

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Isolation and Identification of the fungal isolates

Standard tissue isolation technique was followed to obtain *A. solani* and *F. solani* culture. For this purpose samples of tomato plants showing typical early blight and wilted symptoms were collected from El-Beheira governorate, Egypt.

3.1.1. Isolation of *A. solani*

Tomato leaves showing typical early blight symptoms were cut into small bits measuring about 5 mm and the surface was sterilized in 1% sodium hypochlorite solution for 1 minute, rinsed with sterile distilled water. Pieces were then placed on Potato Dextrose Agar (PDA) and incubated at a temperature of $25 \pm 1^\circ\text{C}$ for 7 days. The developed mycelium was carefully transferred to slant potato dextrose agar (PDA) medium. Pure culture was used as inoculum for further studies.

3.1.2. Isolation of *F. solani*

Roots of wilted tomato plants were washed with tap water. Small pieces of vascular tissues were surface sterilized in 1% sodium hypochlorite solution for 1 minute, then placed in Petri dishes containing Potato Dextrose Agar (PDA). The dishes were incubated at $25 \pm 1^\circ\text{C}$ for 7 days. Pure culture was used as inoculum for further studies.

3.1.3. Identification of fungal isolates

The isolates of *A. solani* and *F. solani* were kindly identified in laboratory at Plant Pathology Department, Faculty of Agriculture, Alexandria University, Egypt based on published descriptions (**Gilman, 1957; Barnett and Hunter, 1972 and Nelson *et al.*, 1983**) of morphological and cultural characteristics of mycelium, conidiophores, conidia and colony morphology.

3.2. Chemicals used:

Different antioxidants, a biocide and chemical fungicides were evaluated and tested against some tomato fungal diseases (early blight and root rot). The used chemicals were as follow:

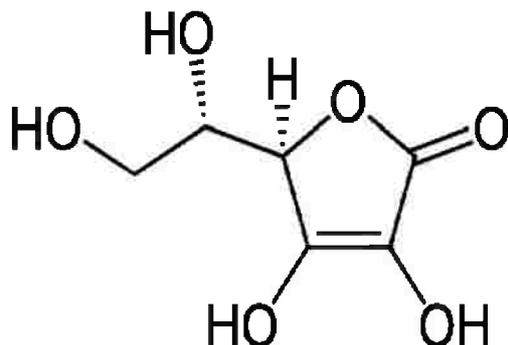
3.2.1 Antioxidants

1. Ascorbic acid

Ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$) is a naturally occurring organic compound with antioxidant properties. It is a white solid material and dissolves well in water to give mildly acidic solutions.

IUPAC name: (5*R*)-[(1*S*)-1,2-Dihydroxyethyl]-3,4-dihydroxyfuran 2(5*H*)-one.

Chemical structure:

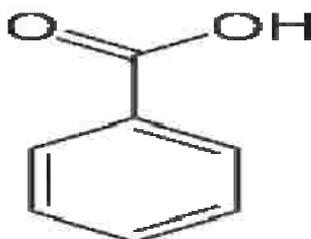


2. Benzoic acid

Benzoic acid ($C_7H_6O_2$) is a colorless crystalline solid and a simple aromatic carboxylic acid.

IUPAC name: Benzoic acid

Chemical structure:



3. Bion[®] (acibenzolar-S-methyl)

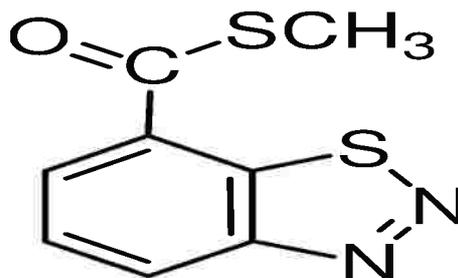
Chemical group: Benzothiadiazole

IUPAC name: *S*-methyl benzo [1,2,3] thiadiazole-7-carbothioate

Biochemistry: Acts as a functional analogue of the natural signal molecule for systemic activated resistance, salicylic acid.

Mode of action: Activates the host plant's natural defense mechanism ("systemic activated resistance" (SAR)) and it has no intrinsic fungicidal activity.

Chemical structure:

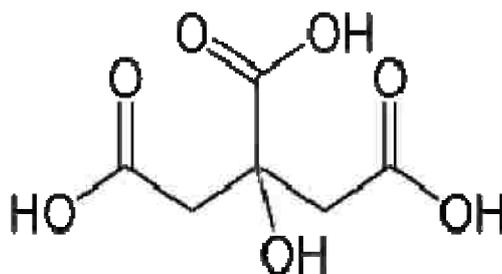


4. Citric acid

Citric acid ($C_6H_8O_7$) is a weak organic acid. It is a natural preservative/conservative which occurs naturally in citrus fruits and is also used to add an acidic or sour taste to foods and drinks.

IUPAC name: 2-hydroxypropane-1,2,3-tricarboxylic acid

Chemical structure:

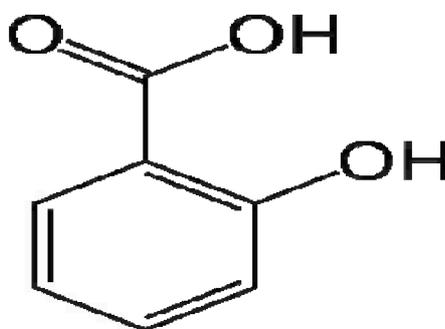


5. Salicylic acid

Salicylic acid ($C_7H_6O_3$) is a monohydroxybenzoic acid, a type of phenolic acid and a beta hydroxy acid. This colorless crystalline organic acid is widely used in organic synthesis and functions as a plant hormone.

IUPAC name: 2-Hydroxybenzoic acid

Chemical structure:



3.2.2 Fungicides

1. Plant Guard[®] (30×10^6 spores/ml)(*Trichoderma harzianum*)

A commercial formulation containing the antagonistic fungus *Trichoderma harzianum* (30×10^6 spores/ml). It is an antifungal biopesticide which competes with pathogenic fungi.

2. Micronized soireil/Samark[®] 70% WP (Sulfur)

Chemical group: Inorganic compounds

Chemical name: Sulfur (S).

Biochemistry: Non-specific thiol reactant, inhibiting respiration.

Mode of action: Non-systemic protective fungicide with contact and vapour action.
It can also acts as acaricide.

3. Ridomil Gold MZ[®] 68% W.P (mancozeb + mefenoxam)

A. Mancozeb

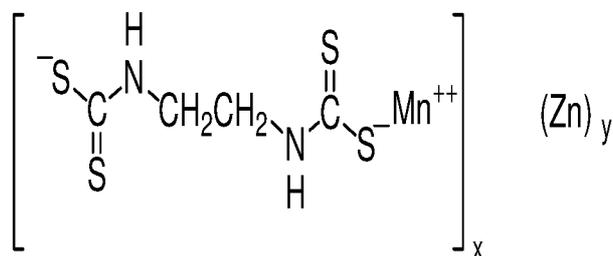
Chemical group: Alkylenebis(dithiocarbamate)

IUPAC name: Manganese ethylenebis (dithiocarbamate) (polymeric) complex with zinc salt.

Biochemistry: Reacts with, and inactivates, the sulfhydryl groups of amino acids and enzymes of fungal cells, resulting in disruption of lipid metabolism, respiration and production of ATP

Mode of action: Fungicide with protective action.

Chemical structure:



$$x:y = 1:0.091$$

B. Mefenoxam

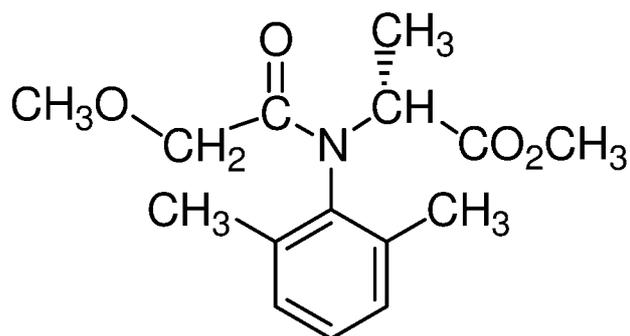
Chemical group: Phenylamide: acylalanine

IUPAC name: Methyl *N*-(methoxyacetyl)-*N*-(2,6-xylyl)-*D*-alaninate; methyl (*R*)-2-[[(2,6-dimethylphenyl)methoxyacetyl]amino}propionate.

Biochemistry: Inhibits protein synthesis in fungi, by interference with the synthesis of ribosomal RNA.

Mode of action: Systemic fungicide with protective and curative action, absorbed through the leaves, stems and roots.

Chemical structure:



4. Tridex[®] 80% W.P (mancozeb)

Mancozeb was previously described in section 3.2.2.3.A

5. Vitavax-200[®] 75% WP (carboxin + thiram)

A. Carboxin

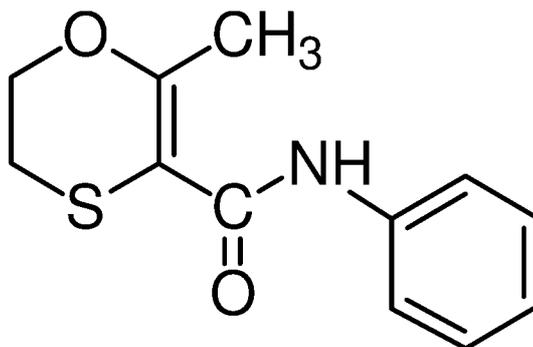
Chemical group: Carboxamide

IUPAC name: 5,6-dihydro-2-methyl-1,4-oxathiane-3-carboxanilide.

Biochemistry: Inhibits mitochondrial function by disrupting complex II (succinate dehydrogenase) in the respiratory electron transport chain.

Mode of action: Systemic fungicide.

Chemical structure:



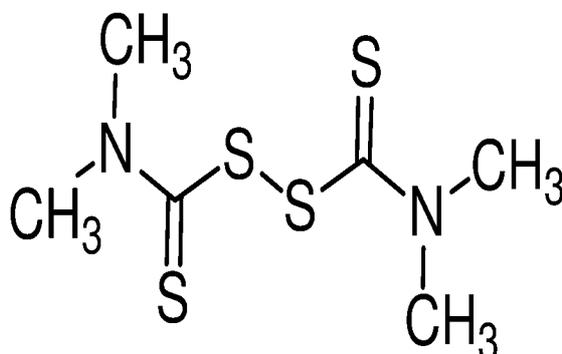
B. Thiram

Chemical group: Dimethyldithiocarbamate.

IUPAC name: Tetramethylthiuram disulfide; bis (dimethylthiocarbamoyl) disulfide.

Mode of action: Basic contact fungicide with protective action.

Chemical structure:



3.3. *In vitro* experiments

3.3.1. The effect of some fungicides and antioxidants on the linear growth of *A. solani* and *F. solani*

3.3.1.1. Preparation of the tested compound concentrations

Five antioxidants, namely ascorbic acid (C₆H₈O₆), benzoic acid (C₇H₆O₂), Bion[®] (bezal 1,2,3 thiadiazole,7 carbothioic acid 5-methylester), citric acid (C₆H₈O₇) and salicylic acid (C₇H₆O₃), as well as one biofungicide, (Plant guard[®] containing the antagonistic fungus *Trichoderma harzianum*, 30 x10⁶ spores/ml), and four fungicides, Ridomil gold MZ[®] 68% (Mefenoxam + Mancozeb), Sulfur(Micronized soreil/Samark[®] 70% WP), Tridex[®] 80% (Mancozeb) and Vitavax-200[®] (Carboxin + Thiram) were evaluated for their effects against the *in vitro* linear growth of *A. solani* and *F. solani*. Different concentrations of the tested compounds were tested as shown in Table 3.

3.3.1.2. Preparation of Czapek Dox Agar medium incorporated with the tested compounds

Fresh sterilized Czapek Dox Agar was prepared and poured in 100 ml flask at rate of 72 ml medium and the concentrations of the tested compound were mixed with CDA media at flasks. Six or seven flasks for each compound were prepared (one flask for each concentration and one flask for control were prepared). Media were poured in sterilized Petri dishes (9 cm diameter), one flask poured in 6 dishes (three dishes for each fungus).

3.3.1.3. Inoculation of fungal disks

All dishes were inoculated in the centre with 8 mm discs taken from the old tested fungi cultures. Three replicated dishes containing CDA media only were used as check and inoculated with tested fungi. All dishes were incubated at 26±2 °C.

Table (3): The tested concentrations of the evaluated acids, plant activator, bio and chemical fungicides.

Treatment	Concentrations (ppm)			
Acids:				
Ascorbic	50	100	200	300
Benzoic	50	100	150	200
Citric	50	100	150	200
Salicylic	50	100	150	200
Plant activator:				
Bion [®]	200	400	800	1000
Biofungicide:				
Plant guard [®] (30 x 10 ⁶ spores/ml)	3.68 x10 ^{2*}	5.47 x10 ^{2*}	7.22 x10 ^{2*}	8.81 x10 ^{2*}
Chemical fungicides:				
Ridomil gold MZ [®] 68% W.P	50	100	200	400
Micronized soredil/Samark [®] 70% W.P	250	500	750	1000
Tridex [®] 80% W.P	50	100	200	400
Vitavax-200 [®] 75% WP	10	50	100	200

* Spores/ml

Table (4): Composition of Czapek Dox Agar medium:

Ingredients	Gms / Litre(Distilled water)
Sucrose (C ₁₂ H ₂₂ O ₁₁)	30
Sodium nitrate (NaNO ₃)	2
Potassium dihydrogen phosphate (K ₂ HPO ₄)	1
Magnesium sulphate(MgSO ₄ . 7H ₂ O)	0.5
Potassium chloride (KCl)	0.5
Ferrous sulphate (FeSO ₄ . 7 H ₂ O)	0.01
Agar	15
Final pH (at 25°C)	7.3±0.2

3.3.1.4. Linear growth measurements

When mycelial growth of control covered the plates, fungal growth diameters were determined for all the tested treatment. The percentage reduction of growth (RG) ratio was calculated according to the following formula (Amer, 1995):

$$RG (\%) = RNT - RT / RNT * 100$$

Where:

RNT = Radius for non-treated media.

RT = Radius for treated media.

Data were subjected to probit analysis (Finney, 1971) where the inhibition of the fungal growth was plotted against the log concentration of the tested chemical and the amount of fungicide required to inhibit 50% of the growth of the fungus (EC₅₀ value) and its fiducial limits were determined.

3.4. *In vivo* experiments

Experiments were carried out in a greenhouse located in Faculty of Agriculture (Saba basha) Alexandria University, during 2013 and 2014 growing seasons on tomato. The material used and methods followed are described below.

3.4.1. Cultivation of tomato plants:

Tomato (*Lycopersicon esculantum* Mill, Master cultivar) seedlings (21 days old) were kindly obtained from one of special nursery of the plastic greenhouse (El-Beheira governorate, Egypt). Tomato seedlings were transplanted in plastic pots (18 cm diameter-disinfested by 7% formalin) containing sterilized soil which consisted of a mixture of clay: sand: betimos (2: 1: 1 v/v) and seedlings were planted at the rate of 4 seedlings/pot. The treatments were replicated three times and the pots were placed randomly in the greenhouse under natural conditions of day length and light intensity and watered regularly to near field capacity with tap water.

3.4.2. *Alternaria solani*

3.4.2.1. Treatment with antioxidants and fungicides

One week after transplanting of the seedlings, plants were sprayed until runoff occurred with the tested antioxidants and fungicides. The five antioxidants (ascorbic, benzoic, citric and salicylic acids and Bion[®]) were sprayed at 100 ppm, as well as one biofungicide, (Plant guard[®]) and three fungicides (Ridomil gold MZ[®] 68%, Micronized sorail/Samark[®] 70% and Tridex[®] 80%) were sprayed with their recommended doses as follows: 250 ml, 200 g, 250 g and 250 g /100 litre, respectively.

On the base of the results obtained from *in vitro* experiments, both salicylic acid and Ridomil gold MZ[®] were chosen for their higher efficiency to be applied as a sequential treatment (one followed by the other after one day) under greenhouse conditions. As a control treatment, water was used instead of the tested compounds solution. Two weeks later, the plants received another spray application with the same compounds concentration as mentioned above.

3.4.2.2. Inoculation of plants

Two weeks after transplanting, the tested plants were inoculated with the tested fungus (*A. solani*) as follows:

Alternaria solani was subcultured on potato dextrose agar at 27± 1°C for 10 days. Fungal spores of 10 days-old cultures were harvested by gentle brushing to separate the spores from the mycelium surface and water was added to obtain a final volume of 500 ml. Spores were counted on a hemacytometer, then spore concentration was adjusted to 10⁶ spores/ml by adding sterile distilled water. Plants were inoculated by spraying the spore suspension until run-off.

3.4.2.3. Disease assessment

Inoculated leaves were kept under observation, disease assessment based on disease incidence and disease severity was done after 40 days from inoculation. The intensity of disease was recorded in each treatment following the score chart 0-7 scale (0= no lesions; 1= trace to 1%; 2= 1 to 5%; 3= 6 to 10%; 4= 11 to 25%; 5= 26 to 50%; 6= 51 to 75% and 7= 76-100% of foliage covered with lesions) proposed by **Chirst (1991)**. The percentage of disease severity was calculated according to the equation:

$$\text{Disease severity (DS \%)} = \frac{\sum(n \times r)}{7N} \times 100, \text{ where}$$

n = number of seedlings of a given disease rating

r = disease severity rating

N = total number of seedlings rated

7 = higher rating value

Disease severity reduction percentage (R) was calculated as follows:

$$\text{Disease severity reduction (R \%)} = \frac{y-x}{y} \times 100$$

Where:

y = mean of disease severity for untreated plants (control).

x = mean of disease severity for treated plants.

3.4.2.4. Enzymes activities assay

For determining the activities of peroxidase (PO) and polyphenoloxidase (PPO) enzymes, leaves were collected at different time intervals (3 and 7 days after inoculation) and immediately frozen in liquid nitrogen and stored at -80 °C until extraction.

3.4.2.4.1. Peroxidase activity

Peroxidase activity was assayed by the method described by **Urbanek *et al.* (1991)** using guaiacol (Guaiacol solution 20 mM which prepared by dissolving 240 mg guaiacol in distilled water to make a final volume of 100 ml) as the substrate. Leaf samples (0.5 g) were homogenized in 1 ml of 0.1 M phosphate buffer (pH 7.0), then centrifuged at 18000 g for 15 minutes and the supernatant were used as enzyme source. The reaction mixture was consisted by adding 25 µl of the crude extract to 2 ml of a solution containing 50 mM sodium acetate buffer (pH 5.2), 20 mM guaiacol and 20 mM hydrogen peroxide (H₂O₂). The mixture was incubated for 10 min at 30°C and then measured spectrophotometrically at the wave length of 480 nm. Na-acetate buffer (pH 5.2) was used as blank. Enzyme activity was determined from the change in absorbance and was expressed as enzyme unit/mg protein as the following equation: Peroxidase activity = OD at 480 nm / mg protein

3.4.2.4.2. Polyphenoloxidase activity

Polyphenol oxidase activity was assayed by the method described by **Mayer *et al.* (1965)** using catechol as the substrate. Leaf samples (0.5 g) were homogenized in 1 ml of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 16 000 g for 15 minutes. The supernatant was used as the enzyme source. The assay mixture consisted of 200 µl of the enzyme extract, 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µl of 0.01 M catechol. Enzyme activity was calculated from the change in absorbance and was expressed as enzyme unit/mg protein as the following equation: PPO units = OD at 420 nm/mg protein.

3.4.3. *Fusarium solani*

3.4.3.1. Treatment with antioxidants and fungicides

The tested compounds were applied as seedling root dipping technique as these roots were dipped for 30 minutes before transplanting. Seedlings root were dipped in each solution of the tested compounds as follows: the five antioxidants (ascorbic, benzoic, citric and salicylic acids and Bion[®]) were treated at 100 ppm, as well as one biofungicide, (Plant guard[®]), and two fungicides, Micronized soireil/Samark[®] 70% and Vitavax-200[®] 75% were treated with their recommended doses (250 ml, 250 g /100 litre and 1g/1 litre, respectively). On base of the results obtained from *in vitro* experiments, both salicylic acid and Vitavax-200[®] 75% were chosen, for their higher efficiency, to apply them in a sequential treatment (one followed by the other after one hour) under greenhouse conditions. As a control treatment, water was used instead of the tested compounds solution. Two weeks later, the plants received foliar spray application with the same

compounds concentration as mentioned above (the sequential treatment were treated as one followed by the other after one day).

3.4.3.2. Inoculation of plants

One week after transplanting, the tested plants were inoculated with the tested fungus (*F. solani*) as follows:

Fusarium solani was subcultured on potato dextrose agar at $27 \pm 1^\circ\text{C}$ for 10 days. Fungal spores of 10 days-old cultures were harvested by gentle brushing to separate the spores from the mycelium surface and water was added to obtain a final volume of 500 ml. Spores were counted on a hemacytometer, then spore concentration was adjusted to 10^6 spores/ml by adding sterile distilled water. Each pot was inoculated with 10 ml of *Fusarium* culture suspension (10^6 spores/ml) as soil treatment for each pot. The control plants were similarly treated with sterile distilled water.

3.4.3.3. Disease assessment

Inoculated plants were kept under observation to determine disease severity after 35 days post-inoculation. Disease severity was recorded based on a modified score described by **Hwang and Chang (1989)**, where 0 = healthy roots, 1 = $\leq 25\%$ root discoloration, 2 = 26 to 50 % root discoloration, 3 = 51 to 75 % root discoloration and 4 = $\geq 76\%$ root discoloration. The percentage of disease severity was calculated according to **Tarabulsi et al. (1998)** as follows:

$$\text{Disease severity (DS \%)} = \frac{\sum(n \times r)}{4N} \times 100$$

Where:

n = number of seedlings of a given disease rating

r = disease severity rating

N = total number of seedlings rated

4 = higher rating value

Disease severity reduction percentage (R) was calculated as follows:

$$\text{Disease severity reduction (R \%)} = \frac{y-x}{y} \times 100$$

Where:

y = mean of disease severity for untreated plants (control).

x = mean of disease severity for treated plants.

3.3.4 Determination of fungicide residues in tomato fruits

At the end of the second season and after 15 days after disease severity assessment for both diseases (early blight and root rot), the plants received another foliar spray application with the same compounds concentration as mentioned above (the sequential treatment were treated as one followed by the other after one day). The fungicide residues in samples of harvested tomato that have been treated with Ridomil gold[®] and Vitavax-200[®] (both were chosen for their higher efficiency against *A. solani* and *F. solani*, respectively) were determined in the Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Food, Agriculture Research Center, Ministry of Agriculture. Quick

and easy method(QuECHERS) was employed for the determination of the fungicide residues using LC-MSMS and GC-MSD instruments(Figure 1).

Sampling was performed by randomly collecting of 1 kg of fruits samples from each treatment (Ridomil gold[®] and Vitavax-200[®]) at 3 and 10 days after fungicides application. The collected samples represent all the plants in the treatment. Samples were placed in the polyethylene bags and transported in iceboxes to the laboratory immediately. In the laboratory, each sample was divided into two sub-samples of 500 g and kept in a freezer at the temperature of -20° C until being analysed.

Loss of fungicide residues percentage (L) was calculated as follows:

$$\text{Loss of fungicide residues (L \%)} = \frac{y-x}{y} \times 100$$

Where:

y = fungicide residue value after 3 days post-application.

x = fungicide residue value after 7 days post-application.

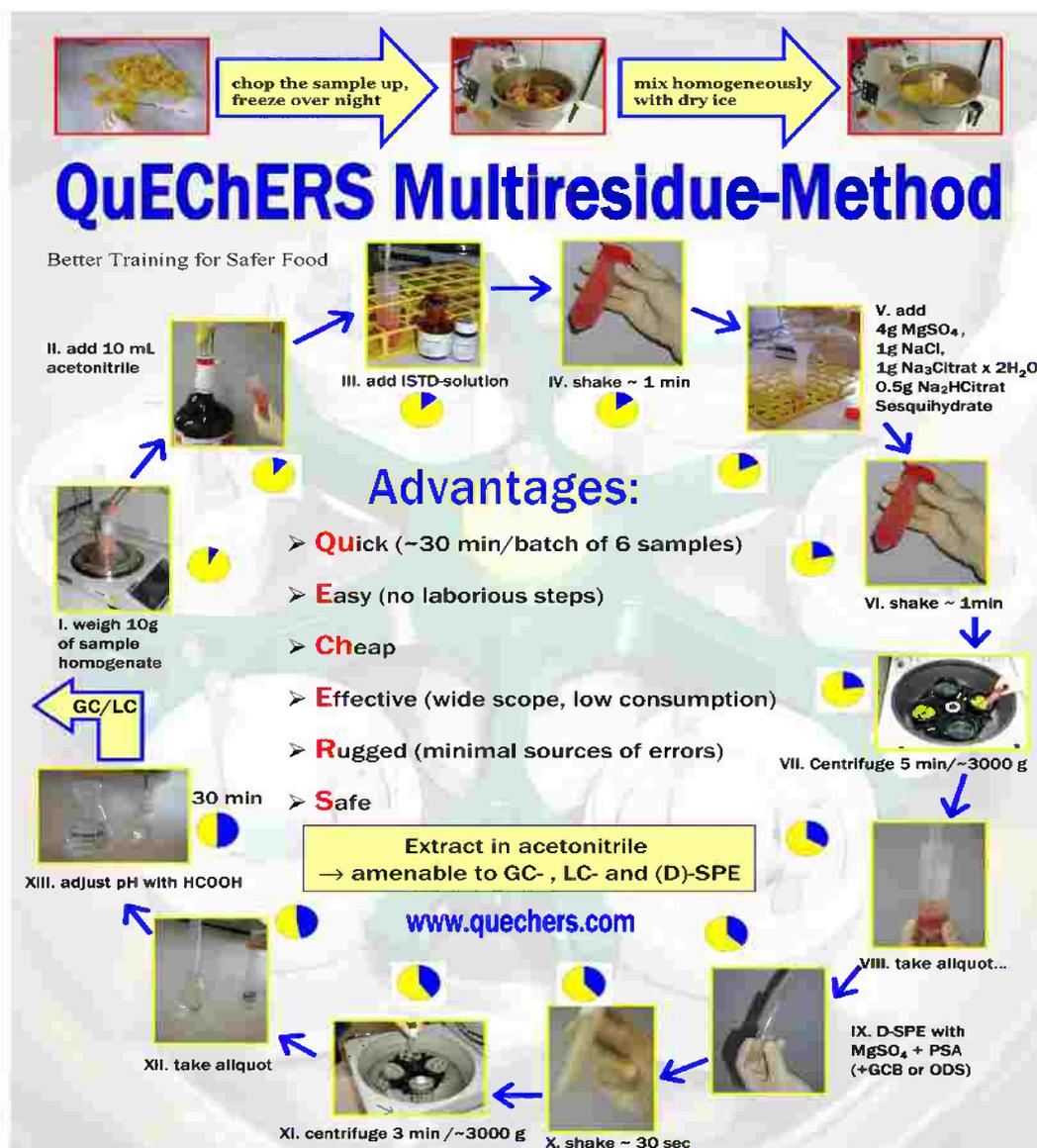


Figure (1): QuEChERS multiresidue-method employed for the determination of the pesticide residues using LC-MSMS and GC-MSD in tomato fruits.

3.4. Statistical analysis

All experiment were set up in a complete randomized design. Analysis of variance (ANOVA) was used to analyse the effect of the antagonistic on linear growth of pathogenic fungi *in vitro*, disease severity and enzymes activities in greenhouse experiment using COSTATE software and Duncan's multiple range test at $P < 0.05$ level was used for means separation (Winer, 1971).