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Physiological Potential of Viral and Viroid Etiology Risks Infection Citrus Trees in Field

Noha K. El-Dougdoug*[1], Mohamed A. Taha[2] and Abeer A. Faiesal[3]

[1]*Botany and Microbiology Department, Faculty of Science, Benha University, Benha, Egypt.*
[2]*Botany and Microbiology Department, Faculty of Science, Zagazeg University, Zagazeg, Egypt.*
[3]*Department of Basic and Applied Agricultural Sciences, Higher Institute for Agriculture Co-Operation, Shoubra EL-Kheima, Egypt.*

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ABSTRACT

To clarify the effect of citrus virus and viroid agents in the etiology of citrus diseases and their potential risk to the citrus trees growth, it will be necessary to study the pathogenicity of agents thoroughly phytochemical and horticultural analysis. Citrus trees cvs. Balady, Grape Fruit, Navel and Valentia distract phytopathological phenomena, could be differentiated into three groups; Gummy bark, stem pitting and bark scaling. These phenomena have known etiology, *Citrus gummy bark viroids* (CGBVd), *Citrus tristaza virus* (CTV) and *Citrus psoriasis virus* (CPsV) based on external symptoms in field lab. tested and indexing in greenhouse. These citrus cvs. trees growth were decline in horticultural characters such as; tree height, trunk diameter, weight of fruit, pH, ascorbic acid, total soluble solids, deformed fruits and yield per infected citrus tree when compared to healthy citrus trees ones. Chlorophyll a and b were decreased in infected leaves, on the contrary carotenoids content were increased. The total sugars content in citrus leaves were decreased related to chlorophyll content. Growth inhibitor ABA was present in a greater amounts infected terminal buds than growth hormones (IAA and GA3). The levels of N, P, K were decreased in infected leaves. The scavenging enzyme activities (catalase, peroxidase and polyphenol oxidases) were increased in infected citrus leaves cv. Navel and healthy ones. On the other hand, the CGBVd, CTV and CPsV agents reduced amylotic and proteolytic hydrolysis enzymes.

***Keywords*:** Citrus, CTV, CPsV, CGBVd, hormones, biochemical content.

Introduction

Egypt at present, among the seventh largest citrus producing countries in the world with a total production of 2.887.599 tons; represented about 40.1% from the total production of fruit crops and represented about 21.8% from the total production of citrus crops. The fruited cultivated area of citrus is 327.838 fed. The exported yield about 630.000 ton, where represented about 21.8% from the total production of citrus crops. This industry, large based in the Delta region, covers about 150.000 ba, is the most important sector in Egypt agriculture and economy (Fahmy *et al.,* 2002; Sofy, 2010).

Among graft transmissible diseases with virus, viroid, phytoplasma or unknown etiology that have been reported from Egypt, CTV, CPsV, *Citrus exocortis virus, Citrus cachexia virus*, CGBVds and *Spiroplasma citri* are most serious diseases and remain the most spread diseases in the country (El-Dougdoug *et al.*, 1997; Ghazal *et al.,* 2008; El-Dougdoug *et al.*, 2009; Sofy, 2010). The effect of infection was determined by assessing performance of infected and non infected trees growing in the field; the infection resulted in small trees with induced canopy yielding reduced crop; fruit quality characteristics were al affected; and infected trees had a poorly developed root system with fibrous roots containing fewer amyloplasts than healthy trees (Bani Hoshemian *et al.,* 2009). The present work was carried out to investigate of some phytopathological phenomena and their relationship to virus and viroid on photochemical, microelements and horticultural characters of citrus trees.

Materials and Method

Sample collection:

The commercial farm and varieties located in different area from Qalubiya governorate were investigated phytopathological phenomena at season2017/2018. Citrus samples (Ten of each bud stick and leaves from Navel variety) were collected based on external symptoms distancing CTV, CPsV and

Corresponding Author: Noha K. El-Dougdoug, Botany and Microbiology Department, Faculty of Science, Benha University, Benha, Egypt. E-mail: nohaeldougdoug@gmail.com

CGBVd, as well as, non-symptomatic trees. The plant materials were collected using tools disinfected domestic bleach diluted to gave 5% sodium hydrochloride solution and stored in a refrigerator bag with ice containers. For apical bud sticks (10-20 cm length) were sampled from the four sides of the selected square areas.

Detection of phytopathological phenomena:

The phytopathological phenomena were diagnosed on citrus tree navel variety samples depending on the external symptoms. Citrus trees displaying the distanced symptoms where trees showing the characteristic gummy bark symptoms, other trees displaying stem pitting, and anther trees showing bark scale. A symptomatic tree from the same trees were also selected.

Biological indexing:

Four blind buds were collected from each quadrant of infected citrus cvs. Balady, Grape Fruit, Navel and Valentia. These blind buds were grafted in Volkameriana lemon as root stock grafting inoculated with eye buds obtained from specific woody indicator plants including Dweet Tongor for CPsV, Mexican Lime for CTV and Etrog Citron for CGBVd. It cut off at 25 cm from the soil surface at the time of inoculation. A healthy and infected control were included in each container. Plants were placed in the greenhouse at 24-27°C high day/18-21°C Min night, for virus multiplication. The previous indicators were weekly inspected for symptoms developments for month as described by Roistacher (1991).

Laboratory indexing:

The lab. indexing was done by using dot blot hybridization (DBH), Double antibodies Sandwich-Enzyme Linked Immuno sorbent Assay (DAD-ELISA) and Tissue print immunoassay (TPIA)**.** The DBH method was used to detect CGBVd using specific probe according to Candresse *et al.*, (1990)**,** DAD-ELISA was applied to detect CPsV using specific polyclonal antibodies according to Clark and Adam (1977), and TPIA was done to detect CTV using CTV-specific polyclonal antibodies according to Garnsey *et al*., (1993).

Morphological and horticulture characters:

Trees leaves and fruits samples of 10 Navel trees (5 infected tree and 5 healthy ones) were collected for studying. Morphological parameters i.e. tree height (m^2); trunk height (cm^2) and trunk diameter (m^3). Horticultural characters were determined as following; number of fruit tree, yield/tree (kg), weight of fruit (g), size of fruit (cm^3), highness of fruit (cm^2); thicken of cortex (mm^2), pH, total acidity, total soluble solids (%) and ascorbic acid (mg/100 mL) according to A.O.A.C. (1990). The total sugars content (mg/ g fresh weight) in the leaves was estimated by adopting the method of Homme *et al.,* (1992).

Determination of photopigments:

The photosynthetic pigments contents (chlorophyll a, b and carotenoids) in fresh leaves were determined by spectrophotometric (Spectronic-601), method described by Moran (1982). The pigments concentration as mg^{-1} fresh weight were calculated using the following equations:

Chl. a (mg^{-1} fresh weight) = 9.784 E663 – 0.99 E644.

Chl. b (mg^{-1} fresh weight) = 21.426 E664 – 4.65 E662

Carotenoids (mg^{-1} fresh weight) = 4.695 E440 – 0.268(A+B)

Determination of total protein:

The total protein content (mg/g fresh weight) in the leaves was estimated by adopting the method of Lowry *et al.,* (1951).

Determination of Scavenging enzymes activity:

Catalase activity was assayed in a reaction mixture composed of phosphate buffer (50 mM pH, 7.0), H_2O_2 (30% w/v) and enzyme crud extract (0.5 mL) according to Kong *et al*., (1999). It was estimated at 470 nm O.D.by spectrophotometer (Spectronic-601) as the change in the optical density g^{-1} fresh weight min^{-1}.

Peroxidase activity was assayed according to Klapheck *et al.,* (1990) in a reaction mixture (3 ml) consist of 0.2 M phosphate buffer, pH 7.0; 20 mM guaiacol and 0.5 mL crude enzyme extract. The

reaction was incubated at 50°C for 3 min and then estimated at 470 nm O.D. by spectrophotometer (Spectronic-601) as the change in O.D g-1 fresh weight mins-1.

Polyphenol oxidase activity was determined in a reaction mixture containing of phosphate buffer (0.2 M, pH 7.0), catechol (100 mM) and crude extract (0.5 mL) and incubated at 50°C for 3min and directly measured using Spectronic-601 spectrophotometer (Coseteng and Lee 1978; Megahed *et al.,* 2019).

Determination of hydrolysis enzymes activity:

Amylase activity was determined in a reaction mixture containing 10 mg/ml soluble starch solution, 0.5 M sodium acetate buffer, pH 5.4 containing 20 mM $CaCl_2$ and 0.5 mL crude extract (Abu Soud *et al.,* 2004). This reaction was incubated at 40°C for 5 min and stopped by adding 5 mL of 5 M acetic acid. The mixture (3mL) was added to potassium iodide (0.01%) iodine (0.001%) which showed blue color and directly measured at 660 nm/15 min. using Spectronic-601 spectrophotometer as change in O.D. g-1 fresh weight.

Determination endogenous hormones:

Determination of endogenous hormones indol acetic acid (IAA), gibberellic acid (GA_3) and abscisic acid (ABS) in the terminal buds of infected and healthy citrus trees were carried out as described by Knegt and Branima (1973). Hormones contents were estimated by HPLC (Arid Land Agric. Research and Services Center, Fac. Agric. Ain Shams Univ., Cairo, Egypt) as follows: Water UbK HPLC, Column Bondapak C18, Dimension 3.9 X 300 mm, Mobile phase MeOH super purity-2% acetic acid, and detection UV water: 486-254 nm

Determination macronutrients:

Total macronutrients were determined in infected and healthy citrus leaves cv. Navel. Total nitrogen was determined by using Kjeldahl digestion method according to Jackson (1973). It was estimated as mg g-1 dry weight of plant tissues. The total phosphorus was determined photometerically in acid solution of digested sample at 660 mm as a method reported by Jackson (1973) by spectrophotometer (Spectronic-601) as mg/g-1 dry weight percentage. Total potassium was determined a mg g-1 dry weight percentage according to Jones *et al.,* (1991) through out measuring in acid solution of digested samples using flam photometer (Model Jenway-Clini-PDP7).

Statistical analysis:

The data were subjected to the proper statistical analysis of variance of a randomized complete block design for comparison between treatments means, using Duncan, (PASW® Advanced Statistics 20, 2010) at 5% level was used. The values recorded in the values of the biochemical analysis are means of three replicates.

Results

Virus and viroid associated with phytopathological phenomena in citrus trees:

The phytopathological phenomena were observed on citrus tree cvs Balady, Grape Fruit, Navel and Valentia distinct different external symptoms that could be differentiated characteristic gummy bark symptoms. A line of reddish- brown, gum-impregnated tissue can be seen around the circumference and especially near the bud union when the bark was scraped; The discoloration and gumming may extend 60 cm or more above the bud union in the bark of the trunk and main branches.; and the dark brown streaks of gum impregnated tissue were seen in both the circumference and in longitudinal sections, fig (1, c).

The infected citrus cvs. Valencia, Balady and Navel were showed bark scaling on the two sides of the trunk and branches; cultivar grapefruit showed bark scaling and gum symptoms on the two sides of the main trunk. The infected citrus trees cvs. Balady and Navel were showed high greenhouse leaves, stunting, heavy crop of small fruits and some trees expressed no field external symptoms but when crushed chips of bark on trunk and branches showing stem pitting normal citrus trees fig (1, a and b)..

Biological indexing:

Some of citrus trees symptom latent which gave the best different wide range of positive results with DAS-ELISA against CPsV were confirmed by biological indexing on indicator plants

(Dweet Tongor) which appeared oak leaf pattern symptoms under greenhouse conditions (24-27°C high day/18-21°C Min night).

(a) Bark scaling and gum symptom on trank and deformed fruit

(b) Cruched chips of bark on trank, stem pitting on branch and small fruits

(c) Line of reddish brown gum impregnated tissue

Fig. 1: Photograme showing the viroid and virus phytopathological phenomena in commercial citrus farms cv. Navel located in Qalubiya governorate at season 2017/2018

The indexing by graft inoculation on Mexican lime detect CTV in trees. It was found the CTV was successfully transmitted after 3 months. The external symptoms as vein clearing, leaf cupping, leaf flecking and stunting. As well as, indexing by graft inoculation Etrog Citron showing leaf epinsety after one month at greenhouse detected CGBVd infected trees.

Lab. indexing:

The imprint of the CTV infected stems was clearly visible with deep purple stained area indicating the presence of CTV in the phloem of stems. The healthy tissues imprint showed no color ones. The infected trees which exhibited specific symptoms as well as symptom gave positive results with tissue print immunoassay using specific monoclonal antibodies of CTV (Fig., 2). CGBVd infected citrus trees were showed gummy bark in stem phloem gave positive results with DBH using specific probe (Fig., 2). The CPsV showing external symptoms bark scaling and gum was reacted by DAD-ELISA using specific polyclonal antibodies

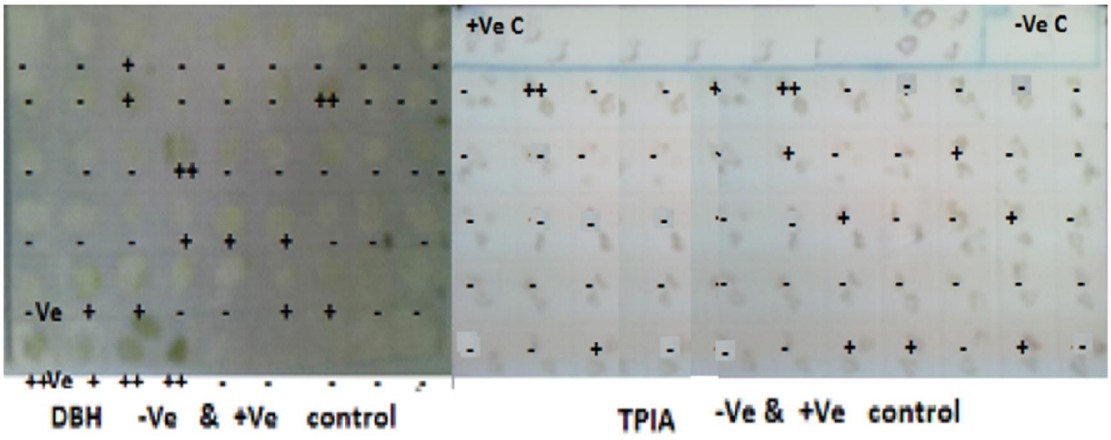

Fig. 2: Photogram illustrate dot blot hybridization assay (DBH) for CGBVd detection using specific probe and tissue print immunoassay (TPIA) for CTV detection using CTV-specific polyclonal antibodies

Horticultural and Navel tree yield characters:

Morphological parameters and fruits characters of citrus cv. Navel infected with viroid and citrus viruses at horticultural ripening were determined (fig., 3). The citrus trees were investigated separately each tree infected with CGBVd, CTV and CPsV. A random 10 trees from each infected and healthy trees ones were taken to determine the horticultural characters. Data showing that, CGBVd, CTV and CPsV infected citrus significantly decreased of tree height, trunk height and trunk diameter compared healthy (fig., 3).

The yield of diseased citrus trees cv. Navel under field condition showed significant reduction of some physical and chemical characters of Navel fruits compared with healthy ones. Low quality was observed on fruits of total soluble solids, L-ascorbic acid and volume of juice and total sugar were significantly decreased in CGBVd, CTV and CPsV infected citrus trees compared to healthy trees ones, while, total acidity (citric acid) (%)revealed to non significant (fig, 3). Diameter of fruit (cm), weight size, highness and thicken of cortex revealed that, non-significant response when compared CGBVd infected citrus with healthy trees, while significant decreased when compared CTV and CPsV infected citrus with healthy trees.

Photopigments contents:

In the present investigation, results clearly reveal a reduction in the photosynthetic pigment levels (chlorophyll a, chlorophyll b). While it was found that, CGBVd, CTV and CPsV due to increase of carotenoids when compared with healthy trees (fig., 4).

Soluble protein content:

Results of the soluble protein contents in citrus leaves cv. Navel were decreased in response to the CGBVd (2.75) and CTV (2.71) infected trees compared to healthy ones. On the other contrary. It was non-significant increased in response to CPsV (300 mg/g dry weight), infected tress compared with healthy ones (2.95 mg/g dry weight).

Scavenging enzymes activity:

The scavenging enzymes (PPO) were non significant effect in response to CGBVd infected leaves when compared with healthy trees. While CTV and CPsV infected citrus leaves are highly significant increased (fig. 5). While the result of peroxidase (POD) activity showed that non significant effect when compared CGBVd and CPsV infected leaves with compared with healthy trees. Except CTV infected leaves significantly increased (fig. 5). In this investigation, catalase (CAT) activity was highly significantly increased in CGBVd, CTV and CPsV infected leaves.

Hydrolysis enzymes activity:

Results in fig. (6) of the present study revealed that hydrolysis enzymes, amylase and protease

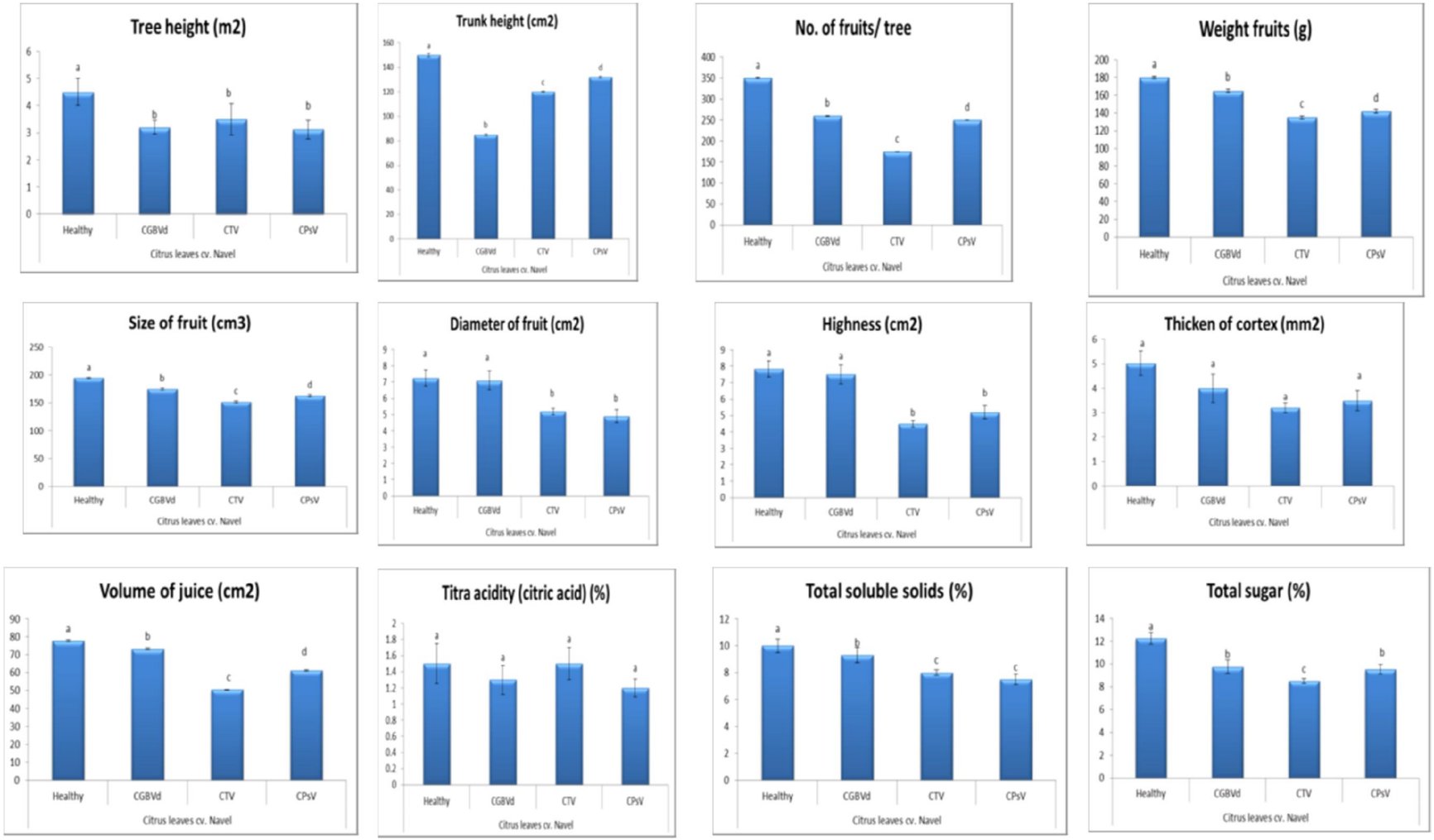

Fig. 3: Histograms statistic analysis showing the effect of of viroid and virus infected citrus tree and fruits on horticultural and tree yield characters

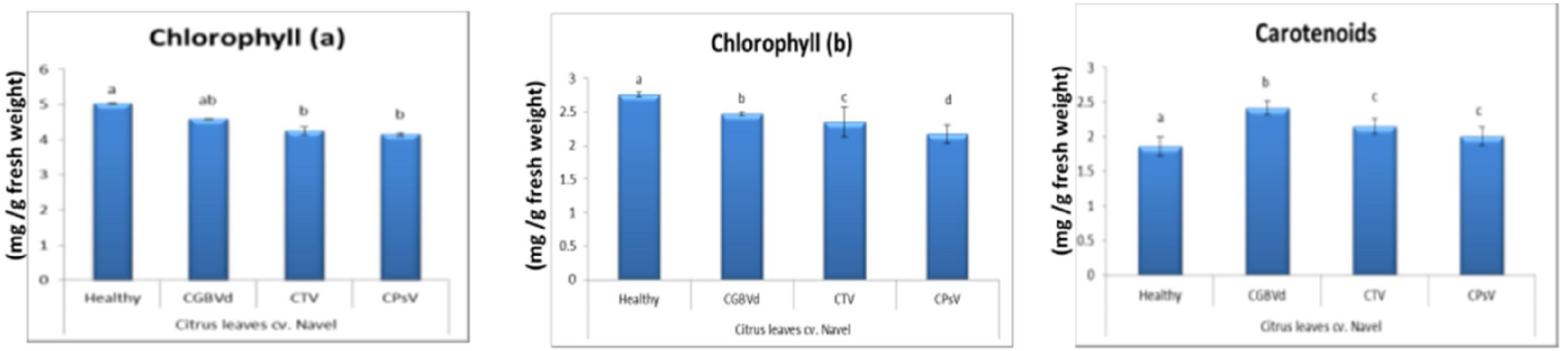

Fig. 4: Histograms statistic analysis showing the effect of viroid and virus infected citrus cv. Navel trees on chlorophyll a, b and carotenoids contents.

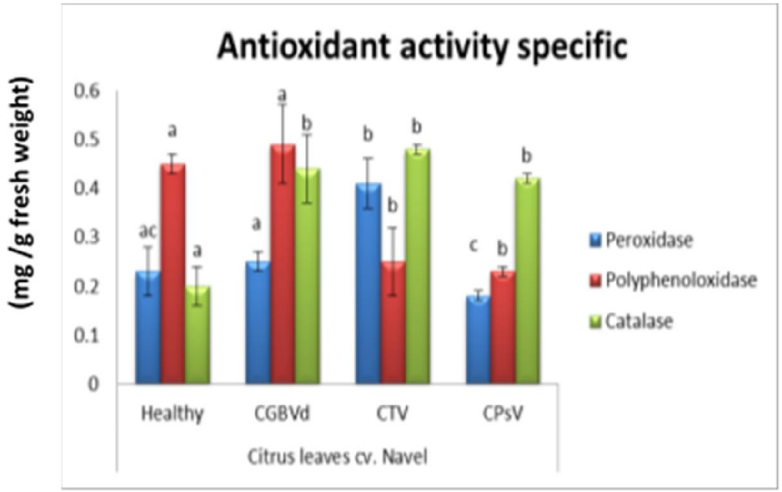

Fig. 5: : Histogram statistic analysis showing the effect of viroid and virus infected trees on antioxidant activity specific content of citrus leaves

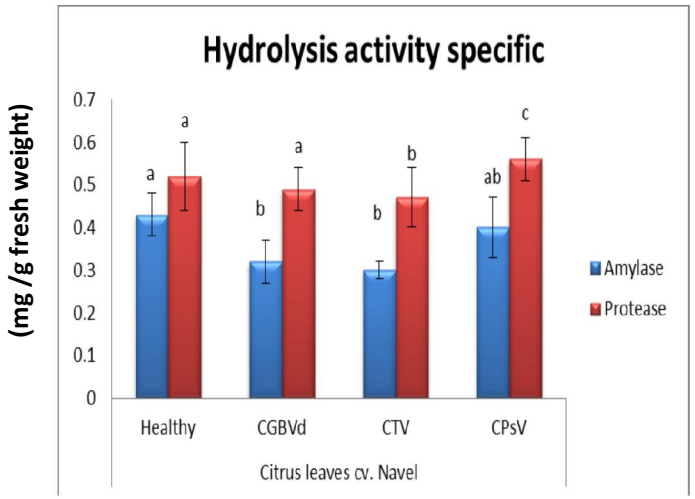

Fig. 6: Histogram statistic analysis showing the effect of viroid and virus infected trees on antioxidant activity specific content of citrus leaves

were reduced non significant effect in response to CGBVd and CTV infected leaves when compared with healthy trees. While CPsV infected citrus leaves are highly significant increased (fig. 6).

Level of endogenous hormones:

In the present investigation, results of growth promoting hormones GA3 and IAA present in significant greater amounts in citrus healthy leaves, while decreased on infected citrus leaves with CGBVd (131.03 and 3.64 mg/g fresh weight), CTV (115.7 and 2.50 mg/g fresh weight) and CPsV (138.7 and 3.01 mg/g fresh weight) respectively (Fig. 7). On the contrary, growth inhibiting hormone ABA was present in greater amounts (11.69 mg/g fresh weight) in CGBVd (1.34 mg/g fresh weight) in CTV and (2.75 mg/g fresh weight) in CPsV infected citrus terminal buds lower amounts (0.91 mg/g fresh weight) in healthy ones (fig. 7).

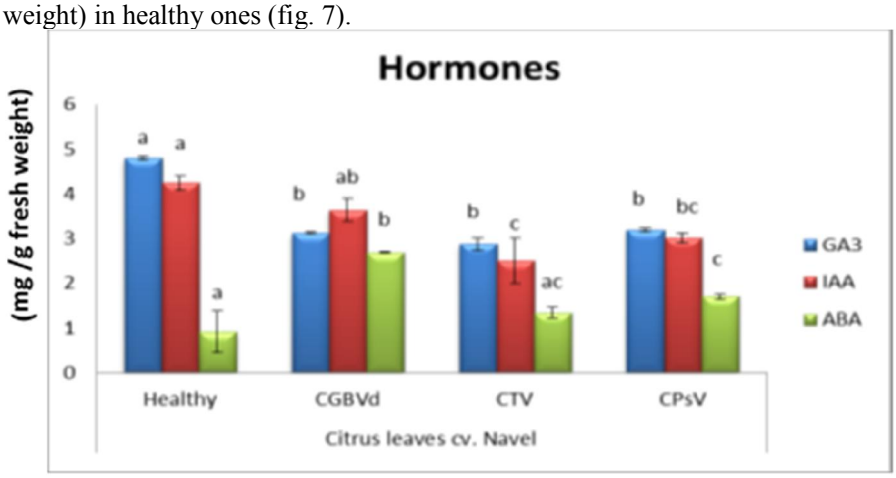

Fig. 7: Histogram statistic analysis showing the effect of viroid and virus on hormones content of citrus leaves.

Macronutrients:

The level of nitrogen, phosphorus and potassium were influenced by viroid and citrus virus agents in citrus leaves where the level of N and K were non significantly decreased in infected citrus leaves. However, p level markedly significant increase when compared to healthy ones (Fig. 8).

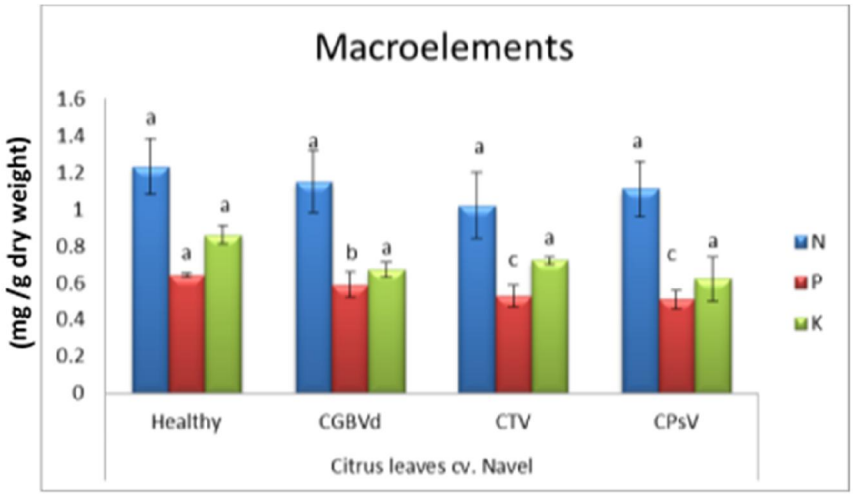

Fig. 8: Histogram statistic analysis showing the effect of viroid and virus on macronutients content of citrus leaves.

Discussion

Among graft transmissible diseases with phytopathological etiology viroid virus and unknown that have been reported from Egypt as well as in the rest of Mediterranean countries, CTV, CPsV,

gummy bark one of the most serious diseases and remain the most spread diseases in the country (Roistacher, 1991; El-Dougdoug *et al.,* 1997; Sofy, 2010; Abdel-Mohsen, 2013).

The relationship between phytopathological phenomena and citrus trees was studied in Egypt therefore this study was carried out on cv. Navel grown in different regions in Egypt. These phytopathological phenomena were detected on citrus trees in the field depending on the external field symptoms which many diseases were diagnosed by Nour Eldin (1959); Roistacher (1991) through specific field symptoms. Citrus tress distinct different external symptoms that could be differentiated into three groups, gummy bark, stem pitting and bark scaling and gum, where these phenomena were described by El-Shorbagy (2007); El-dougdoug *et. al.,* (2010); Abdel-Mohsen (2013).

Ten bud sticks were cut from four different sides of tested tree which collected from different geographical regions and showing phytopathological phenomena. The leaf petioles were printed on nitro cellulose membrane and hybridized with *Hop stunt viroid* (HSVd) Dig labeled cDNA probe. The citrus trees showing gummy bark symptoms have clear positive with HSVd Probe (El-Dougdoug *et al.,* 2010; Sofy, 2010). The trees were indexing by graft inoculation on Etrog citron plant under greenhouse condition. The against reacted with citron where exhibited field leaf epinasty, petiole wrinkle and browning (Sofy, 2010) The citrus trees exhibited bark scaling and gum symptoms were tested serological assay which gave positive results using specific CPsV monoclonal antibodies by DAS-ELISA (EL- Shorbagy, 2007; Sofy, 2008). The citrus trees which showing stem pitting was tested for the presence CTV virus by tissue print immunoassay using specific monoclonal antibodies of CTV (Fahmy *et al.,* 2002).

The citrus trees were indexing by graft inoculation on *Maxican lime* plants under greenhouse condition that against reacted in exhibited yellow (Abdel-Mohsen, 2013). These trees were indexed by graft inoculation on Dweet Tongor, it gave Oak leaf pattern (OLP) symptoms after 25-30 days post inoculation (Fahmy *et al*., 2002; El-Shorbagy, 2007; Sofy, 2008).

These results mean that the citrus trees were infected with CGBVd, CTV and CPsV tested to clarify the role of phytopathological phenomena in etrology of citrus diseases and their potential risk or actued damage to the citrus free growth, it will be necessary for studying the pathogenicity of these gents thoroughly phytochemical analysis and horticultural characters. Photosynthesis is essential for the normal growth and metabolism of plants. It depends on the normal availability of chlorophylls in the chloroplasts and on several enzymes which function during the light and dark phases. A normal functioning of the dark phase results in the production of monosaccharide, disaccharide and polysaccharide by the plant. The photopigments (chlorophyll a, b) of CGBVd infected leaves decreased on the contrary, the carotenoids increased compared to healthy ones. On the other citrus leaves infected with CTV or CPsV decreased in chlorophyll a, b and carotenoids compared to healthy ones. The decreasing in photopigment due to the decreased in total soluble carbohydrates, where significant reduced in CGBVd, CTV and CPsV infected citrus leaves compared to healthy ones, these results were agreement with Jardeny *et al.,* 1965; El-Shorbagy, 2007; Sofy, 2010).

Growth inhibitor ABA was present in a greater amount in CGBVd, CTV and CPsV infected terminal buds than healthy terminal ones. On the other contrary the amounts of growth promoters (IAA and GA3) were present in a greater amounts in healthy than CGBVd, CTV and infected terminal buds tissues. These results were in agreement with the reports of similar changes in amounts of promoter and inhibitor hormones or both in other plant viruese (Diener, 1963; Hank and Feldman, 1972; El-Dougdoug, 1988, El-Shorbagy, 2007; Sofy, 2010).

Soluble protein contents of citrus leaves cv. Navel trees were decreases in response to the infected citrus trees with both of CGBVd; CTV or CPsV. In the same time, proleoytic and amylolytic activities were reduced in response to phytopathological agents infected citrus trees: Epmpared to healthy ones. On the contrary, scavenging enzymes catalase, peroxidase and polyphenol oxidase were increased in citrus leaves response to both CGBVd, CTV or CPsV infection compared to healthy ones. These results have similarly been shown for cachaxin affected trees of clementine mandarin (Jardeny et al., 1965) and citrus sweet orange cv. Navel (Sofy, 2010).

The levels of microelements NPK influenced by the phytopathological agents (CGBVd, CTV and CPsV) where the levels of N, P and K reduced in infected leaves compared to healthy ones, these data were in harmony with Rodriguez and Romano Gallo (1968); El-Shorbagy (2007) and Sofy (2010), they reported that, the level of N, P and K were significantly influenced by *Citrus exocortis viroid* (CEVd), CTV and CGBVd respectively.

In general viroid and viruses infected citrus trees cv. Navel showed a reduced effect on the yield of fruits, this effect was varied depending on the agent and disease severity as well as environmental conditions. The phytopathological phenomena agents due to reduced pH, ascorbic acid, acidity and total soluble solids, as well as small and deformed fruits and total yield per tree comparing to healthy trees ones, these data were agreement with Aronguren *et al.,* (2004); El-Shorbagy (2007) and Sofy (2010), they reported that, cachexia, exocortis, gummt bark, tristizaand bark scaling induced significant shortening of the shoots and reduction of the canopy diameter, high and size with respect to healthy plants. Also, these results were in agreement with Stuchi *et al.,* (2007) and Bani Hoshemian *et al.,* (2009).

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