

# Nitric Oxide Treatment and Induced Genes Role Against *Phytophthora infestans* in Potato

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**Abstract** Effects of sodium nitroprusside (SNP; nitric oxide donor) treatments on enhancement of secondary metabolites production, oxidative stress mediators ( $O_2^-$ ) accumulation and antioxidant defense enzymes of Potato Spunta Sp. suspension culture cells elicited by a fungal extract from *phytophthora infestans* mycelium. The obtained data confirmed the significant increase in various oxidative burst (super oxide anion, hydrogen peroxide and total glutathione) contents. The administration of various NO concentrations strongly decreased hydrogen peroxide concentration and superoxide anion levels. Moreover, the SNP treatments regulate elicitor-induced activation of phenylalanine ammonium-lyase and total soluble phenols accumulation. The highest concentrations of NO donor sodium nitroprusside potentiated elicitor-induced  $H_2O_2$  production. On the other hand, the lowest  $H_2O_2$  contents coincided with elicitation regulated various activities of enzymes superoxide-dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and Phenyl alanine ammonia lyase (PAL) activity, the decrease in  $H_2O_2$  concentration was probably due to a direct reduction interaction of NO- $H_2O_2$ . On the other hand, the addition of these previous NO treatments affects mRNA peroxidase gene expression using RT-PCR techniques. In general, the addition of lower concentrations of nitric oxide reduce the mRNA peroxidase activity on contrary, the higher concentrations induced the

mRNA peroxidase activity, which induce the hypersensitive reactions against fungus infection.

**Keywords** Fungal elicitor · Nitric oxide · Oxidative burst · *Potato Spunta Sp* · Signal pathway

## Stickoxidbehandlung und die Rolle induzierter Gene gegen *Phytophthora infestans* bei Kartoffeln

**Zusammenfassung** Auswirkungen von Behandlungen mit Natrium-Nitroprussid (SNP, Stickoxiddonor) auf die Steigerung der Produktion von Sekundärmetaboliten, die Anreicherung von Mediatoren oxidativen Stresses ( $O_2^-$ ) und antioxidative Abwehrenzyme in Suspensionskulturzellen der Kartoffelsorte *Spunta Sp.*, hervorgerufen durch ein Pilzextrakt aus Myzel der Art *Phytophthora infestans*. Die erzielten Daten bestätigten die signifikante Steigerung verschiedener oxidativer Bursts (Superoxidanion, Wasserstoffperoxid und Gesamtgehalt Glutathion). Die Verabreichung unterschiedlicher NO-Konzentrationen senkte die Wasserstoffperoxidkonzentration und die Superoxidanionwerte stark. Außerdem regulieren die SNP-Behandlungen die Elicitor-induzierte Aktivierung von Phenylalanin-Ammoniak-Lyase sowie die Anreicherung der Gesamtmenge löslicher Phenole. Die höchsten Konzentrationen NO-Donor-Natriumnitroprussid potenzierten die Elicitor-induzierte  $H_2O_2$ -Produktion. Andererseits fallen die geringsten  $H_2O_2$ -Werte mit verschiedenen Elicitor-regulierten Aktivitäten der Enzyme Superoxiddismutase (SOD), Ascorbatperoxidase (APX), Katalase (CAT) und Phenylalanin-Ammoniak-Lyase (PAL) zusammen. Der Rückgang in der  $H_2O_2$ -Konzentration lag wahrscheinlich an der direkten Reduktionsinteraktion mit NO- $H_2O_2$ . Die Hinzufügung dieser o. g. NO-Behandlungen wirkt sich allerdings auch auf die mRNA-

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Peroxidase-Genexpression mit RT-PCR-Techniken aus. Allgemein senkt die Hinzufügung geringerer Konzentrationen Stickoxid die mRNA-Peroxidaseaktivität. Die höheren Konzentrationen induzierten aber die mRNA-Peroxidaseaktivität, was wiederum Überempfindlichkeitsreaktionen gegen die Pilzinfektion induziert.

**Schlüsselwörter** Pilzelicitor · Stickoxid · Oxidativer Burst · Kartoffel *Spunta Sp* · Signalweg

## Introduction

Plants respond to pathogen attack or elicitor treatment by activating a wide variety of protective mechanisms designed to prevent pathogen replication and spreading (Małolepsza and Różalska 2005; Afify et al. 2011a, 2011b). Production of reactive oxygen species (ROS) such as superoxide ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) during so called “oxidative burst” has been considered a central event in activation of disease resistance (Jabs et al. 1997; El-Beltagi et al. 2011, 2012; El-Beltagi and Mohamed 2013) and strengthening of plant cell walls, as well as in triggering hypersensitive cell death and in production of systemic resistance signaling (Vanacker et al. 2000; Hüchelhoven et al. 2003). Nitric oxide has been identified as an essential molecule that mediates hypersensitive cell death and defense gene activation. Nitrosogluthathione is suggested to act as a long-distance phloematic signal in systemic acquired resistance (SAR);  $NO\cdot$  is indispensable to salicylic acid (SA) functioning as SAR inducer (Yu et al. 2001; Song and Goodman 2001). Nitric oxide as a bioactive molecule shows prooxidant as well as antioxidant properties in plants (Delledonne et al. 2002; El-Beltagi et al. 2015). The biosynthetic origin of NO during plant pathogen interactions involves L-arginine conversion into L-citrulline. It has been well established that the elicitor-induced secondary metabolite synthesis of plants requires endogenous signal components such as jasmonic acid (JA) and reactive oxygen species (ROS) (Capaldi and Taylor 1983; Clark et al. 2000). Nitric oxide induces a complementary set of plant defense genes, including two key enzymes of the phenylpropanoid pathway, namely Phenylalanine ammonia lyase (PAL) and chalcone synthase. Furthermore, NO-treated tobacco (*Nicotiana tabacum*) cells were shown to induce the pathogenesis-related protein 1 (PR-1) together with an accumulation of cyclic acid, a key molecule for the expression of systemic acquired resistance (Durner et al. 1998; Crawford and Guo 2005).

The free radical  $NO\cdot$  has a half-life of just a few seconds and can also react with the free radical superoxide  $O_2^-$  to form the reactive molecule peroxyxynitrite ( $ONOO^-$ ), which can lead to the formation of  $NO_2$  and the potent oxidant hydroxyl radical (OH). OH is very strong oxidizing specie

that can rapidly attack biological membranes and all types of bio-molecules such as DNA and proteins leading to irreparable damage, metabolic dysfunction, and cell death (Del-Rio et al. 2003). By itself,  $ONOO^-$  is responsible for tyrosine nitration and oxidation of thiol (Tamir et al. 1993; Lamattina et al. 2003). In plants, the accumulation of NO and  $H_2O_2$  during the hypersensitive disease resistance response (HR) is responsible for the execution of the cell death program (Delledonne et al. 1998; Clarke et al. 2000; and De-Pinto et al. 2002). Among these mechanisms, nitric oxide ( $NO\cdot$ ) is an essential signal for the development of resistance to the invading pathogen. Many defense responses are stimulated by  $NO\cdot$  and the levels of this free radical increase following pathogen attack (Delledonne et al. 1998; Aziz et al. 2004; Kobeasy et al. 2011). Although significant advances have been achieved in understanding the molecular bases of plant pathogen interactions, the mechanism is still not completely clear. This study aims to characterize the role of nitric oxide ( $NO\cdot$ ) treatment in Potato *Spunta Sp* resistance against *Phytophthora infestans*.

## Materials and Methods

### Plant Material and Cell Culture

The plant cell line for the study was initiated from callus induced from the young stems of potato (*Solanum tuberosum*) plants (*Spunta* cultivar). The callus was initiated in a MS-Medium supplemented with 4.0 mg/l of Naphthaleneacetic acid (NAA), 4.0 mg/l of kinetin, 20 g/l sucrose and 8 g/l agar. The callus line had been in culture for 5 months by the time of this study. The suspension culture of the cell line was initiated on a liquid medium similar to that for the callus culture but with 30 g/l sucrose and excluding the agar. The medium was adjusted to pH 5.8 and then sterilized at 121 °C for 20 min.

### Preparation of Mycelium Plugs

*Phytophthora infestans* were isolated from diseased specimens that were collected from diseased fields in Gharbia, Beheira and Kafr elshaikh governorates. Mycelium plugs were prepared from actively growing *Phytophthora infestans* cultures.

### Elicitor Preparation

The fungal elicitor was extracted from the mycelium of a pathogenic fungus, *Phytophthora infestans* as described by Yu et al. (2001). The total carbohydrate content of the elicitor solution was determined by the phenol-sulfuric acid

method using glucose as a standard (Ferrer and Ros-Barcelo 1999).

### Experimental Design and Cell Culture Treatment

Potato cells, 6-day-old cell treated cultures. All parameters and enzymes activity have been monitored through a time course after 0, 6, 12, 48 and 72 h post elicitation and NO donors sodium nitroprusside (SNP) (50, 100, 250  $\mu$ M) treatments. The control received only the elicitor (100  $\mu$ g/ml medium). Potato cell suspension cultures were treated with fungal elicitor (100  $\mu$ g/ml) and various SNP concentrations after 6 days of subculture and after elicitation cells were harvested at time intervals.

Determination of oxidative burst and total glutathione concentration.

### The Production Rate of Superoxide Anion ( $O_2^-$ )

The production rate of superoxide anion ( $O_2^-$ ) was measured by the modified method described by Elstner and Heupel (1976). Fresh mass (200 mg) from culture was homogenized in 1 ml of 50 mM phosphate buffer (pH 7.8) and the homogenate was centrifuged at 10,000 g for 10 min. Then 0.5 ml of the supernatant was added to 0.5 ml 50 mM phosphate buffer (pH 7.8) and 0.1 ml of 10 mM hydroxylamine hydrochloride. After 1 h reaction at 25  $^{\circ}$ C, 1 ml of 17 mM sulfanilamide and 1 ml 7 mM  $\alpha$ -naphthylamine were added to the mixture at 25  $^{\circ}$ C, and after 20 min, the specific absorbance at 530 nm was determined. Sodium nitrite was used as standard solution to calculate the production rate of  $O_2^-$ .

### Assay of Hydrogen Peroxide Concentration

Hydrogen peroxide was measured by the method described by Capaldi and Taylor (1983) with a slight modification. The ground callus in 5% TCA (2.5 ml per 0.5 g callus) with 50 mg active charcoal at 0  $^{\circ}$ C, and centrifuged for 10 min at 15,000  $\times$  g. Supernatant was collected, neutralized with 4 N KOH to pH 3.6 and used for  $H_2O_2$  assay. The reaction mixture contained 200 ml of leaf extract and 100 ml of 3.4 mM 3-methylbenzothiazoline hydrazine (MBTH). The reaction was initiated by adding 500 ml of horseradish peroxidase solution (90 U per 100 ml) in 0.2 M sodium acetate (pH 3.6). Two minutes later 1400 ml of 1 N HCl was added. Absorbance was read at 630 nm after 15 min.

### Determination of Reduced Glutathione

Samples (0.5 g) were added to 2 ml ice-cold 5% (w/v) sulphosalicylic acid solution. The mixture was centrifuged for 30 min at 10,000  $\times$  g, then the supernatants were

collected and immediately assayed. Glutathione was measured with Ellman's reagent (Silber et al. 1992). 300 ml of the supernatant was mixed with 1.2 ml of 0.1 M phosphate buffer solution (pH 7.6). After a stable absorbance reading of 412 nm was obtained, 25 mM 5, 50-dithio-bis (2-nitrobenzoic acid) (DTNB) was added and the increase in absorbance at 412 nm was monitored ( $\Sigma$  412 = 13.6  $mM^{-1}cm^{-1}$ ).

### Determination of Total Soluble Protein

Soluble proteins were measured by the Bio-Rad micro assay modification of the Bradford (1976) procedure using crystalline bovine serum albumin as a reference.

### Determination of Antioxidant Enzymes Activity

#### Preparation of Enzyme Extracts

Samples of 0.25 g was homogenized in 5 ml of 50 mM phosphate buffer pH 7.0 containing 1.0 N NaCl, 1% PVP (Sigma) M.W. 40,000 1mM ascorbate (Sigma) at 4  $^{\circ}$ C. After centrifugation at 15,000  $\times$  g for 15 min the supernatant was collected.

#### Assay of Superoxide Dismutase (SOD) Activity

Superoxide dismutase activity (SOD; EC 1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of NBT using the method of Beauchamp and Fridovich (1971). The 3 ml reaction mixture contained 50 mM phosphate buffer pH 7.8, 13 mM methionine, 75 mM NBT, 2 mM riboflavin, 1.0 mM EDTA and 20 ml enzyme extract. Riboflavin was added last and the reaction was initiated by placing the tubes 30 cm below 15W fluorescent lamps. The reaction was started by switching on the light and was allowed to run for 10 min. Switching off the light stopped the reaction and the tubes were covered with black cloth. Non-illuminated tubes served as control. The absorbance at 560 nm was recorded. One unit of SOD was defined as that being contained in the volume of extract that caused 50% inhibition of the SOD-inhibitable fraction of NBT reduction.

#### Assay of Catalase (CAT) Activity

Catalase activity (CAT; EC 1.11.1.6) was determined by consumption of  $H_2O_2$  using the method of Aebi (1983). The reaction mixture (3 ml) contained 50 mM potassium phosphate buffer pH 7.0, 15 mM  $H_2O_2$  and 50 ml enzyme extract. The reaction was initiated by adding the  $H_2O_2$ . The consumption of  $H_2O_2$  was monitored spectrophotometrically at

240 nm ( $\epsilon = 39.4 \text{ mM}^{-1} \text{ cm}^{-1}$ ) for 3 min. Enzyme activity was expressed in  $\text{mM H}_2\text{O}_2 \text{ min}^{-1}$ .

#### *Assay of Ascorbate Peroxidase (APX) Activity*

Ascorbate peroxidase activity (APX, EC 1.11.1.11) was determined spectrophotometrically by a decrease in the absorbance at 265 nm ( $\epsilon = 13.7 \text{ mM}^{-1} \text{ cm}^{-1}$ ) using the method of Nakano and Asada (1981). The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 5 mM ascorbate, 0.5 mM  $\text{H}_2\text{O}_2$  and enzyme extract. Addition of started the reaction. The rates were corrected for non-enzymatic oxidation of ascorbate by the inclusion of reaction mixture without enzyme extract.

#### **Determination of Secondary Products Based Defense Compounds**

##### *Assay of Phenylalanine Ammonia Lyase (PAL)*

Phenylalanine ammonia-lyase activity (PAL; E.C. 4.3.1.5) was determined based on the rate of cinnamic acid production as described by Ochoa-Alejo and Gomez-Peralta (1993). Briefly, 1 ml of 50 mM Tris-HCl buffer pH 8.8 containing 15 mM of b-mercaptoethanol, 0.5 ml of 10 mM o-salicylic acid, 0.4 ml of double distilled water and 0.1 ml of enzyme extract was incubated at 37 °C for 1 h. The reaction was terminated by addition of 0.5 ml of 6 M HCl and the product was extracted with 15 ml ethyl acetate, followed by evaporation to remove the extracting solvent. The solid residue was suspended in 3 ml of 0.05 M NaOH and the cinnamic acid concentration wherein was quantified with the absorbance 290 nm. One unit of PAL activity is equal to 1 mmol of cinnamic acid produced per min.

##### *Determination of Total Phenols*

The phenolic assay was conducted as per the method of Zieslin and Ben-Zaken (1993). The samples were homogenized at the rate of 0.1 g per 1 ml of 80% methanol and the methanolic extract was kept in a water bath at 70 °C for 15 min with frequent agitation. One ml of methanolic extract was added to 5 ml of distilled water and 250  $\mu\text{l}$  of Folin-Ciocalteu reagent (1 N) was added and the solution was kept at 25 °C for 30 min. Finally, 1 ml of saturated solution of  $\text{Na}_2\text{CO}_3$  and 1 ml of distilled water were added and the reaction mixture was incubated for 1 h at 25 °C. After the blue color development, the absorbance was recorded at 725 nm. Total phenols were determined with the use of an external standard curve and expressed as mg gallic acid/g fresh weight of callus tissues.

#### **RT-PCR Technique of the Selected and Characterized Gene Sequences**

##### *Primers Design*

For the purpose of gene expression study using RT-PCR, four sets of primers were designed for the following selected and characterized gene sequences using DNA star software and Assessment of RNA quality: The integrity of total RNA was assessed by according to the method described by Sambrook et al. (1989).

#### **Monitoring Peroxidase Activity mRNA Gene Expression Using RT-PCR Technique**

##### *Sequences of the PCR Primers*

##### **Peroxidase**

- POX 1: 5' AAG GCT GTG CAG TAT GTG A 3' (19-mer)
- POX 2: 5' AGT GGA GCT TGT TGG AGA G 3' (19-mer)

Size produced after PCR amplification is 277 bp.

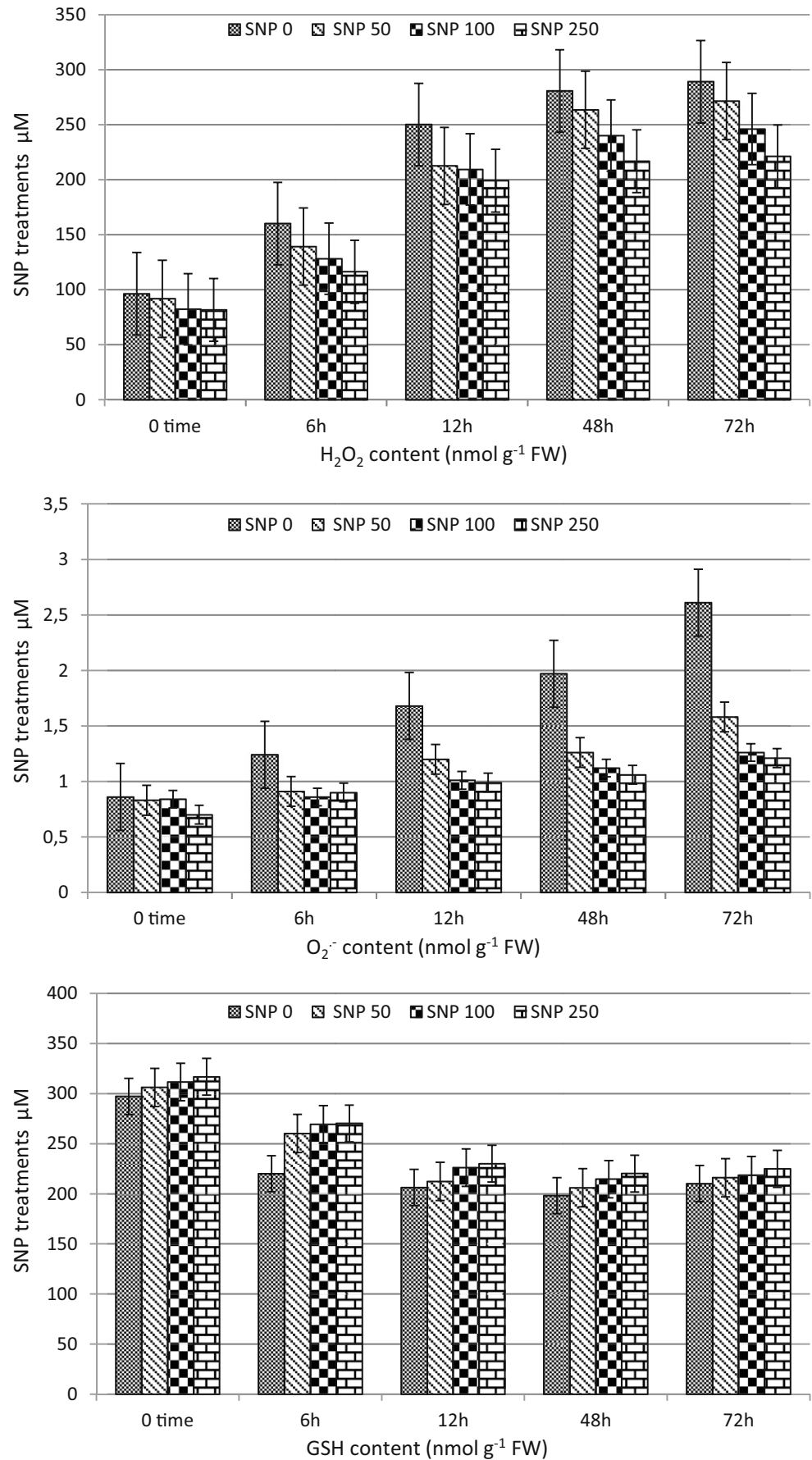
For reverse transcription, 1  $\mu\text{g}$  of total RNA was heated at 65 °C for 5 min. Reverse transcription was then conducted at 37 °C for 1 h. The reaction mixture was composed of 40 units of RNase inhibitor, 0.5 mmol/l of each dNTP, 0.1  $\mu\text{g}$  oligo (dT) 16 primer, reverse transcription buffer, and 3 units of AMV reverse transcriptase. The reverse transcriptase was inactivated by heating to 95 °C for 2 min.

PCR reaction mixtures were prepared on ice; mixtures contained cDNA equivalent to 0.1  $\mu\text{g}$  total RNA, 50 pmol of each primer, 200  $\mu\text{mol/l}$  dNTP, Taq plus 10 $\times$  buffer, and 5 U of Taq plus polymerase. PCR was performed for 25 cycles. The reaction conditions were 94 °C for 1 min, 56.7 °C for 1 min, and 72 °C for 1 min (Ng et al. 2005). The sequences of the PCR primers are shown the semi-quantitative RT-PCR (El-Assal et al. 2004) are presented as a ratio of the integrated optical density of the gene of interest to the integrated optical density of the gene expression (zero time treatments) (Murphy et al. 1990).

##### **Statistical Analysis**

All statistical analyses were carried out using SPSS 10 software. Mean and standard error were descriptive measures of quantitative data using the analysis of variance test (ANOVA) for independent samples. All determinations have been done in triplicate,  $p$ -Values <0.05 were considered significant.

**Fig. 1** Effect of SNP treatments (0, 50, 100 and 250  $\mu\text{M}$ ) on ( $\text{H}_2\text{O}_2$ ,  $\text{O}_2^-$ , GSH) contents elicited suspension culture. Values are expressed as means  $\pm$  SEM ( $n = 3$ )



## Results and Discussion

### Oxidative Burst ( $\text{H}_2\text{O}_2$ , Superoxide Anion and Total GSH Content)

The data presented in (Fig. 1) show that, the elicitation promote oxidative stress and ROS accumulation and the  $\text{H}_2\text{O}_2$  contents ( $\text{nmol g}^{-1}$  FW), there was a positive correlation between the period after elicitation and hydrogen peroxide accumulation. On the other hand, various NO treatments significantly decreased the  $\text{H}_2\text{O}_2$  contents as a direct scavenging activity through oxidative reduction reaction. It has been determined that although wounding does not induce the generation of NO, treatment with NO donors inhibited  $\text{H}_2\text{O}_2$  generation after wounding, and expression of specific wound-induced genes (Delledonne et al. 1998; Cárdenas and Ryan 2002). These results propose that NO generated during pathogenesis might inhibit  $\text{H}_2\text{O}_2$  synthesis and the activation of specific wound-induced signalling pathways (Palavan-Unsal and Arisan 2009).

As shown in (Fig. 1) showed that, the elicitation increased the  $\text{O}_2^-$  in a positive correlation with the time duration, on the other hand, all NO treatments significantly reduce the  $\text{O}_2^-$  contents, and this may be due to its direct reaction with NO to form the peroxy-nirile compounds. Nitric oxide is a highly reactive molecule and the fact of being a free radical allows it to scavenge other reactive intermediates and end chain-propagated reactions. The rapid reaction between  $\text{O}_2^-$  and NO to form the powerful oxidant peroxynitrite ( $\text{ONOO}^-$ ), has been suggested as a deleterious mechanism (Huie and Padmaja 1993). It was also reported, however, that in systems where the toxicity comes predominantly from peroxides, these compounds are much more toxic than (NO and  $\text{ONOO}^-$ ), making NO protective against them (Wink et al. 1993). The treatment with sodium nitroprusside of *P. communis* suspension cultures exposed to stressful action of polyethylene glycol PEG 6000 was accompanied by deceleration of ion leakage, lowering of  $\text{H}_2\text{O}_2$  and superoxide anion  $\text{O}_2^-$  content, and by activation of antioxidant defense enzymes (Zhao et al. 2008).

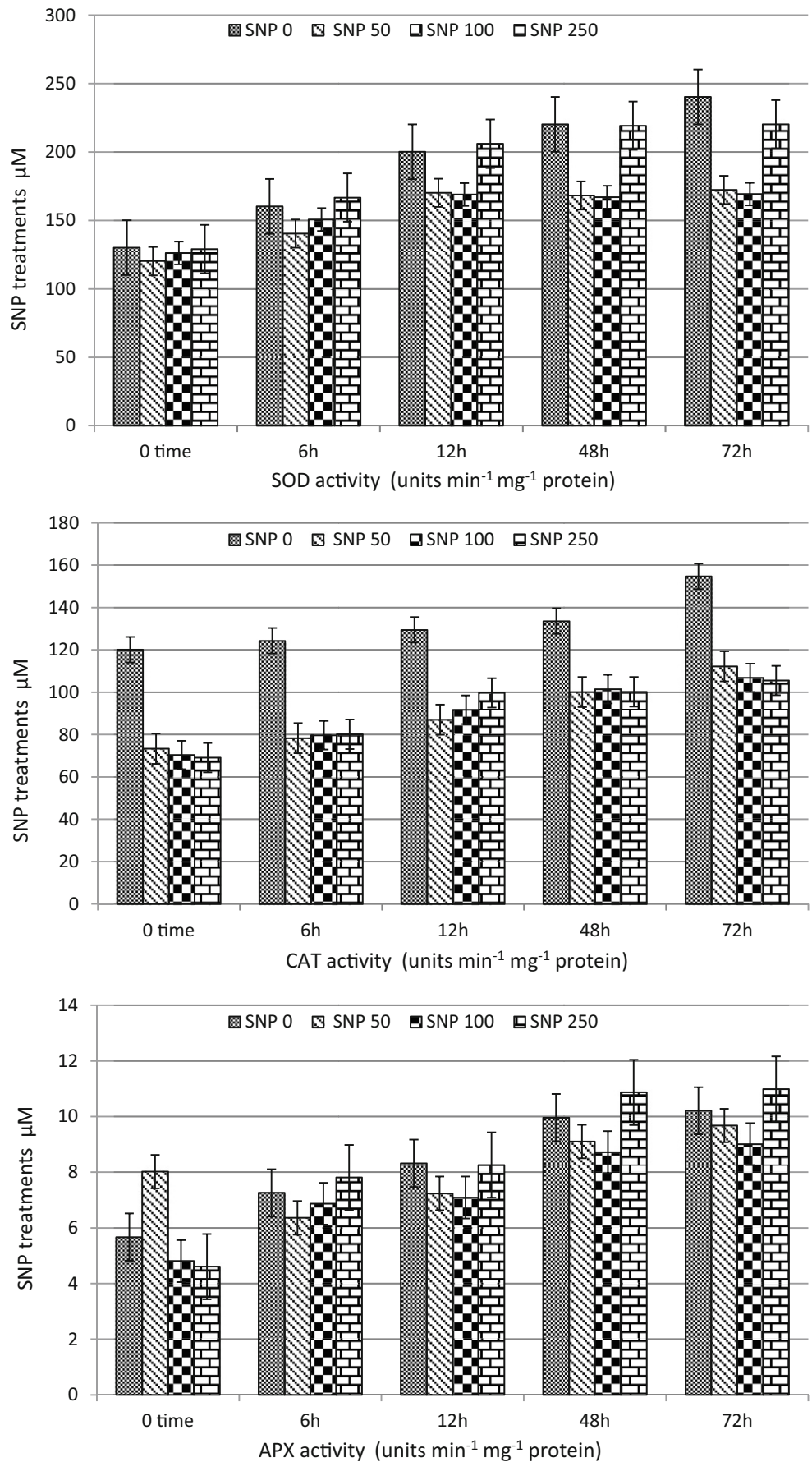
Data in (Fig. 1) show that, the GSH contents decreased by the time duration after elicitation due to the high oxidation stress induced by elicitation process. On the other hand, the addition of NO donors reduced the oxidative burst and in sequence increased the GSH contents compared with the control. The cytoprotective effect of NO was reported to be linked to the NO-dependent regulation of the redox state and the controlling of ROS generation (Arasimowicz-Jelonek et al. 2014a, 2014b). During the incompatible response of potato an enhanced accumulation of ascorbate and total thiol content was observed early after pathogen recognition. This effect was triggered only in *avr P. infestans*-inoculated leaves, indicating fast and precisely con-

trolled redox state re-adjustment. Markedly increased total antioxidative capacity recorded after inoculation with *avr P. infestans* was a symptom of the shifted redox potential to more reductive conditions. Interestingly, *Arabidopsis pad2-1* mutants, with an insufficient GSH supply, were unable to activate defense against the biotrophic *Phytophthora brassicae* or bacterial pathogen *Pseudomonas syringae* (Dubreuil-Maurizi et al. 2011).

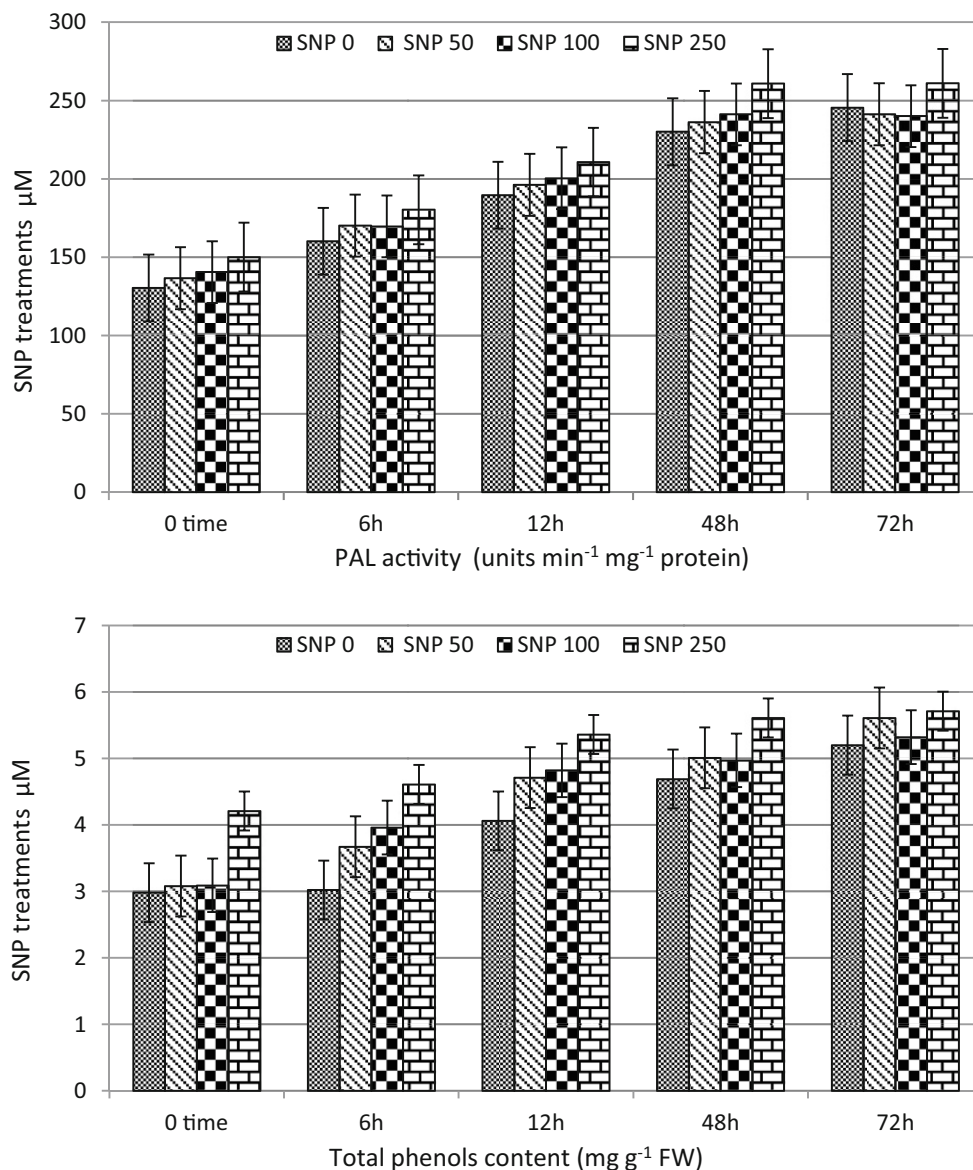
### Antioxidant Enzymes Activity

Fungus elicitation and NO treatments increased the accumulation of defense system related enzymes such as SOD, CAT, APX and PAL then increased the accumulation of phenols, secondary metabolites. So increase in APX and PAL activity might have frequently enhanced the phenol content in challenge inoculated plant cells. Plant responds to elicitation by activating a wide variety of protective mechanisms to prevent pathogen spreading. It has been reported that Fungus elicitation and NO treatments caused unusually strong induction of antioxidative enzymes results in detoxification of ROS and plays a protective role in the interaction between plants and fungi (Alguacil et al. 2003). Increased activity of antioxidant enzymes minimizes the chances of oxidative burst (excessive ROS production), and therefore potato cells might be protected from the oxidative defense system. The relation between NO and ROS in plant disease resistance reaction is very critical according to the amounts and the balance ration. The effect of SNP treatments (0, 50, 100 and 250  $\mu\text{M}$ ) on SOD, CAT, APX activities was determined in elicited suspension culture. As shown in Fig. 2, the SOD activity increased after elicitation by fungus elicitor (100  $\mu\text{g/ml}$ ). On the other hand, the application with 50, 100  $\mu\text{M}$  of SNP reduced the SOD activity compared with control (treated cells only) without SNP but, the highest concentrations of SNP treatment (250  $\mu\text{M}$ ) the SOD activity realized the highest activity. In addition, the elicitation process and SNP treatments trigger a specific activation of APX activity that interfered with the process of hypersensitive reactions against infection ex, lignifications and antibacterial toxic compounds formation during and after the elicitation process. Data in (Fig. 2) revealed that, the promotion of elicitation process on  $\text{H}_2\text{O}_2$  accumulation and CAT activity. On the other hand, the highly protective activity of SNP treatments specially the lowest ones against oxidative burst ( $\text{H}_2\text{O}_2$ ) promoted through elicitation process that reduce the catalase activity compared with the elicited control alone. Suppression of CAT activities and induction of APX and SOD activity may induce the accumulation of a higher level of  $\text{H}_2\text{O}_2$ . A higher concentration of  $\text{H}_2\text{O}_2$  has also been observed in *P. indica*-colonized wheat plants after infection with *B. graminis* f. sp. *tritici* and in maize plants interacting with both *Piriformospora indica* and Fusarium

**Fig. 2** Effect of SNP treatments (0, 50, 100 and 250  $\mu\text{M}$ ) on (SOD, CAT, APX) activities (units  $\text{min}^{-1} \text{mg}^{-1}$  protein) elicited suspension culture. Values are expressed as means  $\pm$  SEM ( $n = 3$ )



**Fig. 3** Effect of SNP treatments (0, 50, 100 and 250  $\mu\text{M}$ ) on PAL activity and total phenols content elicited suspension culture. Values are expressed as means  $\pm$  SEM ( $n = 3$ )



verticillioides (Serfling et al. 2007; Kumar et al. 2009), which supports our hypothesis of accumulation of  $\text{H}_2\text{O}_2$ . These observations suggest the elicitation process and SNP treatments caused a significant alteration in the level of antioxidant enzymes, which probably favours the plant-fungal association and increases resistance to the pathogen. It has been observed in many other plant species that NO stimulates antioxidant enzymes. The inducible effect of the NO donor on the activity of SOD, CAT, and APX was observed in rice and bean plants under drought stress and *Rhizoctonia solani* infection (Shehab et al. 2010; Keshavarz-Tohid et al. 2016). The NO donor increased SOD activity in rice under osmotic stress (Cheng et al. 2002) and increased the activities of SOD and CAT in wheat under oxidized stress by paraquat treatment and heat stress (Hung et al. 2002; El-Beltagi et al. 2016). Also, it is highly possible that the

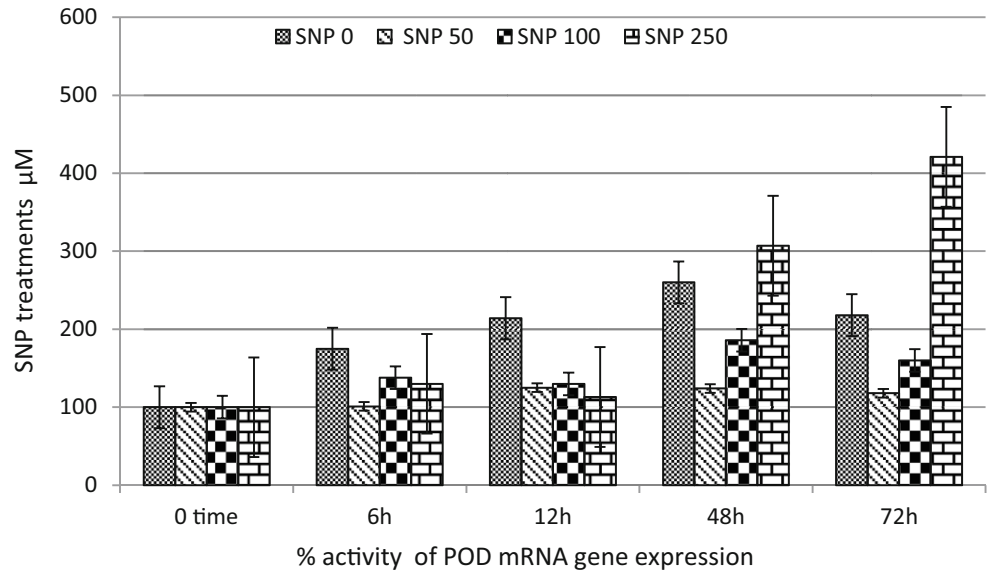
protective effect of NO may result from increased expression of genes encoding active oxygen scavenging enzymes under fungus infection (Mackerness et al. 2001; Qiao and Fan 2008). Collectively, these data highlighted the involvement of NO burst in the induction of redox changes in *P. infestans*-treated potato cells (Abramowski et al. 2015).

### PAL Activity and Total Phenols Content

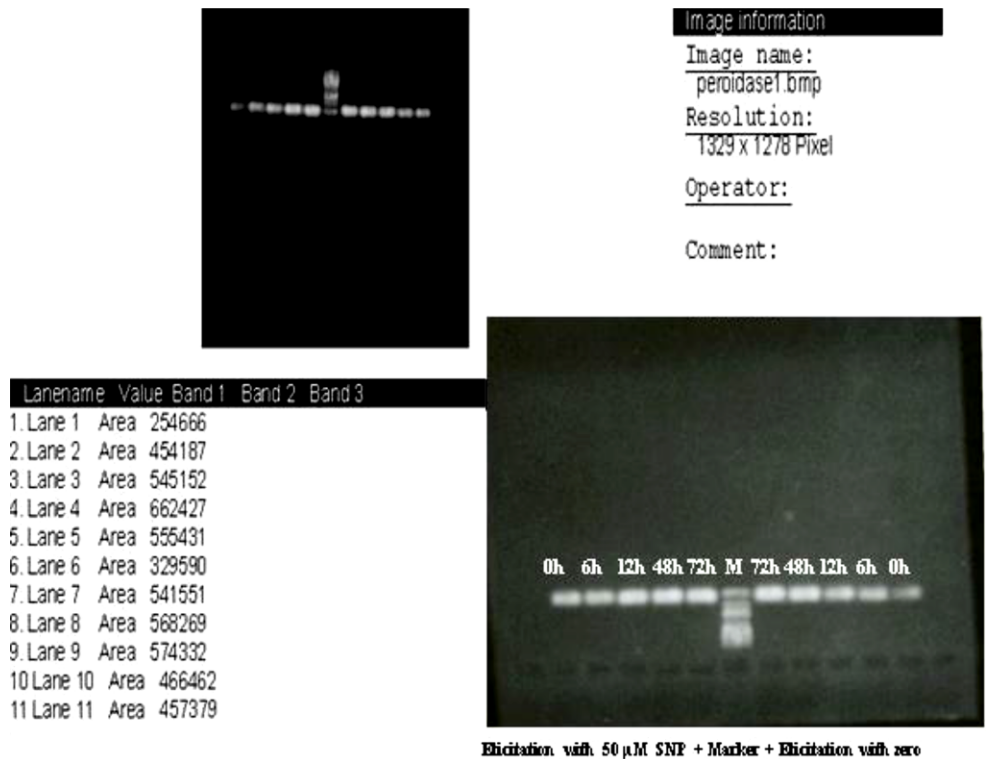
These data in (Fig. 3) revealed that, the highly increase in PAL activity (units/mg protein<sup>-1</sup>) by elicitation which triggered by both NO administration and elicitation process. In addition, there was a positive correlation between NO contents and elicitation and PAL activity. In accordance to Goodman et al. (1967) who found that the increase in APX and PAL activities might have frequently enhanced



**Fig. 4** Effect of SNP treatments (0, 50, 100 and 250  $\mu\text{M}$ ) on relative percentage of integrated optical density of peroxidase mRNA gene expression. Values are expressed as means  $\pm$  SEM ( $n = 3$ )



**Fig. 5** Effect of different SNP doses administration (0, 50  $\mu\text{M}$ ) on integrated optical density of peroxidase activity mRNA gene expression. (The integrated optical densities of peroxidase PCR products were used to calculate the ratio of peroxidase mRNA to the zero time control peroxidase mRNA activity of each treatment)

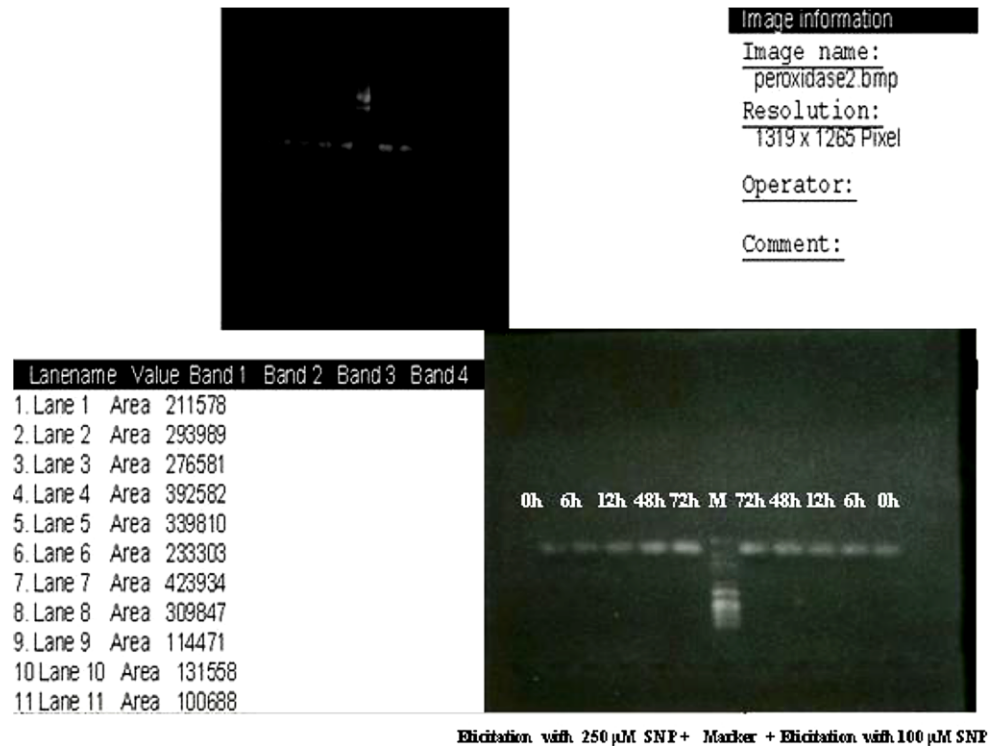


the phenolic contents in challenge inoculated plant cells. These results are in agreement with previous studies who indicated NO treatments improved PAL activity in wheat leaves against heat stress, bean plants against *Rhizoctonia solani* (Guo et al. 2004; Keshavarz-Tohid et al. 2016).

As shown in (Fig. 3) there were a high accumulation of total soluble phenols (mg/g fresh wt.) under elicitation stress. In addition, the highest accumulation has been observed after 72h, on the other hand, all NO treatments

significantly increased the total soluble phenols contents. These data was in accordance with Goodman et al. (1967), who found that multifold increase of phenols after challenging with elicitation in the present study may be due to the excess production of  $\text{H}_2\text{O}_2$  in elicited plant cells through increased respiration (Farkas and Kiraly 1962) or due to the activation of hexose-monophosphate pathway, acetate pathway and release of bound phenols by hydrolytic enzymes. Our results are in agreement with El-Beltagi et al.

**Fig. 6** Effect of different SNP doses administration (100  $\mu$ M, 250  $\mu$ M) on integrated optical density of expressed mRNA of peroxidase gene expression



(2015) who found that NO treatments increased APX, PAL activities and secondary Products (phenolic and flavonoid) contents in *Ginkgo biloba* cell suspension culture. Also, NO treatment significantly promoted PAL activity and inhibited PPO activity, and thus led to higher levels of total phenolics content in banana fruits after cold storage (Wang et al. 2015).

#### RNA of Peroxidase Gene Using Semi Quantitative RT-PCR Technique

The obtained data in Figs. 4, 5 and 6 indicate the results of the semi quantitative RT-PCR which represented as a relative ratio of the integrated optical density of the interested peroxidase gene to the integrated optical density of the zero time treatment of each (Murphy et al. 1990). The integrated optical density of peroxidase RT-PCR products was used to quantify the activity of peroxidase mRNAs induced or suppressed by either elicitation or various combination of SNP concentration elicited cultures represented gel illustrated the partial results of RT-PCR analysis of peroxidase gene expression and the peroxidase gene-expression levels in elicited cultures only which was higher than the zero time cultures when reached the maximum peak after 48 h of elicitation. On the other hand the down regulation of peroxidase activity realized after 72 h of elicitation process. In addition, the peroxidase mRNA gene expression of elicited cultures treated with various concentrations of SNP was also significantly higher than the (zero time) cul-

tures. But, the peroxidase gene expression levels in various SNP treated cultures were significantly lower than the only elucidated cultures (control treatments). Figs. 4, 5 and 6 showed that, elicitation or various SNP treatments induced the mRNA of peroxidase gene expression. Data revealed that, under elicitation condition not only the peroxidase activity induced the enzymatic levels but also in the transcriptional mRNA levels. It could be noticed that, under regulation of various oxidative signaling trigger molecules by elicitation process that could promote the transcription of peroxidase gene. On the other hand the application of low levels of NO donor concentrations (0, 50 and 100  $\mu$ M) negatively affected the mRNA peroxidase gene expression compared with the elicited ones only, that is may be due to the antioxidant activity of NO against various oxidative burst signaling molecules that trigger mRNA peroxidase gene transcription then, in sequence down regulate the levels of peroxidase mRNA gene expression. That is may be due to that, the elevated NO concentrations strongly decreased hydrogen peroxide levels that induce mRNA transcription of peroxidase activity. Moreover, the decrease in  $H_2O_2$  concentration was probably due to a direct NO- $H_2O_2$  interaction. The highest concentrations of NO donor sodium nitroprusside (250  $\mu$ M) potentiated elicitor-induced  $H_2O_2$  production and in sequence mRNA peroxidase gene expression that promote cell death.

In accordance, it is well documented that pathogen or elicitor challenge of a plant led to rapid generation of NO together with ROS; these molecules are commonly present

during different plant-pathogen interactions; however an appropriate balance between ROS and NO production is required to establish effective disease resistance reactions (Delledonne et al. 2002; Wendehenne et al. 2004). Moreover, the relative rates of production (concentration) of NO and ROS may be critical in determining whether NO acts as pro- or antioxidant and is either protective or toxic to plant cells as it was shown by (Orozco and Cárdenas 2002) in tomato plants. The presented data showed that, administration of various SNP treatments to cell suspension culture decreased the peroxidase activity by down regulating the gene expression of peroxidase activity, which contributes to the antioxidant action of SNP treatments on the lower levels. It has not yet been established whether the low concentrations of SNP can scavenge superoxide radical and hydrogen peroxide accumulation as a direct oxidative reduction reaction. On the other hand, Various SNP administrations caused a remarkable decrease in the gene expression level of peroxidase activity. On the other hand, the highly administration of SNP may be oxidized by a direct reaction with  $O_2^-$  superoxide anion to form peroxy nitril molecules that act as a prooxidants that increase the relative activity mRNA gene expression of peroxidase enzymes. Although, much is known about the involvement of both ROS and NO in plant disease defense reactions. Most of the experimental data available on NO detection during plant-pathogen interaction come from the studies of biotrophic pathogens infections (Clarke et al. 2000; Zhang et al. 2003). All results suggest that after a challenge with a virulent pathogen or elicitor NO accumulates in resistant plants and establishes a direct correlation between disease resistance against the biotrophic pathogen and NO. Only a small amount of contradictory data are available regarding the production of NO during the defense response associated with necrotrophic pathogens. The most evident indicated that, the highest NO content was observed in the SNP-supplied plants. NO at low concentration did not counteract higher  $H_2O_2$  generation and that these two compounds present together in cells at appropriate concentrations may be implicated in higher resistance to infection. The higher NO generation as a result of pathogen challenge was observed together with lower  $H_2O_2$  concentration and where infection development was observed. Exposure of cells to artificially high levels of NO markedly increased severity of infection development as expressed by cell viability. Hydrogen peroxide concentration in these treatments was severely diminished compared with that were not supplied with NO donor. It is well documented that pathogen or elicitor challenge of a plant led to rapid generation of NO together with ROS; these molecules are commonly present during different plant-pathogen interactions; however an appropriate balance between ROS and NO production is required to establish effective disease resistance reactions (Delledonne et al. 2002). The same ef-

fects were observed in sweet potato plants. The studies of other ROS: superoxide anion and hydroxyl radical showed that they remained unchanged or increased a little in tomato leaves regardless of NO donor or scavenger supplied indicating that enhanced NO level influenced mostly  $H_2O_2$  concentration in the studied plants. SOD, CAT, and (APX) have been shown to be inhibited by NO (Ferrer and Ros-Barcelo 1999).

## Conclusion

In conclusion, the results indicate that, SNP treatment affect the activities of SOD, CAT, APX and PAL. Participation of the enzyme in further resistance reactions e. g. cross-linking of cell wall proteins, polymerization of lignin precursors with  $H_2O_2$  as substrate for these processes. As activities of the enzymes generating (SOD) and removing (CAT, APX)  $H_2O_2$  in plant cells remained unchanged in the studied plants, the decrease in  $H_2O_2$  concentration was probably due to a chemical reactions between ROS and NO. It is suggested that in plant cells, contrary to animal cells, the main antioxidant action of NO is the reaction with  $O_2^-$  and ONOO<sup>-</sup> formation. It seems that they operate in NO enriched treatments. The results argue strongly that  $H_2O_2$  plays a role in plants protection against infections and provide evidence that NO is a potent antioxidant in plants that can act by directly scavenging ROS.

**Conflict of interest** H.S. El-Beltagi, O.K. Ahmed and G.M.G. Shehab declare that they have no competing interests.

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