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Full Length Research Paper

Water relations of *Pseudomonas solanacearum*

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The growth of 16 strains of *Pseudomonas solanacearum* at different water activities was investigated. These studies were undertaken in order to understand the adaptive mechanisms to osmotic stress of bacteria colonizing plants and to elucidate bacterial persistence when the efforts to eradicate disease fail. Due to inadequate growth of some strains on GYS medium, 3 strains, 66, 64 and 46, were selected for further investigation. These strains showed varied growth patterns on growth media at different water activities, with strain 66 showing the highest tolerance at very low water activity, that is, at 0.970, than the other two strains. The variation observed in the osmotolerance of the strains is an indication of the subtle differences among the strains of the same species.

Key words: *Pseudomonas solanacearum*, water activity, osmotolerance, osmotic stress.

INTRODUCTION

Bacterial wilt disease caused by *Pseudomonas solanacearum,* is one of the most important and widespread diseases of crop plants worldwide. The host range covers a variety of plants including tobacco, tomato, banana, potato, peanut and eggplant (Elphinstone, 2005; Fegan and Prior, 2005; Denny, 2006). It occurs all over the world in the tropics, subtropics, cool and warm temperate regions. In South Africa, *P. solanacearum* is one of the most important pathogens of potatoes. Apart from the typical damage to the plant, that is, wilting and death of infected plants, this bacterium is known to survive for long periods in infested fields. However, the mechanism by which *P. solanacearum* survives is not clearly understood. It has been suggested that, it appears to survive by continually infecting the roots of susceptible or carrier plants, or by colonizing the rhizosphere of non-host plants (Champoiseau et al., 2009). Conventional methods used to eradicate bacterial plant pathogens, such as crop rotation and chemical means (Hayward, 1991), are not very successful with this organism because of its unusual ability to survive in diseased tubers, plant residues and in soil. As *P. solanacearum* is generally soil borne and usually enters the plant via the roots, subsequently spreading in the vascular system, chemical control of the disease is impractical and environmentally unacceptable (Mangin et al., 1999).

In their natural environment, bacteria are exposed to conditions that are stressful both nutritionally and physically. In the soil, bacteria such as *P. solanacearum* are constantly faced with limiting nutrients, unless in rare instances where there may be plant debris or plant residues remaining after cultivation. In addition, soils are often dry out and therefore, the bacterial cell has to find ways of protecting itself against such conditions. Extensive studies have been carried out on nutrient and water stress to find out how bacteria survive under these conditions in nature (Potts, 1994). The limitation of this approach is that one cannot create the extreme water deficit characteristic of the natural environment that is normal to a bacterial cell in the soil. However, these studies do have the advantage of the ease of monitoring the uptake and secretion of various metabolites, and microorganisms can be harvested readily and the cell mass is recovered (Potts, 1994). Understanding adaptive mechanisms to osmotic stress of bacteria colonizing plants is a crucial step in elucidating bacterial persistence when efforts made to eradicate disease fail.

When bacteria are exposed to increased osmotic stress in their environment, such as in the soil, they respond by accumulating biomolecules within the cell in order to prevent cellular dehydration and to maintain intracellular pressure (Pocard et al., 1994). These biomolecules usually do not interfere with macromolecular functions, and are

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Abbreviations: GYS, D-Glucose-YS; $\mathbf{a_w}$**,** water activity.

thus, known as compatible solutes. Compatible solutes such as glycine betaine, proline, proline betaine, trehalose, choline, dimethyl glycine, dimethylsulfoniopropionate, ectoine, glycosylglycerol, and carnitine have been reported to render bacteria such as *Escherichia coli,* osmotolerant, and are thus called osmoprotectants (Kempf and Bremer, 1998; Prescott et al., 1999). Osmoprotectants have been shown to play a key role in the ecology of bacteria, particularly in their survival outside their natural host. Bacteria may accumulate these from the medium or synthesize them *de novo*. In view of the growing body of literature on osmotolerance mechanisms in bacteria (Potts, 1994; Kempf and Bremer, 1998), this is an ideal opportunity to investigate a potentially important aspect of the physiology of *Pseudomonas* species. Knowledge of osmotolerance mechanisms in this genus has potential applications in solving field problems, such as control of spreading of bacterial wilt disease. The role of these compounds in survival, other than that mentioned above, is not clearly understood.

The aim of this investigation is to study the effect of water activity upon the growth of *P. solanacearum*. Experiments were designed to study the growth of *P. solanacearum* at different water activities, and change in turbidity of the bacterial culture was used to follow growth.

MATERIALS AND METHODS

Bacterial strains and growth conditions

All 16 strains of *P. solanacearum* listed here as strain number and place of origin (45, Barkly West; 46, Badplaas; 49, Gamtoos; 55, Nylstroom; 60, Elandsbaai; 62, Rooiwal; 63, Bronkhorstspruit; 64, Roodeplaat; 66, Hankey; 68, Hankey; 70, Davel; 76, Plooysburg; 79, Douglas; 92; Dendron; 94, Piet Retief; 111, Zimbabwe), were isolated from infected potato plants and were obtained from the Vegetable and Ornamental Institute (VOPI) in Pretoria, South Africa. Stock cultures were maintained as suspensions in sterile distilled water at 20°C. These water perms were viable for at least one year. Fresh cultures were prepared by plating aliquots of the suspension onto nutrient agar and transferring fortnightly onto fresh medium. The growth of all the bacterial cultures was carried out in an orbital shaker-incubator (New Brunswick Scientific Company Inc., USA) at 200 rpm and 30°C.

Preparation of growth medium and other reagents

The bacterial cultures were grown in D-glucose-YS (GYS) medium of Prior et al. (1994) which contained the following: Glucose, 1.8 g; Na_2HPO_4, 1.42 g; KH_2PO_4, 0.27 g; $MgSO_4.7H_2O$, 0.24 g; NH_4NO_3, 0.40 g; Yeast extract, 0.30 g, distilled water, 1 L. The medium as it is, is single strength and has a water activity (a_w) value of 0.998 (Mildenhall et al., 1988). A basal salt solution was prepared by dissolving Na_2HPO_4, KH_2PO_4 and NH_4NO_3 in 99% of the final volume of the medium. The pH of the basal salt solution was adjusted to pH 7.2 using concentrated KOH solution before auto-claving. Concentrated solutions (10% w/v) of yeast extract (Difco) and $MgSO_4.7H_2O$ was made and autoclaved separately. The solutions were weighed before and after autoclaving; the difference in weight was replaced by adding an equivalent amount (g) of sterile distilled water. All solutions were sterilized by autoclaving at 101.4 kPa (121°C) for 20 min. Glucose solution (10% w/v) was filter-sterilized. (0.45 µm, Millex-HA filter unit, Millipore, Massachusetts). Sterile solutions of yeast extract, $MgSO_4.7H_2O$ and glucose were added aseptically to a cool, sterile basal medium and made up to the final volume. For the preparation of double strength GYS medium, the above mentioned method was followed but the final volume was reduced by half. All the reagents used in the preparation of media and other solutions were analytical grade.

Determination of the effect of decreasing water activities on the growth of *P. solanacearum* strains.

An inoculum was prepared by taking a loopful of bacterial cells grown on nutrient agar plates and inoculating each of two flasks containing single strength GYS medium (20 ml). The flasks were incubated overnight at 200 rpm in an orbital shaker-incubator at 30°C. A volume (10 ml) of a sterile double strength GYS medium was dispensed into sterile side-arm Erlenmeyer flasks (250 ml). a_w of the flasks were adjusted by adding aseptically, 10 ml of sterile water (0.998 a_w) and 10 ml of sterile NaCl solutions containing appropriate concentrations for a final water activity of 0.995 - 0.970 a_w. The NaCl solutions to be added to double strength basal salts solution were prepared by weighing the appropriate amount of NaCl; 0 g for 0.998, 5.38 g for 0.995, 14.14 g for 0.990, 23.84 g for 0.985, 31.91 g for 0.980, 40.91 g for 0.975 and 49.38 g for 0.970. These were dissolved in distilled water (500 ml), and autoclaved. The difference in weight was replaced by adding an equivalent amount (g) of sterile distilled water. Volumes (10 ml) of the NaCl solutions were added to 10 ml of double strength basal salts medium to adjust water activity. The flasks were inoculated with 1 ml of an overnight culture and growth was monitored by measuring absorbance at 600 nm on a Spectronic 20 spectrophotometer (Bausch and Lomb, New York, USA) until the end of the specified growth period.

RESULTS AND DISCUSSION

When bacteria are grown in a suitable medium and the rate of increase of cellular constituents is constant, they are said to be in a state of balanced growth (Prescott et al., 1999). While such conditions can be approximated in the laboratory, it is unlikely that such conditions last for long in their natural environments. In this study, the initial experiments were concerned with the growth of *P. solanacearum* at different water activities, the cells were in a state of balanced growth during the log phase. Further experiments that were planned for the continuation of this investigation were aimed at perturbing the state of balanced growth by adding varying amounts of NaCl during the mid-log phase of growth. This transition from a suitable growth medium to one in which cells would be subjected to osmotic shock requires optimization in such a way that some growth continues in order that osmoprotectants might accumulate. The final phase of this study was the planned identification of any osmoprotectants present in cells under conditions of unbalanced growth. We report on the initial part of the study.

Many studies have shown that some bacteria are able to withstand conditions that are very low in water activity, that is, at very low humidity, by accumulating various solutes that enable them to maintain cell turgor pressure even at low a_w environments (Kempf and Bremer, 1998;

Table 1. Growth of various strains of P. solanacearum (A600, mean of three replicates) after 9 h in glucose-YS medium.

P. solanacearum strain	Absorbance at 600 nm ± SD
66[a]	1.20 ± 0.009
46[a]	0.70 ± 0.009
62[a]	0.53 ± 0.009
76	0.29 ± 0.008
45	0.23 ± 0.009
111	0.20 ± 0.009
79	0.18 ± 0.009
68	0.16 ± 0.008
49	0.12 ± 0.006
92	0.12 ± 0.007
63	0.09 ±0.007
64	0.09 ± 0.007
94	0.09 ± 0.007
60	0.07 ± 0.004
70	0.07 ± 0.002
55	0.05 ± 0.005

Beales, 2004). These solutes are called osmoprotectants. *In vitro* studies carried out on the survival of bacteria showed that there are certain solutes that may be added to growth medium in order to lower the a_w of the medium. Such solutes are called humectants or osmotica. The food industry has to lower the water activity of food products, particularly tinned food, to inhibit the growth of food borne bacterial pathogens. Addition of solutes, such as NaCl and glycerol, to food stuff is often used to achieve this (Beales, 2004).

Studies on osmotolerance in bacteria have extended to pathogens of plants, especially, *Erwinia chrysanthemi* (Mildenhall et al., 1988; Prior et al., 1994; Gouesbet et al., 1995; Gouesbet et al., 1996). This helps to determine how they survive in dry soil for long periods as saprophytes. Experiments undertaken here on *P. solanacearum* give a glimpse of what may be happening.

Growth of the *P. solanacearum* in GYS medium

All the 16 strains showed satisfactory growth on nutrient broth (Difco Laboratories, Detroit, USA) after 9 h. However, only three of the 16 strains (66, 62 and 46) grew satisfactorily (that is, to an absorbance of > 0.5) after 9 h in the GYS medium (Table 1). It was found that, yeast extract promoted more rapid growth than peptone and casamino acids at similar concentration.

Growth of *P. solanacearum* strain 66, 62 and 46 in GYS at various water activities

The GYS medium was chosen as it has been used extensively in studies of osmotolerance of *E. chrysanthemi* (Mildenhall et al., 1988). Yeast extract supported the highest growth of these *P. solanacearum* strains than peptone and casamino acids, possibly because it contains a richer variety of nutrients, such as vitamin B complex, than other nitrogenous additives. Figures 1, 2 and 3 show the growth curves (absorbance at 600 nm versus time) of strains 66, 62 and 46, respectively, at various water activities. The overall trend of all the three strains is that, the growth rate decreases with increasing concentration of NaCl in the growth medium that is, increasing osmolarity or decreasing water activity of the growth medium. Strain 66 continues to grow, even at water activities as low as 0.970 a_w (Figure 1), although, at a slower rate than at higher water activities. Strain 62 stopped growing at 0.970 a_w (Figure 2) and strain 46 (Figure 3) stopped growing at 0.975 a_w. There is a longer lag phase at lower water activity, the most noticeable being that of strain 62, about 6 h at a_w of 0.975 (Figure 2).

The results obtained show that the growth rate of the three *P. solanacearum* strains declines rapidly with an increase in the osmotic stress of the medium. Strain 66 showed the highest resistance to NaCl as it still grew at 0.970 a_w. By comparison, strain 62 and strain 46 had stopped growing at 0.970 a_w. These results show that, the type of solute used as osmoticum to control a_w has some influence on growth patterns. The lag phase of the three strains in almost all the growth curves in Figures 1, 2 and 3 were similar. The only exception to this was strain 62 whose results showed a longer lag phase of about 6 h at 0.975 a_w. A longer lag phase indicates that, the bacteria (in the case of strain 62) are trying to adapt to the osmotic stress encountered in the medium. Normally, the intracellular water activity of bacterial cells is lower than that of the surrounding medium (Sperber, 1983). This enables the cells to maintain turgor pressure. When the cells are in a medium whose water activity is significantly lower than theirs, they are subjected to osmotic shock, which causes them to lose water rapidly, a process called plasmolysis (Sperber, 1983). During this process, the cells will not grow, and they may die or remain dormant. It seems in the case of strain 62, that the cells remain dormant, adjusting their intracellular water activity so that they can regain turgor pressure, and thereafter, start growing again. To reduce their intracellular water activity, bacterial cells generally accumulate certain solutes (Pocard et al., 1994). They may accumulate these either from the medium or synthesize them *de novo* (Sperber, 1983).

Prior et al. (1987) found that *Pseudomonas fluorescens* had a slower growth rate at 0.980 a_w (NaCl) than at 0.997 a_w, which was the water activity of the medium with no NaCl. Similarly, Figures 1 and 3 show that the growth rates of strain 66 and 46 at 0.980 a_w were slower than at

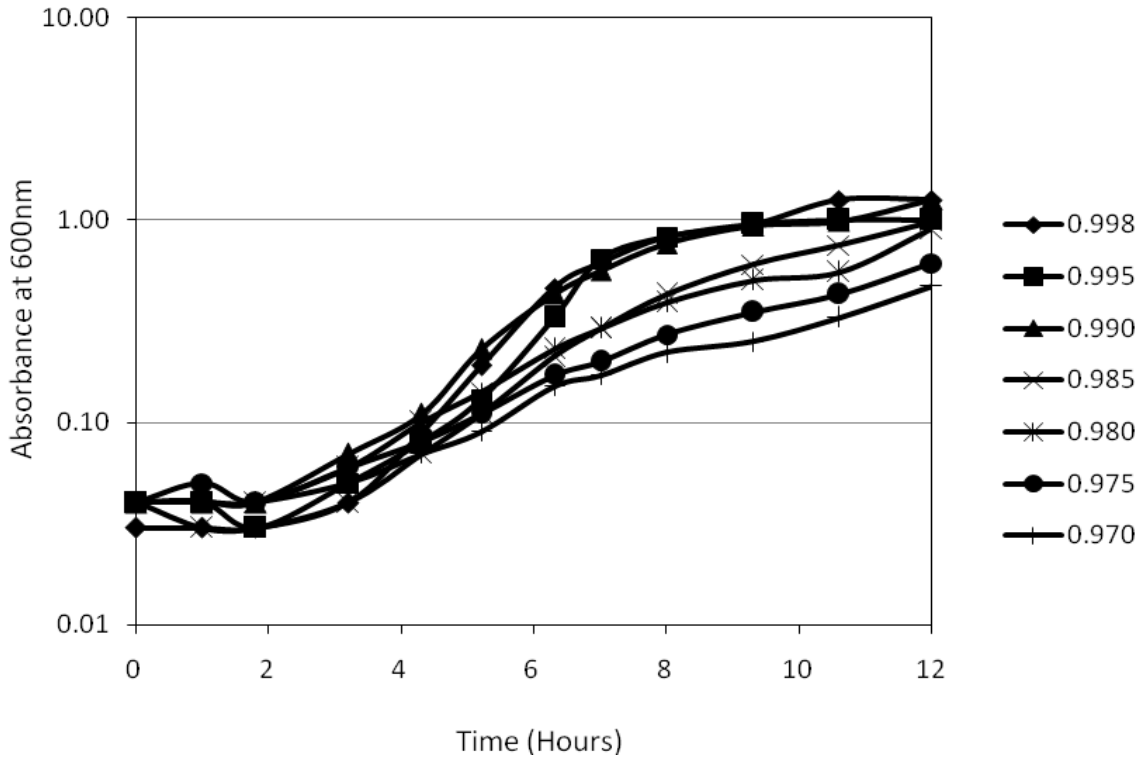

Figure 1. Growth of *P. solanacearum* (66) in GYS medium adjusted with water (0.998 a_w) and NaCl (0.995 - 0.970 a_w) as osmoticum.

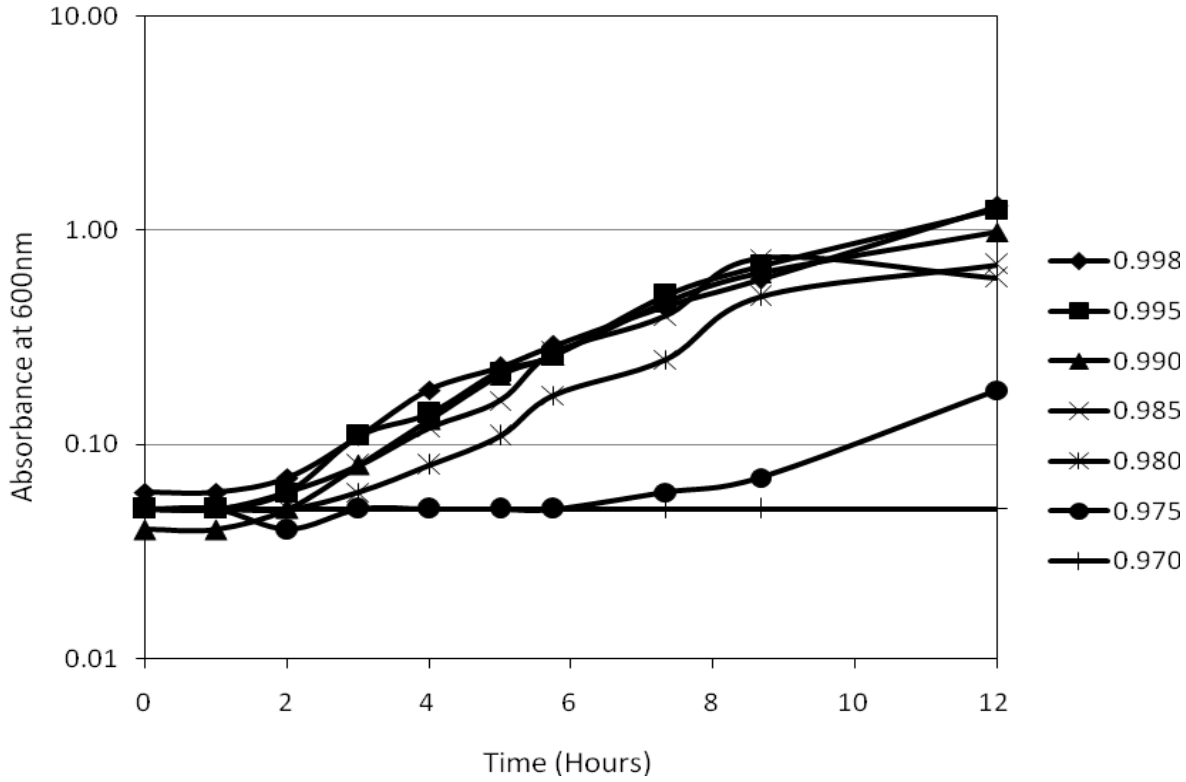

Figure 2. Growth of *P. solanacearum* (62) in GYS medium adjusted with water (0.998 a_w) and NaCl (0.995 – 0.970 a_w) as osmoticum.

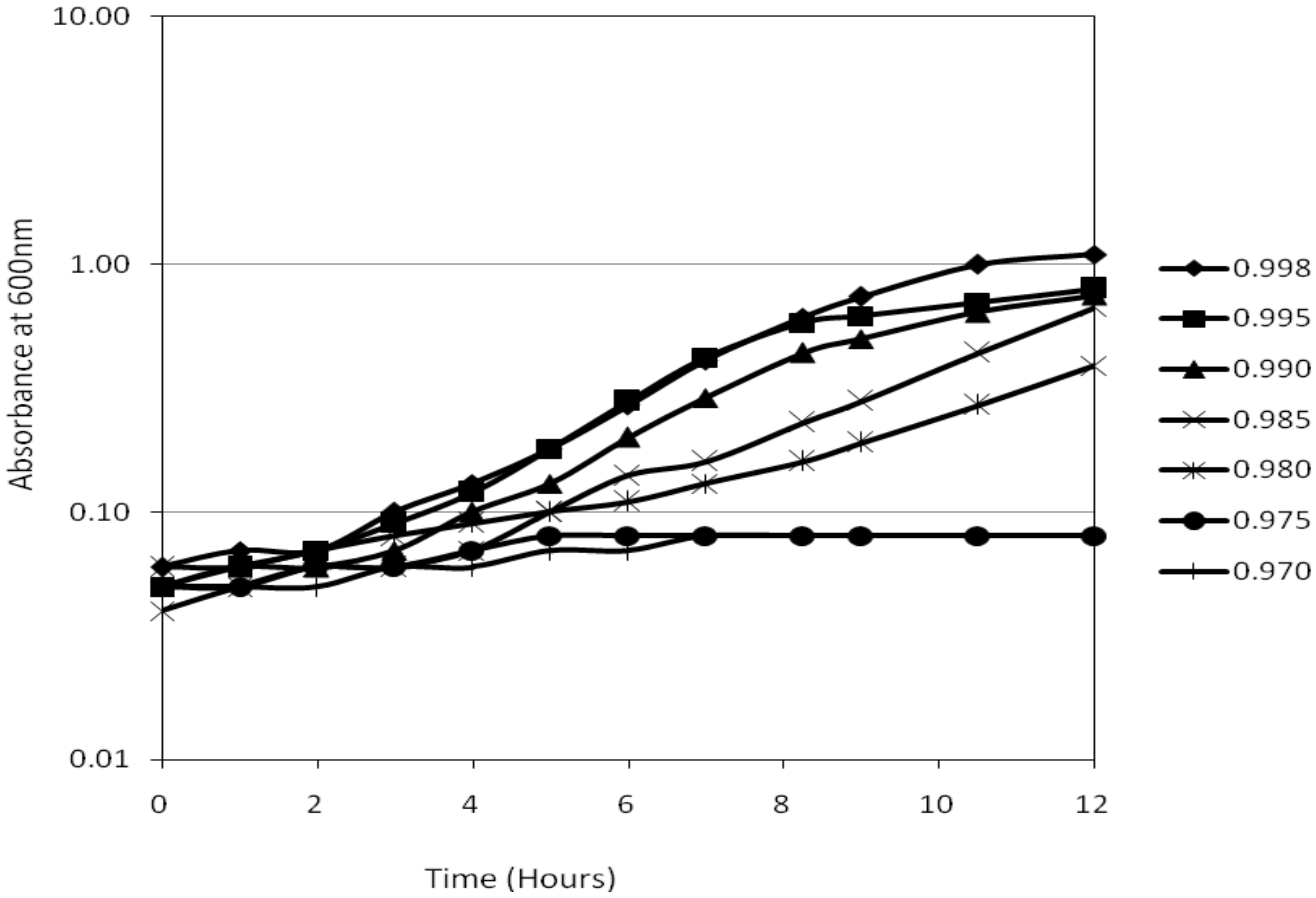

Figure 3. Growth of *P. solanacearum* (46) in GYS medium adjusted with water (0.998 a_w) and NaCl (0.995 – 0.970 a_w) as osmoticum.

0.998 a_w, whilst the growth rate of strain 62 at 0.980 a_w was faster than at 0.998 a_w (Figure 2).

Relationship between growth rate and water activity for strains 66, 62 and 46.

To better illustrate the effect of water activity on the growth of the strains of *P. solanacearum*, growth (absorbance at 600 nm) at 0, 7 and 12 h was plotted against water activity of the medium. The results are presented in Figures 4, 5 and 6. Strain 66 (Figure 4) showed that, it is capable of growth at 0.970 a_w as the absorbance increases from a value of 0.03 at 0 h to 0.170 at 7 h and to 0.470 at 12 h. In contrast, strains 62 and 46 (Figure 5 and 6) showed very little or no growth at 0.970 a_w, even after 12 h. Both of these strains showed reasonable growth after 12 h at 0.980 a_w. Presenting the results by combining the 7 h curves for each strain (Figure 7) and the 12 h curves (Figure 8), again illustrates that strain 66 is still capable of growth at 0.970 a_w.

The relationships shown in Figures 4, 5 and 6 of partial bell-shaped curves are typical of stress-related growth of bacteria (Sperber, 1983). Strain 66 withstands the effect of lowered water activity of the medium better than strains 62 and 46 which stopped growing altogether at low water activity (strain 62 stopped at 0.970 a_w and strain 46 stopped at 0.975). The results in Figures 7 and 8 reaffirm the fact that strain 66 is capable of growing at lower a_w, that is, at 0.970, than the other two strains. This would lead to the next step of the studies, why strain 66 showed more resistance to lowered water activity than other strains? Could it be that, it accumulated certain osmoprotectants that enabled it to withstand lower a_w in the growth medium? Further studies are being pursued with regard to this.

Prior et al. (1987) found that when NaCl is used as a water activity adjuster, *P. fluorescens* accumulated certain amino acids at 0.980 a_w, particularly, glutamic acid. Experiments planned for further studies, would involve the identification of accumulated osmoprotectants by *P. solanacearum* under osmotic stress. The variation observed in the osmotolerance of the strains is an indication of the subtle differences among these strains of the same species and offers an interesting topic for further investigation.

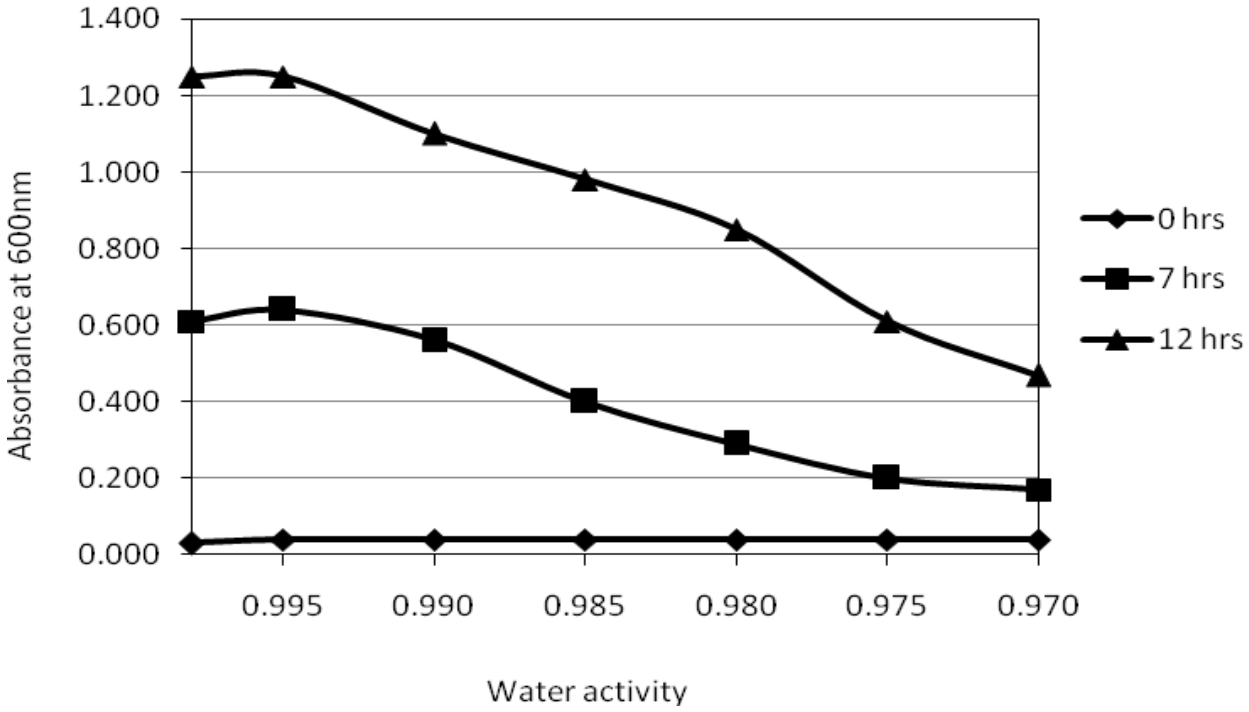

Figure 4. Influence of water activity on the growth of *P. solanacearum* strain 66 at 0, 7 and 12 h.

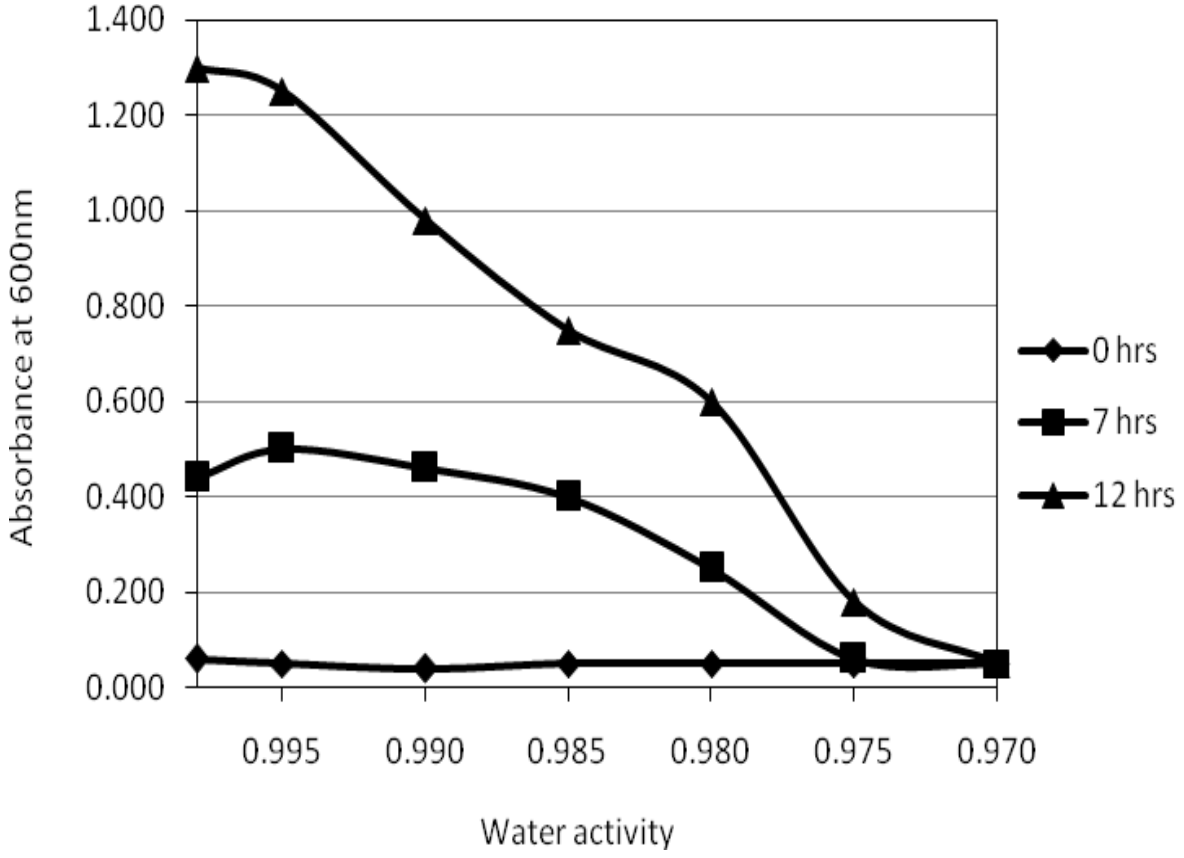

Figure 5. Influence of water activity on the growth of *P. solanacearum* strain 62 at 0, 7 and 12.5 h.

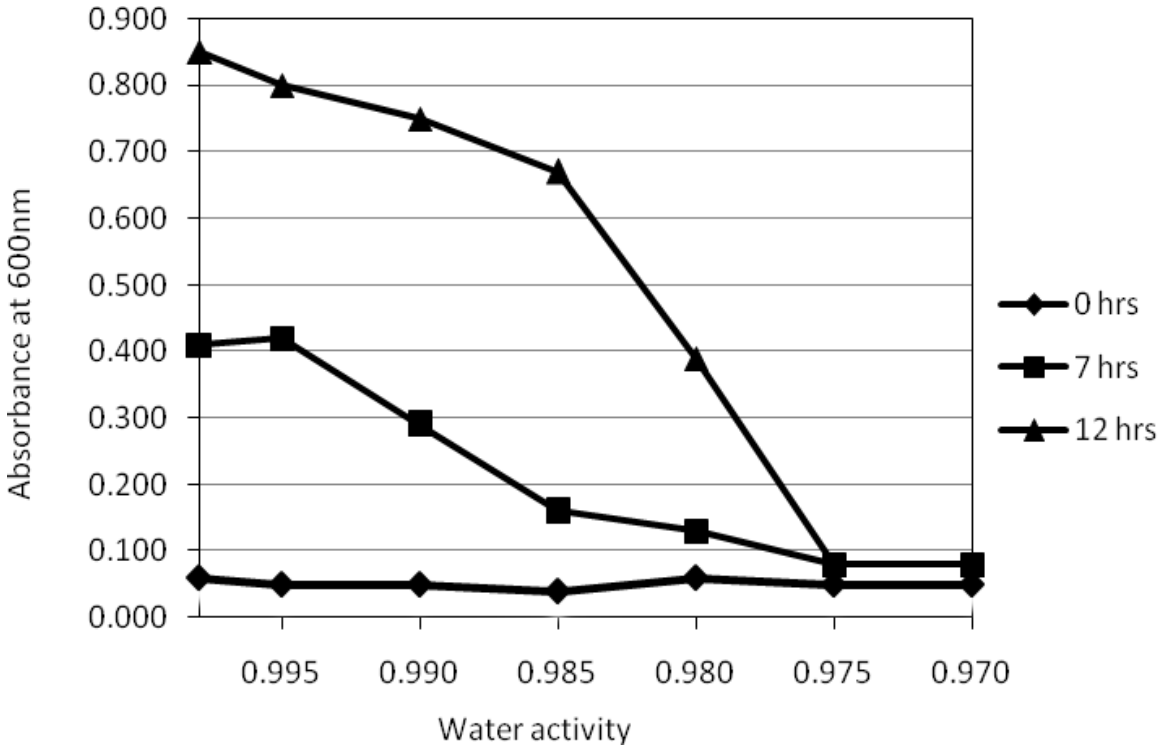

Figure 6. Influence of water activity on the growth of *P. solanacearum* strain 46 at 0, 7 and 12.5 h.

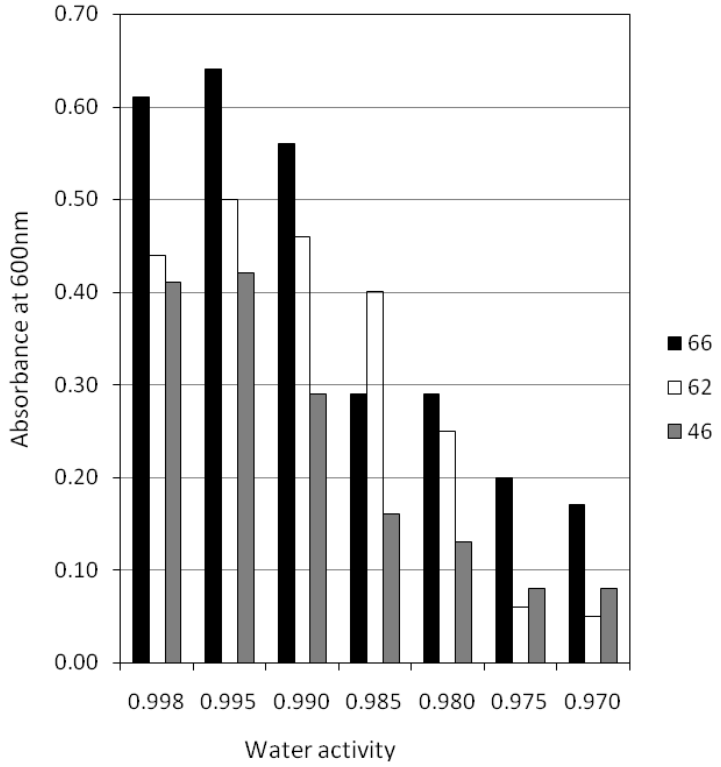

Figure 7. Comparison of the influence of water activity on the growth of *P. solanacearum* strains 66, 62 and 46 respectively, at 7 h.

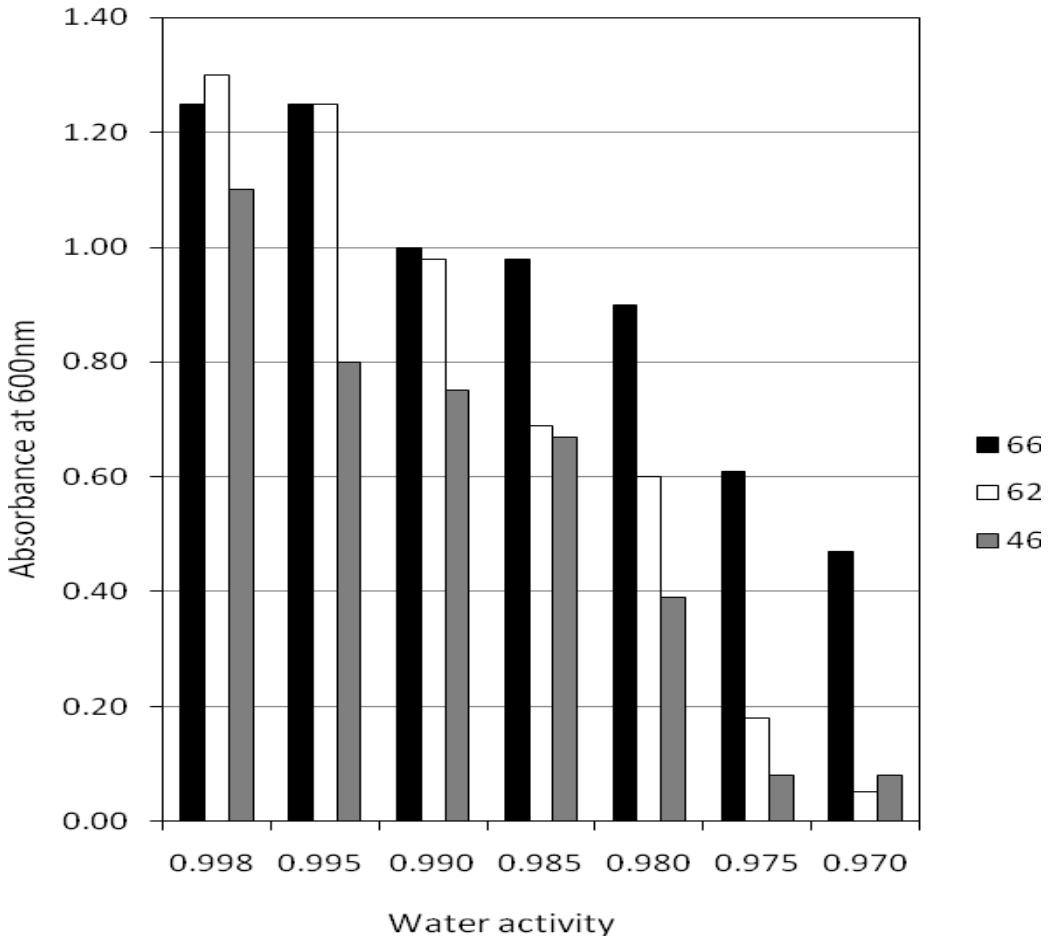

Figure 8. Comparison of the influence of water activity on the growth of *P. solanacearum* strains 66, 62 and 46 respectively, at 12 h.

REFERENCES

Beales N (2004). Adaptation of microorganisms to temperatures, weak acid preservatives, low pH, and osmotic stress: a review. Comprehensive Reviews in Food Sci. Food Saf. 3: 1-30.

Champoiseau PG, Jones JB, Allen C (2009). *Ralstonia solanacearum* race 3 biovar 2 causes tropical losses and temperate anxieties. Plant Health Progress. In press.

Denny TP (2006). Plant pathogenic *Ralstonia* species. In. Gnanamanickam SS (ed) Plant-Associated Bacteria, Springer, Dordrecht, The Netherlands, pp. 573-644.

Elphinstone JG (2005). The current bacterial wilt situation: a global overview. In Alen C, Prior P, Hayward AC (eds) Bacterial Wilt disease and the *Ralstonia solanacearum* species complex, St Paul, MN: APS Press, pp. 9-28.

Fegan M, Prior P (2005). How complex is the "*Ralstonia solanacearum* species complex"? In Alen C, Prior P, Hayward AC (eds) Bacterial Wilt disease and the *Ralstonia solanacearum* species complex, St Paul, MN: APS Press, pp. 449-461.

Gouesbet GM, Jebbar S, Bonnassie N, Hugouvieux-Cotte-Pattat S, Himdi-Kabbab, Blanco C (1995). *Erwinia chrysanthemi* at high osmolarity: influence of osmoprotectants on growth and pectate lyase production. Microbiology, 141: 1407-1412.

Gouesbet GA, Trautwetter S, Bonnassie LF, Wu, Blanco C (1996). Characterization of the *Erwinia chrysanthemi* osmoprotectant transporter gene ousA. J. Bacteriol. 178: 447-455.

Hayward AC (1991). Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 29:65-87.

Kempf B, Bremer E (1998). Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environ. Arch. Microbiol. 170: 319-330.

Mangin BP, Thoquet J, Olivier, Grimsley NH (1999). Temporal and multiple quantitative trait loci analyses of resistance to bacterial wilt in tomato permit the resolution of linked loci. Genetics, 151:1165-1172.

Mildenhall JP, Lindner WA, Prior BA, Tutt K (1988). Elevation and release of cell-associated pectate lyase in *Erwinia chrysanthemi* by lithium and sodium chloride. Phytopathology, 78: 213-217.

Pocard JA, Smith LT, Smith GM, Le Rudulier D (1994). A prominent role of glucosylglycerol in the adaptation of *Pseudomonas mendocina* SKB70 to osmotic stress. J. Bacteriol. 176: 6877-6884.

Potts M (1994). Dessication tolerance of prokaryotes. Microbiol. Rev. 58: 755-805.

Prescott LM, Harley JP, Klein DA (1999). Microbiology, 4th Edition.The McGraw-Hill Co., Inc., New York, USA.

Prior BA, Kenyon CP, van der Veen M, Mildenhall JP (1987). Water relations of solute accumulation in *Pseudomonas fluorescens*. J. Appl. Bacteriol. 62: 119-126.

Prior BA, Hewitt E, Brandt EV, Clarke A, Mildenhall JP (1994). Growth, pectate lyase production and solute accumulation by *Erwinia chrysanthemi* under osmotic stress: Effect of osmoprotectants. J. Appl. Bacteriol. 77: 433-439.

Sperber WH (1983). Infuence of water activity on foodborne bacteria-A review. J. Food Prot. 46:142-150.