Determination of selected dynamic quantities of growing intact seeds of maize

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Abstract. Plant growth and intracellular H⁺ ion kinetics are known to be strictly correlated, although the history of this discovery, which is known as the acid-growth hypothesis, has faced many difficulties and provoked a long-lasting discussion. Simultaneous measurements of the plant cell extension and pH of the incubation medium helped to defend the theory and together with some of the newest physics-based models, offered a new insight at the molecular level. This article focuses on both the biological and physical aspects of plant growth in the presence of endogenous auxin. Our aim was to circumvent the experimental and conceptual pitfalls associated with the standard use of cut and/or abraded coleoptile segments. Therefore, we simultaneously investigated the growth of intact seedlings of maize (Zea mays L.) and pH of the incubation medium. The growth rates were measured by applying a non-invasive technique that records time-lapse images of the macroscopic elongation of the coleoptiles, while changes in the pH were monitored using a pH/Ion meter. In the experiments, we intentionally introduced growth stimulators: indole-3-acetic acid (IAA), fusicoccin (FC), gibberellin acid (GA₃), and a growth inhibitor cadmium chloride (CdCl₂), in order to analyse the resultant effect of both exogenous and endogenous factors.

Key words: Acid growth — Auxin — Cadmium chloride — Diffusion rate — Fusicoccin — Gibberellin — Growth factor — Growth equation

Introduction

Numerous scientific publications have revealed a wide range of compounds that affect the growth and development of plants, the origins of which may be endogenous (such as acids, nucleotides, enzymes and plant hormones) or exogenous (acids, nucleotides, enzymes, vitamins, fungal stimulators, inhibitors, attractants and attracting factors, and many miscellaneous compounds), which may induce a two-fold action – stimulate or inhibit the growth of plants.

Auxins are a class of plant hormones (or plant growth substances) with some morphogen-like characteristics (Lüthen 2015). Auxins have a cardinal role in the coordination of many growth and behavioural processes in a plant’s life cycle and are essential for the development of the plant body. Auxins and their role in plant growth were first described by the Dutch scientist Frits Warmolt Went in 1942 (Went et al. 1942). Went’s research was carried out scrupulously and with great paid attention to the details (Lüthen 2015). His tests, which led to the isolation of indole-3-acetic acid (IAA), the biologically active form of auxin, were essential to the success of further procedures (Tivendale and Cohen 2015). In a plant, auxin acts on the plasma membrane H⁺-ATPase, which secretes H⁺ ions into the cell wall compartment. This pump works on the principle of antiport – it takes up K⁺ ions through the K⁺ channel and expels H⁺ into the wall (Hager 2003). The K⁺ ions diminish the cell wall pH, thus causing the activation of pH-sensitive enzymes and proteins within the wall, which initiates the loosening of the cell wall and extension growth. These processes can be inhibited by the export of K⁺ ions or the addition of K⁺-channel blockers. Conversely, H⁺ pumping and accelerated growth (see “inflation phase” – Pietruszka (2017)) are immediately switched.

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on by the addition of K⁺ ions (Hager 2003). IAA, which was the first plant hormone to be discovered, is the primary natural auxin. IAA is a small molecule, which was formed in cyanobacteria at the beginning of evolution (Hager 2003). The auxin polar transport was discovered in the years between Darwin (1880) and Went (1928). Many researchers investigated molecular mechanisms of the process. PINs, ABCBs, PILS and AUX1 genes, which adjust very well to the earlier concepts and findings, were subsequently discovered. Auxin gradients are the basis of the Cholodny-Went theory (Lüthen 2015). The influence of IAA onto the root growth is, however, opposite; as root cell wall is more sensitive to even infinitesimal concentrations of this hormone, adding IAA at concentration typical for shoot growth stimulation causes strong inhibition of H⁺ pump in the root. Therefore, when analysing the growth of the whole seedling, both effects should be taken into account, particularly in interpretation of the diffusion rates (in fact a complicated self-consistent, iterative model should be constructed).

Fusicoccin (FC) is an organic compound that is synthesised by the fungus *Fusicoccum amygdale*, which causes the hyperpolarisation of the cytoplasmic membrane by stimulating H⁺-ATPase and therefore disrupts the ion transport substrates, cell growth, signal transmission and immune gene transcription. It has a negative effect on plants and causes their death (Ballio et al. 1964). It is widely accepted that the effect of FC relies on the activation of the H⁺ pump thus irreversibly causing a high turgor pressure and a dramatic increase in elongation growth (Hager 2003). Astonishingly, the FC receptor (produced by the fungus) was determined to be a protein that belonged to the 14-3-3 protein family (Marra et al. 1994; Oecking et al. 1994). However, FC binds to neither the 14-3-3 nor to the H⁺-ATPase alone (Oecking et al. 1994). It is stabilised by FC binding, thereby creating an H⁺-ATPase/14-3-3 complex (Oecking et al. 1997). One possible explanation is that this complex induces a conformational change and, in consequence, activation of the ATPase (Baunsgaard et al. 1998).

Gibberellic acid (also called gibberellin A₃, GA and GA₃) is a hormone found in plants and fungi. When purified, it is a white to pale-yellow solid (Silva et al. 2013). GA₃ was first identified as a metabolic by-product of the plant pathogen *Gibberella fujikuroi* (thus the name), which affects rice plants, in Japan in 1935. *Fujikuroi*-infected plants develop bakanae ("foolish seedling"), which causes them to grow so much taller than normal that they die because they are no longer sturdy enough to support their own weight (Riley 1987). However, it should be remembered that although plants produce a low amount of (endogenous) GA₃, this hormone can be produced industrially by microorganisms (Silva et al. 2013). The suggestion that gibberellins operate in the plants by eliminating a restriction of growth was confirmed when it was shown that they induce the deprivation of the growth-inhibiting DELLA proteins (Hedden and Sponsel 2015). The evidence that gibberellin generates DELLA protein degradation by the ubiquitination-proteasome pathway and the isolation of the GID1 GA₃ receptor has made specific knowledge of the early incidents in gibberellin perception and action possible (Hedden and Sponsel 2015).

Among heavy metals, cadmium (Cd) is one of the toxic elements that do not play a vital role and that is therefore unnecessary for living organisms. It has a long biological persistence that cadmium causes leaf rolls, chlorosis and a reduction in root and stem growth (Smeets et al. 2005; Mishra et al. 2006). One of the biochemical changes that occur in plants that are subjected to Cd stress is the production of reactive oxygen species (ROS), which leads to oxidative stress (Bahmani et al. 2012). ROS have a specific role in lipid peroxidation, membrane damage and therefore in the aging of a plant (Zhang et al. 2003). Furthermore, this heavy metal directly affects photosynthesis through changes in chlorophyll biosynthesis and the appropriate development of the chloroplast ultrastructure (Bahmani et al. 2012).

Coleoptile is a pointed protective sheath covering the emerging shoot in monocotyledons such as grasses. In studies by Lüthen et al. (1990), the coleoptiles were abraded of the cuticle. The reason for this was that the cuticle prevented the effect of auxin and other growth factors that affect proton secretion to be compared. Abrasion of the cuticle, or other often-used techniques, is an external interference with growth processes, which induce stress reaction of the plant via changes in the hormone equilibrium. These effects we wanted to avoid and for that reason we proposed an experiment for whole intact seedlings. Moreover, in this research, growth factors came into contact with the seedlings via the roots and are transported within the plant in xylem and phloem. In our experiment, growth factors were present in the medium, and were retrieved with water by the roots (in the root hair zone) and then distributed over the entire plant. Then, they intermingle with endogenous hormones (mainly auxin) resulting in the observable growth effect.

Finally, we should note that one of the physical (abiotic) factors that have the largest impact on the growth of plants and microorganisms is the pH of the environment (and temperature). In chemistry, pH is defined as the negative logarithm of the activity of the hydrogen ion in an aqueous solution. pH is most often expressed as the measure of the concentration of the hydronium ion (Covington et al. 1985). It can be measured, at a fairly constant temperature, simultaneously with the elongation rate of seedling fragments, a coleoptile sections. These data, which are an indication of the transport properties in the whole plant, were our main interest.

For this research, a set of three broadly used plant growth stimulators in a wide range of concentrations, namely auxin, fusicoccin and gibberellins, were selected. For
comparison, cadmium chloride was also used as a growth inhibitor. All results are interpreted in the context of the presence of the endogenous auxin – an integrated system of a growing seedling is submitted to external (chemical) perturbation induced by the effector in question (IAA, GA, FC, CdCl₂).

Materials and Methods

Plant material

Seeds of maize (Zea mays L.) were first soaked in tap water for 2 h, then sown in moist lignin in an incubator. The seedlings were grown in darkness at 27 ± 0.5°C after which four-day-old plants of about 2.5 cm were selected for further treatment – 5 mm below the tip, a segment of 10 mm length was marked with ink and these prepared seedlings were inserted into the incubation medium for each investigated variant. Because of the limited angular range of the CCD camera, only three pieces were selected for observations.

Growth factor solutions

The research was carried out simultaneously for several growth factors: IAA, FC, GA₃ and CdCl₂ in various concentrations, namely 0.5 × 10⁻⁷–10⁻³ M for IAA, 10⁻⁸–10⁻⁵ M for FC, 0.5 × 10⁻⁷–10⁻³ M for GA₃ and 0.5 × 10⁻⁷–10⁻³ M for CdCl₂. The growth factors were dissolved in distilled water. Control experiments were also performed on seedlings grown in artificial pond water, APW (0.1 mM NaCl, 0.1 mM CaCl₂ and 1 mM KCl; pH 5.8).

Measurement and conditions of growth

The growth measurements were performed in the following way: seedlings that were prepared in the manner presented above were grown in fixed environmental conditions – incubation medium, temperature and humidity, in dim green light. Images of the seedlings were recorded every 30 min using a CCD camera (Hama Webcam AC-150). At the same time, the temperature and pH of the incubation medium were measured using two pH/Ion CPI-501 pH meters (one for the control), Figures S1–S2. Although the measuring error did not exceed 0.01, the accuracy level was 0.002 pH according to the manufacturer’s information. The length of the coleoptile segments (initially 10-mm-long fragments indicated by ink spots) was measured in ImageJ program with the accuracy calculated at the ±0.1 mm level. Of the three sets of data (for three growing seedlings), only one, which will henceforth be treated as representative, was chosen for further analysis and two seedlings that were farthest from the mean data were rejected. The relative elongation was calculated using the simple formula \((l_t - l_0)/l_0\) with \(l\) for “length” (\(l_0 = l(t = 0)\)). The experimental data that were obtained by this routine were used in the fitting procedure to interrelate the elongation growth of the coleoptile segments and the growth functional at a constant temperature (Fitexex program, Python code, Zajdel et al. 2016) and are presented in Figures S3–S6. Next, OriginPro 8.5.1 software (Microcal) was used to create the remaining graphs and to perform the calculations. Since only one series of pH measurements was performed for each experimental variant (plus pH for the control), no standard error was calculated and no error bars were put on the plots, see Cumming et al. (2007) for explanation.

The ideal situation would be to have a closed system (in the thermodynamic sense, i.e. with no mass and no energy transfer (outside the measurement chamber), in which all of the environmental conditions could be easily maintained on the same well-defined level. Although humidity was quite well kept at 40%, the most challenging task was to maintain a constant temperature. Temperature is the environmental parameter that explicitly enters the model that was applied in our study, in particular, it has a strong impact on quantities C and D, and therefore it must carefully be kept constant; in our experiment, the average (estimated) fluctuations were about 0.5°C and never exceeded 1°C.

Relative elongation

A formula for the relative volume growth rate, expressed analytically by the growth functional derived by Pietruszka (2012, 2013), which was further elaborated in detail for practical use in Zajdel et al. (2016), was used for data analysis (Supplementary Tables S1–S4):

\[
\frac{V_t - V_0}{V_0} = At + B + C \exp(-\exp(-D(t - t_0)))
\]

where coefficients A, B, C and D are constant in time and V is the expanding volume; \(V_0 = V(t = t_0)\), \(V_T(t) = V(t; T = \text{const})\), where T stands for the temperature in Celsius scale. A time instant \(t = t_0\) denotes the effective particle flow that corresponds to the maximum in the growth rate, see Zajdel et al. (2016). It follows from our earlier considerations (ibid.) that the coefficient A is proportional to the effective pressure \((P - Y)\) and the concentration of the solution; the dimensionless coefficient B has no interpretation. The coefficient C is associated with the ‘amplitude’ of growth. Furthermore, it is related to the temperature or the acidity (pH) of the environment (Pietruszka 2017) and can be adequately described by the Euler beta function (Pietruszka and Haduch-Sendecka 2016). Finally, the coefficient D determines the (volumetric) speed of diffusion (not to be confused with the diffusion...
constant encountered in Fick’s laws) and it can be compared for the different concentrations of the substance that are contained in a medium. In the analysis, the diffusion rate $k_2$, which involves the net transport to/from the cell, was our main interest since (among others) it can be directly inferred from the interpolations of the experimental data. Its value depends on dimensionality such as $[k_2] = [D] = 1/s$ and therefore can be compared between different species in various experimental conditions.

Cross-correlations

In signal processing, cross-correlation is the measure of the similarity of two waveforms as a function of a time delay that is applied to one of them and is also known as the sliding dot product. For the continuous real functions $f$ and $u$, the cross-correlation is defined by the integral

$$
(f * u)(\tau) = \int_{-\infty}^{\infty} f(t)u(t + \tau)dt
$$

where $\tau$ is the time lag. Note, that the cross-correlation takes its maximum at the lag equal to the time delay. The cross-correlation time-derivative reads

$$
\frac{d}{d\tau} (f * u)(\tau) = \frac{d}{d\tau} \int_{-\infty}^{\infty} f(t)u(t + \tau)dt
$$

By assuming $f = pH(t)$ and $u = u(t)$ [\mu m], the cross-correlation derivative (over time delay $\tau$) can be calculated explicitly as follows

$$
\frac{d}{d\tau} (pH * u)(\tau) = \frac{d}{d\tau} \left[ \int_{-\infty}^{\infty} pH(t)u(t + \tau)dt \right] = \\
= \int_{-\infty}^{\infty} pH(t) \frac{d}{d\tau} u(t + \tau)dt = \int_{-\infty}^{\infty} pH(t)u'(t + \tau)dt
$$

where $u' = du/d\tau$ and denotes the growth rate and pH is non-separable variable name.

Results

The relative elongation interpolated by Fitexex program

All growth data were arranged into two columns – time (in hours) and RE (relative elongation – dimensionless) and saved as a .txt file – input for Fitexex program (Zajdel et al. 2016). Next, we started the program and fitted the model (1) to the uploaded data. The following results were then collected: the estimated model parameters $A$, $B$, $C$, $D$ and $t_0$ with errors and saved in report files, while plots of data points and model functions in the form of .png figures, here shifted to the supplementary file and in the central part of the manuscript we present one representative (Fig. 1a). The main important conclusion is that the model fits extraordinarily well. The goodness of fit is expressed by the squared determination coefficient $R^2$, which in our case was very close to 1. This remarkably high value of determination coefficients allowed us to determine the $A$, $B$, $C$ and $D$ co-

![Figure 1](image-url)

**Figure 1.** a. Elongation growth of maize coleoptiles grown in APW (artificial pond water, control growth) and the fitting curve with its two components – linear and sigmoid (the comparable contribution of both branches is clearly visible). b. Derivative of elongation growth reflecting how quickly it takes place. Temporary leaps and drops that indicate strong fluctuations are clearly visible. The high amplitude of these fluctuations results in significant dispersion of the fitting parameters $C$ and $D$, hence the statistical errors, as pointed out in the text. The vertical line shows the position of the inflection point $t_0$, Eq. (1).
efficients with a high fidelity, though the calculated errors exceeded 10% of the actual value of the parameters in some cases, see Tables S1–S4. One may conclude that all of these coefficients are subject to experimental errors of diverse biological and purely mathematical origins thus resulting in dispersion. The main problem in fitting nonlinear models in extrapolation methods is that a visibly small change in the fitted curve may even arise from two significantly different parameters. This especially applies to the parameters that are responsible for the width of the curve or the rate of the increase/decrease, such as D in our model or, e.g. the half-width of resonance curves (Pietruszka and Lewicka 2007). Even a major variation of the D parameter can produce visibly similar curves with very close chi-square test results, and hence relatively large errors (see also the very thorough and comprehensive discussion in Trunstrum et al. (2010)).

Certainly, the dispersion of the experimental points is not of a lesser significance; the point is, however, what are the reasons for such a dispersion (clearly visible in the plot of the calculated derivatives, see Fig. 1b). One putative cause is the effect of transport and diffusion through the root system and random pH fluctuations. We can imagine that immediate leaps in pH or the diffusion rate imply a greater/less elongation growth of the seedling, thus resulting in scattered data and relatively large estimated errors for the fitting parameters. On the other hand, this preliminary data may provide new information about the transport (diffusion) properties of a growing seedling as a whole, especially that the most up-to-date experiments were carried out on the coleoptile segments that were cut from the plant (hence had undergone abiotic stress) and, moreover, the results were averaged for several dozen segments.

All of the results of the presented fitting procedure are collected in Supplementary Information files, Figs. S3–S6. The most interesting information that can be derived from coupling these results with the data for pH over time for different concentrations of the growth factors (see Fig. S3, S9 for FC; Fig. S4, S10 for IAA; Fig. S5, S11 for GA; Fig. S6, S12 for CdCl₂). The reason for this is that, we suppose, the pH of the incubation medium and the growth of the maize seedlings should be correlated as they were measured simultaneously and we accept acid growth theory. Comparison of the data for pH over time to the fitting results should give us the answer to whether or not the elaborated model (Zajdel et al. 2016; Pietruszka 2017) is good and is based on well-defined reasoning. One of its conclusions is that the relative elongation in time has two branches – linear and sigmoid; the linear one originates from (and is strictly correlated to) the constant pH of a medium, while the sigmoid branch – with pH that non-linearly descends in time. We stress that, according to the mentioned model, the linear part is responsible for the stage of the cell wall biosynthesis during plant growth, while the sigmoid part is responsible for diffusion stage and D stands for the depletion rate, while C contains both the production and depletion rates.

From our data for FC one can deduce that the model (Pietruszka 2017) predicts this very well. In the cases in which pH strongly decreases during the entire time period, the nonlinear branch gives the main contribution, see Figs. S3a,c,h and compare them to S9a,c,h. However, when pH decreases strongly only in the first several hours and then remains almost constant (Fig. S3e,f), both branches contribute to the relative elongation curve to a more or less equal extent (Fig. S9 e,f). On the other hand, when the linear branch is strong in the relative elongation RE(t), the pH of the incubation medium is constant (S9g) or has a diverse behaviour (S9b). Only Figs S3/S9 d produce interpretative problems as RE is almost entirely sigmoid, whereas pH has a wide maximum with a long-time plateau, which makes interpretation difficult indeed.

A very similar conclusion can be drawn for the IAA data (Figs. S4 and S10). In the cases in which there is almost no or only a very small contribution of the linear branch in relative elongation (Figs. S4/S10d,e,f,g), pH always declined, while for proportionate contribution of the linear and sigmoid branches, pH is almost constant and even fluctuates over time, see Figs. S4/S10a,b,c).

It is a difficult task to unravel the effect of GA₃ on both relative growth and pH. It seems that this phytohormone, when added exogenously, has little or no effect on pH and a rather small (and quite chaotic) impact on the growth of maize coleoptiles. These behaviours are well known in the literature, see e.g. Stuart and Jones (1978) or Brock and Cleland (1989), although scientists usually study the effect of GA₃ on excised coleoptiles and hypocotyls or on intact dwarf plants, see Chandler and Robertson (1999). It looks as though the impact of GA₃ on pH that is measured simultaneously with growth for different doses is presented for the first time in the literature, see a thorough discussion in the Discussion section.

The influence of CdCl₂ on both of the quantities studies is very interesting and slightly bothersome at the same time. Although Cd is toxic in a wide range of concentrations, it still acidified the incubation medium even though the growth of maize coleoptiles was damped in relation to the control growth. A detailed analysis is as follows: in almost all cases, the linear part of the fitted model is clearly visible (Fig. S6a,b,c,e,f,g,h,j) and is even comparable with the nonlinear part (Fig. S6a,b,c,g,h,j), although in one case it is significantly greater (Fig. S6f). Therefore, it can be concluded that even a high concentration of CdCl₂ has no effect on the biosynthetic aspect of plant growth at least in first two days after application. Next, a very strong decrease of pH is correlated with an almost zero contribution of the linear branch, Figs S6/S12i,d. In the cases of S6/S12f,j, there are very clear and dominating linear branches, but both pH(t) diminish over
the whole time range, although they have a wide plateau, which may be the putative origin of the linear branches. In Fig. S12g,h, the pH is almost constant and the linear part of the model curve is also significant, see Fig. 6g,h.

To sum up, what results from the above considerations is that, generally, there appears to be an almost zero contribution of the linear branch in relative elongation RE(t) together with a strongly decreasing pH, while there is a significant contribution when the pH is linear in entire or almost entire time period. This conclusion strongly supports the model that has been elaborated in earlier studies, Pietruszka (2017).

### Fitting parameters C and D

Basically, only $D$ and partially $C$, provide the most accurate information, because the first one is equal to the depletion rate $k_2$ in the diffusion stage of plant growth (see: newly proposed model, Zajdel et al. 2016) and the second one is related to both the $k_1$ (production) and $k_2$ (depletion) rates (Pietruszka 2012 for details). Moreover, mathematically, $C$ is the amplitude, and therefore it relates to the final volume/length, while $D$ answers the question of how quickly this value can be reached by a growing plant. Therefore, in the central part of our manuscript, we present a full analysis of these parameters, while the remaining parameters ($A$, $B$) were shifted to the supplementary file. Parameters $C$ and $D$, which were calculated for each growth factor, as a function of applied concentration, are presented in Figs. 2 and 3, respectively. We analyzed them simultaneously.

For IAA, the amplitude $C$ reached the highest values within the range of physiological concentrations ($10^{-6}$–$10^{-4}$ M), while at the same time, the depletion rate $D$ decreased in the case of the growth of the control. The interpretation is straightforward. When IAA is applied in physiological concentrations, it does not increase the growth rate of intact maize seedlings very much in the diffusion stage, but does increase their final volume/length rather significantly. This also means that the production rate $k_1$ is much greater that in the control. In a sense, a similar conclusion can be drawn for fusicoccin, although the range of concentration is $10^{-7}$–$10^{-6}$ M and both $C$ and $D$ are greater than in the case of the control. This would suggest that the toxin both quickens the elongation growth and increases the diffusion rate of the (internal) growth factor; this also indirectly supports the model that has been elaborated in earlier studies, Pietruszka (2017).
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points to the diverse action of both growth factors at the biophysical level.

Once again, the action of GA$_3$ causes the biggest problems in interpretation. The fitting parameters $C$ and $D$ change alternately with increasing concentrations, although the total correlation is positive and equals about 0.32. This means that studied effect is moderate; however, it is difficult to read. The fact that the diffusion coefficient is, on average, larger than it is in the control growth is undisputed. Thus, it can be concluded that gibberellins act on plant growth via the diffusion process, as well as through the biosynthesis processes that occur in the plant cell wall, because the linear branch is clearly visible and is significant at most concentrations (see Fig. S5). Simultaneously, at this stage, the task of determining the dependency of the production rate (included in $C$ parameter) with increasing concentrations is a bit troublesome. There are several probable reasons. The first is that GA$_3$ does not influence the elongation growth of monocot coleoptiles as obviously as, e.g. auxin does. The second reason was a consequence of the experimental conditions – the measurements were conducted on intact seedlings. The last reason is that gibberellins do not fully dissolve in water and their uptake by plant roots may be limited.

The $C$ and $D$ parameters, because they are dependent on the cadmium dichloride concentrations, definitely behave less randomly, although one can be astonished by the fact that $C$, which takes a significant part of the growth amplitude, can be even greater than in some cases of stimulated growth. Nonetheless, $C$ is mostly lesser, especially at high concentrations ($10^{-4}$ M and above), and this undoubtedly reflects the toxicity of cadmium and its inhibitory influence on plant growth. Simultaneously, the diffusion parameter $D$, within quite a wide range of concentrations, remained at the same level as in the control growth, although at $0.5 \times 10^{-6}$, $10^{-6}$ and $0.5 \times 10^{-5}$ M it was greater. A very interesting and straightforward conclusion can be drawn from these data. Since the diffusion coefficient describes the depletion of the natural growth stimulators in the intact plant seedling quantitatively, the highest rate of the depletion takes place at the mentioned concentrations, which is opposite to the case of the auxin effect, in which the diffusion coefficient is lower. Parameter $C$ for IAA and CdCl$_2$ in Fig. 2 and $D$ in Fig. 3 behave in an almost perfectly opposite

Figure 3. Parameter $D = k_2(s^{-1})$ of the fitted sigmoid curve as calculated for elongation growth of maize coleoptiles grown under a constant dim green light at 25°C in artificial pond water (APW; control, asterisk in the plot) and under the influence of indole-3-acetic acid (IAA), fusicoccin (FC), gibberellic acid (GA$_3$), and a growth inhibitor cadmium chloride (CdCl$_2$) (a,b,c,d, respectively). See also Fig. 6.
manner and this stimulatory behaviour for IAA and the opposite inhibitory behaviour for CdCl\(_2\) are well known in the literature, e.g. Hu et al. (2013), Farooq et al. (2015), see also Discussion.

This stimulatory/inhibitory behaviour is clearly visible when comparing the fitting parameters \(C\) and \(D\) for the different growth factors applied at the same concentrations, see Supplementary Information and Fig. S7 for the \(C\) parameter and Fig. S8 for the \(D\) parameter. The amplitude (and thus the production rate) is the highest for growth stimulators at their physiological concentrations while they are the lowest for the inhibitors (the heavy metal compound CdCl\(_2\) or phytohormones at high – far above physiological – concentrations) (Fig. S7). In turn, the diffusion coefficient (and thus the depletion rate) is the highest in the cases in which the toxic effects of the growth factors were observed (auxin at a toxic \(10^{-3}\) M concentration, fusicoccin at its toxic concentrations \(\geq 0.5 \times 10^{-5}\) M and CdCl\(_2\)) (Fig. S8).

Cross-correlations

Cross-correlations are helpful for determining the strength of correlations and the time delay between two signals. These were calculated between growth (first signal) and pH (second signal) for IAA, FC, GA\(_3\) and CdCl\(_2\) that were supplemented into the medium at different concentrations. The plots in Figs. 4a–d show that the increased elongation growth of a coleoptile is strictly associated with changes in pH. In addition, we also calculated the cross-correlation derivative, which corresponds to formula (4), using the MicroCal Origin program. The resulting discontinuities, which are clearly visible in Figs. 5a–d, are related to a drop in pH.

In short, the calculated cross-correlation intensity (at zero time delay) for a concentration of \(10^{-3}\) M for IAA, GA\(_3\) and CdCl\(_2\) was smaller than for the control (Figs. 4a,c–d). In these cases, the cross-correlation intensity was also the weakest among all of the investigated data for GA\(_3\) (Fig. 4c). In the case of \(0.5 \times 10^{-3}\) M concentration for CdCl\(_2\), the correlation was stronger than for the control in contrast to IAA and GA\(_3\) (Figs. 4a,c–d). A weak correlation was observed for a \(10^{-4}\) M concentration in all three cases (Fig. 4d). In the case of \(0.5 \times 10^{-4}\) M the correlation was close to the control (Fig. 4a) only for IAA, while for the remaining two growth factors, the correlation was smaller than for the control. The experiments were performed for all four growth factors at a threshold concentration of \(10^{-5}\) M. A weak correlation was observed for all four cases (Figs. 4a–d) at this peculiar concentration, while a strong correlation was detected only

Figure 4. Cross-correlations of elongation growth and pH as a function of time lag \(\tau\) (h) as calculated for maize coleoptiles grown under a constant dim green light at 25°C in artificial pond water (APW) and under the influence of indole-3-acetic acid (IAA), fusicoccin (FC), gibberellic acid (GA\(_3\)), and cadmium chloride (CdCl\(_2\)). Analysed data in Supplementary Figures S3–S6.
Selected dynamic quantities of growing seeds of maize for CdCl$_2$ (Fig. 4d) at $0.5 \times 10^{-5}$ M. The cross-correlation for a concentration of $10^{-6}$ M was similar and close to the control for FC, GA$_3$ and CdCl$_2$ (Figs. 4b–d), while for IAA (Fig. 4a) the correlation at $10^{-6}$ M was weak. Subsequent probes for $0.5 \times 10^{-6}$ M revealed that the correlation was intense exclusively for CdCl$_2$ (Fig. 4d) and the remaining factors resulted in correlations that were similar to the control. The strongest correlation occurred at $10^{-7}$ M for CdCl$_2$ (Fig. 4d), at which the correlation was greater than the control and for IAA, FC and GA$_3$ (Figs. 4a–c), at which was smaller than the control. The correlation intensity at $0.5 \times 10^{-7}$ M was only large for CdCl$_2$ (Fig. 4d). Concentrations of $10^{-8}$ M and $0.5 \times 10^{-8}$ M were probed exclusively for FC (Fig. 4b). The strongest correlation occurred at $10^{-8}$ M (Fig. 4b).

The maximum values for the cross-correlation from Figs. 4a–d are introduced collectively in Supplementary table S5 and Figs. S15a–d. The time delay for the control was nine hours. In the case of IAA for a concentration of $10^{-5}$ M, the time delay was bigger than the control (13 hours) and for other concentrations it was smaller than the control, except for concentrations of $0.5 \times 10^{-3}$ M and $10^{-7}$ M for which the time delay was zero (Supplementary Fig. S15a). The longest time delays for FC than for the control were found at the concentrations: $10^{-5}$ (10.5 h), $0.5 \times 10^{-6}$ (11.5 h) and $0.5 \times 10^{-8}$ (9.5 h) M. The smallest time delay was for $10^{-8}$ M (2.5 h), see Supplementary Fig. S15b. The largest time delay for GA$_3$ was observed at a concentration of $10^{-6}$ M (12.5 h). The other concentrations had a shorter time delay than the control and one was the same as for the control ($0.5 \times 10^{-4}$ M). At a concentration of $10^{-5}$ M, the time delay was zero (Supplementary Fig. S15c). For CdCl$_2$, three concentrations had a longer time delay than the control, namely $0.5 \times 10^{-3}$, $0.5 \times 10^{-4}$ and $10^{-7}$ M. The longest was 11 hours for $0.5 \times 10^{-4}$ M. At a concentration of $0.5 \times 10^{-7}$ M, the time delay was the same as the control (9 h). The other concentrations had a shorter time delay than the control and at a concentration of $0.5 \times 10^{-6}$, it was zero (Supplementary Fig. S15d).

The discontinuities that were noticeable on the cross-correlation derivative in Figs. 5a–d are presented in Supplementary Table S6 and Figs. S16a–d and were interpreted as H$^+$-activity. The biggest H$^+$-activity for IAA occurred for the concentration of $10^{-7}$ M (31.8 $[-\log(a_{H^+})$ per $\mu$m]), which was more than control (28.1), and these are presented in Figs. 5a and S16a. The smallest H$^+$-activity was detected in the concentration of IAA of $10^{-5}$ M (10.7) in the case of FC, the highest H$^+$-activity was at a concentration of $0.5 \times 10^{-6}$ M (40.2) and was also higher than the control. The largest concentration of FC of $10^{-5}$ M (17) had the lowest H$^+$-activity (Figs. 5b and S16b). The highest H$^+$-activity for GA$_3$ was for a concentration of $10^{-6}$ M (36.5), which was

**Figure 5.** Cross-correlation derivative as a function of the time lag for the action of different concentrations of indole-3-acetic acid (IAA), fusicoccin (FC), gibberellic acid (GA$_3$), cadmium chloride (CdCl$_2$) (a,b,c,d, respectively) and for artificial pond water (APW; control).
also higher than the control. This result credibly reflects the fact that 1 µM is the optimal concentration of GA$_3$ for the elongation growth of many species. The smallest H$^+$-activity was at a concentration of 10$^{-5}$ M of GA$_3$ (14.3) and was two-fold smaller than the control (Figs. 5c and S16c). The highest H$^+$-activity for CdCl$_2$ was at a concentration of 0.5 × 10$^{-6}$ M (56.7, for all data), which was also higher than the control. Like in the above-considered cases, the highest concentration of 10$^{-3}$ M of CdCl$_2$ (24.2) produced the value of H$^+$-activity, which was smaller than the control. CdCl$_2$ of 10$^{-4}$ M (15.5) produced the lowest H$^+$-activity (Figs. 5d and S16d).

Discussion

The impact of growth factors on intact growing maize seedlings

Plants are susceptible to many undesired effects. Therefore, this research was carried out on intact coleoptiles for which the results seemed more reliable, although one may suspect a very weak connection between the pH of the incubation medium and coleoptile growth since the roots were immersed in the medium and not the coleoptiles. There is no clear evidence that the pH of a medium is correlated with the acidification of the cell wall in plant organs that are growing with no direct contact with the medium in the literature. It could then be suggested that a decrease in pH is in fact due to the elongation growth of roots, which was not measured, but which did indeed take place, as one can easily see on Fig. S1, which shows the growth of the control. In order to respond to this criticism, let us recall the role of the xylem/phloem in plant growth and development. To date, most research papers have concentrated on the link between cell wall acidification and the elongation of a given plant organ. In these studies, the organs were excised from seedlings and put into an incubation medium in which pH was measured during the entire experiment, e.g. Lüthen et al. (1990). Some researchers focused on the problem of measuring the pH of both the cell wall and cytosol during growth; in particular, they proved that the cytosol is a good buffer with a very stiff pH that is slightly above the neutral 7.0, even when the pH of the incubation medium reaches the uppermost values, Shabala et al. (1997), Shabala (2006). Unfortunately, there is a lack of any experiment in which the pH of the incubation medium and the acidification of the cell wall of an elongating organ in the intact seedling were measured simultaneously. Nonetheless, what results from our knowledge is that the protons that are extruded during coleoptile/hypocotyl/root elongation growth diffuse to the phloem and together with photo-assimilates and nutrients are redistributed throughout the entire plant, see e.g. Larcher (2001) and eventually, through the root system, after which they permeate into the incubation medium, thus resulting in a decrease in pH. Therefore, the measured pH is in fact the
sum effect of the cell wall acidification during the elongation growth of all of the organs of the whole seedling – the coleoptiles, mesocotyl and roots. Therefore, we have to read the data for pH together with the growth rate of the coleoptile segment carefully. In fact, this is the reason why pH always decreases even when toxins or hormones are applied at inhibitory concentrations. Needless to say, the entire system acts in a self-consistent manner.

It must be kept in mind that roots are a good barrier, especially for heavy metals and even when they are added at high concentrations, the reaction of shoots should be retarded and diminished with respect to the roots. Many authors have reported that the first barrier for Cd ions is the cell wall of the root cells, where Cd is immobilised due to their binding to the peptic and hystidyl groups, or in the extracellular space where it is attached by extracellular carbohydrates (see the review of Di Toppi and Gabbrielli (1999) and papers cited therein). In addition, Hart et al. (1998) studied the uptake and accumulation of Cd in bread and durum wheat. They reported that the accumulation of heavy metal ions is several times lower in shoots than in roots and that this effect varies among species. This led the authors to the conclusion that the Cd ions were retained in roots, probably due to sequestration and a decreased xylem loading of Cd and that the investigated species may use these mechanisms to different extents. Interestingly, even after very long-term exposure to Cd (90 days in the work by Hediji et al. 2010), the ratio of root to shoot Cd accumulation was about 3 times in tomato plants, even at a high concentration of $10^{-4}$ M. The accumulation of Cd ions and their increased impact on the elongation of roots is also clearly visible in our experiment (though it was not explicitly measured). When Fig. S1b to a are compared, the maize roots seem to show no elongation growth within the entire two-day period of the experiment in the case of CdCl$_2$, in contrast to the shoot. From this picture, it might then be concluded that Cd primarily inhibits root growth.

The fact that the effects of IAA and CdCl$_2$ are opposite and that this applies to both parameters – C and D, is interesting. Indeed, this fact is well known in the literature, e.g. in Farooq et al. (2015), the researchers studied the influence of cadmium on plant growth and development with or without the addition of the auxin precursor L-TRP to the incubation medium. The addition of L-TRP significantly improved plant growth and yield under cadmium stress. Similarly, Karcz and Kurtyka (2007) investigated the interrelated effect of Cd and IAA on the elongation growth of maize coleoptile segments. They drew several conclusions that are interesting from our point of view. The first is that cadmium in concentrations of even up to $10^{-3}$ M does not significantly change the elongation growth, while higher concentrations decreased the growth two-fold. The addition of IAA to the incubation medium also counteracted the toxic effect of Cd. Our simulation results for the parameters C and D are in agreement with those presented above; they behave in an opposite manner for IAA than for Cd, at concentrations of up to $10^{-3}$ M, above which even IAA inhibits the plant growth.

From our fitting results for parameters C and D, a conclusion can be drawn about the nature of the action of IAA and FC on plant growth. It is clearly visible that the diffusion coefficient D has its minimum value for lower concentrations in the case of FC than IAA. We deducted in the Results section that the minimum would indicate that the action of IAA on the growth of intact seedlings is not so much via the diffusion process as through the biosynthesis of the plant cell wall, and that the diffusion in the physiological concentrations is of lesser significance. A minimum also exists for FC, although at much lower concentrations (less than $10^{-7}$ M) and simultaneously with lower values of the C parameter. Authors typically used 1 μM = $10^{-6}$ M of fusicoccin to determine whether it has a strong influence on plant growth. In this case, both D (diffusion rate) and C (the parameter that contained diffusion and production rate) are higher than in the control. This means that the toxin diffuses much better. This would also explain why FC depolarises the cell membrane of parenchymal cells so quickly, much faster than IAA – the answer is that, at least from the physical point of view, it diffuses much faster and this fact is reflected in the behaviour of the C and D parameters that were obtained from our theoretical considerations and applied to the elongation growth of coleoptiles in intact plant seedlings.

### Linking auxin transport to protons

It is well accepted that auxin stimulates elongation growth by promoting H$^+$ATPase to extrude protons from the cell reservoir through the plasma membrane and against the concentration gradient. The protons are expelled into the intercellular space, thus causing the acidification of the plant cell wall, loosening of the hydrogen bonds in the cellulose microfibrils and promoting the synthesis of expansins – pH-susceptible cell wall enzymes, which again loosen the cell wall structure (Cosgrove 1998; Cleland 2002). As was stressed in an extensive fragment earlier, hydrogen ions are transported throughout the entire plant via the xylem and phloem. They are actively extruded from the cell cytosol and diffuse across a concentration gradient to sieve elements of the phloem, and move toward roots next where, again through H$^+$ATPases, they are actively transported into the incubation medium, which is therefore acidified during the growth of the plant. For a mathematical model see Steinacher et al. (2012).

### Linking fusicoccin transport to protons

Most authors indicate that FC also promotes the rapid elongation growth of higher plants through its action on the H$^+$
pumps in the plant cell membrane, probably by binding to the receptor on the cell membrane, thus causing H+ extrusion to the apoplast (Rollo et al. 1977; Link and Cosgrove 1998). This means that FC and IAA have similar targets; however, as it was pointed above, FC in its "physiological" concentration depolarises the cell membrane potential much faster as it does not have a lag phase. Based on our results, we strongly promote the conclusion that this is due to the faster rate of diffusion in the "diffusion stage". Some authors have even suggested that FC is the substance that better fits the acid growth theory.

**Linking gibberellin transport to protons**

Most studies on the effect of gibberellins on plant growth concentrate on dwarf mutants (treating wild types only as a reference, see e.g., Chandler and Robertson (1999), long-term developmental responses (Little and MacDonald 2003) or their interaction with other hormones (Ockerse and Galston 1967; Weiss and Ori 2007). However, even at this stage some significant conclusions can be drawn and related to the literature. First of all, GA3 causes almost no change in pH (though, in comparison to the control, the amplitude of the fluctuations is greatly increased). This is in a good agreement with some older researches that were conducted by Stuart and Jones (1978) or Brock and Cleland (1989). The main theme of these studies was, among others, to verify the acid growth theory for different plant hormones. Brock and Cleland (1989) came to the conclusion that GA3 promotes elongation growth through mechanisms that do not require the acidification of the apoplast. In terms of our model, this means that gibberellins primarily act in the "biosynthetic stage"; as in almost all of the cases, the linear part of the relative elongation was significant and had a relatively small nonlinear part (though in not all cases; the diffusion coefficient and concentration of GA3 were positively correlated ~0.3). Moreover, as Chandler and Robertson (1999) reported for the elongation of leaf strips, it is difficult to identify its influence on leaf growth since we have no knowledge about the relative contributions of different endogenous GAs, especially at small concentrations of exogenous GA3. In intact seedlings, it is even harder to unravel the nature of the action of gibberellins as the endogenous GAs were biosynthesised in the cells during the entire experiment. Similarly, the dose-response curves for relative elongation growth under the influence of gibberellic acids did not always demonstrate an obvious behaviour as e.g. IAA or FC, for which a typical dose-response curve has a clear optimum at a certain concentration range – below which it has a minor effect while above the optimal concentration, it even inhibits elongation growth. In the case of GAs, the response may be more or less "chaotic". For a case of an atypical, "chaotic" response, see e.g. Fig. 4 in Cowling and Harberd (1999); Fig. 2 in Jacobsen and Olszewski (1993); Fig. 3 in Tanimoto (1987); Fig. 4 in Little and MacDonald (2003) and finally Fig. 4 in King et al. (2001). In turn, for the case of smooth and typical plant hormone response see: Fig. 3 in Chandler and Robertson (1999) for GA3 influence onto barley growth and Fig. 6 in Collet et al. (2000).

**Final comments**

Given the case of (endogenous) auxins, the hormone is produced in large amounts at the tip of the coleoptile and then transported across the plant by the polar auxin transport system (Steinacher et al. 2012). An intact coleoptile is well known to be full of auxin and adding a small amount via the root will not change that level very much. That is the reasons why individuals investigating auxin-induced growth often excise the coleoptile tip. In this case the coleoptiles will deplete in auxin, and when auxin is resupplied, the well-established rapid and impressive auxin-induced bursts in H+ pumping and growth occur. This is a somewhat artificial system, but one can correlate things. Applying auxin to an intact coleoptile change the intracellular auxin concentrations only marginally, and therefore slight effect in growth can be expected; e.g., given the fact that the graphs are recordings of individual experiments one would see no striking effects in Fig S4. We must admit that treating the roots with that much auxin may impair their ability to take up any nutrients and hormones from the incubation solution, as IAA is a strong inhibitor of the H+ pump in the root. Thus possible effects that are perhaps being seen in the data are not necessarily due to the response of the coleoptile to the effector in question (IAA, GA, FC) but possibly reflect secondary effects at the root level. Similarly, treating the roots with 10−5 M FC will increase the uptake of cations from the root, which may affect shoot growth. Also, by looking at the original traces one cannot always see clear trends in the data – this obviously needs further investigations in the future. However, how both calculated coefficients interact, was considered in detail by Zajdel et al. (2016).

As it was stated therein (ibid.), we can identify at least one parameter of the equation (1), namely D (k2) while parameter C can only be used to quantify growth as an equivalent of "growth amplitude" (for further interpretation, in the context of "acid growth", see e.g., Pietruszka and Haduch-Sendecka (2016) or Pietruszka (2017)). C can be roughly associated with k3 (= 1/T2), Zajdel et al. (2016), but it would be valid only in the epoch when a diffusion mechanism is dominant (nonlinear term). In other words both parameters (C and D) may compensate each other, which can be seen in Figs. 2–3 with the aid of Figure 6. We must note that in our approach the system, consisted of a growing seed plus root system responding to the effector in question, forms a device, which returns the result
of self-consistent 'calculations' at the end of the day. And this is the main advantage of our approach.

Also, recent investigation (Barbez et al. 2017) goes along our view, where the final goal is involvement of auxin in root apoplastic pH homeostasis, which is important for root cell expansion, similar to shoot. Authors claim that auxin steers root cell expansion via apoplastic pH regulation in Arabidopsis thaliana. Therefore, our simplistic model consisted of a black-box system (input – black-box – output), can be successfully implemented, even if the actual 'diffusive substance' is unknown yet.

Nonetheless, all the doubts – always present in the interpretation of actually probed data – can be in some measure overcome by the fact that this kind of experiment (on intact seedlings) is much closer to reality than those with the standard use of coleoptile segments. Properly further developed, may be also helpful in agricultural praxis.

Conclusions

In this paper we reported on a new way to apply plant growth factors to intact coleoptiles in an attempt to circumvent the experimental and conceptual problems associated with the standard use of coleoptile segments. The experiment relied on the idea that growth substances, taken up by the root, will eventually end up in the coleoptile and induce growth and acidification responses, though accordingly diminished by the transport across the plant. Apparently, this is not a very efficient method to alter the hormone concentration in the shoot, however, it reflects the actual situation, which is encountered, e.g., in agriculture.

Also, it is often a difficult task to perform simultaneous experiments on macro- and microscopic level in plant science, especially in vivo. In such cases, scientists have to rely on biophysical or mathematical models that are both comprehensible and accurate and whose results are reproducible. We believe that our model (Zajdel et al. 2016) satisfies these conditions and may provide good support in future studies of plant growth and the action of phytohormones (or nutrients) at both the macro- and microscopic levels.

The analytical methods that were introduced in the course of several recent articles enabled us to draw initial conclusions and interpretations about the growth kinetics at the molecular level in the diffusion and biosynthesis stages of growth. We believe that these methods can – to some extent – contribute to the acid growth hypothesis with numbers for intact growing plants, at least in the time scales (domain) where the acid growth theory is supposed to describe events in growth induction.

Undoubtedly, as it has appeared, our model has both requirements and drawbacks. The strongest requirement is that elongation growth must be measured over a quite a long period of time in order to encompass all of the growth phases – acceleration, the linear phase and the cessation of growth. The main drawback is that not all of parameters have a clear and straightforward interpretation. However, this shortcoming offered encouragement for us to undergo the further development of the model in order to enhance its advantages and minimise its drawbacks.

Author contribution statement. MP conceived the paper and introduced analytical methods. MO conceived and performed experiments. MO performed data acquisition. All authors performed data analysis. MP and MO wrote the paper.

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