

Non-linearity of Plant Resistance to Pathogens Induced by N-methylated Compounds

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The N-methylated resistance inducers act within two concentration ranges characterized by suppression of infection rates. Between these active ranges there is always an inactive range (5–7 decimal dilutions) characterized in general by a high rate of infection. This unique phenomenon is called the double immune response, which means a non-linear effect. If the time interval between pre-treatment and inoculation increases the active concentration values for the double immune response shift towards original higher dose values because of the continuous metabolism of methylated inducer in the host plant. The inactive range between the two active dose values remains the same. The multiple pre-treatment of plants with the inducer gives contrasting results: the active dose ranges shifted towards the original lower values. It seems that the two active concentrations of the inducer are always the same. Methylated inducers are potential formaldehyde (HCHO) generators. HCHO generates a time- and dose-dependent immunostimulating activity. HCHO (mainly in bound form) and H₂O₂ can interact and the singlet oxygen and excited HCHO can be formed. The singlet oxygen may participate in the oxidation of water molecules and in biological systems previously unknown oxidants including dihydrogen trioxide and ozone, can be generated. It seems that these reactive molecules – from HCHO to O₃ – form the biochemical basis of the double immune response of plants to pathogens.

Keywords: formaldehyde, hydrogen peroxide, immunization, ozone, resistance, singlet oxygen.

Plants successfully use pre-formed physical and chemical innate defense systems as well as inducible acquired immune strategies to pathogens, although, circulating cells, immunoglobulin molecules and phagocytic processes lack in plant tissues (Menezes and Jared, 2002).

Resistance can be activated by biotic (Hostetter 2000; Klement et al., 2003; Kothari and Patel, 2004) and by abiotic (Alvarez et al., 1998) inducers. Induced resistance means an improvement of the natural (innate) resistance of plants without alteration of their genome. Plant induced resistance is also called local and systemic acquired resistance (SAR) or plant immunity (Kuč, 1982; Menezes and Jared, 2002; Kothari and Patel, 2004). There are special criteria for the verification of induced resistance (Schönbeck et al., 1993). Inducers can be used for induction of resistance without generating an antimicrobial (direct) effect. Systemic induced resistance has been extensively investigated with

attempts made to elucidate its basic processes and chemical-biochemical nature (e.g. Kuć 1982, 1987; Alvarez et al., 1998; Király, 1998; Aver'yanov et al., 2000; Sandermann, 2000; Barna et al., 2003; Klement et al., 2003). The chemical nature of the substances which are responsible for the systemic induced resistance are not well known (Ryals et al., 1996).

Until recently, the mechanisms of antibody-mediated immunity in humans and animals against microorganisms are well known, however until now immunoglobulin-like proteins have not been found in plants. Therefore, it is high time to find common points (e.g. biochemical system) which are valid for immunity in animals as well as in plants. Recent studies open new horizons in this field: antibodies can catalyze the generation of very reactive biological oxidants including dihydrogen trioxide (H_2O_3) and ozone (O_3) from the interaction of singlet oxygen ($^1\text{O}_2$) with H_2O (Wentworth et al., 2000, 2003; Nathan 2002; Babior et al., 2003). The formation of $^1\text{O}_2$ is a crucial step in this system. Both formaldehyde (HCHO) and hydrogen peroxide (H_2O_2) can be formed continuously and are present intracellularly and extracellularly by almost all cells (Tyihák et al., 1994, 1998). These two reactive molecules can interact (also endogenously) and the very reactive $^1\text{O}_2$ and excited HCHO can be formed (Trézl and Pipek, 1988; Tyihák et al., 1994). The formation of $^1\text{O}_2$ may be continuous in all cells at different levels.

The aim of this work is to demonstrate the basic elements of the time- and dose-dependent double immune response of plants to pathogens in relation to the function of HCHO and its reaction products.

Materials and Methods

Chemicals

All chemicals were obtained from Merck Hungary (Budapest, Hungary) and Sigma Co. (St. Louis, USA) as well as REANAL Chemical Co. (Budapest, Hungary).

Plants and pathogens

Bean (*Phaseolus vulgaris* cv. BorLotto, Debreceni tarka, Inka), wheat (*Triticum vulgare* cv. Yubileynaya) and barley (*Hordeum vulgare* cv. GK-Omega) plants were cultivated in commercial compost in the greenhouse held at 24 °C with 4 hours supplementary light (6000 lux) in the morning and evening to give a light period of a least 16 hours per day. The investigations are limited to primary leaves. *Uromyces phaseoli*, *Puccinia recondita* and *Erysiphe graminis* were maintained on bean, wheat and barley plants, respectively, in the greenhouse.

Biochemical immunization

Aqueous solutions of glycine betaine (GB) and other compounds at 10^{-1} – 10^{-14} mol/l were used. The solutions were sprayed onto the leaf surface in case of wheat and barley plants and onto the abaxial leaf surface in case of bean plants. Plants treated with water were used as controls.

Inoculations

Plants were inoculated with an aqueous spore suspension of the pathogens on different days after pre-treatment with inducer solution and then incubated at 20–22 °C for 24 hours at high relative humidity.

Capturing HCHO and O₃ in bean plants (in vivo) with synthetic capturer molecules

To aqueous solution of GB (10⁻¹ mol/l), dimedone (HCHO capturer molecule) and in another experiment Indigo Carmine (O₃ capturer) were given for 0.1% solution. Then the decimal dilution was carried out similar to biochemical immunization process.

Evaluation of data

Pustule densities were assessed 9–10 days after inoculation and disease severity (infection rate) was expressed as the number of pustules cm⁻² in the case of bean plants (using a special, homemade pattern) and as the average number of pustules on a leaf in case of wheat and barley plants. These characteristics were reported to the corresponding values of the control plants to obtain relative percentage data. In order to investigate the effect of inducers on the resistance potential of the plants studied, the dependence of infection rate on the concentration of inducer administered (expressed in negative logarithmic scale) was examined. The mathematical evaluation of the data was carried out by polynomial regression analysis.

Results

A special type of induced resistance concerns the endogenous application of different methylated compounds (e.g. betaines) as inducers of disease resistance in plants. N^ε-trimethyl-L-lysine (TML), as a potential HCHO generator, which promotes animal tumours and healthy cell proliferation and has an immunostimulating activity (Szendé et al., 1970), generates an immunostimulating activity also in plant systems (Tyihák et al., 1989). In an early experiment bean (*Phaseolus vulgaris* cv. Inka) was infected with rust (*Uromyces phaseoli*). The abaxial surface of the primary bean leaves was pre-treated with the aqueous solutions of TML (decimal dilution) at 10⁻³–10⁻¹⁰ mol/l. Plants treated with water were used as controls. Plants were inoculated with an aqueous spore suspension of *U. phaseoli* 6 days after pre-treatment with the inducer (TML) solution and then incubated at 20–22 °C for 24 hours at high relative humidity. Pustule densities were assessed 9 days after inoculation and disease severity (infection rate) was expressed as the number of pustules cm⁻². Bean plants exhibited a dose-dependent double immune response to bean rust when 10⁻³ and 10⁻⁹ mol/l TML pre-treatment was applied. Inhibition of the development of rust colonies was 55% and 65%, respectively (Tyihák et al., 1989).

Figure 1 illustrates the effect of GB (simplest betaine molecule) on the resistance potential of bean plants at different time intervals between pre-treatment and inoculation (2, 5, 7 days). The inactive range means 7 dilutions. It can be seen that a short time inter-

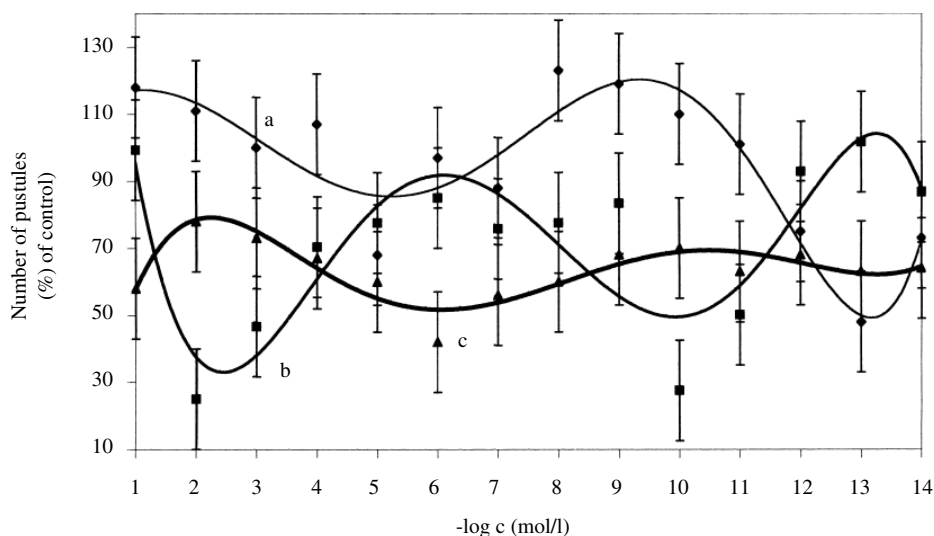


Fig. 1. Effect of day intervals between chemical pre-treatment with GB and inoculation on the rust pustule number in bean

a: 2 days; b: 5 days; c: 7 days

Polynomial equations:

$$2 \text{ days: } Y = 0.0017X^6 - 0.0606X^5 + 0.7637X^4 - 4.0053X^3 + 7.7515X^2 - 4.8619X + 117.02$$

$$5 \text{ days: } Y = -0.0009X^6 + 0.0108X^5 + 0.4752X^4 - 11.176X^3 + 84.876X^2 - 246.41X + 266.83$$

$$7 \text{ days: } Y = -0.0003X^6 + 0.0238X^5 - 0.6316X^4 + 7.7114X^3 - 44.742X^2 + 109.94X - 14.699$$

val (2 days) results in a double immune response at $-\log c = 5$ and 13. If we used a bigger time interval (e.g. 5 days), these two values were as follows: $-\log c = 2$ and 10, that is, the active ranges shifted left because during the relatively long incubation time the metabolism of the inducer (in this case: GB) was considerable. Using a 7 day interval between pre-treatment and inoculation, there was only one active range (practically the original second range shifted towards the higher concentrations; $-\log c = 6$ and the original first active range shifted to the origin; Tyihák et al., 2003).

In case of all three time intervals investigated the statistical evaluation of data showed a functional correlation (polynomial regression as tendency analysis) between the infection and concentration of inducer, however, the active ranges were in general at very narrow concentration ranges.

For the multiple (double, triple) pre-treatments the active concentration ranges shifted right ($\rightarrow$) without a change of the inactive range interval (Tyihák et al., 2003). The two active concentrations are perhaps always the same, namely about 10^{-5} and 10^{-13} mol/l.

It is supposed that the N-methylated compounds as inducers act through a demethylation process in which HCHO and partly or totally demethylated compound can be formed. The results after pre-treatment with diluted formalin solutions of bean plants are as follows: a high concentration of HCHO in formalin solution ($-\log c = 0-2$) practically

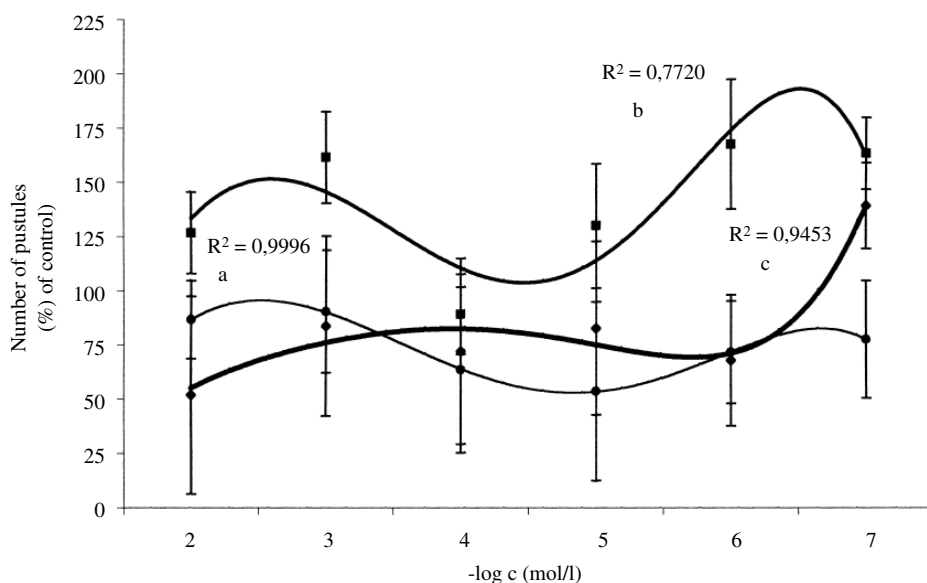


Fig. 2. Effect of GB and its joint effect with dimedone as well as Indigo Carmin on the pustule numbers in bean-*Uromyces* relationship. a: GB pre-treatment; b: GB + dimedone pre-treatment; c: GB + Indigo Carmin pre-treatment

killed the bean plants while a moderate retardation effect occurred up to 10^{-7} mol/l ($-\log c = 7$ which is equal with $3 \mu\text{g}$ of HCHO/l) where a characteristic, considerable retardation effect was observable. This corresponds to the first immune response range. HCHO seems to be a very toxic molecule, however it is also suitable for immunostimulation of plants. (Tyihák et al., 2003).

According to our preliminary results the application of dimedone, as a HCHO capture molecule (classical aldehyde reagent), together with an inducer (e.g. GB) in decimal dilution for pre-treatment of bean plants against *U. phaseoli*, resulted in a decreased immunostimulating activity (Fig. 2) (Tyihák unpublished results, 2004–2005). Elimination or more exactly the decrease of the endogenous level of HCHO formed from the inducer decreased the immune response of bean plants to the pathogen but the tendency of effect is similar to the basic mechanism (Fig. 2). Using Indigo Carmine as an O_3 -capturing molecule in the culture medium (in decimal dilution) a characteristic decrease in the induction of resistance can be observed (Fig. 2). It seems that these very reactive small molecules participate in the resistance mechanism of plants in general.

For the detection of the double immune response in plants other methylated inducers, such as L-carnitine, trigonelline, N,N-dimethyl-L-tyrosine (Tyihák et al., 1989, 1990, 1998) and 1'-methylascorbigen (Kátay and Tyihák, 1998), and other host-parasite relationships (wheat-*Puccinia*, barley-*Erysiphe*) were also used (Manninger et al., 1998).

Discussion

Plants generate a time- and dose-dependent double immune response to different pathogens using N-methylated compounds such as GB, L-carnitine, N,N-dimethyl-L-tyrosine, 1'-methyl-ascorbigen, etc. (Tyihák et al., 1989, 1990, 1998; Manninger et al., 1998; Kátay and Tyihák, 1998). This is supported by our recent observations with different time-intervals and multiple pre-treatments. The actual concentration of the inducer, as a potential HCHO-generator, determines the double immune response.

It is especially interesting that HCHO generates an immunostimulating activity in plants to pathogens. The immunostimulating dose of the aqueous HCHO was observed in a smaller dose than in the case of N-methylated compounds. This means that the continuous demethylation of methylated compounds may be responsible for the immunostimulating activity.

HCHO – similar to hydrogen peroxide (H_2O_2) – is an endogenous, indispensable component of all biological systems (Tyihák et al., 1998). These small molecules can be formed (continuously) intracellularly and extracellularly by almost all cells (Tyihák et al., 1994). HCHO and H_2O_2 can interact in cells or tissues endogenously. In this interaction the very reactive singlet oxygen ($^1\text{O}_2$) and excited HCHO can be formed (Trézl and Pipek, 1988; Tyihák et al., 1994).

It is an important recent observation that antibodies can catalyze the generation of special oxidants including dihydrogen trioxide (H_2O_3) with a ring skeleton and ozone (O_3) in the interaction of $^1\text{O}_2$ with H_2O (Wentworth et al., 2000, 2003; Nathan, 2002; Datta et al., 2002; Babior et al., 2003). It is supposed that HCHO is also a component of the activity process of antibodies and it may be the cause of the formation of $^1\text{O}_2$ in this complex system. One can suppose that the mechanism of the killing action of antibodies and antibiotics (e.g. trans-resveratrol) on the bacterial cells is similar (Tyihák et al., 2004). These very reactive small molecules – from HCHO to O_3 – may be the crucial factors of the innate (natural) resistance of a given biological unit and form a determining common bridge between the innate (natural) and induced resistance in plant and animal organisms.

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