, Deepak, *et al.* 2005, Abogharsal, *et al.* 2006, Mandal, *et al.* 2009**).

In plants, the positive correlation between levels of polyphenoloxidase (PPO) and peroxidase (POD) and the resistance to pathogens is frequently observed. There are some evidences indicating that the activation of POD and PPO plays a crucial role in the biological control and resistance of plants to pathogenic attack (**Thipyapong, *et al.* 2004**, **Chérif, *et al.* 2007**, **She-ze, *et al.* 2008**). It was reported that POD may be some of the elements of the defense systems that are stimulated in plants in response to pathogen infection especially *Fusarium oxysporum* (**Morkunas and Gemerek, 2007**).

Anatomical changes in the vascular elements (protoxylem and metaxylem tissues) were detected in tomato plants infected with *Fusarium oxysporum* f. sp. *lycopersici* or root microflora (**Mueller and Beckman, 1988**). The disease-resistance response correlates with changes in cell biochemistry and physiology that are accompanied by structural modifications. These changes have the potential to significantly improve our knowledge of how plants defend themselves and how plant disease resistance is expressed at the cell level (**Benhamou and Nicole, 1999**).

This work aimed to evaluate the effect of garlic (G) and black pepper (BP) extracts at 4% concentration as resistance inducers against tomato Fusarium wilt disease under glasshouse condition in Kazakhstan.

Also, studying the effects of tested treatments on % wilted plants, wilt disease severity, activity of oxidative enzymes and changes in the anatomy of the conductive elements in leaf petioles.

MATERIALS AND METHODS

Isolation and pathogenicity of FOLs:

Fusarium oxysporium f.sp. *lycopersici* (**FOL**) isolates were isolated from wilted tomato plants collected from different tomato glasshouses in Almaty province of Kazakhstan during May 2008 season. FOLs identification was carried out according to **Nelson, *et al.* (1983), Leslie and Sumerell, (2006)**. Seedlings (4-weeks old) of tomato (cv. Carolina Gold) grown in pots (30 cm in diameter) were inoculated with the prepared spore suspension of tested *Fusarium oxysporium* f.sp. *lycopersici* isolates according to **Beshir, (1991) and Amini, (2009)** where the spore suspensions of tested FOL were prepared and used immediately for inoculating 4-weeks old tomato seedlings by pouring 20 ml of spore suspension (10^6 spores/ml) over stem base of each seedling one week after transplanting. In control (non-inoculated), plain water was used instead of spore suspension. The inoculated tomato plants were kept under glasshouse condition and observed for appearance of wilt symptoms. Two months after inoculation, the wilt disease severity was carried out using a visual 0-4 scale according to **Vakalounakis and Fragkiadakis, (1999)** then the disease incidence was determined according to **Song, *et al.* (2004)**. The obtained data were statistically analyzed using the analysis of variance and the least significant difference at 0.05 was calculated **(Snedecor and Cochran, 1982)**.

Effect of garlic (G) and black pepper (BP) extracts:

Garlic extract (*Allium sativum*) and black pepper (*Piper nigrum*) extracts each at 4% concentration were used as natural resistance inducer treatments for treating 4 weeks-old tomato seedlings (cv., Carolina Gold) immediately at transplanting into plastic pots (30cm. in diameter) and one week before FOL inoculation (Isolate A). Each treatment was performed by three methods *i.e.,* immersing roots (IR) for 10 min., spraying shoots (SS) until dropping or combination between IR and SS methods (IR+SS). The plain water was used instead of inducer treatments for treating tomato seedlings in the control treatment. Spore suspension of tested FOL isolate was prepared and used as mentioned above according to **Beshir, (1991) and Amini, (2009)** All pots were kept under glasshouse conditions. The disease incidence and disease severity was determined two months after FOL challenge as described above.

Induction the polyphenoloxidase and peroxidase enzymes activity in tomato plants previously treated with plant extracts:

The fifth leaves of tomato plants either treated or untreated (at seedling stage) with garlic or black pepper extracts (as previously described) were cut at the leaf base level after two months from FOL inoculation. A weighed samples (1.0 g) of tomato leaves were homogenized with 3.0 ml. of Na phosphate buffer pH 6.8 (0.1 M) and centrifuged at 2°C for 15 min at 17.000g in a refrigerator centrifuge. The clear supernatants were collected and used for assaying activities of the oxidative enzymes *i.e.,* Peroxidase (POD) and polyphenoloxidase (PPO) using a spectrophotometer (SPECTRONIC 20-D) at 27±2°C according to **Chance and Maehly (1955), Taneja and Sachar (1974),** respectively.

Induction changes in the anatomical structures of the leaf petiole in tomato plants previously treated with plant extracts:

Two months after treatments, samples represented petioles of fifth leaves were taken from the main stem basically of each treatment. Specimens were killed and fixed for 48 hr. in FAA solution composed of formalin, glacial acetic acid and ethyl alcohol 70 % at rate of 10:5:85 (by volume), respectively. The selected materials were removed from the FAA solution, washed in 50 % ethyl alcohol, dehydrated in a normal ethyl alcohol series, embedded in paraffin wax (melting point 56°C.), sectioned to a thickness of 15-25 microns, double stained with safranine-fast green, cleared in xylene and mounted in canada balsam **(Willey, 1971)**. Sections were examined microscopically and read to detect anatomical manifestations of noticeable responses resulted from investigated treatments. Light photomicrographs were taken with a digital camera (Panasonic, DMC-FX100, Osaka, Japan) fitted to the microscope. The following anatomical characters were determined for each particular treatment:

{1}- Epidermal layer thickness (μm), {2}- Number of parenchyma layers, {3}- Thickness of parenchyma layers (μm), {4}- Thickness of cortex (μm), {5}- Thickness of outer phloem in the bi-collateral vascular bundle [VB] (μm), {6}- Thickness of cambium in VB (μm), {7}- Thickness of xylem in VB (μm), {8}- Number of xylem vessels in VB, {9}- Thickness of largest vessels in VB (μm), {10}- Thickness of inner phloem in VB (µm), {11}- Length of Vascular Bundle (µm), and {12}- Widest of VB (µm).

RESULTS & DISCUSSION

Pathogenicity test and wilt disease symptoms:

Yellowing symptoms were observed on leaves of tomato plants (cv. Carolina Gold) inoculated with isolates of *Fusarium oxysporum* after 2 months from inoculation. The vascular bundles of infected tomato plant showed dark lines in both sides compared with stems of the healthy plants **(Fig.1)**. This browning of the vascular tissue is characteristic of the disease and can be used for its tentative identification. *F. oxysporum* f. sp. *lycopersici* (FOL) causes severe wilt disease. The browning of the vascular system is characteristic of the disease and generally can be used for identification of the fungal isolates as *Fusarium oxysporum* f. sp. *lycopersici* (**Armstrong and Armstrong, 1968, Jones, 1991, Reis,** ***et al.*** **2005**).

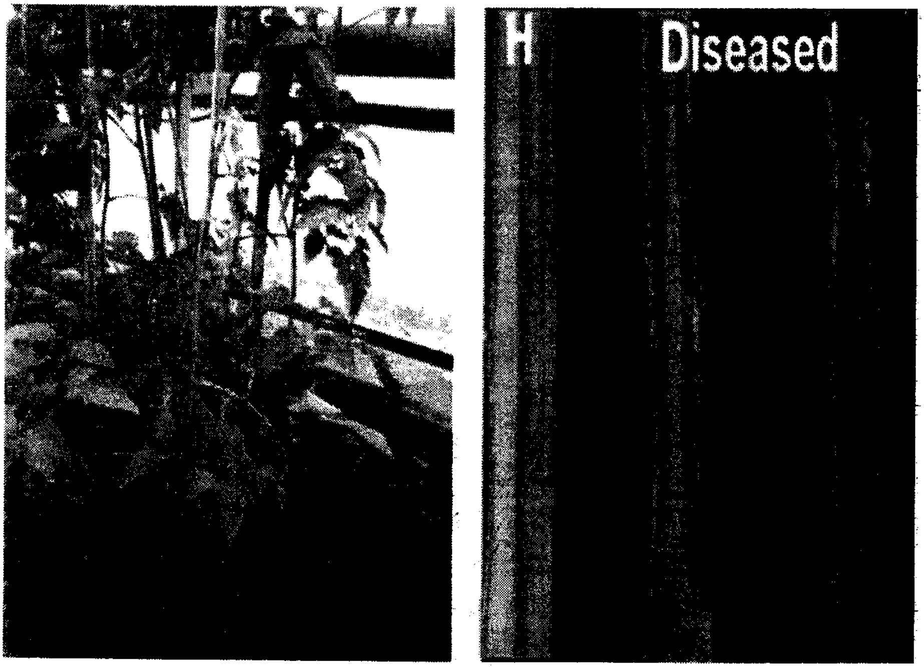

Fig. (1): Wilted tomato plant with yellowing leaf symptoms and dead leaves (left) and vascular discoloration on stem of diseased tomato plants (cv. Carolina Gold) infested with *Fusarium oxysporum* f. sp. *lycopercici* isolate A (right).

Data in **Table (1)** reveal that, the FOLs isolates A and G caused the highest percentage of diseased tomato plants (77.8%) and disease incidence (52.8%). However, isolates H and I seemed to be non-significant when compared with the non-inoculated control which remained disease free. In this respect, **Bost, (2005)** described cv. Carolina Gold as resistant to Fusarium wilt races 1 & 2. The tested Fusarium isolates especially isolates A and G might considered as new isolates of race 1 or 2 or might be race 3. Such findings agree with **Jones, *et al.* (1982)** who observed mature plants with Fusarium wilt symptoms in tomato cultivars possessed resistance to races 1 and 2. Also, **Volin and Jones, (1982)** isolated a new race of *Fusarium oxysporum* f. sp. *lycopersici* from commercially produced plants in western Florida. The principal varieties currently produced commercially are reported to have the 12 gene for race 2 resistance. **Cai, *et al.* (2003)** stated that the collective evidence suggests that race 3 in California originated from the local race 2 population.

Table (1): Percentage diseased tomato plants (cv. Carolina Gold) and disease incidence after two months from inoculation with different isolates of *Fusarium oxysporum*

Tested Fusarium isolates	Diseased plants %	Disease incidence %
A	77.8	52.8
B	55.6	33.3
C	33.3	22.2
D	44.4	25.0
E	33.3	22.2
F	66.7	41.7
G	77.8	52.8
H	11.1	11.1
I	11.1	8.3
Control	**0.0**	**0.0**
L.S.D. at 0.05	21.063	9.173

Effect of garlic and black pepper extracts on the incidence of tomato Fusarium wilt disease:

The data in **Table (2)** reveal that, the garlic extracts reduced the wilt disease by 52.9-100.0% while, the black pepper extracts reduced it by 88.2-100.0% depending upon the application method comparing with the untreated check. In fact, efficiency of the garlic and black pepper extracts in controlling tomato wilt and other plant diseases was reported by several authors (**Attitalla, *et al.* 1998, Aba AlKhail, 2005, Abo-Elnaga and Ahmed, 2006, Lylian, *et al.* 2006, Montes-Belmont and Prados-Ligero, 2006, Deepak, *et al.* 2007**). It was reported that spraying with the aqueous garlic extracts have antibiotic and antifungal properties and suppresses a number of plant diseases, including powdery mildew on cucumbers and, to some extent, black spot on roses (**Quarles, 2000**).

Table (2): Effect of treating seedling with garlic (G) and black pepper (BP) extracts using different application methods on the severity of the tomato Fusarium wilt, 2 months post inoculation with FOL isolate A.

Application methods *	Disease severity %			Reduction % comparing to control**		
	G	BP	Control	G	BP	Control
IR	0.0	1.4	23.6	100.0	94.1	0.0
SS	0.0	0.0	23.6	100.0	100.0	0.0
IR+SS	11.1	2.8	23.6	52.9	88.2	0.0
L.S.D. at 5%	3.93					

* I = Immersing roots, S = Spraying shoots

** Reduction %= (Control - Treatment)/Control x 100

Activity may be due to sulfur-containing compounds such as ajoene or allicin. Garlic releases fungicidal chemicals into the soil. Rotation of garlic with tomatoes, for instance, can reduce the likelihood of soil borne tomato diseases. It was reported that the activity of 7 watery extracts including *Allium sativum* and *Piper nigrum* were commensurable to that of marketed botanical fungicides (**Deepak, *et al.* 2007**).

Activity of the oxidative enzymes:

The recorded data in **Table (3)** indicate that, most tested treatments of garlic and black pepper extracts induced appreciable increase in activities of the polyphenoloxidase (PPO) and peroxidase (POD) enzymes. The increase in activity of the POD enzyme (53.3-640.7%) was more pronounced than the PPO enzyme (4.4-29.8%) comparing to the untreated control. Among tested treatments, immersing roots (IR) in garlic extract and spraying shoots (SS) with black pepper extract induced the highest increase in activity of the PPO enzyme whereas SS with garlic extract and IR+SS with black pepper extract induced the highest increase in activity of the POD enzymes. There are some evidences indicating that the activation of POD and PPO plays a crucial role in the biological control and resistance of plant to pathogenic attack (**Thipyapong, *et al.* 2004, Chérif, *et al.* 2007, She-ze, *et al.* 2008**). In fact, the oxidative enzymes PPO and POD are important in the defense mechanism against pathogens, through their role in the oxidation of phenolic compounds to quinines, causing increasing in antimicrobial activity. Therefore, they may be directly involved in stopping pathogen development **(Quiroga, *et al.* 2000, Shimzu, *et al.* 2006, Melo, *et al.* 2006).**

Table (3): Activity of PPO and POD enzymes in treated tomato leaves (cv. Carolina Gold) with 4% garlic and black pepper extracts, two months post inoculation with FOL isolate A.

Application methods*	Activity (O.D./min/g fresh weight)					
	PPO			POD		
	G	BP	Control	G	BP	Control
IR	47.0	40.8	36.2	21.7	20.7	13.5
SS	40.7	44.7	36.2	100.0	57.9	13.5
IR+SS	26.3	37.8	36.2	40.0	93.9	13.5
Efficiency %**						
IR	29.8	12.7	0.0	60.7	53.3	0.0
SS	12.4	23.5	0.0	640.7	328.9	0.0
IR+SS	-27.3	4.4	0.0	196.3	595.6	0.0

* IR = immersing roots, SS = spraying shoots

** Efficiency %= (Treatment – Control)/Control x 100

Anatomical structure of leaf petiole:

The data in **Table (4)** prove that most investigated anatomical characters of tomato leaf petioles were improved positively in the treated than the untreated tomato plants. Among all investigated characters, the wide of the vascular bundle only was improved positively by all tested treatments (968.4-1656.0 µ) comparing to the untreated control (893.7 µ). Thus, applying the garlic or black pepper extracts for treating tomato seedlings before transplanting induced positive changes in their water conductive elements, reasonably they resist the wilt disease development by facilitating absorbing more water as the plants are need. In fact, the functional water-conducting system, the treachery elements of the xylem, is required to sustain plant growth and development **(Ismail 2004)**. The enlarged number of xylem vessels and width of the vascular bundles caused by the garlic and black pepper extracts might be considered as a probable induced defense mechanism against the tomato Fusarium wilt. It is interest to state that neither conidia nor mycelia of the tomato Fusarium wilt pathogen were detected in leaf petioles of treated and untreated tomato plants.

Table (4): Effect of garlic and black pepper extracts used for immersing (I) roots, spraying (S) shoots and I+S of tomato seedlings on the anatomical structure of leaf petiole after two months treatment and inoculation with the tomato Fusarium wilt pathogen

Tested extracts / Anatomical characters	Application methods						Control
	Immersing roots (IR)		Spraying shoots (SS)		IR+SS		
	G	BP	G	BP	G	BP	
Epidermal layer thick. (µm)	*35.1	*35.1	*36.9	22.7	*37.8	*36.9	28.8
Number of parenchyma layers	*6.0	*5.0	4.0	*5.5	4.0	*5.5	4.0
Parenchyma layers thick. (µm)	*367.2	*392.4	*331.2	286.2	*336.6	*331.2	304.2
Cortex thick. (µm)	*548.1	*603.5	511.2	477.9	*544.5	*533.7	520.7
Outer phloem thick. in V.B.(1) (µm)	71.1	*99.9	*111.6	*81.0	61.7	52.2	74.7
Cambium thick. In V.B. (µm)	*72.0	*59.4	39.6	56.7	45.9	*60.3	59.0
Xylem thick. in V.B. (µm)	435.6	*501.3	307.8	*482.4	395.6	429.3	446.4
Number of xylem vessels in V.B.	*80.0	*65.0	*46.0	*40.0	*36.0	*39.0	32.0
Vessels thick. in V.B. (µm)	*75.4	66.6	65.7	54.9	*78.3	*93.6	68.0
Inner phloem thick. in V.B. (µm)	*101.7	*117.0	52.2	*72.0	*86.0	*79.7	64.8
Length of Vascular Bundle (µm)	*680.4	*777.6	511.2	*692.1	589.1	621.5	644.9
Widest of V.B. (µm)	*1496.7	*1188.9	*968.4	*1656.0	*1244.3	*1620.0	893.7

(1) V.B. = Vascular bundle
* Positive changed character comparing to the control

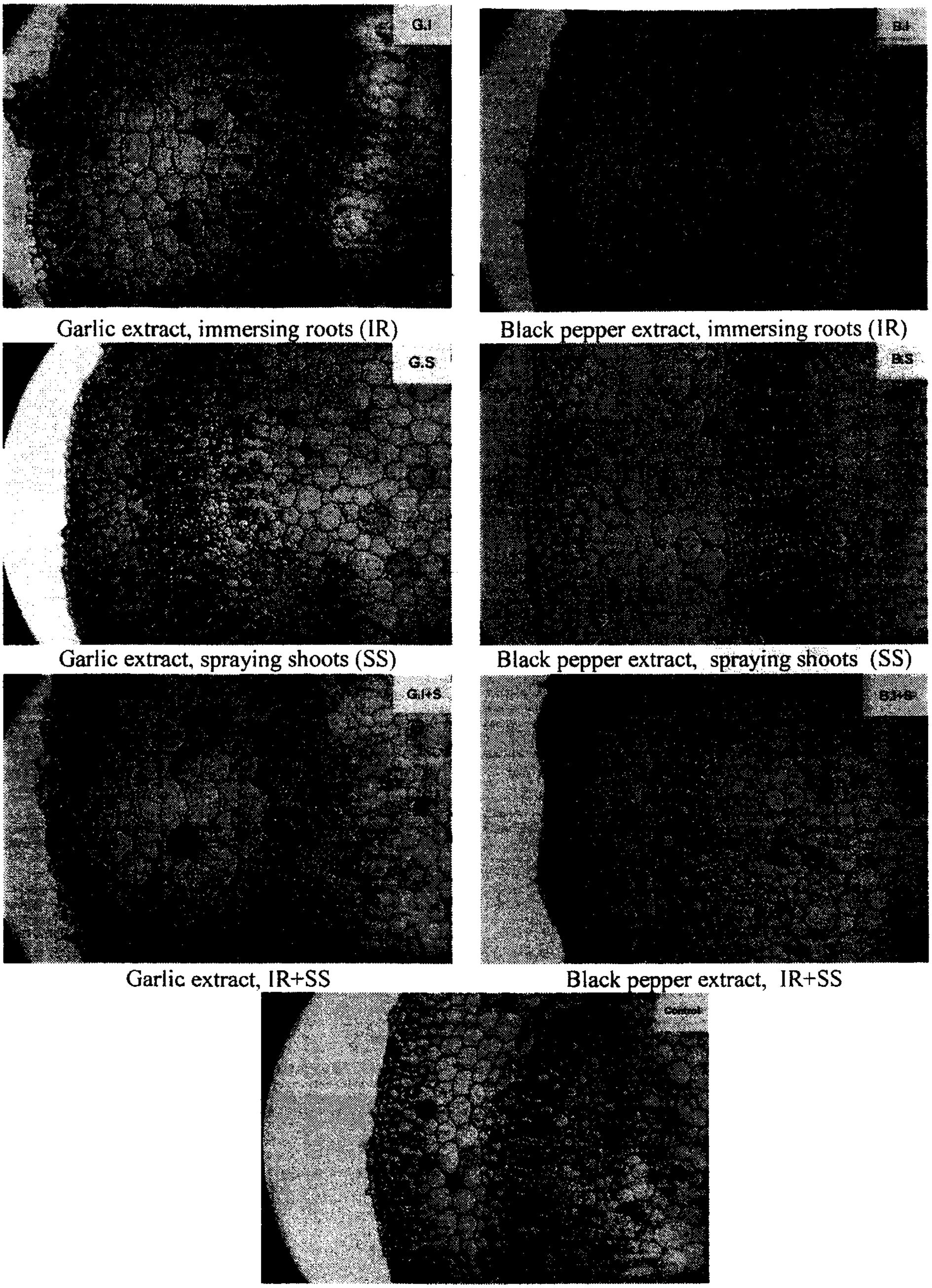

Fig., (1): Anatomical structure of tomato leaf petiole after two months from immersing (IR) roots, spraying (SS) shoots, and IR+SS of tomato transplants with extracts of garlic (left) and black pepper (right) and inoculation with FOL. Control (untreated).

Such findings agree with **Pennypacker and Nelson (1972)** who stated that, no conidia were observed in advance of the mycelium in xylem vessel elements of carnation infected with *Fusarium oxysporum* f.sp. *dianthi*. They added that, the absence of conidia in advance of mycelium in the xylem vessel elements is probably the primary reason for the success of culture indexing as a control measure for Fusarium wilt of carnation. In fact, xylem plays an important role in strengthening plant bodies as well as in transporting water and minerals. It is a complex tissue composed of vessels, tracheids, fibers and parenchyma. In arabidopsis, secondary xylem does not develop in immature fluorescence stems shorter than 10 cm, although primary xylem does exist in them **(Ko, *et al.* 200).**

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مستخلصات الثوم والفلفل الأسود كمحثات مقاومة ضد مرض ذبول الطماطم الفيوزاريومي في كازاخستان

إبراهيم عبد المنعم الفقى،أبايا اورازولى ساجيتوف (*) ، جهاد محمد الهباء

قسم النبات الزراعي – كلية الزراعة – جامعة بنها – مصر

* أكاديمية كازاخستان الوطنية – معهد بحوث وقاية النبات والحجر الصحي – كازاخستان

في هذه الدراسة تم اكتشاف تواجد مرض ذبول الفيوزاريوم المتسبب عن الفطر فيوزاريوم اوكسيسبورم ليكوبرسيسى ولأول مرة على نباتات الطماطم النامية تحت ظروف البيوت الزجاجية في كازاخستان – وقد أدى غمر جذور شتلات الطماطم (صنف كارولينا جولد) فى مستخلص الثوم (تركيز ٤ ٪) أو رش مجموعها الخضرى بمستخلص الثوم أو مستخلص الفلفل الأسود (تركيز ٤ ٪) ثم حقنها بمسبب ذبول الفيوزاريوم إلى منع ظهور أعراض الذبول تماما على نباتات الطماطم حتى بعد شهرين من المعاملة كما أظهرت معظم المعاملات المختبرة زيادة ملحوظة في أنشطة الإنزيمات المؤكسدة (بولي فينول اوكسيديز ، بيروكسيديز) في أوراق النباتات المعاملة مقارنة بغير المعاملة – كما أظهرت الدراسات التشريحية لأعناق أوراق نباتات الطماطم المعاملة (في مرحلة الشتلات) بمستخلصات الثوم أو الفلفل الأسود (تركيز ٤ ٪) تغيرات ايجابية في العناصر الموصلة للمياه وخاصة أوعية الخشب واتساع الحزم الوعائية مقارنة بالنباتات غير المعاملة – تلك التغييرات الإيجابية التى تحدثها المعاملات المختبرة قد تدخل ضمن آليات المقاومة المستحثة والتي تؤدي بدورها إلى مقاومة أو تأخير تطور مرض ذبول الفيوزاريوم في نباتات الطماطم.