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Antifungal Activities of Some Botanical Extracts and Synthetic Compounds Against Down Mildew in Cucumber Plants.

Abeer A El-ghanam[1], Hoballah EM[2], Abdel-Halim KY[3]*, and Sanaa A Massoud[4].

[1]Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt
[2]Agricultural Microbiology Dept., National Research Centre (NRC), Giza, Egypt
[3]*Mammalian & aquatic toxicology Department, Central Agricultural Pesticides Lab. (CAPL), ARC, 12611, Dokki, Egypt.
[4]Fungicies, nematicides and bactericides Department, Central Agricultural Pesticides Lab. (CAPL), ARC, 12611, Dokki, Egypt.

ABSTRACT

Commercial synthetic fungicides have been recommended for controlling phytopathogenic fungi, but they are not considered being long-term solutions, due to concerns of expensive, exposure risks, fungicide residues, and health and environmental hazards. The fungicidal activities of some botanical extracts, discretely or commixed with commercial fungicides in Egypt were examined against downy mildew *Pseudoperonospora cubensis* in cucumber plants. The botanical extracts were obtained by ethanolic extraction of *Laurus camphora* L., *Peganum harmala* L., *Zygophyllum coccineum* L. and *Cymbopogon nardus* plants, while the tested synthetic fungicides antithesis were Gentracur® (propamocarb hydrochloride), Casparytin® (cymoxanil), Moment® (dimethomorph+iprodione) and Lacinia® (cymoxanil+mancozeb). The finding disclosures were recognized along spores germination and disease severity during seasons 2014 and 2015. Administed plants with different treatments induced disease severity in values more than 4.66%. Moreover, Moment® a synthetic fungicide was about to be the outstanding treatment for controlling downy disease compared to others. The mixture decrease residue levels of synthetic compounds down their maximum residue limits. The biochemical quantifications of fruits revealed positively outputs compared with untreated groups. Moreover, the yield increased significantly compared with control. This work viewed approached to mix synthetic fungicides with natural extracts to minimize the residue level in edible parts and improve the quality and yield of crops .
Keywords: Cucumber; downy mildew; plant extracts; fungicides; residue analysis; biochemical quantifications

**Corresponding author*

INTRODUCTION

Cucumber crop (*Cucumis sativus*) is thought out one of the most economical plants of cucurbits family. This vegetable has a documented history of more than 5000 years. However, it is highly influenced by fungal diseases in farms or greenhouses. Under certain conditions, downy mildew spread rapidly causing significant damages. Downy mildew (*Pseudoperonospora cubensis*) is a common fungal disease in cucumbers cultivated in geographically potential places [1]. *Pseudoperonospora cubensis* is one of the most prevalent and distributed foliar fungal diseases of protected cucumber cultivar that considerably reduce the mass production from early spring until autumn seasons and greatly affects both yield and quality. The control of downy mildew disease has been almost exclusively based on the application of chemical fungicides. Several effective fungicides have been recommended for use against this disease, but they are not considered being long-term solutions, due to concerns of expensive, exposure risks, fungicide residues, and health and environmental hazards. Moreover, the development of pathogenic fungi resistance towards commercial synthetic fungicides is a great problem affecting significantly the future of chemical control by fungicides. In recent times, a lot of investigations were done on the use of extracts of medicinal plants for controlling many phytopathogens [2, 3]. Ironically, Many of medicinal plant constituents showed high potential effects against plant diseases such as alkaloids, glycosides, terpenes, terpenoids, different flavonoids and other many natural constituents, which were mostly bred out in many plants such as *Laurus camphora* L., *Peganum harmala* L., *Zygophyllum coccineum* L. and *Cymbopogon nardus* [4-8]. The purposed work aims to 1- Evaluation of antifungal activities of some medical extracts alone or mixed with synthetic fungicides against downy mildew *Pseudoperonospora cubensis*. 2- Determination of residue levels of treatments associated with biochemical quantifications of plants.

MATERIAL AND METHODS

Plant Materials.

The chosen medicinal plants *Laurus camphora* L., *Peganum harmala* L., *Zygophyllum coccineum* L. and *Cymbopogon nardus* collected either selected plant organs from their natural habitats (National Research Center farm in Nubaria village, El-Beheira governorate, Egypt) or herbal market (Cairo herbal market, Harraz Co. for medical plants in Bab El-Khalk area, Cairo, Egypt) were air-dried in shade before being used and ground by a knife mill at 35 mesh sieve (0.5 mm diameter), and kept in dry brown glass bottles. All chosen plants were identified at the herbarium of the National Research Centre in Cairo [6].

Preparation of Plant Extracts.

The selected medicinal plants were extracted by mixing 250 g portion of the dried plant powder with 1 liter of 80% absolute ethanol (95.5%) and 20% water at 75 ˚C using the Soxhlet extractor technique. Initial ethanol extracts were concentrated to the appropriate volume (about 50% of initial volume) in a rotary evaporator at 45 ˚C under vacuum, before being filled in dry brown glass bottles, closed and stored at 4 ˚C until further investigations [6].

Experimental Design

***In vitro* Assay.**

The commercial synthetic fungicides were obtained from trade markets of Egypt as follows: Gentracur® (Gentra Egypt Company); Casparytin® (Sandvally Co.); Moment® (Star Kim Co.) and Lacinia® (Edku Co.), respectively.

The leaf disks screening assay was done as the follows: infected leaves of cucumber plant were collected from the field. Sporangia spores were harvested out of infected leaves using a small paintbrush and water containing 0.01% tween 80. The sporangia spore suspension then filtered through fine nylon membrane 8 µm (Millipore) to remove hyphal aggregates, and then washed twice with sterilized water. The spore's concentration was adjusted to be 10^4 spores per ml using a haemocytometer method as spore counter [9]. Cucumber plant species under search (Beit Alpha hybrid) were used to prepare leaf disks. The leaves below the apex free from disease injury (Microscopic examined by authors) of field plants 1.5 months growth were

surface sterilized using 0.1 sodium hypochlorite, rinsed three times in sterile water, and then leaf disks (15mm in diameter) were cut and dipped in three concentrations for each treatment as shown in Table (1). Sterile water was used as a control treatment. Two ml of sporangia suspension after 24 hr was sprayed on the disks. Then, the sprayed disks were distributed in the Petri dishes (90 mm. in diameter) containing moisture filter paper. All experiments involved four leaf disks as replicates. The disks then were incubated at 20 ˚C in darkness for 24 hr. Spores germination as well as disease incidence were observed using light microscope and subjected to calculate through the following equation:

$$\text{Spore conc.} = \sum \text{spores in four squares} \times 2500 \times \text{dilution factor}$$

Field Assay

Experiments were carried out at Etay El-Baroud Research Station, El-Beheira governorate, Egypt during two seasons (2014 and 2015) in a randomized complete block design with three replicates. The experiment was divided into 75 plots (3.0×3.0 m for each) containing three rows. Seeding rate was as 2 seeds/hole divided for 30 cm between holes. All agricultural practices such as cultivation, irrigation and fertilization were carried out. Plants were weekly sprayed for 3 times distributing in groups from T1to T25 as presented in Table (1). Disease severity was recorded after 7 days of last spraying. In addition, other measurements such as biochemical quantifications and yield were done immediately after fruit's picking.

Disease Severity

Plants were examined periodically for disease status through measurements by using the devised scale (0-5) as adopted by Horsfall and Barratt [10] as follows: 0 scale means no symptoms appear, 1equals 0.1 to 3% of leaf area covered by the infection, 2 equals more than 3 to 10 % of leaf area covered by the infection, 3 equals more than 10 to 25% of leaf area covered by the infection, 4 equals more than 25 to 50% of leaf area covered by the infection and 5 equals more than 75% of the plant growth covered by the infection and the plants turned to be stunted, respectively. The grown plants were periodically examined for disease symptoms to estimate the severity of the disease and the final averages were recorded using the following Formula:

$$\text{Severity \%} = \Sigma (n \times v)/5N \times 100$$

Where: n=number of infected leaves in each category, v=numerical values of each category and N=total number of the infected leaves.

Chemical Analysis

Fungicide Residues

Five g of fruits were homogenized using a polytron homogenizer (Jankes & Kunkel, Gm Hu Co KG) for 1 min. The suspension was mixed with 10 ml of acetonitrile and shaken for 1 min. One g of NaCl and 4 g of $MgSO_4$ were added, vortexed immediately for 1 min and centrifuged at 4000 rpm for 10 min. An aliquot (1 ml) was placed into a micro centrifuge tube (2 ml) containing 50 mg primary-secondary amine (PSA) and 150 mg $MgSO_4$ vortexed for 1 min and re-centrifuged at 6000 rpm for 10 min [11]. The supernatant was subjected for liquid chromatography (Agilent 1260, Japan) equipped with an analytical column; Zordex C_{18} (250×4.6mm×0.5 µm film thickness), auto sampler and quarter pump. A mobile phase of acetonitrile: water (80:20 v/v) was used at a flow rate of 1.2 ml/min. The examined fungicides were subjected on UV detector. The absorbance wavelengths, retention times, limits of detection and recovery percent were adjusted as listed in the results.

Methods and instruments were fully validated as part of a laboratory quality assurances system [12]. The Codex Committee's Criteria for quality assurance were followed to determine the performance of the multi-residue method. Recovery, accuracy and limit of detection (LOD) were determined for every compound.

Mineral Composition

The elements in cucumber fruits were developed using the experimental protocol proposed by Sadzawka et al. [13]. The samples were dried in an oven at 70 ˚C for 24 hr. Then, 0.3 g of dried sample was

mixed with 18 ml of distilled water, 100 ml sulfuric acid (96%) and 6 g of salicylic acid. After 12 hr, the mixture was heated for 2 hr at 200 ˚C and the temperature was increased to 400 ˚C for another 2 hr. Hydrogen peroxide was added to complete the digestion at rates from 5 to 10 ml. Then, 10 ml of water was added and the sample agitated until a clear solution observed. After cooling, the solution was filtered and the volume was adjusted to 50 ml with water.

The essential metals; Cu, Fe, Mn and Zn were estimated on wavelengths; 324.7, 248.3, 279.5, 213.9 nm, respectively, in duplicates by using atomic absorption spectrometer (Spectra AA 220; Variance) at Department of water and soil (Alexandria University, Egypt). The concentration of each metal was calculated as mg/kg dry weight and LOD was calculated as double the standard deviation of a series of measurements. All measurements were closed to the blank absorbance measurement and adjusted to recovery percent for each metal. Appropriate quality assurance procedures and precautions were carried out to ensure reliability of the results. The samples were carefully handled to avoid contamination. Deionizer water was used during preparation and measurements [14].

Biochemical Quantification.

Plant Pigments.

Chlorophyll a, b and carotenoids were determined in samples according to the method of Moran and Porath [15] by using *N, N*-dimethylformamide as an extraction solution. The absorbance was measured at 662, 644 and 440nm for chlorophyll a, b and carotenoids, respectively, by using spectrophotometer (JENWAY 6305 UV/VIS). The pigment's concentrations were estimated as follows: $chlorophyll_a$=9.784×E.662-0.99×E.644; $chlorophyll_b$=21.426×E.644-4.65×E.662 and carotenoides=4.695×E.440-0.268×(chl. a +chl. b), respectively. The concentration was expressed as mg/L.

Total Carbohydrate.

After pigments extraction, the remaining tissues were extracted with 1N NaOH in a boiling water bath for 2 hr. The final extract was used for protein and carbohydrate quantification. Total carbohydrate was determined as glucose by the method of Dubois et al. [16].

Total Soluble Protein.

The protein content was determined according to the method of Lowry *et al.* [17]. Bovine serum albumin was used as a standard.

Total Phenols.

Sample Preparation.

Ten g of fruits were mixed with 90 ml of acetone (70%). The mixture was homogenized for 1 min. The extract was shaken in water bath 20 ˚C for 60 min. Aliquot of sample (1.5 ml) was centrifuged at 2500 rpm for 15 min at 4 ˚C. The supernatant was used for analysis (1st extraction). The residue was re-extracted in same conditions and the supernatant was used for analysis (2nd extraction). Each supernatant (1st and 2nd extraction) was independently measured.

Total Phenolic Content.

The method involves the reduction of folin-Ciocalteau reagent by phenolic compounds forming blue complex. Half ml of extract was mixed with 3.0 ml of distilled water and 0.25 ml of folin-Ciocalteau reagent. Immediately, 0.15 ml of saturated sodium carbonate and 0.95 ml of distilled water were added. Then, the mixture was incubated for 30 min at 37 ˚C. The absorbance was recorded at 765nm. The measurement was compared to a standard curve prepared with gallic acid solution. The total phenolic content was expressed as mg gallic acid equivalents per g of fresh weight [18].

Statistical Analysis.

All the data were presented as a mean value±SE and subjected to analysis of variance (ANOVA). In addition, the means were compared for significance by Lethal Significant Difference (LSD) at 0.05 levels. The statistical analysis was performed using the Costat program [19].

RESULTS AND DISCUSSION

The data of examined fungicides against *P. cubensis* the phytopathogenic obligate fungi were practically used in different rates as presented in Table 1.

Spores Germination

The laboratorial investigation among spores germination was done. Spores germination of *P. cubensis* on cucumber leaf disks treated with different fungicides, in three levels were exhibited in Figure 1. Most of plant extracts were effective in reducing the percentage of *P. cubensis* germination in a range from 0.2 to 3.73% in level 3. On the other hand, the mixtures of botanical with synthetic fungicides as showed in the obtained data reduced the percentage of spore germination in ranges(12.43-5.60), (8.13-2.40) and (0.00-2.90%) for levels 1, 2 and 3, respectively, compared with untreated group which not exceeded than 80.47% in all levels.

Disease Severity.

The data of downy mildew disease development on cucumber plant grown in field during 2014 and 2015 are illustrated in Figure 2. In natural infected plants, downy mildew disease severity increased during growth period as following: the highest values among severity were recorded for T14 to be 65.66, 64.00 and 62.66% at 1st, 2nd, and 3rd sprays during 2014, while the lowest value were recoded for T3 to be 6.16,5.00 and 5.00%, respectively, at the same spraying sequence. Regarding 2015 season, T14 revealed 65.00, 64.00and 64.00 and 63.33%, while T3 recorded 6.66, 5.33 and 4.66% sequence, respectively, as described above. Sprayed plants with different treatments were induced in downy mildew disease severity in values more than 4.66% which registered for T3 at 3rd spray during 2015. Cleary, fungicide, Moment® was considered the best treatment to control downy mildew disease incidence compared with others.

Downy mildew is a rigorous disease in cucumber plants during the low temperature and high dampness season in Egypt. The causal agent; *P. cubensis* has developed resistance to fungicides [20]. Ergo, there is an exigent need to find an alternative mean to control the disease. The results obtained in this study showed that, botanical extract individually or commixed with synthetic fungicides accommodate as an alternative mean to bulwark cucumber plants against P. cubensis as well as reduce environmental pollution.

Plant extracts are a group of substances extracted from different components of plants which contains a great many of compounds with pesticidal properties. These compounds can be obtained from roots, barks, seeds, buds, leaves and fruits. Aromatic plants are especially opulent in these substances and they have been utilized in foods as flavoring agents. Most of these compounds are terpenes which have fungicidal properties [21]. Technically, when toxic components are extracted from the plant and applied on infested crops, these components are called botanical pesticides or botanicals [22]. Different solvents with different methods may be utilized in plant extraction, but it is obligatory to fixate on a standardized extraction method and solvent system for wide variety of researchers working in diverse settings to minimize the variability in the efficacy reports.

Several investigations focused on the use of plant extracts as antifungal agents. Kumer and Tripathi [23] mentioned that extracts of *Eupartrium cannabinum* completely inhibited the mycelia growth of *Pythium de-baryanum*, *R. solani* and *S. rolfsii*. Also, Nirmala et al. [24] showed that essential oil of *Juniperus communis* may be applicable against a range of damping-off diseases. Moreover, Humic and Fulvic acids have been early recorded to have a positive effect against plant pathogens and their cells biological activities [25-27]. *Aloevera* had been found to have antifungal activity against four common postharvest pathogens; *Penicillium digitatum, P. expansum, Botrytis cinerea* and *Alternaria alternata* [28]. The antifungal efficacy of extracts from Halfa bar (*Cymbopogon Proximus*); Ginger (*Zingiber officinale*) and Bay laurel (*Laurus nobilis*) against *A. solani, F. solani,*

F. oxysporum and *Pythium sp* significantly decreased fungal mycelia growth associated with essential oils concentrations [29]. Few studies have focused on the mechanism by which plant extracts and their essential oils inhibit microorganisms. The terpenes contained essential oils are the primary antimicrobials component. Many of the most active terpenes; eugenol, thymol and carvacrol are phenolic in nature. Therefore, it would seem reasonable that their modes of action might be related to those of other phenolic compounds. Essential oils may inhibit enzyme systems in yeasts, including those involved in energy production in cells and synthesis of their structural components [28]. Another recommended concept the high inhibition caused by the fatty acids could be due to their cytolytic activity by being a solvent of cellulose, a constituent of the cell membrane of fungi, while the fatty acids such as oleic acid have the potential of antifungal properties attributed to long chain unsaturation [30]. Disease control resulted in better development of foliage of crop which led to more number of fruits per plant, longer and heavier fruit with higher yield [31, 32]. Khetmalas and Memane [33] used six sprays of Bordeaux mixture® (0.5%) at seven days interval or three sprays of Alliete® (0.2%) at fifteen days interval, followed by three sprays of Mancozeb® (0.3%) at seven days interval starting from thirty days after crop sowing. Despite, the efficacy of selected plant extracts either individual or mixed with synthetic fungicides, limitations may be considered of botanicals using for plant disease management. These concepts may be standardized method, rapid degradation, need the development of formulations and some of chemical components are harmful to human and plants [22].

Fungicide Residue Levels.

The data of synthetic fungicides and their mixture with other botanical extracts are summarized in Table 2. From finding data, all compounds revealed positively response upper roughs of below detection limits (BDL). Moreover, the individual used synthetic fungicides (from T1 to T4) showed response as follows: 1.890, 0.544, 0.346 and 0.509 mg/kg fresh weight for propamocarb hydrochloride, cymoxanil, dimethornorph and cymoxanil+mancozeb, respectively, The above values exceeded the maximum residue levels (MRLs) only in case of propanocarb hydrochloride and cymoxanil. Among mixing cases, T13 revealed only residue level (0.141 mg/kg exceeded than MRLs with set as 0.05 mg/kg fresh weight) compared with other mixtures. However, the order among mixtures which decrease residue levels of synthetic fungicides was as follows: dimethomoroph>cymoanil+mancozeb>cymoxanil>propanocarb hydrochloride, respectively.

Mineral Composition.

Among fruits quality for mineral composition of treatment (e.g. Cu, Fe, Mn and Zn) were quantified as illustrated in Figure 3. The obtained data arise that, Cu, Fe and Zn reached mean values highest than those obtained in untreated group (control). However, Mn was flowed to be under BDL in all treatments compared with untreated group which not exceeded than 1.00 mg/Kg dry weigh. In case of Cu, the level recorded values ranged from 35.23 to 113.16 mg/kg, whereas the highest values was imposed by T2, and the lowest one was recorded for T1 compared with untreated group which not exceeded than 25.47 mg/kg. On the others hand, concentration of Fe accounted for levels highest than others. Moreover, the botanical extracts induced highly levels of Fe content in cucumber fruits in the following order: T7>T6>T5>T8 with values; 2018.92, 899.75, 785.75 and 596.50 mg/kg for T4, T2, T3 and T1, respectively, compared with untreated group (control) which not exceeded than 66.60 mg/kg. However, the mixtures revealed range from 398.75 to 864.00 mg/kg. In case of essential metal (Zn), the levels increased in the following order: botanical extracts >mixtures>individual's synthetic fungicide, respectively.

Biochemical Quantification.

The data of plant pigment e.g. chlorophyll a, b and carotenoids in both leaves and fruits are illustrated in Figures 4 and 5, respectively. In case of cucumber leaves, the all measured pigments revealed the following order 3rd> 1st>2nd stage of growth, respectively, sequencelly with spray programs. Regarding cucumber fruits, all measured pigments were lower than those obtained in leaves, especially in case of carotenoides followed by chlorophyll a and b, respectively. Some of treatments decrease the levels of chlorophyll a and b compared with control which not exceeded than 0.62 and 0.54 mg/L, respectively. Among carotenoids, the pigmented concentration imposed range from 0.45 to 0.81 mg/L compared with untreated group which not exceeded than 0.56 mg/L. The obtained data concerning total carbohydrates, protein and total phenols are illustrated in Figure 6. Synthetic fungicides induced means values lower than untreated group (control), except T2 which exceeded than control. While, T8 reached 17.10 mg/L compared with control

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which not exceeded than 15.71 mg/L. In case of mixture, the highest value was recorded for T17 to be 61.54 mg/L, while the lowest one was recorded for T22 (5.13 mg/L). In case of total protein, the treatments induced values ranged from 1732.57 to 5762.23 mg/L compared with control group which not exceeded than 2263.33 mg/L. All individuals' fungicides imposed mean values higher than their control. However, mixture treatments induced significantly different in their values (*P< 0.01*) as showed in histogram 6C. Regarding total phenols, either synthetic fungicides or botanical extracts revealed mean values higher than 1.11 mg/g fresh weight. However, mixture treatments achieved significantly different (*P<0.01*) in mean values ranged from 0.56 to 2.50 mg/g.

Quality Quantification.

The obtained data of fruits parameters are illustrated in Figure 7. All finding data revealed mean values highest than control. Among number of fruits/plant, individual fungicides raised mean values in range from 20.29 to 22.32 compared with control which not exceeded than 10.41. Other treatments ranged from 11.43 to 19.64. Similarly, in case of fruit length the mean values of individuals were highest than others. On the other hand, fruit weights in all treatments were found in significantly different (*P<0.01*).

CONCLUSION

The plant extracts are valuable source of biologically active molecules possessing antifungal property. Moreover, the mixture with other synthetic compounds minimizes the residue levels of edible parts and improves the yield of crops. Moreover, efficient collaboration or crucial to complete development of an interesting leads to compounds in an exploitable products.

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