ACTA
PHYSIOLOGIAE
PLANTARUM
Vol. 28. No. 2. 2006: 95-100

Relationship between peroxidase activity and the amount of fully N-methylated compounds in bean plants infected by *Pseudomonas savastanoi* pv. *phaseolicola*

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Key words: Choline, Peroxidase, *Phaseolus vulgaris*, *Pseudomonas*, Trigonelline

Abstract

Changes in the level of endogenous formaldehyde (HCHO), some N-methylated compounds (choline and trigonelline) and peroxidase activity were examined in the leaves of bean genotypes (*Phaseolus vulgaris* L.) with different disease-sensitivity during ontogenesis in the stressfree condition and after natural infection by *Pseudomonas savastanoi* pv. *phaseolicola* (until the appearance of lesions). HCHO, as its dimedone adduct, and fully N-methylated compounds were determined by overpressured layer chromatography (OPLC) in different developmental stages and in the infected leaves/leaf discs. Peroxidase activity was measured by a spectrophotometric method. HCHO level decreased with ageing of the primary leaf and accordingly in the leaves at different developmental stages, then increased again in both cases due to the demethylation and methylation processes. Concentration of choline and trigonelline as potential HCHO generators decreased considerably while peroxidase activity increased with ageing of the plants. Comparing the symptomless and the *Pseudomonas* infected leaf discs (with watersoaked lesions) we found a decrease in the level of HCHO, choline and trigonelline and there was detectable increase in the peroxidase activity in the infected leaf tissues. Our findings are in accordance with previously published results that peroxidases play an important role in oxidative demethylation processes. Our hypothesis is that the high level of HCHO in the old leaves can originate from methylated components as the result of peroxidase activity and this high level may lead to the old leaf being resistant to pathogen. This conclusion is supported by the fact that the leaves of susceptible bean genotypes became resistant to *Pseudomonas* while growing older.

List of abbreviations: HCHO, formaldehyde; OPLC overpressured layer chromatography; POD peroxidase

Introduction

In spite of its genotoxic and carcinogen character, HCHO can be found in human (Szarvas *et al.* 1986), animal (Heck *et al.* 1990) and plant tissues (Sárdi and Tyihák 1994, Tyihák *et al.* 1996, Velich and Sárdi 1997). The endogenous HCHO is produced partly by enzymatic demethylation (demethylases, peroxidases) of different N-, S- and O-methylated compounds (Paik and Kim 1980, Kedderis and Hollenberg 1983, Kawata 1983, Huszti and Tyihák 1986, Chelvarajan *et al.* 1993). Since the endogenous transmethylation processes

occur *via* HCHO (Huszti and Tyihák 1986) this compound can be considered as one of the basic constituents of biological systems. Analysis of different plant samples (*e.g*. water-melon, snap been) has shown correlation between the amount of endogenous HCHO and/or the amount of fully N-methylated compounds and the natural disease resistance and/or stress tolerance of the given plant (Sárdi 1994, Velich *et al*. 2000).

In our previous studies some relationships were found between the concentration of HCHO, quaternary ammonium compounds and the biotic stress tolerance of different snap bean varieties with known resistant genes. The resistant varieties, especially in their young trifoliate leaves, have higher concentrations of quaternary ammonium compounds than sensitive genotypes (Sárdi and Velich 1995).

It is supposed that biological systems, like plants, under stress conditions may defend their macromolecular constituents (enzymes, nucleic acids) by methylation with methyl groups originated from endogenous HCHO (László *et al*. 1998, Tyihák *et al*. 1998). This type of induced disease resistance can be achieved by application of endogenous N-, S- and O-methylated compounds for the induction of disease resistance in the host by pretreatment of plants with inducer (N^{ε}-trimethyl-L-lysine in bean plants) before inoculation (Tyihák *et al*. 1989).

It is known that there are different responses in different developmental stages of the plant in the *Phaseolus-Pseudomonas* host-pathogen system (Velich and Szarka 1981). Results of inoculations showed that leaves of susceptible bean genotypes became resistant while growing older (Klement 1982, Velich and Sárdi 1997, Sárdi *et al*. 1999).

Peroxidases not only are indicators of different stress (Tyihák *et al*. 1989), markers of the ontogenetic age of the plant (Sanchez-Romero *et al*. 1993, Albert *et al*. 1998) and important in the identification of different genotypes, but they play a role in demethylation processes and take part in the HCHO cycle (Huszti and Tyihák 1986).

The aim of this work was to determine the effect of the involvement of peroxidase in the enzymatic demethylation processes on the age-dependent resistance (independent of genotype) of *Phaseolus vulgaris* and on the bean-*Pseudomonas* interaction immediately after a natural infection.

Materials and methods

Plant material

Snap bean plants (*Phaseolus vulgaris* L.) were cultivated in commercial compost in a greenhouse. We collected leaf samples at different developmental stages from the primary leaf to the third trifoliate leaf. Marks of samples:

a) I/1, II/1, III/1, IV/1, V/1 - primary leaf of plants in the different developmental stages,
b) IV/1, IV/2, IV/3, IV/4 – leaves from the old to the young in the third trifoliate stage.

The age-dependence of resistance was examined and compared with the resistant and susceptible genotypes in different phenophases. The first trifoliates were inoculated with 10^8 cell/ ml *Pseudomonas savastanoi* pv. *phaseolicola* bacterial suspension. The other plant samples, which were naturally infected with *Pseudomonas* were collected from the field. Leaf discs were taken from the first and second true leaves of plants with differing susceptibility (highly-HS and moderately-MS susceptible lines).

The leaf discs originated from different symptom types: symptomless true leaves as controls (C), watersoaked lesions without a toxic halo in moderately susceptible (MS) varieties, watersoaked lesions with a toxic halo in highly susceptible (HS) plants and young leaves with toxin systemisation (T).

Preparation of samples for OPLC analysis

Leaf tissues were frozen with liquid N_2, powdered and suspended in dimedone solution (0.05 % dimedone in methanol) (*e.g*. 0.3 g plant powder in 0.7 ml of 0.05 % dimedone solution). This suspension was centrifuged at 1500 g for 10 minutes at 4 °C and the clear supernatants were used for OPLC separations (Gersbeck *et al*. 1989). OPLC separations were carried out on OPLC silica gel 80 F_{254} precoated chromatoplates using chloroform - methylenechlo-

ride mixture (35/65,v/v) for formaldemethone determination and i-propanol - methanol - 0.1 M sodium acetate mixture (20/3/30 v/v/v) for quaternary ammonium compounds. Calibration curves were prepared using authentic substances (at λ = 265 nm for formaldemethone and at λ = 525 nm for choline and trigonelline, concentration of standard 1 mg ml^{-1}) which were detected by Dragendorff reagent. Densitograms were taken with a Shimadzu CS-930 scanner.

Sample preparation for analysis of peroxidase activities

0.25 g of leaves were homogenized in 1 ml ice cold 20 mM Tris-HCl extraction buffer (pH 7.8), containing 0.2 mg/ml $MgCl_2$, 10 mg/ml polyvinyl--pyrrolidone, 200 mg/ml sucrose, 3.4 mg/ml potassium metabisulfite, 0.35 mg/ml bovine serum albumin and 100 mg/ml Triton X-100. The crude extracts were centrifuged and the supernatants were analyzed. Peroxidase activities (POD) were measured by spectrophotometric method (Shannon *et al*. 1966) in the presence of H_2O_2 as a substrate and orto-dianizidine as a chromogen reagent ε = 11.3 at λ = 460 nm.

Results

Fig. 1 illustrates our findings, the amount of endogenous HCHO, trigonelline, choline and peroxidase activity on different phenophases in both genotypes. The level of HCHO decreased at first with ageing of the bean plants, but increased again in old leaves of old plants. Concentrations of trigonelline and choline also decreased from young to old leaf, in the different phenophases. The peroxidase activities showed a continuous increase from younger leaves to older ones in both resistant and susceptible genotypes.

A possible explanation for the experienced age-dependent HCHO concentration (originated from fully N-methylated components) is that in young proliferating cells methylation processes are dominant over demethylation ones, while in old leaves oxidative demethylation - in which beside demethylases, peroxidases play a crucial role - overwhelms methylation. The age-related decrease of fully N-methylated compounds as well as an increase in peroxidase activity support the idea that peroxidases are involved in oxidative demethylation.

We have determined the concentration of HCHO and some quaternary ammonium compounds and the activity of peroxidase enzymes in healthy (symptomless: 21C, 87C, 32C, 90C, 137C, 20C, 89C, 2021C) and infected (with toxic halo: 21, 87, 32, 90, 137, 20, 89, 2021) leaf discs of naturally infected bean plants until the appearance of lesions (Fig. 2).

Our results clearly show that the amount of HCHO, choline and trigonelline generally decreased in the infected plant tissues. In the case of highly susceptible varieties the concentration of fully N-methylated compounds decreased in the leaf tissues with watersoaked lesions (89, 2021), but increased again in the leaves positioned above the infected leaves with chlorotic symptoms (89T, 2021T). Peroxidase activity more or less increased in all cases of the bean varieties, however, the rate of increase according to the genetic background (varieties with different sensitivity to *Pseudomonas sp).*

Discussion

Peroxidase activity increased with ageing of leaves, whereas reversely, the concentrations of choline and trigonelline decreased from the youngest leaves to the oldest in a given developmental stage of the plant independent of genotype. The same tendencies were also observed during senescence of a given leaf. A possible explanation for these results is that the methylation processes are more intensive in the rapidly proliferating cells (the youngest leaves). The high level of HCHO observed in young leaves originated from methylation processes (Tyihák and Szőke 1996, Tyihák *et al.* 1996, Albert *et al.* 1998).

The level of choline and trigonelline decreased with ageing of the plant and similarly during the development of a leaf. The content of HCHO also decreased with ageing of bean plants, but increased again during senescence of primary leaves (Fig. 1A) and according to the age of leaves in a given phenophase (Fig. 1B). The relatively high level of

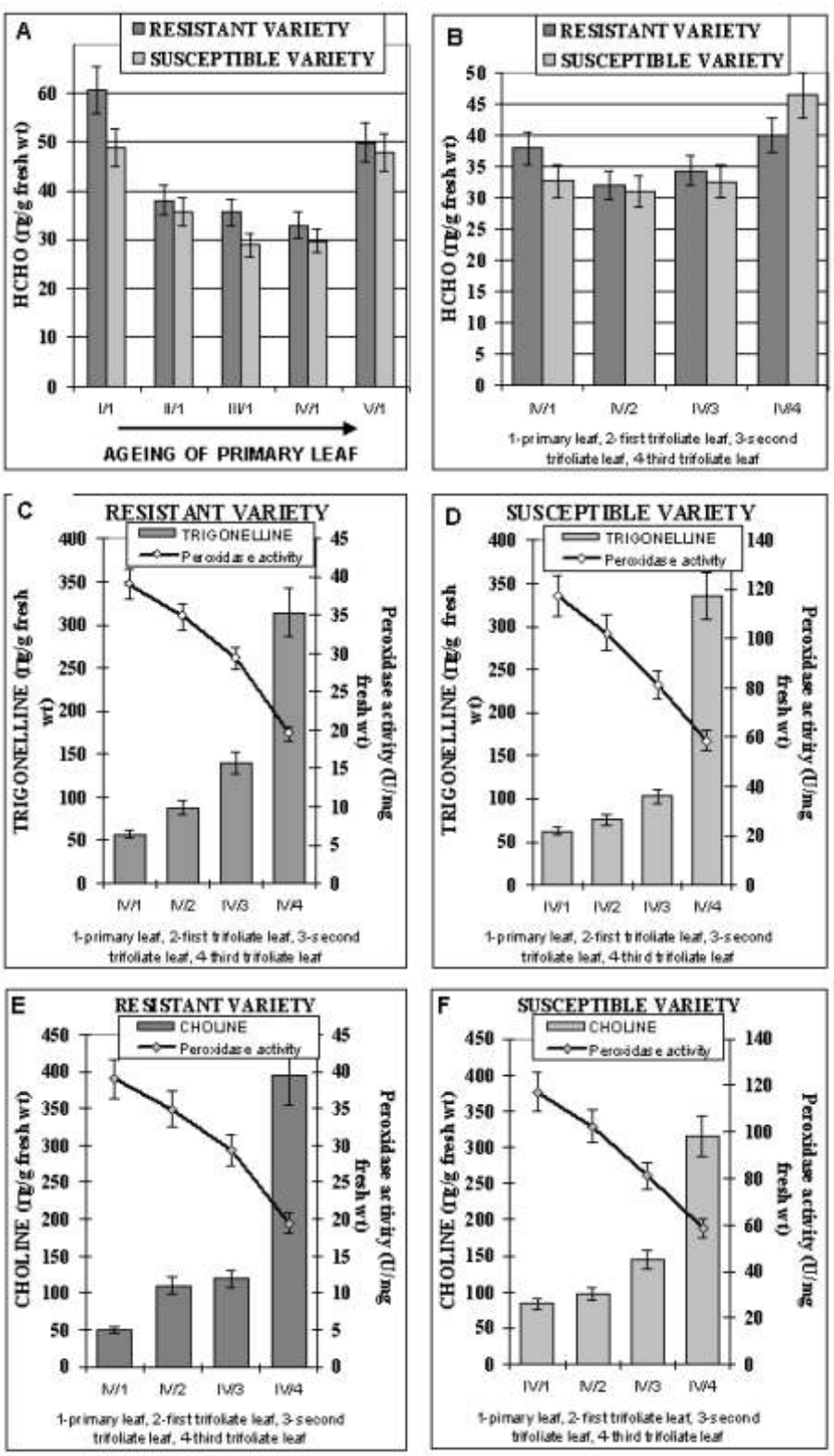

Fig. 1. Endogenous HCHO (A, B), trigonelline (C,D), choline (E,F) (µg/g fresh weight) contents - columns - and peroxidase enzyme acitivities (U/ml) - line - in the different phenological phases of resistant and sensitive bean varieties.
A. I/1, II/1, III/1, IV/1, V/1 - primary leaf of plants in the different developmental stages,
B,C,D, E, F. IV/1, IV/2, IV/3, IV/4 – leaves from the old to the young in the third trifoliate stage.

HCHO in old tissues can originate from demethylation processes of the methylated substances catalysed by peroxidases and/or demethylases (Huszti and Tyihák 1986).

It is known that peroxidase enzymes participate in hydrogen peroxide-dependent demethylation processes and these stress enzymes may play an important role in different stress conditions by catalyzing demethylation processes (Huszti and Tyihák 1986, Kedderis *et al.* 1963). It is supposed that the elevated peroxidase activity observed in old leaves contributed to the accumulation of HCHO in these tissues.

Our hypothesis is that the high level of HCHO in the old leaves can be produced due to peroxidase activity from methylated components and this high level may contribute to the prevention of the old leaf to pathogen attack. This conclusion is supported

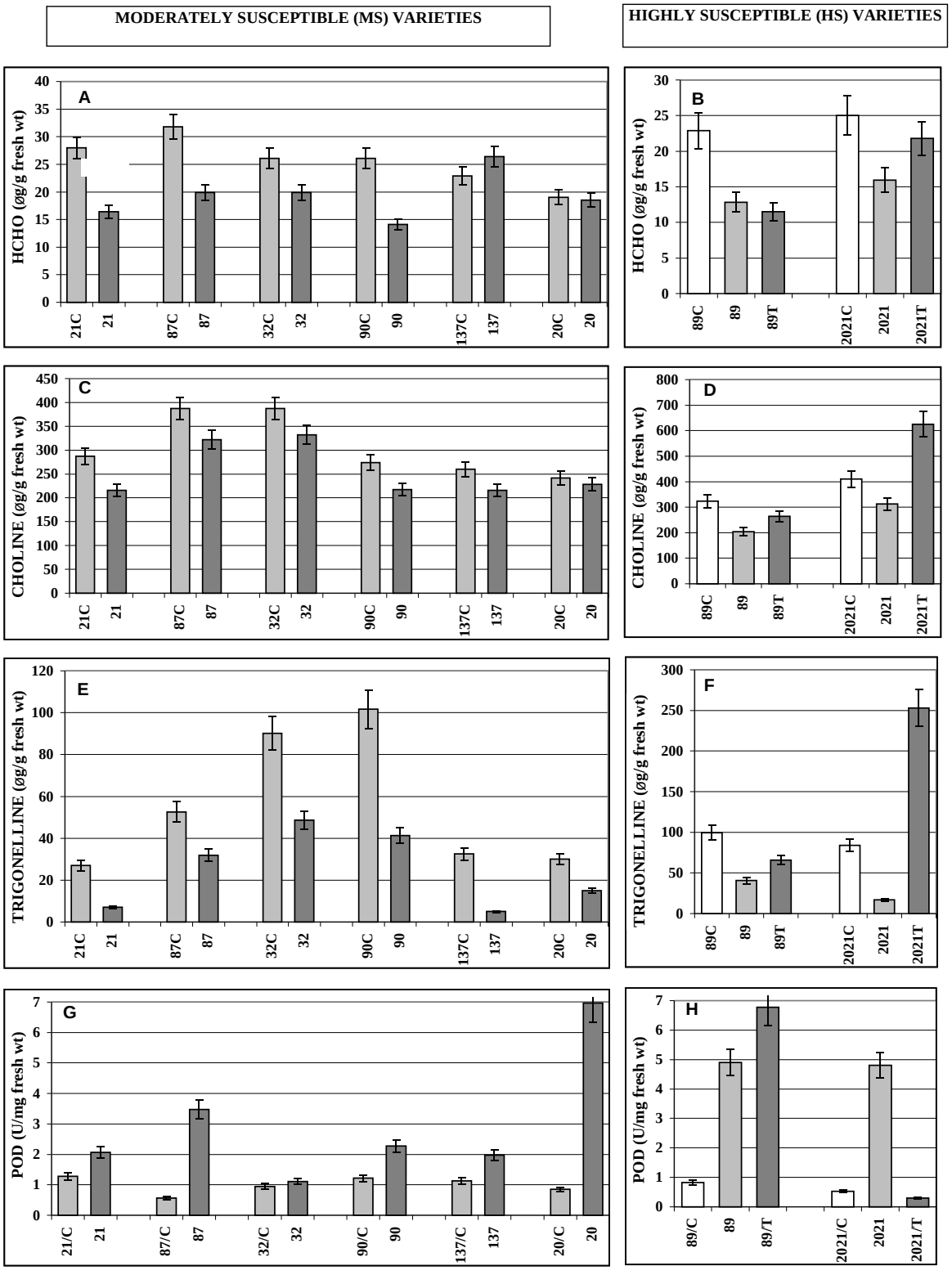

Fig. 2. Effect of *Pseudomonas* infection on the concentration of HCHO (A,B), choline (C,D), trigonelline (E,F) (µg/g fresh weight) and peroxidase activities (G,H) (U/mg fresh weight) in different moderately susceptible (MS) and different highly suspectible (HS) bean genotypes. Labels 21C, 87C, 32C, 90C, 137C, 20C, 89C and 2021C represent the values of control, non-infected leaves; 21, 87, 32, 90, 137, 20, 89 and 2021 represent the values of infected leaves; and 89T, 2021T represent the values of leaves positioned above the infected leaves.

by the fact that susceptibility of leaves is decreased during senescence irrespective of the plant genotype (resistant or susceptible). Senescenced primary leaves of susceptible plants will not show any watersoaked lesion, the well-known symptom of susceptible reactions (Klement 1982, Velich and Sárdi 1997, Sárdi *et al.* 1999).

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Received February 18, 2005; accepted October 28, 2005
edited by M. Stobiecki