

Stenotrophomonas maltophilia - an Emerging Pathogen of Local Varieties of Tomatoes in Bulgaria

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Abstract

Since 2014, an unusual appearance of tomato seeds (*Solanum lycopersicum*) has been observed. The seeds were produced from local tomato genotypes from Bulgaria without visible symptoms of disease. The seeds exhibited small or large brown-grey spots on the surface that affected also the deeper layers. The most affected seeds lacked germinating ability or were unable to produce normal seedlings. Bacteria were derived from exudates, seed coat, endosperm, and embryo. Several tests were carried out to investigate pathogenicity - inoculation of seedlings, seeds, and plants. All isolates were identified as *Stenotrophomonas maltophilia* by BiologTM and after sequencing of the 16S rRNA gene region. *S. maltophilia* was found to be the sole causal agent of the disease in seeds. This is the first report of *S. maltophilia* causing disease of tomato seeds.

Keywords: *Stenotrophomonas maltophilia*, pathogen, tomato, seeds, disease, bald seed

Резюме

От 2014 г. се наблюдават необичайни прояви по семена от локални генотипове домати (*Solanum lycopersicum*) без видими симптоми на заболяване. По повърхността на семената се наблюдават кафяви малки или големи петна, които продължават в дълбочина. Най-засегнатите семена се характеризират с липса на кълняемост или не са в състояние да произведат нормални растения. Бактериите са изолирани от ексудат, семенна обвивка, ендосперм и ембриони. Патогенният потенциал на щамовете е изследван чрез няколко метода - инокулиране на семена и растения в различни фази на развитие. Всички изолирани щамовеса идентифицирани като *Stenotrophomonas maltophilia* чрез системата BiologTM и след секвениране на генетичния регион за 16S rRNA. *S. maltophilia* е установен като единствен причинител на болестта. Това е първото съобщение за *S. maltophilia*, причиняващ болест по семената на домати.

Introduction

Stenotrophomonas maltophilia is plant-associated and has been isolated from tomatoes (*Solanum lycopersicum*), potatoes (*S. tuberosum*), cucumber (*Cucumis sativus*), sunflower (*Helianthus annuus*), maize (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum aestivum*), various weeds, and other plants (Ryan *et al.*, 2009). The bacterium was one of the main species found in tomato roots and tomato rhizosphere in Mexico (Marquez-Santacruz *et al.*, 2010). It has also been isolated from diseased

tomato fruits (Stoyanova and Bogatzevska, 2012) and together with *Xanthomonas* sp. from tomato seeds in Zimbabwe (Sibiya *et al.*, 2003). This study presents identification of *S. maltophilia* as a sole causal agent of disease of tomato seeds.

Material and Methods

Observations

In the late summer and early autumn of 2014, an unusual appearance of tomato seeds (*S. lycopersicum*)

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persicum) was observed in the Plovdiv region of Bulgaria. The seeds were produced from local genotypes with large pink fruits without visible symptoms of disease. The seeds exhibited brown-grey spots or large sections on the surface, or were entirely dark. Severely affected parts lacked the characteristic pappus, making the seed appear bald (Fig. 1). Longitudinal sections revealed that the discoloration was present in the deeper tissues, affecting also the endosperm and the embryo. Bacterial ooze was observed both on the surface and inside the seed. The affected seeds lacked germinating ability or were unable to produce normal seedlings. About 50% of the seeds were infected. In 2015, another large part of the seeds of the same tomato varieties showed identical symptoms. Upon careful examination, ring necrosis on some sepals during the flowering period was discovered. Diseased seeds of variable percentage have been present among the collected seeds in the subsequent years.

Isolations

Bacteria were derived from exudates, coat, endosperm, embryo, sprouting root and germ of the seeds on King's B medium (Klement *et al.*, 1990). Diseased as well as healthy seeds were used for analysis. Cell morphology was observed under light microscope. Basic biochemical characteristics (Gram reaction, anaerobic growth, catalase and oxidase activity) were determined (Schaad *et al.*, 2001).

Identification

Pure cultures were initially identified by the identification system Biolog™ (Biolog™ Inc., USA) with GN2 MicroPlate™ test plates. The bacteria were incubated on BUG agar at 28°C for 24 h

prior to plate inoculation and the procedure was conducted according to the manufacturer's instructions. The software program MicroLog 4.20.05 was used.

Sequencing of the 16S rRNA gene region was performed for confirmation of identification. DNA was extracted from bacterial cultures grown in Luria-Bertrani Broth at 28°C, 200 rpm, overnight by DNeasy Blood & Tissue Purification Kit (QIAGEN GmbH, Germany) according to the manufacturer's instructions. Control of cell density, yield and purity of the extracted DNA was performed by measuring on NanoDrop 2000 spectrophotometer (Thermo Scientific GmbH, Germany). The 16S rRNA gene region was amplified by primers Fgps6 (5'GGAGAGTTAGATCTTGGCTCAG3') and Fgps1509 (5'AAGGAGGGGATCCAGCCGCA3') (Nesmeet *et al.*, 1995). The PCR mixture was held in 25 µl volume and contained 1x PCR buffer, 2.5mM MgCl₂, 50 pmol of each primer, 0.02 mM dNTPs, 1.25U *Taq* DNA polymerase, and 100 ng of DNA. The following amplification program was used: initial denaturation step at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min; and a final extension step at 72°C for 15 min. The PCR products were electrophoretically separated in 1% (w/v) agarose gel in 1x TAE buffer at 100 V for 1h and visualized by the use of ethidium bromide under UV light at 320 nm for length control. The products were purified by PCR Purification Kit (Thermo Scientific GmbH, Germany) according to the manufacturer's instructions. Sequence data was obtained by Macrogen™ (Europe) and analyzed



Fig. 1. Tomato seeds with symptoms of natural infection with *S. maltophilia*

with BLASTN. A distance tree in BLASTN, using the neighbor joining method, was created.

Pathogenicity and epidemiology

Germination ability and symptoms on sprouts of diseased seeds with a different degree of manifestation were observed after incubation in sterile wet chambers at 27°C in dark until germination.

Bacterial suspensions with density 10⁴ cfu/ml from cultures grown on Potato-Dextrose Agar medium (PDA) at 28°C for 24 h were used in the experiments with artificial inoculation. Several methods for studying the pathogenicity of the isolated strains were applied and two tomato varieties were used (cv. Ideal and cv. Trapezitza).

(a) Hypersensitive reaction (HR) test on tobacco: *Nicotiana tabacum* cv. Samsun NN, according to Klement *et al.* (1990). Observations were held periodically between 1st-9th day.

(b) Fast screening for pathogenicity: vacuum inoculation of healthy tomato seedlings at 5-6 leaf stage was performed for 2 x 2 min at -1013 hPa (Jones *et al.*, 1981; Bogatzevska, 1988). Inoculated plants were incubated in nutrient solution, at 18-22°C, in indirect sunlight and examined for pathological changes between 1st-7th day. Healthy seedlings treated in the same manner with distilled water served as negative controls.

(c) Seed inoculation: healthy tomato seeds were artificially inoculated with bacterial suspensions by the vacuum infiltration method (Jones *et al.*, 1981; Bogatzevska, 1988) at exposition 60 min. Inoculated seeds were incubated in sterile wet chambers at 27°C in dark until germination, observed for pathological changes and tested for the presence of the pathogen. Healthy seeds treated in the same manner with distilled water served as neg-

α -cyclodextrin		Xylitol		L-alanine	
Dextrin	■	pyruvic acid methyl ester	■	L-alanyl-glycine	■
glycogen		Mono methyl succinate	■	L-asparagine	■
tween 40	■	Acetic acid	■	L-aspartic acid	■
tween 80	■	Cis-aconitic acid	■	L-glutamic acid	■
N-acetyl-D-galactosamine	■	Citric acid	■	glycyl-L-aspartic acid	■
N-acetyl-D-glucosamine	■	Formic acid	■	glycyl-L-glutamic acid	■
Adonitol		D-galactonic acid lactone	■	L-histidine	■
L-arabinose		D-galacturonic acid		Hydroxy-L-proline	
D-arabitol		D-gluconic acid		L-leucine	■
Cellobiose	■	D-glucosaminic acid		L-ornithine	■
i-erythritol		D-glucuronic acid		L-phenylalanine	
D-fructose	■	α -hydroxybutiric acid	■	L-proline	■
L-fucose		β -hydroxybutiric acid	■	L-pyroglutamic acid	
D-galactose		γ -hydroxybutiric acid		D-serine	
Gentibiose	■	p-hydroxy phenylacetic acid		L-serine	■
α -D-glucose	■	itaconic acid		L-threonine	■
m-inositol		α -keto butyric acid	■	Carnitine	■
α -D-lactose	■	α -keto glutaric acid	■	γ -aminobutyric acid	■
lactulose	■	α -keto valeric acid		urocanic acid	
Maltose		lactic acid	■	Inosine	■
D-mannitol		Malonic acid		Uridine	■
D-mannose	■	Propionic acid		Thymidine	
Melibiose	■	Quinic acid		Phenylethylamine	
β -methyl D-glucoside	■	D-saccharic acid		Putrescine	
D-psicose	■	Sebacic acid		2-amino ethanol	
D-raffinose	■	Succinic acid	■	2,3-butanediol	■
L-rhamnose		Bromo succinic acid	■	glycerol	■
D-sorbitol	■	Succinamic acid		D,L- α -glycerol phosphate	■
Sucrose	■	Glucuronamide		D-glucose-1-phosphate	■
D-trehalose	■	Alaninamide	■	D-glucose-6-phosphate	■
turanose	■	D-alanine	■		

Fig. 2. Metabolic patterns of *Stenotrophomonas maltophilia* strains isolated from tomato seeds

Legend:

- Substrate utilized (positive reaction) by all strains;
- Substrate weakly metabolized by all strains;
- Substrate not utilized (negative reaction) by all strains;
- Substrate utilized or weakly utilized by the strains;
- Substrate not utilized or weakly utilized by the strains

ative controls.

(d) Inoculation of healthy plants in a greenhouse: tomato plants before and during the flowering stages were inoculated by injection. Observation for symptoms up to 20 days after inoculation and isolations from symptomatic/asymptomatic plant parts were carried out.

(e) Inoculation of healthy plants in the field – tomato plants in small green fruits and fruit maturation stages were inoculated by injection. Observation for symptoms up to 30 days after inoculation and isolations from symptomatic/asymptomatic plant parts were carried out.

Results and Discussion

Isolations and identification

The majority of the isolated colonies were non-pigmented, opaque, entire-margined, and did not produce diffusing UV-fluorescent pigment. Pure cultures of these colonies contained gram-negative motile rods, which were aerobic, catalase-positive and showed delayed positive oxidase reaction. Overall, 28 isolates with that type of colonies were obtained in 2014-2016. Some of the strains produced dark brown diffusing pigment on PDA. Some of the strains induced changes in tobacco leaves tissue – chlorosis on the 7th day and HR on the 9th day.

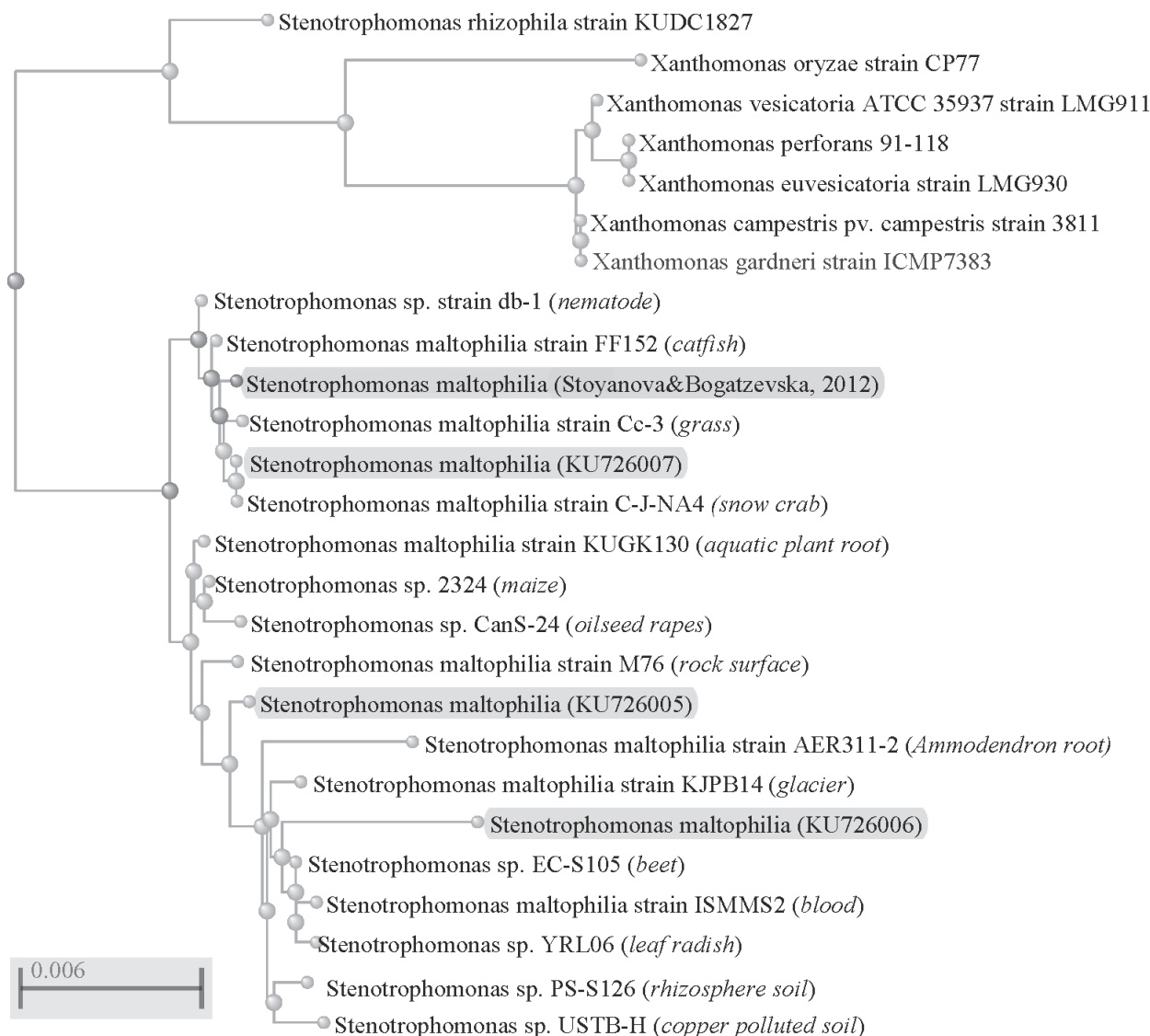


Fig. 3. A distance tree of pairwise comparisons of three *S. maltophilia* strains from tomato seeds and one strain from tomato fruit with other strains from genera *Stenotrophomonas* and *Xanthomonas*

During the isolations in 2014, colonies typical of the phytopathogenic bacteria from the genera *Xanthomonas*, *Pseudomonas*, and *Clavibacter* were not observed. A smaller number of colonies producing diffusing UV-fluorescent pigment, which is typical of the bacteria from genus *Pseudomonas*, were isolated in 2015 and 2016.

All 28 non-fluorescent isolates were identified by the Biolog™ system as *S. maltophilia* with 100% probability and 0.758-0.924 similarity. Metabolic patterns did not generally differ from those published previously (Stoyanova and Bogatzevska, 2012). The strains utilized 34 carbon sources and did not utilize 34 substrates. The strains weakly metabolized five substrates and showed variability in utilization of 22 carbon sources (Fig. 2).

The alignment of the obtained sequences shared 99% identity with *S. maltophilia* strains, which correlated with the identification performed by Biolog™ system. Two of the sequences (from sprouting root isolates) shared 99% similarity with *S. maltophilia*, as well as with unidentified representatives of genus *Stenotrophomonas*. Three of the sequences were deposited in the GenBank under accession Nos. KU726005, KU726006, and KU726007. A distance tree containing a pathogen-

ic isolate from tomato fruit (Stoyanova and Bogatzevska, 2012) for comparison is shown in Fig. 3. *Pathogenicity and epidemiology*

The 28 strains identified as *S. maltophilia* formed dark irregular spots on the 4th-5th day after artificial inoculation by the vacuum infiltration method. The leaf surface between the spots soon became chlorotic and the leaves withered. Ring necrosis on some of the petioles was also observed. All control seedlings remained healthy. Four to five days after inoculation of the seeds, the newly formed sprouts and roots developed spots and necrosis. Control seeds germinated normally.

The tomatoes inoculated before flowering developed as weak and chlorotic plants which did not produce flowers. The older leaves withered prematurely. Cracks appeared around the place of inoculation and longitudinal cuts revealed darkening of the inner tissues in both directions. The leaves gradually began to wither and fell out. The tomatoes inoculated during flowering continued to grow slowly as chlorotic plants and exhibited similar symptoms on the stem and leaves. The flowers darkened and fell out. Examination of the roots of both groups of plants revealed necrosis of the bases, media or top of the roots (Fig. 4).



Fig. 4. Symptoms on tomato plants after artificial inoculation with *S. maltophilia*

The plants inoculated in the field during the small green fruits stage developed a variety of symptoms depending on the cultivar. Again, cracks appeared around the place of inoculation and longitudinal cuts revealed darkening of the xylem tissues in both directions. A cavity in the stem and in the first sprout below the point of inoculation as well as exudates could be observed. The plants of cv. Trapezitza exhibited necrotic spots and ring necrosis on peduncles as well as chlorotic sepals and fell void of the fruits before maturation. The fruits of cv. Ideal grew and matured but developed watery and dark spots that affected also the deeper tissues, as well as brown spots on the sepal tips, cracks and darkened tissues at the base of the peduncles. Symptoms such as dark spots could occasionally be observed on the seeds from these fruits (Fig. 4).

Reisolations were carried out from symptomatic tissues from the stem and the sprouts near the point of inoculation, flower peduncles, sepals, seeds, and roots. *S. maltophilia* was recovered from all symptomatic seeds and plant parts.

S. maltophilia pathogenic strains were isolated from diseased seeds with specific symptoms as well as from healthy seeds from local tomato varieties with large pink fruits. No other pathogens were identified in the samples from 2014. Coexisting with *S. maltophilia* in the coat of the healthy seeds from 2015-2016 was *Pseudomonas fluorescens* and in the coat from diseased seeds - *P. fluorescens* and *P. putida*, but these bacteria lacked the pathogenic potential of the *S. maltophilia* strains. The cotyledons germinated from only slightly spotted seeds with preserved germination potential carried *S. maltophilia* symptomlessly.

During the period 2014-2016, *S. maltophilia* was established to cause symptomless or symptomatic infection of seeds and flowers of local tomato varieties. The seeds with large dark lesions as well as the most infected (entirely dark) seeds were unable to produce healthy germs and plants. However, the slightly diseased seeds produced symptomless sprouts, which were able to grow into mature plants. As the disease has been continuously appearing in the seeds since 2014, it is highly possible that the colonized by the pathogen but symptomless plants act as a source of infection. To our knowledge, this is the first report of *S. maltophilia* causing disease of tomato seeds as a result of a natural self-contained infection.

S. maltophilia was isolated from tomato seeds before - in Zimbabwe together with *Xanthomonas* sp. (Sibiya *et al.*, 2003). Since the isolates were

pathogenic on tomato plants after artificial inoculation, they were considered as opportunistic pathogens. The species was also found to be present in tomato roots and rhizosphere (Marquez-Santacruz *et al.*, 2010), but it is long known to be present in soil, rhizosphere, and waters, and as an endophyte in plants (Ryan *et al.*, 2009). In 2012, we reported pathogenic *S. maltophilia* strains isolated from diseased tomato fruits (Stoyanova and Bogatzevska, 2012). Due to the generous copper-based treatments at that time we assumed that the copper-sensitive *Xanthomonas* spp. was possibly suppressed allowing the resistant *S. maltophilia* to multiply and contribute to symptoms formation (Stoyanova and Bogatzevska, 2012). However, in this previous study, like now, *S. maltophilia* was independently isolated from diseased tomato tissues. Moreover, the symptoms observed on tomato fruits after artificial inoculation of the plants in this study look very similar to the symptoms observed on tomato fruits contaminated with *S. maltophilia* in 2011. This gives us a good reason to reconsider the situation and assume that *S. maltophilia* was not only independently isolated from diseased tomato fruits in 2011 but was probably the sole causal agent as it is in this study.

Recent studies revealed that *S. maltophilia* encodes genes for cellulase, type I, II, IV, V and the twin-arginine transporter (TAT) secretion systems, which are involved in phytopathogenesis, and produces proteases, chitinases, glucanases, DNases, RNases, lipases, and laccases (Ryan *et al.*, 2009). These findings make the phytopathogenic potential of *S. maltophilia* seem not so surprising.

Conclusions

S. maltophilia occurs naturally in local varieties of tomatoes in Bulgaria causing “bald seeds”. As the disease has been continuously observed since 2014 until now, *S. maltophilia* can be considered as an emerging pathogen of local varieties of tomatoes in Bulgaria.

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