

EFFECTS OF SIMULATED RAIN ON *PSEUDOMONAS SYRINGAE* pv. *TOMATO* POPULATIONS ON TOMATO PLANTS

L. Pietrarelli, G.M. Balestra and L. Varvaro

Dipartimento di Protezione delle Piante, Università della Tuscia, Via S. Camillo de Lellis, 01100 Viterbo, Italy

SUMMARY

A rain simulator was developed to study the effects of rain on the biological and the epidemiological aspects (distribution, epiphytic survival) of *Pseudomonas syringae* pv. *tomato* populations in the tomato canopy. Two different kinds of rain were simulated. Rain events, relative humidity, drip-irrigation and temperature were automatically controlled and monitored in a growth chamber. Simulated rainfall intensity varied with nozzle size, water pressure at the nozzle, and height of the nozzle above the plot surface. Spray nozzles formed drops of 1.6 mm or 2.7 mm in diameter. Rain was simulated on tomato plants already colonized by *P. s.* pv. *tomato* (plants with speck symptoms where bacteria were inoculated 5 days before the rain event) and on plants not colonized by the pathogen (inoculated 3 h before the rain event). After each rain simulation, the horizontal and vertical distribution and epiphytic survival of *P. s.* pv. *tomato* were observed, for two weeks. Drops 2.7 mm in diameter were more effective in distributing bacteria over short distances and drops 1.6 mm in diameter more effective in long-distance distribution. Higher rain intensity reduced epiphytic survival of *P. s.* pv. *tomato* when the host-pathogen relation was precarious (non-colonized tomato plants) but when the pathogen was already established (colonized plants), higher rain intensity increased bacterial growth.

Key words: leaf surface, phyllosphere, epiphytic survival, bacterial distribution, bacterial growth, raindrops.

INTRODUCTION

Bacterial speck of tomato (*Solanum lycopersicum* L.), caused by *Pseudomonas syringae* pv. *tomato* [(Okabe) Young, Dye, and Wilkie], is one of the most serious bacterial diseases of the tomato plant, and can reduce both yield and quality in fresh market and processed

tomatoes (Varvaro and Guarino, 1983). The pathogen, as a leaf resident *sensu* Leben (1965), survives by using substances leaching from the leaf and organic matter transported by the wind (Schneider and Grogan, 1977). As with other epiphytic bacterial pathogens (Kinkel, 1997, Wilson *et al.*, 1999), *P. s.* pv. *tomato* survives in particular leaf sites such as depressions between epidermal cells, sub-stomatal chambers and around trichomes (Varvaro *et al.*, 1993). One of the factors which affect the growth and dispersal of epiphytic microorganisms is rain (Costantinidou *et al.*, 1990; Madden, 1992, 1997). The average size of rain-drops is 2-3 mm, ranging from 0.2 to 5.5 mm, and the terminal velocity of an average-sized drop is about 6.5-8 m s⁻¹ (Huber *et al.*, 1998). The diameter of a rain-drop depends on different meteorological factors, and collision/coalescence, rain-drop breaking and evaporation, may occur (Madden, 1992, 1997; Huber *et al.*, 1998).

The effect of rain on bacterial growth and population dynamics has been studied particularly on *Pseudomonas syringae* (Lindemann and Upper, 1985; Hirano, *et al.*, 1987, 1995, 1996; Hirano and Upper, 1991, 2000; Costantinidou *et al.*, 1990; Lindow, 1996).

However, to date, the effects of rain on *P. s.* pv. *tomato* populations has not been studied: surprising considering the world importance of the tomato and the damage that *P. s.* pv. *tomato* can cause.

This study aims to evaluate the effect of rain on the spatial distribution of the pathogen (horizontal and vertical) and on the epiphytic survival of *P. s.* pv. *tomato* in the canopy of the tomato crop.

MATERIALS AND METHODS

Bacterial strain. One isolate of *P. s.* pv. *tomato* (10PST21) from the culture collection of the Department of Plant Protection, University of Tuscia (VT), Italy was used. This came from field tomato plants with typical leaf symptoms. Cultures were stored at 4°C on nutrient agar slants supplemented with 2% glycerol (NAG) and cultured on King's medium B (KB) (King *et al.*, 1954) containing 100 mg per mL rifampicin (KBr) at 25±2°C for 24 h for inoculum production.

Plants and bacterial inoculation. Thirty-day-old tomato plants cv 'S. Marzano' were used. The tomato plants were grown in a greenhouse in 15-cm-diameter pots containing a sterilized mix of soil:sand:peat (2:1:1, by volume) and watered daily by drip-irrigation. A mineral solution (NPK 20-20-20+B+Cu+Fe+Mn+Mo+Zn 1-5-30-10-10-10, respectively) at 2g l⁻¹ was distributed weekly into the pots to maintain optimum nutritional conditions.

Temperatures were maintained at 25±2°C and 15±2°C day/night, respectively, while relative humidity (RH) was maintained at 50-70% under natural light conditions. The plants were approximately 30 cm high (4 leaf nodes) at the time of inoculation.

P. s. pv. *tomato* suspensions in sterile distilled water were prepared from 24 h-old bacterial cultures and adjusted to 10⁸ colony-forming units (cfu) ml⁻¹ using a spectrophotometer at 600 nm. The suspension was sprayed on tomato plants with a CO₂-pressurized handheld sprayer equipped with a large orifice (Δ 1.4mm, Tee-Jet 8004) nozzle operating at a pressure of 2.8 g/cm² to produce large spray droplets. The spray nozzle was kept close (20-30 cm) to the plants to reduce bacterial movement by aerosol from the application site.

In the chamber, some plants were subjected to the rain simulation 3 h after inoculation, and labeled as not colonized (NC); those colonized (C), were submitted to rain simulation after typical symptoms had occurred (5 days).

Rain simulator. The simulator was adapted to the dimensions of the experimental area (a greenhouse chamber of 2.5 × 2.5 × 2.5 m) and operated at different pressures to generate rain-drops of two diameters. Each rain simulator consisted of a spray nozzle, a PVC pipe (4 m length), a pumping system, an on-off valve to eliminate nozzle drip problems, electric-valves, an adjustable flow meter to regulate rain intensity, and a pressure gauge. The pipe (2 cm in diameter) connected the source (distilled water in a storage barrel outside the experimental chamber) to the experimental area. The pipe was located 2.5 m above the experimental area, in the centre of the chamber ceiling.

The parameters applied during rain simulations were indicated by Spraying System (Co. Wheaton, Illinois, USA) and related to the characteristics of the spray nozzles utilized: a "full jet" type, 3/8 HH-20W, (type A, 9.09 l/min at 103 kPa) and type 1/4 HH-10 (type B, 3.78 l/min at 69 kPa). The nozzles were chosen for their uniformity of droplet distribution (Tossell *et al.*, 1987). Nozzle A gave droplets 1.6 mm in diameter compared to the 2.7 mm diameter of nozzle B.

Natural rainfall with peak rates that meet or exceed 1 mm/min are associated with bacterial population increase as observed for *P. syringae* on bean plants (Hirano *et al.*, 1996).

To simulate natural rainfall with an intensity of 0.9 mm/min of 20 min-duration, rain A (droplets 1.6 mm in diameter) was produced at 96 kPa water pressure, delivered from 1.10 m above the tomato canopy, with an intensity of 1.3 mm/min, 78° spray angle and 30 min long.

To simulate natural rainfall with an intensity of 0.5 mm/min of 18 min in duration, rain B (droplets 2.7 mm in diameter) was produced at 69 kPa water pressure, delivered from 1.10 m above the tomato canopy, with an intensity of 0.8 mm/min, 63° spray angle and 30 min long (Tossell *et al.*, 1987; Leone and Pica, 1993b).

Day and night temperatures were 25±2°C and 15±2°C, respectively. The mean RH was maintained at 50% by using a prototype automatic drip irrigation system controlled by MET (Multimedia Engineer Technologies, Bruxelles, Belgium).

By increasing rain intensity and duration it was possible to reproduce a similar kinetic energy and a terminal velocity of the drops (Leone and Pica, 1993a, 1993b). In this study, by using a similar amount of water, the rain simulations were utilized as a model for field conditions.

Environmental parameter measurements. Digital and analog data (temperature, relative humidity, rain duration) were recorded by a data logger at 1 min intervals.

Experimental area. Four concentric circular areas (A0, A1, A2, A3) were created.

Four inoculated tomato plants were placed in the middle of each area, in A0; it had a 20 cm diameter with three concentric buffers of 30 cm radius each (A1, A2, A3). The entire experimental area was 2 m in diameter, with 8, 18 and 27 healthy tomato plants placed in A1, A2 and A3, respectively, to obtain the same plant-density (17 plants/m²) (Fig. 1). As negative control, identical layouts

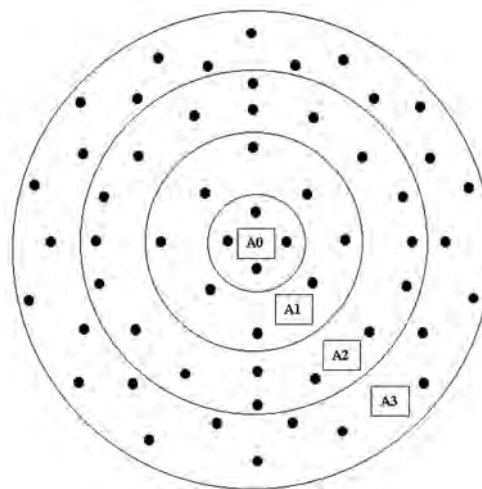


Fig. 1. Schematic representation of the experimental area: **A0** (4 tomato plants inoculated by 10⁸ cfu ml⁻¹ of *P. s.* pv. *tomato* suspension); **A1** (8 healthy tomato plants); **A2** (18 healthy tomato plants); **A3** (27 healthy tomato plants); all areas had the same plant density (17 plants/m²).

(four inoculated tomato plants in A0 and, A1, A2, A3 areas around), were used without any rain simulation.

Effect of rain on horizontal distribution of the bacteria. To study the effect of rain on horizontal spread, for each test, 12 plants were inoculated with a bacterial suspension (10^8 cfu/mL). Four of these plants were kept as controls in another chamber under the same conditions as the experimental area and rain (A and B) was simulated for 30 min. Both groups of plants were monitored for one month after inoculation. Each test was repeated three times.

At 1, 3, 5, 10 and 15 days after rain simulation, leaf samples were collected randomly. Each leaf sample included 8, 16, 36 and 54 leaflets from the A0, A1, A2 and A3 areas, respectively. To evaluate the influence of the rain on the inoculated area (A0), leaf samples were taken before and immediately after the rain. Each leaf sample was placed in a sterile flask with an appropriate amount of sterile distilled water (20 to 200 mL depending on leaf dimension) and washed in an orbital shaker for 1 h at 150 rpm. Serial dilutions of the washing water were plated (0.1 mL/dish) twice on KBr medium. Plates were incubated at $25 \pm 2^\circ\text{C}$ for 48h, and, based on morphological characteristics, *P. s. pv. tomato* colonies were counted using a stereomicroscope. The mean numbers of colonies per plate was calculated to determine the bacterial presence in the original suspension. These estimates were normalized by measuring leaf area with a Delta T Area Meter Device (Decagon Devices Inc. Pullman, WA). During each experiment, to verify possible plant contamination with other fluorescent bacterial populations that colonize tomato phyllosphere (*P. fluorescens*, *P. putida*), 10% of recovered colonies were submitted to the oxidase test (Sands, 1990).

Effect of rain on vertical distribution of the bacteria. To study the effect of rain on vertical distribution, rain simulations were conducted for a 20-day period and repeated three times. In these experiments only plants already colonized (C) were used. Leaf sampling was the same as described above, but leaves were only collected twice, at distal (1st) and basal (3rd) node level. The replica print method (Leben *et al.*, 1970) for qualitative analysis and leaf washing methods for quantitative analysis, were used, on leaflets chosen at random. Each leaflet was replica-printed (both surfaces), to evaluate bacterial distribution at different leaf positions. Leaflets were pressed onto KBr medium plates, which were then incubated at $25 \pm 2^\circ\text{C}$ and observed under a stereomicroscope after 48-72h.

In addition, single leaflets were placed in sterile tubes with an appropriate amount of sterile distilled water (5 to 10 mL depending on leaf dimension) and washed in an orbital shaker for 1 h at 150 rpm. Serial dilutions of the washing water were plated (0.1 mL/dish) twice on KBr

medium. Plates were incubated at $25 \pm 2^\circ\text{C}$ for 48h, and then *P. s. pv. tomato* colonies were counted using a stereomicroscope. Similarly, in order to study the horizontal distribution of the pathogen, the same methods were used to estimate the *P. s. pv. tomato* cfu/cm² tomato leaf.

Effect of rain on epiphytic survival of *P. s. pv. tomato* populations. To verify the possible effect of rain (A and B) duration (10, 20 and 30 min) on the epiphytic survival of the bacterium, the same methods were applied. Also, in order to collect the water run-off a sterile funnel was fixed on a cylinder under single leaflets of 3 random leaflets per plant to quantify the cfu/cm². The tests were done on C and NC plants (ten plants/treatment).

Statistical analyses. Each experiment was carried out three times. Analysis of variance and means comparison tests were performed using the Statistical Analysis Systems (SAS Institute Inc. Cary, N.C.) software.

RESULTS

Effect of rain on horizontal distribution of *P. s. pv. tomato*. After rain A on NC plants, in area A0 1 day after rain simulation, bacterial numbers were reduced; in area A1 on NC plants after 7 days, numbers were similar to those of area A0 (Fig. 2, NC). In areas A2 and A3

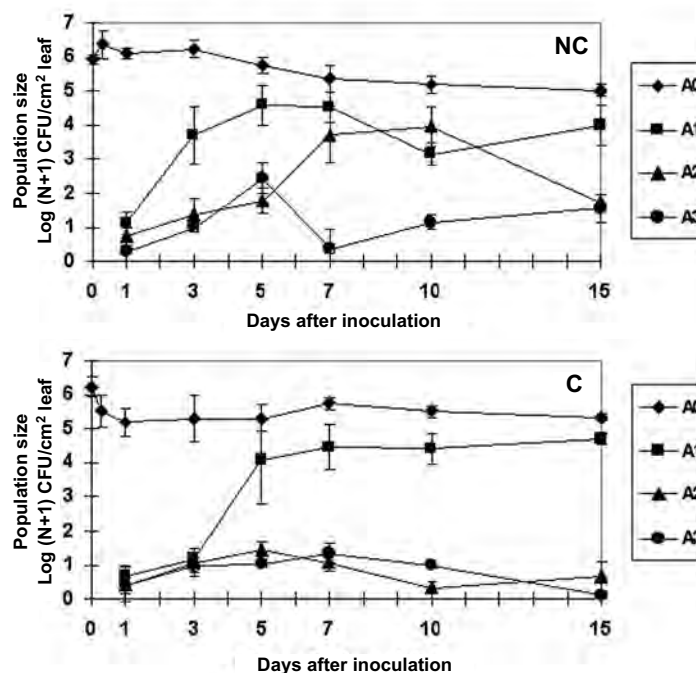


Fig. 2. Populations of *P. s. pv. tomato* in the tomato canopy in different areas (A0, A1, A2, A3), after rain A simulation (with 1.6 mm diameter rain drops) on non-colonized (NC), and on colonized (C) hosts, (see text). The vertical bars represent the standard error of the mean log-transformed population sizes at the given sampling time. Each point represents the mean bacterial population size determined from three replicate experiments.

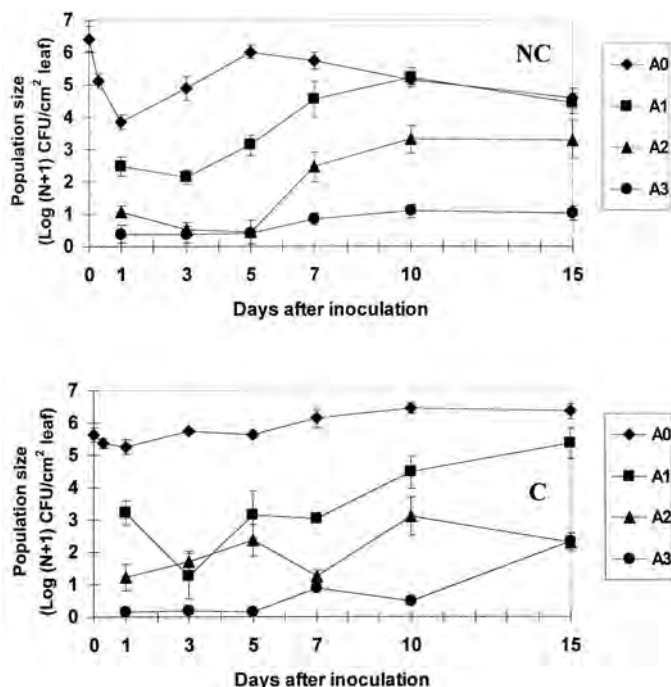


Fig. 3. Populations of *P. s. pv. tomato* in the tomato canopy in different areas (A0, A1, A2, A3) after rain B simulation (with 2.7 mm diameter rain drops) on non-colonized (NC), and on colonized (C) hosts, (see text). The vertical bars represent the standard error of the mean log-transformed population sizes at the given sampling time. Each point represents the mean bacterial population size determined from three replicate experiments.

on NC plants 1 day after rain simulation, values were below 1×10^1 cfu/cm² leaf (Fig. 2, NC).

After rain A on C plants, in area A0 1 day after rain, bacterial numbers decreased from 5.4×10^6 cfu/cm² leaf to 3.5×10^5 cfu/cm² leaf, reaching 2.9×10^5 cfu/cm² leaf at day 15 (Fig. 2C). In area A1 3 days after rain, numbers tended to increase (1.4×10^4 cfu/cm² leaf at day 5 and 6.2×10^4 cfu/cm² leaf at day 15 (Fig. 2, C). In areas A2 and A3 numbers reached the highest values at day 5 and day 7, respectively (Fig. 2C).

After rain B on NC plants, in A0 area bacterial numbers decreased; in A1, A2 and A3 areas, numbers were 8×10^4 , 1.8×10^3 and 1×10^1 cfu/cm² leaf, at day 15, respectively (Fig. 3NC).

After rain B on C tomato plants, in A0 area bacterial numbers increased until to 4.2×10^6 cfu/cm² leaf at day 15 (Fig. 3C). At day 15, in A1 area numbers were 2.9×10^5 cfu/cm² leaf and in A2 and A3 areas 4.1×10^2 cfu/cm² leaf (Fig. 3C).

All bacterial colonies submitted to the oxidase test were negative. No *P. s. pv. tomato* were recorded on plants in areas A1-A3 in experiments without rain simulation.

Effect of rain on vertical distribution of *P. s. pv. tomato*. Replica printing showed qualitative effects of

rain A on vertical distribution in different areas (A1, A2, A3); bacterial colonies were found at both leaf surfaces after two weeks at both the 1st and 3rd node (Fig. 4).

After rain B, distribution of bacterial colonies increased on the lower leaf surface at 3rd node leaf level, especially in areas A1 and A2 (Fig. 5).

Effect of rain on epiphytic survival of *P. s. pv. tomato*. During rain A, mainly within 20 min on NC hosts, the bacteria were washed away from leaves (from 2.1×10^6 to 4.8×10^4 cfu/cm² leaf) (Fig. 6A). On C hosts after the same rain simulation, population sizes increased (from 9.3×10^5 to 2.5×10^6 cfu/cm² leaf) (Fig. 6A).

During rain B on NC hosts, bacteria tended to be removed from leaves (from 5.1×10^6 to 2×10^5 cfu/cm² leaf) while, on C plants, no clear differences in population size were recorded (from 6×10^5 to 5.3×10^5 cfu/cm² leaf) (Fig. 6B).

On C plants one month after inoculation, the bacterium was able to rebuild its population up to 10^5 cfu/cm² per leaf. On NC host plants, populations were reduced by increasing the duration of rain B (from 3.3×10^6 to 1×10^5 cfu/cm² leaf), while no clear change was recorded on the C plants.

DISCUSSION

This study shows that epiphytic populations of *P. s. pv. tomato* are influenced by rain events with different characteristics. This seems to depend partially on the diameter of rain drops as related to their kinetic energy (Huber *et al.*, 1988).

According to previous studies (Hirano *et al.*, 1996; Sabaratnam and Beattie, 2003), the increase of *P. s. pv. tomato* seems to be related to peak rainfall intensities triggering growth of bacterial leaf pathogens.

Bacterial population sizes decreased 70-90 % on NC plants after rain (to 2×10^5 and to 5.8×10^4 cfu/cm² leaf, in A0 areas, after rain A and B, respectively) while they increased on C plants (from 10^5 to 10^6 cfu/cm² leaf). Rain caused an increase in the population of bacteria already established inside the host, while those bacteria with a precarious relation to host plants (non-colonized) were washed away.

Our results show that bigger rain drops influence the distribution of bacteria for a short distance around the inoculum source, while smaller rain drops affected long distance distribution.

In vertical distribution experiments with rain B (2.7mm in diameter), the lower surface at 3rd node leaf level (basal) was better colonized than with rain A (drops 1.6 mm in diameter) probably because drop fragmentation helps bacteria to return by means of vapor to the leaves.

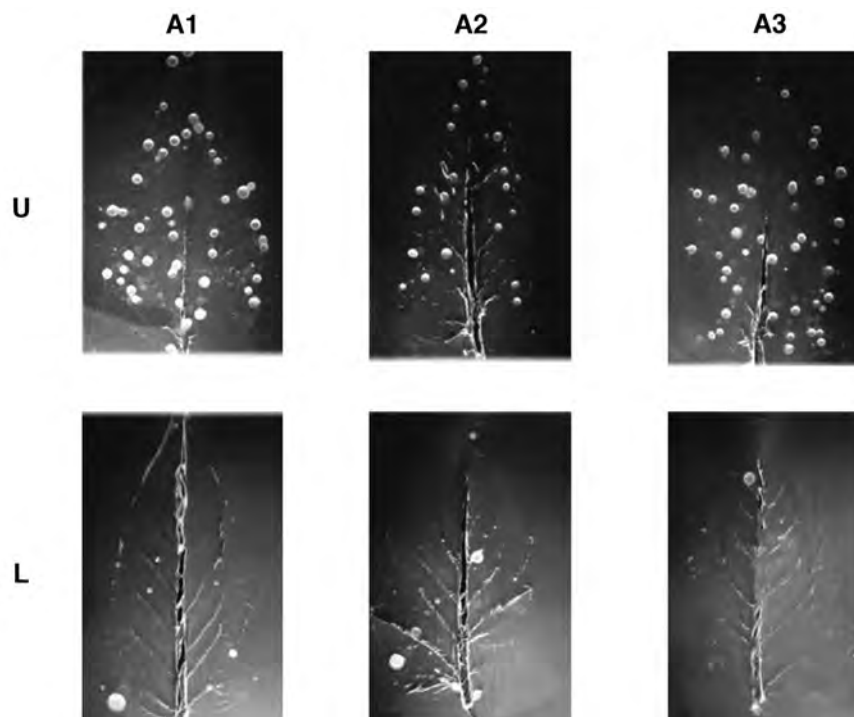


Fig. 4. Colonies of *P. s. pv. tomato* on tomato leaflets replica-printed on KBr medium 3 days after type A rain simulation. The bacteria were more abundant on the upper leaf surface (U) than on the lower surface (L), at both 1st and 3rd node leaf levels. During the first week the colonies were distributed on the upper leaf surface and after two weeks were also found on the lower surfaces at both the 1st and 3rd node. After 1 day at 1st and 3rd node level, in area A1 numbers were 3.9×10^5 and 3.1×10^5 cfu/cm² leaf, respectively; in A2 and A3 areas were 2.2×10^3 and 1.8×10^2 cfu/cm² leaf. One week later population sizes had not appreciably changed at different node levels in different experimental areas (A1-A3).

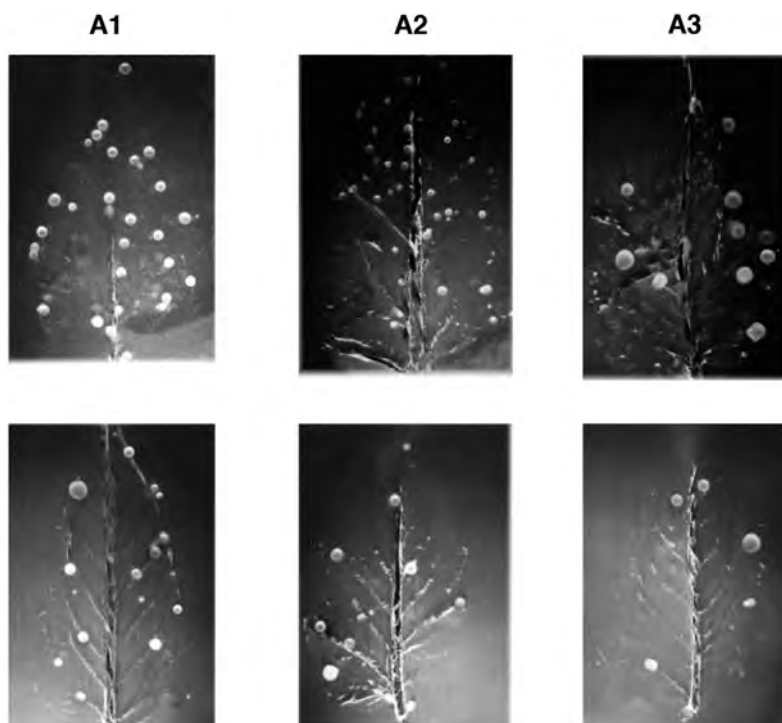


Fig. 5. Colonies of *P. s. pv. tomato* on tomato leaflets gently pressed replica-printed on KBr medium 3 days after type B rain simulation. The bacteria were uniformly distributed on the upper surfaces of 1st and 3rd node leaves especially on the lower leaf surface at 3rd node leaf level, especially in areas A1 and A2. After 1 day at 1st and 3rd node level, in area A1, numbers were 6.3×10^5 and 5.2×10^5 cfu/cm² leaf respectively; in areas A2 and A3, were 4.1×10^3 and 2.7×10^2 cfu/cm² leaf. One week after rain B simulation, numbers were 4.5×10^5 , 3.5×10^4 and 2.2×10^2 cfu/cm² leaf, in areas A1, A2 and A3, respectively.

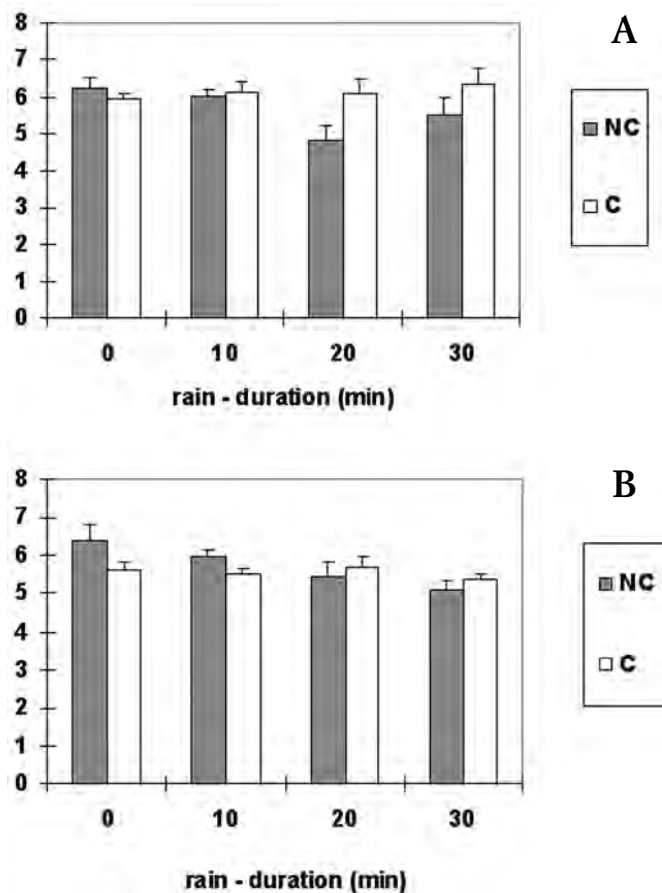


Fig. 6. *P. s.* pv. *tomato* populations on non-colonized (NC) and colonized (C) tomato canopy after different durations of simulated rain **A**: 1.6 mm diameter drops; **B**: 2.7 mm diameter drops). The vertical bars represent the standard error of the mean log-transformed population sizes recorded at the 1st and 3rd node leaf level at the given sampling time. All the results were significant ($P < 0.05$). Each bar represents the mean bacterial population size determined from three replicate experiments.

The results obtained show that rain action on *P. s.* pv. *tomato* populations seems to be related to host-pathogen interaction at the time of the event. In addition, it shows that rain is involved in lateral dissemination of bacteria and, when rain is particularly heavy prior to the emergence of symptoms. Moreover, rain was shown to influence the micro-climate in the host phyllosphere, producing favourable conditions for survival and reproduction of the pathogen (Beattie and Lindow, 1995; Upper and Hirano, 2002; Upper *et al.*, 2003).

Under natural rainfall almost all bacteria on the leaf surface may be washed away, but those in protected sites can re-build their populations thanks to favorable conditions (absence of antagonists, discharge of toxic substances, high relative humidity, temperature) (Hirano *et al.*, 1987; Lindow and Brandl, 2003; Upper *et al.*, 2003).

Future studies will better explain the effects of rain

on non-pathogenic epiphytes, on incompatible pathogenic populations (Wilson *et al.*, 1999) and on the ability of particular plant species to support large bacterial populations (Morris *et al.*, 1997; Monier and Lindow, 2003). As with fungal spores (Fitt *et al.*, 1986; Pielaat *et al.*, 1998; Paul *et al.*, 2004) more studies, related to the effects of rain on the behavior of bacteria, will help in developing new models combining theoretical and physical parameters and will take into account the frequency and characteristics of rain events during the growing season.

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