

Effect of some immunomodulators and zeatin on susceptibility of wheat to powdery mildew

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Abstract: The effects of zeatin, salicylic acid, and plant oligoadenylates on susceptibility of wheat seedlings to powdery mildew and the shape of dose-response curves for immunomodulation were studied. Salicylic acid and zeatin produced complex dose-responses with upregulation or downregulation of plant susceptibility. Under different experimental conditions, the dose-response curve for zeatin varied in shape from a curve with a maximum to a curve with a minimum of susceptibility to powdery mildew. More complicated curves with a resistance region framed with regions of increased susceptibility were also found. Plant oligoadenylates inhibited the development of powdery mildew at optimal concentrations. The increasing concentrations of salicylic acid and plant oligoadenylates, when combined with zeatin, led to a gradual change in the shape of the zeatin concentration curve. Treatment with salicylic acid and plant oligoadenylates in these experiments may simulate plant responses under biotic and abiotic stresses. Thus, exogenous zeatin was applied to model changes in the phytohormone metabolism. The complex multiphase nature of dose-response is proposed to cause a kind of random-number generator that produces variability in the physiological and immunological status of plants in natural conditions. Variation in susceptibility of individual plants may be crucial for the survival of infected plants and the stabilization of plant-pathogen interactions.

Key words: Complex dose-response, zeatin, salicylic acid, plant oligoadenylates, immunomodulation, powdery mildew, wheat

1. Introduction

Treatment with bioactive products may induce a resistance in plants to infection. According to the definition common in the medical sciences, an immunomodulator is a compound that interacts with the immune system and upregulates or downregulates the host response (Tzianabos, 2000). However, sometimes the effects of immunomodulators are unstable or even ambiguous, thereby preventing their widespread use in plant protection (Ozeretskovskaya and Vasyukova, 2002). Treatment with a high concentration of salicylic acid (SA), a classic resistance inducer, stimulated susceptibility to late blight in potato (Vasyukova et al., 1996). Moreover, induced resistance with low concentrations of SA was replaced with sensitivity after a certain period of time. Paradoxically, the treatment of initially resistant plants with some adaptogens can reduce their adaptive potential (Gudkovskii et al., 2002). Applying stress protectors upregulated or downregulated plant resistance in leaves of different ages or infected with different pathogens (Smolin and Saveliev, 2007; Khairullin et al., 2009). A prerequisite for effective disease control mediated by induced resistance is the use of “appropriate elicitors at the right time and at the right frequency for responsive crop cultivars” (Walters et al., 2013). However, this is not always easy to put into practice.

The direction of immunomodulation of exogenous bioactive products seems to depend on the physiological status of plant and is controlled by plant hormones. A considerable number of microbial pathogens produce phytohormones and could manipulate plant defenses (Kazan and Lyons, 2014; Shen et al., 2018). A considerable number of pathogenic microorganisms are capable of producing cytokinins (Hu and Rijkenberg, 1998; Chanclud et al., 2016). Treatment with exogenous cytokinins may simulate the natural modulation of cytokinin metabolism in infected plants. However, experimental data on immunomodulation with these substances have been contradictory. Thus, a cytokinin may be either a negative (Cole et al., 1970; Edwards, 1983; Vizarova, 1987) or a positive (Levin, 1984) regulator of plant susceptibility. Recent studies have shown that exogenous application of cytokinins or a change in their concentration in transgenic plants can have an ambiguous immunomodulating effect due to influencing the SA pathway of resistance (Argueso et al., 2012; Naseem et al., 2014, 2015; Arnaud et al., 2017). In this case, cytokinin-activated B-type response regulators (ARRs) stimulate the SA pathway, including the expression of specific markers similar to PR1 (Choi et al., 2010; Jiang et al., 2013). In contrast, A-type regulators, which are also

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activated by cytokinins, reduce resistance to pathogens due to downregulation of SA-dependent responses. In addition, the activation of cytokinin signaling can reduce innate immunity to *Pseudomonas syringae* in *Arabidopsis* by inhibiting accumulation of the flagellin receptor FLS2 (Hann et al., 2014).

At least some of the problems may be studied in the framework of the complex concentration curve. Dose-response curves for different processes are usually described by a monotonic increasing or decreasing function. However, multiphase dose-responses with opposite effects for low and high doses are also common in animals and plants (Calabrese and Blain, 2009). Some concentration effects were described with an optimum (bell-shaped or U-shaped) curve. For enzymatic reactions, U- or inverted U-shaped dose-response curves appear as the result of a 2-site model of substrate inhibition in which one binding site is inhibitory (Hutzler and Tracy, 2002).

Indole acetic acid in growth medium stimulated colonization of a resistant tobacco callus by *Phytophthora parasitica*, but development of the fungus sharply decreased when the phytohormone was outside the optimal range (Haberlach et al., 1978). Other examples of such dose-responses are the effect of kinetin and kinetin-riboside on amaranthine accumulation (Vallon et al., 1989), and cytokinin action on indole acetic acid oxidase in tobacco callus tissues (Lee, 1971).

A dose-response curve with 2 optimum concentrations was obtained for the action of epibrassinolide on the growth of wheat roots (Shakirova et al., 2002). Inhibition of *Magnaporthe grisea* appressoria by hydrogen peroxide had the main maximum at 10 mM and one or two peaks at 10^{-8} to 10^{-6} M or less (Aver'yanov et al., 2007). Some elements of a dose-response with several extremes were present in the action of pectinase and chitosan on electrolyte leakage from cells, metabolic efficiency, phenolic content, and catalase in the callus of winter oilseed rape (Plazek et al., 2003). There were multiple local extremes on dose-response curves of the action of 6-benzylaminopurine and kinetin on chlorophyll content in barley leaves (Schistad and Nissen, 1984). Earlier, the author observed dose-response for cytokinin immunomodulation in the powdery mildew-wheat plant pathosystem, which had a resistance region flanked by two regions of increased susceptibility (Babosha, 2009, 2012). Similar cytokinin dose-response curves were obtained for the number of normal appressoria and for the size of halos induced by the powdery mildew fungi at the sites of entry (Babosha et al., 2009).

Two metabolic processes with substrate inhibition connected in series were suggested to produce concentration curves of complex shape (Gurevich, 2001). Positive and negative feedback loops are quite common

in hormonal regulatory systems (Vlot et al., 2009), and should manifest themselves in complex concentration dependencies for a variety of bioactive compounds. The fact that they have rarely fallen into the field of view of previous researchers could be explained by technical problems, the need for a large number of trials with different concentrations, and the difficulty of statistical validation of the effects.

A mathematical model that reproduced multiphase dose-response curves of different shapes including curves with two maxima and minima has been developed (Babosha, 2009). According to this model, even minor changes in the values of cytokinin supply or degradation may promote unpredictable changes in the shape of the dose-response curve. A numerical simulation demonstrated the possibility of a shift along the X-axis of one of the peaks and gradual transformation of curves with a maximum of resistance to curves with a maximum of susceptibility. Experimental verification of the model showed similar variations in the shape of zeatin dose-response curves after changes in mineral nutrition or simultaneous treatment with thidiazuron.

There are investigations that have also demonstrated dramatic changes in the shape of the dose-response curve. Normal callus tissue of *Arabidopsis* and each of several hormone autonomous tumor lines of tobacco and *Arabidopsis* displayed a unique set of growth responses to exogenously supplied growth regulators, ranging from monotonous decreasing dose-response curve to a curve with a maximum (Hansen and Meins, 1986; Campell and Town, 1991).

Though numerous reports have attributed monotonous dose-response inhibition to cytokinins in root growth, a plateau-like region in the middle part of the concentration curve has been observed in several studies (Plakidou-Dymock et al., 1998; Kuroha et al., 2002). The inhibition of the growth of *Arabidopsis* seedlings by chitosan also has a plateau-like region on the concentration curve (Lopez-Moya et al., 2017). The low concentration of chitosan, which corresponded to the plateau-like region, simultaneously promoted maximum expression of the auxin transport gene *PIN1* and the auxin signaling gene *ARF1*. Such a region was also inherent for cytokinin inhibition of tobacco seedlings of the wild type (Werner et al., 2001). However, overexpression of the cytokinin oxidase genes from *Arabidopsis thaliana* resulted in partial resistance of transgenic roots to cytokinin and the disappearance of the plateau-like segment on the inhibition curve (Werner et al., 2001). On the contrary, the dose-response curve for cytokinin inhibition in roots of wild-type *Arabidopsis* had no plateau-like region (To et al., 2004). Multiple insertion mutants with T-DNA insertions in 6 of the type-A *Arabidopsis* cytokinin response regulators (ARRs)

were constructed and showed progressively increasing sensitivity to cytokinin. The appearance of the plateau-like region was correlated with simultaneous loss-of-function of both *arr3* and *arr4* alleles. Interestingly, the T-DNA insertion mutant that affected the RPN12a subunit of the *Arabidopsis* regulatory particle demonstrated the unexpected ability of cytokinin to stimulate the elongation of mutant roots at low concentrations (Smalle et al., 2002).

Plants are known to exist in unstable environments. Abiotic stress or elicitors of pathogen origin may induce protective reactions. Thus, some degree of induced resistance is usual for plants under field conditions (Walters et al., 2013). SA accumulates in plant tissues following pathogen infection and induces SA-mediated defense signaling pathways (Gaffney et al., 1993). Early synthesized stress compounds should affect metabolism of cytokinins and, accordingly, the shape of concentration curves for various manifestations of the activity of exogenous cytokinins. Such a situation could be modeled when a plant is exposed to mixtures of zeatin and additional immunomodulators.

The objective of this study was to investigate concentration-response dependences of some bioactive substances and zeatin in wheat leaves infected with powdery mildew.

2. Materials and methods

2.1. Plant material and growth conditions

Wheat seeds (*Triticum aestivum* L. 'Zarya') were germinated in rollers of filter paper in Knop's solution. Seedlings were cultivated in greenhouse conditions at 20–25 °C under natural illumination from the end of February to June with additional illumination (3400 lx, 16-h photoperiod).

2.2. Pathogen inoculation

Two-week-old plants were inoculated with the pathogen *Blumeria graminis* f. sp. *tritici* (formerly *Erysiphe graminis* f. sp. *tritici*) by gently shaking infected plants over the seedlings. A special modification of the inoculation procedure using several new bunches of infected plants provided a uniform infection, as described in an earlier study (Babosha, 2009). Only the first true leaves of seedlings were used to count numbers of colonies as they have the most equalized initial susceptibility.

2.3. Treatments with inducers

Immediately after inoculation, bunches of seedlings in filter paper rolls were placed in Knop's solution with the addition of the test substances. For the first 2 days of treatment, the experimental plants were placed in a moist chamber.

The stock solution of zeatin (Sigma) was prepared by dissolving 1 mg in 1 mL of 96% ethanol. Plant oligoadenylates (POAs) were synthesized from ATP using a plant enzyme preparation immobilized on dsRNA

cellulose, followed by removal of most unreacted ATP on a DEAE-cellulose column and collecting the fraction eluted with 0.4 M NaCl, as described earlier (Babosha et al., 1991). POA stock concentration (4 mkM) was estimated from the ATP extinction coefficient. The stock solution of salicylic acid (SA) in distilled water (6 mg/mL) was neutralized with NaOH to prevent an effect on the pH value of culture medium.

As the quantity and quality of conidial inoculum were constant in the same experiment because of the inoculation method, the surface density of colonies represents estimates of plant susceptibility in experimental variants. Total numbers of colonies were counted on 7.5-cm-long segments of abaxial and adaxial sides of the leaf, and leaf widths were measured using a binocular microscope 5–7 days after inoculation. For each treatment, an average value for the surface density of colonies was obtained from 8–20 leaves (15–30 leaves in the control). The sums of values on the abaxial and adaxial sides of leaves were expressed as mean numbers of colonies per cm² or as a percentage of the average value of control cases.

2.4. Statistical analysis

ANOVA was performed, followed by Newman–Keuls, Dunn, or Dunnett post hoc tests. The visual extreme point of a complex dose-response dependence was marked as statistically significant if there was at least one point simultaneously on both the ascending and descending branches (for higher and lower concentration, rightward and leftward on the abscissa), which had a statistically significant difference from the ordinate extremes at $P < 0.05$ (Babosha, 2012). An unpaired t-test as a softer estimation and Newman–Keuls or Dunn tests as a stricter estimation of reliability for these comparisons were used. MS Excel 2003 and S-Plus software were used for statistical computations.

3. Results

Obviously, the internal physiological state of plants and powdery mildew inocula varies. This may result in concentration curves with a slightly different shape and with a different general direction of immunomodulation when treated with exogenous substances. Several typical zeatin curves in Figure 1 demonstrate the main varieties in the form of cytokinin concentration curves, which we observed earlier among more than 70 curves obtained with different cytokinins and with changing experimental conditions.

Line 1 in Figure 1 represents a dose-response curve with a region of increased resistance flanked by 2 regions of increased susceptibility. However, on a similar curve (line 4) with 2 maxima of susceptibility, there is no immunomodulating activity at the minimum point between them. Two of the curves were observed entirely

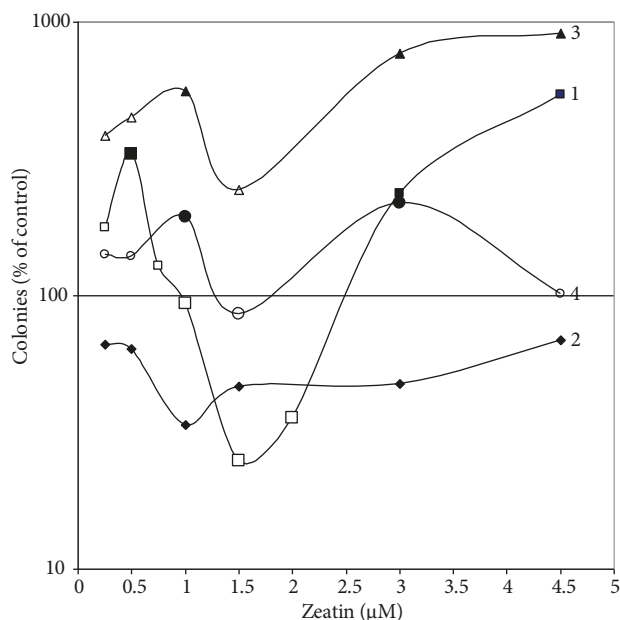


Figure 1. Variability of dose-response curves of zeatin for the number of powdery mildew colonies (percentage of untreated control) obtained in different experiments. Wheat seedlings were placed on Knop solutions supplemented with different amounts of zeatin immediately after inoculation. The mean numbers of colonies per cm² in control leaves (100%) were 1.0, 53.8, 0.7, and 24.1 for curves 1–4, respectively. LSD values (least significant difference at $P = 0.05$) expressed in percentage of corresponding control values were 100.8, 54.2, 362.5, and 42.9 for curves 1–4, respectively. The filled markers designate data points significantly different from untreated controls at $P < 0.05$ according to Dunnett tests. The large markers designate significant extremes according to Newman–Keuls tests at $P < 0.05$.

in the regions of positive (line 3) or negative (line 2) immunomodulation. Despite the high variability, the shape of the curves had a statistical justification in each independent experiment (Figure 1). Most of the visual extreme points had significant differences from untreated controls or from the points of higher and lower cytokinin concentration. Thus, the treatment with cytokinin may result in both a significant increase and a significant decrease in plant resistance in the same (line 1) or in different experiments (lines 2 and 3). All of the concentration dependences were nonlinear and nonmonotonic and had a minimum of relatively increased resistance flanked either with distinct maxima or with regions of relatively higher susceptibility.

The results of 3 independent experiments for testing the activity of SA in our model system are presented in Figure 2. The immunomodulatory effect of the substance strongly depended on the concentration. The dose-response curves were not monotonous and were similar in shape to zeatin

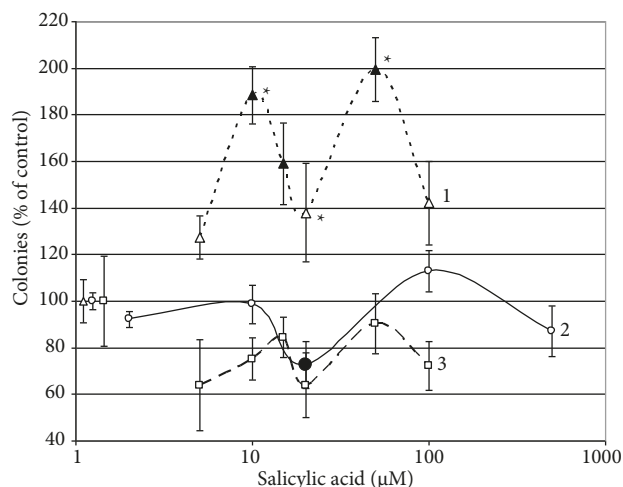


Figure 2. Effect of salicylic acid (SA) on the number of powdery mildew colonies (percentage of untreated control \pm SE). Wheat seedlings were placed on Knop solutions supplemented with fixed amounts of SA immediately after inoculation. 1, 2, 3: Results of 3 independent experiments. The mean numbers \pm SE of colonies per cm² in control leaves (100%) were 5.1 ± 0.5 , 86 ± 3 , and 1.5 ± 0.3 for curves 1–3, respectively. The separate points with ordinates 100% on the left show the error values in untreated controls (percentage). The filled markers designate the data points that are significantly different from untreated controls at $P < 0.05$ according to Dunnett tests. The large marker designates a significant extreme according to the Newman–Keuls test at $P < 0.05$. The asterisks designate extreme points significantly different from the other points with both higher and lower concentrations according to unpaired t-test ($P < 0.03$).

curves. Visual minima at 20 μ M SA were observed in all 3 experiments. Additionally, all of the curves had 2 visual maxima of varying degrees of severity at the same or similar concentrations of SA. All 3 extremes of the upper curve were separated from adjacent points with significant differences (unpaired t-test). A nearly 2-fold increase in the number of pathogen colonies compared to untreated controls was observed at the maxima. On curve 2, there was a minimum at 20 μ M SA that was statistically different from the points of higher and lower concentration when a Newman–Keuls test was used for multiple comparisons. Only this concentration of SA showed significant inhibition of powdery mildew colonies when compared with the untreated control. SA concentrations exceeding this level had no significant inhibition and even showed a nonsignificant stimulating effect. On the contrary, all of the concentrations of SA in experiment 3 were inhibitory, but their value did not exceed statistically significant levels. Thus, the differences in the general direction of immunomodulation in independent experiments were associated with different general positions along the Y-axis of similar concentration curves.

Inhibition of powdery mildew development was also observed after wheat seedlings were placed in Knop solution supplemented with different amounts of POAs. The dose-response curves showed a nearly U-shaped form (Figure 3) with significant inhibiting effects on colony densities at the points of visual minima. The mean numbers of pathogen colonies in the points of visual minimum and maximum on curve 1 were statistically different from the values at higher and lower concentrations according to the unpaired t-test. Thus, curve 1 demonstrated some signs of a more complex shape. However, the stimulation in the putative maximum was not significant when compared to the untreated control. The visual minimum on curve 2 (10^{-10} to 10^{-11} M) included 2 experimental points, which were both significantly different from the untreated control. In this test, most of the putative ascending branch of the minimum seems to be outside the investigated concentration range.

Changes in the form of the immunomodulation curve of zeatin in the presence of fixed amounts of SA are shown in Figure 4. The single-agent dose-response dependence for zeatin in this experiment was defined with one peak curve. However, upon treatment with a high enough concentration of SA (100 μ M), a curve with 2 maxima and minima was observed. The first maximum appeared to be the same as the other two curves but shifted to the right. The second one was new.

SA alone at 20 μ M induced resistance, consistent with the data in Figure 2. However, in combination with zeatin, this concentration loses its activity. There were no essential differences in the number of powdery mildew colonies between the points of dose-response curves of zeatin with or without 20 μ M SA. Furthermore, 100 μ M SA without cytokinin produced only a nonsignificant decrease (Figure 2, line 3, and Figure 4) or increase (Figure 2, lines 1 and 2) of plant susceptibility to the pathogen. However, 100 μ M SA showed strong upregulation of susceptibility at 1 and 3 μ M zeatin when compared to the data of concomitant zeatin concentrations without SA. One may suppose that the experimental model with the application of exogenous cytokinin represents the physiological status of the plant with increased cytokinin concentrations. Thus, immunomodulation activity of SA may depend on cytokinin regulation.

Subsequent experiments with mixtures of zeatin and POAs were carried out in 2 variants: 1) 16/8 h of light/dark cold fluorescent illumination (Figure 5A); 2) natural illumination (the end of February–March) alone (Figure 5B). Change in illumination served as an additional agent for producing variations in plant physiological status.

In these experiments, seedlings treated with POAs (0.04–4 nM) alone showed sensitivity to infection varying in range from increased susceptibility to increased

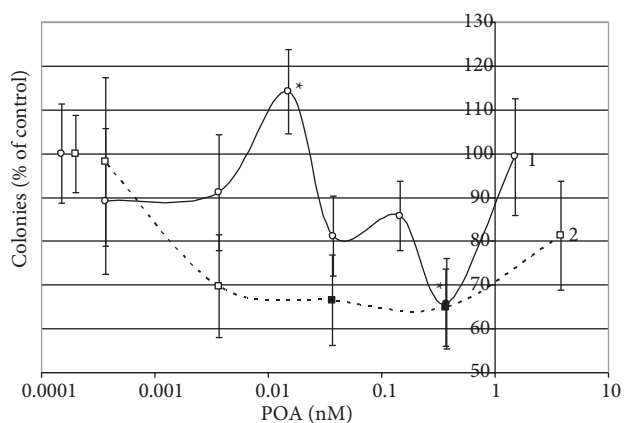


Figure 3. Effect of plant oligoadenylates (POAs) on the number of powdery mildew colonies (percentage of untreated control \pm SE). Wheat seedlings were placed on Knop solutions supplemented with different amounts of POAs immediately after inoculation. 1 and 2: Results of 2 independent experiments. The mean numbers of colonies \pm SE per cm^2 in control leaves (100%) were 1.6 ± 0.2 and 69.7 ± 6.1 for upper and lower curves, respectively. The separate points with ordinates 100% on the left show the error values in untreated controls (percentage). The filled markers designate the data points significantly different from untreated controls at $P < 0.05$ according to Dunnett tests. The large marker designates a significant extreme according to the Newman-Keuls test at $P < 0.05$. The asterisks designate extreme points significantly different from other points with both higher and lower concentrations according to unpaired t-test ($P < 0.02$).

resistance. A dose-response curve of colony numbers for exogenous zeatin alone looked like a curve with a minimum under insufficient natural illumination and like a resistance zone flanked by two regions of increased susceptibility under additional illumination (Figures 5A and 5B, line 1). Thus, in the first experiment, induction of susceptibility generally dominated, and in the second one, resistance to the infection increased.

On simultaneous treatment with an incremental range of POA amounts, more or less gradual changes of the curve shape were observed. The expressiveness of some peaks also changed.

1) Under additional fluorescent illumination (Figure 5A), the dose-response curves of cytokinin alone or in combination with small amounts of POAs looked like curves with minima flanked by maxima on the left and a region with relatively higher susceptibility on the right. Increasing POA concentrations caused the shift of the left peak position to the right and increasing susceptibility at 4.5 μ M zeatin. Treatment with POAs at the greatest concentration resulted in a curve with only one well-expressed maximum at 1 μ M zeatin.

2) Under natural illumination (Figure 5B), the U-shaped concentration curves of zeatin with visual

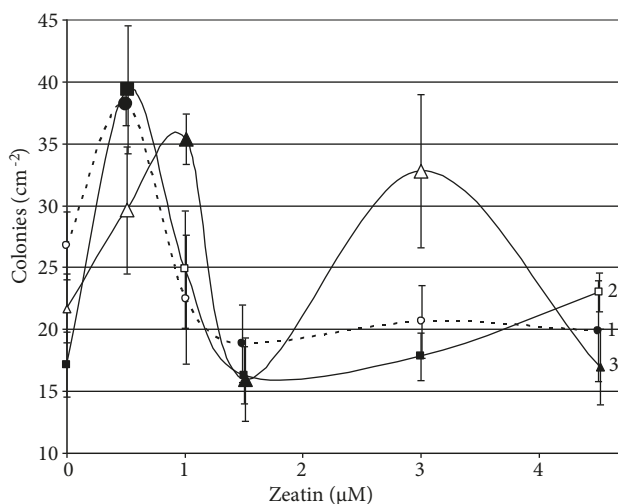


Figure 4. Effect of SA on the shape of dose-response curves of zeatin for the number of powdery mildew colonies (the mean numbers \pm SE of colonies per cm^2). The wheat seedlings were placed on Knop solutions supplemented with 0 (1), 20 (2), and 100 (3) μM SA in combination with fixed amounts of zeatin immediately after inoculation. The filled markers designate the data points significantly different from untreated controls at $P < 0.05$ according to Dunnett tests. The large marker designates a significant extreme according to the Newman-Keuls test at $P < 0.05$.

minimum transformed to curves with a minimum and at least one maximum when POAs were added. Both extremes on each of these curves were well expressed and significantly different from the points on both the ascending and descending branches. The ordinates of all 3 minima and one of 3 maxima on the curves with the addition of POAs were significantly different from untreated controls.

4. Discussion

The results obtained indicate that all examined substances are active as immunomodulators in the model system used. The dose-response functions for POAs and SA were not monotonic, and in some respects they closely resembled complex concentration dependence for cytokinins. They were multiphase and had 1–3 extremes varying in magnitude. Application of SA and zeatin produced trials with both significant upregulation and downregulation of plant susceptibility (Figures 1 and 2). With the simultaneous use of two immunomodulators, it is possible to consider the effect of fixed amounts of one of them on the concentration dependence of the other.

In the present study, the combined action of SA or POAs and zeatin resulted in similarly ranging dose-response curves of zeatin (Figures 2, 4, and 5). The number of extremes, their positions on abscissa axes, and

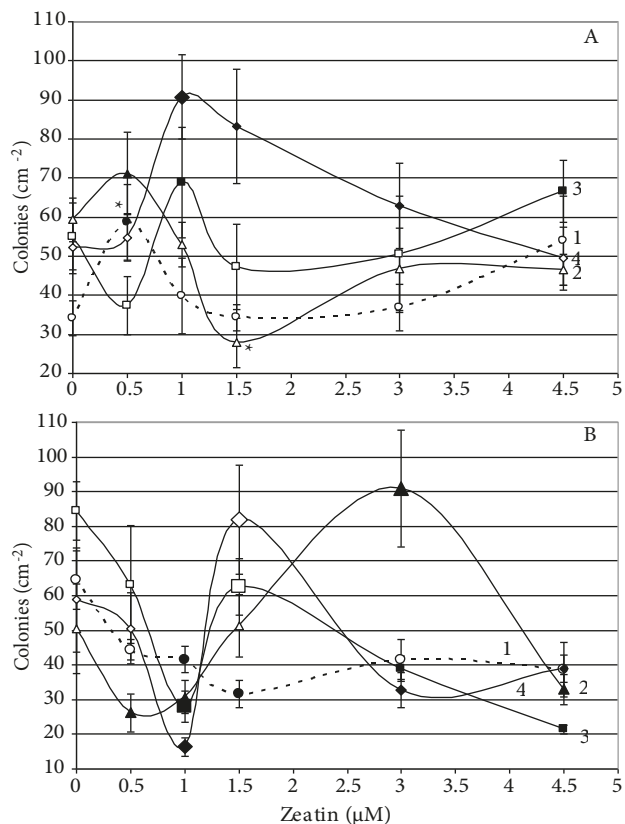


Figure 5. Effect of 0 (1), 0.03 (2), 0.3 (3), and 3 (4) nM POAs on the shape of dose-response curves of zeatin for the number of powdery mildew colonies (the mean numbers \pm SE of colonies per cm^2). A – Additional 16/8 h light/dark cold fluorescent illumination; B – natural illumination. The filled markers designate data points significantly different from untreated controls at $P < 0.05$ according to Dunnett tests. The large marker designates a significant extreme according to the Newman-Keuls test at $P < 0.05$. The asterisks designate extreme points significantly different from points with both higher and lower concentrations according to unpaired t-test ($P < 0.02$).

peak-to-peak values varied. Additional illumination not only changed the shape of the dose-response curves but also strongly increased the number of powdery mildew colonies upon treatment with zeatin, thereby displacing concentration curves almost entirely above the level of untreated control seedlings.

Salicylates are known to be hormonal regulators of disease resistance (Raskin, 1992; Uzun et al., 2017). Cytokinin-activated transcription factor ARR2 interacts with SA response factor TGA3 in *Pr1* activation and promotion of plant immunity (Choi et al., 2010). SA regulates many aspects of plant growth and development (Vlot et al., 2009), but its activity in cytokinin tests is not known. However, salicylate-induced changes in cytokinin metabolism may affect the cytokinin dose-response curve.

POAs seem to be plant analogs of animal 2',5'-oligoadenylates (2-5A). POAs have biological properties similar to those of 2-5A, but different chemical structures (Cayley et al., 1982). 2-5A mediates antiviral action of interferon in animal tissues (Samuel, 2001) and shows antiviral activity in several plant tests (Devash et al., 1984). 2-5A treatment was used for plant virus elimination by meristem tip culture (Konovalova, 1990). Application of POAs also improved survival and morphogenesis of potato meristem explants, with increased frequency of virus eradication (Babosha et al., 2002). The authors observed both positive and negative immunomodulation with POAs in virus-infected potato plants.

2-5A and POAs, as well as human interferon, were shown to have cytokinin-like activity in cytokinin bioassays (Tal'yanskii et al., 1987; Ladygina and Babosha, 1996). Because interferon and 2-5A are very different in structure from cytokinins, these compounds are not strictly supposed to be cytokinins and their activity was due to their influence on cytokinin synthesis (Tal'yanskii et al., 1987). Indeed, the use of interferon and 2-5A in optimal and relatively low concentrations resulted in a few times increase of cytokinins in tobacco leaves. However, a further increase of interferon concentration had an opposite effect (Kulaeva et al., 1992).

Correlations between cytokinin-like and antivirus activity for some other compounds are a surprising finding in this field (Matolcsy et al., 1968). It has been observed that cytokinins can exert an antistress effect, and vice versa, many stress protectors manifest cytokinin properties in some bioassays (Zhyrmyskaja et al., 1989; Lukatkin et al., 2007). It can be assumed that the antistress activity of a certain class of plant adaptogens is due to their influence on the shape of the dose-response dependence for the immunomodulatory activity of endogenous cytokinin.

In the case of independent action on pathogen infection, additional immunomodulators in combination with zeatin should produce a parallel shift of the whole cytokinin or the immunomodulator dose-response curves on the effect scale (a response-additive model). Dose-response curves that were similar in shape but located at different levels on the vertical axis were obtained in several independent experiments with the use of cytokinin (Figure 1) and SA (Figure 2), and in experiments with different lighting (Figure 5). However, in neither the experiments of Figures 4 and 5 nor in our other experiments were such displacements observed when trying to modify the shape of the zeatin concentration curve using bioactive additives.

Another type of additivity may originate from cytokinin-like properties of POAs. Additional quantities of cytokinin should produce a parallel shift of the whole concentration curve on the dose scale to the left without changes in its shape (a dose-additive model). On the

contrary, the addition of substances with anticytokinin activity, apparently, is capable of shifting the curve to the right. Similar shifts to the left or to the right are expected for suitable induced changes in initial levels of endogenous cytokinins.

Despite POAs also being compounds with cytokinin-like activity, their action as an additional portion of cytokinins could only partially explain the experimental data. In the case of POAs, we observed only individual maxima or minima shifting while others did not move. Treatment with POAs and SA also changed peak-to-peak values and promoted the appearance of additional maxima, making fundamental changes in the shape of the cytokinin concentration curve. These data are consistent with earlier findings with the effect of thidiazuron, which has innate cytokinin activity but causes a substantial alteration in the shape of the dose-response curve for zeatin and exhibits little or no positive or negative additivity with zeatin action (Babosha, 2009).

The level of endogenous cytokinins is known to depend on illumination (Hammerton et al., 1998). Changing in illumination in our tests affected the shape of zeatin immunomodulation curves and displaced them on the ordinates axis. However, the simple summation of endogenous and exogenous phytohormones should produce only a horizontal shift of the curve along the abscissa axis. Taking into account the stimulating effect of SA in Figure 4 observed at higher concentrations of cytokinins, it is possible that curve 1 in Figure 2 with the predominantly pathogen-stimulating activity of SA could be obtained by using the experimental plants with higher average values of cytokinins or relevant activity of protein regulators of cytokinin response. However, even if so, the mechanism of the displacement on the effect axis is more complex than a simple summation of the effects of SA and cytokinins.

In spite of an apparent diversity of dose-response curves, there was regularity in their variations. The obtained dose-response curves for cytokinins and some other immunomodulators may be arranged into 3 basic groups: 1) curves with a susceptibility maximum (line of 4 nM POA in Figure 5b); 2) curves with a resistance maximum (Figure 3, line 2 in Figure 1); 3) more complicated curves with a susceptibility minimum flanked by 2 susceptibility maxima (or regions of increased susceptibility) and derivatives from the last type. All of the observed modifications seem to have the same origin. Incremental amounts of additives produced gradual changes in dose-response curves of zeatin. Combinations of different levels of illumination and of POA concentrations also produced U- or inverted U-shaped dose-response curves of zeatin and a number of transition forms (Figure 5). Similar variability in the shape of dose-response curves was

observed in Figure 1 in independent experiments under very similar conditions due only to their natural variation.

As physiological status directly or indirectly depends on phytohormones and, in particular, on cytokinins, exogenous treatments with different cytokinin concentrations might simulate different *in vivo* hormonal and physiological statuses of a plant. The activation of plant defense reactions by exogenous and endogenous elicitors mediates changes in the resistance to pathogens or tolerance to abiotic stresses. Supposing that microbes and microbial elicitors are an inherent part of the plant's natural environment, the results of simultaneous treatments with immunomodulators and cytokinin to some extent may reflect a variability in plant resistance specified by different biological and physiological conditions in the intact plant. Both the complex nature of the dose-response curves and the variability of the shape of these curves could be suggested as reasons for previously noted differences in experimental data regarding immunomodulation in plants. Due to this mechanism, at least some types of resistance inducers have the innate ability for bidirectional immunomodulation. Thus, for practical usage of some immunomodulators, stability of their dose-response in variable conditions should be studied.

On the other hand, nonlinearity and the complex multiphase nature of dose-response may cause a kind of

random-number generator that produces unpredictable changes in the physiological and immunological status in plants when metabolism is modified through local microbial elicitation or abiotic stresses. This variability in susceptibility to pathogens may apparently be generalized on the level of the individual plant and individual cell. The peak-to-peak values of susceptibility in these cases should even be above the average variations commonly obtained in experiments. Experimental concentration curves seem to be a result of averaging dose-responses that are very different in shape inherent to the individual plants and cells. The more diverse the experimental plants, the smoother and less detailed the obtained dose-response curves should be. One may hypothesize that such variability in individual responses plays an important role in stabilizing plant-pathogen interactions. Heterogeneity in plant-host susceptibility gives an opportunity for pathogens to find vulnerable plant cells among a population of relatively resistant plants. Conversely, certain susceptible plants get a chance to survive severe infection.

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