SOME EPIDEMIOLOGICAL ASPECTS OF *RALSTONIA SOLANACEARUM*

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Abstract

Pathogenic potential of *Ralstonia solanacearum* isolated from different sources was determined. Mixed inoculum of potato tuber isolates caused the highest wilt severity, on inoculation in tomato seedlings, compared to those from the above ground potato stems. Isolates recovered from *Rumex dentatus* and *Solanum nigrum* induced severe wilt similar to those recovered from potato tubers. Isolates from soil, irrigation water, and potato foliage showed limited pathogenic potential. of Plating isolates on SMSA medium revealed phenotypic change from virulent (vi) to the known avirulent (av) form. The latter form, however, showed pathogenic potential on tomato seedlings, thus referred to as atypical (at) form. Isolates stored in sterilized tap water amended with easily assimilated organic material survived for only 110 days, indicating a detrimental effect of the treatment.

The pathogen persisted for 6 months in either sandy or clay soil maintained at 75% water holding capacity (WHC) and temperature ranging from 28.8°C to 15.2°C (July to December, respectively). Under the same temperature conditions and in dry soil, the pathogen survived in sandy soil for 6 months as well, with very high densities in December. On the other hand, densities in dry clay soil were extremely low after 5 months *i. e.,* in November, with higher densities of the pathogen in sandy soil compared to those recorded in clay soil. The biofertilization with a biosystem microorganisms product (EM) showed seasonal fluctuation in densities of *R. solanacearum*. In January and February, however, *R. solanacearum* could not be detected in the assayed samples.

Key words : *Ralstonia solanacearum*, atypical (at), virulent (vi), avirulent (av), survival in water, survival in soil, preferential host organ effect, phenotypic change in colony morphology , effect of organic material on persistence.

INTRODUCTION

Potato brown rot (*Ralstonia solanacearum*) was reported in Egypt by Briton-Jones in 1925. The disease is being found wherever potatoes are grown either in Egypt or in Europe. In addition to the solanaceous hosts, certain plant species of other families were reported as hosts to race 1 of the pathogen. This race has not been recorded in Egypt, thus no disease problems on other solanaceous crops could be observed in the country.

Drought and high temperature which prevailed in Europe twelve years ago has effected greater incidence of the disease across the continent. Such a pandemic spread in Europe, following temporary changes in weather conditions, highlighted the indigenous presence and pointed to the possible introduction from Europe into Egypt for the following : (1) the mass importation of tuber seeds from Europe, over past decades, for summer crop plantation, (2) potato race 3 (biovars II) being the only dominant strain both in Europe in Egypt (3) no wild types of potato could neither be found, nor been reported in the past or present, in Egypt.

It is not surprising to see several disease outbreaks in Europe, even in countries with the most developed potato production technologies. The recorded outbreaks in different parts of Europe and on different potato cultivars have pointed to the influence of the dry weather that prevailed in Europe in some years on disease development (Janse, 1996 and Farag , 2000)

In Egypt, certain bacteriological and pathological studies were carried out (Farag, 1970 and 1976) along with disease surveys (Mickail *et al.*, 1974). Subsequent studies were made (Farag *et al.*, 1999 and 2004) on persistence in soil and irrigation water.

This work was undertaken to study certain aspects related to disease epidemiology.

MATERIALS AND METHODS

1. Source, identity and pathogenicity of isolates:

Phenotypically virulent (vi) forms of *R. solanacearum* were isolated from potato tubers, potato stems, weeds, soil and irrigation water.

Plant organs and weeds collected from the traditional potato districts in Talia village, Minufiya Governorate, were thoroughly washed and surface disinfected by flaming. Isolation was made on the Semi Selective Medium of South Africa (SMSA medium) described by Englebrecht (1994) and as modified by Elphinstone *et al.* (1996).

Soil and irrigation water samples collected from Minufiya and Ismailia Governorates were also used. Soil suspension (10^{-2}) was vigorously shaken for 2 hours in a cooling system. Serial dilutions were made for decimal plate count technique on SMSA medium (Wenneker *et al.*, 1999).

Water samples (50ml x 4 replicates) centrifuged at 10.000 rpm for 15 minutes at 15°C, using Biofuge 28RS Heraeus Spatech Centrifuge. The sediment was resuspended in 1 ml phosphate buffer (0.01 M), vortexed and then plated on the modified SMSA medium. Incubation conditions, Immunofluorescence antibody stain (IFAS) and pathogenicity tests were made according to Janse (1988 & 1996) and Wenneker *et al.* (1999).

Identity of isolates was made by polymerase chain reaction (PCR) using the forward primer OLI-1 5` GGG GGT AGC TTG CTA CCT GCC-3` and the reverse primer Y- 2 5` CCC ACT GCT GCC TCC CGT AGG AGT-3` according to Seal *et al.* (1993). The fatty acids profile was kindly made at the Plant Protection Service, Wageningen. The Netherlands, while BOX-PCR and Taq-Man assay were made at the Central Science Laboratory, York, UK (Stead, 1992, Louws *et al.*, 1994 and Weller *et al.*, 2000).

The pathogenic potential of the isolates obtained from the above-mentioned sources were determined by inoculating tomato seedling (3 leaves) grown under greenhouse conditions. For this purpose, the most pathogenic isolates from a given source (3 representatives) were grown separately on glucose nutrient agar (GNA) medium for 3 days at 28 °C, and mixed together before inoculation into the leaf axis of the seedling. Ten plants were inoculated for each source and control treatments were made. The disease progress was determined according to Winstead and Kelman (1952) describing the wilt symptom in the plant as follows:

0 = no symptoms, 1 = one or two leaves wilted,

2 = three leaves wilted , 3 = four or more leaves wilted and 4 = plant died.

2. Phenotypic changes in colony morphology of isolates stored in still water:

Phenotypically virulent *R. solanacearum* isolates, selected from SMSA isolation plates, were used to study the development of avirulent forms in stored suspensions. Three isolates recovered from each of the different sources were mixed together and inoculated into sterilized tap water. Incubation was made at ambient temperature and plating was made after one year on SMSA medium. One ml from each stock suspension was used and serial dilutions were made. Three replicates were prepared for each dilution and counts/ml were determined on SMSA medium. IFAS, PCR, BOX-PCR, Taq-Man and pathogenicity were carried out after storage in water suspensions.

3. Effect of organic matter on persistence of *R. solanacearum* in still water:

Phenotypically virulent isolates were used to study the effect of organic matter on the viability of the pathogen. Composite inoculum representing each source, was inoculated in 250 ml/flasks (1 ml/flask) containing different volumes (10, 30, 40 and 45 ml/ flask) of sterile tap water. Volumes of glucose nutrient broth medium (GNB) were aseptically added to give a final volume of 50 ml . Control treatments, were also prepared. The bacterial density in each treatment was determined at zero time and periodically for up to 210 days on SMSA medium by the serial dilution method.

4. Survival in soil:

Clay and sandy soils from the traditional potato districts in Nubaria and El-Minufiya Governorates were dispensed in 25 cm plastic pots. Inoculation was made with a collection of 16 virulent *R. solanacearum* isolates propagated in King's medium broth (King *et al.*, 1954) for 3 days at 28°C. The soil moisture was kept constant at 75% by a tensiometer after inoculation with 200 ml/pot (10^7 cells/ml) of the mixed broth culture. The zero time count determination was made and repeated periodically at monthly intervals to the end of the experiment. The decimal plate count technique was used and plating was made on the semi selective medium SMSA (Elphinstone *et al.*, 1996). Another trial was made as mentioned before but under dry conditions.

The survival of *R. solanacearum* in sandy and clay soil amended with 200 ml/pot of the EM (Effective microorganisms) product was also tried under the same conditions.

RESULTS AND DISCUSSION

1. Preferential organ effect on isolate pathogenic potential:

Results in Table (1). show that a composite inoculum of tuber isolates caused 80% infection in inoculated tomato seedling. The determined wilt severity was 52.5%, compared to 15.0% resulting from the inoculum prepared from potato stem isolates. Soil and water isolates showed 17.5 and 15.0%, wilt severity, respectively. Isolates recovered from weed plants, namely *Rumex dentatus* and *Solanum nigrum* showed high infection percentage of 80% and a severity of 50% comparable to those originally isolated from potato tubers.

The differences in pathogenic potential of *R. solanacearum* isolates were reported earlier by Farag (1970 and 1976). The preferential effect on the potential of isolates recovered from either potato tubers or above ground stems is documented in the present work. Greater severity of isolates recovered from potato tubers compared to those from above ground stems is found.

The weeds *Solanum nigrum* and *Rumex dentatus* were reported among the natural hosts for race 3 biovar II (Farag *et al.*, 2004). Such results are confirmed in the present work, as the pathogenic potential of the weed isolates is comparable to those from potato tuber. Therefore, the above mentioned weeds should be taken into consideration when epidemiological aspects of the disease are studied. The presence of these weed hosts would play an important role in the persistence and carry over of the pathogen and would also affect the efficacy of crop rotation in the disease control.

Table 1. Percentage of infection and wilt severity induced by mixtures of *R. solanacearum* isolates collected from five different sources.

Isolate's code	Source of isolates	Infection %	Wilt severity %
Tu (Tu_1+Tu_2+Tu_3)	Potato tuber	80.0	52.5
St (St_1+St_2+St_3+St_4)	Potato stem	20.0	15.0
So (So_1+So_2+So_3)	Soil	30.0	17.50
Wt (Wt_1+Wt_2+Wt_3)	Water	20.0	15.0
*Wd (Wd_1+Wd_2+Wd_3)	Weed	80.0	50.0

* *Rumex dentatus* Tu : potato tuber Wt : water So : soil

* *Solanum nigrum* St : potato stemWd : weed

2. Phenotypic changes in colony morphology of isolates stored in still water:

The development of phenotypically avirulent forms, in the population of virulent one(s), originally isolated from the sources under study and kept at ambient temperature for one year, is shown in table (2). Water suspension of typical virulent isolates were surveyed for atypical (at) forms. It is interesting to note a complete (at) phenotypical change, similar to the avirulent colony morphology in certain isolate sources. The percentage of (at) colonies in the stored isolates, originally isolated from soil, irrigation water and potato stem were 100%, indicating complete change to the atypical form. The only exception may be recognized for tuber and weeds isolates. Such morphologically changed isolates, however, showed typical cell morphology as described in IFAS test, of isolates before storage for one year. Moreover, wilting in tomato plants under green house conditions was recorded. It is important to note that the isolates recovered from potato tubers and weeds showed relatively smaller proportion of (at) colony form, being 78.8% and 64.9%, respectively.

Despite the phenotypic change in colony morphology after one year in tap water, it is interesting to note that the isolates in concern did not show any pathological differences (Table 2). Moreover, the IFAS reaction, PCR pattern, BOX-PCR and Taq-Man were quite similar for all isolates (Figures 1,2 and 3).

The development of large proportion of (at) on SMSA with their pathogenic potential, similar to the (vi) on inoculation in tomato seedlings, was reported by Farag *et al.* (2004). They reported that the (vi) and (at) forms were identical in PCR pattern, BOX-PCR, Taq-Man and pathogenicity. This indicates non genetic change from (vi) to (av) and makes diagnosis, based solely on colony morphology on SMSA medium, controversial (Farag *et al.*, 2004).

Table 2. Percentage of typical and atypical forms of *R. solanacearum* and pathogenicity after one year storage in tap water (counts x 10^4).

Habitat		Count	%	Pathogenicity
Potato tuber	Typical	4.3	21.2	+
	Atypical	16.0	78.8	+
Stem	Typical	-	-	-
	Atypical	130.0	100.0	+
Weeds	Typical	200.0	35.1	+
	Atypical	37.0	64.9	+
Soil	Typical	-	-	-
	Atypical	10.0	100.0	+
Water	Typical	-	-	-
	Atypical	1.7	100.0	+

* Isolates from different sources at zero- time were phenotypically virulent.

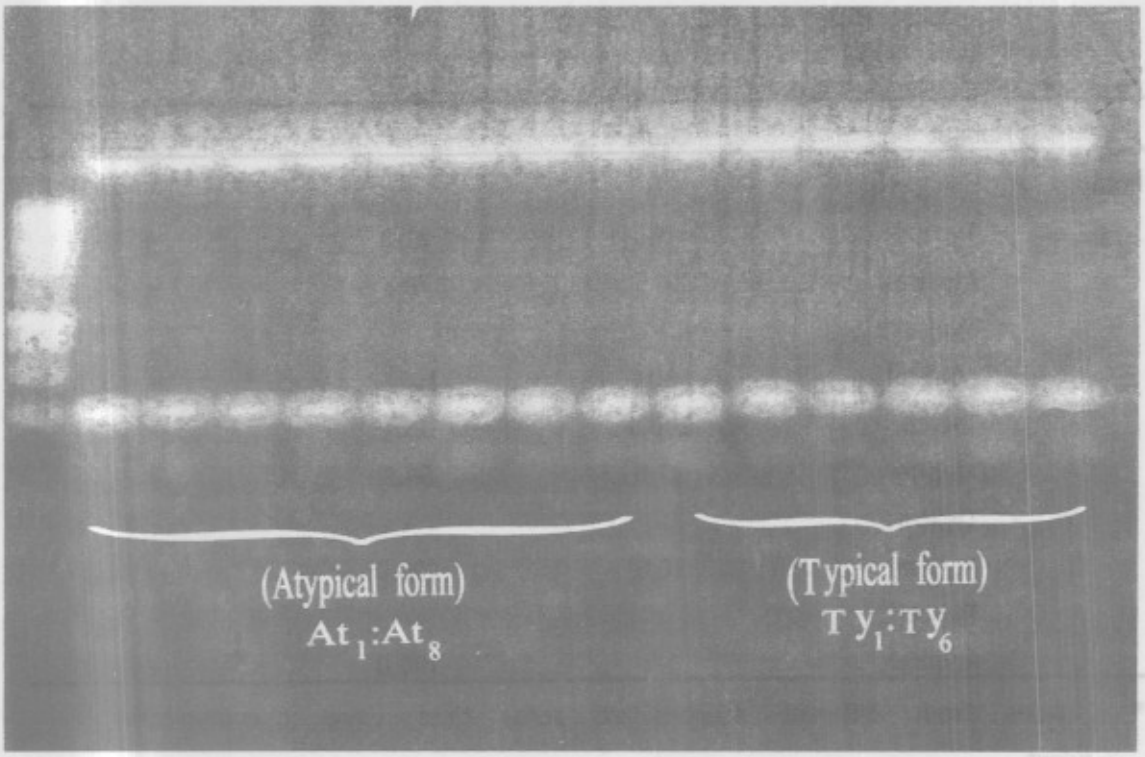

Figure 1. PCR of atypical and typical isolates of *Ralstonia solanacearum*

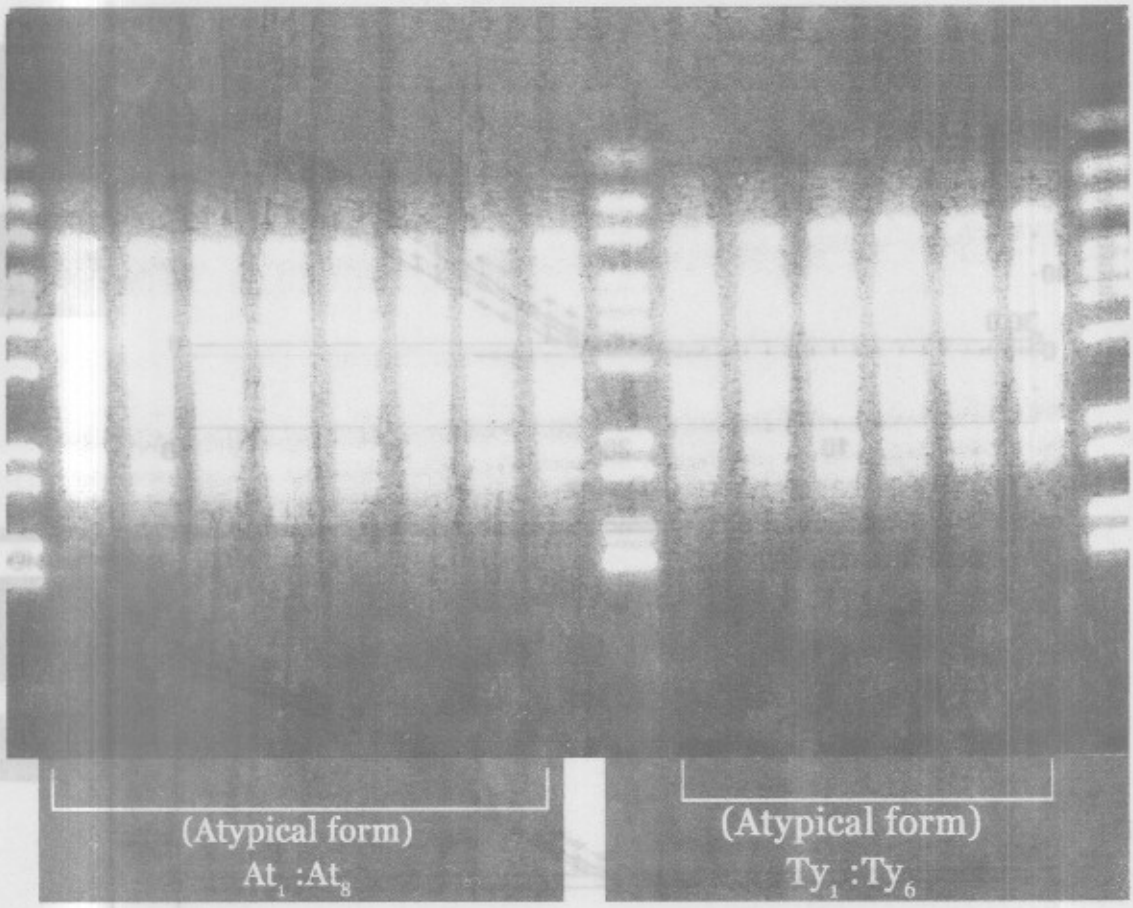

Figure 2. BOX-PCR to atypical and typical isolates of *Ralstonia solanacearum*

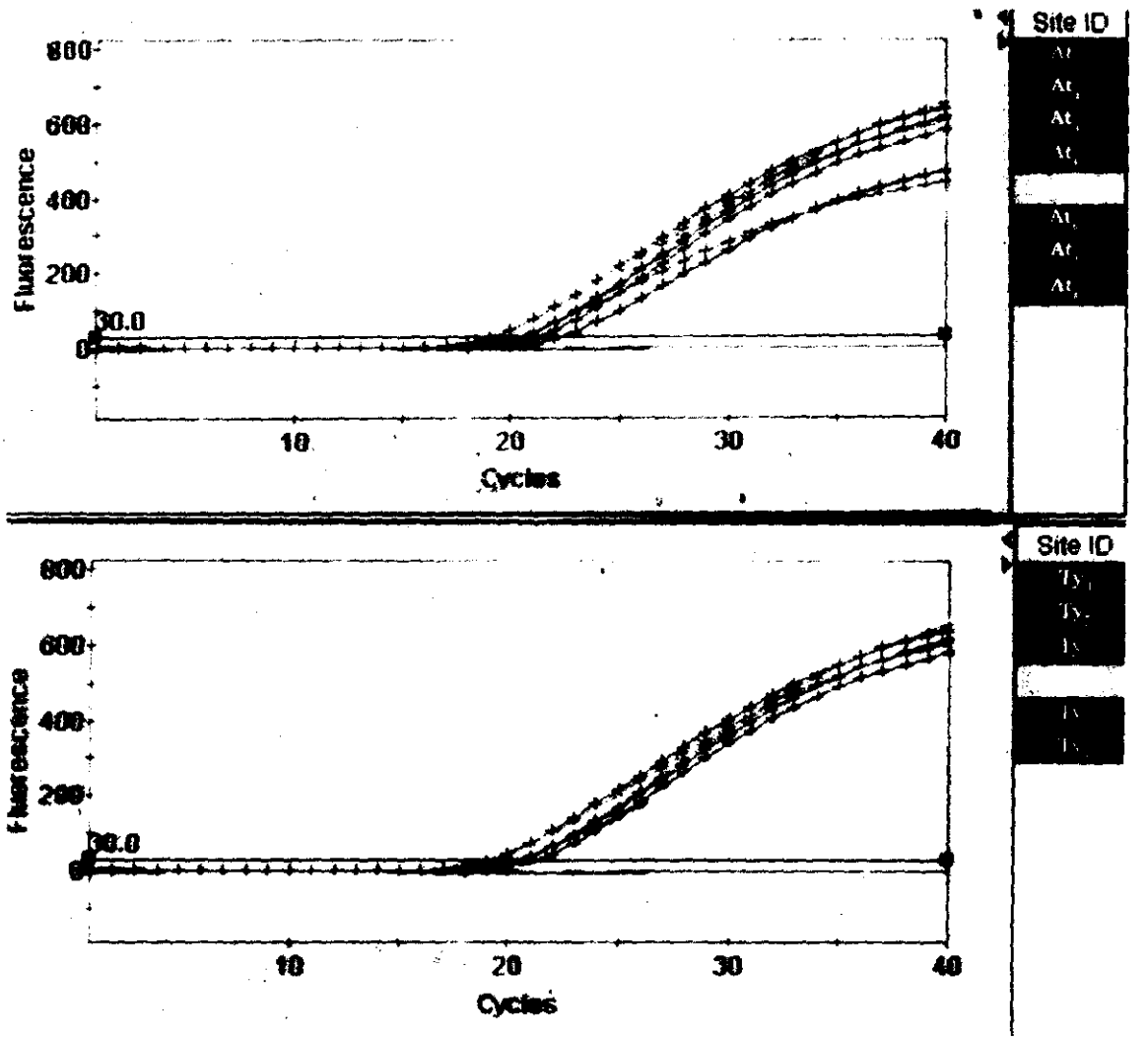

Figure 3. Taq-Man assay of atypical and typical *Ralstonia solanacearum* isolates.

3. Effect of organic matter on persistence of *R. solanacearum* in still water :

The survival of the pathogen in still (non streaming) tap water is shown in table (3). The mixture of *R. solanacearum* isolates kept in plain water developed dense atypical uniform growth on SMSA medium after 210 days. Increasing the organic matter, in the form of glucose nutrient broth (GNB) from 5 ml/flask (10%) up to 20 ml/flask (40%) decreased the survival to 170 days. Further increase in organic matter to 40 (80%) and 50 (100%) ml GNB/flask decreased survival to 110 days.

According to the results accumulated herein, the long survival of the pathogen in plain water and the deleterious effect of organic matter supplements, could be concluded. such a reduced survival may be attributed to the higher rate of metabolism and the accumulation of inhibitory by products.

The organic matter or farmyard manure amendment, was advised for brown rot control (Farag, 1976). The effect was attributed to the possible increase in the antagonistic soil microflora. In the present work, however, the easily assimilated organic materials exerted a direct deleterious effect on the persistence of *R. solanacearum* away from the influence of any microbial antagonism. This observation may be important in studying the epidemiological aspects for brown rot control and explain the shorter survival under these conditions.

Table 3. Effect of easily assimilated organic matter on the persistence of *R. solanacearum* in still water.

(Log numbers)

Treatment	% GNB	time duration (days)										
		0-time	7	14	21	30	45	75	110	140	170	210
(50 ml) water	0	0	7.03	7.51	7.61	7.82	7.90	7.60	7.40	7.20	7.10	7.10
(45 ml) water + (5 ml) GNB	10	0	8.67	9.81	9.80	9.80	9.20	8.60	8.30	8.10	7.90	0
(40 ml) water + (10 ml) GNB	20	0	9.21	9.40	9.60	6.90	6.90	6.90	6.90	5.80	5.50	0
(30 ml) water + (20 ml) GNB	40	0	9.30	9.40	9.40	9.20	8.20	7.20	7.20	7.20	6.0	0
(10 ml) water + (40 ml) GNB	80	0	9.50	7.60	7.50	7.00	7.00	6.90	4.60	0	0	0
(50 ml) GNB	100	0	11.00	9.82	8.90	8.40	8.40	8.40	6.40	0	0	0

4. Survival of *R. solanacearum* in soil :

Table (4). shows the densities of *R. solanacearum* in clay and sandy soil infested with the pathogen over eight months of monitoring starting from July 2003. It is interesting to note that sandy soil showed higher densities of the pathogen compared to those recorded is clay soil in July, August and September. The recorded densities at the aforementioned dates were 310 x 10^4, 0.04 x 10^4 and 0.04 x 10^4 cfu/g in sandy soil samples, respectively. The corresponding figures in clay soil were 190 x 10^4, 0.03 x 10^4 and 0.02 x 10^4 cfu/g for the same intervals, respectively. In October and November, however, the clay soil showed greater increase in densities compared to the sandy soil which may explain the recorded high disease incidence, in the Delta, in different surveys carried out in Egypt. The unprecedented increase in *R. solanacearum* densities in December in sandy soil (4300.0 x 10^4 cfu/g) compared to that recorded in clay soil (147.0 x 10^4 cfu/g) may as well explain the new records on greater incidence of the disease in the new potato districts being planted in December. On the other hand, densities in dry clay soil were extremely low after 5 months (November).

It is found that soil biofertilization with effective microorganisms (EM) showed seasonal fluctuation in densities of *R. solanacearum* different from that recorded in non-biofertilized ones in October and November with clay and sandy soil, respectively. In January and February, however, no *R. solanacearum* could be detected in the assayed samples.

The recorded low densities of *R. solanacearum* in clay soil in July, August, and September may be attributed to the kinetics of attraction of soil colloids and soil minerals in hot climates and/or the observed deleterious effect of organic matter reported herein. The greater increase in October in clay soil may coincide with lower soil temperature that results in decreasing attraction forces of clay colloids. The present work did not consider the fraction of viable but nonculturable form (VBNC) that may possibly explain the differences in pathogen densities between sand and clay (Grey and Steck, 2001). The driest soil contained generally higher number of viable *R. solanacearum* than did the wetter treatments. Thus, provided the pressure potential remains constant, some strains of *Ps. solanacearum* in certain soils are not sensitive to dry soil conditions (Moffett *et al.*, 1983).

Table 4. Densities of the pathogen (cfu/g dry soil) in two soil types infested with *Ralstonia solanacearum*.

(Counts x 10^4)

Intervals / Treatment	Months after inoculation							
	July	August	September	October	November	December	January	February
Clay soil								
Control	0	0	0	0	0	0	0	0
Inoculated *R. solanacearum*	190.0	0.03	0.02	88.0	185.0	147.0	0	0
Inoculated *R. solanacearum* + EM	160.0	0.26	0.04	263.0	78.0	0	0	0
Sandy soil								
Control	0	0	0	0	0	0	0	0
Inoculated *R. solanacearum*	310	0.04	0.04	0.30	6.0	4300.0	0	0
Inoculated *R. solanacearum* + EM	430	0.30	0.06	0.20	43.0	4150.0	0	0
Dry soil								
Clay with *R. solanacearum*	200.0	0.007	0.002	316.0	71.0	0	0	0
Sandy with *R. solanacearum*	370.0	0.014	0.005	11.0	899.0	2333.0	0	0

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بعض النواحى الوبائية لبكتيريا "رالستونيا سولاناسيرم"

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اختلفت عزلات "رالستونيا سولاناسيرم" فى قدرتها المرضية على حسب المصادر المأخوذة منها ، حيث ثبت أن العزلات المأخوذة من درنات البطاطس والحشائش أشد فى قدرتها المرضية عن تلك المأخوذة من الماء والتربة وسيقان البطاطس النامية فوق سطح التربة . كما اتضح أن حشيشة الحميض *Rumex dentatus* وعنب الديب *Solanum nigrum* من العوائل الطبيعية للسلالة ٣ طراز II من البكتيريا "رالستونيا سولاناسيرم" .

وقد تبين تكون أعداد متزايدة من الطرز غير المطابقة (at) والشبيهه بالطرازات الغير حادة مرضياً (av) على بيئة (SMSA) الملقحة من معلق بكتيرى فعال مرضياً ومخزن كمعلق مائي على درجة حرارة الغرفة لمدة طويلة . هذه الطرز غير المطابقة (at) أظهرت قدرة عالية على إحداث الذبول في شتلات الطماطم الملقحة فى السيقان على الرغم من مظهرها الغير فعال وكانت مشابهة تماماً للطراز الحاد مرضياً فى اختبارات الوميض الفلورسنتى المعروف باسم IFAS ، PCR ، BOX- PCR ، Taq-Man كذلك يجب الإشارة إلى أن هناك اختلاف مابين العزلات على حسب اختلاف مصادرها فى قدرتها على التحول الى الطراز الغير مطابق حيث تحولت كل العزلات المأخوذة من التربة وماء الري وسيقان البطاطس بالكامل الى الطراز الغير مطابق (ولكنه فعال في إحداث الإصابة) ولكن العزلات المأخوذة من درنات البطاطس والحشائش ظهر بها كلا الطرازين المطابق والغير مطابق . ومع ذلك لم يكن هناك اختلاف بينهما في إحداث القدرة المرضية على نباتات الطماطم .

تناول هذا البحث أيضاً تأثير إضافة مادة عضوية بسيطة مثل بيئة الجلوكوز المغذية السائلة بمقادير مختلفة الى ماء صنبور معقم وأخذت عينات بصفة منتظمة حتى (٢١٠يوم) . وكان أول اختفاء للميكروب بعد (٤٠ يوم) في التركيزات العالية بينما باقي التركيزات الأقل استمرت البكتيريا

فيها إلى (١٧٠يوم) باستثناء المعاملة التي بها الماء فقط بدون أي إضافة مادة عضوية حيث استمرت البكتيريا أكثر من (٢١٠ يوم) .

ولقد أظهرت الدراسة بقاء البكتيريا حية فى كلا من التربة الطينية والرملية لمدة ستة أشـــهر بعد التلقيح تحت ظروف تنظيم الرطوبة الأرضية عند ٧٥% فى درجات حرارة التربة من يوليو حتى ديسمبر (٢٨,٢°م و ١٥,٢°م). كذلك تمت هذه الدراسة على التربة الجافة لمعرفة تأثير الجفاف على بقاء الميكروب فبقيت البكتيريا فى التربة الرملية لمدة ستة أشهر وأظهرت كثافة عالية جداً فى شـــهر ديسمبر ، أما فى الأرض الطينية الجافة فقد أحتوت على كثافة منخفضة فى شهر نوفمبر أى أن بقائها استمر لمدة خمسة أشهر .

كذلك تم التلقيح بلقاح حيوى الـــ EM مع الـــ *R. solanacearum* لمعرفة تأثيره على أعـــداد هذا الميكروب فأظهر زيادة ملحوظة جداً فى شهرى أكتوبر ونوفمبر فى كلا مـــن الأرض الطينيـــة والرملية على الترتيب وذلك مقارنة بنظيره الغير ملقح بالـــ EM .