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Title: Phenotyping of bacterial spot pathogens from the Balkan Peninsula

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Applicant: Dr. Taca Vancheva, Institut de Recherche pour le Développement, Montpellier (FR), tacavancheva@gmail.com

Host: Prof. Dr. Ulla Bonas, Martin-Luther-Universität, Halle (Saale) (DE), ulla.bonas@genetik.uni-halle.de

Background:

Bacterial spot (BS) is the most common, and one of the most destructive diseases of tomato and pepper plants in Bulgaria and Macedonia. The disease is caused by three species of *Xanthomonas*: *Xanthomonas euvesicatoria* (incl. the formerly separate species *Xanthomonas perforans*), *Xanthomonas gardneri*, and *Xanthomonas vesicatoria*. Over the last years, hundreds of isolates were sampled and characterized. Most pepper isolates belong to *X. euvesicatoria*, while most isolates from tomato belong to *X. vesicatoria*. To cause disease, most plant-pathogenic xanthomonads, incl. BS pathogens, depend on a type III protein secretion system and the set of effector proteins that are collectively injected into the plant cell. Of particular interest are Transcription Activator-Like (TAL) effectors, which act as gene activators inside the plant cell. Two distinct TAL effectors have been described in *X. euvesicatoria*: AvrBs3 and AvrBs4. The ability of many avirulent bacteria to elicit the hypersensitive reaction (HR) depends upon the "hypersensitive response and pathogenicity" (*hrp*) genes, which encode the type III secretion system that injects effector proteins into the host cell. AvrBs3 is recognized in pepper plants (*Capsicum annuum*) by the *Bs3* resistance gene, and AvrBs4 is recognized in pepper plants (*Capsicum pubescens*) by the *Bs4C* resistance gene. AvrBs4 is also recognized in tomato plants by a gene termed *Bs4*, which is not related to *Bs4C* in pepper.

Objectives/ Purpose of the visit:

Previous genetic screens initially identified bacterial *avr* genes among a collection of bacterial spot pathogens (*X. euvesicatoria*), isolated from different pepper-producing regions in Bulgaria and Macedonia. The purpose of the visit was to functionally characterize the strains with respect to resistance reactions. For this, the strains were inoculated into pepper and tomato lines (described below).

- *C. annuum* ECW-30R pepper (with *Bs3*)
- parental line ECW (without *Bs3*)

• *C. pubescens* PI line 235 047 (with *Bs4C*)
• *C. pubescens* PI line 585 270 (without *Bs4C*)
• Moneymaker tomato (with *Bs4*)
• Moneymaker with the *Lycopersicon pennellii bs4* allele crossed in (i.e. without *Bs4*).

Description of the work:

Six-week old pepper and tomato plants were infiltrated into the lower side of the leaves using a needleless 1 mL-syringe with bacterial suspension at optical density 600 nm (OD_{600}) of 0.3. The strains 85-10 (pGGXI::*avrBs3*) and 85-10 (pL3-*avrBs4*), provided by Prof. Bonas, were used as positive controls. Water and strain *X. euvesicatoria* 85-10 served as controls. After infiltration, the plants were kept in a culture chamber for 48 to 72 hours and the presence or absence of an HR at the infiltration site was scored (Fig 1).

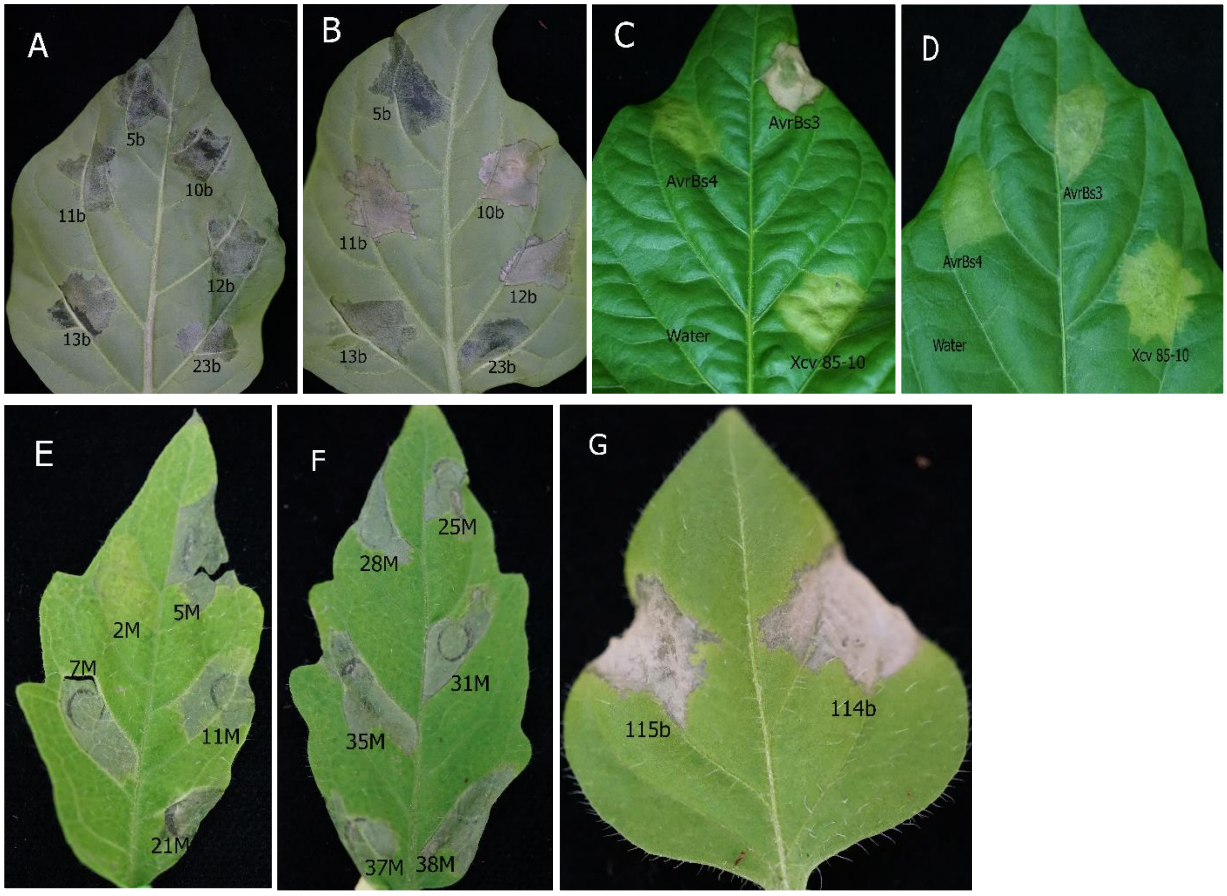

Fig. 1. Infected leaves showing presence or absence of the HR

X. euvesicatoria strains inoculated into ECW; B- *X. euvesicatoria* strains inoculated into ECW-30R; C- controls inoculated into ECW-30R; D- controls inoculated into ECW; E- *X. euvesicatoria* strains inoculated into Moneymaker (*bs4*); F- *X. euvesicatoria* strains inoculated into Moneymaker; G- *X. euvesicatoria* strains inoculated into *C. pubescens* PI line 235 047 (*Bs4C*).

Main results:

All 132 strains were screened for presence or absence of *avrBs3* and *avrBs4* effector genes via PCR with primers specific for the N- or C-terminal regions of the TAL effector genes The majority of the strains appear to contain at least one of the two genes and 49% of the strains appear to contain even both genes (Fig. 2).

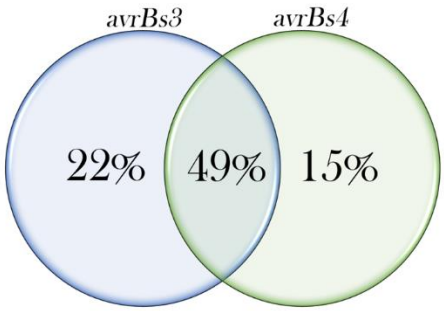

Fig. 2. Venn Diagram showing the percentage of strains with *avrBs3* or *avrBs4* only or with both effector genes.

91.4% of the strains that possess the ***avrBs3*** gene induced the HR in pepper ECW-30R. Interestingly, 8.6% of the strains in which the gene *avrBs3* appeared to be present (based on the diagnostic PCR assay) were not recognized in ECW-30R. This could be due to mutation and inactivation of the *avrBs3* gene. Recognition was observed for 23.7% of the strains that were negative in the diagnostic PCR for *avrBs3.* This recognition may be explained by the fact that these strains have a TAL effector, which was typed by PCR as *avrBs4* but may have undergone recombination resulting in *avrBs3* activity (strains 35M, 50M, 95b).

For evaluation of the ***avrBs4*** gene, we used tomato and pepper plants. Surprisingly, 55.7% of the strains were recognized in the control plants (Moneymaker carrying the *L. pennellii bs4* allele) and their analysis in Moneymaker tomato (*Bs4*) plants did not reveal information on the function of *avrBs4* in the corresponding strains. 69.6% of the remaining strains behaved as expected, i.e., those with a diagnosed *avrBs4* gene triggered the HR and those without did not trigger an HR. Notably, 10 strains with a diagnosed *avrBs4* gene did not trigger the HR, perhaps due to mutational inactivation. Moreover, two strains with *avrBs3* but without a diagnosed *avrBs4* gene triggered the HR, a finding that could be explained by a possible recombination event resulting in *avrBs4* activity. It should also be kept in mind that the tomato *Bs4* gene can recognize C-terminally truncated versions of AvrBs4 and AvrBs3 variants with internal in-frame deletions.

When the strains were evaluated on *C. pubescens* PI line 235 047 plants (*Bs4C*), 64.1% of the strains behaved as expected, i.e., those with a diagnosed *avrBs4* gene triggered

the HR and those without did not trigger an HR. Notably, 17 strains with a diagnosed *avrBs4* gene did not trigger the HR, perhaps due to mutational inactivation. Moreover, 22 strains with *avrBs3* but without a diagnosed *avrBs4* gene triggered the HR, a finding that could be explained by a possible recombination event or other types of mutations resulting in *avrBs4* activity.

Interestingly, 28% of the **strains that lack *avrBs3* and *avrBs4*** genes induced the HR in ECW-30R, Moneymaker and *C. pubescens* PI line 235 047 plants, 17% in *C. pubescens* PI line 235 047 and Moneymaker, and 5% only in ECW-30R. Perhaps another avirulence gene is present in these strains that is recognized by another *R* gene(s) in the different plant cultivars. For instance, ECW-30R is a near-isogenic line of ECW resulting from seven backcrossings of ECW as the recurring parent against resistant progeny from a cross between susceptible ECW plants x the resistant *C. annuum* PI 271 322 from India. It is thus conceivable that another gene from the PI 271 322 was co-introduced into ECW-30R.

- Future collaboration with the host institution (if applicable):
  The sending and the receiving institutions expressed their interest in continuing to work on TAL effectors from *X. euvesicatoria*.
- Projected publications/articles related to or resulting from the STSM:
  A manuscript on the genetic diversity of *X. euvesicatoria* strains from the Balkan Peninsula is being drafted and will include these data.
- Confirmation by the host institution of the successful execution of the STSM (this must consist of a signed letter from the host institution):
  Signed letter from the host institution will be submitted in parallel.
- Other comments (if any):
  The applicant wishes to express her gratitude to the COST Action of having supported this valuable stay at the host institution, which will help in her career development as an early-career investigator.