The Role of Water in Epiphytic Colonization and Infection of Pomaceous Flowers by *Erwinia amylovora*

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**ABSTRACT**


Detached crab apple flowers were used as an experimental model to investigate the effect of relative humidity (RH), free moisture, and water potential (Ψw) on the interaction between *Erwinia amylovora* and pomaceous flowers. Flowers were maintained at 24°C with the cut pedicel submerged in a sucrose solution. The bacterium multiplied on inoculated flower stigmas at between approximately 55 and 100% RH but not in the floral cup (hypanthium) until the RH was higher than 80%. To study the effect of free moisture, stigma-inoculated flowers were kept wet for different periods. Flowers became diseased only with wetting, and incidence was high (77%) even when water application was immediately followed by a 52-min drying period. In other experiments with hypanthium-inoculated flowers, RH or sucrose concentration in holding vials was varied to affect Ψw of flower nectar and ovary tissue. Population size of *E. amylovora* in the hypanthium increased with nectar Ψw following a sigmoidal curve (R² = 0.99). Disease incidence and severity, however, were more closely related to ovary Ψw (R² = 0.85 and 0.91, respectively) than to bacterial population size (R² = 0.25 and 0.67, respectively) as fitted to the quadratic equation. Maximum disease incidence and severity occurred at an ovary Ψw above ~2.0 MPa, and disease severity continued to increase above ~1.0 MPa. These results were confirmed with detached flowers of Delicious apple and d’Anjou pear. A practical implication is that disease might be partly managed in arid climates by limiting soil irrigation water during bloom and early fruit set.

**MATERIALS AND METHODS**

**Pathogen strain and plant material.** The pathogen strain used was a nalidixic acid-resistant derivative of *E. amylovora* strain Ea153 obtained from K. Johnson (13), Oregon State University. The bacterium was cultured on nutrient yeast dextrose agar (nutrient broth, 8 g; yeast, 5 g; dextrose, 5 g; agar, 15 g; and deionized water, 1 liter) for 24 h at 24°C, and cell suspensions were prepared in 10 mM potassium phosphate buffer (pH 7.0) and 0.03% Tween 20.

Manchurian crab apple trees of 1.6 cm minimum stem diameter (Van Well Nursery, Wenatchee, WA) were induced to bloom in a greenhouse as described previously (17). Newly opened flowers with some or all anthers not dehisced were collected for laboratory experiments. The detached flowers were maintained with the cut surface of the pedicel submerged in a sucrose solution contained in a 2-ml vial. Vials with flowers were supported in plastic tube racks enclosed in 4-liter plastic containers held at 24°C. RH was established by flooding the bottom of the containers with 1 liter of a glycerol solution, glycerol concentration being dependent on the desired RH (12).

**Relative humidity and epiphytic colonization.** Detached flowers were placed in holding vials with 10% sucrose, and RH was maintained at 40, 50, 60, 70, 75, 80, 85, 90, 95, or 100%.
After 48 h of incubation, flowers were inoculated with *E. amylovora*. Approximately 0.1 to 0.2 µl of inoculum suspension (10⁷ CFU/ml) was applied per flower by touching a droplet to each stigma (normally five per flower) to form a thin film of inoculum volume per flower was estimated on the basis of total volume lost from a micropipet after inoculating a series of flowers. Consistency was verified by determining initial population sizes, which were at about 10⁵ CFU per flower. In a separate experiment, each flower was inoculated by pipetting 0.5 µl of inoculum suspension (2.0 × 10⁸ CFU/ml) directly into the hypanthium. After incubation for 24 h, cells of *E. amylovora* on flower stigmas or hypanthia were recovered by placing the respective tissues in sterile microcentrifuge tubes containing 1 ml of sterile buffer (10 mM potassium phosphate, pH 7.0). Stigmas of each flower were collected along with portions of the supporting styles. The hypanthium was partially isolated by removing the corolla, calyx, and pedicel. Tubes were vortexed briefly and placed in a sonication bath for 60 s. Samples were again vortexed, and serial dilutions spread on CCT medium (10) amended with nalidixic acid (100 µg/ml). After 3 or 4 days of incubation at 24°C, bacterial colonies were counted to determine population size.

**Free moisture on flower surfaces.** Flower stigmas were inoculated as described previously. After 24 h at 85% RH, population size of *E. amylovora* on stigmas of sampled flowers was determined as described above before wetting the remaining flowers with 15 µl of sterile water (plus 0.25% Tween 20) that flowed over the stigmas and into the hypanthium. Control flowers were not subjected to wetting or a change in RH. Wetted flowers were allowed to air dry in a laminar flow hood immediately or after a period of 2 or 24 h at 100% RH. Average drying time was 52 to 60 min. After drying, flowers were again maintained at 85% RH. At 24 h after drying, population size of *E. amylovora* on stigmas and hypanthia of sampled flowers was determined as described earlier. The remaining flowers were evaluated for disease incidence and severity 5 days after initiation of wetting. Severity was based on a rating scale from 0 to 5 (0 = no necrosis; 1 = detectable necrosis; 2 = up to one half of ovary necrotic; 3 = between one-half and whole ovary necrotic; 4 = necrosis extending beyond ovary and into pedicel; 5 = like 4, but bacterial ooze visible).

**Water potential of nectar and flower tissue.** RH and sucrose concentration in flower holding vials were varied with the expected result that flowers would have a relatively wide range. In one test, flowers were maintained in 10% sucrose and RH was varied from 40 to 100% in increments mentioned previously. In a second test, flowers were held in 0, 5, 10, 15, 20, or 25% sucrose and 85 or 100% RH. In both experiments, flowers were incubated for 48 h to allow water to equilibrate before pipetting 0.5 µl of the inoculum suspension (10⁹ CFU/ml) into the hypanthium. *Ψw* of sampled flowers was determined on the day of inoculation by removing a cross-sectional disk 0.5 to 1.0 mm in thickness from near the longitudinal center of the ovaries and placing it in a vapor pressure osmometer (Model 5520, Wescor Instruments, Logan, UT). Five days after inoculation, flowers were evaluated for disease incidence and severity as described.

In a separate test, procedures were the same with the following exceptions. Flowers were held in 10% sucrose, and RH ranged from 80 to 100% with increments of 2.5%. Nectar of 10 sampled flowers per treatment was collected with a micropipet and pooled, and average nectar volume per flower was calculated. From the pooled nectar sample, a volume between 3.0 and 5.0 µl was used to estimate total sugar concentration with a refractometer, and 2 µl was used to determine water potential with the osmometer. In addition to disease evaluations, population size of *E. amylovora* in the hypanthium was determined 24 h after inoculation.

**Confirmatory tests with apple and pear.** Flowers were collected from individual Delicious apple and d’Anjou pear trees located near Cashmere, WA. The detached flowers, maintained as before using 10% sucrose in holding vials and RH at 75, 85, or 95%, were inoculated with *E. amylovora* by pipetting 0.5 µl of the inoculum suspension (10⁹ CFU/ml) directly into the hypanthium. Population size, ovary water potential, and disease severity were determined as described.

**Replication and data analysis.** For a given experiment, flowers were collected from one or several trees and randomly placed in different chambers, one for each environmental treatment. In all cases, population size of *E. amylovora* on the flower stigmas or hypanthium was determined using 10 flowers per treatment. A separate set of 10 flowers was used when evaluating disease development. Ovary *Ψw* was determined for each of five sampled flowers per treatment. Sugar concentration and *Ψw* of nectar were determined with one pooled sample of nectar collected from 10 flowers per treatment. All experiments were repeated, and data from two trials were pooled. Single values for sugar concentration and nectar *Ψw* for each treatment in the two trials were averaged. Standard errors were calculated for population size, ovary *Ψw*, and disease severity. Nonlinear regression and analysis of variance were performed (SigmaPlot 4.0 software, Jandel Scientific, San Rafael, CA).

**RESULTS**

**Relative humidity and epiphytic colonization.** Population size of *E. amylovora* on flower surfaces after 24 h was studied in relation to RH. Although data for both stigmas and hypanthia closely fitted a sigmoidal curve (Fig. 1), the two curves were distinctly different. As RH increased from 40 to 100%, the bacterial population size on stigmas increased gradually from a population of nearly log 3.0 CFU to greater than log 6.0 CFU per flower (Fig. 1). At between 55 and 80% RH, population size and RH appeared to have a log-linear relationship. In contrast, the population size in hypanthia remained small and did not increase until RH was above 80%. In the RH range from 85 to 95%, the population increased from about log 3.0 CFU to log 6.0 CFU per flower.

**Free moisture on flower surfaces.** Water was applied to flowers to assess its importance in the spread of *E. amylovora* from the stigma to the hypanthium and its relevance to the initiation of disease. All flowers were inoculated on stigmas, and only those subsequently subjected to wetting became infected (Table 1). The bacterial population size on stigmas of both wetted and nonwetted flowers remained high (mean values ranged from 6.7 to 7.7 log CFU per flower). After wetting, the bacterium was present in hypanthia in populations ranging from 5.3 to 7.4 log CFU per flower, but was barely detectable in hypanthia of nonwetted flowers. At the detection limit of 2.0 log CFU per flower, 2 of 20 nonwetted flowers showed populations of *E. amylovora* in the hypanthium of 2.0 and 2.3 log CFU.

![Fig. 1. Relationship between relative humidity (RH) and population size of *Erwinia amylovora*. A, Stigmas. B, Hypanthium of detached flowers. Inoculated flowers were incubated at 24°C for 24 h at the specified RH. Data were fitted to a sigmoidal curve: $f = y_0 + a [1 + \exp(-{(x-x_0)/b})]$, $P < 0.0001$. Bars represent standard error.](image-url)
Water potential of nectar and flower tissue. In initial tests to determine the effect of $\psi_w$ of flower ovary tissue on disease development, a wide $\psi_w$ range was established by varying RH or the sucrose concentration in flower-holding vials. Ovary $\psi_w$ increased with an increase in RH (Fig. 2A) from 40 to 100% and decreased with an increase in sucrose concentration in flower-holding vials from 0 to 25% (Fig. 2B). When sucrose concentration was varied at two different RH levels, an interaction was apparent. The effect of sucrose concentration on ovary $\psi_w$ was greater at 85% RH than at 100% RH (Fig. 2B). Data were best fitted to the quadratic equation.

Following direct inoculation of hypanthia with E. amylovora, disease incidence and severity of flowers were closely related to ovary $\psi_w$ (Fig. 3), with data best fitted to sigmoidal curves. When $\psi_w$ was dependent on RH, a log-linear relationship was evident at the approximate $\psi_w$ range from –2.3 to –1.5 MPa (Fig. 3A and B). When $\psi_w$ was dependent on sucrose concentration, however, the log-linear phase was from about –3.2 to –2.3 MPa (Fig. 3C) or from –2.7 to –2.0 MPa (Fig. 3D).

The study was then directed toward the critical RH range affecting nectar and ovary $\psi_w$. As RH increased from 80 to 100%, the volume of nectar in hypanthia increased from 2.5 to 33.0 µl per flower (Fig. 4A). Concurrently, sugar concentration decreased from 49.8 to 3.5% (Fig. 4B) and nectar $\psi_w$, measurable at 85% and higher, increased from –8.0 to –0.4 MPa (Fig. 4C). Nectar sugar concentration and nectar $\psi_w$ were closely related ($R^2 = 0.999; P < 0.0001$). These interrelationships involving nectar were best shown using the quadratic equation; however, data relating bacterial population size to nectar $\psi_w$ closely fitted a sigmoidal curve (Fig. 4D).

As the population size of E. amylovora in nectar increased, disease incidence and severity appeared to increase (Fig. 5A and B). In the same experiment, disease development was evaluated in relation to ovary $\psi_w$ as dependent on RH (Fig. 5C to F). Disease incidence and severity were more closely related to ovary $\psi_w$ than to bacterial population size in the hypanthium (Fig. 5).

Confirmatory tests with apple and pear. Tests were conducted to determine whether the relationship between ovary $\psi_w$ and disease in crab apple flowers is similar to that for other pomaceous plants. Detached apple and pear flowers held at 75% RH had a mean ovary $\psi_w$ of –2.77 and –2.52 MPa, respectively (Table 2), and no disease occurred. At 85% and 95% RH, however, ovary $\psi_w$ ranged from –1.21 to –1.35 MPa, and flowers exhibited necrosis typical of blossom blight. Disease incidence and severity were greater at 95% than at 85% RH. Results were consistent with those of experiments with crab apple flowers (Fig. 3).

![Fig. 2. Water potential of flower ovary as related to A, relative humidity (RH) in surrounding air and B, sucrose concentration in flower-holding vial. RH was varied while holding flowers in 10% sucrose, and sucrose concentration was varied while holding RH at 85% or 100%. O = 85%, $\triangle = 100%$. Data were fitted to a quadratic curve: $f = y_0 + a*x^2+b*x+c$. A, $P < 0.0001$; B, $P = 0.0002$ (85%) or $P = 0.017$ (100%). Bars represent standard error.](image)

**DISCUSSION**

The availability of water is critical in the sequence of events leading to infection by E. amylovora in pomaceous flowers. During initial establishment of the bacterium on flower surfaces, water in the vapor form is an important factor but not the only determinant of moisture availability. In the field, soil moisture affects host $\psi_w$, which relates to the secretion of fluids from stigmatic and hypanthial tissues. When detached flowers were maintained at one RH level and the ovary $\psi_w$ was varied with sucrose concentration in holding vials, moisture associated with these flower tissues was greater when the ovary $\psi_w$ was relatively high (data not shown). Although data in the present study may not be used to predict epiphytic growth of the bacterium in the field on the basis of RH alone, the results illustrate fundamental differences in the effect of RH on bacterial colonization of the stigma and hypanthium.

The bacterium could multiply on the stigma at a lower RH than in the hypanthium, consistent with the observation by Thomson (26) that E. amylovora occurs predominantly on the stigmas of pomaceous flowers under dry conditions in the western United States. Thomson also found bacterial populations on inoculated pear stigmas increased to between $10^6$ and $10^7$ CFU per flower, whether RH was high (70 to 100%) or low (less than 20 to 30%), but populations on inoculated pear hypanthia declined, particularly when RH was low. The stigmatic surface remains moist under low RH partly because of the presence of hydrophilic compounds that augment the uptake of moisture needed for pollen germination (6). Under low RH, bacterial survival is probably better in apple or crab apple hypanthia than in pear hypanthia, which are comparatively more open and more subject to desiccation. This difference in survival was offered as an explanation of why a single-cell inoculation of an apple hypanthium resulted in infection but similar attempts to infect pear failed (7). At low RH, multiplication in hypanthia may be limited by a lack of moisture. Above a critical RH, nectar is produced, and as RH increases, nectar volume enlarges, corresponding to a simultaneous decrease in sugar concentration and an increase in nectar $\psi_w$. Nectar $\psi_w$ and bacterial population size were intimately related ($R^2 = 0.999, P = 0.0005$). Thus, as RH increases, the microenvironment of the hypanthium becomes increasingly favorable for growth of E. amylovora.

The general relationship of RH to nectar volume and sugar concentration was recognized long ago (3,11), and early researchers assumed that this phenomenon fully explained the relationship

<table>
<thead>
<tr>
<th>Wetting duration (h)</th>
<th>Drying duration (min)</th>
<th>Stigmatic population size (log CFU)</th>
<th>Hypanthial population size (log CFU)</th>
<th>Disease incidence (%)</th>
<th>Disease severity (0–5 rating)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>0</td>
<td>$6.69 \pm 0.13$</td>
<td>$0.24 \pm 0.15$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>52</td>
<td>$6.66 \pm 0.10$</td>
<td>$5.31 \pm 0.24$</td>
<td>76.7</td>
<td>3.37 \pm 0.47</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>$6.62 \pm 0.20$</td>
<td>$6.38 \pm 0.12$</td>
<td>90.0</td>
<td>3.98 \pm 0.33</td>
</tr>
<tr>
<td>24</td>
<td>53</td>
<td>$7.73 \pm 0.06$</td>
<td>$7.42 \pm 0.05$</td>
<td>100.0</td>
<td>4.27 \pm 0.14</td>
</tr>
</tbody>
</table>

* No = no water was applied after inoculation. 0 = water was applied, and flowers were immediately allowed to air dry. For longer wetting, flowers were held at 100% relative humidity before drying.

* Population size per flower was determined 24 h after flowers were dry. Disease evaluations were made 5 days after the start of wetting.
between RH and disease incidence (8,25). Several investigators studied the capacity of *Erwinia amylovora* to grow and survive in artificial nectar (1,9,11,18,25). The current study is the first attempt to examine in vivo population size of *Erwinia amylovora* and sugar concentration of natural nectar.

Nectar sugar concentrations in the detached crab apple flowers were within the reported range of nectar sugar for pomaceous flowers (3,11,27). Sugar composition in the nectar of detached flowers typically consisted of sucrose, glucose, and fructose in the ratio of 1:2:2, respectively, regardless of conditions (data not shown). This ratio was closer to what has been reported for pear nectar (4,28) than for crab apple or apple nectar (4,5) in which sucrose was the predominant sugar. Bacterial population size in different synthetic nectar solutions having the same total sugar concentration was previously shown to decrease with a decrease in the ratio of disaccharide to monosaccharide sugar and a corresponding decrease in *Ψ*$_{w}$ (18). Also in that study, differing concentrations of total sugar in solution were similar in their effect on bacterial multiplication whenever *Ψ*$_{w}$ was the same. The curve for bacterial population size as related to nectar *Ψ*$_{w}$ (Fig. 4D) is very similar to that for “relative multiplication rate” in vitro and *Ψ*$_{w}$ (20) on the basis of data transformed from Shaw (22) who expressed water availability in artificial media as “equivalent relative humidity.”

Results of laboratory simulation of flower wetting were consistent with those of Thomson (26), who concluded that colonization of stigmas by *Erwinia amylovora* under dry conditions did not lead to blossom blight unless rain facilitated movement of the pathogen to the hypanthium where infection generally occurs. Thomson suggested the role of rain in moving bacteria is more important than its role in diluting nectar as emphasized previously (11,14). Following a wetting event, it is unknown whether bacteria generally multiply in the nectar prior to invasion or immediately enter nectarthodes and infect flowers. Both scenarios probably occur. If the number of bacterial cells spreading to the hypanthium is relatively low, multiplication is probably necessary before infection is likely.

Flowers inoculated on stigmas became infected only when subject to wetting, which led to the presence of relatively large populations of the pathogen in the hypanthium. Infection occurred even when the application of water was immediately followed by a 52-min drying period. Thus, disease may result from shorter periods of wetness than previously thought. In Washington State, vegetative wetting is not considered to present a significant risk until leaf wetness readings indicate a duration of 3 h or more (23). The laboratory methods employed here, which included the use of a surfactant at low concentration, insured the movement of water from stigmas to hypanthium. Longer periods of vegetative wetness may be required under normal field conditions before a significant number of bacterial cells spread to the hypanthium.

Results showed that disease incidence and severity increased with an increase in ovary *Ψ*$_{w}$ whether ovary *Ψ*$_{w}$ was manipulated by varying RH or sucrose concentrations in flower-holding vials. The ovary *Ψ*$_{w}$ range at which disease occurred extended lower when *Ψ*$_{w}$ was dependent on sucrose than on RH. Although this difference is not understood, it may be inferred that ovary *Ψ*$_{w}$ interacts with other factors related to disease development in flowers. In either case, however, maximum disease incidence and severity occurred when ovary *Ψ*$_{w}$ was above −2.0 MPa. Results were consistent with confirmatory tests with detached apple and pear flowers and with previous studies involving other pomaceous tissues. Shaw (22) demonstrated that disease development in excised pear shoots and fruit and in shoots of potted apple and pear trees was related to “intercellular relative humidity,” an obsolete parameter closely related to *Ψ*$_{w}$. An approximate transformation of Shaw’s data to *Ψ*$_{w}$ (data not shown) indicated that maximum disease expression generally occurred above −2.0 MPa. In addition, Suleman and Steiner’s (24) data showed that apple leaf necrosis was maximized when the solute potential of extracted leaf fluid exceeded −1.3 MPa.

In the critical RH range from 80 to 100%, the more comprehensive evaluation of interrelationships involving water revealed that disease incidence and severity were more closely related to ovary

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**Fig. 3.** Relationship between water potential in flower ovaries and disease incidence and severity. Water potential was varied by altering A and B, relative humidity; or C and D, sucrose concentration in flower-holding vial. Data were fitted to a sigmoidal curve $f = a[1 + \exp(-(x-x_0)/b)]$. All regressions were highly significant ($P < 0.004$). Bars represent standard error.

**Fig. 4.** Relationship between relative humidity and A, nectar volume; B, nectar sugar concentration; and C, nectar water potential. D, Relationship between nectar water potential and population size of *Erwinia amylovora* in the flower hypanthium. Detached crab apple flowers were maintained with peduncle in 10% sucrose. A, B, and C data fitted to a quadratic curve $f = y_0 + ax^2 + bx^2$; D, data fitted to a sigmoidal curve $f = y_0 + a[1 + \exp(-(x-x_0)/b)]c$. Regressions had a significance of $P < 0.001$, except in A ($P < 0.015$).
ψ than to bacterial population size in the hypanthium. The trend in disease development in the upper part of the ψw range was noteworthy. Although disease incidence reached 100% at an RH of 90% or higher in the laboratory, disease severity continued to increase along a quadratic curve as ovary ψw increased from –1.8 to greater than –1.0 MPa.

On the basis of these laboratory experiments, it could be assumed that declines in ovary ψw of flower tissues in the orchard reduce their susceptibility to blossom blight. According to Klepper (15), the ψw of pear shoots in an orchard usually fluctuates between about 0 and –3 MPa. If the ψw range is similar for flowers, the incidence and rate of blossom blight development would vary widely depending on factors related to ψw in host flowers. A practical implication is that blossom blight might be managed to some degree in arid regions, particularly at sites with sandy soils, by limiting the amount of irrigation water applied to the soil during the period of bloom and early fruit set.

The importance and role of ψw in nectar and flower tissue are difficult to separate and fully assess experimentally because of their interrelation. In this study, both were affected by RH. During initial infection, *E. amylovora* was probably multiplying simultaneously in nectar and hypanthial tissue. The data indicated that ψw of the flower tissue was more important than that of the nectar. Either factor, however, could be critical to fire blight incidence depending on environmental conditions.

**Fig. 5.** Disease development as related to A and B, population size in the flower hypanthium; C, D, E, and F, ovary water potential. Detached crab apple flowers were maintained with peduncle in 10% sucrose. Data were fitted to a quadratic curve \( f = y_0 + ax + bx^2 \). Regressions had a significance of \( P < 0.003 \), except in A, \( P = 0.17 \) and B, \( P = 0.015 \). Bars represent standard error.

**TABLE 2.** Relationship between water potential in ovaries of detached apple and pear flowers and disease development caused by *Erwinia amylovora*

<table>
<thead>
<tr>
<th>Flower Type</th>
<th>Relative humidity (%)a</th>
<th>Ovary water potential (MPa)b</th>
<th>Disease incidence (%)</th>
<th>Disease severity (0–5 rating)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple</td>
<td>75</td>
<td>–2.77 ± 0.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Apple</td>
<td>85</td>
<td>–1.83 ± 0.10</td>
<td>52.63</td>
<td>1.74 ± 0.41</td>
</tr>
<tr>
<td>Apple</td>
<td>95</td>
<td>–1.39 ± 0.04</td>
<td>55.00</td>
<td>2.20 ± 0.46</td>
</tr>
<tr>
<td>Pear</td>
<td>75</td>
<td>–2.52 ± 0.09</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pear</td>
<td>85</td>
<td>–2.15 ± 0.07</td>
<td>25.00</td>
<td>0.95 ± 0.21</td>
</tr>
<tr>
<td>Pear</td>
<td>95</td>
<td>–1.35 ± 0.05</td>
<td>80.00</td>
<td>3.60 ± 0.80</td>
</tr>
</tbody>
</table>

a Detached flowers were maintained under different relative humidities by adding glycerol solutions to the closed chamber containing the flowers.
b Flowers were sampled to determine water potential with an osmometer immediately before inoculation of the flower hypanthium, and flowers were evaluated for disease 5 days after inoculation of the hypanthium. Standard error is indicated.

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