Research Article **ISSN: 2349 – 4492**

Asian Journal of Research in Biological and Pharmaceutical Sciences

Journal home page: www.ajrbps.com

https://doi.org/10.36673/AJRBPS.2020.v08.i04.A16

IN *VITRO* INVESTIGATION OF THE INFECTION MECHANISMS AND STRATEGIES OF *PENICILLIUM DIGITATUM* (PERS. FR) ON CITRUS FRUITS

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ABSTRACT

In *vitro* experiments have been conducted to study and investigate the infection mechanisms and the strategies of penetration invasion and colonization behavior of *penicillium digitatum*. Artificial inoculation with *P. digitatum* spores harvest firstly from growing colonies on Sabroid agar. The ability of P. digitatum to infect four citrus varities with same virulence after application three methods of artificial inoculation. Through cooling experiment we found the optimum growth temperature ranged between 25-27°C. Study the effect of depth and wound location on the previous citrus varities does not show any significant impact on infection behavior in varities sampled. After study natural micro-flora associated with tested fruits surface. Three types of bacteria are identified: *Staphylococcus hominis, Staphylococcus haemolyticus* and *Staphylococcus warneri*. After artificial inoculation with Penicillium digitatum with inoculum density (10 the washed fruits speed infected compared with non - washed fruits with dispersal time about 12 hours. In test of three types of juice (Peel Juice - Sac Juice - Whole fruit), all tested juice from citrus varities studied showed *P. digitatum* growth with more rapidly on peel juice compared with other two types of juice extracts, which make ensure that the tested fungus was this obligate aerobic. Through intervene modulation to change acidic medium of studied fruits by inject two concentrations of apple vineiger and solution of sodium bicarbonate, solution of sodium chloride and sterile distill water as control showed that there is no role in prevent infection by *Penicillium digitatum* but just delaying infection time about five days by used solution of Sodium bicarbonate Na2HCO3.

KEYWORDS

Citrus fruits, *Penicillium sp* and Postharvest diseases.

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INTRODUCTON

Citrus cultivation is an important commercial and industrial agronomic activity worldwide. Citrus fruit is widely consumed, both as fresh fruit and as juice, not only for its flavor but also because of its vitamin C and antioxidant content. Citrus is the main fresh fruit exported by the Australian horticultural industry, with fruit mainly grown in New South

Wales (NSW), Queensland, South Australia and Victoria. The main markets for Australian citrus are fresh fruit (domestic and export), fresh juice (domestic and export) and drink manufactured from frozen concentrated juice. In 2002/2003 record export volumes of 167.000 tonnes were achieved, with an estimated gross value production of AUD$201 million (Australian Citrus Limited, 2009). In particular, South Australia exported fresh oranges to the value of $58.1 million in 2007 (Primary Industries and Resources SA, 2010). Citrus is a non-climacteric fruit, therefore it has a relatively long shelf-life. Its cultivation is an important commercial agronomic activity worldwide. Citrus fruits are the most valuable fruit crop in international trade, and include; Mandarins, Sweet oranges, Limes and grapefruit (Erminawati Wuryatmo, 2011)[1].

Citrus fruits have an essential component of some of the human nutritional requirements like Vitamins, Minerals and Organic acids. Preservation of these products, however, is one of the central problems encountered by producers worldwide. The postharvest losses of fruit and vegetable stands at 20-40% in the average (Irtwange, 2006)[2].

Citrus fruits are a major export commodity of Egypt, with production estimated to be 2.5 million tonnes / year. The most common and serious postharvest diseases of citrus fruits are green and blue moulds, caused by *Penicillium digitatum* and *P. italicum*, respectively (Plaza *et al*, 2003)[3]. During April 2009, oranges (*Citrus sinensis*) from three Egyptian cultivars Baladi, Sukhary, and Abu-surra were collected from commercial markets and packinghouses in the Giza Governorate. After 3 weeks storage at room temperature and high relative humidity, a morphologically distinct Penicillium spp. was observed as a mixed infection with *P. digitatum and P. italicum* (Holmes *et al*, 1993)[4]. The pathogen was isolated on potato dextrose agar (PDA), and identified as *P. ulaiense*, according to its morphological and cultural characteristics. *Penicillium ulaiense* was distinguished from *P. digitatum* by its blue-grey spore mass and from *P. italicum* by its ability to form coremia (1-7 mm tall) with white stalks (Youssef, *et al*, 2010)[5].

Citrus fruits are infected with many fungal disease especially after harvest in the orchard which transported to the supermarkets and developed in stored from between these disease: Penicillium rots, this pathogen caused 90% of citrus losses during the storage (Henik, *et al*, 2012)[6].

Post-harvest diseases account to about 50% losses in fruits stored in poor storage conditions especially under high humidity. They are posing a major problem to the agriculture industry (Agrios, 2005)[7]. Citrus fruits are among the crops susceptible to post-harvest diseases caused by fungi under poor storage conditions (Ogawa *et al*, 1995)[8]. The most important fungi causing post-harvest diseases include: *Penicillium spp*, *Aspergillus spp*., *Alternaria spp.*, *Botrytis cinerea*, *Monilinia lax* and *Rhizopus stolonifer* (Harbant Singh *et al*, 2011)[9].

Postharvest fungal diseases result in significant economic losses in the citrus industry. The most common postharvest fungal diseases affecting citrus fruits worldwide are green mould, blue mould and sour rot which are caused by the filamentous fungi *Penicillium digitatum* (Pers.: Fr.) Sacc., *Penicillium italicum* Wehmer and *Geotrichum citri-aurantii* Link ex Pers. Respectively (Plaza *et al*, 2003[10], Cunningham *et al*, 2007[11] and Droby *et al*, 2006[12]). These pathogens may infect fruit in the packing-house, in transit, in storage and in the market (Erminawati Wuryatmo, 2011)[1].

To control of postharvest disease evaluated the effect of temperature and time on oranges decay development of *Penicillium digitatum* and *Penicillium italicum,* the most postharvest diseases of citrus fruit. The assays carried out in inoculated 'Valencia late' oranges showed in both seasons an excellent control of both pathogens, when fruits were exposed to treatments at 40ºC for 18 h, and stores for 5 days at 5ºC plus 7 days at 20ºC. Concerning quality changes slightly effects were observed on fruits submitted to curing treatment. These results suggest that this treatment could be an environmental friendly alternative to chemical fungicides in oranges packinghouses (Carla Nunes, *et al*, 2007)[13].

Aim of the study

- Pathogenicity test by application Kock Pastulate.
- Study the *Penicillium* behavior (mode of action).

MATERIAL AND METHODS

Inoculum preparation

After one week of incubation growing of *Penicillium spp.* was growing on Sabroid Agar and forming small separate colonies. Almost harvested all the spores were harvested from the on the surface of the media using sterile glass rod and putted it in a clean test tube contain 10 ml of sterile distilled water then closed and mixed well (Original inoculum). The density of spores suspension was equivalent to approximately 10-9 spores/ml (Palou, *et al*, 2001)[14].

From original inoculum three serious dilution have been prepared (10^{-1} , 10^{-2} , 10^{-3}) (Edward Ntui Okey, 2015)[15] number of spores suspension have been determined for each dilution , by putted 1ml from each dilution on clean slides and calculated under the microscope. E.g: The preparation of first dilution (10^{-1} spores/ml), Putted 9ml sterile distill water in clean test tube and added 1ml from original inoculum then closed and mixed well. In our study each group from samples injected with different inoculums (isolate), some groups injected with inoculum from lemon, another groups injected with inoculum with orange, grapefruits respectively to investigate all of these inoculum given same symptoms or non.

Pathogenicity test and artificial inoculation

A total 320 matured healthy citrus fruits which have been collected from studied fruits were by washing 160 fruit samples with chlorox 1% in 10 liter sterile distal water and 160 non washed 80 samples from each one saved in refrigerator at (5°C) for (10) hour and another 80 samples kept at room temperatures for 10 hour and all of these samples were wounded by sterile metal cork borer, 3 holes with 1cm deep on 40 samples have been made and another 40 samples bored 2cm deep in juice sacs in 3 different sites in middle and lower part of each single fruit and all of 320 samples injuried in 2 sites in the peel upper and middle (Downes, *et al*, 2001)[16]. All injured or wounded fruits were artificially inoculated by three methods (Figure No.1).

Direct injection of inoculum

107 samples inoculated with first dilution (10^{-1}) and another 107 samples inoculated with second dilution (10^{-2}) and last 107 samples inoculated with third dilution (10^{-3}) (Edward Ntui Okey, 2015)[15] injected 1.5ml from each dilution in to the holes and peel injuries by sterile syringe, the point of inoculation was sealed with petroleum jelly (sterile Vaseline) to prevent contamination. The inoculated fruits were then placed in carton boxes to create a humid environment to indice rots and incubated at 27°C for 7 days with daily observation for symptoms. A control was also setup for each citrus fruits.

Spray the inoculum

Four samples were taken from each tested of fruits studies two fruit samples were injuried by sterile knife in all direction in peel and juice Sacc and another two samples wounded with the same way which maintained previous then spray all samples with the first dilution (10^{-1}) conidia/ml the point of the inoculation sealed with sterile vaseline then put the samples in carton boxes to create a humid environment to stimulate moulds and keep it to incubated at 27°C for 7 days with daily observation for symptoms. A control was also setup for each samples without injuried and spray with the dilution (Figure No.2).

Contaminated by spore of *Penicillium spp.* directly

Two type of fruit samples were taken from studied fruits and wounded with the same way which maintained previous then used sterile needle with cotton to take spores from *Penicillium spp.*, growth on petri dish directly and putted these spores in the wounded then sealed point of inoculation with sterile vaseline and put the samples in carton boxes to create a humid environment to stimulate rots and kept to inoculated at 27°C for 7 days with daily observation for symptoms. A control was also setup for each citrus fruits.

In each method used different source of *Penicillium digitatum* isolated from different type of citrus fruits to preparation inoculum sources (Figure No.3).

Test the affectivity of different artificial inoculation methods

In this experiment three methods of artificial inoculation were applied on some healthy samples of citrus fruits after wounded (3.1) as following: Firstly direct injection with three type of *Penicillium digitatum* spore suspension, Secondly spray fruit surface with first dilution (10^{-1} spores/ml), Thirdly putted *Penicillium* spores

directly from old *Penicillium* growth in petri dish. This study to known what the best and quick method causing infection or all of these experiment will appositely in the infection mechanism, to test the arrival way of inoculum and detect the amount of spores needed to cause initial infection.

Effect of cooling period - room temperatures - air atmosphere and infection severity on growth of *P. digitatium*

Around sample of fruits were studied after putted in refrigerator at 5°C for 10 hours and another samples kept at a humid and room temperature for 10 hour then all of these samples injuried and injected with 3 different dilutions of *Penicillium* spore suspension 10^{-1}, 10^{-2} and 10^{-3} spores/ml, which explained before (3.1), and another samples from each type of studied fruits putted in air atmosphere after injuried only without injection (artificial inoculation) with pervious dilutions. This experiment will research the effect of different temperatures degrees on growth of *Penicillium* and infection severity. In all experiments in our study used samples from each citrus varities as control (without injuries and artificial inoculation).

Test the different wounds depth and location on Penicillium growth

This experiment was carried out by making five wounds on tested all citrus fruit surface by done two wounds in upper and middle part of peel and 3 holes in the upper, middle and lower part of juice sacs. With two depths 1cm and 2cm by using sterile metal borer, in this experiments measured the thickness of peels for all studied fruits. This test was objected to investigate the wound depth in juice sac and location on fruit peel are appositeness and susceptible for grown and activity of inoculated *Penicillium* spores. Moreover, to detect *Penicillium spp*. become more active when reached and close contact to the peel or near to the central axis on the juice sacs, also to known which thickness of peel more suitable for growth and activity *Penicillium spp*. (Figure No.4).

Study the natural micro flora found on fruit peel surface of fruits and how the effect on Penicillium growth

In this study 10 samples from each citrus fruit were selected randomly from each citrus varities, five fruits were washed with 1% chlorox in 10 liter sterile distilled water and another five samples unwashed. All samples were injuried and inoculated with the same methods which maintained previously (3.1) all fruits were placed in clean carton boxes to create a humid conditions and kept at room temperature 27°C for 7 days.

To identification unknown microflora found on the peel of tested fruit samples. Washed 3 samples from all studied fruits with sterile distilled water only, the washed suspension from all types of studied fruits with S.D.W kept in closed sterile test tubes, seven petri dishes of Nutrient Agar were prepared containing 1ml of extracted microflora suspension was added to the surface of solid Nutrient Agar media to each single plates and distributed in zigzag fashion. All inoculated petri dish were kept at 37°C for 24 hour. Waiting the growth for these unknown microflora. Resolution was take place by using selective media (Macconkey agar) and to distinct and recognize the growing isolated microflora.

Identification was carried out by using phonex™ system, for bacteria and for fungi identification species.

This study aimed to explain the roles of wild natural microflora on growth and activity of *Penicillium spp*. either stop, stimulate or delaying the artificial infection processes.

Study effect of juice extract from citrus fruits on growth and activity of *Penicillium spp*

Preparation of juice from peels, sacs and whole fruit were carried out from seven healthy fruit samples. Each fruit peels, sacs and whole fruit were separated and cutted to small peaces then putted in blender to obtainer extracts and transferred to sterile plastic container for each types of juice. By using sterile cork borer we transfer 1 piece from *Penicillium* colonies to each juices in plastic container to contaminated and kept the plastic container at room temperature for (7) days with daily observation (Figure No.5). The aim of study to investigated which part of the fruit more suitable for growth the fungus isolated.

Modification of hydrogen ion concentration (pH) in healthy tissue of citrus fruits to study infection density mechanism of *Penicillium spp*

In this tested some compound were used to change and modify the natural pH of host tissues. Three types of compounds were used first by natural pure

and solution of apple vinegar, second by solution of sodium bicarbonate and thirdly by solution of sodium chloride and finally used sterile distilled water as control, determination pH three materials by using strip detector.

Pure apple vinegar is done by two ways

First way by direct injected 2ml of concentration apple vinegar 4-5% pH (4.5) in each inject 0.2ml by sterile syringe in all type of fruits studied , kept fruits for absorption the apple vinegar for 7-10 minute and then injuried and contaminated with methods mention in 3.1.1.

Second way by prepared solution from 50ml apple vinegar 4-5% pH (5) in 50ml S.D.W in glass flask then mixed well and injected studied samples with 2ml by sterile syringe in each inject 0.2ml then repeat work maintained previous.

The second material solution of sodium bicarbonate (NA2HCO3) pH (8) by adding 13gram sodium bicarbonate to 50ml S.D.W in glass flask then mixed well and repeat work mention above.

The third material solution of sodium chloride (Nacl) "salt food" pH (7.5) by adding 10gram Nacl to 50ml S.D.W in glass flask then mixed well and repeat work mention above. A control for this study S.D.W pH (7) applied same work maintained previous.

RESULTS

The results of the direct injection with different inoculum density of *Penicillium spp.* spores

The results of the experiment with three different types of artificial contamination appears the same obtained result on all the four citrus varities in the typical symptoms to infect by mould caused by *Penicillium spp.* and there is different appears in the time period to manifestness initial typical symptoms by three types inoculum source 10^{-1}, 10^{-2}, 10^{-3} spores/ml with disperse 12 hour between them.

The early infection area (Initial symptoms) appears after 48 hour by first dilution and after 60 hours by second dilution and 72 hours by third dilution. By the second method of artificial contamination after 8 days (metal borer) 12 days (sterile Knife) and by third method of artificial contamination after 7 day, as a softening of the tissue about the point of injection after 48 hours in Mandarine and Grapefruit, in the third days in Blader, sweet, sour, navel and in fourth day in the lemon which turn into water soaked area and slightly discolored spot from ¼ to ½ inch (2.5cm) in diameter then the softening watery spot developed after 1 to 1.5 days at 27°C±1 to weight mycelia appears in the surface which produced olive green spores when the grown fungal is about 1 inch in diameter then developed after one day to green fungal growth but retains margin is white (1-20) mm, then increased the fungal growth area gradually on the fruits surface to spread the olive green spores to covered all fruits from 10-12 days then the spores developed to hypha growth from 13-19 days, after that appears from 3-9 integrated white circles with cotton textures and the last symptoms in all infected fruits becomes more softening and laxly with black color and they have bad smell, all of inoculum source used in our study given same symptoms (Figure No.6 to Figure No.13).

Calculation of inoculum density of Penicillium spores suspension

The result of three serial spore dilutions 10^{-1}=10 spores/ml 10^{-2}=10, 10^{-2}=10^{-3} approximately. The objectives of this experiment to capability of *Penicillium spp.* spore for causing infection and the longivity of symptom appearance the result on this experiment show differences in time period to exposure initial typical symptoms as following: After 48 hour by first dilution, after 60 hours by second dilution and 72 hours by third dilution (Figure No.14 to Figure No.16).

The role of refrigerate storage (cold storage) in infection

The aims of these experiments to compare the effect cooling and non cooling period (time) to four citrus varities, the fruits which kept in reifridge under 5°C show early appearance of green mold symptoms compared with fruits storage in room temperature 25-27°C show the delay appearance of symptoms green mold with dispersal time about 12 hour, while the samples kept in the outdoor which just injuried without injection with *Penicillium* spores and control samples (uncontaminated) do not show any appearance of green mold symptoms after kept for long time.

The result study of peel thickness of four types of studied fruits

The result obtained from studying peel thickness shoe appear that the *Penicillium digitatum* able to penetrate easily through thin or thick fruit peel but take different time so that the time factor is play importance role to appear the typical symptoms of green mold. Table No.1 and (Figure No.17) from the depth and location experiments which done or carried out on four citrus varities.

The resulting growth was superficial not deep just only on surface (Aerobic area). The best and rapid growth of *Penicillium* appears in thin peel in mandarine, grapefruits then blood, sweet, sour, navel and the last in thick peel of lemon respectively (Figure No.18). Although the lemon is last fruits infected with *Penicillium digitatum*. But it is the first fruit covered completely with growth of *Penicillium digitatum* before another studied fruits. Do not observational different effect in the sites of the five wounds on upper, middle and lower surface of the peel fruits which ensure any wounds on the peel suitable and facilitated entry and growth of *Penicillium digitatum.* Enhance, the holes in the juice sac it's the sites for absorb the water and nutrient materials. There is no different in the depth of wounds 1cm and 2cm.

Study natural surface microflora on the surface citrus fruit

The conclusion obtained from study natural microflora which found on the surface of different citrus fruits by comparing washing and non-washing fruits collected from the same resource. The washing fruits (free of microflora) show rapaid growth than non-washing fruits (with microflora) with disperse time 12 hours. From the washing suspension of citrus fruits different type of bacteria was found and identified by using phonex system™ named as *Staphyloccous hominis, Staphyloccous haemolyticus, Staphyloccous warneri.* As mention in the table (2) which represent the natural microflora on surfaces studied citrus fruit (Figure No.19 and Figure No.20).

The observation *Penicillium digitatum* growth for three types of peel juice, sac juice and whole fruit juice (peel + sac)

The observed results of peel juice, sac juice and whole fruit juice (peel + sac) after contented with *P. digitatum* spore and kept at room temperature 27°C, the first initiation *P. digitatum* growth was observed after 48 hours in all peel citrus varities except blood orange which does not show any growth, Table No.4-5). While in the sac juice does not show growth in sour orange, mandarine and grapefruits, observed growth in sour orange and lemon only in the third types of juice.

After ten days obtained completely growth in peel juice on all types of studied fruits, while sacs juice and whole fruits juice have been spottily growth except sac juice of grapefruit do not show any growth for long time (Figure No.21-23).

The effect of modulation pH tissue of hosts by some materials

After injection solution of sodium bicarbonate the initial symptoms was appears after 5 days at room temperature. while after injected the others chemical materials solution sodium chloride, concentration and solution of apple vinegar and sterile distill water the clear symptoms of *Penicillium* green mold have been observed after 48 hours with observation black color only in samples injected with apple vinegar (Figure No.23).

DISCUSSION

The modulation of the citrus fruits tissues environment and the activation of mechanism of cell death induction. This implies that diffusible factors that have a direct or indirect phytotoxic activity are released by the pathogen. This conclusion by Oladele and Owolabi (2016)[17] was similar to our experiment of pathogenicity. The explanation of successful infection of *P. digitatum* to all citrus varieties after artificial inoculation through mechanical wounds on peel surface, may due to the differences of peel thickness between orange types, Mandarine, Lemon, and grapefruits , which clear the thickness of peel studied fruits have important role in delay or enhance the infection citrus fruit with *P. digitatum* this differences may be attributed the presence of chemical compounds e.g volatile compounds, ascorbic and citric acids which change the acidity of peel substrate. The thickness of peel is considered to be a character of importance in many citrus fruits.

It was noticed that peel thickness of Naval, Sour, Sweet and Bloody oranges decreased with increasing storage period, this decrease was significantly affect the defense mechanism and

concentration of chemical compounds as phenol, citric acid, ascorbic acids and lingenine, some physical, nutritional and functional properties of the fruit have play dramatic roles in facilitation of spore germination, penetration invasion and infection. Increase respiration level, sugar content and the antioxidant capacity, play major indirect roles in the enzymatic peeling because they affect the possibilities of shelf life time (storage) and the quality of fruit juice, this conclusion was proved by (Perez *et al,* 2005)[18].

Saprophytic growth on different substrate ability of all species can lead to extinction of the host and even allow the pathogen to persist in the absence of its host. The absence and lack of genetic breeding programs in citrus trees to develop and improve fruit quality and quantity while still remaining on mother tree will increase the resistance ability of post-harvest fruits to combat the infection of virulent Penicillium strains. Virulence is associated with rapid intra-host growth rates ultimately leading to rapid inter - host transmission specially for fungi like Penicillium have a high reproductive potential in large plant host range (Abd-Allah, *et al*, 2012)[19]. Virulence is a measure of the relative capacity a microbe to cause damage to a host. And high virulence is associated with rapid intra host growth rots, virulence of *Penicillium digitatum* increased by acidity of the host tissue, when fruits exposure to high density of *Penicillium digitatum* spores the capacity of virulence is increase (Joseph, *et al*, 2007)[20].

Each pathogens require special nutrient (chemical component) for growth found in specific plant without others. There for, each pathogen infect specific host plant without the other (Erminawati Wuryatmo, 2011)[1].

From the molecular point of view pectin, cellulose and hemicelluloses are mean chemical compounds of living cells will enhance *P. digitatum* spore to produce large amount of extra cellular enzymes to degreed and decompose the previous compounds in virulence manner, avoiding depriving any other microbic competitor specially at the beginning of invasion. Also the previous certain compounds are responsible for the harding and adherence of the peel (Skin) to the inside fruit tissues.

These fore both pectinases and celluloses are needed for the enzymatic peeling. The celluloses are probably needed for the release of the pectinases of the albedo and the pectinases contribute to the hydrolysis of the polysaccharides of the cell wall (Ismail *et al*, 2005)[21]. However the space or the gape and the adherence of the peel to the fruit and it is thickness are different according to the citrus varities like Mandarine has thin peel with little space between peel and inside tissues will allow fungus to spread in quickly in short time compared with lemon or orange Atmospheric temperature and change in pH to acidity level will drastically affect the Penicillium invasion (Abd El-Morsie *et al*, 2008)[22].

The resultant of pathogenicity test, which include the change of wound location and depth on fruit surface will investigates strong and quick ability of Penicillium to reach the wound position in short time followed by successful penetration through citrus fruit peel, in this study show there is no different in two depth 1cm, 2cm and any wounded in peel suitable and facilitate entry, growth of Penicillium digitatum and investigate this fungus growth in peel surface of fruit and absorb the nutrient material, water, from tissue of juice sacs which ensure this fungus from aerobic fungi. In addition to that, this experiment show abundance of inoculum potential of vital Penicillium spores in air.

The prolongation of the marketing stage resulted in increased fruit weight loss and physiological changes of fruit tissues. The mean of penetration by post-harvest pathogens (Penicillium) in involved in the opportunistic type of infection in which the pathogens penetrate through a natural wound or one that occurs mainly after harvest or following storage stresses. This type of penetration may be exploited by the same pathogens that penetrate directly as well as others that require a breached or weak end cuticle. However although pathogens may differ in their initial mechanisms of penetration. The colonization mechanism of the pathogens that penetration through wounds or directly are the same, in this study appears there is no different between methods of wounded by sterile knife or sterile metal borer which both facilitate the penetration of *P. digitatum* to cause infection.

Penetration by post-harvest pathogens through wounds in fruits resulted in earlier appearance of symptoms than direct penetration. This may indicate the Penicillium species are able penetrate wax layers of citrus fruits surface there layers do not seen to pose a serious barriers to penetration. In this case Penicillium well increase the incidence of infection increased thickness of the citrus fruits cuticle layer modulated the susceptibility to fungal attack (Domsch *et al*, 1980). Since most of fruits and vegetables have thick cuticles it has been suggested that pathogens might secrete surfactants in the form of proteins that reduce surface hydrophobicity and dissolve the wax layer pathogenesis related genes have long been known to be expressed only when the pathogen is inside the host once the host barriers have been overcome and the initial penetration has taken place, the pathogen e.g. Penicillium switches from the biotrophic to the nerotrophic stage. The changes in due the transformation from a quiescent to an active infection in which cell death occurs and initial symptoms (Green mold) are observed.

The optimum temperature for mould growth is 27°C, no growth occurs above 30°C and growth is slow below 10°C, the cooling fruits for 10 hours at 5°C enhance the activity of growth enzymes inside fungal cell and sometimes break the dormancy (Fu-Wen Liu, 2010)[23].

Efforts have been made to minimize the losses through developing better understanding of this biology of post-harvest diseases as well as by developing adequate post-harvest handling technologies and control strategies (Prusky and Gullino, 2010)[24].

The experiment of which have been done in this research about modulation of environment pH of inside healthy of citrus fruit tissues show that all of used compounds (concentrate and solution of apple vinegar, solution of bicarbonate sodium , solution of sodium chloride and sterile distal water used as control) with different value does not prevent or reduce of *Penicillium digitatum* growth but just late the infection by used solution of bicarbonate sodium for 5 days, while the other materials does not have any effect which exposure the typical symptoms. In all tested citrus fruits which indicate the virulence of *Penicillium digitatum* was related to gene characters for each *Penicillium* strains. The results indicate tissue pH is an important parameter in aqueous environments, so it affects the activates of enzymes and determines the expression virulence genes inside the host.

Modulation of pathogenicity factor these post-harvest pathogenicity fungi modulate the host pH as a basis for expression of virulence factor during the colonization of the target host tissues. Pathogens may modulate their virulence by local acidification or alkalization the host tissue e.g. colonization of acidified citrus and apple tissues by *Penicillium spp.* was enchareed by low pH.

Application of neutralizing solutions depending on the type of pathogen. This approach is important for the control of post-harvest disease because it's directly effect on the germination of conidia and fungal colonization (Askarne L, *et al*, 2013)[25].

Modulation of environmental pH of host tissues is as important parameter in aqueous environments, since it affect the activities of enzymes and determinations the expression of virulence genes inside the host (Prusky and Yakoby, 2003)[26]. At change in the ambient pH during fungal attack may be a critical factor in the expression of pathogenicity factor (Skaria, *et al*, 2003)[27].

The effect of the pathogen on ambient pH the pathogen itself can dynamically after the local pH to fit its enzymatic arsenal with level of pathogenicity being related to the efficiency of the pH change. This ability lies behind the terms "alkaline fungi" and "acidic fungi" in case of all Penicillium species infection of citrus fruits such as *P. expansum, P. digitatum, P. italicum* (Hong-Yin Zhang*, et al*, 2004)[28] use tissue acidification in their attack realized by accumulation of organic acids and/or H+ excretion e.g *Botrytis cinerea* decrease the host pH by secreting significant amounts of oxalic acid, while gluconic and citric acids are mainly secreted by P. expansum acidifies the tissues to pH levels of 3.5 to 4.0 (Goldbach, *et al*, 1997)[29].

oxalic citric and gluconic acids exhibited strong Ca2+ chelating activities that weaken the plant cell wall by altering its mineral balance and their by affect the stability of cell membrane and cell wall pectate polymers (Cunningham and Taverner, 2007)[11].

Table No.1: Peel thickness of citrus fruit varities

S.No	Varities	Thickness of peel (cm)
1	Blood orange	0.4cm
2	Sweet orange	0.6cm
3	Sour orange	0.3cm
4	Navel orange	0.6cm
5	Mandarine	0.3cm
6	Lemon	0.7
7	Grapefruits	1.0cm

Table No.2: Citrus fruit varities, types of bacteria, confidence value and inoculum density

S.No	Varities	Type of Bacteria	Confidence value	Inoculum density
1	Blood	*Staphyloccous hominis*	98%	0.5
2	Sweet	*Staphyloccous hominis*	98%	0.5
3	Sour	*Staphyloccous hominis*	99%	0.5
4	Navel	*Staphyloccous hominis*	99%	0.5
5	Mandarine	*Staphyloccous hominis*	90%	0.5

Table No.3: Citrus fruit varities, Peel juice, Sac juice, and whole (Peel + Sac juice)

S.No	Varities	Peel juice	Sac juice	Whole fruit juice (Peel + Sac juice)
1	Blood orange	-	+	-
2	Sweet orange	+	+	-
3	Sour orange	+	-	+
4	Navel orange	+	+	-
5	Mandarine	+	-	-
6	Lemon	+	+	+
7	Grapefruits	+	-	-

(+) Growth and (-) Non growth.

Table No.4: The measurement of pH peel juice, pH sac juice and pH whole fruit juice

S.No	Varities	Peel pH	Juice pH	Whole pH
1	Blood	6	5	6
2	Sweet	5	6	6
3	Sour	5	5	5
4	Navel	5	5	6
5	Mandarine	5	6	6
6	Lemon	6	6	6.5
7	Grapefruits	5.5	6	6

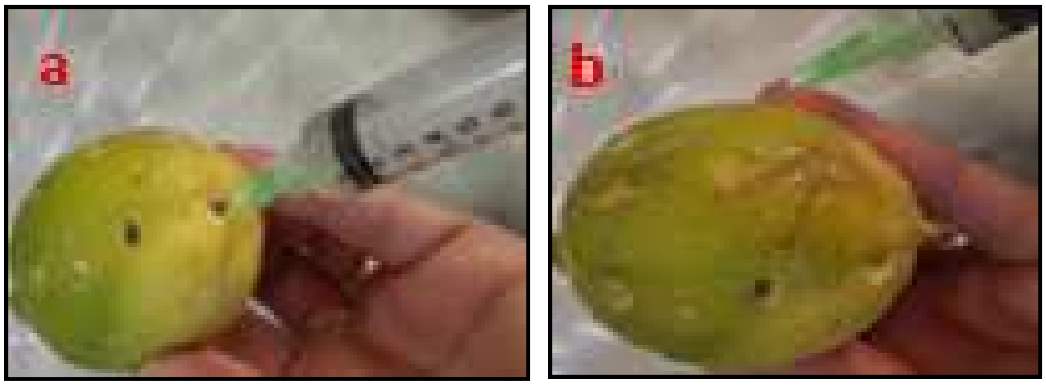


Figure No.1: Pathogenicity test and artificial inoculation

Figure No.2: a) Artificial inoculation by spray dilution, b) Injuires by sterile knife, c) Injuires by sterile metal borer

Figure No.3: Artificial inoculation by direct put of spores

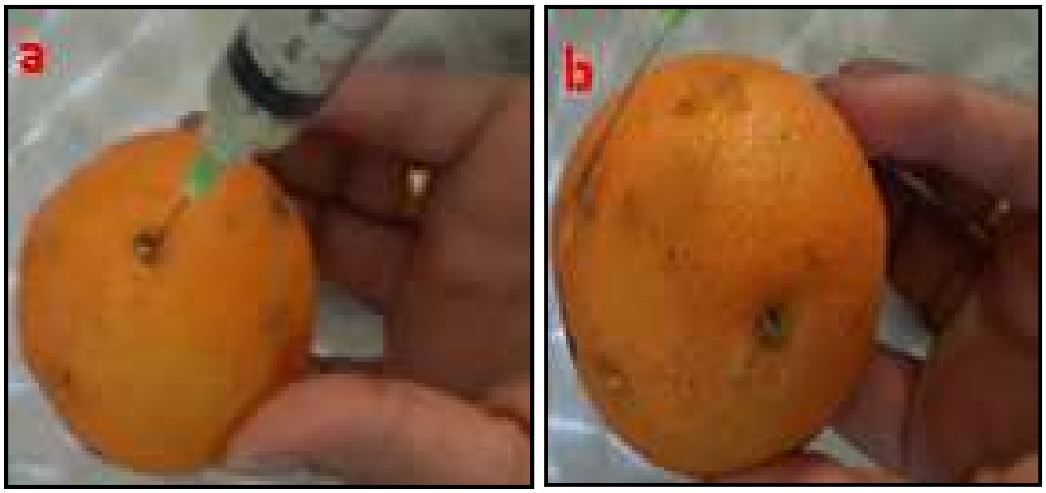
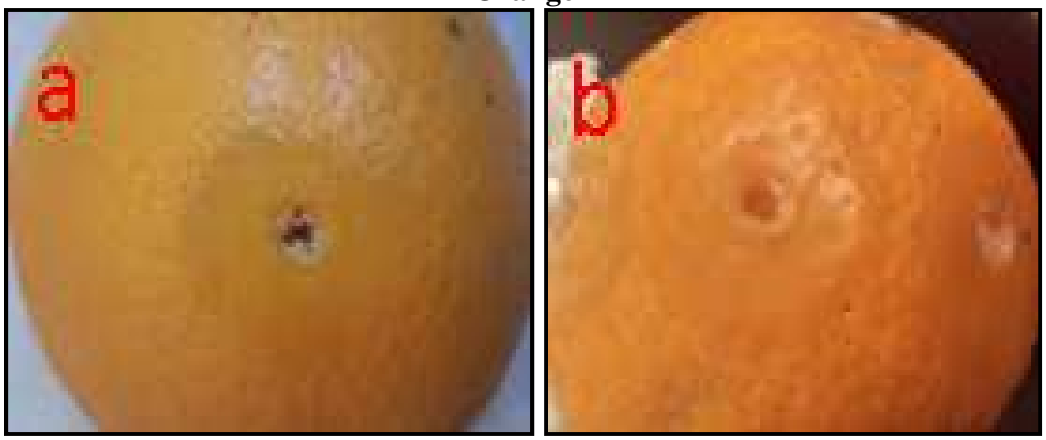


Figure No.4: Artificial inoculation by direct injection of inoculum, a) In the hole of juice sac, b) In peel surface of the healthy orange fruit

Figure No.5: Contaminated three types of juice with *Penicillium* growth, a) The Lemon, b) The Blood Orange



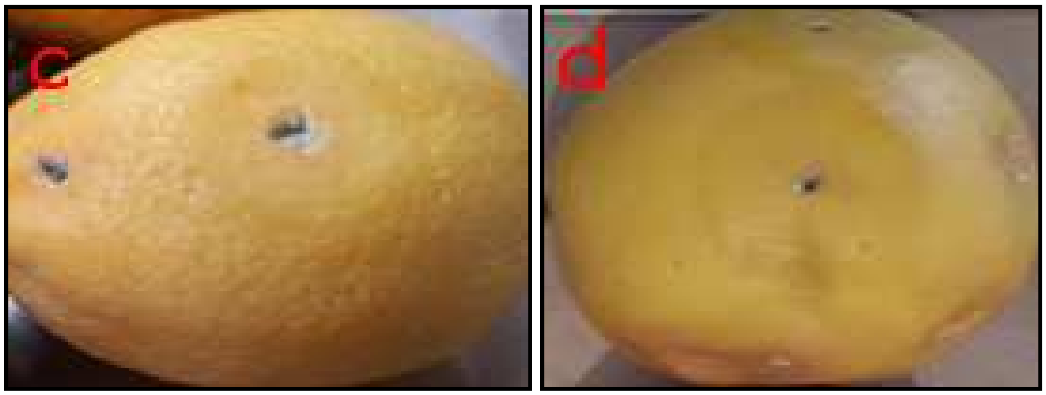


Figure No.6: Water soaked area, a) In Orange, b) In Mandarine, c) In Lemon fruit and d) In Grapefruits

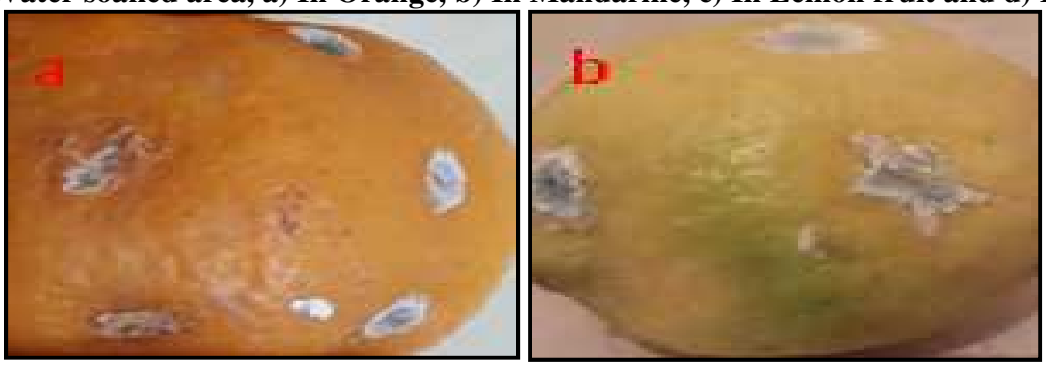


Figure No.7: Appearing the white mycelia after inoculation with *Penicillium spp,* a) After three day on Mandarine and b) After 5 days in Lemon

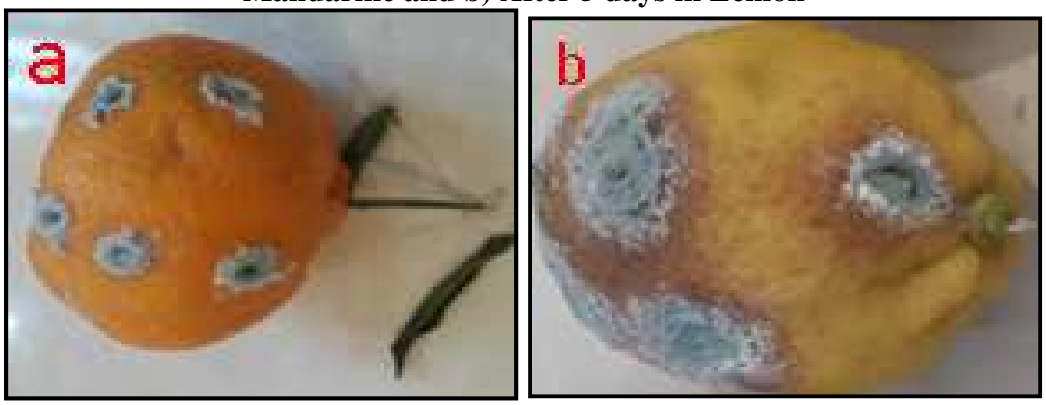


Figure No.8: Production of olive green spores by injected *Penicillium spp*. on a) Mandarine after 4 days and b) Lemon fruit after 6 days

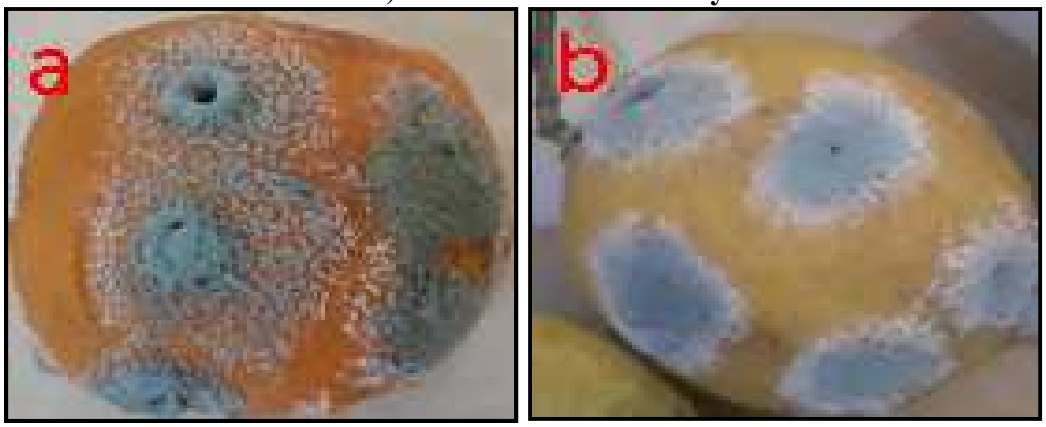


Figure No.9: Gradually spread of *Penicillium spp.* growth with retain white margin a, a') In mandarine from (5 - 7 days), b, b') In lemon from (6 - 8 days)

Figure No.10: Gradually spread *Penicillium spp.* growth with retain white margin (*) in Grape fruits after 7 days

Figure No.11: a) Initial manifestness of hypha growth after 11 days, b) Spread hypha growth on the peel surface after 16 days

Figure No.12: Integrated white circle with cotton texture on the fruit surface after 18 days

Figure No.13: Some types of fruits after period of infection

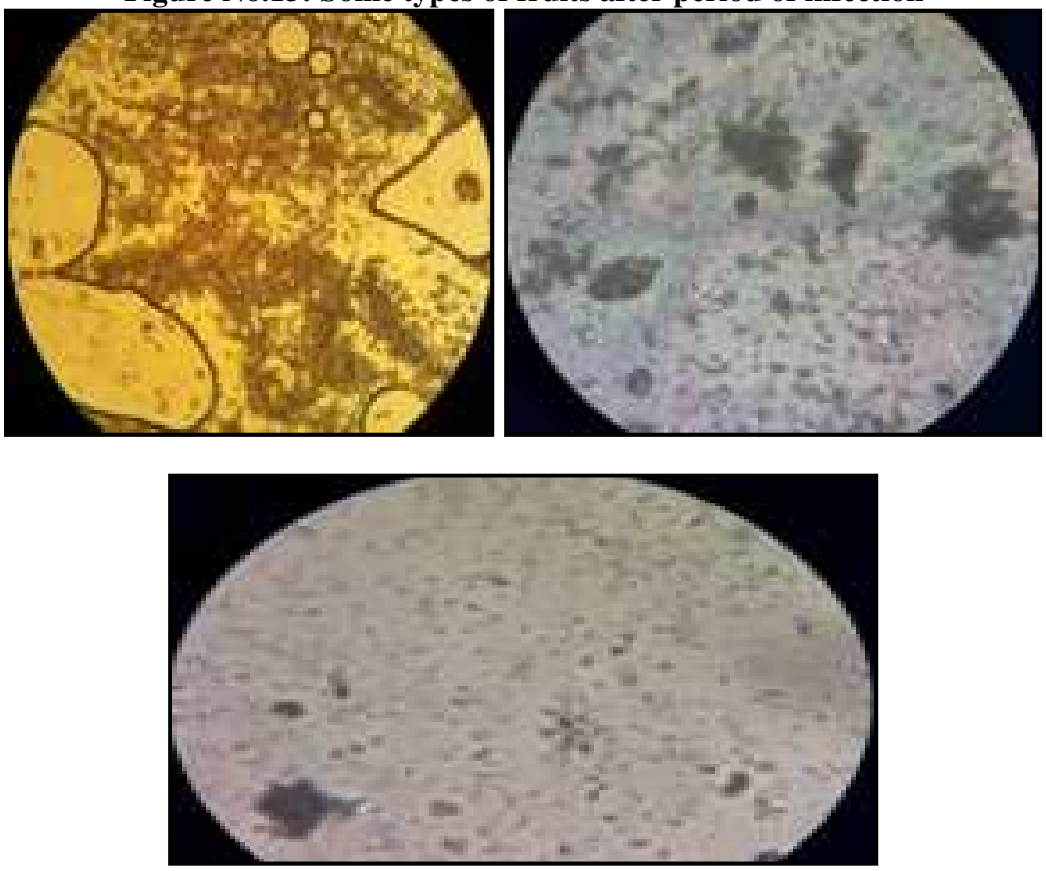


Figure No.14: a) Density of *Penicillium* spores in first dilution, b) Density of *Penicillium* spores in second dilution, c) Density of Penicillium spores in third dilution

Figure No.15: The differences between three types of inoculum density

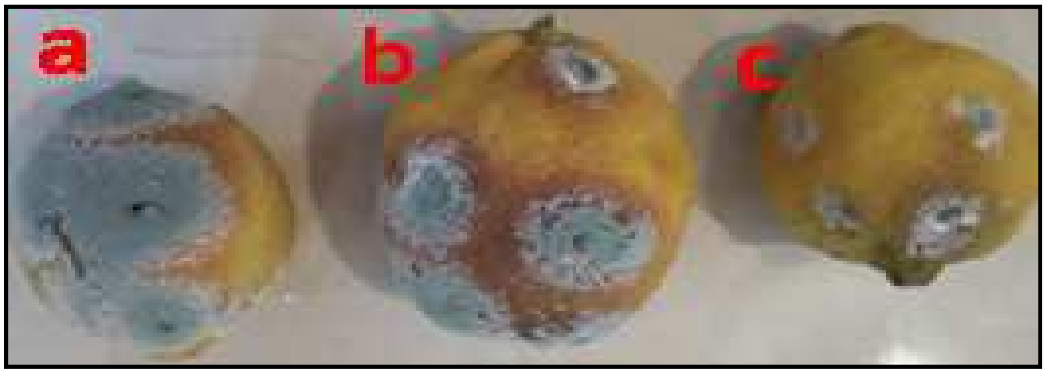


Figure No.16: Different growth by different concentration of three dilution types from inoculum density, a) First dilution, b) Second dilution, c) Third dilution

Figure No.17: Cross section in the infected fruit explains growth of *Penicillium digitatum* in the studied citrus fruit after 26 days

Figure No.18: Cross section in studied fruits after 2 month

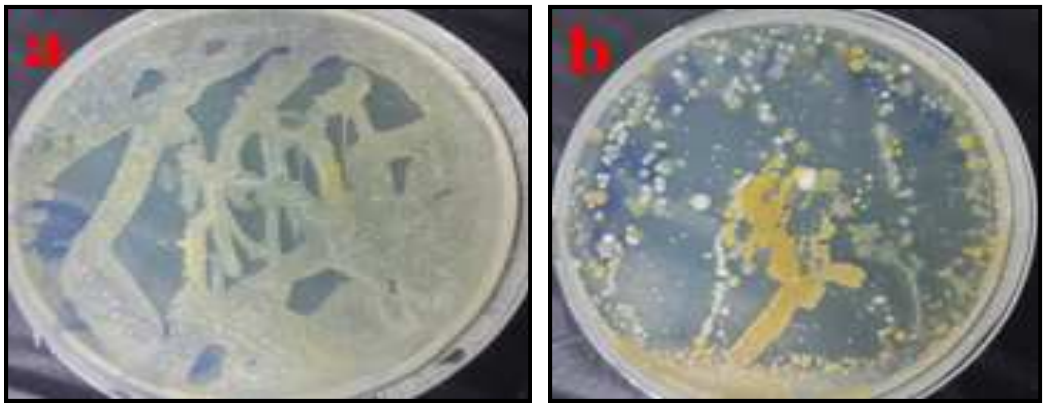


Figure No.19: Bacterial growth isolated from surface studied fruits in nutrient agar for a) Grapefruits, b) Blood orange

Figure No.20: Bacterial growth isolated from surface of Navel orange in the nutrient agar

Figure No.21: Growth of *P. digitatum* spores on three types of juice lemon fruit and effect of time factor on growth of *P. digitatum* after 10 days

Figure No.22: Infected peel juice of studied fruits with *Penicillium* spores after 10 days

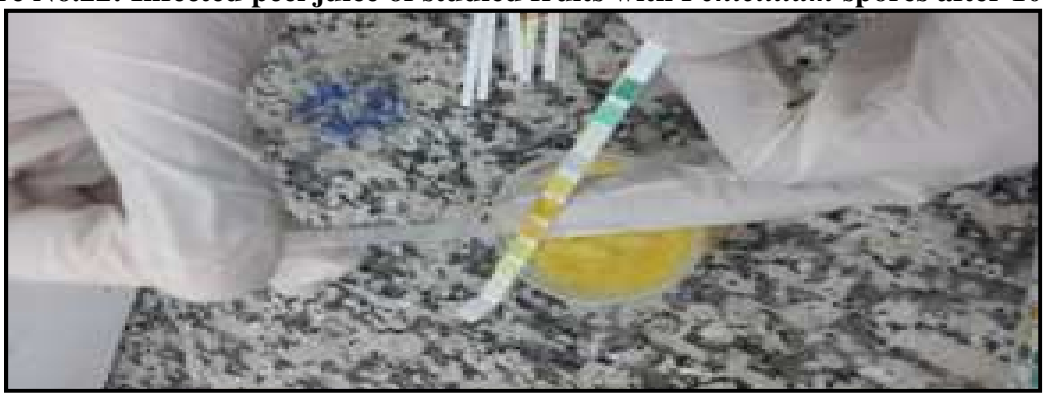


Figure No.23: The measurement of pH peel juice, pH sac juice and pH whole fruit juice

CONCLUSION

This study show the all three methods from artificial contamination were used can cause the infection to all studied citrus varities with same typical symptoms but different in the time period to manifestnes initial typical symptoms by three kinds from inoculum source (3 series dilution 10^{-1}, 10^{-2}, 10^{-3} spores/ml with dispersal time 12h between them *P. digitatium* can grow at 5°C inside host tissues which it indicate to enzymatic activity inside fungal tissues. Also it can grow more rapidly in thin peel of mandarin which it have little space between peel and inside tissues compared with other studied varities, this indicate to the thickness of peel have role to delay or enhance the infection with *P. digitatium.* Moreover, there is no differ in the location and depth of wounds to facilitated entry of this fungus. Chemical compounds of peel studied fruits can change the acidity of peel substrate and causing it them easy to adherence with extracellular enzyme produced by *P. digitatium.* From study of nature microflora on the surface of studied fruits, showed the presence of identified bacteria by phonex system™ e.g *Staphyloccous hominis* can delay the infection with *P. digitatium* about 12h compared with washed samples. Through modulation environmental pH inside healthy tissues samples, showed the used compound did not prevent or reduce of *P. digitatium* growth but just late the infection for 5 days by solution of Sodium bicarbonate.

ACKNOWLEDGEMENT

The authors wish to express their sincere gratitude to Department of Botany, Faculty of Sciences, Benghazi University, Libya for providing necessary facilities to carry out this research work.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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Please cite this article in press as: Nagwa S. A. Alraaydi *et al. In vitro* investigation of the infection mechanisms and strategies of *penicillium digitatum* (pers. Fr) on citrus fruits, *Asian Journal of Research in Biological and Pharmaceutical Sciences,* 8(4), 2020, 128-146.