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Biochemical and Histochemical Responses of Nonhost Resistance in Cucurbits to the Compatible and Incompatible Powdery Mildew Pathogens

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ABSTRACT

Resistance shown by an entire plant species to a wide range of bacterial, fungal and viral pathogens is already mentioned as nonhost resistance (NHR) phenomenon. Here, the defense responses of cucumber, squash and pumpkin inoculated with two different pathogens of powdery mildew of compatible, which belong to genera *Sphaerotheca fuliginea* (Schlecht. Fr.) and incompatible, which belong to genera *Leveillula taurica*, cause powdery mildew of pepper were investigated. No visible symptoms were observed as a result of inoculation of cucurbits with nonadapted (incompatible) pathogen, while severe symptoms were occurred as a result of inoculation with compatible pathogen. Disease severity percentage was increased in all host plants inoculated with compatible pathogen relative to nonhost plants inoculated by incompatible pathogen. Levels of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) which are the major forms of reactive oxygen species (ROS) were increased and accumulated early after 1, 2 and 3 days from inoculation in the nonhost as compared with host plants. Activities of antioxidant enzymes such as catalase (CAT), peroxidase (POX) and polyphenol oxidase (PPO) in all tested nonhost plants inoculated with incompatible pathogen 1, 2 and 3 days after inoculation were less than host plants inoculated with compatible pathogen at the same time intervals. It seems that accumulation of ROS early in nonhost plants has the key role of inhibiting or killing the incompatible pathogen. Understanding the resistance responses of host and nonhost plants for identifying similarities and differences is needed for the practical application in crop improvement.

Keywords: nonhost resistance, reactive oxygen species; antioxidants; powdery mildew; cucurbits

INTRODUCTION

In most countries of the world, species of cucurbits are native and cultivated in every country especially in Egypt where crop plants can be grown. Edible species of cucurbits include cucumber; squash and pumpkins have many nutrients such as starch, carbohydrates, proteins, fibers, minerals and vitamins (Tang *et al*. 2010) in addition antioxidants and anti-inflammatory benefits for humans. These important plants can be seriously damaged by powdery mildew pathogen which belongs to genera *Sphaerotheca fuliginea* (Schlecht. Fr.). Research for new resistance mechanisms is needed to protect cucurbits from serious and economic damage. How nonhost plants suppressing and inhibiting or even killing the incompatible pathogen, this is still unexplored mechanism or/mechanisms according to our knowledge from published results which have been achieved in this field. Nevertheless, some preliminary and promising results were obtained and indicated that ROS have an essential act which might be partially related with inhibiting or killing the pathogen in nonhost resistance. However, little information is known about the mechanisms of defense responses in cucumber, squash and pumpkin to nonadapted pathogens, especially when they have economical and biological importance. Nonhost resistance against probable pathogens has been considered as substantial and interesting phenomenon for the reason of the complete effect and apparent considerable protection. Two types of nonhost resistance explained by Mysore and Ryu (2004) shown against bacteria and fungi. When no visible symptoms are appeared on plants in response to pathogen invasion that is called type I of nonhost resistance. In contrast, type II results in quick response of hypersensitive reaction with initiating of cell death. Nonhost resistance mechanisms happened when the entire plant species are resistant to all genetic variants of a pathogen (Heath 1997; Nürnberger and Lipka 2005). Phenomenon of nonhost resistance gives us a new approach for thinking why the nonhost plants remain healthy and are immune to the vast majority of potential pathogens which cannot suppress the plant defense mechanisms. Very little information are currently published about the precisely mechanisms of nonhost resistance in contrast with the mechanisms of host resistance (Dangl and Jones 2001; Schulze-Lefert and Bieri 2005). It is commonly assumed that nonhost resistance could be overworked by plant breeders because of its durability and effectiveness for plant immunity over time for improving disease resistance among host species (Heath 2000). Additionally, susceptibility process happened when a compatible pathogen success to suppress the defense mechanisms or avoiding recognition by delivering appropriate effectors to the host plant (Schulze-Lefert and Panstruga 2003). Recent studies have been shown that there is a relationship between nonhost resistance and molecular architecture but still remained largely unexplored (Zellerhoff *et al*. 2010). Nevertheless, few articles are available about biochemical studies of nonhost resistance as wall-associated defenses and callose containing pappilae, which exist to produce physical barriers to infection (Heath *et al.* 1992; Hückelhoven 2007; Zellerhoff *et al*. 2010) and accumulation of hydrogen peroxide burst (H_2O_2) a major form of ROS (Schweizer 2007). Reactive oxygen species are generated and accumulated usually extracellularly with an oxidative burst when plant cells respond to microbial pathogens or elicitors (Bolwell 1999).

The major point of the present work aimed a clarifying how the mechanisms of nonhost working in defense responses in cucumber, squash and pumpkin to two different powdery mildew pathogens by the morphological, biochemical and histochemical investigations, which may be useful to improve disease resistance in host plants. The present study could participate and provide an evidence of the importance of plant defense responses during the earliest stages of inoculation in nonhost plants to powdery mildew pathogens.

MATERIALS AND METHODS

Plant materials:

Cucumber (Cucumis sativus L.) seeds of cv. Beta-Alpha, squash (Cucurbita pepo L.) cv. Askandrane and pumpkin (Cucurbita mixta Pang.) cv. Balady were obtained from Horticultural Research Institute (ARC), Egypt. Thirty seeds of each cucurbit cultivar were sown into an 84-cell plastic tray filled with sterilized peat moss. Four weeks after seeding, seedlings were transplanted into plastic pots (25 cm diameter) filled with sterilized peat moss and maintained in the greenhouse during the winter months where conditions were optimal for powdery mildew development. Temperature was 28±5 °C approximately,12 hours (approx.) photoperiod supplemented by natural light and relative humidity was 80±5%.

All experiments were conducted during 2016 in greenhouse of Sakha Research Station (ARC) and laboratory of Plant Pathology and Biotechnology at Agricultural Botany Dept., Faculty of Agriculture, Kafrelsheikh University.

Source of plant pathogens:

Two different pathogens of powdery mildew of compatible, which belong to genera Sphaerotheca fuliginea (Schlecht. Fr.), cause powdery mildew of cucurbits (infected cucumber cv. Baracoda) and incompatible, which belong to genera Leveillula taurica, cause powdery mildew of pepper (infected pepper cv. Top-Star) were obtained from greenhouse of Sakha Research Station. Complete infected leaves of cucumber covered with the pathogen were collected and immediately used for inoculation through dusting it on the healthy host plant leaves of cucumber, squash and pumpkin four weeks old from transplanting. Also, complete infected leaves of pepper covered with the pathogen were collected and immediately used for inoculation through dusting it on the healthy nonhost plant leaves of cucumber, squash and pumpkin. All plants used for inoculation were sprayed with water before the dusting process to make water film for affixing the conidia.

Assessment of the disease severity:

Disease severity percentage was determined (percentage of leaf area per each plant covered with powdery mildew lesions). Disease severity percentage of cucumber, squash and pumpkin inoculated with S. fuliginea (compatible) and L. taurica (incompatible) were recorded after inoculation process and estimated on scale of 0 to 5 described by Sen and Kapoor (1974). For disease severity assessment, eight plants of each replicate were recorded visually 1, 2, 3 and 4 weeks after inoculation. The 0 to 5 scale was based on increasing disease severity where 0 = no infection, 1 = 1-10%, 2 = 11-25%, 3 = 26-50%, 4 = 51-75% and 5 = 76-100% of leaf area covered. Index of disease severity (DSI) started from 1 (no infection) up to 100 (total leaf area covered by powdery mildew) was calculated by using the following formula according to Kim *et al*. (2000):

$$\mathbf{DSI} = \frac{\Sigma \text{ disease ratings of each plant}}{\text{total No. of plants rated} \times 5} \times 100$$

Histochemical analysis of reactive oxygen species:

Classic photometric assay for production of superoxide (O2•−) through leaf tissues based on the ability of O2•− to reduce nitro blue tetrazolium (NBT) as a histochemical staining procedure was done as mentioned below. Purple discoloration of NBT was visualized for detection of O2•−. Freshly collected leaves of cucumber, squash and pumpkin (control, inoculated host and inoculated nonhost) were cut into small discs (approx. 2 cm2) and vacuum infiltrated according Hagborg, (1970) protocol. According to Ádám *et al*. (1989) the purple discoloration of leaf tissues was visualized after adding 10 mM potassium phosphate buffer (pH 7.8) with 0.1 w/v % NBT (Sigma–Aldrich, Germany). Infiltrated leaf discs with NBT were replaced from the above NBT solution and incubated for 20 min. under natural daylight and then replaced into clearing solution consists of 0.15 % trichloroacetic acid (wt/vol) in ethanol: chloroform by ratio 4:1 (vol/vol). Samples of leaf discs were replaced once from old clearing solution to a newly fresh one during 48 h of incubation at room temperature according to procedure of Hückelhoven *et al*. (1999). For next evaluation, treated leaf discs were immersed in 50 % glycerol after incubation period. Quantification of discoloration of leaves was done using a ChemiImager 4000 digital imaging system (Alpha Innotech Corp., San Leandro, USA). For detecting the hydrogen peroxide (H2O2) production in leaf tissues, discs of leaves were vacuum infiltrated with 0.1% 3-3'-diaminobenzidine (DAB) after adding 10 mM Tris buffer (pH 7.8) as a classic photometric assay. Samples of leaf discs were transferred from the solution and incubated for 2 hours under daylight at room temperature. Finally leaf discs were cleared as described above and the intensity of brown color was estimated according to protocol of Hückelhoven *et al*. (1999). Histochemical analysis for detection of O2•− and H2O2 was done at 1, 2 and 3 days after inoculation process. During the experiment work, all above mentioned tests were three times repeated.

Biochemical assays of antioxidant enzymes:

Three milliliters of 50 mM TRIS buffer (pH 7.8) with 1 mM EDTA-Na2 and polyvinylpyrrolidone (7.5%) were used for homogenization of 0.5 g detached freshly cucumber, squash and pumpkin leaves (ground in liquid nitrogen) inoculated with two powdery mildew pathogens [control (sprayed only by water), S. fuliginea (compatible) and L. taurica (incompatible)] for the measurement of antioxidant enzymes activities 1, 2 and 3 days after inoculation. Subsequently, centrifugation was carried out on 12000 xg for 20 min at 4 °C for all homogenates. Supernatant of each homogenate was replaced for measuring the total soluble enzyme activity using spectrophotometer (UV-160A, Shimadzu, Japan) at 25 °C.

Peroxidase (POX) activity

According to the method of Hammerschmidt *et al*. (1982), the POX activity was measured in presence of guaiacol and hydrogen peroxide by changing the absorbance of ultraviolet (470 nm) every 30 sec intervals for 3 min. Supernatant obtained from leaf extract (50 µl) was mixed with 60 µmoles of guaiacol, 50 µmoles of sodium acetate buffer (pH 5.6) and 8 µmoles of hydrogen peroxide in a final volume of 3 ml. Changing of absorbance per min. per gram fresh weight was calculated for one unit of peroxidase enzyme activity.

Catalase (CAT) activity

Catalase activity was measured spectrophotometric according to the protocol of Aebi (1984). Decreasing the

absorption of ultraviolet at wave length 240 nm for 3 min was recorded for breakdown of hydrogen peroxide enzymatically. One unit of catalase enzyme activity was calculated by absorbance changing /min. /g fresh weight. Fifty microliters of supernatant from leaf extract were mixed with 100 µl of hydrogen peroxide, 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) in a final volume 2.15 ml.

Polyphenol oxidase (PPO) activity

According to the protocol of Coseteng and Lee (1978), PPO activity was measured by changing the ultraviolet absorbance at 420 nm. Mixture of 0.1 M phosphate buffer (pH 6.0), 20 mM of catechol solution and 0.05 ml of the enzyme solution was adjusted to final volume of 2.95 ml. one unit of polyphenol oxidase activity was calculated by changing of absorbance per min per gram fresh weight.

Statistical analysis

Complete randomized design was used for all experiments conducted under the greenhouse conditions with three replicates. Obtained data represent the mean ± SD.

RESULTS AND DISCUSSION

Disease symptoms and severity of host and nonhost pathogens:

Inoculated cucumber, squash and pumpkin plants with S. fuliginea (compatible) showed high susceptibility reaction to infection through formation of powdery mildew colonies (lesions) on leaf surfaces (Fig. 1 A, C and E). During the experiments period, some plant leaves were totally covered by powdery lesions in progress of time. These symptoms were similar to those symptoms of powdery mildew obtained by Gupta *et al*. (2001). In contrast, the inoculated cucumber, squash and pumpkin plants with L. taurica (incompatible) showed high resistance against pathogen infection with no visible symptoms (Fig. 1 B, D and F). This reaction against pathogen inoculation is completely in agreement with the explanation mentioned by Mysore and Ryu (2004) about the nonhost resistance type I.

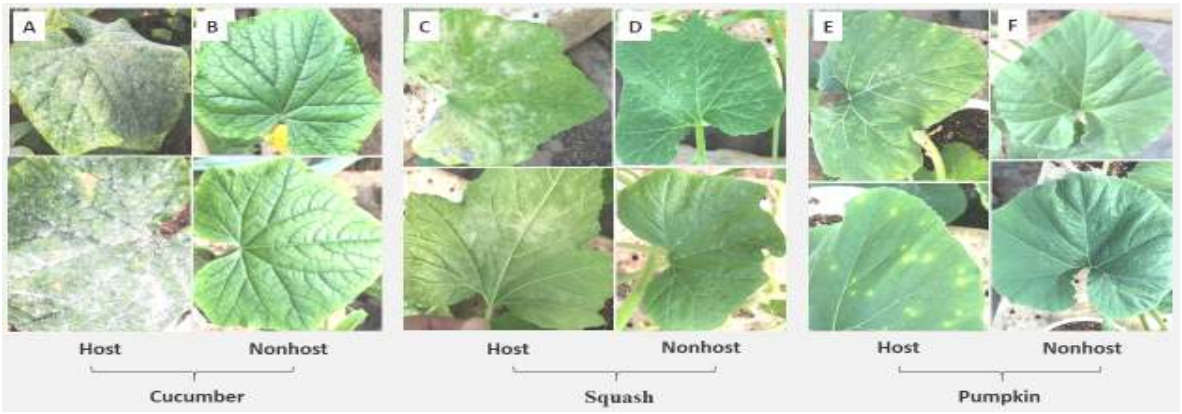

Fig. 1. Disease symptoms of host and nonhost plants of cucumber, squash and pumpkin 21 days after inoculation (dai) with *S. fuliginea* (compatible pathogen) and *L. taurica* (incompatible pathogen). A: Host cucumber inoculated with *S. fuliginea.* B: Nonhost cucumber inoculated with *L. taurica*. C: Host squash inoculated with *S. fuliginea.* D: Nonhost squash inoculated with *L. taurica*. E: Host pumpkin inoculated with *S. fuliginea.* F: Nonhost pumpkin inoculated with *L. taurica*.

Formation of disease symptoms may be happened successfully (in the susceptible host) through suppression of the plant resistance defense responses by several pathogen effectors. On the other hand, nonhost plant resistance responses are not suppressed at all by a certain pathogen effectors. This defense leads to that several factors and effectors of pathogen have been reported by O'Connell and Panstruga (2006) to assist in neutralization of the plant defense mechanisms. Disease symptoms were significantly appeared in the host plants compared to the nonhost plants which had no symptoms at all (Fig. 1). Recently, some obtained results indicated that the inoculated nonhost plants *Brassica rapa* contained more effectors induced by *Hyaloperonospora arabidopsidis* than in inoculated arabidopsis plants which are host of the same pathogen. Pathogen effectors might play an essential role in nonhost plants (Fabro *et al*. 2011). Susceptibility process happened when a compatible pathogen success to suppress the defense mechanisms or avoiding recognition by delivering appropriate effectors to the host plant (Schulze-Lefert and Panstruga 2003). Disease severity percentage was significantly increased in all host plants (cucumber, squash and pumpkin) inoculated with adapted pathogen *S. fuliginea* compared to the nonhost plants inoculated with nonadapted pathogen *L. taurica* (Fig. 2).

Histochemical analysis of reactive oxygen species:

Reactive oxygen species (ROS) production in cucumber, squash and pumpkin is one of the earliest responses following successful pathogen recognition in host and nonhost plants inoculated with *S. fuliginea* and *L. taurica* respectively. Intensity of brown discoloration caused by H_2O_2 and purple discoloration caused by $O_2^{\bullet-}$ indicate to levels of ROS generated in plant leaves inoculated with compatible and incompatible pathogens (Fig. 3).

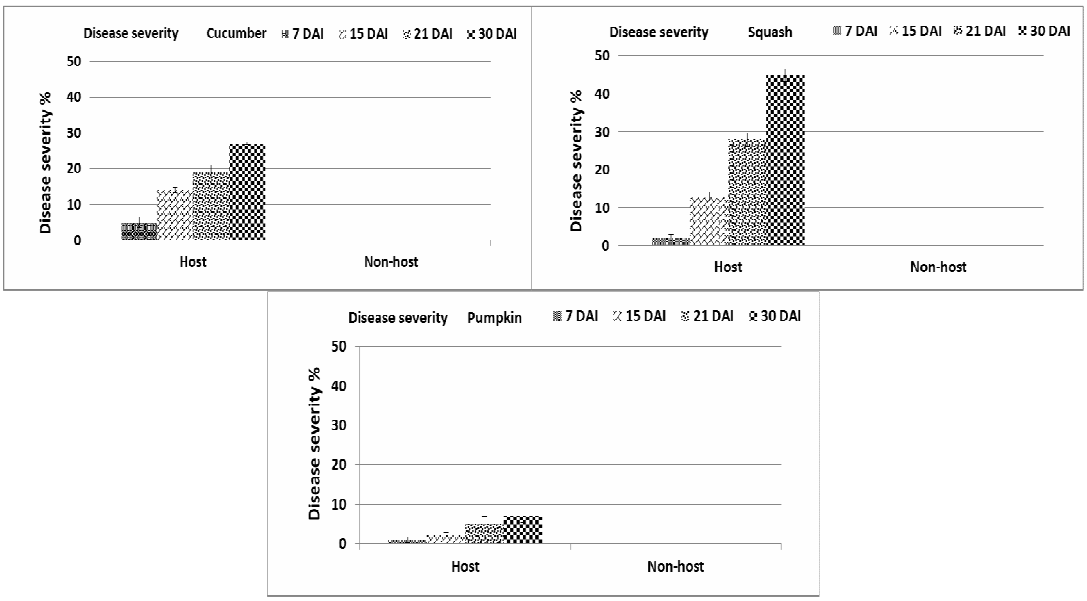

Fig. 2. Disease severity percentage of host and nonhost plants of cucumber, squash and pumpkin recorded 7, 15, 21 and 30 days after inoculation (dai) with *S. fuliginea* (compatible pathogen) and *L. taurica* (incompatible pathogen). Host: cucumber, squash and pumpkin plants inoculated with *S. fuliginea.* Nonhost: cucumber, squash and pumpkin plants inoculated with *L. taurica*.

Fig. 3. Brown discoloration caused by H_2O_2 and purple discoloration caused by $O_2^{\bullet-}$ of host and nonhost plant leaves of cucumber, squash and pumpkin 1, 2 and 3 days after inoculation (dai) with *S. fuliginea* (compatible pathogen) and *L. taurica* (incompatible pathogen).

Levels of ROS mainly H_2O_2 and $O_2^{\bullet-}$ (Fig. 4, 5 and 6) significantly accumulated early 1 day after inoculation (dai) in all nonhost plants of cucumber, squash and pumpkin inoculated with *S. fuliginea* (compatible pathogen) compared with plants inoculated with *L. taurica* (incompatible pathogen).

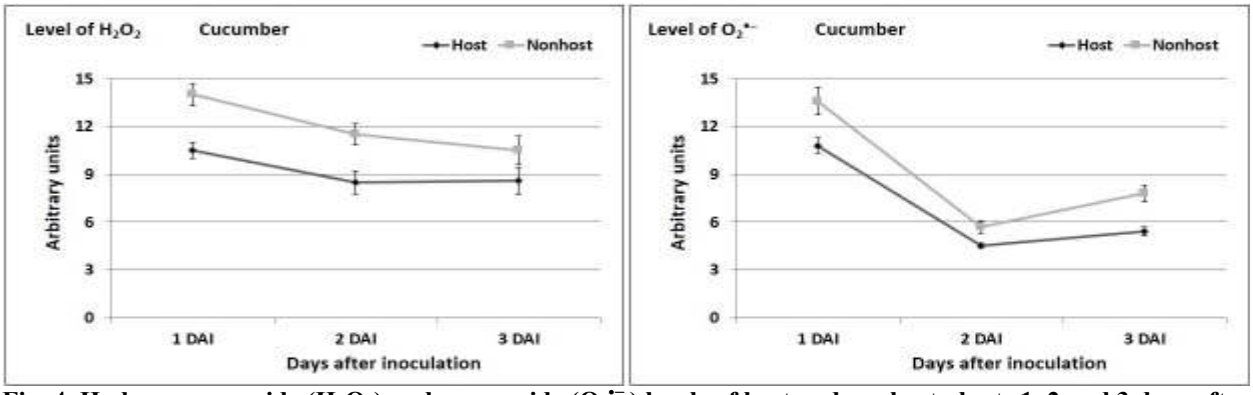

Fig. 4. Hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$) levels of host and nonhost plants 1, 2 and 3 days after inoculation (dai) with compatible and incompatible powdery mildew pathogens. Host: cucumber plants inoculated with *S. fuliginea* (compatible pathogen). Nonhost: cucumber plants inoculated with *L. taurica* (incompatible pathogen).

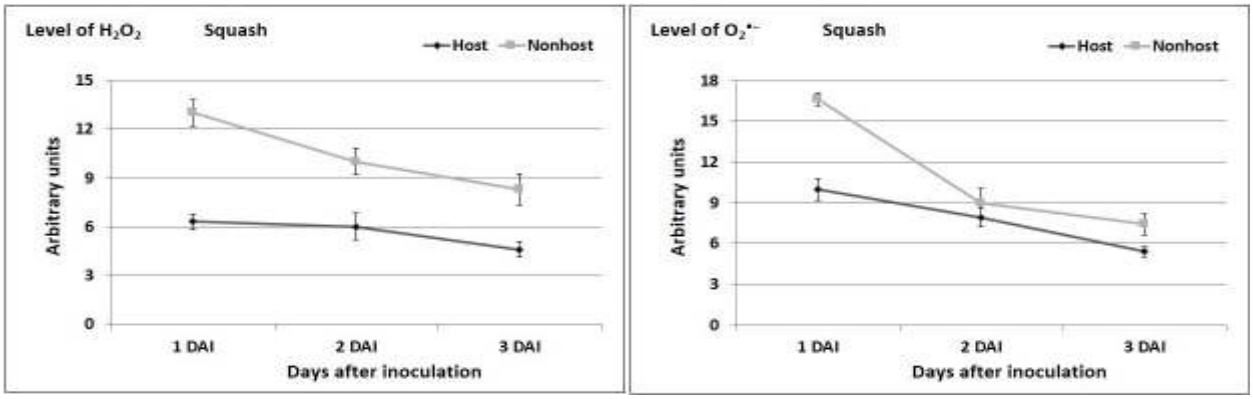

Fig. 5. Hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$) levels of host and nonhost plants 1, 2 and 3 days after inoculation (dai) with compatible and incompatible powdery mildew pathogens. Host: squash plants inoculated with *S. fuliginea* (compatible pathogen). Nonhost: squash plants inoculated with *L. taurica* (incompatible pathogen).

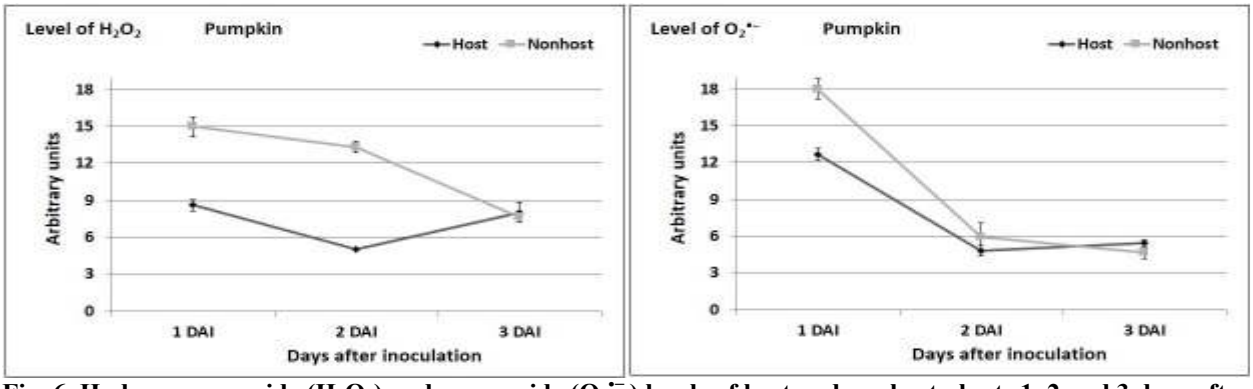

Fig. 6. Hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$) levels of host and nonhost plants 1, 2 and 3 days after inoculation (dai) with compatible and incompatible powdery mildew pathogens. Host: pumpkin plants inoculated with *S. fuliginea* (compatible pathogen). Nonhost: pumpkin plants inoculated with *L. taurica* (incompatible pathogen).

Biochemical assays of antioxidant enzymes:

Activities of antioxidant enzymes such as catalase, peroxidase and polyphenol oxidase were not increased in all tested nonhost plants of cucumber, squash and pumpkin inoculated with incompatible pathogen *L. taurica* 1, 2 and 3 days after inoculation compared with host plants inoculated with compatible pathogen *S. fuliginea* at the same time intervals (Fig. 7, 8 and 9). Interestingly, the antioxidant enzymes activities in host plants were decreased at the 2nd and 3rd day from inoculation with

compatible pathogen *S. fuliginea* except PPO in cucumber was a little bit increased. These obtained results are in agreement with results of Ketta (2015) who found that the down-regulation of antioxidants may be partially related with the soybean susceptibility to sudden death syndrome fungus infection.

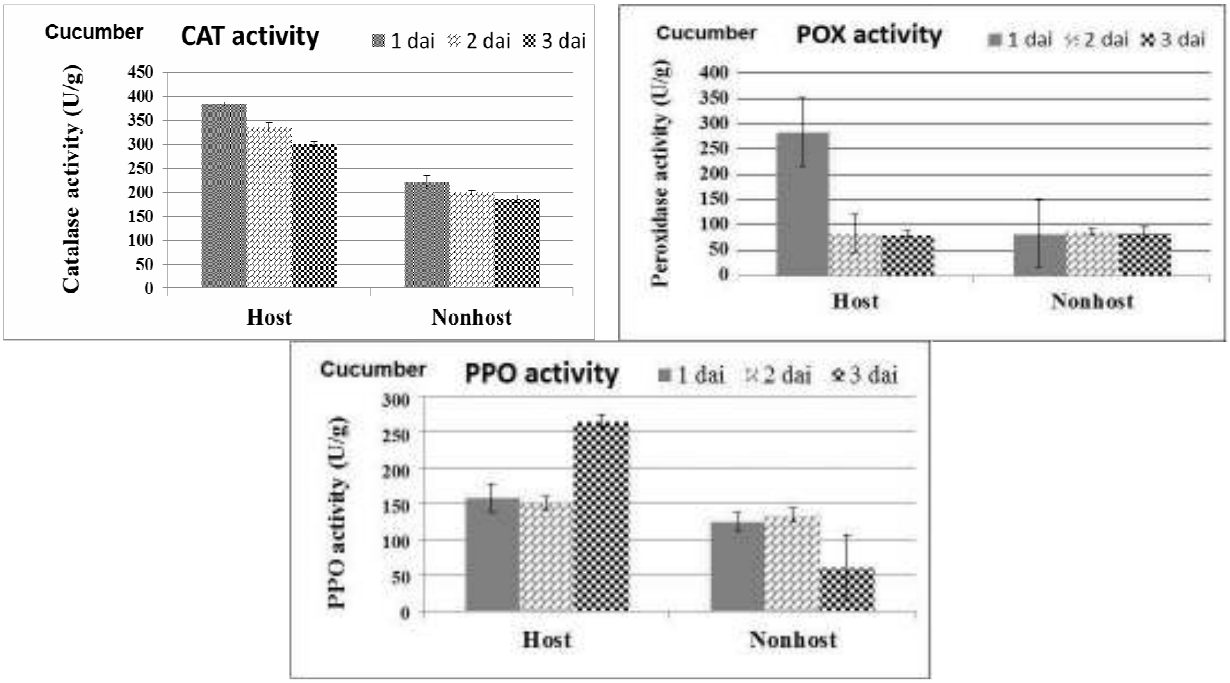

Fig. 7. Antioxidant enzymes (catalase, peroxidase and polyphenol oxidase) activities in host and nonhost of cucumber plant leaves 1, 2 and 3 days after inoculation. Host: cucumber plants inoculated with *S. fuliginea* (compatible pathogen). Nonhost: cucumber plants inoculated with *L. taurica* (incompatible pathogen).

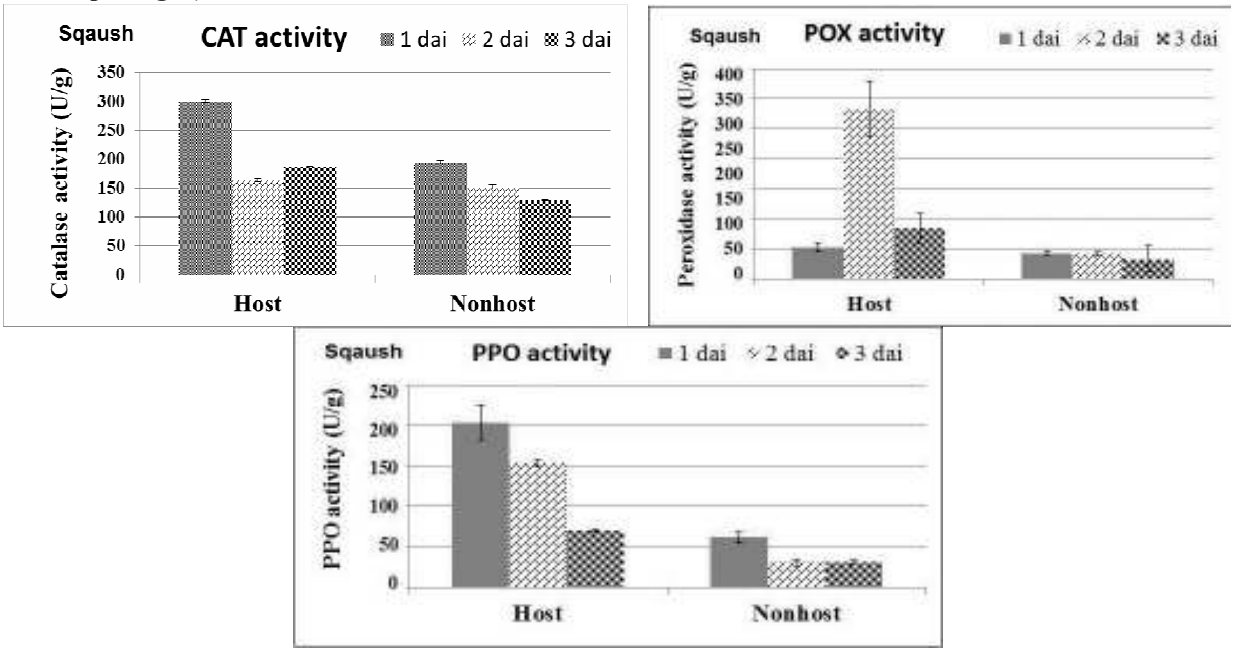

Fig. 8. Antioxidant enzymes (catalase, peroxidase and polyphenol oxidase) activities in host and nonhost of squash plant leaves 1, 2 and 3 days after inoculation. Host: squash plants inoculated with *S. fuliginea* (compatible pathogen). Nonhost: plants inoculated with *L. taurica* (incompatible pathogen).

Disappearance of symptoms or even reaction of hypersensitivity in nonhost plants of cucumber, squash and pumpkin inoculated with incompatible pathogen *L. taurica* might be related with the rapid and early formation and accumulation of ROS such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) toward quick recognition of plant pathogen. The quick ROS-accumulation toward pathogen invasion might propose to coordinate various defensive walls against the pathogens (Torres *et al*. 2006). Accumulation of ROS in the pathogen invasion site is harmful to the pathogen (Lamb and Dixon 1997) and plants in the same time protecting themselves by producing of antioxidant complex for avoidance the damage of ROS (Lebeda *et al*. 2001).

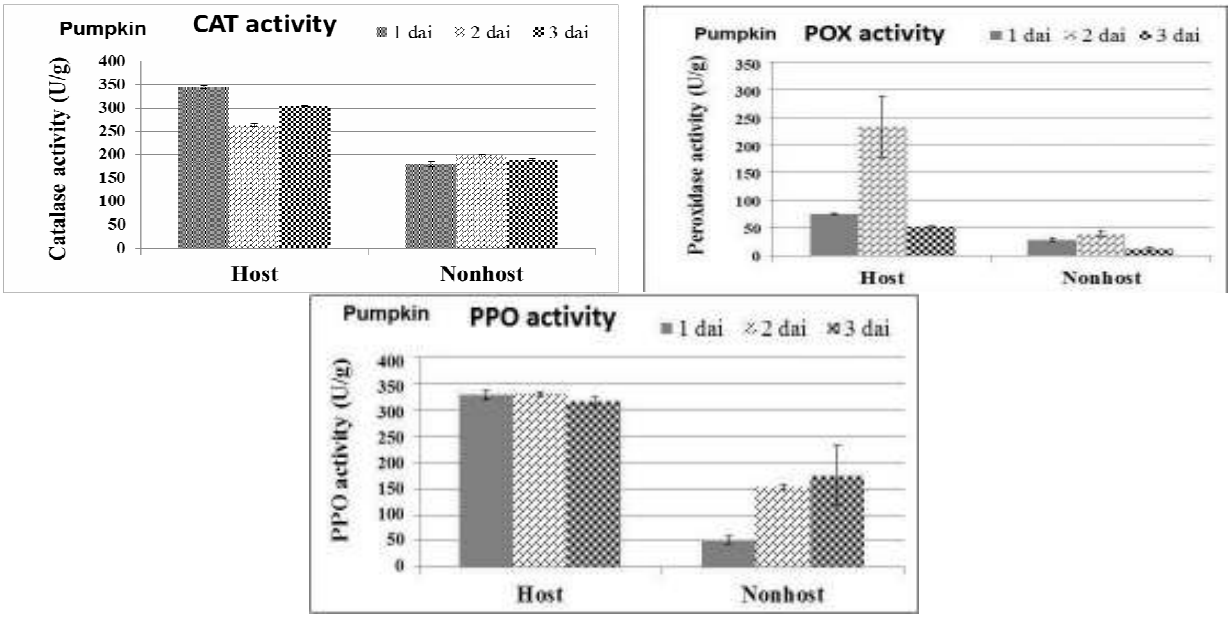

Fig. 9. Antioxidant enzymes (catalase, peroxidase and polyphenol oxidase) activities in host and nonhost of pumpkin plant leaves 1, 2 and 3 days after inoculation. Host: pumpkin plants inoculated with *S. fuliginea* (compatible pathogen). Nonhost: pumpkin plants inoculated with *L. taurica* (incompatible pathogen).

CONCLUSION

Increment and accumulation of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) early after 1, 2 and 3 days from inoculation in the nonhost plants as compared with host plants were observed. In contrast, all tested nonhost plants inoculated with incompatible pathogen 1, 2 and 3 days after inoculation were not increased in activities of antioxidant enzymes such as catalase (CAT), peroxidase (POX) and polyphenol oxidase (PPO) compared with host plants inoculated with compatible pathogen at the same time intervals. From the obtained results it could be suggested that early formation of superoxide and hydrogen peroxide in all nonhost plants of cucumber, squash and pumpkin might play an essential act in suppressing or even killing the incompatible pathogen. Improved understanding of nonhost resistance mechanisms will be helpful in searching and producing plants able to produce and accumulate of ROS early in response to pathogen recognition. Understanding the host and nonhost resistance responses to identify similarities and differences is needed for the practical application in crop improvement.

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الاستجابات البيوكيميائية والهيستوكيميائية الخاصة بمقاومة غير العائل في القرعيات لمسببات امراض البياض الدقيقي المتوافقة والغير متوافقة

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المقاومة التي تظهرها الأنواع النباتية بأكملها ضد مجموعة واسعة من مسببات الأمراض البكتيرية والفطرية والفيروسية قد سبق تعريفها كظاهرة المقاومة (لغير العائل). الردود الدفاعية من الخيار والكوسة والقرع العسلي بعد تلقيحها باثنين مختلفين من مسببات أمراض البياض الدقيقي واولها المتوافق اللذي ينتمي إلى جنس *Sphaerotheca fuliginea* (Schlecht. Fr.) وغير المتوافق واللذي ينتمي إلى جنس *Leveillula taurica* المسبب للبياض الدقيقي في الفلفل، تم التحقق منها. لم تلاحظ أي أعراض واضحة نتيجة تلقيح القرعيات الثلاثة مع المسبب المرضي غير المتوافق، في حين ظهرت أعراض واضحة نتيجة التلقيح بالمسبب المرضي المتوافق. زادت الشدة المرضية في جميع النباتات العائلة والملقحة بالمسبب المرضي المتوافق مقارنة بالنباتات غير العائلة والملقحة بالمسبب المرضي غير المتوافق. مستويات السوبرأكسيد وفوق أكسيد الهيدروجين اللّذان هما أحد أبرز مشتقات الأكسچين الحرة زادت وتراكمت بعد 1 و 2 و 3 أيام من التلقيح في النباتات غير العائلة مقارنة بالنباتات العائلة. إنخفض نشاط الإنزيمات المضادة للأكسدة مثل الكتاليز، البيروكسيديز والبولي فينول أكسيديز في جميع النباتات الغير عائلة والملقحة بالمسبب المرضي غير المتوافق بعد 1 و 2 و 3 أيام من التلقيح بنسبة اكبر من النباتات العائلة والملقحة بالمسبب المرضي المتوافق خلال نفس الفترات الزمنية. يبدو أن تراكم مشتقات الأكسچين الحرة في وقت مبكر في النباتات الغير عائلة له الدور الرئيسي في تثبيط أو قتل المسبب المرضي غير المتوافق. فهم استجابات المقاومة في النباتات العائلة وغير العائلة لتحديد أوجه الشبه والاختلاف ضروري جدا من أجل التطبيق العملي في تحسين المحاصيل الزراعية.