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EFFECT OF SOME ORGANIC ACIDS ON GROWTH, YIELD, OIL PRODUCTION AND ENHANCING ANATOMICAL CHANGES TO REDUCE FUSARIUM WILT OF *Nigella sativa* L. PLANT

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Abstract

The present research was conducted during 2017/2018 and 2018/2019 growing seasons in the Plant Pathology Research Institute, Giza, Egypt to investigate the effect of organic acids each alone (*i.e.* citric, lactic and malic acids at concentrations: 700, 1000 and 1300 ppm) compared with the untreated control on the growth, fruiting, seed yield, volatile oil production and fixed oil percentage of black cumin; *Nigella sativa* L. plant under artificial infection with *Fusarium oxysporum* the causal agent of wilt disease. All mentioned organic acids reduced the mycelium growth of *Fusarium oxysporum* on Potato Dextrose Agar media (PDA) plate. This reduction was noticeably increased with increasing in concentration of all organic acids under study. In pot experiment, lactic and malic acids were more effective than citric acid in controlling the disease. All concentrations of lactic and malic acids (especially at 1300 ppm concentration) gave the lowest percentage in disease severity. The results indicated that the individual application of those acids significantly increased the plant height, number of branches/plant, number of capsule/plant, as well as fixed and volatile oil percentages compared to infected control in the two seasons. Also, malic acid was the most effective treatment at concentration 1300 ppm in both seasons. Moreover, the highest percentages of limonene and thymoquinone (the main constituents of volatile oil) were achieved with this treatment. *Fusarium oxysporum* caused a negative effect on anatomical structure of *Nigella sativa* L. stem. Infected stems showed reduction in thickness of all internal stem tissues. The highest concentration of all acids used caused an enhancement in stem anatomical features and internal tissue. Infected plants treated with high concentration of malic acid recorded high thickness in epidermis, xylem vessels diameter as well as phloem thickness compared to healthy and infected plants.

Key words: *Nigella sativa* L., wilt, yield, organic acids, oil, stem anatomical features

Introduction

Black cumin (*Nigella sativa* L.), a member of the Ranunculaceae family growing on the Mediterranean coasts, is an annual herb with beautiful blue or white flowers. *Nigella sativa* L. forms capsule fruits that contain trigonal seeds (Randhawa and Alenazi, 2016). *Nigella sativa* L. seeds and their oil have long history of folklore usage in various systems of medicines and food (Khalid and Shedeed, 2015). Moreover, *Nigella sativa* L. is reported to possess numerous pharmacological properties as antithypertensive, liver tonics, antidiarrheal and antimicrobial (Ahmad *et al.,* 2013). *Nigella sativa* L. seeds contain more than 100 natural compounds; *i.e.* nigellone, thymoquinone, phytosterols, fatty acids, vitamins and minerals (Boseila and Messalam, 2011).

Wilt diseases are the most widespread and destructive soil borne diseases, which attack large number of plant species worldwide. Wilt symptoms expressed when the causal pathogens infect the roots of susceptible plant and plug the water conducting tissues, causing about 90% loss in the yield (Patoky *et al.,* 2000 and Deacon, 2004). The genus *Fusarium* includes the important wilt causal fungal pathogens. Dubey (1995) recorded Fusarium wilt disease of *Nigella sativa* L. in India. Hilal *et al.,* (1994) published the first report on root diseases of *Nigella sativa*

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L. in Egypt. They isolated *Fusarium moniliforme*, *F. oxysporum*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Alternaria* spp. and *Nigrospora* spp. Pathogenicity tests indicated that *F. oxysporum* and *M. phaseolina* were the most virulent on (*Nigella sativa* L.). El-Wakil and Ghoneem (1999) isolated internal and external seed-borne fungi and showed that *Fusarium oxysporum, F. solani* , *Verticillium* sp. and *F. moniliforme* were widespread with a higher percentage of pre-and post-emergence damping-off developed as a result of seed inoculation.

Chemical fungicides may not always be desirable; excessive and improper use of these fungicides presents a menace to the health of humans, animals and environment. Many of these chemicals are also too expensive for poor farmers (Shabana *et al.,* 2004). In this study, three organic acids (citric, lactic and malic acids) were tested as safe alternatives to those chemical fungicides.

Organic acids are weak acids and do not dissolve completely in soil solution. They can be classified into two major groups based on their molecular weight; the high and low molecular weights. Organic acids belong to the 2nd group, usually contain one to three carboxylic functional groups. Citric, lactic and malic acids are examples of this group (Mimmo *et al.,* 2008 and Jiang *et al.,* 2012). Citric acid is vital organic acid, which serves as the source of carbon skeleton and cellular energy (Da Silva, 2003). Lactic acid plays as a plant growth regulator (Kinnersely *et al.,* 1990). While, malic acid is an organic dicarboxylic acid found in all living organisms; it is involved in many biochemical pathways and plant defense mechanisms (Gowda *et al.,* 2004). Kinnersely *et al.,* (1990) showed that the growth increased in marigold and sunflower when tissue cultures were treated with citric, lactic and malic acids as they have shown promising antimicrobial properties. Therefore, they have been tested for their use against food borne pathogens (Dickson, 1992 and Eswaranandam *et al.,* 2004).

The objective of this work was to study the effect of the separately exogenous application by citric, lactic and malic acids on enhancing the growth and increasing the yield as well as the oil production, in addition to the anatomical characteristics to reduce Fusarium wilt of *Nigella sativa* L. plant.

Materials and Methods

Isolation and identification of seed-borne fungi

Seed samples of *Nigella sativa* L. were collected from the commercial markets located in different governorates of Egypt (Assiut, Giza and Qalubiya) during the season 2017/2018 and used in this study. These experiments were conducted at Plant Pathology Research Institute.

The detection of seed-borne mycoflora was carried out following the rules of the international seed testing association (ISTA, 1993). One hundred seeds for each sample were tested using the standard blotter method.

The incubated seeds were examined under a stereoscopic light microscope after 7 days of incubation for the presence of fungi and their morphological characteristics were recorded. Whenever necessary, a compound light microscope was used to confirm identification by examining the morphology of fungal structures including conidia and conidiophores...etc. The seed-borne fungi were identified using the Commonwealth Mycological Institute, Key Surrey, England (CMI) description sheets, (Singh *et al.,* 1991). Using a stereoscopic microscope, isolated hyphal tips from *Fusarium* spp. cultures were cut and cultivated on potato dextrose agar plates using the tips of heat-stretched capillary tubes. Pure cultures of the fungi were obtained and all isolates were preserved on PDA slants then stored in a refrigerator at (4ºC).The percentage of frequency for the isolated fungal species was calculated according to the following equation:

$$\text{Frequency\%} = \frac{\textit{No.of colonies per each fungus}}{\textit{Total no.of colonies for all fungi}} \times 100$$

The antifungal activity of citric, lactic and malic acids on the linear growth of the tested fungi

Citric acid (99.5%), lactic acid (95%) and malic acid (98%) were obtained from EL-Gomhoriya Company for Trading Chemicals and Medical Appliances (GOMAC).

Stock solutions of 700, 1000 and 1300 ppm of citric, lactic and malic acids, respectively were prepared at laboratory. From the stock solutions 1 ml was transferred into 9 cm sterilized Petri dishes and amended with 8 ml PDA media. The prepared plates were then inoculated after solidification with 0.5cm grown fungal disc taken from 7 days old culture of each tested fungus at the center of the plate. PDA medium plates free from acids were kept as control. Five plates were used as replicates for each particular treatment. All plates were incubated at 25+2ºC. Once the fungus mycelium of any of the tested fungi filled up the plates of any concentration, the fungal radial growth (cm) was measured for all treatments. The percentages of the fungal growth inhibition were calculated according to the following formula:

$$\textbf{Inhibition (\%) = C-T×100/C}$$

C = Growth diam. (cm) of control and T = Growth diam. (cm) of treatment.

Pathogenicity test

Three *Fusarium oxysporum* isolates were used in this experiment. Each isolate was separately allowed to grow in 500ml bottles, each containing about 200g of sorghum seeds soaked overnight in distilled water. The bottles were sterilized and inoculated, each with one fungal disc (5 mm) taken from the edges of the desired *F. oxysporum* isolate and incubated at 25±1°C for two weeks (El-Abeid *et al.*, 2020). The experiment consisted of four treatments, each was prepared in triplicates and each replicate consisted of nine pots (27 seeds). Pots (30 cm-diam.) filled with sterile clay and washed sand (1:1v/v) were infested with 3% (w/w) of the desired pathogen and control pots were inoculated with sorghum free pathogen. The infested pots were irrigated two times, during the winter season. Daily observations for germination and symptoms of pre- and post-emergence damping off and wilted plants were recorded after two months of post inoculation.

Stimulating activity of some organic acids for plant growth, yield, oil production, disease severity and anatomical changes that reduce Fusarium wilt in *Nigella sativa* L. plant

Experiment site

These experiments were conducted at Plant Pathology Research Institute during two successive seasons 2017/2018 and 2018/2019.

Experiment layout

The layout of the experiment was designed in complete randomized blocks included eleven treatments, each treatment was replicated three times and every replicate consisted of nine pots (1plant/pot).

The analysis of variance (ANOVA) was conducted and the means of the treatments were compared using L.S.D. at 5%, the statistical analysis according to (Snedecor and Cochran, 1980).

Plant materials and chemical fertilization

The seeds of *Nigella sativa* L. were obtained from the Experimental Farm of Medicinal and Aromatic Plants Research Department, El- Kanater El- Khairia and were sown on 22th October of the two seasons. Chemical fertilizers (NPK) were added as ammonium sulphate (20.6% N), calcium superphosphate (15.5% P_2O_5) and potassium sulphate (48%K_2O) at the recommended level in three doses. The 1st dose was for all phosphorous amount which was added during soil preparation, the rest (NK) were applied in the two equal doses, the 1st was applied 45 days after sowing and the 2nd was added 30 days later.

Treatments

Pots (30cm-diam) filled with sterile clay and washed sand (1:1v/v) were infested with 3% (w/w) of the pathogen whereas healthy control pots were inoculated only with barely free pathogen and all the pots were irrigated twice. Citric, lactic and malic acids were tested at different concentrations (700, 1000 and 1300 ppm) for controlling wilt disease caused by *Fusarium oxysporum*. Moreover, these organic acids were added three times over a course of time. The 1st was applied after sowing and repeated twice after 30 days interval.

1. Infected control plants grown in soil infested with *Fusarium oxysporum* and sprayed with tap water.

2. Healthy control plants grown in un-infested soil and sprayed with tap water only.

3. Plants grown in soil infested with *Fusarium oxysporum* and treated with citric acid at concentrations 700, 1000 and 1300 ppm each alone.

4. Plants grown in soil infested with *Fusarium oxysporum* and treated with lactic acid at concentrations 700, 1000 and 1300 ppm each alone.

5. Plants grown in soil infested with *Fusarium oxysporum* and treated with malic acid at concentrations 700, 1000 and 1300 ppm, each alone.

Data recorded

The following data were recorded

1. Disease severity. The degree of wilt was evaluated based on the index value of 0 to 3 visual scale according to (Vakalounakis and Fragkiadakis, 1999). The disease severity (DS) values were calculated based on the following formula:

DS (%) = [sum (class frequency×score of rating class)]/[(total number of plants)×(maximal disease index)]×100.

2. Plant height (cm).

3. Number of branches/plant.

4. Number of capsule/plant. 5. Seed yield/plant (g).

6. Seed index as weight of 100 seeds.

7. Volatile oil percentage was determined according to (British Pharmacopeia, 1963).

8. Chemical composition of volatile oil, the main constituents of *Nigella sativa* L. seeds volatile oil were determined by subjecting oil samples of the 2nd season to gas liquid chromatography (GLC) analysis as recommended by (Hoftman, 1967 and Bunzen, 1969).

9. Fixed oil percentage was estimated according to (A.O.C.S., 1964).

Samples preparation for anatomical studies

Samples of the middle internode of the main stem were taken throughout the 2nd season at the age of 10 weeks. Tested materials were killed and fixed in FAA (10 ml formalin- 5 ml glacial acetic acid- 85 ml ethyl alcohol) for at least 48h, rinsed in 50% ethyl alcohol and dehydrated in butyl alcohol series. Specimens were embedded in paraffin wax. Samples were sectioned in a rotational microtome. For staining process, double stained with crystal violet-erythrosin was applied. Finally, sections were cleared in xylene and mounted in Canada balsam (Nassar and El-Sahar, 1998).

Results and Discussion

Isolation and identification of seed-borne fungi

Isolation, identification and frequency of the associated fungi

The microscopic examination indicated that four different fungi were belonging to four different taxonomical fungal groups were isolated from the collected seeds from Assiut, Giza and Qalubiya governorates, These fungi were morphologically identified as *Alternaria alternata, Fusarium oxysporum, Epicoccum* sp. and *Nigrospora* sp. table 1. The frequency of isolated fungi ranged from 0 to 80%. *Fusarium oxysporum* was commonly recorded in all *Nigella sativa* L. collected samples and showed the highest occurrence in Qalubiya governorate, being 48%. These findings are in agreement with (Mohamed *et al.,* 2017) who indicated that Fusarium wilt of *Nigella sativa* L. caused by *F. oxysporum* was commonly recorded in *Nigella sativa* L. seed samples collected from Qalubiya governorate. On the other hand, *Nigella sativa* L. wilt is among the major diseases that caused by a soil and seed borne vascular wilt pathogen "*Fusarium* sp." Those data are also in consistent with previous studies (Fatima and Khot, 2015) who indicated that *F. oxysporum* was commonly present in all their forty collected samples of *Nigella sativa* L. It is well Known that *Nigella sativa* L. wilt caused by *F. oxysporum* is one of the most important diseases in Egypt (Sharma and Meena, 2012). However, *Alternaria alternata* showed the highest frequency in samples collected from Assiut and Giza governorates, being with 40- 80%, respectively.

Table 1: Frequency of fungi isolated from seeds of *Nigella sativa* L. collected from different governorates.

Governorates	Assiut,(A)	Giza (G)	Qalubiya (Q)
Fungi	**Frequency(%)**	**Frequency(%)**	**Frequency(%)**
Alternaria alternata	40	80	38
Fusarium oxysporum	10	2	48
Epicoccum sp.	16	0	0
Nigrospora sp.	34	18	14
Total	100	100	100

The antifungal activity of citric, lactic and malic acids on the linear growth rate of *Fusarium oxysporum* Assiut isolate

Data presented in table 2 show the differences in activity of the tested organic acids, *i.e*. citric, lactic and malic acids against *Fusarium oxysporum*.

In general, it was noticed that the increasing in organic acids concentration result in a visible increment in the percentage of the mycelial growth inhibition. Where, it was noticed that for the three organic acids, maximum influence was observed at 1300 ppm. On the other hand, citric and malic acids showed the highest inhibition for the *Fusarium oxysporum* mycelial growth values (82% and 75.5 %, respectively) followed by lactic acid being 74.6%. In this regard, some studies indicated that citric acid inhibited growth of filamentous fungi and all pathogens studied rather than yeasts (Shokri, 2011). Several researches also documented the inhibitory activity of acids, such as saturated fatty acids, formic and propionic acids, lactic acid and medium chain fatty acids against numerous microorganisms (Cherrington *et al.,* 1990; Dibner and Buttin, 2002).

Table 2: The antifungal activity of citric, lactic and malic acids on the linear growth rate of the tested fungi.

Treatments	Linear growth (cm.) *Fusarium oxysporum**	Inhibition%
Control (water treated)	9.0	0
Citric acid (100 ppm)	3.6	60
Citric acid (700 ppm)	3.1	65
Citric acid (1000 ppm)	1.9	79
Citric acid (1300 ppm)	1.6	82
Lactic acid (100 ppm)	2.8	68.4
Lactic acid (700 ppm)	2.5	71.8
Lactic acid (1000 ppm)	2.4	73.3
Lactic acid (1300 ppm)	2.3	74.6
Malic acid (100 ppm)	3.5	61.7
Malic acid (700 ppm)	2.9	67.8
Malic acid (1000 ppm)	2.5	73.3
Malic acid (1300 ppm)	2.2	75.5
L.S.D.at 5%	0.85	---

* *Fusarium oxysporum* Assiut isolate (A)

Pathogenicity test

Data concerning the incidence of Fusarium wilt at different stages [pre- and post-emergence damping-off, wilted plants and survivals] are shown in table 3. The pathogenic capability of *F. oxysporum* isolates on *Nigella sativa* L. plants were tested. In general, data presented in table 3 indicated that all fungal isolates were able to infect *Nigella sativa* L. plants. *Fusarium oxysporum*[A] Assiut isolate showed the highest percentage of disease incidence % on *Nigella sativa* L. plants. This highly pathogenic isolate was selected as the most virulent to complete the study.

Another study in Egypt stated that there were differences among the various pathogenic fungi that are transmitted by seeds in their effect on germination and seedling growth. Seeds treated with *F. oxysporum* caused a higher incidence of seedling infection than using the soil infestation technique (El-Wakeel and Ghoneem, 1999). Tawfik and Allam **(**2004) isolated Fusarium wilt isolates and found significant variations in disease incidence in cumin and some isolates were nonpathogenic. Variation was reported in mycelial growth in addition to the pathogenicity of nine isolates of *Fusarium oxysporum* f. sp. *cumini* in India (Champawat and Pathak, 1989).

Table 3: Percentages of infection of *Nigella sativa* L. plants grown in soil infested with various *Fusarium oxysporum* isolates.

Fungi	Disease incidence %			
	Pre	**Post**	**Wilted plants**	**Survival Plants**
F. oxysporum[A]	33.3	7.4	37	22.3
F. oxysporum[G]	11.1	0	11.1	77.8
F. oxysporum[Q]	22.2	3.7	29.6	44.5
Control	0.0	0.0	0.0	100
L.S.D. at 5%	30.71	3.06	29.34	23.0

Stimulating activity of the organic acids for plant growth, yield, oil production, reduction disease severity and anatomical changes that reduce Fusarium wilt in *Nigella sativa* L. plant

This experiment was conducted to study the influence of organic acids treatments on the reduction of severity of Fusarium wilt in *Nigella sativa* L. plant that was associated with increasing growth, fruiting, seed yield and volatile oil production as well as fixed oil percentage.

Effect of citric, lactic and malic acids on percentage of disease severity

In pot experiment, *Nigella sativa* L. plants were treated with three different concentrations (700, 1000 and 1300 ppm) of three organic acids namely citric, lactic and malic acids as described under "Materials and Methods" to study their effect on the percentage of disease severity. Results in table 4 showed that the tested organic acids caused significant reduction in the disease severity (%) compared with infected control grown in soil infested with *Fusarium oxysporum*. Efficacy of the tested organic acids was found to be variable according to the concentration and the type of the tested acids. Lactic and malic acids at all concentrations especially at 1300 ppm were the most effective treatments where they scored the lowest disease severity percentages, 3.7 % at 1st season and 5.9 % and 7.4 %, respectively at 2nd season. Meanwhile, citric acid recorded the highest percentages of the disease severity, 8.1 % at 1st season and 7.4 % at 2nd season, respectively).

Shokri (2011) showed that some organic acids as citric acid were more active against filamentous fungi which may be related to the different structures of the fungal cell walls. The main target of organic acids and its relatives are cell wall and membrane proteins. If proteins are considered to contribute to the transport through the membrane, the first-named organisms may be affected to a lower concentration. In conclusion, citric and tartaric acids are active antifungal agents *in vitro*. On the other hand, the effectiveness of malic acid because it is dicarboxylic acid and therefore has active sites. It also has the ability to prevent the development of *Fusarium oxysporum*. Also lactic acid, is an alpha-hydroxy acid due to the presence of a hydroxyl group adjacent to the carboxyl group. Hsiao and Siebert (1999) referred that chemical and physical properties of organic acids which reduces the development and growth of microorganisms.

Table 4: Effect of citric, lactic and malic acids on Fusarium disease severity percentage % in *Nigella sativa* L .plants during 2017/ 2018 and 2018/2019 seasons.

Treatments	Disease severity %	
	1st season	**2nd season**
Infected control	30.4	35.8
Healthy control	0	0
Citric acid (700 ppm)	24.4	21.5
Citric acid (1000 ppm)	17.0	11.1
Citric acid (1300 ppm)	8.1	7.4
Lactic acid (700 ppm)	4.4	11.1
Lactic acid(1000 ppm)	11.1	6.7
Lactic acid (1300 ppm)	3.7	5.9
Malic acid (700 ppm)	10.4	14.1
Malic acid (1000 ppm)	5.9	7.4
Malic acid (1300 ppm)	3.7	7.4
L.S.D. at 5%	15.11	11.56

Infected control = *Nigella sativa* L. plants grown in soil infested with *Fusarium oxysporum* isolate [A] and without any treatments.

Healthy control = *Nigella sativa* L. plants grown in soil uninfested with *Fusarium oxysporum* isolate [A] and without any treatments.

Effect of organic acids on vegetative growth

Plant height and number of branches/plant

Effect of citric acid

Data in table 5 showed that increasing the concentration of citric acid significantly increased plant height and number of branches/plant as compared to infected control in the two seasons. The best results were obtained by using citric acid at 1300 ppm; giving (64.00 and 68.33 cm) and (9.00 and 9.33 branches/plant) in the both seasons. In contrast the lowest values were observed in infected control. Such effect may be attributed to organic acids properties, their molecules auto (ox-reduction), they scavenge the free radicals (Singh *et al.*, 2010). Also, citric acid as non-enzymatic antioxidant destroying the free radicals and protecting plant from injury could result in prolonging the shelf life of plant cells and improving growth characters (Sadak and Orabi, 2015).

Effect of lactic acid

Data in table 5 indicated that in general, *Nigella sativa* L. growth showed stimulation in response to lactic acid expressed as an increment of both plant height and number of branches/plant. However, the best results (76.00 and 81.33 cm) and (11.00 and 11.33 branches / plant) were recorded with 1300 ppm in the two seasons. Infected control (without any treatment) gave the lowest values in both seasons. These results may be due to the ability of lactic acid to assimilate nutrients (Kinnersely *et al.*, 1990) Also, such effect may be attributed to the exogenous application of lactic acid resulted in noticeable reduction of mycelium growth (Humer *et al.*, 2016).

Effect of malic acid

Concerning the effect of malic acid, it was found that *Nigella sativa* L. plants treated with malic acid at 1300 ppm showed a significant increase in both plant height and number of branches/plant as compared to infected control in the two seasons. The highest values of these characters were (87.67 and 90.67 cm) and (13.33 and 13.67 branches/plant) in both seasons. However, the lowest values were noticed from infected control followed by healthy control in this concern table 5. These results are in harmony with (Talebi *et al.*, 2014) mentioned that foliar application of malic acid significantly increased growth of *Gazania rigens* L. These results may be explained by findings of (Lopez–Bucio *et al.*, 2000) reported that malic acid has the potential to facilitate the absorption of phosphorus and iron from the soil.

Effect of organic acids on fruiting (number of capsules/plant)

Effect of citric acid

All concentrations of citric acid; 700, 1000 and 1300 ppm significantly increased number of capsules/plant in both seasons compared to infected control. The best results were observed in the plants treated with citric acid at 1300ppm, followed by healthy control in the two seasons. The differences between infected and healthy controls were highly significant in both seasons table 6. This may be due to citric acid role in mitochondria that creates cellular energy by oxidative phosphorylation reactions (Wills *et al.*, 1981**)**. Additionally, antioxidants have the ability to control many plant diseases (Lin *et al.*, 2007) and stimulate the growth and division of plant cells (Johnson *et al.*, 1999).

Effect of lactic acid

Lactic acid at 1300 ppm significantly increased number of capsule/plant as compared to infected control. The lowest values recorded in case of infected control in the two seasons table 6. Higgins and Brinkhous (1999) mentioned that growth and morphology of fungi are influenced by the pH of media. The application of organic acids result a decrease in pH value, this may influence mycelium growth by acidifying the cell, which will consume a great amount of energy to maintain the intracellular pH homeostasis (Kang *et al.*, 2003).

Table 5: Effect of citric, lactic and malic acids on vegetative growth of *Nigella sativa* L. plants during 2017/ 2018 and 2018/2019 seasons.

Treatments	Growth characters			
	1st season		2nd season	
	Plant height (cm)	Number of branches /plant	Plant height (cm)	Number of branches /plant
Infected control	41.00	4.33	46.67	5.00
Healthy control	62.33	8.33	65.00	8.64
Citric acid (700 ppm)	53.33	7.00	54.00	7.33
Citric acid (1000 ppm)	56.33	7.67	58.67	8.00
Citric acid (1300 ppm)	64.00	9.00	68.33	9.33
Lactic acid (700 ppm)	71.33	9.67	76.00	10.00
Lactic acid(1000 ppm)	73.67	10.33	79.33	10.67
Lactic acid (1300 ppm)	76.00	11.00	81.33	11.33
Malic acid (700 ppm)	80.00	11.67	84.33	12.00
Malic acid (1000 ppm)	84.67	12.33	85.67	12.67
Malic acid (1300 ppm)	87.67	13.33	90.67	13.67
L.S.D.at 5%	2.21	0.63	1.64	0.64

Infected control = *Nigella sativa* L. plants grown in soil infested with *Fusarium oxysporum* isolate [A] and without any treatments.
Healthy control = *Nigella sativa* L. plants grown in soil uninfested with *Fusarium oxysporum* isolate [A] and without any treatments.

Table 6: Effect of citric, lactic and malic acids on fruiting (number of capsules /plant) of *Nigella sativa* L. plants during 2017/ 2018 and 2018/2019 seasons.

Treatments	1st season Number of capsules /plant	2nd season Number of capsules/plant
Infected control	15.33	17.00
Healthy control	31.00	32.00
Citric acid (700 ppm)	20.33	22.33
Citric acid (1000 ppm)	24.33	25.67
Citric acid (1300 ppm)	32.67	34.67
Lactic acid (700 ppm)	37.00	40.33
Lactic acid(1000 ppm)	42.00	43.33
Lactic acid (1300 ppm)	45.33	47.00
Malic acid (700 ppm)	48.33	50.00
Malic acid (1000 ppm)	52.33	54.00
Malic acid (1300 ppm)	56.00	57.33
L.S.D. at 5%	1.87	2.09

Infected control = *Nigella sativa* L. plants grown in soil infested with *Fusarium oxysporum* isolate A and without any treatments.

Healthy control = *Nigella sativa* L. plants grown in soil uninfested with *Fusarium oxysporum* isolate A and without any treatments.

Effect of malic acid

The application of malic acid gradually increased number of capsules/plant in the two seasons. The most effective treatment was 1300 ppm, which gave (56.00 and 57.33 capsules/ plant) in the 1st and 2nd seasons, respectively. The lowest values were observed in infected control table 6.These results are in agreement with (Jafari and Hadavi, 2012) revealed that plant growth parameters as well as tolerance to powdery mildew were increased by the combination of 0.3 % citric acid and 0.1% malic acid. Such effect may be attributed to that endogenous organic acids are the source of both carbon skeleton and energy for cell used in respiratory cycle and other biochemical pathways. Malic acid is metabolized in plant mitochondria by reaction of malic enzyme (Day and Hanson, 1977). Malate is a common reserve anion playing a role in plant vacuole as counter ion for K and Ca (Ting, 1981).

Effect of organic acids on seed yield and seed index as weight of 100 seeds

Effect of citric acid

The exogenous application of citric acid significantly increased seed yield /plant and seed index as weight of 100 seeds compared with infected control in both seasons. The best results were recorded when the plants treated with 1300 ppm, giving (9.96 and 10.34 g /plant) and (0.636 and 0.649 g/100 seeds) in the two seasons. Moreover, healthy control occupied the second rank in this concern, while the lowest values were obtained from infected control in the two seasons table 7. The improving effect of citric acid may be attributed to its effect on stimulating the bio synthesis of carbohydrates (El – Badaway, 2013).

Table 7: Effect of citric, lactic and malic acids on seed yield (g/ plant) and seed index as weight of 100 seeds of *Nigella sativa* L. plants during 2017/ 2018 and 2018/2019 seasons.

Treatments	1st season		2nd season	
	seed yield (g)/ plant	seed index as weight of 100 seeds	seed yield (g)/ plant	seed index as weight of 100 seeds
Infected control	7.92	0.402	8.37	0.415
Healthy control	9.69	0.625	10.06	0.637
Citric acid (700 ppm)	9.13	0.608	9.54	0.625
Citric acid (1000 ppm)	9.30	0.624	9.64	0.631
Citric acid (1300 ppm)	9.96	0.636	10.34	0.649
Lactic acid (700 ppm)	10.39	0.643	10.62	0.656
Lactic acid(1000 ppm)	11.40	0.652	12.47	0.669
Lactic acid (1300 ppm)	12.69	0.658	12.86	0.680
Malic acid (700 ppm)	12.90	0.665	13.04	0.698
Malic acid (1000 ppm)	13.09	0.674	13.24	0.738
Malic acid (1300 ppm)	14.02	0.701	14.54	0.747
L.S.D. at 5%	0.22	0.009	0.23	0.008

Infected control = *Nigella sativa* L. plants grown in soil infested with *Fusarium oxysporum* isolate A and without any treatments. Healthy control = *Nigella sativa* L. plants grown in soil uninfested with *Fusarium oxysporum* isolate A and without any treatments.

Effect of lactic acid

It was obvious that the plants treated with lactic acid at 1300 ppm showed a significant increase in seed yield /plant (12.69 and12.86 g/plant) and seed index as weight of 100 seeds (0.658 and 0.680 g / 100 seeds) as compared to infected control in the two seasons, while infected control recorded the lowest values table 7. These results may be due to that lactic acid inhibited mycelium growth of *Fusarium oxysporum* and biosynthesis of mycotoxin (Hassan *et al.,* 2015).

Effect of malic acid

All doses of malic acid significantly enhanced seed yield /plant and seed index as weight of 100 seeds compared with infected control in both seasons. The heaviest seed yield/plant and seed index as weight of 100 seeds (14.02 and 14.54 g/plant) and (0.701 and 0.747g/100 seeds) in the two seasons were observed as a results of treating malic acid at 1300 ppm. The infected control gave the lowest values table 7. These results may be attributed to the positive effect of malic acid on photosynthesis process (Darandeh and Hadavi, 2012). Also, the application of malic acid completely inhibited growth of *Fusarium oxysporum* (Hassan *et al.,*2015).

Generally, from the above data, the previous conclusion of the effectiveness of malic acid compared to lactic acid and citric acid was found to come true in stimulating *Nigella sativa* L. growth in term of plant height, number of branches, number of capsules, seed yield and seed index as weight of 100 seeds.

Effect of organic acids on volatile oil production

Volatile oil percentage

Effect of citric acid

Nigella sativa L. plants treated with citric acid at high concentrations (1000 and 1300 ppm) significantly increased volatile oil percentage in seeds over infected control in both seasons. The highest volatile oil percentage was obtained from citric acid at 1300 ppm which gave; (0.117and 0.128%) in the 1st and 2nd seasons, respectively, followed by healthy control. However, the lowest values (0.043 and 0.047%) were recorded by infected control in both seasons table 8. Similar results were observed by (Mendez- Albores *et al.*, 2005) reported that 97% degradation of initial concentration of aflatoxin contaminated maize or even a complete removal (depending on initial concentration) through the application of aqueous citric acid. Also, mentioned that the acidified samples were effective in decreasing negative side effects of aflatoxins on toxicity, mutagenicity without compromising the nutritional and organoleptic quality of the feed.

Table 8: Effect of citric, lactic and malic acids on volatile oil percentage % of *Nigella sativa* L. plants during 2017/ 2018 and 2018/2019 seasons.

Treatments	Volatile oil percentage %	
	1st season	2nd season
Infected control	0.043	0.047
Healthy control	0.107	0.115
Citric acid (700 ppm)	0.062	0.065
Citric acid (1000 ppm)	0.085	0.098
Citric acid (1300 ppm)	0.117	0.128
Lactic acid (700 ppm)	0.143	0.153
Lactic acid(1000 ppm)	0.157	0.160
Lactic acid (1300 ppm)	0.170	0.172
Malic acid (700 ppm)	0.175	0.178
Malic acid (1000 ppm)	0.183	0.187
Malic acid (1300 ppm)	0.188	0.192
L.S.D. at 5%	0.021	0.015

Infected control =*Nigella sativa* L. plants grown in soil infested with *Fusarium oxysporum* isolate ^{A} and without any treatments. Healthy control = *Nigella sativa* L. plants grown in soil uninfested with *Fusarium oxysporum* isolate ^{A} and without any treatments.

Effect of lactic acid

Lactic acid had a significant effect on volatile oil percentage of *Nigella sativa* L. seeds in both seasons as compared to infected control. In this regard, lactic acid at 1300 ppm gave the best results (0.170 and 0.172 %) in the 1st and 2nd seasons, respectively. Followed by lactic acid at 1000 ppm concentration, the differences between the two concentrations were not significant in both seasons. The lowest values were obtained from infected control in the two seasons table 8. These results may be due to that acidic solutions have the ability to destroy the mycotoxins (Jalili *et al.*,2011).

Effect of malic acid

The application of malic acid at 1300 ppm significantly increased volatile oil percentage of *Nigella sativa* L. seeds in the two seasons as compared to infected control. The values were (0.188 and 0.192%) in the 1st and 2nd seasons, respectively. The differences between the two treatments; 1000 and 1300 ppm were not significant. However, the lowest values of volatile oil percentage were obtained from infected control (0.0 43 and 0.047%) in both seasons table 8. Such effect may be due to that malic acid inhibited growth of *Fusarium oxysporum* by the presence of COOH group (Hassan *et al.*, 2015). Also, organic acids serve as an alternative to hormonal treatments these organic acids improve the growth.

Volatile oil components

The results of GLC analysis of volatile oil during the 2nd season were shown in table 9. Limonene was identified as the major component in volatile oil ranged from 12.92 to 17.95%, the second main compound, thymoquinone ranged from (6.68 and12.45%).Treating *Nigella sativa* L. plants with malic acid at 1300 ppm was most effective treatment which gave the highest percentages of limonene and thymoquinone (17.95 and 12.45 %), respectively. The application of lactic acid at 1300 ppm occupied the second rank (14.23and 10.73%), followed by citric acid at 1300 ppm. The lowest values (12.92 and 6.68%) were obtained from infected control, respectively. However, healthy control recorded; (13.12 and 10.08 %).These results are in agreement with the finding by (Salem and Awad Alla, 2017).

Table 9: Effect of citric, lactic and malic acids on volatile oil components of *Nigella sativa* L. plants during 2018/ 2019 season.

Treatments / Components	Infected control	Healthy Control	Citric acid at 1300 ppm	Lactic acid at 1300 ppm	Malic acid at 1300 ppm
αPinene	10.76	9.05	11.00	6.22	8.96
βPinene	3.69	8.05	8.08	4.93	8.02
Myrcene	2.76	7.67	3.83	10.22	7.65
P-Cymene	12.65	10.97	6.75	13.94	10.95
Limonene	12.92	13.12	13.63	14.23	17.95
γTerpinene	7.35	3.70	5.43	6.20	8.18
Thymoquinone	6.68	10.08	10.15	10.73	12.45
Terpinene-4-ol	6.43	8.07	7.53	9.65	10.41
Carvacrol	7.76	5.52	5.29	5.17	6.01
Trans-anethol	1.37	5.76	5.89	6.87	7.08
Unkown	27.63	11.01	22.42	11.84	2.34

Infected control =*Nigella sativa* L. plants grown in soil infested with *Fusarium oxysporum* isolate ^{A} and without any treatments.

Healthy control = *Nigella sativa* L. plants grown in soil uninfested with *Fusarium oxysporum* isolate ^{A} and without any treatments.

Effect of organic acids on fixed oil of *Nigella sativa* L.

Effect of citric acid

It was observed that all concentrations of citric acid significantly increased fixed oil percentage as compared to infected control in the two seasons. The best results (26.74 and 27.51%) were recorded with the plants received citric acid at 1300 ppm in the 1st and 2nd seasons, respectively. The lowest values were recorded in case of infected control in both seasons table 10.

Effect of lactic acid

It was found that the same trend previously observed in case of citric acid was also detected with lactic acid at 1300 ppm significantly increased fixed oil percentage compared with infected control in both seasons. The lowest values were recorded with infected control. Lactic acid was more effective than citric acid in increasing fixed oil percentage table 10.

Effect of malic acid

All doses of malic acid significantly increased fixed oil percentage compared with infected control in the two seasons. The maximum values achieved with the highest dose in both seasons. However, the lowest values were observed in case of infected control table 10.

Table. 10: Effect of citric, lactic and malic acids on fixed oil percentage (%) of *Nigella sativa* L. plants during 2017/ 2018 and 2018/2019 seasons.

Treatments	Fixed oil percentage (%)	
	1st season	2nd season
Infected control	19.53	19.82
Healthy control	25.38	26.15
Citric acid (700 ppm)	20.42	21.20
Citric acid (1000 ppm)	22.56	23.48
Citric acid (1300 ppm)	26.74	27.51
Lactic acid (700 ppm)	28.57	29.16
Lactic acid(1000 ppm)	29.48	30.17
Lactic acid (1300 ppm)	29.70	30.36
Malic acid (700 ppm)	29.93	30.53
Malic acid (1000 ppm)	30.42	30.84
Malic acid (1300 ppm)	30.56	31.04
L.S.D. at 5%	0.17	0.13

Infected control =*Nigella sativa* L. plants grown in soil infested with *Fusarium oxysporum* isolate A and without any treatments. Healthy control = *Nigella sativa* L. plants grown in soil uninfested with *Fusarium oxysporum* isolate A and without any treatments.

It is important to confirm that malic acid was the most effective than lactic and citric acids regarding volatile oil production and fixed oil percentage as well as all growth characters. This effectiveness of malic acid may be due to that it is unsaturated acid containing double bond between two carboxylic groups; thus it has three active sites. Therefore, it has the ability to greatly inhibit mycelium growth of *Fusarium oxysporum* (Pathogenic fungus) and facilitate nutrients uptake.

As for lactic acid, it has two functional groups (carboxylic and hydroxyl).This chemical structure inhibits the activity of pathogenic fungus and increases plant growth. However, citric acid was less effective may due to that it is a saturated acid decarboxylation of citric acid that occurs enzymatically easily through catabolic reactions. These results are in line with (Hsiao and Siebert, 1999) mentioned that the inhibitory effect of organic acids on microbial growth was related to the sites and number of functional groups. Organic acids are not homologous series but vary in the numbers of carboxylic groups, hydroxyl groups and carbon – carbon double bonds in the molecule. Thus, their properties correspond to polar groups, the number of double bonds and molecular size.

Anatomical studies

Cross sections through the middle internode of the healthy stem of the black cumin (*Nigella sativa* L.) plant are given in table 11 and Fig. (1A). The cross section showed normal anatomical structure. The stem transection surrounded by uniseriate epidermal layer covered with thick cuticle and possess glandular trichomes. The epidermis is tangentially elongated. The epidermal layer under laid by the cortex. Cortex characterized by the presence of chlorenchyma above the groups of fibers intercepted by collenchyma cells. Groups of extaxylary fibers were noticed above each vascular bundle. The vascular bundles appeared in a ring conjoined by highly thick- walled lignified cells. The bundle is collateral consists of phloem, xylem and cambium layer between them. The vascular region is followed by extended layers of parenchymatious cells. Pith, small cavities arised at the center of stem transection. The anatomical description of stem is more or less in accordance with (Salim *et al.*, 2016).

Data in table 11 and Fig. (1B) illustrate the effect of *Fusarium oxysporum* on the anatomical structure of *Nigella sativa* L. stem at the age of 10 weeks compared to uninfected healthy one. As shown, the infected stem recorded reduction in both stem and pith thickness. The percentages of reduction were; 8.99 and 3.74%, respectively. In addition, the small pieces of the *Fusarium oxysporum* structures appear clearly penetrate the infected stem tissues Fig. (1B). Moreover, the infected plant showed reduction in both fibers layers thickness and number. Thickness of the vascular bundle was reduced as a result of reduction induced in both xylem and phloem tissue thickness. Worthy to mention that the vessels, diameter was negatively affected also by the infection compared to the healthy plants, the percentage of reduction in xylem, phloem tissue thickness and vessel diameter were; 19.25, 26.68 and 30.96%, respectively. The deficiency occurs in the anatomical structure of the infected plant as a result of pathogen penetration through the plant root system and colonization it's vascular system causing blockage of vessels and water stress, (Ortiz *et al.*, 2014). These results explain the negative impact of *Fusarium oxysporum* on plant morphology and yield.

Regarding the effect of citric, lactic and malic acids on infected *Nigella sativa* L. plant, the highest concentration caused an enhancement in stem anatomical features and internal tissue, An increment was recorded in stem and pith diameter by such concentrations (Fig.1C,D & E). Relative to infected plants, the percentages of increments in stem diameter were; 22.00, 51.36 and 31.82% for citric, lactic and malic acids, respectively. Concerning pith the percentages of increase over the infected stems were; 46.02, 34.73 and 30.29 %, respectively. The thickness of fiberous tissue (adjacent to the bundles) was increased in plant stems treated with lactic and malic acids followed by those treated with citric acid compared to healthy and infected plants. The lateral results clearly confirmed a mechanical support against the fungal pathogen under study. Moreover, plants treated with malic acid at concentration1300 ppm showed clearly differentiated and having normal shape of epidermal and cortex layers. Moreover, this treatment recorded high thickness in epidermis, xylem vessels diameter as well as phloem thickness were also increased compared to healthy and infected plants. The percentages of increment in epidermis thickness, xylem vessels diameter and phloem tissue relative to healthy plants were; 8.71, 10.38 and 24.84% for malic acid treatment. This explains the increased occurred on morphological and anatomical traits as a result of this treatment.

So, it could be concluded that the increment occurred in the anatomical features of the treated plants with the three organic acids may be due to the inhibitory effect of these organic acids to *F. oxysporum*, which is in agreement with (Hassan *et al.*,2015).

Table 11: Effect of citric, lactic and malic acids on anatomical structure of *Nigella sativa* L. plants stem grown in soil infested with *Fusarium oxysporum.*

Histological Characters	Healthy pl.	Infected pl.	± % to healthy pl.*	1300 ppm Citric acid	± % to infected pl.*	1300 ppm lactic Acid	± % to infected pl.*	1300ppm malic acid	± % to infected pl.*
A.v. stem diameter(μ)	2174.19	1978.61	- 8.99	2413.96	+ 22.00	2994.86	+ 51.36	2608.25	+31.82
A.v. Epidermis thickness (μ)	21.13	14.89	- 29.53	18.96	+ 27.33	17.09	+ 14.78	22.97	+ 54.26
A.v. Cortex thickness (μ)	141.29	88.33	- 37.48	131.19	+ 48.52	125.14	+ 41.67	127.70	+ 44.57
A.v. fibers thickness (μ)	81.64	71.18	- 12.81	93.22	+ 30.96	112.62	+ 58.22	93.93	+ 31.96
A.v. V. bundle diameter(μ)	169.05	145.29	- 14.05	195.85	+ 34.79	220.89	+ 52.03	162.58	+ 11.90
A.v. xylem thickness (μ)	114.96	92.82	-19.25	108.07	+ 16.43	145.04	+ 56.26	129.04	+ 39.02
A.v. Xylem vessels diameter(μ)	26.68	18.42	-30.96	21.46	+ 16.50	24.32	+ 32.03	29.45	+ 59.88
A.v. phloem thickness(μ)	64.48	47.16	- 26.68	72.51	+ 53.75	68.49	+ 45.23	80.50	+ 70.69
A.v. pith diameter(μ)	1390.85	1338.86	-3.74	1955.00	+ 46.02	1803.87	+ 34.73	1744.46	+ 30.29

*± % to healthy pl. =percentage of increase or reduction to healthy plant.

*± % to infected pl. = percentage of increase or reduction to infected plant.

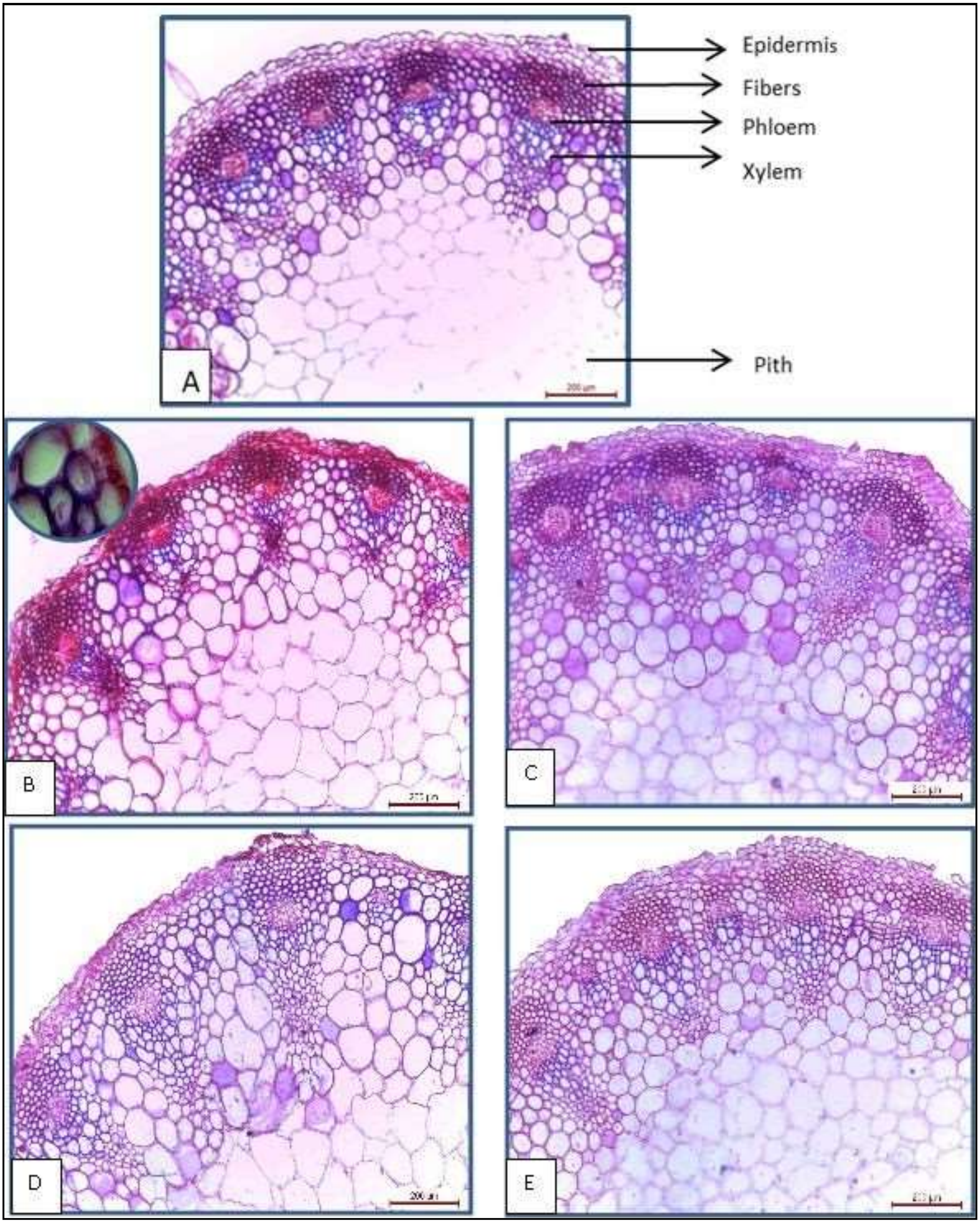

Fig. 1: Cross sections in stem of *Nigella sativa* L. plants infected with *Fusarium oxysporum* showed the effect of organic acids. A) Healthy plant B) Infected plant C) Infected plant treated with 1300ppm citric acid D)Infected plant treated with 1300ppm lactic acid E)Infected plant treated with 1300 ppm malic acid.

Recommendation

From the results mentioned above, it could be concluded that the application of malic acid at 1300 ppm was the most effective treatment. It was safe and effective against Fusarium wilt disease of (*Nigella sativa* L.) plant caused by *Fusarium oxysporum*. Also, it recorded the highest values of plant growth, fruiting, seed yield, volatile oil production and fixed oil percentage under wilt disease infection. Therefore, treating (*Nigella sativa* L.) plants with malic acid at 1300 ppm could be recommended.

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