Water Relations of Erwinia chrysanthemi: Growth and Extracellular Pectic Acid Lyase Production

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Growth of Erwinia chrysanthemi in a glucose/yeast extract/salts medium, adjusted to various water activities \( (a_w) \) with NaCl, was not observed below 0.970 \( a_w \). Growth rate increased when \( a_w \) was lowered from 0.998 to 0.990, below which growth rate again declined rapidly. Similar results were obtained using mannose to adjust \( a_w \). Growth rate declined linearly between the limits of 0.998 \( a_w \) and 0.970 \( a_w \) on a sodium polypectate/yeast extract/salts medium adjusted with mannose. Extracellular pectate lyase (PL) production on the sodium polypectate/yeast extract/salts medium increased when \( a_w \) was lowered from 0.998 \( a_w \) to 0.990 \( a_w \) using NaCl as \( a_w \) adjuster. However, when either mannose or lactose was used to adjust \( a_w \) over this range, PL production decreased considerably. PL was more stable in the presence of NaCl than with lactose or mannose. The significance of the differential effects of NaCl as opposed to organic \( a_w \) adjusters on PL production is discussed.

INTRODUCTION

Although plant pathologists have recognized the importance of moisture in the infection of plants by pathogenic micro-organisms for over a century (Colhoun, 1973) there have been relatively few quantitative studies on the amount of moisture required for the infection process and subsequent disease development. Over the past decade, the water relations of fungi have been studied (Cook & Papendick, 1972; Dubé et al., 1971; Duniway, 1976, 1977; Griffin, 1977; Ioannou et al., 1977; Mozumder et al., 1970). However, apart from the work of Shaw (1935) scant attention has been paid to phytopathogenic bacteria.

Bacterial stalk rot of maize (Zea mays L.) caused by Erwinia chrysanthemi Burkholder, McFadden and Dimock has characteristically been observed on sprinkler-irrigated maize (Hoppe & Kelman, 1969; Kelman et al., 1957; Mildenhall, 1974). The association of free water with the development of stalk rot suggested that E. chrysanthemi was sensitive to desiccation, a point which prompted us to investigate its water relations. One of the most important enzymes involved in the soft rot caused by E. chrysanthemi is pectate lyase (EC 4.2.2.2) (PL), also known as polygalacturonic acid transeliminase (Bagley & Starr, 1979). Factors which influence either the production or the secretion of this enzyme may have an important bearing on the development in vivo of E. chrysanthemi in resistant and susceptible maize. The availability of water has been shown to affect the growth, respiration, enzyme synthesis and other physiological functions of many micro-organisms (Christian & Waltho, 1964; Prior, 1978; Prior & Kenyon, 1980; Troller & Stinson, 1975, 1978).
The term water activity ($a_w$) as defined by Scott (1953) has been widely accepted for describing the effects of solute or water removal on the growth and physiology of micro-organisms. The $a_w$ is directly related to relative humidity (RH):

$$a_w = \frac{P}{P_0} = \frac{\%RH}{100}$$

where $P$ is the vapour pressure of liquid and $P_0$ is the vapour pressure of pure water. Water activity is also directly related to water potential, a term that has been used in several studies on fungi (Duniway, 1976):

$$\psi = (RT/V) \ln a_w$$

where $\psi$ is the water potential, $R$ is the ideal gas constant, $T$ is the absolute temperature and $V$ is the mole volume of water. Although most $a_w$ studies have been done in liquids, the results are similar to those observed for solid surfaces (Shaw, 1935) and this led us to select liquid culture media for the study of water relations of *E. chrysanthemi*. The objective of this study was to determine the effect of $a_w$ upon growth and extracellular PL production by *E. chrysanthemi*.

**METHODS**

*Organism and inoculum*. A local isolate of *E. chrysanthemi* (Mildenhall, 1974) which has been deposited in the collection of Professor A. Kelman, Department of Plant Pathology, University of Wisconsin, Madison, U.S.A., was used. Stock cultures maintained on nutrient agar at 20°C were transferred monthly.

Late-exponential phase cells from a culture which had been seeded with exponential-phase cells were used as inoculum in all experiments; 1 ml inoculum was added to each flask. Inoculum was grown on the same carbon source as that being investigated, namely either glucose or sodium polypectate (NaPP).

*Medium*. Scott's medium (1953) was modified by omitting casitone and increasing the quantity of yeast extract. The medium (YS) contained the following ingredients: Na$_2$HPO$_4$, 1.42 g; KH$_2$PO$_4$, 0.27 g; MgSO$_4$, 7H$_2$O, 0.24 g; NH$_4$NO$_3$, 0.4 g; yeast extract, 0.9 g; H$_2$O, 1000 g. This yielded a medium of pH 7.6, and no further adjustment was necessary. Either NaPP or glucose was used as carbon source at a concentration of 1.8 g in 1000 g H$_2$O. Sodium polypectate was obtained from Nutritional Biochemical Corp., yeast extract from Difco and mannose from Riedel-de Haën, Hannover, F.R.G. All other chemicals were obtained from Merck.

*Water activity adjusters*. Either NaCl (glucose-YS medium) or mannose (glucose-YS and NaPP-YS media) was used to adjust $a_w$ for growth studies. NaCl was autoclaved in the glucose-YS medium. Mannose was filter sterilized (Millipore) into the autoclaved NaPP-YS medium whereas glucose was filter sterilized with the mannose into the autoclaved YS medium. Mannose and glucose were dissolved in half the volume of water and the YS medium autoclaved in the other half.

Water lost during autoclaving was replaced. With the exception of NaCl, the amount of solute required to prepare media of various $a_w$ values (Table 1) was determined from a standard curve obtained by measuring the $a_w$ of various molal concentrations of the solute using a Wescor psychrometer (Prior *et al.*, 1977). NaCl concentrations were calculated from the data of Robinson & Stokes (1955). Since the $a_w$ of the YS medium was 0.998, the amount of solute required for a given $a_w$ was corrected by 0.002.

*Growth studies*. Side-arm Erlenmeyer flasks (250 ml) containing either 50 ml medium (NaCl) or 25 ml medium (mannose) were used for all experiments. The cost of mannose necessitated smaller culture volumes. In all experiments flasks were incubated at 30°C on a Gallenkamp orbital shaker (180 rev. min$^{-1}$; 30 mm throw). Water activity was checked at the end of each experiment and never varied significantly from the $a_w$ at the start of the growth study. Growth was measured by recording the $A_{620}$ on either a Bausch & Lomb Spectronic 20 spectrophotometer or a Klett-Summerson colorimeter with a no. 62 filter.

*Enzyme studies*. Since PL is induced by NaPP (Moran & Starr, 1969), this compound was used as the main carbon source in all media. Mannose, lactose and NaCl were employed as $a_w$ adjusters, and were sterilized separately by filtration. Each 250 ml side-arm Erlenmeyer flask contained 25 ml medium. Samples (1 ml) were removed immediately after inoculation and thereafter at suitable intervals as indicated by $A_{620}$ measurements. The samples were centrifuged at 9000 g in a Beckman model B Microfuge at room temperature for 2 min, followed by immediate cooling on ice. The supernatant fluids were assayed for PL activity at 230 nm (30°C) on a Beckman model 35 spectrophotometer, with, as substrate, 0.4% (w/v) polymeric anhydrogalacturonic acid (Merck) in 50 mM-Tris/HCl buffer (pH 8.5) containing 1 mM-Ca$^{2+}$. Enzyme activity was calculated from a molar extinction coefficient of 4800 l mol$^{-1}$ cm$^{-1}$ (MacMillan & Phaff, 1966), one unit of enzyme activity being defined as the production of 1 µmol of unsaturated uronide product min$^{-1}$. 
Water relations of *Erwinia chrysanthemi*

### Table 1. Water activity of solutes at 25 °C

<table>
<thead>
<tr>
<th>(a_w)</th>
<th>Lactose (monohydrate) (mol kg(^{-1}))</th>
<th>Mannose (anhydrous) (mol kg(^{-1}))</th>
<th>NaCl (mol kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.995</td>
<td>0.35</td>
<td>0.26</td>
<td>0.15</td>
</tr>
<tr>
<td>0.990</td>
<td>0.70</td>
<td>0.52</td>
<td>0.30</td>
</tr>
<tr>
<td>0.985</td>
<td>1.04</td>
<td>0.81</td>
<td>0.46</td>
</tr>
<tr>
<td>0.980</td>
<td>1.38</td>
<td>1.10</td>
<td>0.61</td>
</tr>
<tr>
<td>0.975</td>
<td>1.72</td>
<td>1.40</td>
<td>0.76</td>
</tr>
<tr>
<td>0.970</td>
<td>2.08</td>
<td>1.70</td>
<td>0.91</td>
</tr>
</tbody>
</table>

**Enzyme stability studies.** The stability of PL in a NaPP-YS medium at various \(a_w\) values without the organism was determined by adding 2 ml of cell-free dialysed supernatant fluid (20 units PL ml\(^{-1}\)) to 25 ml medium. The mixture was incubated as described in the growth studies. Samples removed at suitable intervals were assayed for PL as described above. The cell-free dialysed supernatant fluid was prepared by growing *E. chrysanthemi* until stationary phase as described above, in YS medium containing 20 g NaPP l\(^{-1}\) without \(a_w\) adjuster. The cells were removed by centrifugation and the supernatant fluid was dialysed twice against 50 mM-Tris/HCl buffer (pH 8.5) for 24 h at 4°C.

### RESULTS

**Evaluation of \(a_w\) adjusters**

The ideal solute for adjusting \(a_w\) would have the following properties: (a) freely soluble in water, (b) not utilized as a growth substrate, (c) does not induce or repress PL production, (d) is not inhibitory at high concentrations to the micro-organism or its extracellular enzymes, and (e) relatively inexpensive. No perfect compound was found among the solutes tested; however, mannose was selected as the most suitable for our work (Table 2). On NaPP-YS medium, NaCl and lactose were used within their particular constraints.

**Effects on growth**

When the \(a_w\) of glucose-YS medium adjusted with NaCl was lowered from 0.998 to 0.990, specific growth rate \(\mu\) increased (Fig. 1). Thereafter \(\mu\) declined rapidly until 0.970 \(a_w\) below which no growth was observed. A similar effect was observed when mannose was used to adjust \(a_w\). On NaPP-YS medium adjusted with mannose, \(\mu\) declined linearly with \(a_w\), although significant growth still occurred at 0.970 \(a_w\).

### Table 2. Evaluation of solutes for adjusting the \(a_w\) of growth media

<table>
<thead>
<tr>
<th>(a_w) adjuster</th>
<th>Glucose*</th>
<th>Sodium polypectate*</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0</td>
<td>0</td>
<td>Sodium polypectate precipitates in medium at 0.980 (a_w)</td>
</tr>
<tr>
<td>Lactose</td>
<td>70</td>
<td>55</td>
<td>Poorly soluble at 0.985 (a_w). Extracellular PL not repressed</td>
</tr>
<tr>
<td>Polyethylene glycol 400</td>
<td>0</td>
<td>0</td>
<td>No growth in presence of glucose or sodium polypectate</td>
</tr>
<tr>
<td>Sucrose</td>
<td>73</td>
<td>57</td>
<td>Extracellular PL repressed†</td>
</tr>
<tr>
<td>Glycerol</td>
<td>91</td>
<td>71</td>
<td>Rapidly metabolized</td>
</tr>
<tr>
<td>Mannose</td>
<td>36</td>
<td>29</td>
<td>Slowly metabolized, extracellular PL not repressed†</td>
</tr>
</tbody>
</table>

* Added at a concentration of 1.8 g l\(^{-1}\).
† J. S. S. Gray & J. P. Mildenhall, unpublished results.
Effect of $a_w$ on the specific growth rate of *Erwinia chrysanthemi* grown in glucose-YS medium with $a_w$ adjusted with NaCl (□) or mannose (▲), and in NaPP-YS medium with $a_w$ adjusted with mannose (●). Growth was determined by measuring increases in turbidity ($A_{620}$). Each point represents the mean of six determinations.

**Effect of $a_w$ on accumulation of extracellular PL**

In the control flasks (0.998 $a_w$), PL levels increased exponentially with growth (Figs 2-4) until the stationary phase. When mannose was used to adjust $a_w$, a lag period, which increased with the reduction of $a_w$, was observed in the accumulation of extracellular PL. Furthermore, the maximum levels of PL attained were lower at lower $a_w$ values, and at 0.980 $a_w$ no PL was detected in the medium (Fig. 2).

When lactose was used to adjust $a_w$, growth rate decreased slightly down to 0.990 $a_w$ (Fig. 3). The maximum PL level, however, declined rapidly over this $a_w$ range: the yield at 0.990 $a_w$ was only 16% of the control (0.998 $a_w$), whereas growth at 0.990 $a_w$ was 55% of the control. Therefore the decline in PL level could not be ascribed solely to a decrease in growth. Furthermore, PL level declined rapidly once the culture had reached late-exponential phase.

The specific growth rate decreased when $a_w$ was lowered from 0.998 $a_w$ to 0.990 $a_w$ by adding NaCl. In contrast, however, to the effect observed with mannose and lactose, PL level decreased at 0.995 $a_w$ and thereafter increased at 0.990 $a_w$ (Fig. 4). At 0.990 $a_w$ PL production was stimulated.

Some PL activity at time 0 (Figs 2-4) was due to a carry-over of enzyme in the inoculum.

**Effect of $a_w$ on enzyme stability**

The activity of PL decreased with time (Fig. 5). The enzyme was most stable in NaPP-YS (0.990 $a_w$, NaCl) medium, with 88% activity remaining after 90 min as compared to 76% activity in the control (0.998 $a_w$). PL activity decreased more rapidly when $a_w$ was adjusted with lactose (0.990 $a_w$) and mannose (0.980 $a_w$), and only 31 and 24% activity, respectively, remained after 90 min.

**DISCUSSION**

Both the nature of the medium and the nature of the $a_w$ adjuster may profoundly influence the effect of $a_w$ on growth and extracellular PL production. Because NaPP precipitated in the presence of high concentrations of NaCl, this adjuster could not be used for studies below 0.990 $a_w$ in NaPP-YS medium. Lactose was unsuitable for adjusting $a_w$ below 0.985 because of poor solubility and turbidity in the medium. The selection of a suitable organic compound for adjusting $a_w$ was complicated because of the large number of compounds metabolized by *E. chrysanthemi* (Goto, 1979).
Fig. 2

Fig. 3

Fig. 4

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Figs 2–4. Relation of growth (○) to pectate lyase activity (▲) in the culture medium of *Erwinia chrysanthemi* at various $a_w$ values (indicated in the figures) adjusted with mannose (Fig. 2), lactose (Fig. 3) and NaCl (Fig. 4). Each point represents the mean of three determinations.
The minimum $a_w$ for growth of *E. chrysanthemi* on glucose-YS (NaCl) medium was between 0.970 and 0.965 $a_w$, because the organism failed to grow at 0.965 $a_w$ over a 2 week period (J. P. Mildenhall & B. A. Prior, unpublished data). Growth at $a_w$ values below 0.970 on glucose-YS (mannose) and NaPP-YS (mannose) was not investigated. The linear decline of growth rate with $a_w$ on NaPP-YS (mannose) (Fig. 1) is similar to the results of Shaw (1935) who cultivated *Erwinia amylovora* in beef extract/peptone broth adjusted to various $a_w$ values with sugars. Growth of *E. amylovora* at 0.990 and 0.980 $a_w$ was 30% and 5%, respectively, of the control (0.999 $a_w$). The stimulation of growth rate between 0.998 and 0.990 $a_w$ on glucose-YS (NaCl; mannose) is similar to the effect of $a_w$ (NaCl) observed on *Staphylococcus aureus* (Scott, 1953); *S. aureus* is more halotolerant than *E. chrysanthemi*, the minimum $a_w$ for growth being 0.860.

The greater levels of PL produced when NaCl rather than lactose or mannose were used to adjust the medium to 0.990 $a_w$ could, in part, be explained by the greater stability of PL in the presence of NaCl (Fig. 5). The enzyme was even more stable at 0.990 $a_w$ (NaCl) than at 0.998 $a_w$. Evidence for poor stability of PL in lactose or mannose is also shown by rapid decreases in the enzyme levels in the culture broth once the stationary growth phase was reached (Figs 2 and 3). PL instability in lactose and mannose could have been further accentuated by the culture conditions: shaking has been shown to inactivate enzymes by shear stress (Reese, 1980).

PL instability is, however, unlikely to account for the lack of extracellular PL production at 0.980 $a_w$ (mannose). This lack of production could be a water activity effect per se or an inhibition of the transport of the enzyme across the cell envelope. Troller & Stinson (1978) found that the extracellular activity of deoxyribonuclease, lipase and catalase was suppressed following $a_w$ reduction using NaCl, whereas protease activity increased three- to fourfold between 0.996 and 0.940 $a_w$ and decreased sharply thereafter. No major changes in cell permeability at lowered $a_w$ have been reported (Gould & Measures, 1977) but ionic effects might stimulate release of extracellular enzymes (Oteng-Gyang *et al.*, 1980). A study of PL production below 0.990 $a_w$ (NaCl) might be achieved with oligogalacturonic acids in various states of polymerization, since the galacturonic acid monomer itself does not induce this enzyme (Tsyumu, 1977).

The suppression of PL production by *E. chrysanthemi* between 0.995 and 0.990 $a_w$ (mannose and lactose) is unlikely to be due to catabolite repression. Unpublished work by J. S. S. Gray, J. P. Mildenhall & R. A. Bassett showed that lactose or mannose at 5 g l⁻¹ stimulated extracellular PL production when *E. chrysanthemi* was grown in the presence of NaPP at 5 g l⁻¹.
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The investigation by Shaw (1935) on the infection by E. amylovora of pear and apple shoots held at various relative humidities revealed a tenfold decrease in the number of infected shoots after inoculation at 98% RH (0.980 $a_w$) compared with 100% RH. Disease development at 97% RH was minimal and no infection occurred at 96% RH. An extension of our studies to the host/parasite interaction is envisaged whereby the effect of $a_w$ on infection of maize by E. chrysanthemi may enable one to differentiate between resistant and susceptible cultivars.

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