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Effect of temperature and pH level on growth of bacterial wilt causing *R. solanacearum* under *in vitro* condition

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Abstract
Bacterial wilt is one of the major diseases of all solanaceous plants. The disease is known to occur in the wet tropical, subtropical and temperate regions of the world. The disease is caused by the bacterium *Ralstonia solanacearum* (Smith) Yabuuchi *et al*, previously known as *Pseudomonas solanacearum*. In Chhattisgarh state, Bacterial *wilt* of solanaceous crop caused by *R. solanacearum* has been grouped under Race 1 and Biovar 3 in the previous study, which attacks wide range of crop plants, ornamentals and weeds in extremely dangerous conditions at 25-28 ºC temperature and 5.5-6.6 pH in field conditions. Whereas, other groups of races of *R. solanacearum* have more severe in 35-37 ºC temperatures range. Soil temperature and pH is a major factor affecting the wilt causing bacterial and fungal pathogens and soil microbial community. In the present study, four temperature ranges i.e. 20 ºC, 25 ºC, 30 ºC and 35 ºC and 10 pH levels i.e. 4, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 on *R. solanacearum* were tested under *in vitro* condition and found that the population of *R. solanacearum* (443.33 cfu) were significantly higher on 6.50 pH level at 30ºC after 96 hours of inoculation on TZC medium and the population of *R. solanacearum* was not able to grow in 4 pH at 20 ºC, 25 ºC, 35ºC and 40 ºC.

Keywords: *R. solanacearum*, Temperature, pH, Bacterial wilt of solanaceous crop

Introduction
The disease is most severe at 24-35 °C soil temperature and a high soil moisture percentage of 80% above. It is found that the growth of *R. solanacearum* bacterium is strongly inhibited at below 5 ºC and above 40 ºC temperature. Soil moisture and temperature are commonly optimum during the periods of wet weather or rainy seasons and are associated with high disease severity. Among this soil moisture is also one of the major factors affecting reproduction and survival of the *R. solanacearum*. *R. solanacearum* is a typical soil borne bacterium, the occurrence; spread and prevalence of this wilt causing bacteria are closely related to soil pH and other properties of soil. Continuous cropping of susceptible host plants and use of chemical fertilizers in excessive amount have led to the degradation of soil physicochemical properties.

Review of Literature
Temperature and pH are the most important factors that affect host-pathogen interactions. Temperature has been reported to have a direct effect on the pathogen. Most bacterial strains of *R. solanacearum* grow optimally at 30-32ºC; they do not multiply at 4 or 40^{0}C and growth is highly inhibited at 2% NaCl. They produce fluidal, irregularly round, white colonies with pink centers on 2,3,5-triphenyl tetrazolium chloride-amended (TZC) medium (Kelman 1954) [7]. In many studies, it was observed that the optimal growth temperature for most of the bacterial strains of *R. solanacearum* falls between 28-32 ^{0}C (Hayward, 1964; Schaad *et al.*, 2001) [5, 13]. Kalman (1954) reported approximate minimum and maximum growth temperature levels would be 8 to 20 ºC and 37 to 39 ºC, respectively and recording pH requirements, in general *R. solanacearum* growth was inhibited in acid media but favoured in alkaline conditions. Disease severity was significantly greater at 32.2ºC then at 26.6ºC in tomato cultivars resistant to *R. solanacearum* (Krause and Thurston, 1975). Ciampi and Security (1980) [2] reported that most of the *R. solanacearum* strains were non-pathogenic below 20ºC. Temperature of 30 to 35ºC was associated with an increase in severity of bacterial wilt in several hosts. The ability of pathogens to survive in the soil in the absence of a host for extended periods as well as in the protected niche of a weed's rhizosphere was also evaluated. Some soil types suppress the pathogen as the soil moisture determines the antagonistic population levels, which compete with *R. solanacearum.*

High temperatures (i.e. 30-35 ºC) promote occurrence of bacterial wilt disease caused by *R. solanacearum*, whereas soil temperatures below 20 ºC are not suitable for the disease (Gadewar *et al.*, 1993; Wang and Lin, 2005) [4, 15]. Champoiseau and Momol (2008) [1] suggested that high temperatures (29 to 35 ºC) played a major role in pathogen growth and disease development. Fajinmi and Fajinmi (2010) [3] reported that temperature was an important factor which affected the growth and aggressiveness of pathogens and expression of symptoms in the plant. Wilt disease is most severe on plants when temperature ranges between 25 to 35 ºC and its aggressiveness decreases, either above 35 ºC or below 18ºC temperature. The disease symptoms rarely appear below 18ºC temperature. Sahu *et al*. (2017) [12] conducted a temperature tolerance test for evaluating the thermal death point of *R. solanacearum*. It indicated that the pathogen survived up to 30ºC. Its growth deteriorated in the 30-40 ºC temperature range beyond this range; the pathogen did not show any growth. The results revealed a 37ºC thermal death point for *R. solanacearum*.

Materials and Methods

The single and interaction effects of factors (*e.g.* temperature and pH) were studied on the growth of bacterial isolates simultaneously. The pure bacterial cultures were multiplied separately in TZC broth. The culture was prepared by inoculating a loopful of bacterial cultures from stock culture to 100 ml TZC broth contained in 250 ml conical flask and incubated for 48 hours at 28ºC. Fifty µl of diluted bacterial culture was poured onto the surface of TTZ agar medium taken in sterilize petridishes containing different pH *viz.*, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. The bacterial suspension was uniformly spread with the help of a sterilized glass spreader so as to obtain well distributed bacterial colonies. The inoculated plates were incubated at different temperatures *viz.;* 20ºC, 25ºC, 30ºC, 35ºC and 40ºC for 96 hours. Observation was recorded on the number of colony forming units appearing after the incubation period at 48, 72 and 96 hours. To determine single or interaction effects of factors (*e.g.*, temperature and pH), data were subjected to square root transformation and statistical analysis using simple CRD (Completely Randomized Design). Since significant interactions were observed between factors, the level of one factor was compared at each level of the other factor.

Result and Discussion

The effect of four temperature range i.e. 20ºC, 25ºC, 30 ºC and 35ºC at 10 pH level i.e. 4, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 were evaluated on the growth of *R. solanacearum* under *in vitro*. The obtained data regarding influence of different temperature and pH level on the multiplication of *R. solanacearum* were significantly differing among different temperature and pH levels. The results are presented in table 1 and figure 1. At 20ºC temperature, the growth of *R. solanacearum* bacteria was significantly varied on different pH levels after 48 hours of inoculation on TZC medium. The growth of *R. solanacearum* was significantly higher on 6.50 pH (385.00 cfu) was statistically on par with level of 6.00 pH (369.33 cfu), 7.00 pH (354.33 cfu), 7.50 pH (348.67 cfu) and minimum cfu was in 4.50 pH (0.00 cfu). Whereas, 72 hours after inoculation, the growth of *R. solanacearum* bacteria was significantly higher on 6.50 pH (410.33 cfu) was statistically on par with 6.00 pH (408.00 cfu), 7.00 pH (396.67 cfu), 7.50 pH (391.33 cfu) and minimum cfu was in 4.50 pH (0.00 cfu). Finally at 96 hours after inoculation, the growth of *R. solanacearum* bacteria was significantly higher recorded with 435.33 cfu on 6.00 pH level was statistically on par with 7.50 pH (434.67 cfu), 7.00 pH (434.33 cfu), 6.50 pH (430.33 cfu), 8.50 pH (391.00 cfu) and minimum cfu was in 4.00 pH (0.00 cfu). At 25 ºC temperature after 48 hours of inoculation, the growth of *R. solanacearum* bacteria was significantly higher on 6.50 pH level (246.00 cfu) was statistically on par with 6.00 pH (229.33 cfu), 5.50 pH (192.33 cfu) and minimum cfu was in 4.50 pH (0.00 cfu). Whereas, at 25ºC temperature after 72 hours of inoculation, the growth of *R. solanacearum* bacteria was significantly higher on 6.00 pH level (289.67 cfu) was statistically on par with 6.50 pH (264.00 cfu), 5.50 pH (239.67 cfu) and minimum cfu was in 4.00 pH (0.00 cfu). Finally after 96 hours of inoculation, the growth of *R. solanacearum* bacteria was recorded significantly higher on 6.00 pH level (389.00 cfu) was statistically on par with 6.50 pH (371.33 cfu), 5.50 pH (358.33 cfu), 7.00 pH (327.00 cfu), 8.50 pH (294.00 cfu), 8.00 pH (293.33 cfu) was followed by the pH of 7.50 (283.67 cfu), 5.00 pH (104.33 cfu), 4.50 pH (22.33 cfu) and minimum cfu was in 4.00 pH (0.00 cfu). At 30ºC temperature after 48 hours of inoculation, the maximum and statistically significant growth of *R. solanacearum* bacteria was recorded at 6.50 pH level (431.00 cfu) and minimum cfu was at 4.00 pH (3.67 cfu). Whereas, at 30ºC temperature after 72 hours of inoculation, the growth of *R. solanacearum* bacteria was significantly higher on 6.50 pH level (434.33 cfu) was statistically on par with 7.00 pH (392.33 cfu), 6.00 pH (389.33 cfu) and minimum cfu was in 4.00 pH (7.00 cfu). Finally at 30 ºC temperature after 96 hours of inoculation on TZC medium, the growth of *R. solanacearum* bacteria was significantly higher on 6.50 pH level (443.33 cfu) was statistically on par with 5.50 pH (426.33 cfu), 6.00 pH (416.00 cfu), 7.00 pH (404.00 cfu), 5.00 pH (382.00 cfu) and minimum cfu was in 4.00 pH (11.33 cfu). At 35 ºC temperature after 48 hours of inoculation on TZC medium, the growth of *R. solanacearum* bacteria was recorded significantly higher on 6.50 pH level (215.67 cfu) was statistically on par with 7.50 pH (192.67 cfu) and minimum cfu was in 4.00 pH (0.00 cfu). Whereas at 35ºC temperature after 72 hours of inoculation on TZC medium, the growth of *R. solanacearum* bacteria was recorded significantly higher on 6.50 pH level (287.33 cfu) was statistically on par with 7.50 pH (258.33 cfu), 7.00 pH (256.67 cfu) and minimum cfu was in 4.00 pH (0.00 cfu). Finally at 35ºC temperature after 72 hours of inoculation on TZC medium, the growth of *R. solanacearum* was significantly higher on 6.50 pH level (322.00 cfu) was statistically on par with 7.00pH (316.33 cfu), 7.50pH (291.67 cfu) and minimum cfu was in 4.00pH (0.00 cfu) at 35ºC after 96 hours of inoculation.

In the present investigation the growth of *R. solanacearum* was completely checked in 4 pH at 20ºC, 25ºC, 35ºC and 40ºC except 30ºC. However the growth of *R. solanacearum* was slightly increased to 4.5pH in 20ºC to 35ºC temperature range. This result supports the previous finding that bacterial growth is suppressed under low pH conditions (Rousk *et al*., 2009) [11]. They reported that all microbial variables including bacteria, fungi nematodes, etc. were universally inhibited at or below 4.5pH. Probably inhibition of growth may be due to the release of free aluminum. Earlier, similar findings were also reported by Michel and Mew (1998) [9] that the growth of all strains of *Ralstonia* was suppressed at pH level of 3, 10, and 11 and strongly reduced at 4 and 9 pH ($P< 0.001$) whereas the growth

reduction was weak at 5 and 8 pH and normal growth of all strains occurred at 6pH. Similar observations on pH were made by Li *et al*., 2017 [8] that the *R. solanacearum* was able to grow within the pH range 4.5-8. They reported that the *R. solanacearum* growth was highest at 5.0-6.0 pH whereas alkaline pH conditions (e.g., pH 8.0 and 8.5) significantly slowed the growth of *R. solanacearum*. These results indicate that acidic conditions (pH 4.5 - 6.5) favour *R. solanacearum* growth. *R. solanacearum* could not grow below pH 4.0 but grew well on or above 4.5 pH.

They suggested that pH 4.4 is the minimum threshold for *R. solanacearum* normal growth. In the present investigation maximum cfu of *R. solanacearum* bacterium was recorded in the combination of 30ºC with 6.5 pH as compared to all the temperature and pH combinations under the study, whereas *R. solanacearum* cfu was significantly higher in varied pH level (6 to 8.5 pH) at 25ºC. Above findings were supported by Suryawanshi *et al*. (2016) [14] that the growth of *R. solanacearum* bacteria at different temperature (5 to 35 with 5 interval) and pH levels (4 to 10 with 0.5 interval) indicated significant differences and the temperature of 30ºC and pH 6 to 7 were found optimum for maximum growth of *R. solanacearum*. Wang and Lin (2005) also reported that temperature of 30-35ºC is conducive for the disease occurrence of bacterial wilt whereas soil temperature < 20ºC was not found suitable for the disease. Prior *et al*., (1996) [10] observed that *R. solanacearum* rapidly moves through the plant at a temperature above 25 ºC, thus the temperature of >28 ºC is ideal for the growth of the bacteria both in plants and on nutrient medium as evident in the present study. These results are in conformity with the results of Hayward, (1991) [6] that the temperature between 30 and 35 ºC significantly increases wilt severity in tobacco and other hosts.

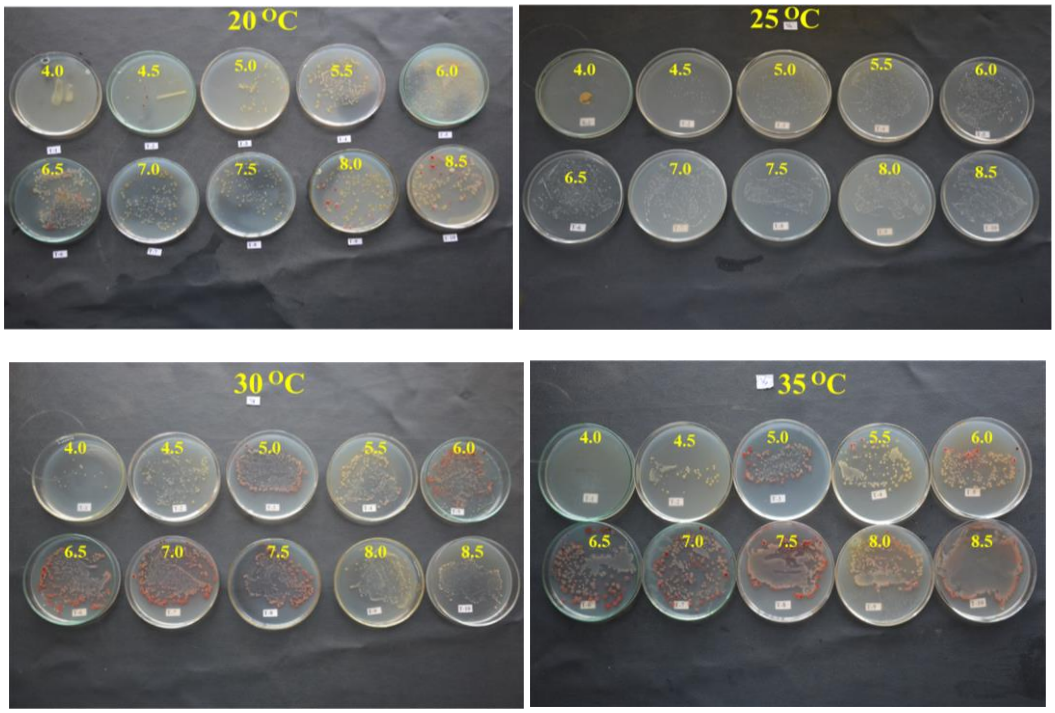

Fig 1: Effects of temperature and pH level on growth of bacterial wilt bacteria (*Ralstonia solanacearum*) under *in vitro* condition

Table 1: Effects of temperature and pH on growth of bacterial wilt bacteria (*R. solanacearum*) under *in vitro* condition

pH level	Bacterial population at 10^{-7} cfu / ml on different temperature ranges											
	20 °C			25 °C			30 °C			35 °C		
	48 hr.	72 hr.	96 hr.	48 hr.	72 hr.	96 hr.	48 hr.	72 hr.	96 hr.	48 hr.	72 hr.	96 hr.
4.0	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	3.67 (2.13)	7.00 (2.80)	11.33 (3.49)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
4.5	0.00 (1.00)	0.00 (1.00)	45.67 (6.81)	0.00 (1.00)	4.00 (2.09)	22.33 (4.49)	123.33 (11.12)	166.00 (12.91)	246.33 (15.70)	32.67 (5.72)	90.00 (9.44)	119.00 (10.90)
5.0	135.67 (11.62)	165.33 (12.85)	211.33 (14.50)	20.67 (3.31)	33.00 (5.47)	104.33 (9.89)	347.33 (18.66)	358.67 (18.96)	382.00 (19.57)	89.33 (9.50)	149.00 (12.24)	218.67 (14.81)
5.5	312.00 (17.67)	345.00 (18.59)	375.67 (19.40)	192.33 (13.89)	239.67 (15.50)	358.33 (18.93)	309.33 (17.61)	368.33 (19.20)	426.33 (20.67)	103.33 (10.21)	169.67 (13.06)	237.00 (15.42)
6.0	369.33 (19.24)	408.00 (20.22)	435.33 (20.88)	229.33 (15.17)	289.67 (17.04)	389.00 (19.73)	354.00 (8.83)	389.33 (19.75)	416.00 (20.40)	125.67 (11.24)	194.00 (13.85)	269.33 (16.44)
6.5	385.00 (19.63)	410.33 (20.27)	430.33 (20.77)	246.00 (15.71)	264.00 (16.27)	371.33 (19.30)	431.00 (20.78)	434.33 (20.86)	443.33 (21.05)	215.67 (14.61)	287.33 (16.97)	322.00 (17.97)
7.0	354.33 (18.85)	396.67 (19.93)	434.33 (20.85)	129.33 (11.41)	193.00 (13.80)	327.00 (18.07)	368.33 (19.21)	392.33 (19.83)	404.00 (20.12)	163.33 (12.82)	256.67 (16.05)	316.33 (17.81)
7.5	348.67 (18.69)	391.33 (19.81)	434.67 (20.87)	117.33 (10.87)	165.33 (12.84)	283.67 (16.85)	329.33 (18.09)	338.67 (18.39)	372.00 (19.28)	192.67 (13.92)	258.33 (16.10)	291.67 (17.11)
8.0	271.67	304.00	345.33	108.67	142.33	293.33	321.33	339.67	355.00	157.67	195.00	226.33

	(16.50)	(17.45)	(18.60)	(10.46)	(11.96)	(17.05)	(17.94)	(18.44)	(18.85)	(12.59)	(13.99)	(15.08)
8.5	294.67 (17.19)	343.67 (18.56)	391.00 (19.80)	142.33 (11.93)	182.67 13.54)	294.00 (17.17)	308.33 (17.59)	310.67 (17.65)	347.33 (18.65)	117.00 (10.83)	168.00 (12.96)	188.67 (13.76)
CD	1.32	1.17	1.32	2.41	2.23	2.83	1.32	1.38	1.68	1.55	1.79	1.02
C.V.	5.44	4.57	4.71	14.83	11.87	11.58	4.70	4.77	5.50	8.79	8.31	4.24

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