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Original article

Histopathological and biochemical aspects of grafted and non-grafted cucumber infected with stem rot caused by *Fusarium* spp.

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ABSTRACT

Cucumber grafting has been used in Egypt recently to induce soil diseases tolerance. The impact of various grafting techniques on the vulnerability of grafted cucumber seedlings to Fusarium which stimulates the stem rot was investigated. Consequently, the anatomical and physiological studies were carried out on the diseased and healthy grafted cucumber seedlings, comparing with the non-grafted ones. *Fusarium equiseti* (MW216971.1) caused a severe stem rot of the grafted seedling through affecting the connection area of the different grafting methods, leading to complete seedling death. The hole insertion grafting method significantly exhibited the highest diseases incidence (100%), and mean disease severity index (5) when inoculated with *F. equiseti*. The pathogen remarkably affected the graft union area causing tissue discoloration and decay. The levels of antioxidant enzymes and total phenols were significantly enhanced in the diseased grafted and self-rooted cucumber. However, the diseased grafted cucumber recorded significantly the highest values of the antioxidant enzymes activities and total phenolic content when compared with the self-rooted ones. The results of SDS-PAGE profile revealed variations in the leaves protein profile of the grafted and self- rooted seedlings in response to *Fusarium* infection. Taken together, grafting cucumber onto a resistant rootstock using the splice technique can alleviate the stem rot severity caused by *Fusarium* spp. by enhancing the histological, physiological and molecular defense response of the grafted seedling.

1. Introduction

Cucumber (*Cucumis sativus* L.) is considered as a popular summer vegetable crop in Egypt, beside the commercial production in the greenhouses per the whole year. The cucumber total cultivated area in Egypt, reached (16.104 k ha.) producing (364.571 K tons.) for all the growing seasons of 2019 (FAO, 2019). Cucumber plants suffer from different soil-borne pathogens during various growth stages such as, *Fusarium* spp., resulting considerable yield reduction (Chehri et al., 2011, Mohammed and Hasan, 2018; Aldakil et al., 2019; Al-Fadhal et al., 2019; El-Komy et al., 2021). Aldakil et al. (2019) witnessed a crown rot resulting yellowing and wilting of cucumber leaves caused by *F. equiseti*, impacting roughly 80% of the commercially grown cucumber. The root and stem rots which resulted from *Fusarium* species, formed a yield-limiting factor in Egypt's intense cucumber production.

Therefore, cucumber grafting has become a common practice that is used as a substitute for soil chemical treatments to manage soil-borne diseases in many Mediterranean countries, particularly after 2005 when the use of methyl bromide was banned (Al-Debei et al., 2012). Cucurbit grafting has recently expanded across Egypt at a breakneck pace (Kamel and Taher, 2021). It is considered an ecofriendly technique that is highly recommended for integrated disease management systems. The impact of cucurbits grafting includes not only improved disease resistance, but also increasing abiotic stress tolerance and enhancing crop production (Lee et al.; 2010; Papadaki et al.; 2017; Aslam et al., 2020). The most frequent cucurbit grafting methods are the hole insertion grafting (HIG), tongue approach grafting (TAG), and splice grafting (SG) (Miao et al., 2019). In cucumber, HIG and TAG are the most common, whereas SG is the easiest for commercial production (Lee et al., 2010; Huang et al., 2015; Miao et al., 2019). Root and stem base rots still occur prevalently in the grafted cucumber greenhouses in Egypt. Han et al. (2012) observed a severe crown rot on the grafted cucumber caused by *Fusarium solani* f. sp. *cucur-*

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bitae, resulting dark brown, water-soaked lesions at the stem base of the naturally infected plants.

Previous studies focused on the physiological and histological changes associated with cucumber grafting (Xu et al., 2015; Miao et al., 2019), nevertheless, changes occurred in the grafted seedlings due to infection remain unclear. Grafting, as a wounding stress, activates antioxidant defense schemes through elevating the reactive oxygen species (ROS) level (Xu et al., 2015; Miao et al., 2019). This stress can be alleviated in part by complex defensive antioxidative systems, which include antioxidant enzymes as POD, PPO and CAT (Xu et al., 2015; Reyad et al., 2021). Production of defense antioxidases in the grafted seedlings can also promote disease resistance. Non-enzymatic biochemicals, as phenolic compounds, also have a significant part in decreasing the oxidative stress in grafted seedlings. Phenolic compounds are commonly produced as way of a plant's defensive systems in response to biotic or abiotic stressors and are engaged in various metabolic activities (Mittler, 2002).

Long distance proteins transport in the grafted plants across the graft connection impacts their development by enhancing the photosynthesis, the metabolism and the energy production, as well as plays a key role in stresses resistance (Rasool et al., 2020). Proteins involved in signal transduction such as antioxidant-related proteins, positively regulates the defense process when grafted plants are under stress to stimulate other resistance pathways (Xu et al., 2018; Zhang et al., 2021). Thus, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) technique was carried out and compared. The protein profile analysis revealed variations in the grafted cucumber scions in response to pathogen stress, compared with the self-rooted seedlings.

Changes associated with cucumber grafting onto resistant rootstocks have been elucidated previously (Xu et al., 2018; Miao et al., 2019). However, information related to grafted cucumber histology and physiology under the impact of the virulent soil-borne pathogens, remains limited. Hence, this study focuses on characterizing the most important histopatholgical changes occurred in the infected graft union area of various grafting methods. In addition, analyzing the antioxidant enzymes and total phenols changes in the grafted seedlings after infection. Variations in the leaves protein ectrophoretic banding patterns were included as well.

2. Materials and methods

2.1. Effect of Fusarium spp. on cucumber grafted onto Supershintosa rootstock using different grafting techniques

2.1.1. Source of Fusarium isolates

Two pathogenic *Fusarium* spp., previously isolated from naturally infected grafted cucumber plants showing root and stem rot symptoms, were used in this study. *Fusarium proliferatum* (MW242871) was isolated from diseased cucumber plants, which grafted onto Strongtosa rootstock and grown at Gamasa, Dakahlia governorate, Egypt. As well as *Fusarium equiseti* (MW216971.1) was obtained from diseased cucumber plants, grafted onto Supershintosa rootstock and grown at Belbis, Sharkia governorate, Egypt.

2.1.2. Plant materials and growth conditions

The cultivation of the rootstock and scion seeds, as well as the grafting process were conducted at (Roots Nursery for Grafted Vegetables Seedlings) located in the New El-Nobaria city Cairo–Alexandria desert road, Egypt, in August 2019. As a scion, the cucumber cultivar (*Cucumis sativus* L. Barracuda F1 hybrid from Seminis Seed Company) was utilized. Supershintosa (F1 hyprid of *Cucurbita maxima* Duch × *Cucurbita moschata* Duch) from Egypt's Technogreen company was used as a rootstock. Seeds of the scion and rootstock were sown trays containing commercial organic substrates (Peatmoss: Vermiculite: Perlite mixed at the rate of 1:1:1 v/v/v). The environmental conditions of germination and growth were 25–28 °C, 85–90% relative humidity and 15 h of daylight (Miao et al, 2019; Noor et al., 2019). Grafting was performed once the scion had fully produced the first true leaf and the rootstock had begun to develop the first true leaf (Mohamed et al., 2014).

2.1.3. Grafting process

The three grafting methods (Hole insertion grafting, Approach grafting and Splice grafting) were carried out as described by Lee et al. (2010); Mohamed et al. (2014); El-Gazzar et al. (2016); Miao et al. (2019) and Noor et al. (2019) (Fig. 1). All grafts were carried out by one operator. For the graft union healing, grafted plants were relocated to a humidity chamber where they were kept at a temperature of 26/20 °C day/night with a relative humidity of 80–95% for around 7 days. Following the healing process, grafted plants were then transferred to the greenhouse and maintained at 24–30 °C until the scion and rootstock are well linked. The grafted and non-grafted seedlings were divided for achieving the following treatments: (1) Inoculation with *F. proliferatum* (2) Inoculation with *F. equiseti* (3) Check control. Ten replicates were used for each particular treatment. Non-grafted cucumber was used for comparison.

2.1.4. Seedlings inoculation and disease assessment

Fusarium proliferatum and *F. equiseti* were inoculated separately, using the barely-sand medium as described by Al-Fadhal et al., 2019, in plastic pots of 20-cm-diameter, containing 2 kg sterilized sand and clay mixtures (1:1), along with the un-infested controls. One seedling was cultivated per pot for each aforementioned treatment with 10 replications for each one, arranged in a factorial design of two factors. The inoculated and control treatments were grown in the glasshouse of Plant Path. Dept., Fac. Agric., Zagazig Univ. with day and night temperatures of 30–32 °C and 23–30 °C, respectively and watered every 2 days to field capacity. The seedlings were examined every seven days for symptoms development until 4 weeks after inoculation, where the plants were pulled out, and observed for rot symptoms. Diseases incidences, disease severity index and the appeared symptoms on the grafted and non-grafted seedlings, were recorded for each isolate. Disease incidence was calculated according to Wu et al.

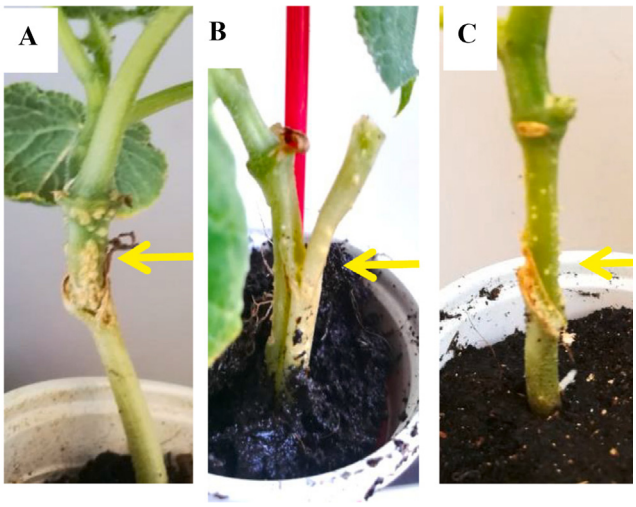

Fig. 1. The graft union connection of the different grafting methods, after the healing and hardening stages. **(A)** Hole insertion grafting, **(B)** Approach grafting **(C)** Splice grafting.

(2006), while the disease severity was determined using the scale of Bletsos (2005) which was modified by Seo and Kim (2017).

2.2. Histopathological studies on the grafted cucumber union area

The Anatomical studies were conducted to follow the pathological changes in the graft union area tissues of different grafting methods. Five samples of the healthy and diseased grafted seedlings (showing graft junction rot caused by *F. equiseti*) were harvested for this study along with the healthy and infected non-grafted cucumber and rootstock seedlings.

Transversal sections were carried out at the graft union region between the rootstock and the scion of the three different grafting methods (Hole insertion grafting, Approach grafting and Splice grafting) after the appearance of rot symptoms on the conjunction area. The samples of grafted seedlings were trimmed between 3 mm above and 3 mm below the graft junction. For the self-rooted cucumber and rootstock seedlings, the histopathological studies were conducted on the stem base (3 mm below the cotyledons) of diseased and healthy seedlings after 30–35 days from sowing. The obtained materials of graft unions of the grafted seedlings as well as, the scion and rootstock stems were cleaned well with tap water, killed and fixed in FAA solution (85 ml ethyl alcohol 70%, 10 ml formalin and 5 ml glacial acetic acid) for 48 h. The samples were then washed of ethanol, and then cleared by transferring in concentrations of absolute ethanol and xylene at the rate of (3:1–1:1–1:3 and 100% xylene). Sectioning and fixation was performed according to Nassar and El-Sahhar (1998). The sections were examined by light microscope (Model: LEICA ICC50 HD) supplemented with photographic unit.

2.3. Biochemical factors affecting stem rot diseases resistance of the grafted cucumber seedlings

The biochemical changes of the Barracuda cucumber, occurred due to grafting on Supershintosa rootstock, were studied and compared with the non-grafted cucumber seedlings and the rootstock. Peroxidase, polyphenol oxidase and catalase activities, and the total phenols were determined in the diseased and healthy grafted and non-grafted seedlings. The antioxidant enzymes activities and the total phenol content were evaluated in the Analysis and Measurements lab., Central lab. of Plant Path. Inst., at the Agric. Res. Center.

2.3.1. Plant materials and treatments

Different plant materials were used in this analysis [Supershintosa rootstock (RS), Cucumber seedlings grafted on supershinosa using splice grafting technique (G) and self-rooted cucumber scion (Sc)]. Enzymatic activities and total phenols were assessed in healthy seedlings (H) along with diseased ones (D) infected with stem rot caused by *F. equiseti*. Newly grown leaves were taken from seedlings at the same age for extraction. An average of three replicates has been carried out.

2.3.2. Preparation of enzyme extracts

For each sample, 0.5 g fresh weight of leaves was immediately ground with a pestle in an ice-cold mortar with 4 ml 50 mM phosphate buffer (pH 7.0). The homogenates were centrifuged at 12,000 rpm for 20 min at 4 °C and the supernatant was utilized to determine enzymes activities (Mishra et al., 2006).

2.3.3. Determination of peroxidase activity (PO)

Peroxidase activity was evaluated as the rise in absorbance generated by guaiacol oxidation at 470 nm in spectrophotometer Milton Roy Spectronic (Polle et al., 1994).

2.3.4. Determination of polyphenoloxidase activity (PPO)

The PPO assay was performed according to (Aquino-Bolaos and Mercado-Silva, 2004).

2.3.5. Catalase activity assay

The procedure of Bergmeyer (1974) was used to determine Catalase activity, as the decline in absorbance produced by the decrease in H_2O_2 removal at 240 nm in spectrophotometer Milton Roy Spectronic 601.

2.3.6. Determination of total phenols:

Determination of total phenols was carried out as described by (Snell and Snell, 1953). Standard curve for catechol was constructed and total phenols were extrapolated. Data presented as mg/g.

2.4. Protein analysis by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

The proteomic analysis was performed at the Biotechnology Lab. At the Cairo University Research Park, Faculty of Agriculture (CURP), according to (Laemmli, 1970). It was performed to determine the leaves protein profile changes due to the infection of the grafted cucumber seedlings, compared with the own-rooted ones.

2.4.1. Plant materials and protein extraction

Newly grown leaves were taken from the healthy (H) and diseased (D) seedling of supershintosa rootstock (RS), grafted seedlings (G) and cucumber scion (Sc) to study their protein fingerprints. To separate the proteins to small sub-units, 10 g of leaves samples were extracted using the protein extraction buffer (0.6 M Tris HCL buffer-pH 6.8 mixed SDS and βmercaptoethanol). The mixture was centrifuged at 12,000 rpm for 10 min at room temperature. Then supernatant were transmitted into new 1.5 ml Eppendorf tubes and kept at −20 °C until gel electrophoresis.

2.4.2. Sample loading, electrophoresis and gel staining

For each one of slot gels, 15 μl of each leaves extract sample was loaded, electrophoresis and gel was stained using the coomassie brilliant blue (CBB) according to (Kim and Cho, 2019). Electrophoresis was conducted at 100 V until the bromophenol blue (BPB) reached to the bottom of gel plate. Then the gel was destained by washing with a solution containing acetic acid, methanol and water in the ratio of 5:20:75 (v/v), so that the blue color of the coomassie brilliant blue (CBB) disappears and the electrophoresis band on gels clearly visible.

2.4.3. Gel analysis and data processing

The gels were photographed and the protein bands were scored. The electrophoretic products compared with protein bands of a ladder, from the Bio- Rad manual for the Gel Slab Dryers model 483.2.6. The protein bands on SDS gel were compared using gel documentation (G: Box) (SYNGENE model 680 XHR) made in UK. Patterns of the SDS-PAGE bands were scored as: (0) for absent or (1) as present, and each was treated independently. Protein diversity was determined by comparing the banding patterns of all tested plant materials (Kim and Cho, 2019).

2.5. Statistical analysis

Data Analysis of the previous experiments was conducted according to Snedecor and Cochran (1980) using Statistic complete V. 9 program for ANOVA and LSD analysis. The means were compared using Least Significant Difference (LSD) and differences at $p< 0.05$ were considered to be significant Duncan (1955).

Table 1
Effect different grafting methods on the stem rots caused by *F. prolifertum* and *F. equiseti* of cucumber grafted on to Supershintosa rootstock compared with the non-grafted cucumber.

Grafting techniques / Fungi	Hole insertion grafting			Splice grafting			Approach grafting			Non-grafted Cucumber		
	DI %	Disease Severity Index	Mean DSI	DI%	Disease Severity Index	Mean DSI	DI%	Disease Severity Index	Mean DSI	DI%	Disease severity Index	Mean DSI
		0 1 2 3 4 5			0 1 2 3 4 5			0 1 2 3 4 5			0 1 2 3 4 5	
F.proliferatum	80	0 2 0 4 4 0	3 [b]	50	5 2 3 0 0 0	1.3 [cd]	70	3 0 0 3 2 2	2.7 [bc]	100	0 0 0 0 0 5	5 [a]
F. equiseti	100	0 0 0 0 0 10	5 [a]	80	2 0 0 0 6 2	3.4 [b]	80	2 0 0 0 0 8	4 [b]	100	0 0 0 0 0 5	5 [a]
Negative control	0	10 0 0 0 0 0	0 [d]	0	10 0 0 0 0 0	0 [d]	0	10 0 0 0 0 0	0 [d]	0	10 0 0 0 0 0	0 [d]

LSD for the disease severity index (DSI) at P = 0.05:
Fungi = 0.51 Grafting technique = 0.64 Fungi × Grafting technique = 1.43
Values of disease severity index (DSI) followed by different letters are significantly different at p = 0.05 according to Duncan's multiple range test. (DI) = Disease Incidence (DSI) = Disease severity index was the mean disease severity value on 10 artificially inoculated grafted and non-grafted seedlings, assessed with a 0–5 visual scale modified by Seo and Kim (2017).

3. Results

3.1. Effect of Fusarium spp. on cucumber grafted onto Supershintosa rootstock using different grafting techniques

Table 1 and Figs. 2–4 show the reaction of grafted cucumber, against *Fusarium* spp., comparing with the self-rooted one. The non-grafted cucumber was highly susceptible to root and stem rot disease. It exhibited significantly the highest diseases incidence and mean disease severity index when inoculated with both inspected *Fusarium* spp., being (100% DI and 5 DSI).

The same performance of susceptibility was observed once the hole insertion grafting was applied in the rootstock-grafted seedlings inoculated with *F. equiseti* resulting in (100% DI and 5 DSI) (Figs. 2 and 3A–C). Also, *Fusarium equiseti* caused crown and stem rots on cucumber seedlings grafted using splice and approach grafting methods (Fig. 3D, E). The pathogen caused (80% DI and 3.4 DSI) on the seedlings grafted using the splice grafting method and (80% DI and 4 DSI) when approach grafting method was applied.

On the other hand, *F. proliferatum* exhibited significantly lower disease incidence and severity index on the grafted seedlings comparing with *F. equiseti* (Figs. 2 and 4). The inoculated seedlings showed (80% DI and 3 DSI) when the hole insertion method was used, followed by the approach grafting method (70% DI and 2.7 DSI) without significant difference (Fig. 4A, B, E and F). Seedlings grafted using the splice grafting proved to be much more resistant to *F. proliferatum* as it exhibited the least values being, (50% DI and 1.3 DSI) (Fig. 4C).

Fusarium equiseti caused a severe stem rot of the grafted seedling that affected the graft union area, leading to complete seedling death as cucumber scions were unable to receive water and nutrients from the rootstocks (Figs. 2 and 3). While, *F. proliferatum* affected mostly the rootstock hypocotyl and roots (Fig. 4). The graft junction being placed near, or below the soil surface can be easily infected with *Fusarium* spp. (Fig. 3B, D and E) as well as. (Fig. 4A, C, E and F). Thus, grafted and self- rooted cucumber seedlings infected with stem rot caused by *F. equiseti*, were used for further histopathological studies.

3.2. Histopathological studies on the grafted cucumber union area

Samples of diseased and healthy graft union area obtained from seedlings, grafted using different methods, were investigated

Fig. 2. The effect of both *Fusarium* spp. on the grafted Cucumber (grafted using three different grafting techniques) after 2 weeks of inoculation.

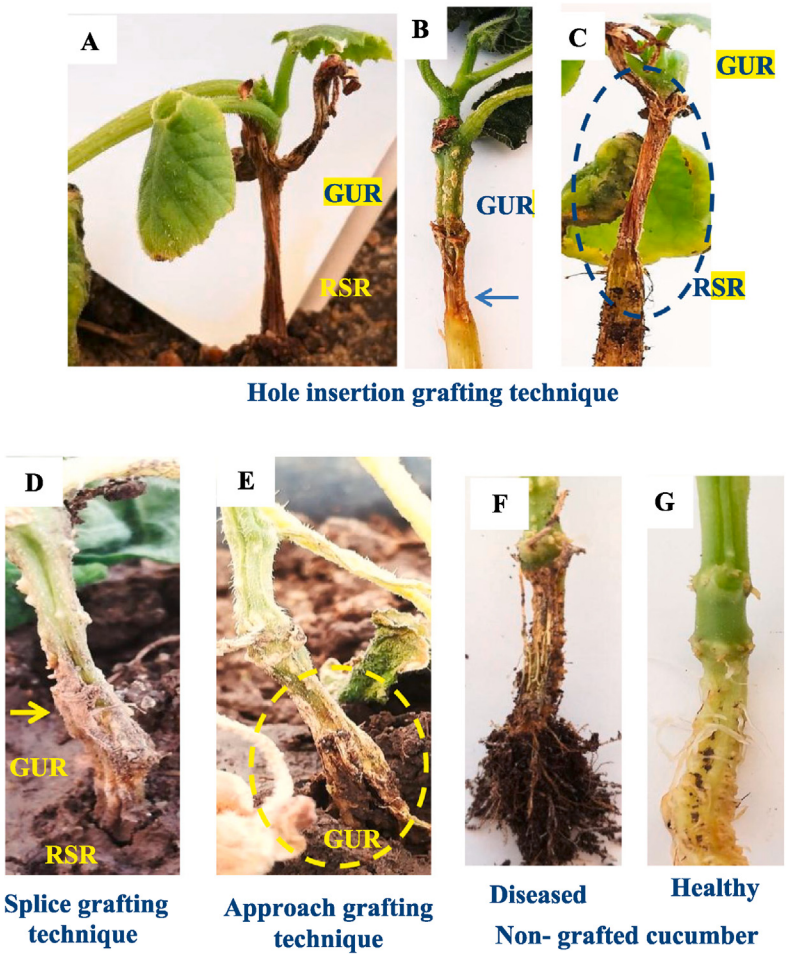

Fig. 3. Symptoms of *Fusarium equiseti*. on Cucumber (grafted using three different grafting techniques) after 4 weeks of inoculation, compared with the non-grafted seedlings. **Abbreviations: RSR**: Rootstock Rot **GUR**: Grafting Union area Rot**.**

microscopically. As well as, samples of diseased and healthy self-rooted scion and rootstock were obtained for comparison (Figs. 5 and 6). It was observed that, *F. equiseti* caused tissue discoloration, disintegration due to the development of pathogen in the different graft conjunctions and production of pectic enzymes in infected tissues. The pathogen growth and its lysis effect can be restricted to the superficial layers of cortex (Fig. 5, A4, A5 and C5). While in certain junctions, these phenomena developed in the whole graft union reaching the vascular tissues, causing the complete tissue lysis and malformation (Fig. 5, B4, B5 and C4). In the healthy grafted seedlings, callus was observed in the contact region of both sides of the graft partners to make connection between scion and rootstock (connection area CA) (Fig. 5 A3). Callus tissue between scion and rootstock differentiates to new small vascular connection elements (xylem and phloem) as shown in Fig. 5 (B3). The histological study revealed also the growth of the adventitious roots from the susceptible scion in combination grafted using the approach method (Fig. 5, C3). Scion' adventitious roots penetrate the soil and induce disease by passing resistant rootstock, which can lead to infection of the graft union resulting seedling death (Fig. 5, C4). Adventitious rooting was also observed clearly in the self-rooted cucumber scion (Fig. 6, D1), which was severely affected with the stem rot pathogen causing significant morphological changes (Fig. 6, D2).

Conversely, the non-grafted rootstock sections showed a resistant reaction against the pathogen as the tissues were not affected (Fig. 6, E1 and E2).

3.3. Biochemical factors affecting stem rot diseases resistance of the grafted cucumber seedlings

Data in Table 2 show that, the values of the antioxidant enzymes activities and total phenol content differed between the rootstock-scion grafted seedlings and self- rooted rootstock and scion. In addition, these values differed in the healthy and diseased seedlings of each inspected plant material. The activities of PO (peroxidase), PPO (polyphenol oxidase), CAT (catalase) and the (PC) phenolic contents were significantly enhanced in the grafted cucumber seedlings when compared with the non-grafted ones. As well as, higher levels of antioxidant enzymes activities and phenolic content were recorded in diseased seedlings compared with the healthy ones (Table 2). In this concern, the diseased grafted cucumber seedlings recorded significantly the highest values of the antioxidant enzymes being, 2.900, 0.046, 2.908 U min^{-1} mg^{-1} protein for PO, PPO and CAT as well as the highest total phenolic content being 52.731 mg/g. For PO activity, the diseased rootstock seedlings showed significant increase of enzyme activity (2.554 U min^{-1} mg^{-1} protein) compared with the healthy seedlings (1.429 U min^{-1} mg^{-1} protein). The same trend of PO elevation was observed in the grafted seedlings. However, non-significant PO induction was recorded in the self-rooted cucumber scion after infection being (1.285 U min^{-1} mg^{-1} protein) in healthy seedlings and (1.847 U min^{-1} mg^{-1} protein) in diseased seedlings. Polyphenol oxidase and catalase activities were also significantly elevated in the grafted cucumber seedlings after infection, nevertheless,

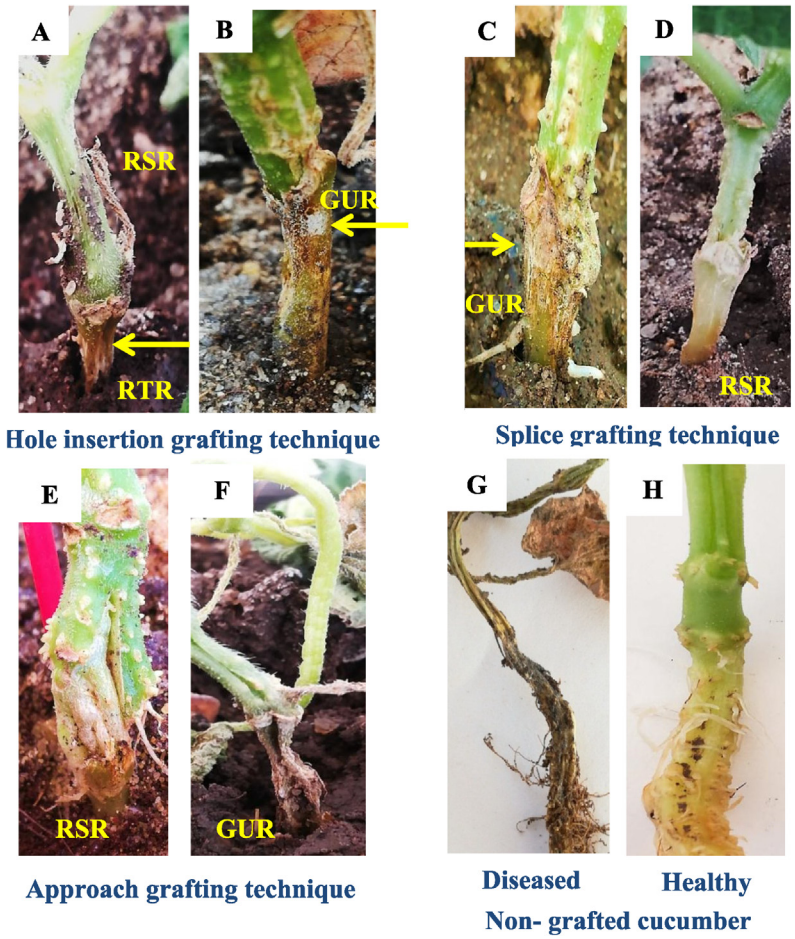

Fig. 4. The symptoms of *Fusarium proliferatum*. on Cucumber (grafted using three different grafting techniques) after 4 weeks of inoculation, compared with the non-grafted seedlings. **Abbreviations: RSR**: Rootstock Rot **GUR**: Grafting Union Area Rot**.**

insignificant increase of both enzymes was observed in the non-grafted rootstock and cucumber scion after infection. Furthermore, the phenolic compounds content was significantly increased in all inspected plant materials after infection.

3.4. Protein analysis by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Leaves protein profile changes due to the infection of the grafted cucumber seedlings, compared to non-grafted rootstock and scion seedlings was determined and presented in (Figs. 7 and 8) and Tables 3 and 4. Table 3 shows that, variations occurred in protein profiles of the different plant materials due to the pathogen infection. The analysis of SDS-PAGE revealed 16 protein bands with different molecular weights ranged from 20 to 170 kD. These bands were commonly detected among the diseased and healthy plant materials. The protein bands of all different plant materials were varied in numbers after infection. In rootstock seedlings, the total number of protein bands reduced from (13 to 10) after infection, while the bands increased from (7 to 11) and from (11 to 12) in the self- rooted and grafted cucumber seedlings, respectively. The healthy rootstock revealed the highest total number with 13 bands. The healthy self-rooted cucumber scion displayed the lowest number with 7 bands.

Data in Table 4 shows the protein bands changes of the inspected plant materials after the infection. The data revealed differences in protein bands between the control and infected treatments. The Supershintosa rootstock showed 1 positive unique band appeared after infection with size 45 kD and 4 negative bands with sizes 20, 22, 66 and 150 kD. The self- rooted cucumber scion displayed the highest number of positive unique bands (4) after infection with sizes (26, 30, 120 and 170 kD) and no positive bands were detected. On the other hand, the grafted cucumber leaves showed only 2 unique bands with molecular weights (20 and 26 kD) and one negative band with molecular weight 62 kD. The unique protein band with molecular weight 62 kD appeared only in the healthy grafted cucumber seedlings, while it was absent in all inspected treatments. It was also observed that, four protein bands appeared in the healthy grafted cucumber leaves, while they were absent in the self- rooted cucumber scion.

4. Discussion

Grafting can be an efficient approach to combat soil-borne fungal diseases. In Japan, Korea, and Greece, cucumber grafting has been used to combat Verticillium and Fusarium wilt. The interspecific *C. maxima* × *C. moschata* is the most widely used rootstock species around the world for grafting cucumbers (Lee and Oda, 2003). Because of the vast range of cucumber scion–rootstock interactions formed by grafting, response of the grafted seedling to pathogens is difficult to investigate (Leonardi and Romano, 2004; Al-Debei et al., 2012).

Graft compatibility is a complex reaction that involves a variety of anatomical and physiological interactions. The success in grafting method is largely determined by the selection of appropriate scion and rootstock combinations, the use of appropriate

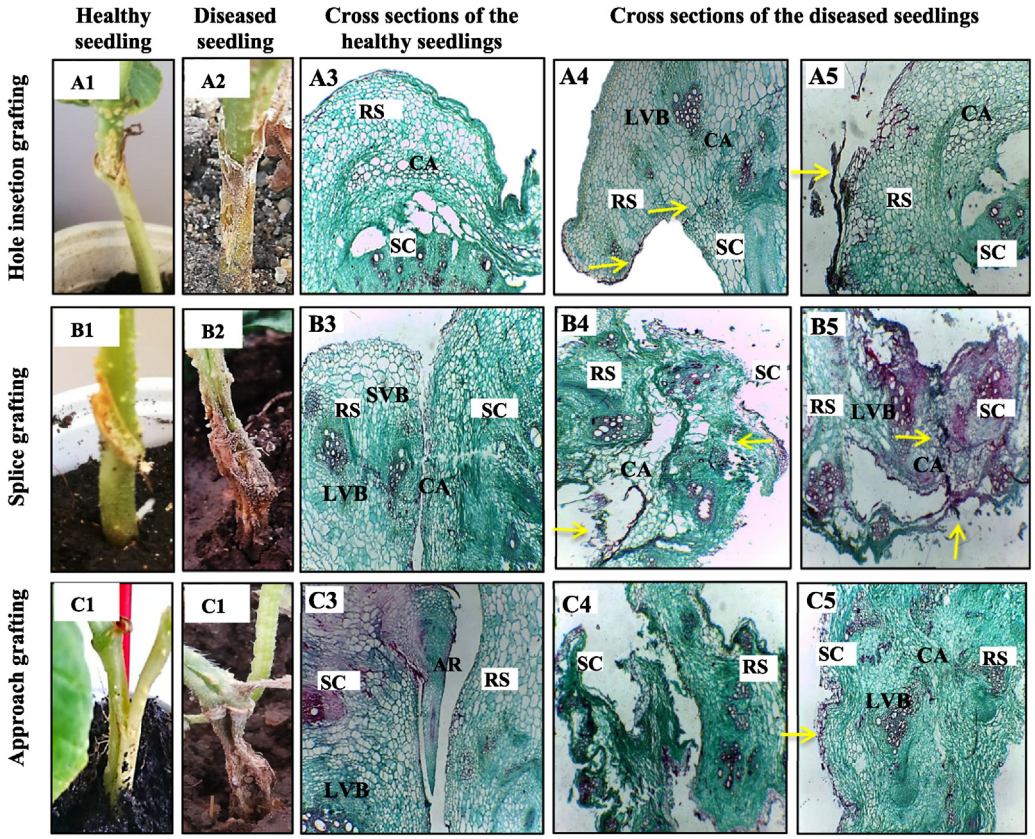

Fig. 5. Cross sections of the grafting union area after 4 weeks from inoculation of cucumber F1 (scion) grafted onto Supershintosa rootstock seedlings using three different grafting techniques (Oc. 10x * Obj. 4x). **Abbreviations RS)** Root stock **SC)** Scion **CA)** Connection Area **LVB)** Large Vascular Bundle **SVB)** Small Vascular Bundle **AR)** Adventitious Roots from the Scion. **The Yellow arrows** show the disruption of the diseased tissues indicating the periderm and cortex tissue discoloration due to the infection. **A4, A5 and C5)** The superficial layers of periderm and cortex tissue discoloration and disintegration. **A4)** Infection progress through the graft union area **B4, B5 and C4)** The pathogen revealed a pattern of progression through the connection area, parenchyma and the vascular tissues showing marked disruption, disintegration and lack of connection between the root stock and the scion.

grafting methods and the maintenance of grafts (Aloni et al., 2008). Rapid callus establishment in grafting place, vascular cambium differentiation, secondary xylem and phloem formation, and eventually vascular linkage between scion and rootstock are all important aspects (El-Gazzar et al., 2017). This in turn, impacts the grafted transplants survival percentage and the rapid resumption of root and shoot growing subsequently, influences the grafted seedlings' resistance performance (Rasool et al., 2020).

Various cucumber grafting methods have been developed. The most successful techniques are **T**ongue **A**pproach **G**rafting (TAG), **H**ole **I**nsertion **G**rafting (HIG), and **S**plice **G**rafting (SG) (Lee and Oda, 2003; Miao et al., 2019; Noor et al., 2019). Splice grafting is a very simple approach that achieves rapid and strong connect, enhanced graft union area, and good compatibility, when compared to other grafting procedures (Khankahdani et al., 2012; El-Gazzar et al., 2017; Noor et al., 2019). On the other hand, Miao et al. (2019) observed that, the scion was tightly connected to the rootstock at the graft union of plants grafted using HIG and TAG, compared with SG. The applied grafting method had an impact on the grafting success rate. For example, when the top grafting method was utilised instead of the tongue approach grafting method, the cucumber plants had a lower survival percentage (Al-Debei et al., 2012). The differences in grafting success rates achieved by various grafting techniques could be due to variances in the contact surface between the cambium layers of both the scion and rootstocks, which could lead to scion death. These results agree with Lee and Oda (2003).

Unfortunately, Al-Debei et al., 2012 observed that, the survival ratio of the grafted cucumber seedlings was decreased because of infection with soil-borne pathogens. They indicated that plants grafted on Strongtosa and Shintosa supreme had 100% survival percentage, where 32–43% were attacked by *Rhizoctonia solani* and *Pythium*. The present results indicated that cucumber grafting methods and the virulence of *Fusarium* species can significantly influence the disease incidence and severity of the inoculated seedlings, as evidenced by Table 1 and Figs. 2, 3 and 4. Different grafting methods resulted in varied structural development of the graft union formation, subsequently varied histopathological features. The pathogenic mechanisms of *Fusarium* spp. are also very complicated and varied, possibly because of the effect of several pectolytic enzymes. This fungus generates a lot of pectic enzymes, which are involved in the degradation of host tissues. A large variety of *Fusarium* spp. produce inducible pectinases (Zamani, et al., 2001). In general, pectic enzymes produced by *Fusarium* spp. are necessary for the grafted seedlings infection and disease progression. *Fusarium* spp. invaded epidermis, ground tissue and vascular system of the diseased seedlings. The pathogen can reach the grafting union area through the rootstock infection leading to seedling death. The pathogen also can directly penetrate the grafted seedlings through the wounds in the graft junction area between the rootstock and cucumber scion. The severity of infection increases when the grafted seedlings were inoculated with *F. equiseti* compared with *F. proliferatum*, thus seedlings infected with *F. equiseti* were used for further histopathogical and biochemical studies (Seo and Kim, 2017).

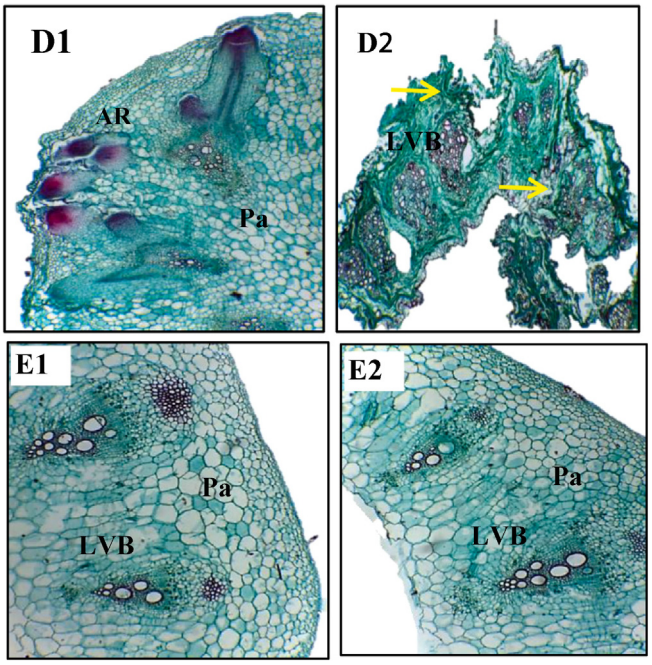

Fig. 6. Cross sections on the stem base of the healthy and diseased self-rooted cucumber and supershintosa seedlings after 4 weeks from inoculation (Oc. 10x * Obj. 4x). **D1)** Healthy scion **D2)** Diseased scion **E1)** Healthy rootstock **E2)** Diseased rootstock**. The Yellow arrows** show the disruption of the diseased tissues. **Abbreviations LVB)** Large Vascular Bundle. **AR)** Adventitious Roots from the Scion. **Pa)** Parenchyma. The Yellow arrows show the disruption of the diseased tissues. **D2)** The periderm and cortex tissue discoloration and disintegration due to the production of lytic enzymes in the infected tissues causing the complete rot of the susceptible scion tissues.

The histopathological studies indicated that the pathogen caused tissue discoloration and disintegration in the infected grafting unions (Fig. 5). This might be due to the production of pectic compounds. The same outcomes were obtained by (Shahriar et al., 2011). This harmful effect can be in the superficial layers of cortex, or in whole graft junction reaching the vascular stele connection. Three main structural phases of graft formation between scion and rootstock have been documented, (1) ruptured cells collapse to form a necrotic layer, (2) cells proliferate to form callus, and (3) callus cells differentiate into vascular tissue to reconnect the phloem and xylem across the graft junction (Miao et al., 2019). In each grafting technique, grafting development includes the establishment of a necrotic layer and its successive stock and scion cohesion, then the differentiation of graft-connecting vascular tissue and cambium (Xu et al., 2015). The formation of necrotic layer was attributed to the tissues that were wounded during cutting and was considered as a protective way to prevent pathogen invasion. The necrotic layer appeared 3–5 days after grafting (Miao et al., 2019). According to these findings, the graft wound healing by generating the necrotic layer, as well as the level of pathogen pectic compounds can be considered as two major factors of the graft union infection in the grafted cucumber seedlings (Fig. 5) (Seo and Kim 2017; Miao et al., 2019).

Variations were noticed in the enzymes activities and total phenols following the infection of the grafted seedlings, and this could affect the stem rot disease incidence and severity. Grafting, as a wounding stress, activates antioxidant defense mechanisms. It has the potential to disrupt the dynamic balance of reactive oxygen species (ROS) metabolism in plants. Grafting may elevate the ROS level of cucumber seedlings, and this was also observed in grafted melon (Aloni et al., 2008). Increased ROS may prevent the development of pathogenic microorganisms at the damage site, allowing the wound to lignify more quickly (Rasool et al., 2020). This could be a positive response to the external damage of the plant itself. Recent researchers have found that the activities of defense-activated enzymes in grafted seedlings of other species were higher than in self-rooted seedlings, suggesting that this could be advantageous in removing ROS produced by wounding and protecting plants from grafting damage (Xu et al., 2015). Several antioxidases play a part in the xylem lignification of the recently differentiating vascular system after the graft connection (Quiroga et al., 2000). The increased activities of defensive antioxidant enzymes in the cucumber seedlings grafted onto resistant rootstock reflected their strong scavenging capability for ROS, which improved their resistance to the stem rot pathogen. After grafting, the antioxidative redox enzymatic system (PO, PPO, and CAT) could efficiently keep ROS at a constant level. Because of the wounding reaction, PO, PPO, and CAT activities were greatly increased after 1 day grafting, according to Miao et al. (2019). This fact was indicated in the present work, as the values of PO, PPO, and CAT were elevated in the grafted cucumber seedlings being, 1.457, 0.016 and 2.507 U min^{-1} mg^{-1} protein, respectively when compared to the non-grafted cucumber being, 1.285, 0.009 and 2.476 U min^{-1} mg^{-1} protein (Table 2). Plants, on the other hand,

Table 2
Biochemical factors affecting stem rot disease resistance of the grafted cucumber seedlings compared with the non-grafted cucumber and rootstock seedlings.

Treatment		PO activity Δ_{470} U min^{-1} mg^{-1} protein	PPO activity Δ_{390} U min^{-1} mg^{-1} protein	CAT activity Δ_{240} U min^{-1} mg^{-1} protein	Total Phenolic compounds (mg/g)
RS	H	1.429 ^{c}	0.030 ^{b}	2.533 ^{b}	24.478 ^{f}
	D	2.554 ^{ab}	0.030 ^{b}	2.544 ^{b}	46.693 ^{b}
G	H	1.457 ^{c}	0.016 ^{c}	2.507 ^{b}	33.968 ^{d}
	D	**2.900** ^{a}	**0.046** ^{a}	**2.908** ^{a}	**52.731** ^{a}
SC	H	1.285 ^{c}	0.009 ^{c}	2.476 ^{b}	31.272 ^{e}
	D	1.847 ^{bc}	0.012 ^{c}	2.525 ^{b}	36.448 ^{c}
LSD $P = 0.05\%$		1.0487	0.0135	0.1984	1.1931

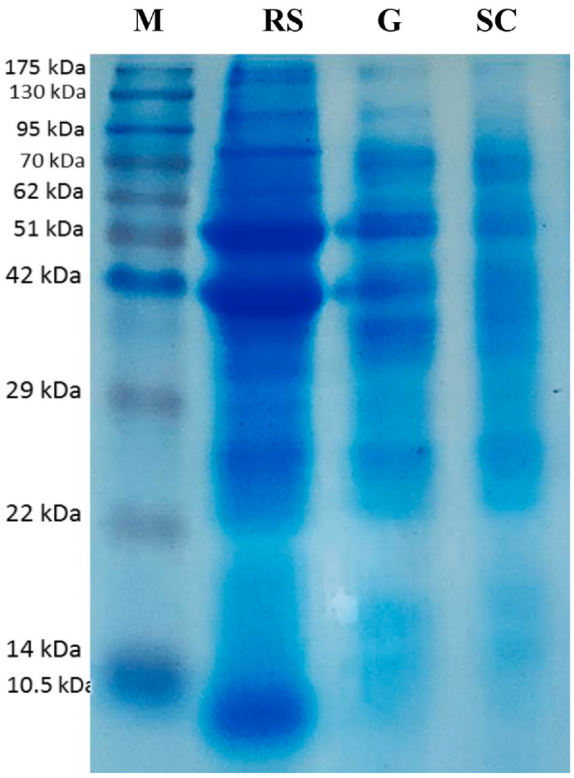

Fig. 7. The SDS-PAGE profile of the healthy grafted cucumber seedlings and self-rooted scion and rootstock. **RS**: Rootstock. **G**: Grafted seedling. **SC**: Cucumber scion.

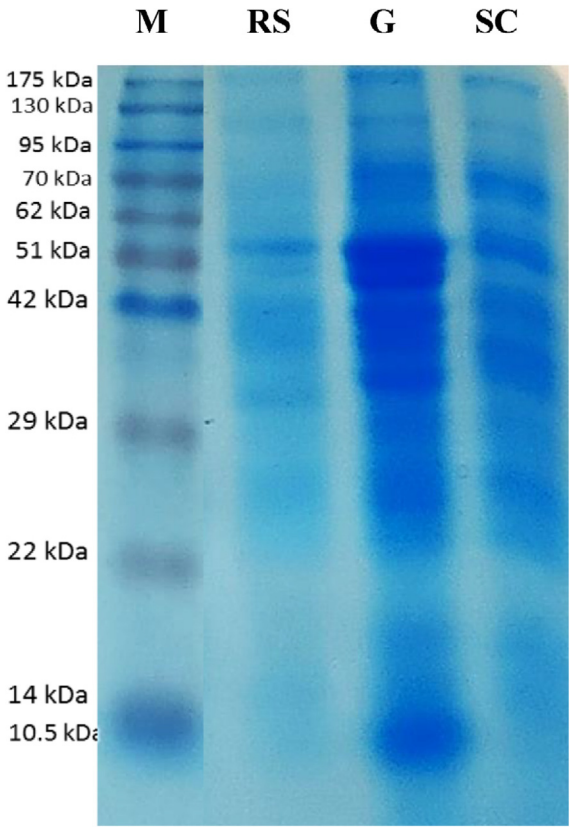

Fig. 8. The SDS-PAGE analysis for the total proteins of the diseased grafted cucumber seedlings and self-rooted scion and rootstock. **RS**: Rootstock. **G**: Grafted seedling. **SC**: Cucumber scion.

Table 3
The scoring data for SDS-PAGE protein analysis of the grafted and non-grafted cucumber seedlings comparing with the self-rooted scion and rootstock before and infection with *F. equiseti*.

MW	Rootstock		Scion		Grafted rootstock\scion	
	H	D	H	D	H	D
170	1	1	0	1	1	1
150	1	0	0	0	0	0
120	1	1	0	1	1	1
82	1	1	1	1	1	1
66	1	0	0	0	0	0
62	0	0	0	0	1	0
55	1	1	1	1	1	1
45	0	1	0	0	0	0
40	1	1	1	1	1	1
35	1	1	1	1	1	1
30	1	1	0	1	1	1
26	1	1	0	1	0	1
24	1	1	1	1	1	1
22	1	0	1	1	1	1
21	0	0	1	1	1	1
20	1	0	0	0	0	1
Total	13	10	7	11	11	12

Table 4
Total number of negative and positive unique bands in the in grafted and self-rooted cucumber scion and rootstock occurred due to the infection with the stem rot pathogen.

Plant materials	Negative band	Positive band
Rootstock	4	1
Self-rooted cucumber scion	0	4
Grafted rootstock\scion	1	2

apply resistance to reduce the impact of pathogens or their damaging components. Changes in antioxidant activity as a result of pathogen infection are referred to constitutive enzymatic reactions to pathogen attack (Table 2) (Moghbeli et al., 2017). Here, we report significant variations between the grafted and self- rooted cucumber in the antioxidant enzymes activities and polyphenols. As well as, significant induction of these antioxidant factors was observed after infection in both grafted and self-rooted cucumber, confirming their role in decreasing the disease severity.

Peroxidase contributes to the generation of reactive oxygen species (ROS), which are directly harmful to pathogens, and indirectly reduces pathogen transmission by increasing the cross-linking and lignification of plant cell walls. Peroxidase catalyzes the synthesis of lignin and other oxidative phenols, which are involved in the creation of defense barriers and cell structure strengthening. The activities of peroxidase and polyphenol oxidase are found to rise in response to pathogen infection (Madadkhah et al. 2012). Polyphenol oxidase, in similar way, help to minimize the ROS damage by scavenging free radicals, changing cell wall composition, and collecting antimicrobial secondary metabolites, all of which are significant in systemic acquired resistance (Torres et al. 2006). Peroxidase and PPO are important components of the pathogen defense system as they participate in the oxidation of polyphenols to quinones, which increases antimicrobial activity and inhibits pathogen growth. In response to infection, both susceptible and resistant cucumber hybrids increased their levels of PPO and PO activity, as well as PCs. However, the activity increase in the resistant hybrids was more considerable, therefore it could

be assumed a biochemical indicator for resistance (Madadkhah et al., 2012). The activity of CAT was also dramatically elevated in rootstock-grafted plants when they were infected with *Fusarium* sp. Increased CAT activity in rootstock-grafted plants may aid to scavenge the toxic levels of ROS caused by biotic and abiotic stress, as well as increase plants' tolerance (Liu et al., 2014).

Flavonoids are non-enzymatic molecules that may have a significant role in reducing oxidative stress. Plants' defensive systems against injury and infection usually include phenolic compounds (Evrenosoglu et al., 2010). They are frequently produced under biotic or abiotic stresses, and they have a role in a variety of metabolic processes (Xu et al., 2015). Thus, Phenolic compounds were always synthesized at the graft interface (Pina and Errea, 2008) and can be considered as chemical indicators for the early recognition of graft compatibility. Phenols escape from the vacuole into the cytoplasmic matrix where they are oxidized by POD and PPO (Hartmann et al., 2002). The present findings for PO, PPO, and CAT activities, as well as the rise in PCs following infection strongly suggest that they play a role in the grafted cucumber's direct defense mechanisms against the pathogen (Miao et al., 2019; Reyad et al., 2021).

Results of The SDS-PAGE revealed variations in the leaves protein profile of the grafted and self- rooted cucumber as well as variations were observed in all inspected plant materials due to stem rot infection. Four protein bands appeared in the healthy grafted cucumber leaves, while they were absent in the self- rooted cucumber scion. It is suggested that might be related to the grafting process, confirming graft-induced changes via rootstock-scion interactions that improve the quantity and quality of grafted plants (Tsaballa et al., 2021). Proteins induced by grafting can enhance the photosynthesis, the Carbohydrate metabolism, energy production, the biosynthesis of protein and nucleic acid as well as the defense response against the biotic and a biotic stress. Proteins involved in defense response such as antioxidant-related proteins (peroxidase) and catalase are responsible for the regulation ROS level when plants are under stress to stimulate other resistance pathways (Xu et al., 2018, Zhang et al., 2021).

Rasool et al. (2020) reported long-distance protein transport across the graft union in grafted plants, which regulates their growth and development and also plays a key role in stress resistance. Researchers confirmed the movement of proteins, hormones, RNAs, and secondary metabolites over long distances by grafting different genotypes to each other (Goldschmidt, 2014; Tsaballa et al., 2021). Proteomic analysis in the grafted cucumber scions in exposed to heat stress revealed the accumulation of 77 distinct proteins linked with critical processes like photosynthesis, energy metabolism and nucleic acids synthesis, which reduced the negative impact of heat stress on scion growth (Xu et al., 2018). The transportation of the DNA expression molecules, like RNA and proteins, through the vascular system can also be responsible for graft-induced molecular alterations. (Tsaballa et al., 2013; Wang et al 2017; Tsaballa et al., 2021).

The present study also revealed differences in protein bands between the control and infected treatments. Alternations in the types and levels of cell wall proteins, proteinase inhibitors, hydrolytic enzymes as well as pathogenesis-related proteins and phytoalexin biosynthetic enzymes appear to have a significant function in Fusarium wilt defense (Klessig et al., 1998). To combat against cellular metabolism stress that is triggered by *Fusarium* spp., plants respond by reprograming their transcriptome, proteome, which changes the amounts of several proteins. Stress responsive proteins are responsible for the detoxification of a variety of stressors (Yuan et al., 2011). Also, defense/stress-response proteins are thought to have a function in graft healing (Muneer et al., 2016).

5. Conclusion

Fusarium spp. remarkably affected the grafted cucumber seedlings in Egypt, causing severe stem rot through affecting at the connection area, leading to complete seedling death. Our study provides histopathological, physiological and molecular evidences of the grafted and non-grafted plants' response to Fusarium stem rot. The results confirmed the role of antioxidant enzymes and total phenols in the defense reaction of the grafted seedling. Variations in the leaves protein profile of the grafted and self-rooted ones after Fusarium infection may indicate the role of some proteins in adapting this biotic stress.

CRediT authorship contribution statement

Soha Sabry: Writing – review & editing.**Ahmed Z.Ali:** Writing – review & editing.**Dawlat A. Abdel-Kader:** Writing – review & editing.**Mohamed I. Abou-Zaid:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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