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Effect of extracellular calcium, pH and borate on growth oscillations in *Lilium formosanum* pollen tubes

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Abstract

Calcium ions (Ca^{2+}), protons (H^+), and borate ($B(OH)_4^-$) are essential ions in the control of tip growth of pollen tubes. All three ions may interact with pectins, a major component of the expanding pollen tube cell wall. Ca^{2+} is thought to bind acidic residues, and cross-link adjacent pectin chains, thereby strengthening the cell wall. Protons are loosening agents; in pollen tube walls they may act through the enzyme pectin methylesterase (PME), and either reduce demethylation or stimulate hydrolysis of pectin. Finally, borate cross-links monomers of rhamnogalacturonan II (RG-II), and thus stiffens the cell wall. It is demonstrated here that changing the extracellular concentrations of Ca^{2+}, H^+ and borate affect not only the average growth rate of lily pollen tubes, but also influence the period of growth rate oscillations. The most dramatic effects are observed with increasing concentrations of Ca^{2+} and borate, both of which markedly reduce the rate of growth of oscillating pollen tubes. Protons are less active, except at pH 7.0 where growth is inhibited. It is noteworthy, especially with borate, that the faster growing tubes exhibit the shorter periods of oscillation. The results are consistent with the idea that binding of Ca^{2+} and borate to the cell wall may act at a similar level to alter the mechanical properties of the apical cell wall, with optimal concentrations being high enough to impart sufficient rigidity to the wall so as to prevent bursting in the face of cell turgor, but low enough to allow the wall to stretch quickly during periods of accelerating growth.

Key words: Borate, calcium, cell wall, pectin, oscillations, pH, pollen tube.

Introduction

It is well known that Ca^{2+}, H^+, and borate in the extracellular medium are essential for the growth of pollen tubes (Steer and Steer, 1989; Welch, 1995), although their targets for action have not been entirely resolved, particularly since Ca^{2+} and H^+ participate in both intracellular and extracellular processes. More recently, the phenomenon of oscillatory growth in pollen tubes (Pierson *et al*., 1996) and the measurement of accompanying oscillations in intracellular gradients and extracellular fluxes of Ca^{2+} and pH (Messerli and Robinson, 1997; Holdaway-Clarke *et al*., 1997; Feijó *et al*., 1999) have led to the proposal that the cell wall is a major player in the process of pollen tube elongation (Holdaway-Clarke *et al*., 1997). It is important to note that the cell wall at the tip consists mainly of pectic polysaccharides, with few or no cellulose microfibrils (Heslop-Harrison, 1987), and that all three ion species, in one way or another, interact with pectin and control its mechanical properties. Therefore changes in the extensibility of pectin, as brought about by extracellular Ca^{2+}, H^+, and/or borate, may control oscillatory pollen tube growth.

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During pollen tube growth, pectins, which are a mixture of complex polysaccharides characterized by 1,4-linked α-D-galactosyluronic acid residues (Ridley *et al*., 2001), are secreted primarily as methylesters (Lennon and Lord, 2000; Li *et al*., 1997, 2002), and subsequently de-esterified by the enzyme pectin methylesterase (PME) in the cell wall (Li *et al*., 2002). Although not yet demonstrated in pollen tube cell walls, it is presumed that Ca^{2+}, as in other systems, reacts with acidic residues on homogalacturonan pectin and cross-links adjacent chains forming the 'egg-box' configuration, and that this reaction imparts rigidity to the cell wall (Carpita and Gibeaut, 1993). H^+, on the other hand may promote a more plastic and extensible wall; for example, acidic pH decreases the activity of PME (Moustacas *et al*., 1986), thus reducing the number of carboxyl residues and the amount of Ca^{2+} cross-linking. Low pH may also enhance the activity of acidic isoforms of PME (Li *et al*., 2002), which together with pectin hydrolyases, cause the degradation of pectin gels (Bordenave, 1996). Borate, like Ca^{2+}, most probably imparts rigidity to the cell wall since recent work shows that it forms 1:2 diol ester linkages between apiosyl residues of rhamnogalaturonan II (RG-II) monomers (Fleischer *et al*., 1998, 1999; Ishii *et al*., 2001; Matoh and Kobayashi, 1998; Ridley *et al*., 2001). Here H^+ may help to strengthen the wall since lower pH promotes RG-II dimer formation *in vitro* (O'Neill *et al*., 1996).

In order to dissect the distinct contributions of Ca^{2+}, H^+, and borate, the effects of changing the extracellular concentrations of these ions on pollen germination and on growth rate oscillations were examined. It is shown here that varying the extracellular concentrations of these ions have distinct effects on pollen tube germination, average growth rate and the shape of growth rate oscillations. The similarity of effects of extracellular Ca^{2+} and borate on the relationship between period of oscillation and growth rate supports the idea that cross-linking of the pectins in the cell wall by Ca^{2+} and borate participates in the regulation of pollen tube growth oscillations.

Materials and methods

Pollen germination

Fresh *Lilium formosanum* pollen was obtained from plants grown in a growth chamber. Anthers were removed, placed in 1 ml germination medium in a 1.5 ml tube on a rotary device and allowed to germinate for at least 1.5 h. The control medium consisted of 7% sucrose, 0.1 mM KCl, 0.1 mM $CaCl_2$, 1.6 mM H_3BO_3, and 15 mM MES buffer adjusted to pH 6.0 with KOH. Test media with varying pH, or concentrations of $CaCl_2$ or H_3BO_3 were constructed with all other parameters of the media the same as for the control. The experimental concentrations were generally as follows; pH: 4.5, 5.0, 5.5, 6.5, 7.0; $CaCl_2$ (in mM): 0.01, 0.05, 0.1, 0.5, 1.0, 10; H_3BO_3 (in mM): 0.32, 0.8, 1.6, 3.2, 8.0, 16.0. All images were acquired using a Princeton Instruments Micromax CCD camera attached to a Nikon Diaphot 300 inverted microscope, controlled by MetaMorph software (Universal Imaging).

Germination experiments

In germination experiments, six anthers from one plant were taken and placed in each of several different media: the control medium and the test media with different experimental concentrations of either $CaCl_2$, H_3BO_3 or pH. After 1.5 h, pollen was placed on slides and images acquired with a low power (4×) objective on the microscope. Germination frequency was determined by counting the number of germinated and non-germinated grains in the images taken using the count objects feature of MetaMorph. Approximately 100 pollen grains were counted for each treatment. Each experiment was repeated at least three times.

Bulk growth rate experiments

To determine the effect of altering extracellular concentrations of protons (H^+), $CaCl_2$, and H_3BO_3 on the bulk growth rate of pollen tubes, the pollen from up to six anthers from a single flower were germinated in a single tube containing 1 ml of control medium per anther on a rotary device. After at least 1 h of germination, low power digital images of approximately 100 tubes were acquired for later measurement. The solution with medium and pollen was then divided into smaller aliquots, the pollen allowed to settle to the bottom of each tube, and the germination media suctioned off using a pipette and replaced with media with the experimental pH or concentrations of H_3BO_3 or $CaCl_2$. The pollen was then germinated for at least another hour, before samples were removed to slides for imaging, and the time between these later samples and the first sample was noted. MetaMorph software was used to measure the lengths of at least 100 pollen tubes in each sample, and the average pollen tube growth rate was calculated for each different solution.

Oscillatory growth measurements

The growth characteristics of pollen tubes displaying oscillating growth rates (those longer than 1000 μm) were investigated in various levels of pH, $CaCl_2$ and H_3BO_3 by initially germinating pollen in the control medium for at least 1 h before changing the medium to a test solution. Pollen tubes were given at least 1 h in the new solution to adjust to the new condition before being plated onto a slide with a thin layer of 1.2% agarose (Sigma type VII-low gelling point) made from media containing the same experimental conditions. Once the agarose had gelled, the pollen tubes were allowed to recover for at least 30 min. The tubes were observed with a Nikon inverted microscope (Diaphot 300) with a 40× oil immersion objective (NA 1.3), and images acquired every 0.5–2 s. The 'track objects' feature of MetaMorph was used to track the growth rate of pollen tube tips and generate data files that could be imported into Microsoft Excel or Microcal Origin for further processing and graphing. The characteristics of the growth rate oscillations, including average rate and period of oscillation, were determined for each pollen tube imaged. An indication of the amplitude of the oscillation was obtained by using the standard deviation of the growth rates measured at each time point in a time-lapse experiment; in a sinusoidal oscillation, the standard deviation of points acquired at regular intervals much shorter than the period of oscillation is related to the amplitude of the oscillation by the following equation: Amplitude=$2S^2$=2(variance).

Statistical analysis

The data were analysed by the general approach of linear statistical inference and analysis of variance as set out in Rao (1965). Pollen germination proportions were arcsine transformed to assure homoskedasticity prior to analysis.

Results

Effects of extracellular Ca^{2+}, pH and borate on pollen germination

Figure 1 reveals that variations in extracellular pH across the experimental range had more dramatic effects on pollen germination than either Ca^{2+} or borate. In Fig. 1a the data indicate that germination rate is optimal when Ca^{2+} is in the range 0.1–1.0 mM, and that it falls off only moderately either below 0.1 mM or above 1.0 mM. However, even the extremes of 0.01 mM and 10 mM support better than 50% germination. Optimum germination (i.e. 55–65%) is supported by a rather wide range of pH (4.5–6.0), however, above 6.0 the process is increasingly inhibited and at 7.0 is only 12% (Fig. 1b). Extracellular borate appeared to have little effect on germination over the range of concentrations tested (Fig. 1c). Although a cursory inspection suggests that 1.6 mM borate is optimal, these data are not significantly different (P >0.05) from those obtained at higher or lower concentrations.

Effects of extracellular Ca^{2+}, pH and borate effects on growth rates in tubes <1000 μm

During the early phases of elongation following germination, and before they reach approximately 1000 μm, pollen tubes do not exhibit the characteristic oscillatory growth behaviour observed in longer tubes, rather they grow at a steady rate with minor fluctuations about the mean (Pierson *et al*., 1996). Realizing that there are different phases during the growth of the lily pollen tube, the effects of extracellular Ca^{2+}, pH and borate on tubes less than 1000 μm were tested first. An overall comparison of Figs 2 and 3 immediately reveals that the shorter tubes (Fig. 2) grow much more slowly than the longer, oscillatory tubes (Fig. 3). Focusing on the short pollen tubes, it is evident that increases in both Ca^{2+} and pH have a marked inhibitory effect on the rate. With Ca^{2+} the rate drops from 0.05 μm s^{-1} at 0.5 mM to less than 0.005 μm s^{-1} at 10 mM (Fig. 2a). The change with elevating pH is equally dramatic showing a decline from 0.083 μm s^{-1} at pH 6.0 to 0.018 μm s^{-1} at pH 7.0 (Fig. 2b). With borate, 1.6 mM supports the fastest bulk growth rates in these non-oscillating pollen tubes (Fig. 2c). However, we fail to detect the trends that are apparent in both calcium and pH, and thus the minimum (0.32 mM) and maximum (16.0 mM) levels of borate yield nearly the same bulk growth rates.

Effects of extracellular Ca^{2+}, pH and borate on growth oscillations in tubes >1000 μm

Longer pollen tubes displaying oscillating growth rates were measured individually. Figure 3a, b, and c shows that changes in the levels of all ions affect growth, with marked inhibitions occurring as the concentration of Ca^{2+} (Fig. 3a) and borate (Fig. 3c) increased, or that of H^+ (Fig. 3b) decreased. For Ca^{2+} a maximum growth rate of 0.27 μm s^{-1} was observed at 0.05 mM, which then declined to 0.07 μm s^{-1} at 10 mM, while for borate the maximum values extend from 0.27 μm s^{-1} at 3.2 mM to 0.1 μm s^{-1} at 16 mM. In

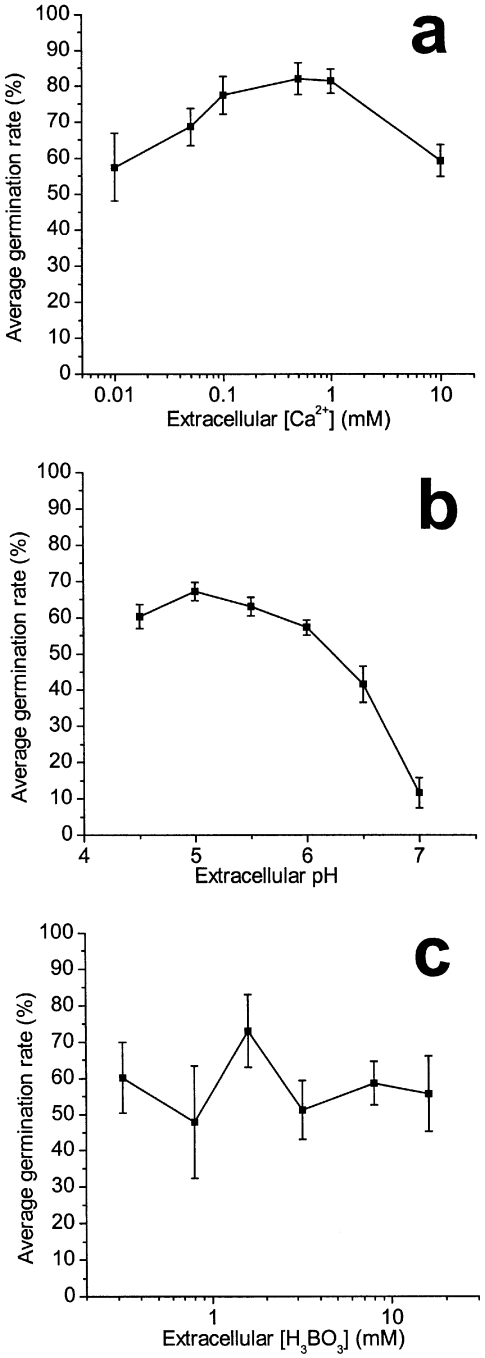


Fig. 1. The effect of extracellular calcium (Ca^{2+}), pH, and borate (H_3BO_3) on pollen germination. (a) Ca^{2+} concentration was set at 0.01, 0.05, 0.1, 0.5, 1.0, and 10 mM; results are from four replicates. (b) pH was set at 4.5, 5.0. 5.5, 6.0, 6.5, and 7.0; results are from six replicates. (c) H_3BO_3 concentration was set at 0.32. 0.8, 1.6, 3.2, 8.0, and 16 mM; results are from seven replicates. Replicates above consist of approximately 100 grains each.

passing, it was noted that the average growth rate was fastest in the presence of 3.2 mM extracellular borate, which is twice the concentration used in the standard, control medium. With pH there is a modest decline in growth rate from a maximum of 0.26 μm s^{-1} at pH 5.5 to 0.175 μm s^{-1} at pH 6.5. However, at pH 7.0 (Fig. 3b) growth drops to nearly zero (data not shown); because of this lack of growth, pH 7.0 has not been included in the subsequent analyses.

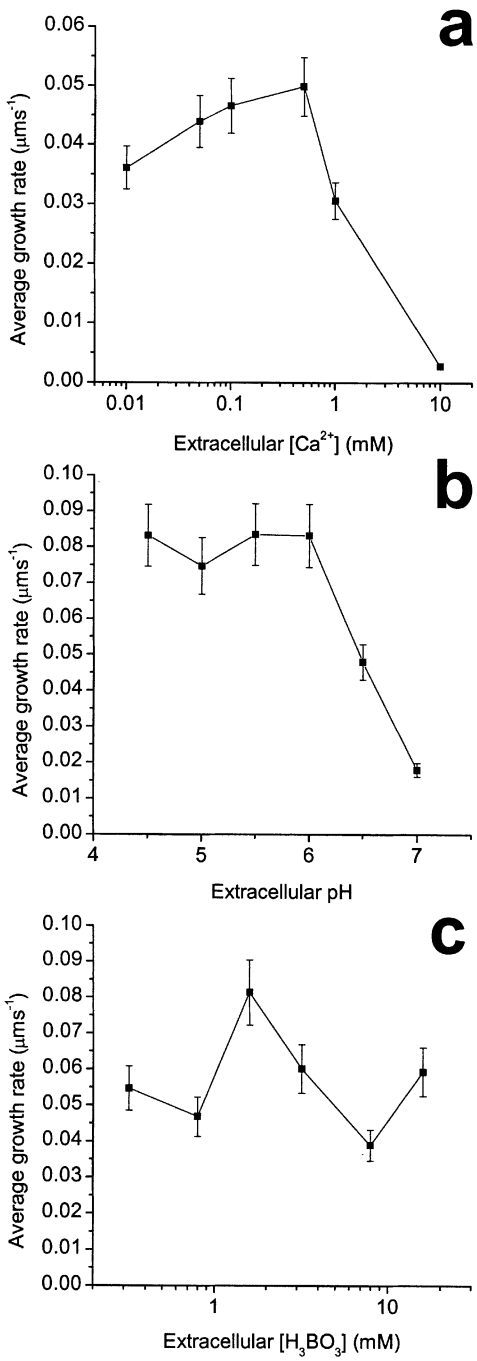


Fig. 2. The effect of extracellular Ca^{2+}, pH and H_3BO_3 on bulk growth rates of pollen tubes <1000 μm. (a) Ca^{2+} concentration was set at 0.01, 0.05, 0.1, 0.5, 1.0, and 10 mM; results are from five replicates. (b) pH was set at 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0; results are from four replicates. (c) H_3BO_3 concentration was set at 0.32, 0.8, 1.6, 3.2, 8.0, and 16 mM; results are from four replicates. Replicates above consist of approximately 100 pollen tubes each.

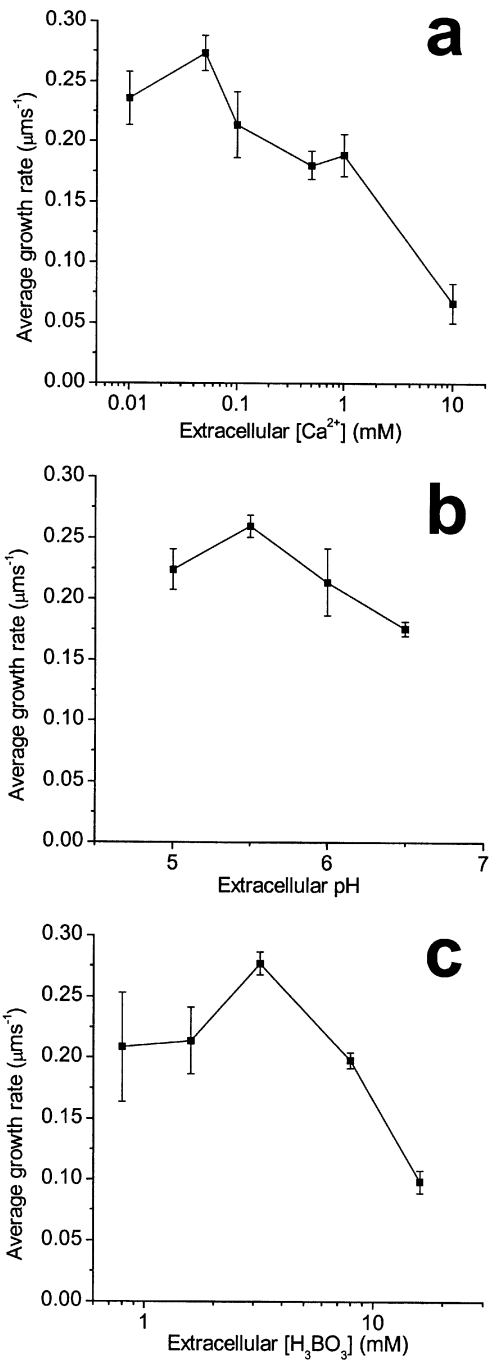


Fig. 3. The effect of extracellular Ca^{2+}, pH and H_3BO_3 on the average growth rates of oscillating pollen tubes (>1000 μm). (a) Ca^{2+} concentration was set at 0.01, 0.05, 0.1, 0.5, 1.0, and 10 mM (n=5). (b) pH was set at 5.0, 5.5, 6.0, and 6.5 (n=7). (c) H_3BO_3 concentration was set at 0.8, 1.6, 3.2, 8.0, and 16 mM (n=7).

Examination of the effects of these three ions on the period and amplitude of growth oscillations reveals that borate generates dramatic changes (Fig. 4). The two concentrations shown, 3.2 mM and 16 mM, produced, respectively, the maximum and minimum average growth rates (0.29 μm s^{-1} and 0.1 μm s^{-1}) and shortest and longest periods of oscillation (68±11 s and 24±2 s). The relative amplitude of oscillation in 16 mM borate was approximately twice that observed in 3.2 mM borate. By contrast with borate, changes in Ca^{2+} and pH appear to have relatively little effect on the period and amplitude of growth oscillation (Fig. 5), although Ca^{2+}, like borate, does produce somewhat shorter periods of oscillation at intermediate concentrations, and longer periods at both higher and lower concentrations (Fig. 5a, c, respectively). The amplitude of the oscillations is most influenced by borate (Fig. 5c).

When the results of period versus growth rate for oscillating pollen tubes under changing Ca^{2+}, pH, or borate are pooled, it becomes apparent that there is a relationship between these parameters for Ca^{2+} and borate, but not pH (Fig. 6). A curve fit of the plot for all Ca^{2+} data revealed a very highly significant linear relationship between period of oscillation and average growth rate (P <0.00129, Fig. 6a). By contrast, curve fits of pooled results for all extracellular pH levels reveal no significant relationship between period of oscillation and average growth rate (Fig. 6b). The borate data set also revealed a very highly significant linear relationship between period and amplitude of oscillation (P <0.0001); however, a hyperbolic fit is a highly significantly better fit than the linear fit (F = test, P <0.001, Fig. 6c).

Discussion

Different phases of pollen growth have different ionic requirements

It is noteworthy that varying the concentrations of Ca^{2+}, H^+, and borate in the extracellular medium has distinct

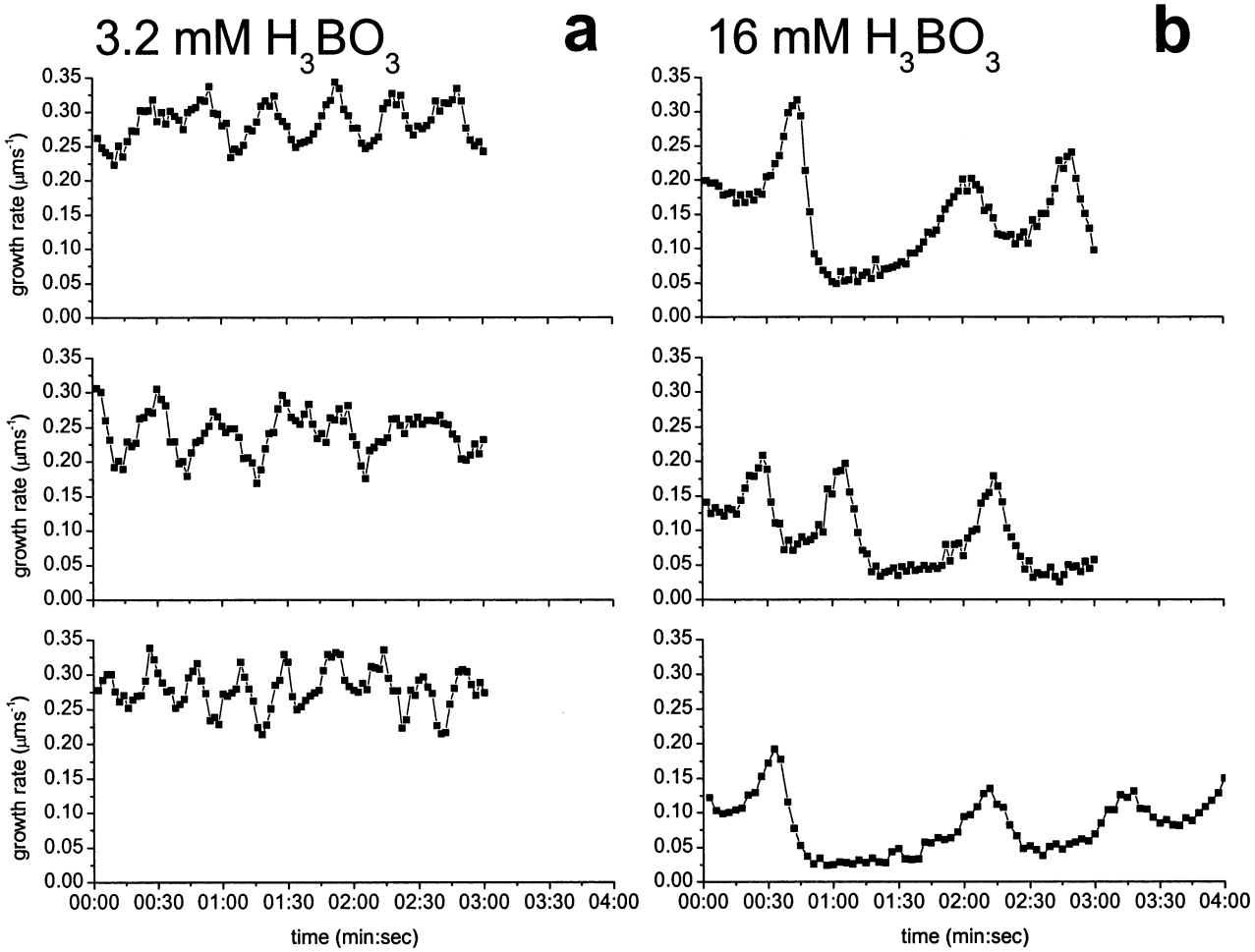


Fig. 4. The effect of two different extracellular concentrations of H_3BO_3 on the period and amplitude of growth rate oscillations. (a) Three typical examples of growth oscillations in the presence of 3.2 mM H_3BO_3. (b) Three typical examples of growth oscillations in the presence of 16 mM H_3BO_3. Comparison of (a) and (b) reveals that rapid growth is accompanied by a short oscillation period and low amplitude, whereas slow growth is accompanied by a long oscillation period and large amplitude.

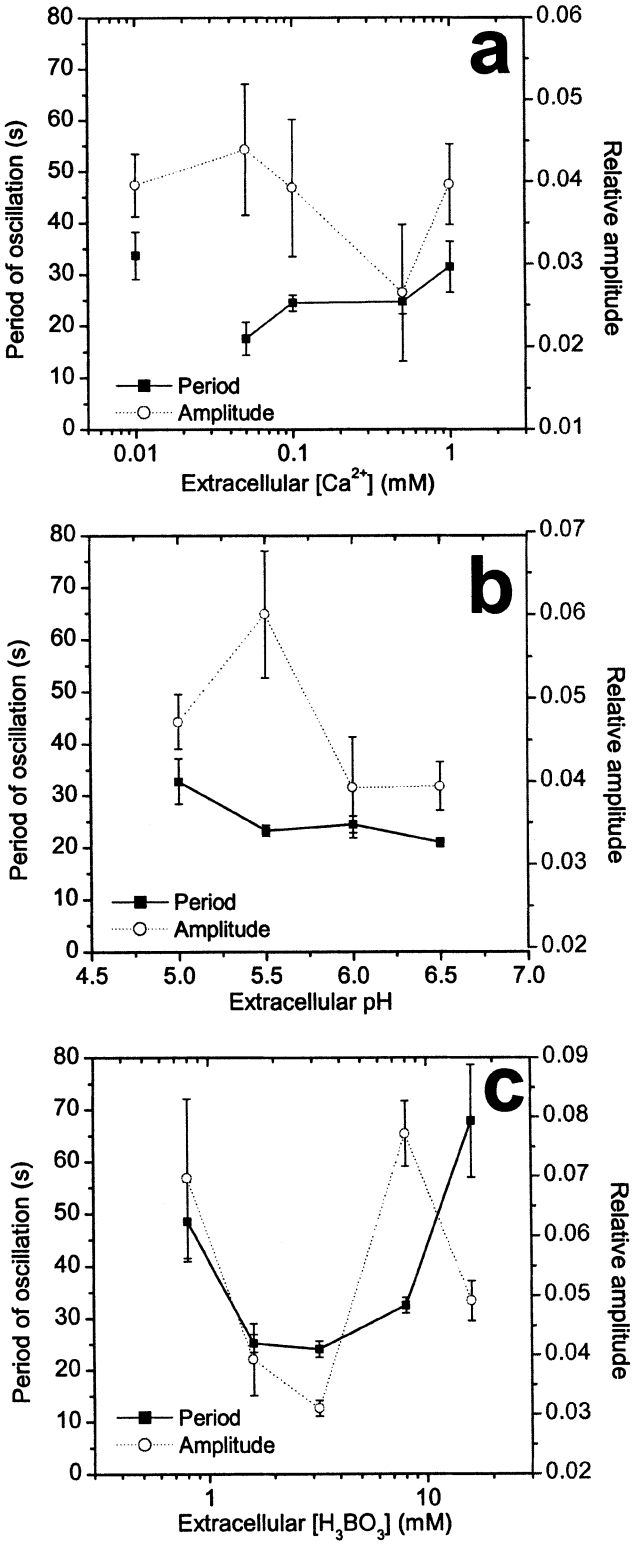
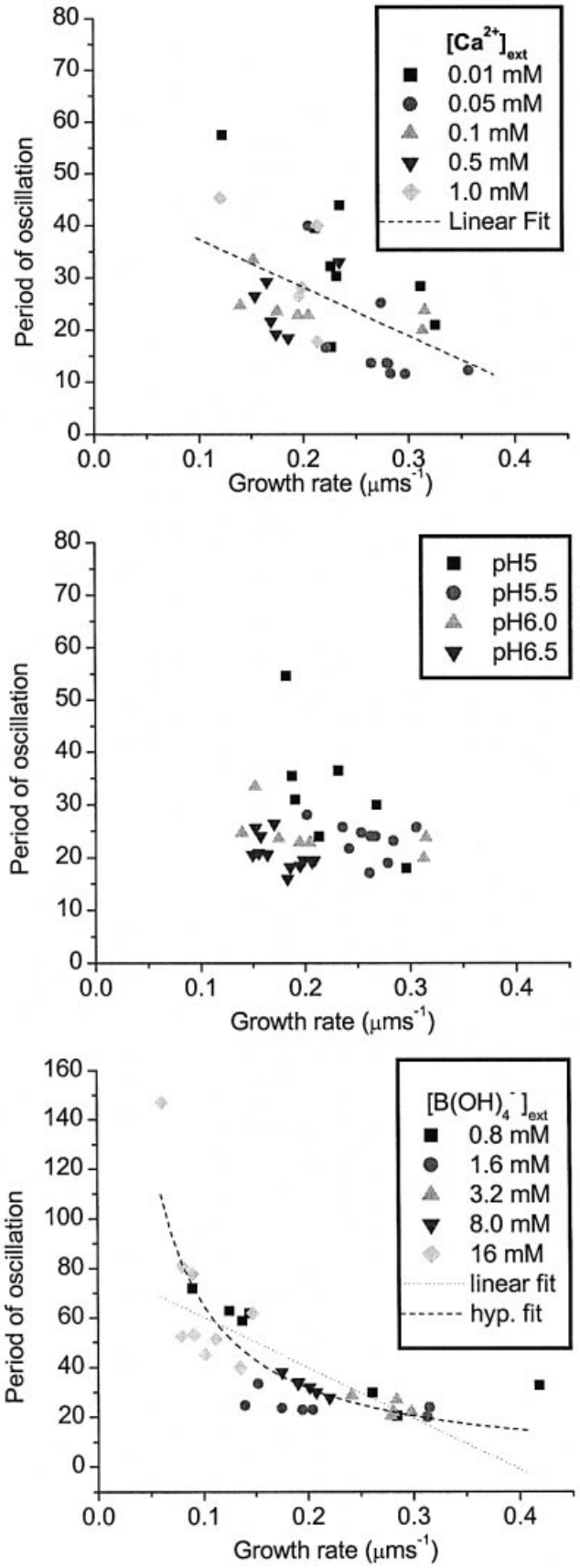


Fig. 5. The effect of extracellular Ca^{2+}, pH and H_3BO_3 on the average period and amplitude of growth rate oscillations. (a) Ca^{2+} concentration was set at 0.01, 0.05, 0.1, 0.5, 1.0, and 10 mM (n = 5). (b) pH was set at 5.0, 5.5, 6.0, and 6.5 (n = 7). (c) H_3BO_3 concentration was set at 0.8, 1.6, 3.2, 8.0, and 16 mM (n = 7).

effects on pollen tube germination rates, growth rates in shorter (non-oscillating) tubes, growth rates in longer (oscillating) tubes, and on the characteristics of growth rate oscillations (Figs 1, 2, 3). Germination rate and average growth rate of shorter tubes was more sensitive to changes in Ca^{2+} and pH, than to borate, whereas the longer, oscillating tubes were severely inhibited by higher Ca^{2+} and borate, while pH had relatively little effect, except at pH 7.0 where it completely stopped growth. These results are consistent with there being three classes of pollen tube growth behaviour as described by Feijó *et al.* (2001): (1) spiking, which occurs at the onset of tube growth or germination, (2) statistically stable growth in which there are random, small fluctuations about the average, as is observed in *Lilium* and *Hemerocallis* tubes that are less than 1000 μm, and (3) oscillatory growth in which there are sustained, quasi-sinusoidal oscillations in the growth rate as is seen in *Lilium* tubes that are greater than 1000 μm. The shorter, non-oscillating pollen tubes also exhibit substantially slower average growth rates than their longer, oscillating counterparts (compare Figs 2 and 3). Taken together these observations support the notion that the oscillations are a consequence of the pollen tube growing as fast as possible, in which the tube comes close to but narrowly avoids the disaster of extending the wall so far and fast as to cause a breach.

Borate cross-linking of RG-II is possibly a major player in the phenomenon of oscillatory growth

In the oscillatory phase, which is of primary interest because the pollen tube does most of its growing in this manner, both Ca^{2+} and borate exert strong influences over the average growth rate. Given the ability of Ca^{2+} and borate, in different cell wall systems, to cross-link pectin chains, and rigidify the cell wall (Ridley *et al.*, 2001) it may be readily understandable that both these ions, when applied to pollen tubes, reduce growth rate at elevated concentrations. Protons, by contrast, can contribute to both the loosening and strengthening of the cell wall. Studies from non-pollen tube systems indicate that acidic pHs inhibit the activity of certain PME isoforms and thus reduce de-esterification and the number of Ca^{2+}-pectate bridges (Moustacas *et al.*, 1986). Low pH also enhances the activity of acidic isoforms of PME which, together with pectin hydrolases, can cause the random degradation of pectin gels and weaken the cell wall (Bordenave, 1996). However, low pH enhances the *in vitro* formation of RG-II borate ester dimers (O'Neill *et al.*, 1996), which *in vivo* may lead to a strengthening of the cell wall. When extrapolated to the growing pollen tube it seems plausible that H^+ will have a similar dual activity on the apical cell wall. On the one hand, low pH may reduce Ca^{2+}-pectate cross-bridges and stimulate pectin gel degradation, thus weakening the apical cell wall. On the other hand, low pH may enhance the formation of RG-II borate esters and



contribute to cell wall stiffening. These opposing responses to low pH may explain why we fail to see a marked effect of proton concentration on pollen tube oscillatory growth. Ca^{2+}, aside from its role in pectate cross-links, can also contribute to maintaining RG-II dimers (Matoh and Kobayashi, 1998).

The correlation of rate and period for pooled Ca^{2+} (Fig. 6a) and borate data (Fig. 6c), but not for pH (Fig. 6b) further indicate that Ca^{2+} and borate have a similar mode of action, which, we propose, is cross-linking of pectins. The relationship between period and average growth rate is logical, as a stiffer wall will yield more slowly and thus produce longer periods between growth rate peaks, and an overall slower average growth rate. Further investigations using different combinations of Ca^{2+}, H^+, and borate should provide more information on the relationship of these ions in determining cell wall extension.

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Fig. 6. Plots showing the relationship between period versus growth rate for oscillating pollen tubes. (a) Ca^{2+}: the dashed line is a linear fit of the pooled data showing a highly significant relationship between the period of oscillation and the average growth rates across all tested concentrations of Ca^{2+}. (b) pH: there is no clear relationship between period of oscillation and growth rate. (c) H_3BO_3: the dashed line is a hyperbolic fit of the pooled data, whereas the dotted line is a linear fit. The hyperbolic fit is significantly better than the linear fit.

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