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Induction of Systemic Acquired Resistance in Tobacco Plants against Tobacco Mosaic Virus by Local Inoculation and Benzothiadiazole (BTH)

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Lower leaves of tobacco (*Nicotiana tabacum* L. cv. Xanthi-nc and cv. NahG) were inoculated (first inoculation) with tobacco mosaic virus (TMV). Systemic acquired resistance (SAR) were induced in the remote (upper) leaves of Xanthi-nc against the second inoculation with TMV. Levels of reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) were reduced in the remote inoculated leaves which exhibited SAR. As a result, few necroses only were produced in the resistant upper leaves of cv. Xanthi-nc. ROS and tissue necrotization in the transgenic cv. NahG (unable to produce SAR) were increased in the upper leaves. As a result of the first inoculation treatment, activities of the antioxidant enzymes catalase (CAT) and dehydroascorbate reductase (DHAR) and gene expression level (using RT-PCR) of CAT, superoxide dismutase (SOD) and DHAR were significantly increased in the remote upper leaves of Xanthi-nc only and not in NahG. Benzothiadiazole (BTH) treatment increased ROS levels and total phenolic contents at 6, 12 and 24 hours after inoculation (hai) followed by significant reduction of ROS levels and tissue necrotization 36 and 48 hai with TMV in the upper leaves either in cvs. Xanthi-nc or NahG. Activities of CAT and DHAR as well as the gene expression level of CAT, SOD and DHAR were increased significantly in the upper inoculated cv. Xanthi-nc and NahG leaves treated with BTH. So, virus suppression and a limited number as well as size of necrotic lesions caused by TMV seem to be correlated with the level of ROS and phenolic contents. The result indicated that BTH induced ROS and phenolics early after inoculation which inhibit virus multiplication and as a mild stress it immunized plants against the pathogen by the elevated levels and activities of the antioxidant enzymes. This might support the application of low concentration of ROS (H_2O_2) to increase the plant resistance against pathogens. ROS and antioxidants play a pivotal role in TMV-induced and suppressed cell death response, respectively. One can conclude that BTH has a key role in SAR even in the NahG tobacco (which is unable to produce SAR), thus we recommend giving more attention to apply BTH in several crops either in the greenhouse or in field experiments to protect plants against viral infection.

Keywords: Antioxidants, acquired resistance, benzothiadiazole, TMV and tobacco plants.

When the resistant tobacco *Nicotiana tabacum* L. cv. Xanthi-nc (NN genotype) was inoculated with tobacco mosaic virus (TMV), local cell death was induced, resulting in systemic acquired resistance (SAR) to subsequent (second) inoculation in leaves remote (upper) to the initial inoculation sites (Sticher *et al.*, 1997 and Hafez *et al.*, 2004). As a result of SAR induction against TMV in the resistant tobacco hosts, few and small necroses appeared in response to the second inoculation with TMV (Ross, 1961) and salicylic acid (SA) level increased in both of the inoculation site and in the remote tissues (Malamy *et al.*, 1990). Exogenous application of salicylic acid (SA) or its functional analogues, such as benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), and 2,6-dichloroisonicotinic acid (INA) exogenously also increased the resistance against TMV (Friedrich *et al.*, 1996; Hafez *et. al.*, 2004) and powdery mildews (Hafez, 2005). However, TMV-inoculated tobacco NahG plants (NN genotypes) transformed with the salicylic acid hydroxylase gene can not induce SAR because of SA conversion to catechol (Delaney *et al.*, 1994 and Mur *et al.*, 1997). According to this SAR block in NahG, it is assumed that SA is a necessary and sufficient signal molecule for induction of SAR. Tobacco inoculated with TMV and showing necroses (cell death) were produced and exhibited an oxidative burst (Mur *et al.*, 1997). The recognition site of an invading pathogen resulted in the activation NAD (P)H oxidase and superoxide (O_2^-) production (Jabs *et al.*, 1996). Superoxide dismutase catalysed O_2^- to H_2O_2 spontaneously which play an important role in defence mechanisms and programmed cell death (Lamb and Dixon, 1997). ROS sometimes damaged the cellular compartments either in the pathogenic or the host cells, depending on the level of ROS sufficient to initiate cell death (Hafez *et al.*, 2009). Several investigators have indicated a preferential association between the ROS accumulation and the degree of cell death (Jabs *et al.*, 1996 and Kiraly *et al.*, 2008). According to this model, antioxidative mechanisms which scavenge and neutralize ROS were shown to be suppressed during the early steps of TMV-induced cell death in tobacco (Fodor *et al.*, 1997). Similarly, low oxygen pressure, which inhibits the accumulation of ROS, was found to prevent tissue necrotization in TMV-inoculated tobacco (Mittler *et al.*, 1996). In addition, transgenic tobacco over expressing ferritin gene (iron-storage protein), the numbers of virus-induced necrotic lesions were suppressed because the free iron was bound by this ferritin, as a result, production of hydroxyl radical (OH˙) which is the most harmful ROS, was inhibited (Deák *et al.*, 1999). ROS are counterbalanced by endogenous antioxidant applications (Hafez, 2005 and Kiraly *et al.*, 2008). In the upper uninoculated leaves of Xanthi-nc plants the antioxidants SOD, glutathione reductase, glutathione S-transferase activities and levels of glutathione were induced one week after the SAR induction by TMV inoculation of the lower leaves (Fodor *et al.*, 1997). In these upper uninoculated leaves which the SAR induced the antioxidative defence systems could be triggered by a microoxidative burst (Fodor *et al.*, 2001 and Hafez *et al.*, 2004). SA accumulation is a prerequisite for these antioxidative defence systems, because antioxidants failed to be activated in remote leaves of TMV-inoculated NahG transgenic tobacco which cannot accumulate SA (Király *et al.*, 2002).

Therefore, BTH was applied to induce SAR in tobacco as compared with SAR induction by a previous TMV inoculation either in cvs. Xanthi-nc or NahG tobacco. To clarify the SAR mechanisms $O_2^{\cdot-}$, H_2O_2 levels, total phenolic contents, activities and gene expression (RT-PCR) of the antioxidant enzymes in the remote (upper) leaves were studied. As expected, $O_2^{\cdot-}$, H_2O_2 levels and total phenolic contents should be increased very early after the inoculation to inhibit the virus. This may cause the antioxidants to be elevated and thus suppress tissue necrotization. This may support the immunization theory in plants by using exogenous application of H_2O_2 (ROS) to induce the antioxidative defence system against biotic and abiotic stresses (Gechev *et al.*, 2002; Hafez *et al.*, 2008 and Bacso *et al.*, 2009).

Materials and Methods

Plant materials:

Tobacco (*Nicotiana tabacum* L.) cultivars Xanthi-nc (wild-type) and NahG (transgenic) were grown under greenhouse conditions. This experiment was held in the Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary. Cultivars used in this study were provided by Syngenta, Agricultural Biotechnology Research, and Research Triangle Park, NC, USA. Seedlings of the above mentioned cultivars were sown in plastic pots (10 or 15 cm diameter) containing a mixture of soil:peat:sand (2:1:1, v/v/v) and grown under greenhouse conditions at 18-23°C, with 16 hours photoperiod per day using supplemental light by mercury lamps (HQL) with a light intensity of 160 mE m^{-2} s^{-1} and 75-80% relative humidity.

TMV inoculation:

The U1 strain of TMV was maintained on the Samsun (nn) susceptible cultivar of tobacco (*Nicotiana tabacum* L.). For mechanical virus inoculation, TMV-infected leaves, were homogenized in tap water. Carborundum was used as an abrasive for both virus and mock inoculations. Two days after inoculation with TMV, visible necrotic lesions (HR) in Xanthi-nc and NahG cultivars were recorded.

Treatments with TMV locally and BTH:

For SAR induction, the third and fourth true leaves of tobacco plants were inoculated with TMV (first inoculation) at the age of 8 weeks as described by Fodor *et al.* (2001). Challenge (second inoculation) was carried out in the fifth and sixth upper leaves two weeks later, after the development of SAR. Mock-inoculated plants were used as controls.

Aqueous solution of 0.3 mM Benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), (Syngenta, Agricultural Biotechnology Research, Research Triangle Park, NC, USA) was ectopically applied by spraying of the whole plant, using a pump aerosol sprayer in a formulation containing 50% (w/w) active ingredient. BTH-treated leaves were inoculated by TMV one week after the treatment.

Detection of $O_2^{\cdot-}$ and H_2O_2:

Superoxide and H_2O_2 were visualised as a purple coloration of nitro blue tetrazolium (NBT) and a reddish-brown coloration of 3,3-diaminobenzidine (DAB), respectively. Leaf discs (2 cm in diameter) were vacuum infiltrated with 10 mM

potassium phosphate buffer (pH 7.8) containing 0.1% (w/v) NBT (Sigma–Aldrich, Steinheim, Germany) according to Ádám *et al.* (1989) or 0.1% (w/v) DAB (Fluka, Buchs, Switzerland). NBT- and DAB-treated samples were incubated under daylight for 20 min and 2 hours, respectively and subsequently cleared in 0.15% (w/v) trichloroacetic acid in ethanol:chloroform 4:1 (v/v) for 1 day (Hückelhoven *et al.*, 1999). Cleared samples were washed with water and placed in 50% glycerol prior to evaluation. Discoloration of leaf discs resulted by NBT or DAB staining was quantified using a ChemiImager 4000 digital imaging system (Alpha Innotech Corp., San Leandro, USA).

Detection of H_2O_2 using xylenol orange dye:

To detect H_2O_2 spectrophotometrically with a peroxidase independent reaction, a xylenol orange based method was used according to the method of Gay *et al.* (1999). Leaf tissue (0.25 g) was ground in a mortar in boric acid/borax buffer (pH 8.4) mixed up 0.61 g boric acid in 150 ml water and 0.95 g borax in 50 ml water. The homogenate was centrifuged (12000 rpm, 15 min). 200 ml supernatant was combined with 1 ml xylenol orange solution.

The xylenol orange solution was prepared just before usage by mixing 100 ml of solution A (25 mM $FeSO_4$, 25 mM $(NH_4)_2SO_4$, 25 mM H_2SO_4) and 10 ml of solution B (125 mM xylenol orange from Sigma and 100 mM sorbitol). After 30 minuts incubation at 25°C, the absorption of A_{560} was estimated using a spectrophotometer. For quantification of the measured xylenol orange values, a standard curve was done by applying dilution series of H_2C_2 as described by Hafez *et al.* (2009).

Determination of TMV concentration:

Enzyme-linked immunosorbent assays (ELISA) were used for determining TMV concentration according to Clark and Adams (1977) and Tóbiás *et al.* (1982) using a TMV kit of Bioreba (Art. No. 190412 and 190422). The extinction values were measured with Multiskan ELISA Reader at 405 nm.

Determination of phenol contents:

To assay the total phenolic contents, tissue sample (0.2 g) was homogenized with 10 of ice-cold 1% HCl-methanol solution and then centrifuged at 4°C for 10 min at 12,000 rpm according to Pirie and Mullins (1976).

The measurements in the supernatent was at 280 nm. Total phenolic compounds content was expressed as µMol gallic acid/g FW.

Biochemical assays of antioxidant enzymes:

For enzyme assays in tobacco plants, 0.5g leaves were homogenized at 0-4°C in 3 ml of 50 mM Tris buffer (pH 7.8), containing 1 mM EDTA-Na_2 and 7.5% polyvinylpyrrolidone. The homogenates were centrifuged (12,000 rpm, 20 min, 4°C), and the total soluble enzyme activities were measured spectrophotometrically in the supernatant. All measurements were carried out at 25°C, using the model UV-160A spectrophotometer (Shimadzu, Japan).

Activity of catalase (CAT):

Activity of CAT (E.C. 1.11.1.6) was determined spectrophotometrically according to Aebi (1984).

Activity of dehydroascorbate reductase (DHAR):

Activity of DHAR (E.C. 1.8.5.1) was determined spectrophotometrically as described by Asada (1984).

Gene expression of antioxidants:

Total RNA isolation from tobacco and barley plants:

Samples (200mg fresh leaves) from upper tobacco leaves (Xanthi and NahG) which SAR was induced either by the first inoculation with TMV or BTH treatments were used 36 hours after the second inoculation with TMV. Total RNA isolation from plant was done as described by Hafez (2005).

Determination of RNA concentration spectrophotometrically:

The absorption maximum of RNA is at a wavelength of 260 nm, while that of proteins at 280 nm in the UV range. Assays of RNA concentration were done at 260 and 280 nm (the latter for indication of protein contamination). The ratio of absorbance values were measured at 260 and 280 nm (A_{260}/A_{280}) indicates purity of the RNA sample, values between 1.6 and 2.0 are acceptable. RNA samples were diluted 100× in sterile distilled water to 500 ml total volume. Assays were carried out in 1ml quartz cuvette, sterile distilled water was used as a reference.

Formaldehyde-agarose gel electrophoresis of plant RNA:

RNA integrity and concentration was checked by formaldehyde-agarose gel electrophoresis (Rapley and Manning, 1998). The integrity and concentration of ribosomal RNA (rRNA) bands visible in the gel is an indication of the quality and amount of mRNA present in the samples.

Electrophoresis running buffer was prepared from a 10× stock (0.2 M 3-(N-Morpholino)propane sulfonic acid, MOPS, pH 7.0 adjusted with HCl; 0.1 M sodium acetate; 10 mM EDTA, pH 8.0). A formaldehyde-agarose gel was used for electrophoresis (1 % w/v agarose and 5% v/v formaldehyde in gel).

Samples were prepared by denaturing 1-5 mg of plant RNA at 95°C for 5 min then placing the RNA samples on ice and adding an equal volume of 2× RNA loading buffer (Fermentas).

Electrophoresis was done at 50-60 V until rRNA bands have moved ca. two thirds of the way along the gel (4-6 V/cm length of gel). rRNA bands were visualized under a UV transilluminator at 254 nm.

Gene expression analysis on the mRNA level:

For gene expression analysis on the mRNA level, a two step reverse transcription-polymerase chain reaction (RT-PCR) procedure was applied (Fermentas) according to manufacturers instructions. Primers used in the RT-PCR assays were as the following:

5´-CGGAATCCACGAGACTACATAC-3´ (5´ primer [forward]) and 5´-GGGAAGCCAAGATAGAGC-3´ (3´ primer [reverse]) for a 230-bp tobacco

actin (*NtAct*) cDNA fragment (Genbank accession X69885); 5′-GCCGTCCTTAGCAGCAGT-3′ (5′primer) and 5′-ACAAGCAACCCTTCCACC-3′ (3′primer) for a 420-bp tobacco cytosolic Cu/Zn superoxide dismutase (*NpSOD1*) cDNA fragment (Genbank accession X55974); 5′-TCCGCTTGATGTGACTAAA-3′ (5′primer) and 5′-TCCACCCACCGACGAATA -3′ (3′primer) for a 501-bp tobacco catalase (*NgCAT1*) cDNA fragment (Genbank accession AF006067); 5′-CAAGGCTCACGGACCATA-3′ (5′primer) and 5′-ACTTCCTGCGAAACAACG-3′ (3′primer) for a 277-bp tobacco cytoplasmic dehydroascorbate reductase (*NtDHAR*) cDNA fragment (Genbank accession AY074787).

Results

Effect of SAR induction on number and size of necrotic lesions:

Inoculation of lower leaves of tobacco cv. Xanthi-nc with TMV (first inoculation) induced SAR in the upper (remote) leaves against the second (challenge) inoculation with TMV. As a result of SAR induction the number and size of visible necroses were reduced significantly. However, TMV inoculation (first inoculation) could not induce SAR in cv. NahG tobacco and the number and size of necroses were not reduced (Fig. 1). BTH treatment induced SAR against the second TMV inoculation in the upper leaves either in cvs. Xanthi-nc or NahG plants successfully and also reduced the number and size of necroses significantly (Fig. 1).

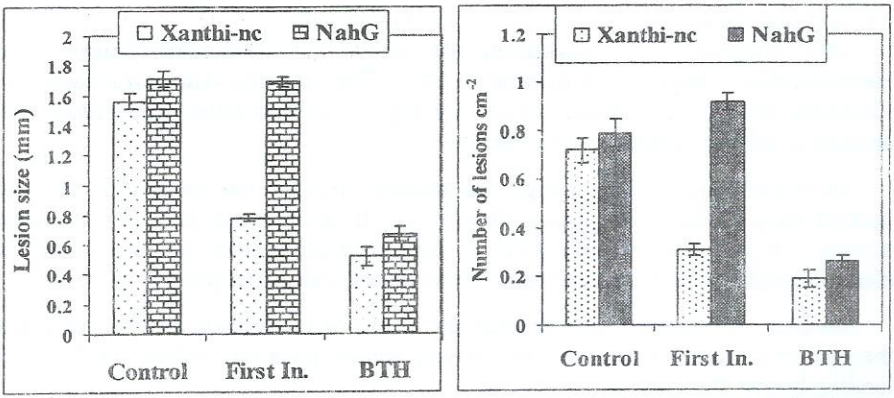

Fig. 1. Effects of SAR induction on the number and size of necrotic lesions in TMV-inoculated upper leaves (Xanthi-nc and NahG tobacco) 36 hours after inoculation. Control: upper leaves inoculated only with TMV. First In.: upper leaves were inoculated with TMV (second inoculation) two weeks after the first inoculation of the lower leaves. BTH: tobacco plants were sprayed with 0.3 mM BTH one week before inoculation of the upper leaves with TMV. Values are means ± SD of four leaves from three independent experiments.

Effect of SAR on the levels of ROS in uninoculated upper leaves upon TMV inoculation of the lower leaves:

Levels of $O_2^{\cdot-}$ and H_2O_2 were significant reduced in Xanthi-nc plants after the induction of SAR however, it did not reduce in the upper leaves of TMV-inoculated NahG tobacco (Fig. 2), which is consistent with the inability of NahG plants to activate antioxidative defence after TMV inoculation.

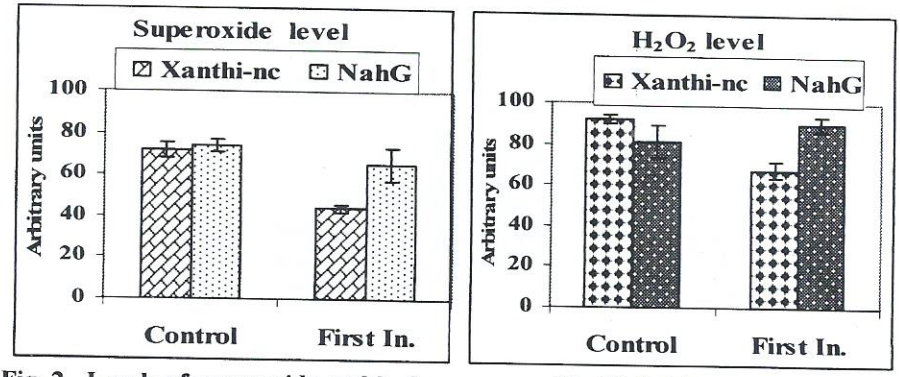

Fig. 2. Levels of superoxide and hydrogen peroxide (H_2O_2) in uninfected upper leaves of tobacco Xanthi-ne and NahG plants 2 weeks after inoculation of the lower leaves by TMV. Control: mock inoculated leaves. First In.: third and fourth true leaves inoculated with TMV (first inoculation only). Means of three independent experiments ± SD represent the density of purple formazan precipitate of NBT staining (superoxide) and reddish-brown DAB staining (H_2O_2).

Effect of SAR on the levels of ROS in TMV-inoculated upper leaves upon TMV inoculation of the lower leaves:

Inoculation of tobacco leaves with TMV only (which no SAR induced) resulted in a significant increase in tissue necrotization, $O_2^{\cdot-}$ and H_2O_2 levels (Figs. 3, 4 & 5). NBT and DAB staining were more intensive in NahG tobacco, which cannot develop SAR and produce large necrotic spots.

When SAR was induced by the first TMV inoculation, $O_2^{\cdot-}$ and H_2O_2 levels were significantly decreased and smaller oxidative burst (necroses) in the remote leaves of Xanthi-nc tobacco were produced as compared to plants in which SAR was not induced (Figs. 4 and 5). However, neither $O_2^{\cdot-}$ nor H_2O_2 were decreased significantly in NahG plants during the challenge TMV inoculation (Figs. 4 and 5).

BTH-treated leaves of Xanthi-nc and NahG tobacco in which the SAR was already induced, the accumulation of $O_2^{\cdot-}$ and H_2O_2 increased significantly early 6 and 12 hours after inoculation (hai) in the case of $O_2^{\cdot-}$ and 6, 12 and 24 hai in the case of H_2O_2. Levels of $O_2^{\cdot-}$ and H_2O_2 were decreased significantly in BTH treated leaves as compared with other treatments 36 and 48 hai (Figs. 4 and 5). Interestingly enough that BTH was able to decrease $O_2^{\cdot-}$ and H_2O_2 levels even in the NahG tobacco 36 and 48 hai as compared with TMV treatments (Figs. 4 and 5).

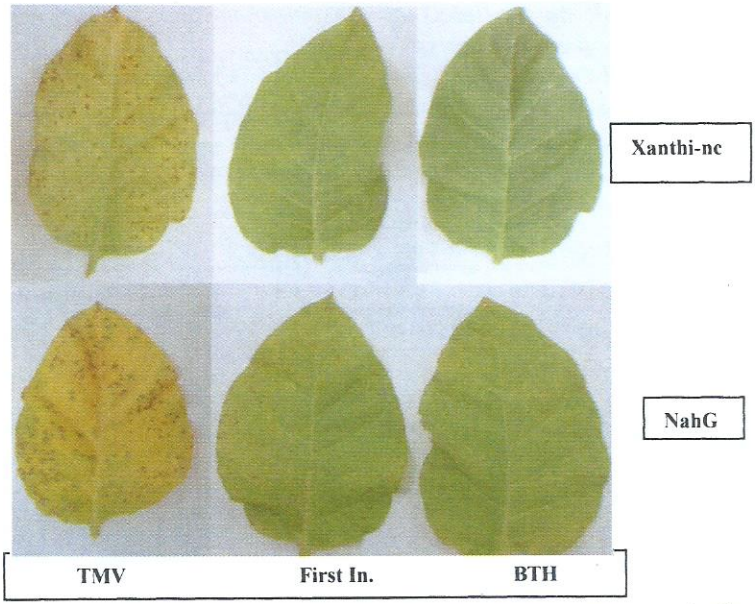

Fig. 3. Effects of SAR induction on TMV-inoculated upper leaves and tissue necrotization in tobacco (Xanthi-nc and NahG) 4 days after inoculation. TMV: upper leaves inoculated only with TMV. First In: upper leaves were inoculated with TMV (second inoculation) two weeks after the first inoculation of the lower leaves. BTH: tobacco plants were sprayed with 0.3 mM BTH one week before inoculation of the upper leaves with TMV.

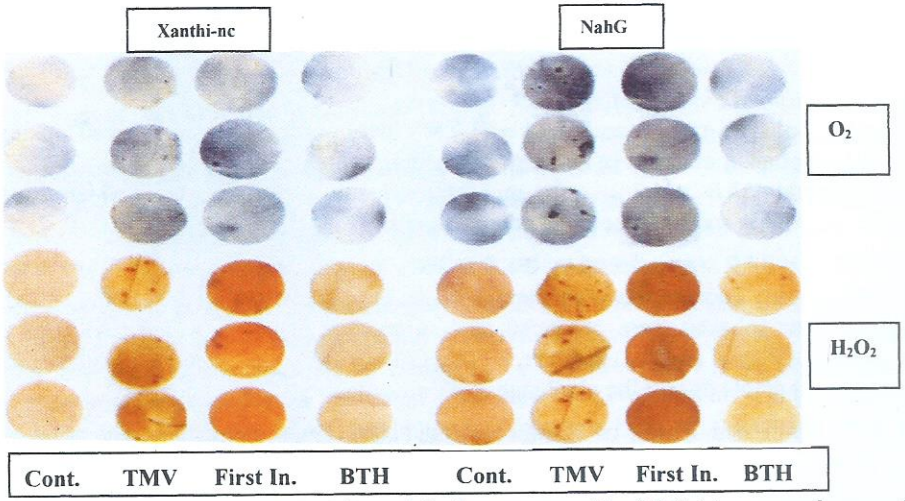

Fig. 4. Levels of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) in upper leaves of tobacco Xanthi-nc and NahG inoculated with TMV (challenge inoculation) 2 days after inoculation. Control: leaves were uninoculated. TMV, First In. and BTH: (see Fig. 3). NBT and DAB staining were used to measure the level of O_2 and H_2O_2 respectively.

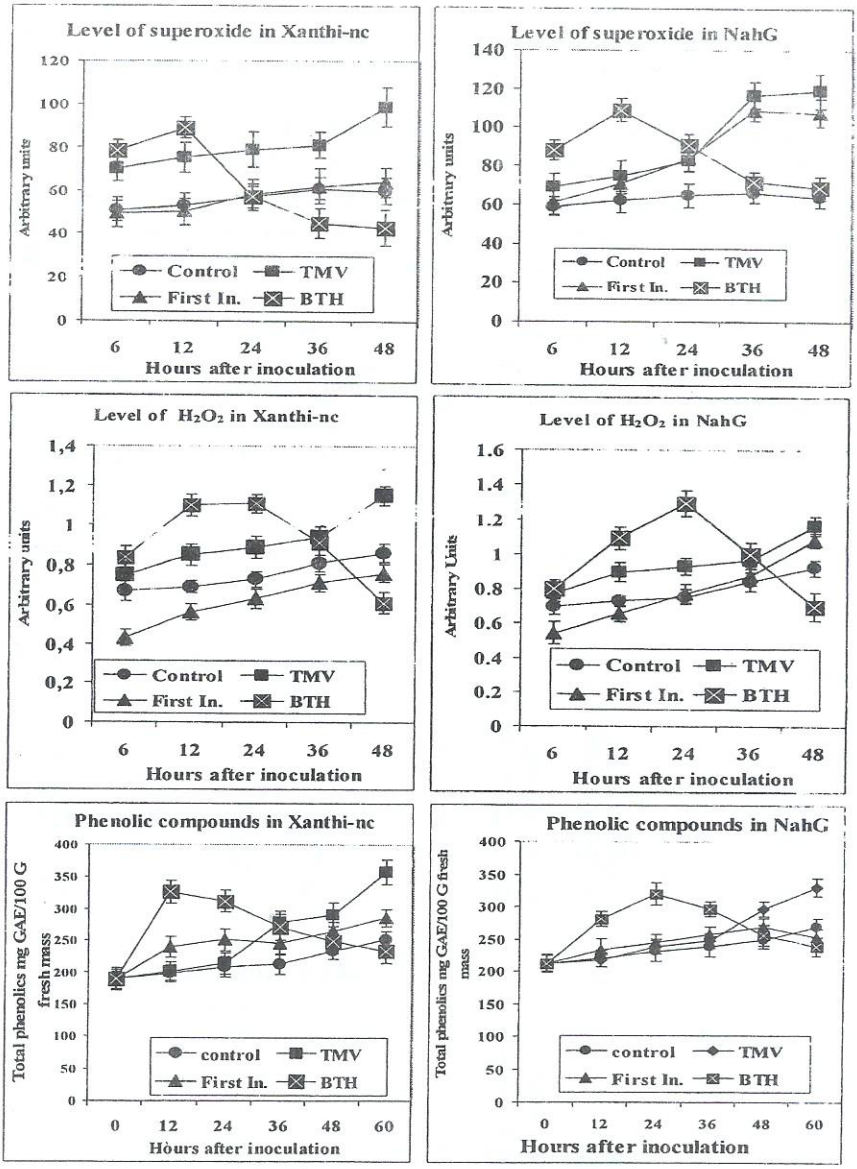

Fig. 5. Levels of superoxide using NBT staining, hydrogen peroxide (H_2O_2) using xylenol orange and total phenolic contents in the upper leaves of tobacco Xanthi-nc and NahG inoculated with TMV (challenge inoculation) 6, 12, 24, 36 and 48 hours after inoculation. TMV, First In. and BTH: (see Fig. 3). Means of three independent experiments ± SD are shown.

Effect of SAR on the total phenolic contents in TMV-inoculated upper leaves upon TMV inoculation of the lower leaves:

Total phenolic contents were increased significantly early 6, 12 and 24 hours after inoculation in the remote leaves of tobacco (Xanthi-nc) and tobacco (NahG) which treated with BTH as compared to TMV only (no SAR) and TMV (first inoculation) treatments which SAR was induced. After that total phenolic contents significant decreased 36, 48 and 60 hai compared to other treatments (Fig. 5).

BTH was able to increase significantly $O_2^{\cdot-}$ and H_2O_2 levels as well as phenolic contents early 6, 12 and 24 hai which may play an important role for inhibiting the virus concentration either in tobacco Xanthi-nc or NahG as compared to the other treatments (Fig. 5).

Effect of SAR on the activity and gene expression of the antioxidant enzymes in TMV-inoculated upper leaves upon TMV inoculation of the lower leaves:

Activities of catalase (CAT) and dehydroascorbate reductase (DHAR) were increased significantly in the remote leaves of tobacco Xanthi-nc but not in NahG treated with BTH and TMV (first inoculation) as compared to control and TMV treatments 2, 3 and 4 days after inoculation (Fig. 5). However, BTH treated tobacco (NahG) was able to exhibit significant increasing of the antioxidant activities (CAT and DHAR) even in this transgenic tobacco NahG which SAR did not induce (Fig. 6).

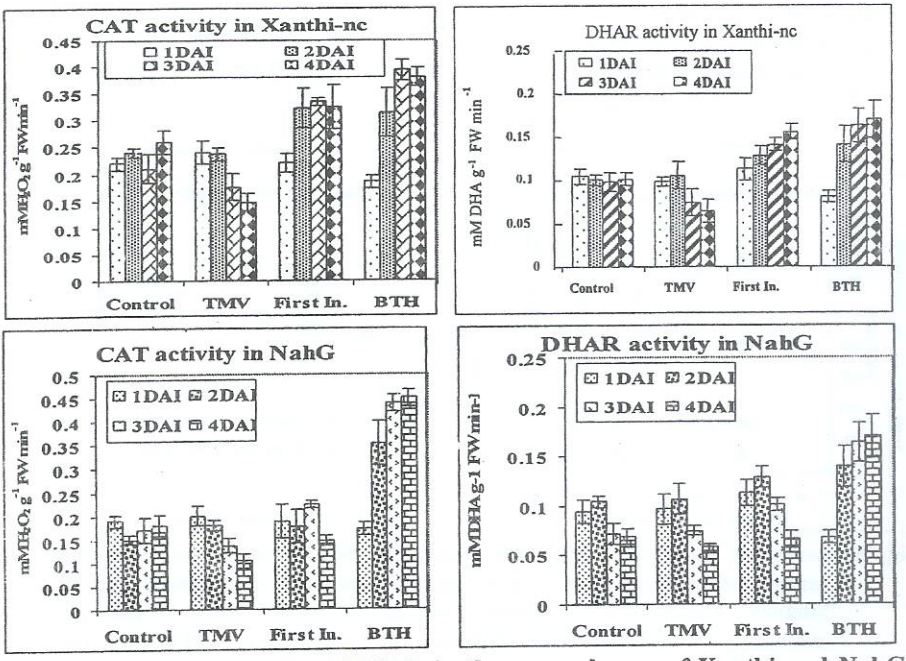

Fig. 6. Activities of CAT and DHAR in the upper leaves of Xanthi and NahG tobacco 1, 2, 3 and 4 days after inoculation (DAI) with TMV (see Fig. 2). Means of three independent experiments ± SD are shown.

Expression of the genes *NtCAT1*, *NtSOD* and *NtDHAR* in tobacco Xanthi and NahG plants were induced significantly in the upper leaves when SAR was induced by BTH treatment as compared to other treatments at 48 hours after the second challenge inoculation (Fig. 7). However, when SAR was induced by the first inoculation of the lower leaves one only strong induction of the genes *NtCAT1*, *NtSOD* and *NtDHAR* in Xanthi tobacco, but in NahG tobacco no significant induction was recorded at 48 hours after the second challenge inoculation (Fig. 7).

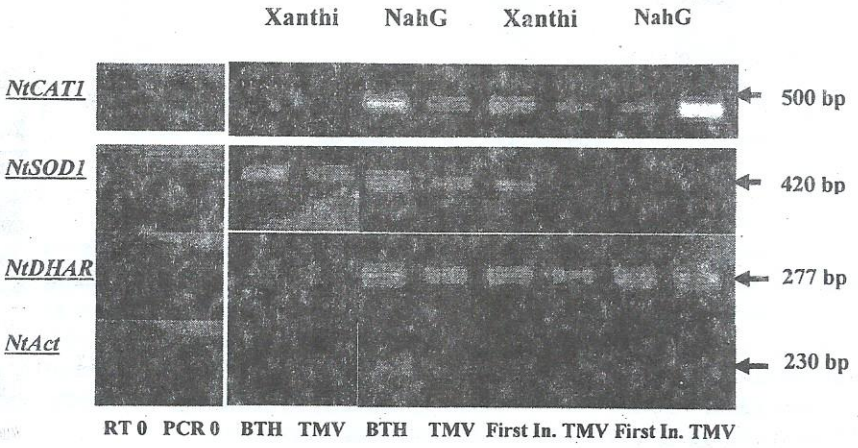

Fig. 7. Expression of *NtCAT1*, *NtSOD* and *NtDHAR* genes in tobacco Xanthi-nc and NahG upper leaves 48 hours after inoculation with TMV. BTH: leaves were sprayed with BTH (0.3 mM) one week before inoculation of upper leaves. TMV: upper leaves were inoculated only with TMV. First In.: upper leaves were inoculated with TMV (second inoculation) 2 weeks after the first inoculation on the lower leaves. RT 0: negative control of reverse transcription (no RNA template present); PCR 0: negative control of PCR reaction (no cDNA template present). Gene expression assays were done using two steps reverse transcription-polymerase chain reaction (RT-PCR) procedure. A tobacco actin gene (*NtAct*) served as a reference of gene expression.

Effect of SAR induction on the TMV concentration:

When SAR was induced by first inoculation of the lower leaves, TMV concentration was not reduced significantly in the upper leaves. Similarly, when tissue necrotization was strongly suppressed by leaf infiltration with a mixture of the antioxidant enzymes, *i.e.* superoxide dismutase and catalase, virus concentration was not reduced. But in BTH-treated tobacco leaves TMV concentration was reduced either in cvs. Xanthi-nc or NahG (Fig. 8).

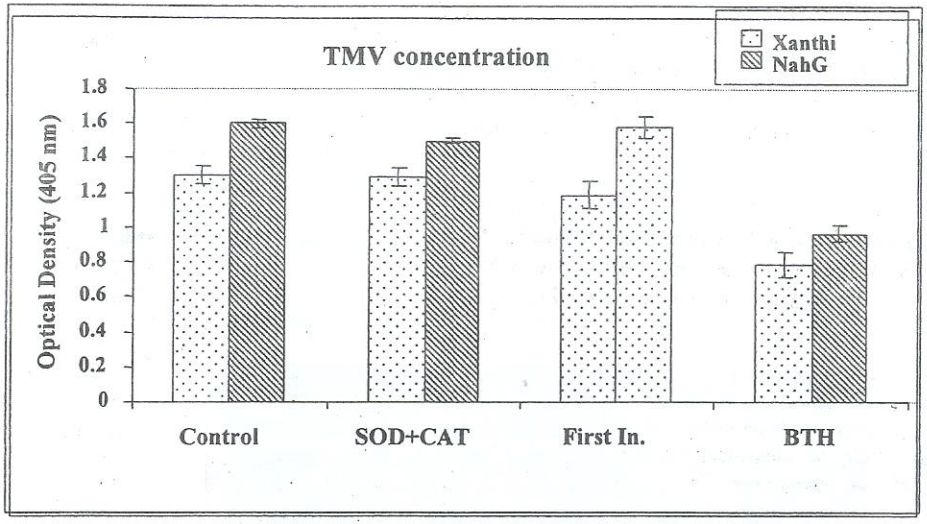

Fig. 8. Concentrations of tobacco mosaic virus (TMV) in the upper leaves extracts (10 time dilutions) in Xanthi-nc and NahG tobacco using ELISA test 12 hours after second inoculation. Control: upper leaves inoculated only with TMV. SOD+CAT: leaves inoculated with TMV and immediately injected with a mixture of superoxide dismutase and catalase (3000 and 5000 units /leaf, respectively) by a syringe (no SAR). Means of three independent experiments ± SD are shown.

Discussion

Systemic acquired resistance (SAR) was noticed in the upper tobacco Xanthi-nc leaves by inoculating the lower leaves (first inoculation) with TMV, however in tobacco NahG SAR was not induced because of salicylic acid hydroxylase gene which converted SA to catechol (Delaney *et al.*, 1994 and Mur *et al.*, 1997). BTH induced SAR in tobacco Xanthi-nc and even in NahG (no SAR induced by first inoculation) because BTH has activated the SAR signal transduction pathway downstream of SA accumulation (Friedrich *et al.*, 1996). In Xanthi-nc plants which can activate antioxidative defence, levels of O_2 and H_2O_2 was reduced when antioxidants was elevated. Levels of O_2 and H_2O_2 were not reduced in NahG because NahG tobacco plants can not activate antioxidative defence after TMV inoculation (Király *et al.*, 2002). NBT and DAB staining were more intensive in NahG tobacco, which cannot develop SAR and produce large necrotic spots (Gaffney *et al.*, 1993) however, this tissue necrotization was reduced when NahG tobacco leaves were treated with BTH. When tissue necrotization were reduced, it always accompany with reduced level of $O_2^{\cdot-}$ and H_2O_2. This suggests that stronger tissue necrotization is correlated with the higher levels of $O_2^{\cdot-}$ and H_2O_2. Similar results were obtained from detection of monodehydroascorbate radical (ROS) by paramagnetic resonance spectroscopy, which is a useful probe for oxidative stress (Fodor *et al.*, 2001).

BTH increased levels of O_2, H_2O_2 and total phenolic contents early after the inoculation with TMV either in Xanthi-nc or in NahG, this perhaps play important role for inhibiting virus multiplication. This result support the idea that when ROS applied exogenously at early time after inoculation, pathogens may be inhibited significantly (Hafez and Kiraly, 2003 and Hafez, 2005). The significant increasing of phenolics could also suppress the virus multiplication. The results are similar with those of Dao *et al.* (2009) who found that levels of phenolic metabolites were reduced significantly in BTH-treated *Arabidopsis*. The increased level of ROS (O_2^- and H_2O_2) has a dual role in resistance against the virus: firstly, virus inhibition by ROS, secondly, ROS as a mild stress could immunize the plants and induce the antioxidants enzymes (Bacso *et al.*, 2009). High levels of ROS early increased significantly the antioxidant enzymes 36 and 48 hours after the inoculation. Gechev *et al.* (2002) have shown that pre-treatment of tobacco plants by a low concentration of H_2O_2 (a mild oxidative stress), stimulates a few antioxidant enzymes and produces tolerant plant to abiotic stresses. Furthermore, Vandenabeele *et al.* (2003) demonstrated that this mild oxidative stress might induce expression of several genes associated with up-regulation of several signal transduction components and tolerance against abiotic stresses. Recently, it was shown that tobacco Xanthi-nc were immunized by a low concentration of H_2O_2 against TMV, *Pseudomonas syringae* and *Botrytis cinerea* (biotic stresses) accordingly, tissue necrotization were also suppressed (Hafez *et al.*, 2008; Bacso *et al.*, 2009). Induction of SAR by TMV or SA resulted in elevated levels of the antioxidants in Xanthi-nc tobacco (Fodor *et al.*, 1997). Activities of SOD, glutathione S-transferase, glutathione reductase and level of reduced glutathione were elevated in tobacco plants in which SAR was established by TMV inoculation (Fodor *et al.*, 1997). Systemic microoxidative burst triggers the activation of the antioxidative defence machinery in TMV-inoculated tobacco (Fodor *et al.*, 2001). Thus, accumulation of SA seems necessary for the subsequent activation of antioxidants. As a consequence of the large SA-dependent increase of antioxidant defence, there is a transient shift in the redox balance inside the tobacco leaves towards more reduced state. BTH increased significantly the activities of CAT and DHAR and expression of the genes *NtCAT1*, *NtSOD* and *NtDHAR* in the upper leaves of tobacco Xanthi-nc and NahG 36 and 48 hai.

Similar results were obtained when SAR was induced by first inoculation of TMV in the first leaves only in Xanthi-nc plant. Several studies indicated that SA and functional analogues like BTH and INA caused the ROS accumulation through the mitochondrial electron transport inhibition (Norman *et al.*, 2004) or antioxidant enzymes (Hafez, 2005). It was suggested that H_2O_2 induces SA accumulation which enhances the accumulation of H_2O_2 (Van Camp *et al.*, 1998). As a result of H_2O_2 and SA accumulation microbursts may generate intensify and spread the H_2O_2 signal which is required for oxidative cell death and establishment of SAR. It was also found an early burst of ROS and transient inhibition of antioxidant defence 1-2 days after inoculation of Xanthi-nc leaves with TMV (Fodor *et al.*, 1997and Fodor *et al.*, 2001), followed by a massive induction of antioxidants (Fodor *et al.*, 1997).

Obtained results also showed that enhancing antioxidative capacity reduced levels of ROS in Xanthi-nc tobacco which induced SAR 2 weeks earlier. This response did not occur in NahG tobacco, which cannot accumulate SA. Thus, it

would seem that the reduced level of ROS is SA-dependent. Evidently, plant cells overcompensate the initial oxidative stress caused by pathogen inoculation or chemical treatment with both enzymatic and non-enzymatic antioxidants that can scavenge ROS during a subsequent challenge inoculation. These results suggest that activation of antioxidant defence in leaves possessing SAR can successfully inhibit cell death. This hypothesis is supported by the findings that establishment of SAR enhanced resistance to tissue necrotization caused by heavy metal stress or paraquat which generates ROS (Strobel and Kuć, 1995). In this latter case, the SAR suppressed only the necrotic symptoms because pathogens were not included in this experiment. Furthermore, previous experiment in the laboratory has shown that SAR was effective in lessening the degree of necrotization caused by droplets of 0.1 % $HgCl_2$ (Goodman *et al.*, 1986). Infiltration of TMV-inoculated leaves of Xanthi-nc tobacco with a mixture of antioxidants (superoxide dismutase and catalase) strongly suppressed the necrotic lesion formation but did not affect pathogenic growth (Hafez, 2005). An early result showed that exogenously applied antioxidants inhibited the TMV-induced tissue necrotization in a local lesion tobacco (Solymosi *et al.*, 1959). It was also suggested that SAR was expressed by a reduction in necrotic symptoms caused by viruses in addition to a restriction of virus multiplication (Balázs *et al.*, 1977). Interestingly, BTH were able to inhibit the virus multiplication due to the elevated level of ROS and phenolics early after the inoculation. The results also suppose that in TMV-inoculated tobacco leaves possessing SAR necrotic lesions will be smaller due to the effect of elevated antioxidant defence. Another explanation suggests that SA inhibits multiplication and spread of TMV. In NahG tobacco, virus particles and TMV-induced necrotization often spread through the petiole to the stem and the adjacent leaves (Delaney *et al.*, 1994 and Mur *et al.*, 1997). Furthermore, disease symptoms, virus yield and virus spread decreased in SA-pretreated tobacco plants with and/or without the resistance gene *N* (Naylor *et al.*, 1998). These two explanations of the mechanisms of SAR could be reconciled if one considers that NahG plants expressing SA-hydroxylase have very low levels of SA, while exogenous application of SA results in a very high concentration of SA in plant tissues. One can suppose that TMV multiplication is not influenced significantly in the upper leaves by a relatively low increase in SA after induction of SAR by TMV (Malamy *et al.*, 1990), while extremely high concentration of SA has important influence on virus spread and multiplication.

Conclusion

Applying BTH, a synthetic analogue of SA to clarify the effect and mechanisms of this chemical inducer of resistance on the levels of O_2^- and H_2O_2. BTH is able to induce SAR in both wild-type Xanthi-nc and transgenic NahG plants (Friedrich *et al.*, 1996).

Evidently, BTH treatment inhibited the lesion size of both wild-type Xanthi-nc and NahG tobacco leaves upon inoculation with TMV when applied as a foliar spray. BTH increased the ROS level and phenolics early, as a result induction of the

antioxidants were occurred later and therefore tissue necrotization were suppressed. Furthermore, pre-treatment with BTH inhibited accumulation of O_2^- and H_2O_2. Thus, our results support the hypothesis that lower number and size of tissue necrotization is due to the lower accumulation of ROS and elevated level of the antioxidants in TMV-inoculated tobacco leaves possessing SAR.

We recommend applying BTH, mild stresses and low concentration of ROS such as H_2O_2 which can be used in the practical field and safety use in the organic food production to immunize and protect plants against viral, bacterial and fungal pathogens.

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استحثاث المقاومة الجهازية المكتسبة فى نباتات الدخان لفيروس موزايك أوراق الدخان بواسطة العدوى الموضعية والبنزوثياديازول

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أدت عدوى الأوراق السفلى لصنفى نباتات الدخان (*Nicotiana tabacum* L., cv. Xanthi-nc and cv. NahG) بفيروس موزايك الدخان (TMV) إلى استحثاث المقاومة الجهازية المكتسبة (SAR) فى الأوراق العليا البعيدة عندما تصاب بالفيروس (العدوى الثانية). ونتيجة استحثاث المقاومة الجهازية المُكتسبة فى الأوراق العليا للصنف سانتى (Xanthi-nc) البعيدة أصبحت أكثر مقاومة حيث تكون فقط عدد قليل من النيكروزس و ذلك نتيجة لإنخفاض مستوى مشتقات الأكسجين الحرة (ROS) مثل السوبرأكسيد (O_2^-) و فوق أكسيد الهيدروجين (H_2O_2).

أما فى صنف الناخ جى (NahG) غير القادر على استحثاث المقاومة (SAR) فإن مستوى مشتقات الأكسجين الحرة إرتفع إرتفاعاً معنوياً فى الأوراق العليا وزادت عدد و حجم النيكروزس عندما أعديت الأوراق بالعدوى الثانية. استحثاث المقاومة (SAR) بالعدوى الأولية فى الصنف سانتى أدى إلى إرتفاع معنوى فى نشاط إنزيمات الكتاليز(CAT) و الديهيدروأسكوربيت رداكتيز(DHAR) و باستخدام تقنية البيولوجيا الجزيئية (RT-PCR) وجد أيضاً إرتفاع فى مستوى التعبير الجيني لنفس الإنزيمات السالفة الذكربالإضافة للسوبر أكسيد ديسميوتيز (SOD) وهنا الإرتفاع فى مضادات الأكسدة لم يلاحظ فى صنف الناخ جى لأنه غير قادر على استحثاث المقاومة.

أدت معاملة صنفى الدخان سانتى و ناخ جى بمركب البنزوثياديازول (BTH) إلى إرتفاع مستوى مشتقات الأكسجين الحرة ومركبات الفينولات إرتفاعاً معنوياً فى الأوراق العليا فى وقت مبكر ٦ و١٢ و٢٤ ساعة بعد العدوى بالفيروس أعقب ذلك مباشرة إنخفاض متدرج و معنوى فى مستوى مشتقات الأكسجين الحرة ومركبات الفينولات بعد ٣٦ و ٤٨ ساعة من العدوى للأوراق العليا وكان هذا الإنخفاض مصحوباً بإرتفاع معنوى فى نشاط إنزيمات CAT وDHAR وأيضاً إرتفاع فى مستوى التعبير الجينى للإنزيمات السابقة بالإضافة لإنزيم الـ SOD.

لذلك فإن تثبيط الفيروس وقلة عدد وحجم النيكروزس مرتبط بمستوى مشتقات الأكسجين الحرة ومركبات الفينولات وأن مشتقات الأكسجين الحرة و مضادات الأكسدة تلعب دور هام و حيوى فى استحثاث أو تثبيط تكون النيكروزس فى الخلايا المصابة بالفيروس.

و الجديد فى البحث هو أن الـ (BTH) استحثت مشتقات الأكسجين الحرة ومركبات الفينولات مبكراً بعد العدوى مما أدى إلى تثبيط تضاعف الفيروس وهذا بدوره يعتبر محفزاً ومثيراً فى زيادة مناعة النباتات بإنتاج وزيادة مستوى و نشاط مضادات الأكسدة. وهذه النتيجة تدعم تطبيق إستعمال بعض مشتقات الأكسجين الحرة مثل فوق أكسيد الهيدروجين(H_2O_2) بتركيز منخفض لزيادة مناعة النباتات ضد المسببات المرضية.

إجمالاً يمكن القول بأن مركب الـ (BTH) له دور محورى فى المقاومة المستحثة حتى فى الأصناف التى لم يمكن إستحثاث المقاومة فيها مثل الصنف ناخ جى الذي يحتوى على جين يحلل حمض الساليسليك لذلك نوصى بالتطبيق المعملى و الحقلى بإستعمال ال BTH وH_2O_2 لزيادة مناعة ومقاومة ووقاية النباتات من الأمراض الفيروسية.