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BIOLOGICAL CONTROL OF TWO BACTERIAL DISEASES ATTACKING TOMATO PLANTS BY TWO DIFFERENT ANTAGONISTIC MICRO-ORGANISMS

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ABSTRACT

A considerable bacterial and fungal populations are lived on the aerial surfaces of tomato plants even at the age of 8 weeks.Two epiphytic bacterial strains and two epiphytic yeast ones proved to be highly antagonistic against the tomato pathogens (*Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *Vesicatoria)in vitro* studies. One of the most antagonistic bacterial strains belongs to the genus *Bacillu* sand the other one belongs to the genus *Pseudomonas*.The two most antagonistic yeast strains taxonomically belong to the genera *Aureobasidium* and *Cryptococcus*.

On the base of their in vitro antagonism, the most effective antagonistic bacterium, i.e. *Pseudomonas* sp. and the most effective yeast strain, i.e. *Cryptococcus* sp. were tested in pot experiments. Both the tested bacterium and the yeast strain showed a significant antagonistic effect against *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria*. The preventive effect of the two selected microbes on the bacterial speck and bacterial spot diseases was exhibited in a preliminary pot experiment too.

Keywords: Tomato, Biological control, *Rhizobacteria*, *Pseudomonas*

INTRODUCTION

Tomato is an outstanding vegetable crop allover the world. It is found both in land farms and in gardens for consuming as well as for processing. Temperate zone shares are especially favourable for tomato. A satisfactory crop of top-grade tomatoes can be obtained only from well-nourished plants that are free of disease. Thus, a careful attention should therefore be given to the disease control and culturing methods.

The plant resistance to the disease did not show fixed status. Bacterial *speck* and *spot* diseases are the two most serious diseases of tomato world-wide.

In sustainable agriculture and horticulture, the use of pesticides is to be limited because of their hazardous contaminations to the man and environment. Therefore, the biological control should be put into practice even in the near future, especially in the greenhouse cultures.

Additionally, some diseases, e.g. the bacterial speck and bacterial spot hardly can be controlled by any 'traditional' method.

Bacterial speck disease is caused by *Pseudomonas syringae* pv. *tomato*. In the recent years, this disease has been much more prevalent than bacterial spot, often in crops. (Agrios, 1988).The disease is most noticeable on the fruit, where it causes numerous dark-brown spots, 1-2 mm in

diameter. Sometimes the lesions are larger and more irregular, closely resembling those kind of bacterial spot. On the leaves, bacterial speck causes dark brown to black spots that are usually slightly larger than those on the fruit. Infection occurs most abundantly after heavy rains that splash the bacteria to all parts of the plant.

Bacterial spot disease of tomato is caused by *Xanthomonas camptstris.* pv. *vesicatoria.* It is widely spread and causing considerable injury to the leaves and stems, especially to the seedlings; however, the disease is most noticeable by its effect on the fruit. On the leaves, the symptoms appear as small (about 3 mm), irregular, purplish grey spots with a black center and a narrow yellow halo. The infection of flower and green fruit causes a serious blossom drop. No effective pesticides are known to the control and the disease is impossible to control once it appears in the field. (Jones *et al.*, 1995).

Recently, the successful resistance can be achieved by application of genetically transformed non-pathogenic strains of the same bacterial pathogens (Martin, 1994 and Salmeron *et al.*, 1994). Breeding of resistant tomato plants is often based on the natural resistance of some tomato varieties and their genetic study (Stockinger and Walling, 1994).

Taking all these above mentioned facts into consideration, the aim of the present research was to investigate the bacterial and yeast populations on tomato leaf surface, isolation of a larg amount of bacterial and yeast colonies. Also *in vitro* testing of the representative isolates for their antagonistic activity against the pathogens *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria*; was carried out. In addition to, *in vivo* experiments of the most effective bacterial and yeast isolates were involved.

MATERIALS AND METHODS

Tomato plants:

To investigate the epiphytic bacterial and fungal populations of two cultivars of tomato plants, i.e. Korall and Robot (*Lycopersicum esculentum* L.) the tested plants were grown in the pots under the greenhouse conditions and used at the age of 8 weeks.

Causal organisms of tomato diseases:

Isolates of *Pseudomonas syringae* pv. *tomato* (the causal organism of bacterial speck) and *Xanthomonas campestris* pv. *vesicatoria* (the causal organism of bacterial spot) were given by the Hungarian National Collection of Industrial and Agricultural Microorganisms.

Isolation of epiphytic microorganisms

The diseased samples of tomato plants were placed in a physiological solution enriched with a 0.1% yeast extract and 0.01% Tween 80 and shaken on an orbital incubar for 10 minutes. The washings were diluted to 10^1-10^3 ranges for mycological studies and 10^4-10^7 ranges for bacteriological culturing. 0.1 ml of each above-mentioned dilutions was plated on Petri plates with nutrient agar (Difco) and Martin's agar containing

rose bengal and streptomycin. After 2 days of incubation at 25°C, the bacterial colonies were counted and picked up for subcultures. After 5 days of incubation the fungal colonies were counted and yeast colonies were isolated for subculturing.

Taxonomical characterisation of isolates:

The characterisation of the bacterial isolates was carried out according to the selected methods of Cowan and Steel (1974), while of the yeast isolates was performed according to the method of Barnett *et al.* (1990).

Chemicals and diagnostics:

The referring methods are listed in the followings:

Cell Morphology-Colony Morphology and Pigmentation-Spore Staining-Oxidation and Fermentation of Glucose-Oxidase Test-Catalase Test-Casein Hydrolysis-Hydrolysis of Gelatin-Hydrolysis of Starch-Nitrate-reduction-Assessing Growth in the Presence of Cyclohemicide-Detecting Production of Extracellular Starch-like Compounds - Urea Hydrolysis Test - In vitro Testing of Antagonism (Difco Manual, 1984; Hugh and Leifson, 1953; Cowan and Steel, 1974; Oxdid Manual, 1982)

Investigation of the antagonistic strains in vivo experiments

Inoculation Technique

Healthy tomato plants, cv. 'Korall' (aged 8 weeks) and growing vigorously were selected for the pathogenicity tests. To prepare the inoculum, cells from YDC slant cultures were grown in a nutrient broth, and adjusted to give 106-107 CFU/ml, according to the Dye's method (1962). For getting definite leaf spots, the upper parts of stems or young petioles of young leaves were punctured with a sharp needle in a several locations. Using a single liquid sprayer that provided a fine moist, the whole plant was lightly sprayed (preferable on a turntable for even coverage) with the inoculum of bacterial suspension prepared as above. The control plants were similarly wounded and sprayed using a sterile sprayer containing diluent only. The plants were still moist they were placed in a moist chamber (The temperature was controlled at about 21-23°C). The plant surfaces remained wet continuously without much runoff. Light was supplied during daylight hours either naturally or by artificial lights without effecting the temperature. The wet plants were covered with a plastic bag. After 48 hours, the plants were transferred to the greenhouse at 25-27°C for developing of symptoms. Symptoms were recorded whether the infection occurred at random parts or only at wounded sites.

Evaluation of plant infections

The infected young tomato plants as experimental models of both bacterial speck and bacterial spot can be quantitatively evaluated. The symptoms on the leaves were considered to be positive when at least one simple spot was appeared through the incubation period.

Statistical evaluations

Statistical analyses were performed with One-Way Analysis of Variance and Correlation Analysis. Calculations were carried out with Statgraph 5.0 software.

RESULTS

Epiphytic bacterial and fungal populations:

Remarkable bacterial and fungal populations were isolated from the phylloplane of tomato plants (Fig.1). The total numbers of bacterial cells were $6.9x10^6$ and $9.7x10^6$ colony forming units (CFU) on the aerial surface of cv. *Robot* and cv. *Korall*, respectively. The total numbers of yeast cells were $4.6x10^4$ and $6.5x10^4$ on the two cultivars, respectively.

Taxonomic characteristics of bacterial isolates

Two bacterial isolates, namely BTo8 and BTo11 were selected to a preliminary taxonomic characterisation. Data obtained are presented in Table (1). It was found that, the cells of the isolate BTo8 were rod-shaped, Gram positive, with a diameter of 2 µm, they were motile, forming endospores. The strain grew strictly aerobically, with an oxidative breakdown of carbohydrates, a positive cytochrome C oxidase and catalase test. It can hydrolyse casein, gelatin and starch did not reduce nitrate. It can grow on citrate agar, did not require any growth factor, and did not produce pigment on King' B agar.

Concerning with the other isolate, i.e. BT011, it was noticed that their cells were also rod-shaped, Gram negative, with a diameter of one µm; they were motile, without endospores. The strain grew strictly aerobically, with an oxidative breakdown of carbohydrates, a positive cytochrome c oxidase and catalase test. It hydrolysed casein, but not gelatin and starch; also did not reduct nitrate. It grew on citrate agar, does not require any growth factor. The strain produced an intensive fluorescence on King' B agar (Table 1).

Table (1): Taxonomic characteristics of bacterial isolates that isolated from tomato phylloplane

Characteristics	Code number of bacterial isolates	
	BTo8	BTo11
Cell morphology	rod-shaped	rod-shaped
Diameter	2 µm	1 µm
Motility	+	+
Endospores	+	-
Gram stain	positive	negative
Growth	strictly aerobic	strictly aerobic
Breakdown of carbohydrates	oxidative	oxidative
Cytochrome c oxidase	+	+
Catalase	+	+
Casein hydrolysis	+	+
Gelatin hydrolysis	+	-
Starch hydrolysis	+	-
Denitrification	-	-
Growth factors	not required	not required
Growth on citrate agar	+	+
Other characteristics		
King' B agar	no pigment	fluorescent pigment

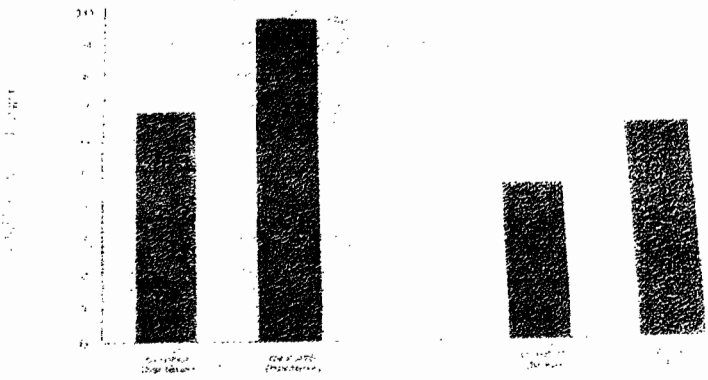

Fig (1): bacterial and fungal populations on the leaf surface of tomato plants

Taxonomic characteristics of yeast isolates

Two yeast isolates, YTo2 and YTo4 were selected for a preliminary taxonomic characterisation. Data in Table (2) reveal that, the colonies of the isolate YTo2 were black on Glucose-Pepton Agar medium.

Table (2): Taxonomic characteristics of antagonistic yeasts isolated from tomato phylloplane

Characteristics	Code number of yeast isolates	
	YTo2	YTo4
Cultural characteristics		
Colonies colour	black	pink
Vegetative reproduction	filaments, budding	budding only
Sexual reproduction	not	not
Physiological characteristics		
Fermentation	none	none
Growth		
D-Glucose	+	+
D-Galactose	+	+
L-Sorbose	+	-
Sucrose	+	+
Maltose	+	+
Arbutin	-	+
Lactose	-	-
Raffinose	+	+
Inulin	+	-
Glycerol	+	-
D-Mannitol	-	+
myo-Inositol	-	+
D-Gluconate	+	+
Succinate	+	+
Citrate	+	+
Ethanol	-	+
Nitrate	+	-
w/o vitamins	-	-
0.1% Cycloheximide	-	-
Starch formation	-	+
Urea hydrolysis	+	+

The colonies consisted of septate hyphae with an abundant formation of lateral budding cells. The strain has not got a sexual reproduction. It did not ferment any carbon source; utilized D-glucose, D-galactose, L-sorbose, sucrose, maltose, raffinose, inulin, glycerol, D-gluconate, succinate, citrate as sole carbon source, nitrate as sole nitrogen source, and did not utilize arbutin, lactose, D-mannitol, myo-inositol, ethanol. The strain requires different vitamins, hydrolyses urea, did not form starch from glucose, and did not resist cycloheximide.

Dealing with the other isolate "YT04" their colonies were mucous, pink coloured on Glucose-Pepton agar. The colonies consisted of only ovoid budding cells. The strain has not got any sexual reproduction. It did not ferment any carbon source; utilized D-glucose, D-galactose, sucrose, maltose, arbutin, raffinose, D-mannitol, myo-inositol, D-gluconate, succinate, citrate and ethanol as sole carbon source; did not utilize L-sorbose, lactose, inulin, glycerol as sole carbon source, nitrate as sole nitrogen source. The strain requires different vitamins, hydrolysed urea, formed starch from glucose, and sensitive cyclohemicide (Table 2).

In vitro antagonistic effect of the epiphytic bacteria and yeasts on the phytopathogenic bacteria Pseudomonas syringae pv. tomato and Xanthomonas campestris pv. vesicatoria

Ten epiphytic bacterial isolates were selected to evaluate their *in vitro* antagonistic effect. Six of them showed an antagonistic effect against the pathogen *Pseudomonas syringae* pv. *tomato*, the effect manifested 0.33-7.66 mm of growth inhibition zone, while, two of them exhibited a real antagonistic (3.66, 7.66 mm). on the other hand, twelve bacterial isolates showed an antagonistic effect against the pathogen *Xanthomonas campestris* pv. *vesicatoria*, the effect manifested 0.33-8.00 mm of growth inhibition zone. The same aforementioned two tested isolates showed a real antagonistic (3.66, 8.00 mm of growth inhibition zone).

Five epiphytic yeasts isolates were selected for testing their in vitro antagonistic effect. All of them showed antagonism against both tested bacteria, but only two of them proved to be highly antagonistic (4.33, 9.33 mm of inhibition zone) (Table 3 and Fig 2)

Effect of epiphytic bacteria and yeast on the tomato disease symptoms

On the base of bacteria and yeast antagonism degree *in vitro*, one isolate of bacteria (BT011) and other one of yeast (YT04) were tested for their antagonism on tomato plants.

In the tomato plants which were previously treated with the suspension of living epiphytic bacterium cells, only, 1.7 leaves were diseased after the infection with the pathogen *Pseudomonas syringae* pv. *Tomato* (Figs.3&5) and 2.3 leaves after the infection with the pathogen *Xanthomonas campestris* pv. *vesicatoria*. (Figs. 4&6)

In the tomato plants which were previously treated with the suspension of living epiphytic yeast cells, only 2.3 leaves were diseased after the infection with the pathogen *Xanthomonas campestris* pv. *vesicatoria*(Figs.4&8). and 2.0 leaves after the infection with the pathogen *Pseudomonas syringae* pv. *tomato* (Figs. 3&7)

Table (3): *In vitro* antagonistic activity of selected bacteria and yeasts against the growth of two pathogenic bacteria of tomato plant using nutrient agar medium (growth inhibition zones in mm)

Epiphytic isolates	P. l.	s. ll.	pv.t. lll.	X`	X.c. l.	pv.ll.	v. lll.	X`
Bacteria								
BTo1	1	0	2	1.00	1	0	0	0.33
Bto2	0	1	1	0.66	0	0	1	0.33
BTo3	0	1	1	0.66	0	1	0	0.33
BTo4	0	0	0	0.00	0	0	0	0.00
BTo5	0	0	0	0.00	0	1	1	0.66
BTo6	1	0	0	0.33	0	1	0	0.33
BTo7	0	1	1	0.66	1	0	0	0.33
BTo8	4	3	4	3.66	2	4	5	3.66
BTo9	0	1	0	0.33	0	2	0	0.66
BTo10	0	0	0	0.00	0	0	1	0.33
BTo11	7	7	9	7.66	8	7	9	8.00
BTo12	1	2	0	1.00	0	1	1	0.66
BTo13	0	0	0	0.00	0	0	0	0.00
BTo14	1	1	2	1.33	2	1	0	1.00
Yeast								
YTo1	1	2	1	1.33	1	1	2	1.33
YTo2	5	4	4	4.33	4	6	5	5.00
YTo3	1	2	1	1.33	1	2	0	1.00
YTo4	10	9	9	9.33	8	10	9	9.00
YTo5	1	2	1	1.33	1	1	1	1.00
LSD 5%				1.49				1.83

P.s. pv.t. = Pseudomonas syringae pv. Tomato X.c. pv. v. = Xanthomonas campestris p.v. vesicatoria

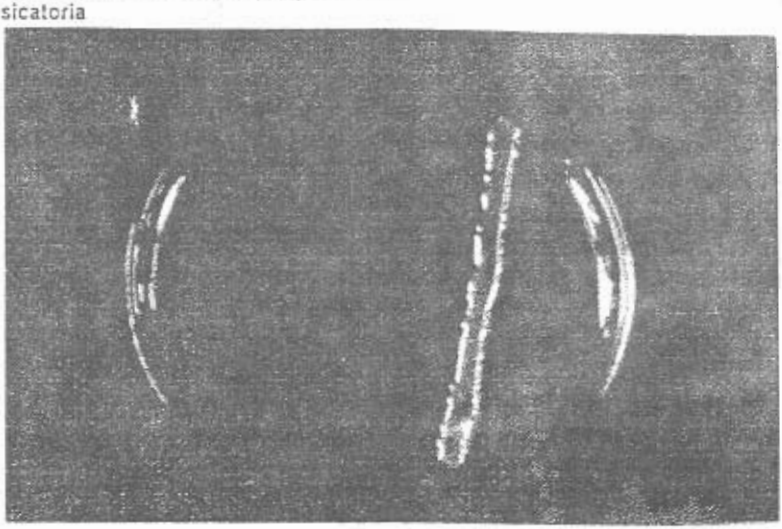

Fig (2): *In vitro* antagonistic effect of the yeast strain YT03 against two tomato pathogen (glucose-peptone agar medium) after 3 days of incubation

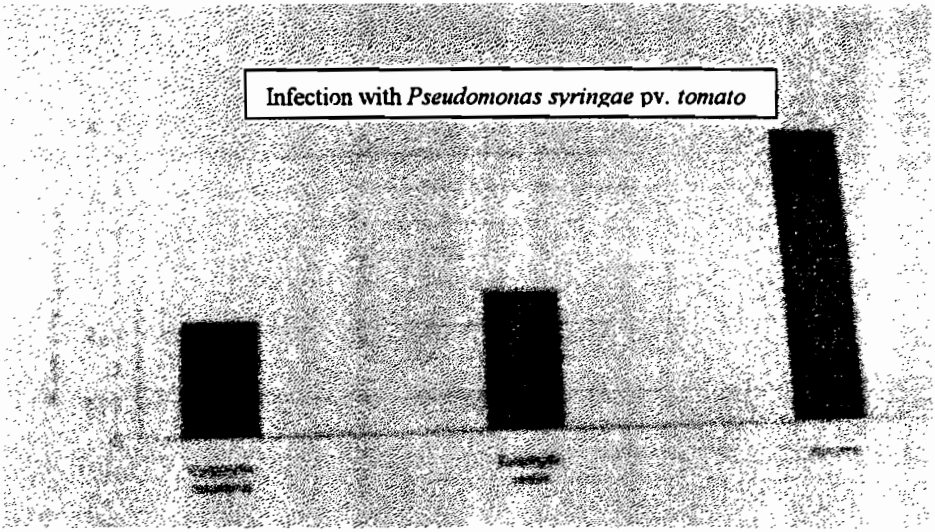

Fig (3): The preventing effect of the epiphytic bacterium (BT011) and epiphytic yeast (YT04) on the tomato plants modelling bacterial speck disease.

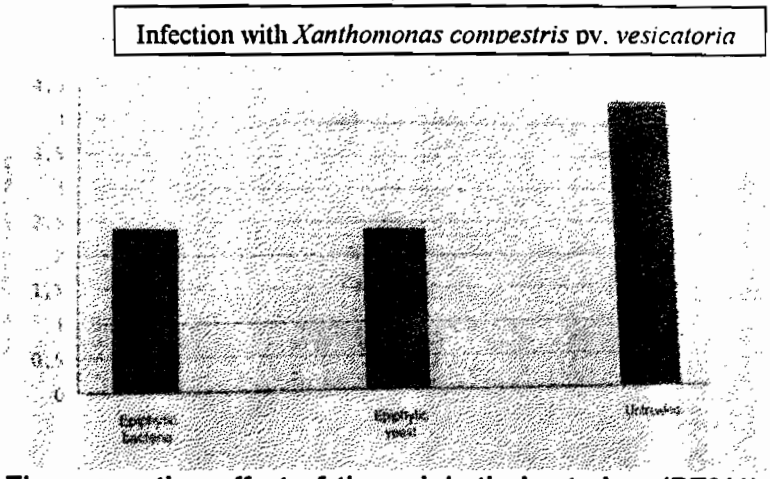

Fig (4): The preventing effect of the epiphytic bacterium (BT011) and epiphytic yeast (YT04) on the tomato plants modelling bacterial spot disease.

Fig (5): Tomato plants treated with the epiphytic bacterium strain (BT011) and infected with the pathogen *Pseudomonas syringae* pv. *tomato*

Fig (6): Tomato plants treated with the epiphytic bacterium strain (BT011) and infected with the pathogen *Xanthomonas campestris* pv. *vesicatoria*

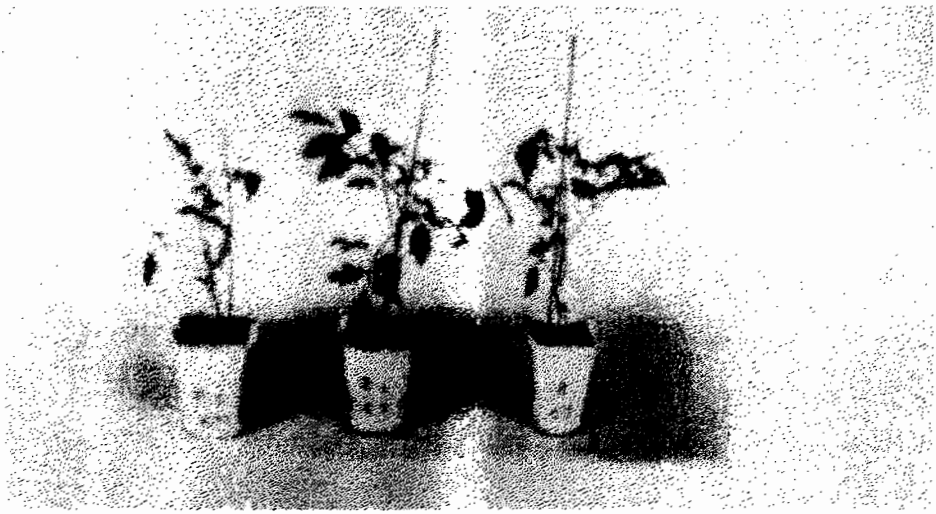

Fig (7): Tomato plants treated with the epiphytic yeast strain (YT04) and infected with the pathogen *Pseudomonas syringae* pv. *tomato*

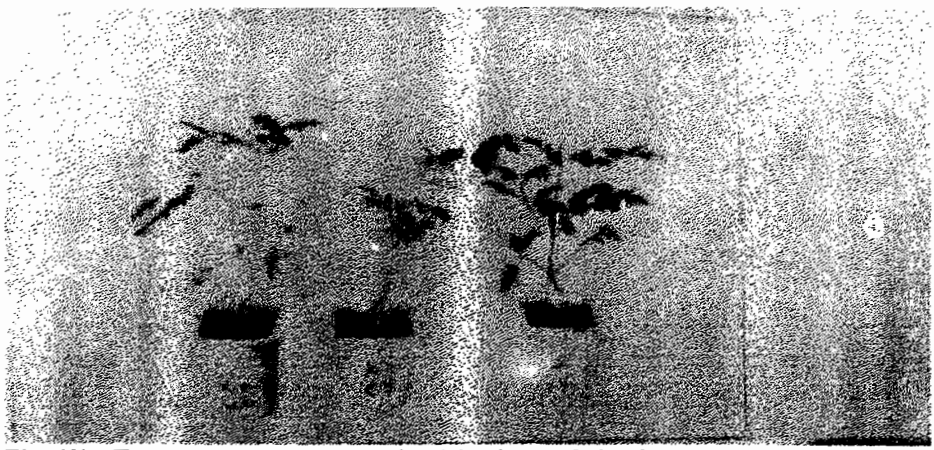

Fig (8): Tomato plants treated with the epiphytic yeast strain (BT04) and infected with the pathogen *Xanthomonas campestris* pv. *vesicatoria*

DISCUSSION

There were significant populations of bacteria and fungi are lived on the surface of tomato plants aged 8 weeks. There was a correlation between the tomato cultivars and bacterial populations (R=0.9848), while between the fungal populations of cv. Robot and cv. Korall there was no significant difference.

The pioneer character is supposed on the base of the dimensions of both bacterial and fungal populations.

In numerous taxonomic characteristics (motility, catalse reaction, casein hydrolysis, etc.) it could be estimated the same, while the bacterial isolates were differed in some other characteristics (Gram staining, cell morphology, endospore-forming, gelatine hydrolysis, etc.).

On the base of rod-shaped, Gram positive cells, strictly aerobic growth, forming endospores, hydrolysis of casein, gelatin and starch the strain BTo8 is considered to belong to the genus *Bacillus*.

On the base of rod-shaped, Gram negative cells, strictly aerobic growth, not forming endospores, hydrolysis of casein, but gelatin and starch not and forming characteristic fluorescence pigment on King' B agar, the strain BTo11 is considered to belong the genus *Pseudomonas*; moreover, to the group section I, with the species *Pseudomonas* fluorescence.

On the base of the black colonies, filamentous and lateral budding vegetative reproduction, the lack of sexual reproduction, the lack of the ability of anaerob fermentation, the yeast strain (YTo2) is considered to belong to the genus *Aureobasidium*. It could utilized a broad spectrum of carbon sources (D-glucose, D-galactose, L-sorbose, sucrose, maltose, raffinose, inulin, glycerol, D-gluconate, etc.); the nitrogen source is nitrate, and it can hydrolyse urea. The most common species of the genus *Aureobasidium pullulans* are in highly frequent number of the phylloplane microflora all over the world. (Davenport, 1976).

On the base of the pink-coloured, mucous colonies, ovoid budding cells as only vegetative organs, the lack of sexual reproduction, the lack of any fermentation, and the presence of an amyloid capsule, the yeast strain (YTo4) is considered to belong to the genus *Cryptococcus*. It could utilized a long series of carbon sources (D-glucose, D-galactose, sucrose, maltose, arbutin, raffinose, D-mannitol, inositol, D-gluconate, succinate, etc.), cannot utilize nitrate as nitrogen source, and requires some vitamins. The strain must be positioned near the species *Cryptococcus hungaricus*. With the pink coloured cell wall and the thick capsule these cells can resist to physical and chemical effects on the surface of tomato plants.

Two strains of 14 epiphytic bacteria and two other of 5 epiphytic yeasts proved to be highly antagonistic. A large number of the tested strains had some smaller activity with the *in vitro* method of inhibition zone. The physiological and molecular mechanism of the antagonistic activity must be really different, but the highly antagonistic ones could be promising in biological control. Although microbal species has an antagonistic (amensalistic, antibiotic, etc.) activity in majority, the selection of the appropriate strains needs a comprehensive research work.

A significant preventive effect was found after previousley treatment with the selected epiphytic microbes, there was no significant difference between the effect of the bacterial and yeast strains. Also, there were no differences between the target pathogens *Pseudomonas* and *Xanthomonas*.

Although the young tomato plants proved good models of bacterial speck and bacterial spot, the comprehensive experiments of biological control even in the greenhouse should involve the treatment of other plant organs too.

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المقاومة الحيوية لأثنين من الأمراض البكتيرية التي تصيب نباتات الطماطم بواسطة كائنين من الكائنات المضادة.

سعد الدين فتحى بن عامر و دوبى اتشابا

قسم البيوتكنولوجا والميكروبيولوجيا الزراعية جامعة سيزنت إستافان - جودولو

توجد أعداد كبيره من الكائنات البكتيرية و الفطرية على سطح نباتات الطماطم حتى عند عمر ثماني أسابيع. و قد أثبتت الدراسات أنه هناك عزلتين من مجموعة البكتيريا و كذلك عزلتين من مجموعه الخمائر كان لهما درجه عاليه من التضاد للمسببات المرضية المحدثة لأمراض الطماطم تحت الدراسة (بسيدوموناس سيرنجى المسبب لمرض الـ Speck واكسانزوموناس كمبيتيرس المسبب لمرض التبقع). و من الجانب الأخر فقد تبين أن العزلتين من البكتيريا أحداهما يتبع جنس باسيليس و العزلة الأخرى تتبع جنس بسيدوموناس في حين أن عزلتي الخميرة يتبعا جنس أروباسيديم وكريبتوكوكس.

ومن منطلق نشاط تلك الكائنات في المعمل فقد أختبر تأثير أعلى هذه العزلات قدره على التضاد في الصوبة على نباتات الطماطم و تبين أن لهذه الكائنات قدرة عاليه على تثبيط المسببات المرضية المحدثة لأمراض الطماطم تحت ظروف الصوبة.