INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2006/08–1–47–51
http://www.fspublishers.org

Induction of Resistance in *Phaseolus vulgaris* Against TNV by Salicylic Acid and Kinetin

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ABSTRACT

Aim of this study is to explain the role of exogenous application of salicylic acid and kinetin in increasing *Phaseolus vulgaris* resistance against tobacco necrosis virus (TNV). Two-week-old *Phaseolus vulgaris* plants treated with 0.1, 0.5 and 1 mm salicylic acid and 0.05, 0.1 and 0.5 mm kinetin, showed partial inhibition of the accumulation of local lesion virus in infected primary bean leaves. This inhibition was accompanied with an increase in the peroxidases activity, especially at the low concentration used of early treatment salicylic acid and kinetin compared with the un-treated control plants. In addition, the accumulation of total soluble protein contents were increased in comparison with the untreated control plants.

Key Words: Kinetin; *Phaseolus vulgaris*; Salicylic acid; Virus resistance

INTRODUCTION

Plant diseases may caused by environmental stresses, genetic or physiological disorders and infectious agents such as viroids, viruses, bacteria, fungi and other pathogens, all are responsible for enormous economic loss. The ability of plant to stop invasion of the pathogen depends on the presence of preformed barriers. Plants have a natural way of defending against pathogen attack by an array of biochemical responses. The plant hormones, e.g. salicylic acid (Naylor *et al*., 1998; Murphy *et al*., 1999; Ton *et al*., 2001) and kinetin (Clarke *et al*., 1998, 2000 & 2002) are each involved in the regulation of basal resistance against different pathogens. Previously, Clarke *et al*. (1998) and Galis *et al*. (2004), showed that exogenous application of salicylic acid and cytokinin or dehydrozeatin induced resistance against white clover mosaic virus in *Phaseolus vulgaris* plants. Salicylic acid is a natural phenolic compound present in many plants and participates in the regulation of physiological processes in plants. Also, it is an important component in the signal transduction pathway and involved in local and systemic resistance to pathogens (Delaney *et al*., 1995). In addition, concentration of endogenous salicylic acid increases at the site of hypersensitive response and acts as a transducer signal for activation of defense response (Delaney *et al*., 1994). Thus, the exogenous applied of salicylic acid is required for the expression of resistance, as well as for the enhancing the defensive capacity of tissues with acquired resistance (Ryals *et al*., 1994 & 1996). On the other hand, Clarke *et al*. (1999) reported that, virus titer increased rapidly 3 d after inoculation, attaining a maximum level at d 5, this increase accompanied the decline in the endogenous cytokinin content may allow the increase of virus titer in bean and lead to the senescence of infected leaves. Thus we have recently shown that the exogenous application of the cytokinins to bean (*Phaseolus vulgaris*) led to an inhibtion of virus replication (Clarke *et al*., 1998). Furthermore, Sano *et al*. (1994 & 1996) and Sano and Ohashi (1995) have implicated the cytokinins as components of the plant defense signal transduction pathway. Similarly, Dhingra *et al*. (1991) reported that kinetin, zeatin and 6 -benzylaminopurine (6 -BAP) reduced virus replication of potato virus Y (PVY) in infected potato callus.

Plant disease resulting from virus infection is thought to be caused by metabolic changes in particular sets of plant cells during the establishment and replication of the virus (Goodman *et al*., 1986). This pathogen-induced oxidative burst is known to participate in the hypersensitive reaction where plant cells in the direct vicinity of an infection undergo programmed cell death in order to eliminate the most immediate source of energy and nutrients for invading pathogens (Greenberg *et al*., 1994). Major reactive oxygen species scavenging mechanisms of plant include superoxidase dismutase, catalase and peroxidase, etc. and the balance between their activities in the cells is crucial for determining the steady state level of superoxide radicals and hydrogen peroxide (Bowler *et al*., 1991). Peroxidases represent a class of enzymes widely distributed throughout the plant kingdom. As multifunctional enzymes, peroxidases have a key role in several stress-related physiological processes such as disease resistance (Montalbini *et al*., 1995; Wojtaszek, 1997). Pathogensis-related proteins may be considered as stress proteins produced in response to, necrotizing infections by viruses, viroids, fungi and bacteria (Van Loon, 1989). Induction of acquired resistance and pathogensis-related proteins is often accomplished by spraying plants with salicylic acid solution

(Van Loon, 1997). Furthermore, by comparison of salicylic acid–and dihydrozeatin-induced gene expression that include pathogenesis-related protein, while salicylic acid treatments induced accumulation of defense related genes, the plants wick-fed with dihydrozeatin did not show a significant change in expression profile of any of these genes (Clarke *et al*., 1998).

The aim of this work was to study effect of infection of bean plants by tobacco necrosis virus on peroxidase activity, pattern of isoenzymes and induction of pathogen related proteins under the salicylic acid and kinetin applications.

MATERIALS AND METHODS

Plant and pathogen. Bean seeds (*Phaseolus vulgaris* L. cv. Contender) were obtained from Horticulture Institute, Agriculture Reserch Centre, (ARC), Dokki, Egypt. The seeds surfaces were sterilized with sodium hypochlorite solution (5%) and then, seeds planted in pots containing 700 g of soil and plased in a green-house under a 16 h potoperiod of 20 - 25^{0}C.

Tobacco necrosis virus (TNV) was obtained from Taha (1996). The virus isolate was maintained and propagated by serial inoculations in bean plants (*Phaseolus vulgaris* L. cv. Contender) according to the method suggested by Anfoka and Buchenauer (1997). Primary leaves of bean plants were dusted with carborandum (600 meshes) and after inoculation with TNV the leaves were washed with water and plants placed in the green-house. Five days later leaves with abundant necrotic local lesions were ground in a mortar with 0.05 M sodium phosphate buffer (pH 7.0) in a ratio of 1:1 (w/ v). The homogenate was then filtered and used as crude-inoculum.

Salicylic acid and kinetin treatments. Two-week-old plants with almost fully expanded primary leaves were divided in to 4 groups. The first group was sprayed with water as control plants, the second group was for virus inoculation only, the third group of plants was sprayed with series of salicylic acid concentrations (0.1, 0.5 & 1 mm) of whereas the fourth group was sprayed with 3 does of kinetin (0.05, 0.1 & 0.5 mm). The last two groups of plants were then inoculated with the virus.

Virus inoculation. Three days after salicylic acid and kinetin treatments, primary leaves of two-week-old plants were mechanically inoculated with TNV-inoculum. Crude-TNV inoculum diluted ten folds with the buffer before use. This dilution found to give a reasonable number of discrete local lesions in bean plants. Chemical analysis was done using primary un-treated leaves (as control) and primary infected leaves with or without salicylic acid and kinetin treatments at the 3rd day.

Total protein content. For determination of protein content, fresh primary leaves (0.1 g) were homogenized in a chiled (4^{0}C) mortar using a buffer containing: 50 mm Tris-HCl, pH 7.0. After centrifugation at 12100 g for 1 h at 4^{0}C, the supernatant filtered and then tranferred to Eppendorf tubes and the samples were kept on ice at 4^{0}C. Total soluble protein content was measured spectrophotometrically (Lowry *et al*., 1951) using bovine serum albumin as standard.

Analysis of isoperoxidase pattern. One gram of bean primary leaves was ground on ice in a mortar using 0.1 m Tris-HCl, pH 7.0 containing 0.002 m cysteine. The homogenate was centrifuged at 15000 rpm and 4^{0}C for 5 min. Native Polyacrylamide gel electrophoresis was performed in 7.5% acrylamide slab gel. The gels were run at 25 mA per gel for 6 h at 4^{0}C with 0.025 m Tris-HCl 0.192 m glycine buffer, pH 8.9. Peroxidase isoenzymes were then dectected according to (Brewer, 1970).

Peroxidase activity. Peroxidase activity in the extracts of primary leaves was assayed spectrophotometrically at 30^{0}C by following the increase in absorbance at 470 nm. 50 mm of exctracts prepared as previously described was added to a mixture of 40 mm potassium phosphate (pH 7.2), 0.1 mm EDTA, 0.5 mm guiaiacol, and 0.3 mm hydrogen peroxide (Wakamatsu & Takahama, 1993). The kinetic evolution of absorbency at 470 nm was calculated using the extinction coefficient (26.6 mm^{-1} cm^{-1} at 470 nm). The reaction was carried out for 3 min. One unit of peroxidase activity represents the amount of enzyme catalysing the oxidation of 1 µmol of guaiacol in 1 min.

The data obtained in three replicates were analysed by one way of ANOVA analysis.

RESULTS AND DISCUSSION

Primary leaves inoculated with TNV showed a marked increase in the number of local lesions beginning 3 days after virus inoculation (Table I). Pre-treatment of bean primary leaves with salicylic acid and kinetin caused a reduction in the number of local lesions, especially at the medium and low doses of salicylic acid or kinetin, respectively. These results are in agreement with those of Reglinski *et al*. (1997), they demonstrated that pre-treatment of kiwifruit leaves with salicylic acid and 4 -chlorosalicylic acid caused a reduction in the size of lesions arising from infection by *Sclerotinia sclerotiorum*. Similarly, in kiwifruit, pre-harvest application of salicylic acid enhanced resistance to wound infection by *Botrytic cinerea* in the immediate post-harvest period (Poole & Mcleod, 1994). Meena *et al*. (2001) reported that foliar application of salicylic acid (1 mm) reduced significantly late leaf spot disease intensity but however increased the pod yield in groundnut. In contrast, from the results obtained for the primary leaves infection by TNV after spraying treatment with salicylic acid and kinetin (Table I, Fig. 1), the number of local lesions and size of pathogen was more apparent especially at high concentration of salicylic acid (1.0 mm) and kinetin (0.5 mm) used. These results are in agreement with Van Loon and Antoniw (1982), which suggested that, salicylic acid at high concentrations may be induced the full set of systemic

acquired resistance (SAR) genes. Similarly, Galis *et al*. (2004) reported that the increases caused by µM/ L concentration of cytokinins may reflect a high-cytokinin stress condition of the plants rather than genuine signaling responses. In contrast to cytokinins, 1 mm/ L salicylic acid induced resistance during the early stage of WCIMV infection. In an attempt to partially or completely alleviated of pathogenic effect, it was found that pre-treatment with salicylic acid and also kinetin induced resistance in primary leaves of bean against local necrosis virus probably by the lack of relevant resistance gene (Clarke *et al*., 1998; Naylor *et al*., 1998).

Bean plants which treated with salicylic acid and kinetin were examined for the induction of peroxidases and total soluble proteins. In present study (Table II), the activity of peroxidase increased when bean plants were inoculated with TNV alone compared with un-treated bean plants as control. Similar results have been reported with other plant-virus interactions like tobacco and *Tobacco necrosis* virus, bean and *Peanut mottle* virus, and in cucumber infected with *Cucumber mosaic* virus (Clarke *et al*., 2002). This might have been due to the change in the antioxidant status of cell as a result of the variations in activities of enzymes dismutase (SOD), catalase (CAT), and peroxidase (POD) that are responsible for warding off active oxygen species in the cells (Adam *et al*., 1995). Analysis of primary leaves infection with virus after treatment with salicylic acid and kinetin revealed that the peroxidase activity was markedly elevated (Table II). The increasing in peroxidase activity and the intensity and thickness of the bands with isoenzyme pattern were observed as early as 3 days after application of these compounds especially at high concentrations levels used compared with un-treated bean plants as control (Table II & Fig. 2). These elevated in peroxidase activity and the intensity of the bands with isoenzymes were more pronounced in pre-treatment with salicylic acid than treatment with kinetin. These results are in agreement with Van Loon and Antoniw (1982), suggested that in tobacco, 1 mm of salicylic acid proved to be at the border of toxicity, was elevated the stress by increasing the peroxidase activity. Similarly, Chen *et al*. (1995) reported that intracellular H_2O_2 accumulation activities H_2O_2-scavenging enzymes such as catalase and peroxidase in plants, in which salicylic acid is required for induction of these antioxidant enzymes (Alvarez *et al*., 1998). Recently, the application of cytokinins dihydrozeatin (DZ) and DZ riboside to the xylem of bean plants led to a reduction in virus at the point of double-stranded RNA synthesis and prevented the normal virus-induced, which decrease in free-radical-scavenging enzymes (Clarke *et al*., 1998).

In Table III, primary leaves inoculated with TNV alone showed a marked increase in the accumulation of total soluble protein contents compared with un-treated bean plants as control. These results are in agreement with those of Van Loon (1989) reported that, plant when exposed to various environmental stress such as necrotizing infection induced synthesizing sets of specific proteins (PRs). Upon treatment of bean leaves with salicylic acid and kinetin, the accumulation of total soluble protein contents are increased. These increased were more pronounced in pre-treatment with salicylic acid than treatment with kinetin compared with un-treated bean plant as control. The results of this study also indicate that, pre-treatment with salicylic acid induces a range of defense genes that include pathogenesis-related proteins (Bowles, 1990). Furthermore, by

Table I. Effect of different concentrations of salicylic acid and kinetin on number of local lesions (per leaf) in bean plants inoculated with TNV

Treatment	Concentration (mM)	Local lesions (per leaf)
Virus-inoculated plants	0.0	134.67
Salicylic acid	0.10	100.00**
+	0.50	41.10**
virus-inoculated plants	1.00	107.29**
Kinetin	0.05	52.00**
+	0.10	81.40**
virus-inoculated plants	0.50	120.00*
L. S. D. at 5%		11.53
L. S. D. at 1%		16.05

* Significant differences;

** Highly Significant differences as compared with control**.**

Table II. Peroxidase activity (mM/ g fresh weight) in bean plants inoculated with TNV under salicylic acid and kinetin treatments

Treatment	Concentration (mM)	Peroxidase activity (mM/ g fresh weight)
Untreated plants	Control	17.13
Virus-inoculated plants	0.0	26.30
Salicylic acid	0.1	166.87**
+	0.5	308.05**
virus-inoculated plants	1.0	310.68**
Kinetin	0.05	59.03**
+	0.1	55.00**
virus-inoculated plants	0.5	77.67**
L. S. D. at 5%		21.76
L. S. D. at 1%		30.30

* Significant differences;

** Highly Significant differences as compared with control.

Table III. Effect of different concentrations of salicylic acid and kinetin on total soluble protein contents (mg/ g fresh weight) in bean plants inoculated with TNV

Treatment	Concentration (mM)	Protein content (mg/ g fresh weight)
Untreated plants	Control	2.91
virus-inoculated plants	0	10.57**
Salicylic acid	0.1	9.90**
+	0.5	12.23**
virus-inoculated plants	1.0	29.40**
Kinetin	0.05	4.87*
+	0.1	7.06**
virus-inoculated plants	0.5	6.14**
L. S. D. at 5%		1.47
L. S. D. at 1%		2.05

* Significant differences;

** Highly Significant differences as compared with control.

Fig. 1. Effect of different concentrations of salicylic acid and kinetin on size of pathogen in bean plants inoculated with TNV. Control (1), infected virus (2), salicylic acid (3, 4, 5), kinetin (6, 7, 8)

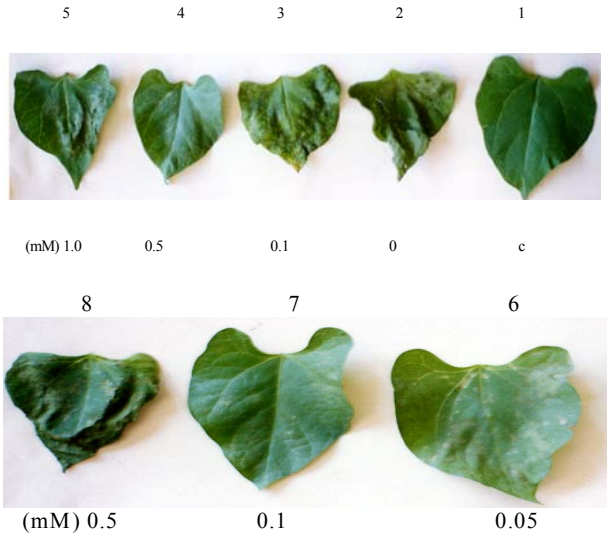
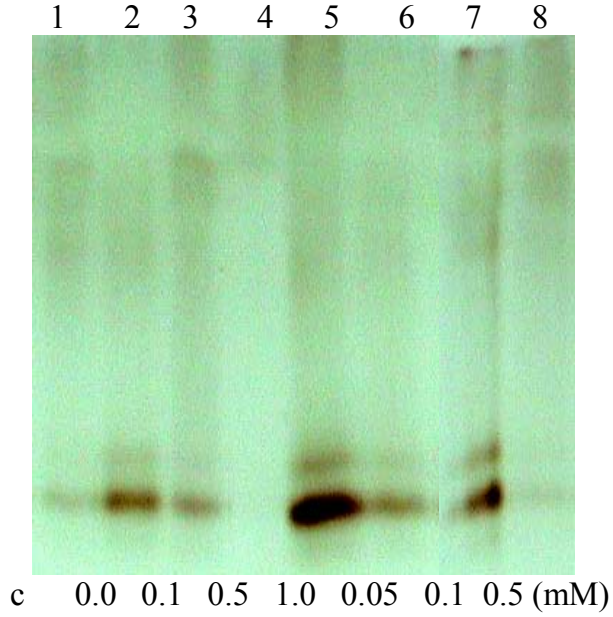


Fig. 2. Effect of different concentrations of salicylic acid and kinetin on peroxidases isoenzyme pattern in bean plants inoculated with TNV. Control (Lane 1), infected virus (Lane 2), salicylic acid (Lane 3, 4, 5), kinetin (Lane 6, 7, 8)



comparison of salicylic acid and dihydrozeatin treatment for induction of gene expression. SA treatments induced accumulation of defense-related gene expression, wheras plants wick-fed with DHZ did not show significant changes in the gene expression (Clarke *et al*., 1998). Van Loon *et al.* (1994) reported that, some of the pathogenesis-related proteins (PRs) possess a potential antipathogenic activities. Constitutive expression of individual PRs in transgenic plants can lead to reduced pathogen growth and symptom expression (Ryals *et al*., 1994).

CONCLUSION

In summary, the present study demonstrates the induction of peroxidases in bean primary leaves in response to treatment with salicylic acid and kinetin. Pretreatment with salicylic acid and kinetin also induced a considerable resistance in bean to challenge inoculation with TNV. Hence it is possible that increased resistance in bean against TNV after application of salicylic acid or kinetin may be related to the accumulation of pathogenesis-related proteins.

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(Received 20 November 2005; Accepted 26 December 2005)