

**MOLECULAR AND PATHOLOGICAL STUDIES ON BACTERIAL WILT DISEASE
CAUSED BY**

Ralstonia solanacearum

OF POTATO AND TOMATO CROPS

By

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5. SUMMARY

Bacterial wilt of tomato and potato caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al.*, (Syn. *Pseudomonas solanacearum* E.F. Smith) is one of the most important diseases of tomato and potato causing complete failure of some crops in upper Egypt governorates and lower Egypt governorates. Studies on various aspects of the disease viz., occurrence, survey, symptomatology, pathogenicity, cultural, morphological, biochemical and molecular variability amongst *R. solanacearum* isolates, and disease management were undertaken and the results obtained are summarized as follows.

- A survey was conducted throughout seventeen Egyptian governorates and the percentage of disease incidence and disease severity were calculated during 2014-2015, transplanting season.
- Bacterial wilt disease was distributed over the entire tested tomato and potato field and over all averages of disease incidence and disease severity were 9.88 & 3.01%, for tomato and 11.30 & 4.12% for potato in upper Egypt governorates while, in lower Egypt governorates were 12.30 & 4.00%, for tomato and 18.52 & 5.75% for potato respectively. In tomato the highest mean (10.29%) bacterial wilt incidence in tomato was observed in Luxor governorate followed by Assuit (10.21%) and Al- Minia governorates (10.10%), while Al- Giza governorate tested field recorded the lowest significantly percentage of disease incidence being 9.41% followed by Sohag and Al- Fayoum governorates 9.59 and 9.69% respectively. While in lower Egypt , Al- Behiera governorate, fields recorded a higher disease incidence and severity than all governorates, the highest disease incidence were 15.28% followed by Al-Daqahlia, Al- Gharbia, Kafr Al- Shikh and Al- Sharqia governorates which were 13.23, 13.14, 12.15 and 12.14% respectively.
- In potato bacterial wilt of potato was observed in the early growth or during tuberization and continued up to crop maturity. Al- Minia governorate tested fields were recorded the highest significantly percentage of bacterial wilt disease followed by Beni Sueif and Al- Giza governorates being 12.12, 11.86 and 11.04%, respectively, While Sohag and Al- Fayoum governorates tested fields recorded the lowest significantly percentage of disease incidence being 10.64 and 10.85%, respectively. In Al- Behiera governorate fields a higher disease incidence and severity was observed than other lower Egypt governorates areas (23.20 and

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- 7.68%) respectively followed by Al- Ismailia, Al- Qalioubia and Al- Gharbia governorates which were (20.49 and 6.53%); (20.01 and 5.94%) and (19.88 and 6.14%), respectively.
- 2356 bacterial isolates were isolated from infected tomato, potato stems, potato tubers and soil samples collected from seventeen Egyptian governorates. 1045 isolates (44.36%), obtained from infected tomato plants, 1065 isolates obtained from potato stems and tubers (45.20%) and 246 isolates obtained from soil samples (10.44%). At least three isolates were chosen to represent all districts and hosts based on their cultural characteristics on TZC medium.
 - At the end of the preliminary screening, 614 out of 2356 isolates were chosen to represent all the surveyed districts of all governorates.
 - The highest virulent isolates (150 isolates) were chosen to represent the host and geographical location and submitted to successive round of experiments.
 - All isolates of *Ralstonia solanacearum* were able to produce wilt symptoms in tomato and potato plants, 10 isolates showed the highest virulence were selected for further studies.
 - *R. solanacearum* (R2352) recorded the highest infection % where it gave 100% with (53.87 and 23.65% disease severity) on tomato and (49.91 and 20.08% disease severity) on potato at 35 day post inoculation the sterilized and non- sterilized soil respectively, with bacterial wilt pathogens. *R. solanacearum* (R766) came in second rank where it caused 100% infection with (52.07 and 22.88% disease severity) on tomato and (49, 84 and 19.81% disease severity) on potato at 45 day post inoculation followed by *R. solanacearum* (R2033). The least infection% was recorded by *R. solanacearum* (R436) where it recorded (49.79 and 19.64% disease severity) on tomato and (46.56, 84 and 16.07% disease severity) on potato followed by *R. solanacearum* (R137), compared with the un-inoculated control under greenhouse conditions.
 - Studies on morphological characters revealed that the bacterial isolates were rod shaped, gram negative, non-spore forming and motile. Based on symptomatology, cultural, morphological and biochemical characteristics and pathogenicity test, the test pathogen was identified as *R. solanacearum* and its further identity was confirmed.
 - The temperature of 25 and 30^o C were found optimum for the growth of pathogen as the highest growth were recorded at this temperature level followed by 15, 20 and 35^oC. However, the pathogen grew at temperature of 15^oC, but it failed to grow at the lowest and highest extreme temperatures of 5, 45 and 50^oC.

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- The bacterial isolate can survive within a pH range of 4-10, though there is very low growth at pH-4 and at pH-10 but in between the pH there was luxuriant growth. At pH -3 the growth of the isolate was ceased.
- *R. solanacearum* isolates showed variation growth of different concentration of sodium chloride. They showed abundant growth at 1%, normal growth at 2%, scant growth, at 3% and 4%, no growth at 5%, 6%, 7%, 8% and 9% in nutrient broth.
- All *R. solanacearum*-isolates gave positive reaction with tests of KOH test, catalase activity, oxidase activity, nitrate reduction, citrate utilization, However, all isolates of *R. solanacearum* gave negative reactions when tested for production of fluorescent on King B, gelatin liquefaction, starch hydrolysis, levan production, H₂S production, indole production, urease reduction and potato soft rot, and most of them are positive reaction with test of potato brown rot.
- *R. solanacearum* isolates oxidized disaccharides (Lactose, Maltose, Cellobiose, Sucrose and Glucose) and sugar alcohols (Mannitol, Sorbitol and Dulcitol.)
- Oxidization and utilization disaccharides and hexahydric alcohols tests differentiated all the strains into five biovars I, II, III, IV and V.
- Eggplant (*Solanum melongena* L.) var., Marwa, pepper (*Capsicum annum* L.) var., El- Madina and geranium (*Pelargonium graveolens* L.) plants were inoculated with the most virulent *R. solanacearum* isolates (10 isolates encoded: R137, R436, R488, 610, R766, R859, R993, R2033, R2231 and R2352) to determine their host range. All tested plants were differed in their susceptibility. Eggplant was the most susceptible to infect with bacterial wilt strains, followed by pepper and geranium which were 27.17, 19.14 and 17.44% severity respectively.
- Twenty varieties of tomato and twenty five cultivars of potato were screened under greenhouse condition against bacterial wilt caused by *Ralstonia solanacearum*. All tested *R. solanacearum* strains caused bacterial wilt disease symptoms on tomato varieties and potato cultivars compared with the un-inoculated control. All tested tomato varieties and potato cultivars were susceptible to infection either grown in sterilized and un-sterilized soil. The tested varieties and cultivars were significantly varied in this respect. Also, the infectivity of a tested *R. solanacearum* isolate seemed to be varied in the sterilized and un-sterilized soil.

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- As for sterilized inoculated soil, none of tomato varieties were found to be resistant against tomato bacterial wilt. Two varieties (Nairouz and Super Streem B) were found moderately susceptible and four varieties (737, Super marmand, 186 and El-Basha) were found susceptible varieties and fourteen (765, 935, N23, Beto, Beto 086, Crestal palas, Elissa, Gold stone, G.S., Loujain, Marmand, Marwa, Nada and Nema) showed high susceptibility location severity index of 0.61–0.9. In un-sterilized inoculated soil of the 20 varieties tested, six varieties (737, 010, 186, El-Basha, Nairouz and Super Streem B) had a mean disease index of less than 0.51 (moderately susceptible), while nine varieties (765, 935, N23, Beto 086, Crestal palas, Elissa, Loujain, Marwa and Nema) scored a mean index of less than 0.61 (susceptible). Five tested varieties (Beto, Gold Stone, G.S., Marmand and Nada) showed high susceptibility location severity index of 0.61–0.9.
- The highest scores of disease severity (Extremely susceptible) on the potato cultivars Silina (1.18), Diamont (1.05), Santana (1.03) and Flora (0.95). While five cultivars (Picasso, Penllini, Nicola, Spunta and Vivaldi) were found susceptible and sixteen potato cultivars were found highly susceptible in sterilized inoculated soil. In the un-sterilized soil, 10 cultivars (Amarin, Arizona, Bamba, Picasso, Pellini, Cara, Donia, Nicola, Spunta and Vivaldi) showed moderately susceptible location severity index of (0.41- 0.50). While eight cultivars (Barren, Benlour, Elictra, Gelatica, Kenza, Margrita, Mondial and Sinora) were found susceptible against potato wilt disease. While, Diamont, Draga, Elmoudo, Flora, Krouz, Santana and Silina cvs. were highly susceptible to bacterial wilt disease.
- There were very close similarity without any variation among the all tested plants and the positive control (*R. solanacearum* identified by Brown Rot Project in Egypt) one under investigation to confirm that these tested bacterial infections are *R. solanacearum*.
- Phylotype specific multiplex PCR revealed that 10 *R. solanacearum* strains from different locations of Egypt belonged to Phylotype II as 288 and 372 bp amplicon was observed in all the strains when Phylotype specific multiplex (Pmx- PCR) products of these strains were subjected to electrophoresis on 1.5% Agarose gel.
- Total counts of tomato and potato rhizosphere microorganism were (201×10^4 , 309×10^6 and 161×10^5) and (170×10^4 , 259×10^6 and 141×10^5), for fungal (*Trichoderma* spp.); bacterial (*Bacillus* and *Pseudomonas*) and actinomycetes isolates, for tomato and potato respectively.

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- The antagonistic isolates were identified and coded as *Trichoderma harzianum* isolates (TH17, TH43, TH58, TH69 and TH71), *T. viride* isolates (TV77, TV89, TV105, and TV113) and *T. koningii* isolates (TK121, TK135 and TK141), *Bacillus subtilis* isolates (B9, B23, B45 and B103), *Pseudomonas fluorescens* isolates (Pf13, Pf 47, Pf 83, Pf 98, Pf 107 and Pf 118) and *Streptomyces* spp. (*S. fumigatisclerotis* (St14), *S. rochei* (St19), *S. avermitilis* (St27), *S. griseus* (St33), *S. griseviridis* (St38), *S. violaceusniger* (St40) and *Streptomyces* spp., (St41). These isolates were used for biological control studies.
- A screening of 568 bacterial isolates isolated from tomato (309 isolates) and potato (259 isolates) healthy plants rhizosphere; these isolates were isolated from different localities revealed that, ten screened bacterial isolates; four *Bacillus subtilis* (B9, B23, B45 and B103) and six *Pseudomonas fluorescens* (Pf13, Pf 47, Pf 83, Pf 98, Pf 107 and Pf 118) isolates resulted to be the most interesting isolate in terms of *Ralstonia* strains growth inhibition.
- The antagonistic effect of bacterial isolates Pf 118 and B103 were more effective on the reduction of growth and increasing the inhibitory effect on the tested *Ralstonia* strains than other isolates, followed by B9 and B23 (23.65 and 22.93 %) and the lowest inhibition was exhibited by Pf13 (20.67%).
- Four out of 161 strains for tomato and 3 out of 141 strains for potato were chosen and submitted to successive round of experiments. The highest antibacterial activity against *Ralstonia* strains was recorded for *S. griseus* (St33), *S. fumigatisclerotis* (St14) and *S. griseviridis* (St38), which exhibit inhibition 24.78, 24.89 and 24.20 %, respectively. While, *Streptomyces* spp., exhibit lowest inhibition (20.71 %).
- Out of 371 fungi tested, 12 *Trichoderma* isolates showed the highest antagonistic property against the pathogen. *T. harzianum* (TH58) recorded the highest reduction average followed by *T. harzianum* (TH43) and *T. harzianum* (TH17), which were 30.69, 30.63 and 30.56 % respectively.
- All *Ralstonia* strains were significantly inhibited by all different bacteriocin produced from lactic acid bacterial strains used in this study, the highest antibacterial activity against *Ralstonia* strains was recorded for Lab100, Lab2, Lab58 and Lab107, which exhibit clear zone diameter 21.08, 20.93, 20.70 and 20.54 mm, respectively. While, bacteriocin from strain Lab105, exhibit clear zone diameter 20.33 mm.

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- The maximum inhibition zone of 16.13mm diameter was observed in clove oil followed by cinnamon oil which showed inhibition zone of 15.56 mm diameter, whereas, garlic oil and eucalyptus oil showed inhibition zone of 14.71 and 14.92 mm diameter against *R. solanacearum*. R2352 *Ralstonia* strain was the most sensitive strain to all tested essential oils, followed by R993 strain, while R2033 and R436 *Ralstonia* strains were the least sensitive to all tested essential oils.
- All tested concentrations of chitosan have significantly reduced the growth of *Ralstonia* strains compared to the control. Reduction of growth tested strains increased as the concentrations of chitosan are increased; the highest antibacterial activity against *Ralstonia* strains was obtained with 4.50% chitosan concentration, which exhibit clear zone diameter 17.56mm.
- All treatments of induce resistance compounds significantly reduced the growth of all *Ralstonia* strains. The highest antibacterial activity of the salt and antioxidant compounds used in this study was 16.75 and 16.08 mm for salicylic acid and ascorbic acid, respectively, followed by copper sulphate and potassium sorbate, which recorded the antibacterial activity, 14.96 and 14.29 mm, respectively. Clear zone diameter increased as the concentrations of mineral salt compounds and antioxidants are increased.
- All the tested bio-agents isolates significantly reduced the average percentages of bacterial wilt incidence for both tomato and potato plants. In general, wilt incidence was reduced more by *Streptomyces griseus* (St33) followed by *Streptomyces fumigatisclerotis* (St14) than by other treatments for both tomato and potato wilt. Similar trend was also observed concerning treatments applied as a soil drench and dipping significantly reduced disease infection and disease severity by (19.16 and 11.31 %), in tomato and (19.99 and 10.22%), in potato respectively.
- Under artificial infection all bacteriocin-like substance treated tomato and potato plants showed significantly decreased on wilt disease incidence than positive control.
- Treatments with bacteriocin from Lab100 strain was the most effective against bacterial wilt disease incidence in tomato which were (17.77 % infection and 10.18% severity) followed by bacteriocin from Lab 58 strain (24.44 infection and 10.81% severity) respectively, while in potato bacteriocin from Lab102 strain was the most effective against bacterial wilt disease incidence followed by bacteriocin from Lab100.

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- Under greenhouse conditions, treatments with chitosan significantly reduced the average of infection percentage (IP) and disease severity (DS) of tomato and potato comparing to the positive control.
- The average of infection percentage (IP) and disease severity (DS) reduced from 100 and 53.21% in control to 46.66 and 18.05% in tomato and 40.00 and 12.70% in potato respectively.
- Tested chitosan was most effective against the bacterial wilt disease when it applied as pre & post- transplantation which were (42.22 and 22.63%) and (40.00 and 18.66%) incidence for both tomato and potato respectively.
- All tested essential oils have significantly ($P \leq 0.05$) reduced tomato and potato bacterial wilt incidence and severity compared with positive control.
- Tested essential oils were most effective against bacterial wilt disease when they were applied as pre& post-transplanting treatments.
- Applications of essential oils as pre- transplanting treatments were moderately effective against the disease and were less effective when they were applied as post- transplanting treatments.
- Clove oil was the most efficient treatment in controlling the disease followed by thyme oil and cinnamon oil in both tomato and potato.
- Salicylic acid (15 and 10 mM) was the most effective in reducing the bacterial wilt disease infection which were in concentration 15 Mm, 24.44 and 14.00% infection and severity in tomato. In potato salicylic acid (15 mM) and ascorbic acid (15 Mm) concentrations were the most effective in suppressive potato bacterial wilt which were 20.00, 9.36, 22.22 and 9.94% infection and severity respectively.
- Pre& post-transplanting treatments with induce resistance compounds were the most effective against bacterial wilt disease which were followed by pre then post-transplanting treatments in tomato and potato.
- All the treatments of bio- agents mixture or in combination with other treatments proved significantly superior over control. Among them, the highest percent plant infection was found in to (control) which was 100%, the most effective treatments in protective effect of tomato against bacterial wilt disease were T10 (TH58+ TH69+ Thyme) and T7 (TH58+ TH69+ Chitosan 4.5%) which recorded 15.55% infection.

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- The combination treatments T22 (TH58 + TH69 + Benzoic acid 15 mM) and T15 (TH58 + TH69 + Cooper sulphate 10 Mm), showed the lowest disease suppression for tomato which were 57.78 and 57.77% respectively.
- Similar trend was also observed concerning potato wilt infection, T10 and T7 Treatments are the most effective in protective of potato against development of wilt disease, which reduced the wilt by 13.33%, while T22 and T15 showed the lowest disease suppression which were 48.88 and 46.66% respectively.
- In general, using any mixture of two trichoderma isolates or combination cause significant protective effect against the bacterial wilt, compare with positive control treatment.
- Antagonistic bacterial isolates suppressed disease when mixed together with the pathogen through the soil or when seedlings were treated with the strains before transfer into soil infested with *R. solanacearum*.
- T7 (Pfl18+ B103 + Chitosan 4.5%) and T10 (Pfl18+ B103 + Thyme oil) recorded the highest suppressive efficacy of bacterial wilt in tomato and potato which were in tomato (26.66 and 33.33%) respectively. While in potato were 28.89 and 31.11% respectively. Combination of antagonistic bacterial isolates with bacteriocin like- substances (T5, T6 and T4) recorded the lowest effect for both tomato and potato wilt infection which was in tomato 57.77, 53.33 and 51.11% respectively. While, in potato recorded 55.55, 51.03 and 46.66% respectively. The lowest bacterial wilt severity was recorded in T7 and T10 11.18 and 11.34%for tomato and 10.16 and 10.89% for potato.
- All combined treatments with *Streptomyces* spp., isolates significantly decrease the wilt infection. T3 (St14+St33) and T7 (St14+ St33 + Chitosan 4.5%) treatments were the most effective in protective of tomato against development of wilt disease, which reduced the wilt by (20.70 and 20.85 % infection) and (8.53and 13.22% severity) respectively. T21 and T15 treatments recorded the lowest suppressive efficacy of bacterial wilt in tomato which recorded 50.48 and 46.85 % infection and 14.48 and 13.22% severity respectively.
- Similar trend was also observed concerning potato wilt infection, T3 (St14+St33) and T7 (St14+ St33 + Chitosan 4.5%) treatments were the most effective in protective of potato against development of wilt disease. Also, T22 and T15 treatments recorded the lowest suppressive efficacy of bacterial wilt in potato.

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- All the treatments recorded disease infection, over untreated control and it was ranged from 15.55 to 57.77%, as against 100% in untreated control in tomato. While disease severity ranged from 8.07 to 19.47% as against 53.21% in untreated control in tomato. T7 (Lab58+ Lab100+ Lab102+ Chitosan 5%) which was 15.55 and 8.07% infection and severity respectively. This was followed by treatments *viz.*, T3 (Lab58+Lab100+ Lab102) and T8 (Lab58+Lab100+ Lab102+ Clove) . T15 (Lab58+ Lab100+ Lab102 + Cooper sulphate10 mM) (44.44 and 14.71%) and T13 (Lab58+ Lab100+ Lab102+ Potassium sorbate 10 Mm) (44.44 and 14.16%) were found least effective with comparatively minimum percent disease control.
- In potato, T7 (Lab58+ Lab100+ Lab102+ Chitosan 5%) recorded the highest suppressive efficacy of bacterial wilt in potato (13.33 and 8.99%) followed by 15.55, 9.11.20.00 and 9.33 % in T3 and T8. Also, T15, T14 and T13 treatments recorded the lowest suppressive efficacy of bacterial wilt in potato which were (60.00, 17.71, 53.33, 16.80, 48.89 and 15.81%) respectively.