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RESEARCH PAPER

In vitro efficiency of some plant extracts, bio-agents, and fungicides on *Alternaria solani*, the causal agent of early blight of potato in Erbil, Iraq

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A B S T R A C T:

Early blight of tomato and potato caused by *Alternaria solani* is considered a destructive disease wherever the crops are grown. Losses due to the disease, under favorable conditions, are considered high. Several control measures have been practiced to manage the disease. These include chemical, biological, and the use of plant extracts. This study aimed to assess four selected plant extracts, three bio agents, and four modern fungicides against *A. solani* in vitro. After the isolation and identification of the pathogen, a complete randomized design in four replicates was conducted in the laboratory. The lab experiment conducted using Petri plates by amending fresh potato dextrose agar with plant extracts and fungicides then inoculated with the pathogen and then incubated for one week. To evaluate the efficacy of bioagents against *A. solani*, dual culture technique was performed in vitro. The results showed that from plant extracts, pomegranate extract was the most efficient showing an inhibition of 55.75%. Among the bioagents, *Trichoderma harzianum* showed greater efficacy displaying an inhibition of 76.5%. Both Pristine and Difecor were the most effective synthetic fungicides that inhibited the pathogen by 100%. It can be concluded that an integration based on different methods could be the best option to manage the disease and, therefore, reduce the usage of chemical fungicides which have worldwide environmental concerns.

KEY WORDS: *Potato blight, Isolation, Biocontrol, Identification, Disease Management*.
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1.INTRODUCTION :

Tomatoes (*Lycopersicon esculentum*) and potatoes (*Solanum tuberosum*) are both members of Solanaceae, a large plant family with more than 3,000 species (Djaman *et al.*, 2022). They are considered as warm-season crops and the most consumable vegetables in the world (O'brien *et al.*, 1998). In Iraq potato production was more than 190,000 t with a total cultivated area of 7950 ha on average 23.996 t h-1 (Hossain *et al.*, 2016). According to Ministry of Agriculture and Water Resources data in the Iraqi Kurdistan region, potato production in the Kurdistan Region exceeded 150,000 metric tons in 2016.

Alternaria solani is the prevalent pathogen that causes early blight in tomatoes and potatoes. The disease is more serious under wet or humid conditions. Early blight on potatoes causes major yield losses in most growing regions of the world. Losses due to the disease vary enormously from 5 - 78% (van der Waals *et al.*, 2004).

When the disease is present, leaves, stems, petioles, twigs, and fruits are affected. This causes defoliation, twig drying out, and early fruit drop, which reduces fruit yield by 50% to 86%.(Soni *et al.*, 2015) .

This disease can only be effectively managed by implementing an integrated strategy management. The disease is primarily controlled by bio-agents, plant extracts, and foliar fungicides (Tsedaley, 2014). *Trichoderma harzianum* and *Pseudomonas fluorescens* -based biological control also affects *A. solani* (Ramanujam *et al.*, 2015). *Bacillus subtilis*, a plant-beneficial bacterium, has been studied extensively for its antifungal properties. (Zhang *et al.*, 2020).

Numerous extracts of higher plants were found to possess antibacterial, antifungal, and insecticidal properties in the laboratory tests. Bioactive compounds extracted from plants have great potential for controlling pathogenic fungi (Miah *et al.*, 1990). Pomegranate extract has a high growth inhibition rate against *A. solani* in vitro (Mostafa *et al.*, 2021). In comparison to synthetic

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pesticides, plant metabolites and plant-based pesticides appear to be one of the best alternatives due to their minimal environmental impact and consumer risk (Varma and Dubey, 1999). Plant extracts have antimicrobial activity in vitro and in vivo for controlling early blight and other plant diseases. (Wszelaki and Miller, 2005).

The use of chemical fungicides, despite global concerns, also plays a vital role in disease management whether alone or integrated with other methods. (Kemmitt and tomato., 2013). Integrated use of fungicides, microbial biocontrol agents, and plant extracts has proven effective against a broad spectrum of plant pathogens (Amadioha, 2003);(Bowers and Locke, 2004). This work was intended to assess a variety of methods including plant extracts, beneficial microorganisms, and some modern fungicides to control early blight disease on tomatoes and potatoes.

2.Materials and methods

2.1Disease survey and sampling

The disease survey was performed in May of 2021 in three locations in Erbil Province including Qushtapa (south of Erbil), Shamamk (south west of Erbil), and Gainj (north of Erbil). Leaf samples of potato and tomato, showing early blight (EB) symptoms, were taken randomly from several fields. The samples were placed in plastic bags and transferred to the laboratory, where they were stored at 4°C in a refrigerator until isolation.

2.1.1.Isolation of the pathogen

The fungus was isolated from infected leaf samples by cutting them into 0.5*0.5cm pieces, surface sterilized with 70% ethanol for approximately 2 minutes, then washed three times with sterilized distilled water, and placed on freshly prepared potato dextrose agar (PDA) medium. To inhibit bacterial growth, 200 mg/L of streptomycin was added to the medium before powering them in petri plates. The inoculated plates were incubated for seven days at 25°C. To maintain fungal growth, new plates were sub-cultured with fresh fungal growths. (Dhingra and Sinclair, 2017).

2.2.Identification of the pathogen

2.2.1.Morphological identification

Microscopic slideswere prepared from fungal growths and tested with compound microscope at 400X, to identify the isolated fungi according to the available classification Keyes (Pitt and Hocking, 2009, Ellis, 1971, Lackner *et al.*, 2014). The identification depended on measuring some distinguishing fungal characteristics such as hyphae, conidia, and conidiophores (Dhingra and Sinclair, 2017). The fungal pathogen was characterized depending of hyphal width and colour, the shape and divisions of conidia and the conidial beak, and the existence of short conidiophores.

2.2.2.Molecular identification of the pathogen

In order to confirm the fungal pathogen identification, polymerase chain reaction (PCR) was developed to determine the pathogen's diagnostics. The method is regarded as accurate and precise, complementing microscopic descriptions. To do this, DNA extracted from the pure cultures of *A. solani* grown on PDA media according to the protocol set by the manufactures Genomic DNA extraction Kit (FATG/Korea). The target DNA of *A. solani* was amplified using species-specific primers: AS1(5-GCTCCCACTCCTTCCGCGC-3) and AS2 (5 GGAGGTGGAGTTA-CCGACAA-3) designed from the β-tubulin gene and used by (Kumar *et al.*, 2013). In 25 l of reaction volume in PCR tubes, DNA was amplified. The master mix contained 12.5 l of 2x PCR master mix, 1 l of each forward and reverse primer, 2 l of DNA template, and 8.5 l of DNase-free water to achieve a final volume of 25 l. The temperature profile of the PCR protocol consisted of an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 40 seconds, primer annealing at 58 °C for 45 seconds, an extension at 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes.

To create 1% agarose gels, 1g of agarose powder was suspended in 1 Tris-Borate-EDTA (TBE) and dissolved in a microwave oven. After the solution cooled to 60°C, Fluro Safe dye was added (Fisher Scientific, USA). The gel was poured into a plastic frame with wells created by inserting a comb after it was mixed. After adding loading dye (Bromophenol blue) to the DNA samples, the wells were loaded with 5l of each template and the ladder using a micropipette. Positive controls were also included in the gel electrophoresis run. The electrophoresis was performed at 100V for 45 minutes, until the dye moved halfway between the wells and the end of the gel. The gel was photographed under ultraviolet (UV) illumination, and the band sizes were compared to a ladder to confirm the product size.

2.3.In vitro assessment of plant extracts

The efficacy of Pomegranate (peels), Mandarine (peels), Dodonaea (leaves), and coper extracts were evaluated. Plant parts were dried, ground

into powders, and then dissolved in sterilized distilled water at the rate of 1 and 10 g/L according to (Raza *et al.*, 2016). The mixture was shacked by magnetic stirrer for 8 h at medium speed. then filtrated through Buchner Funnel using 11cm filter paper. Two different concentrations (1000 ppm and 5000ppm) were made from the stock solutions. The food poisoning techniques were utilized, and the selective concentrations were mixed with the PDA. Each plate's center was inoculated with 0.5 cm mycelial plugs replicating four times then incubated at 25^{o}C until the mycelial growth in the untreated plates covered the plates. Mycelial growth was measured using a ruler in two dimensions and then averaged. The inhibition percentage was calculated following this formula:

Inhibition (%) = (GC – GT) x 100/GC

Where:

GC = Mycelial growth in the untreated control

GT = Mycelial growth in the treatment

2.4.In vitro assessment of the bio-agents

Using the bio agent fungus *T. harzianum* and the bacteria *Bacillus subtilis* and *Pseudomonas* f*luorescens*, dual culture technique was performed in vitro to evaluate the biological control of *A. solani*. The plates were divided into two halves, the first half was inoculated with a circular mycelium plug (5 mm) taken from the margin of new *A . solani* cultures while the other half was inoculated with new cultures of *T. harzianum*, in the same manner. The mycelium plugs were placed on the plates face down. Regarding the bacteria, *B. subtilis*, and *P. fluorescens* were placed by streaking a sterilized loop on PDA medium opposite *A. solani (El-Tanany et al., 2018)*.and negative controls contained the pathogen only. Each treatment consisted of four replicates; plates were then incubated at 25 degrees Celsius for two weeks. Radial growth was measured at two different angles at 90 degrees using a digital caliper and the averages were calculated after the 5mm mycelium plug . Finally, the measurements were recorded and then the antagonistic effect of bio agents was determined using the following formula:

$$\text{Antagonistic effect} = \frac{\text{A}-\text{B}}{\text{A}} \times 100$$

Where: A= the diameter of mycelial growth of pathogenic fungus in the control

B= the diameter of mycelial growth of the pathogenic fungus with the bio agents

3. In vitro assessment of the fungicides

to assess chemical fungicides in vitro, four modern fungicides which were Pristine (Pyraclostrobin 12.8% + Boscalid 25.2%), Zoxi (Azoxistrobin 25% SC), Foliogold (Metalaxyl 37.5% + Chlorothalonil 50%), and Difecor (Deficonazole 25%). For each fungicide, a recommended rate from the company was used by pouring the required amount to the lquid agar before solidification. Four plates for each fungicide were inoculated with 5 mm of fungal discs using cork borer. The the plates were incubated at 25^{o}C until the full growth of the untreated control. The growth inhibition was measured by measuring the growth of the fungus using radial growth was measured at two different angles at 90 degrees using a digital caliper and the averages were calculated after the 5mm mycelium plug was deducted. The inhibition percentage was calculated in the same was followed in plant extracts.

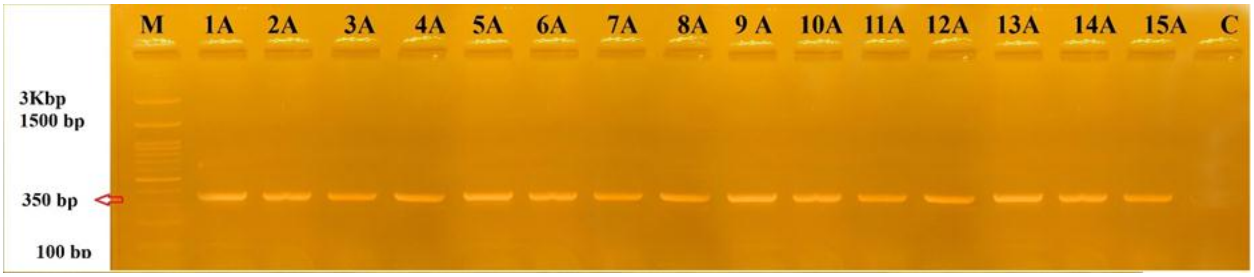
Data analysis

All data were statistically analyzed by using StatGraphics software package version (15) and the comparisons between means were performed by using least significance difference (LSD) at $P \leq 0.05$.

Result and discussions

Isolation and Identification of the pathogen

The isolated fungus from surveys was identified and the initial diagnosis, based on morphological and microscopical examination showed the fungus was *A. solani*. The fungal pathogen has septate and dark-colored mycelium, conidia were multicellular, brown to pale, the number of transverse septa were ranged from 5 -11um, the beaks were incurved with the length of 4.5 – 11.25 um. The colony color was dark brown with circular margin growth and concentric zonation. To confirm the identification of the pathogen, a molecular technique employing PCR with a specific primer was used, and the results confirmed that the fungus was present in all of the survey isolates, was *A. solani* (Figure 1).



(Figure 1): the PCR gel electrophoresis of *A. solani* showing the band sizes of 350 bp.

3.1In vitro assessment of plant extracts

The results showed that the effect of plant extracts was variable on the growth of *A. solani*. The highest inhibition was by Pomegranate axtract (55.76%) which had no significant differences with Mandarin extract but had significant differences with Dodonaea extract and Coper. Dodonaea had the least effect on the inhibition of the growth of the fungus (figure 2). However, there are no differences between the concentrations used in the experiment (figure 3).

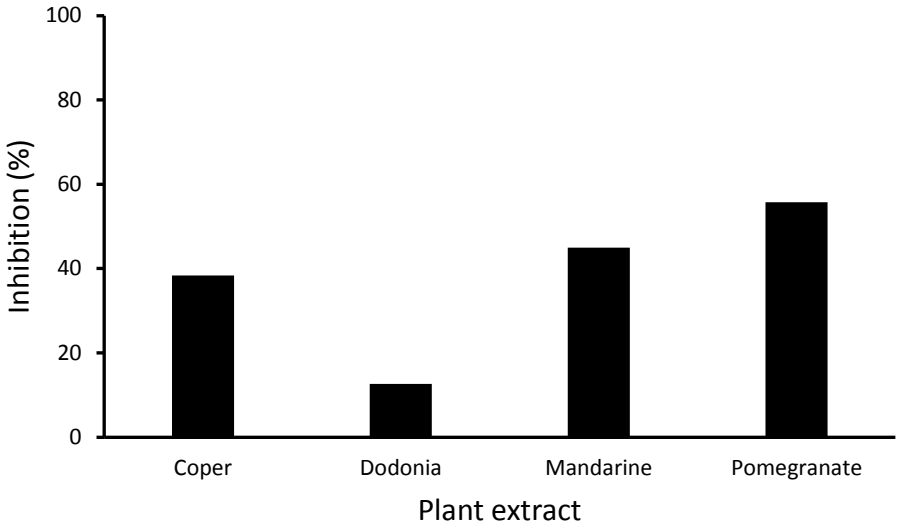
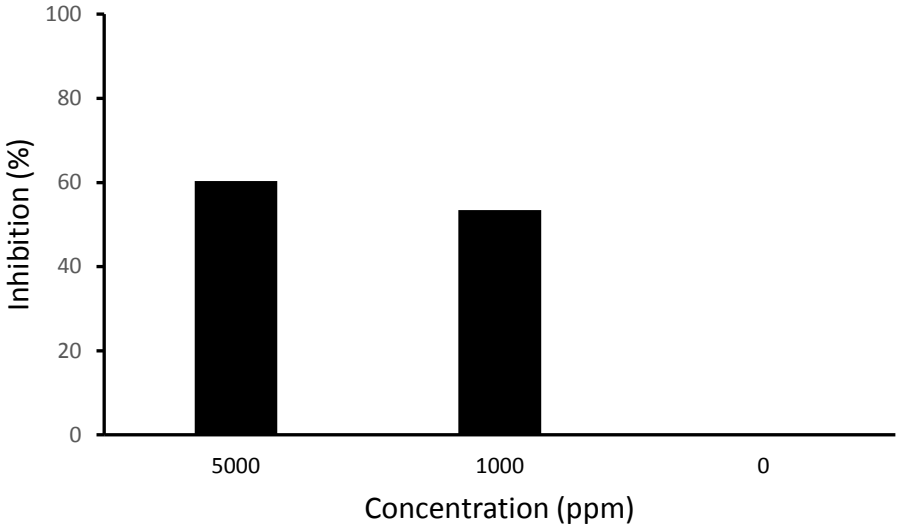


Figure 2: Effect of different plant extracts on *A. solani* growth.



Figur3: Effect of different concentrations of plant extract on the growth of *A. solani*

3.2In vitro assessment of bioagents

The results showed that the effect of the bio agents were variable on the growth of *A. solan* (figure 4). The highest inhibition (76.5%) was by *T. harzianum* which had a significant difference with other bio-agents followed by *B. subtilis* (51.9%) and then *P. fluorescence* (44.8%).

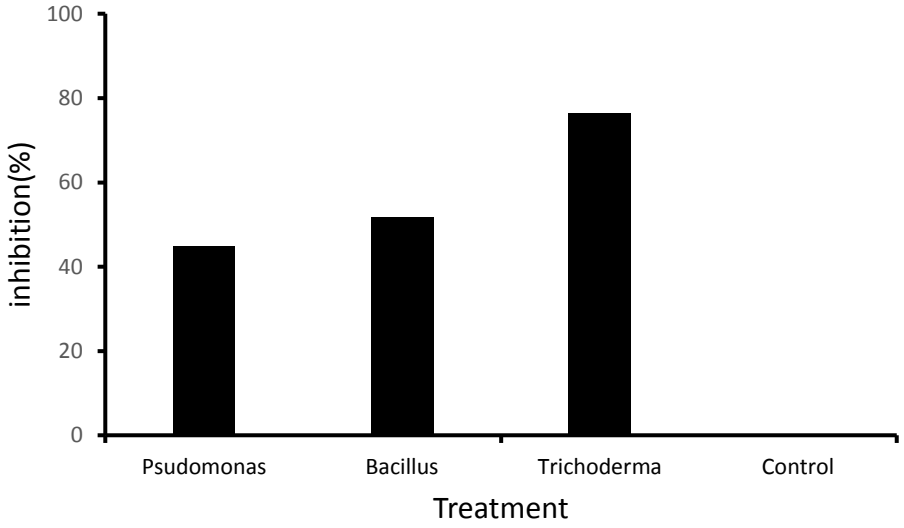


Figure 4: Effect of different bio-agents on the growth of *A. solani*

4.In vitro assessment of the fungicides

The results of using fungicides in vitro showed that both Pristine and Difecor had the highest inhibition (100%) on the growth of *A. solani* in vitro compared with other fungicides (figure 5). Moreover, Foliogold was significantly better than Azoxystrobin for controlling the growth of *A. solani*, where their inhibition values of them were 56.9 and 40%, respectively.

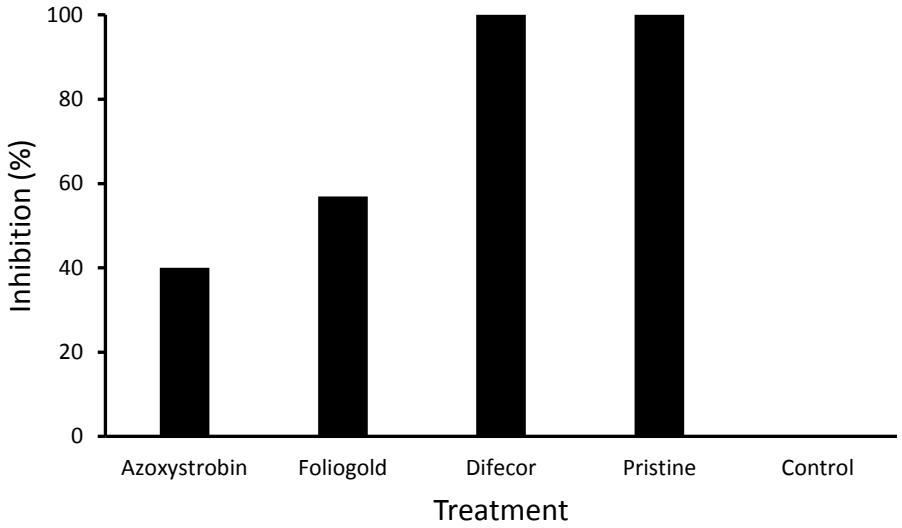


Figure 5: The in vitro effect of different fungicides on the growth of *A. solani*

5.Discussions

In the present study, we attempted to identify an effective plant extract dose, biocontrol agents, and fungicides against *A. solani*, the causal agent of tomato early blight. The ultimate objective of the recent research in this field has been the creation of alternative control strategies to reduce reliance on synthetic fungicides. Aromatic secondary metabolites, such as phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins, and coumarins, can be synthesized by plants. (Cowan, 1999). Components with phenolic structures, such as carvacrol, eugenol, and thymol, exhibited potent antimicrobial activity. These classes of compounds possess antimicrobial properties and serve as plant defenses against pathogenic microorganisms. (Das *et al.*, 2010). Phenolic compounds are aromatic benzene rings with one or additional hydroxyl groups made by plants mostly for protection against anti-stress (Bhattacharya *et al.*, 2010). Furthermore, phenolics are the major unit of secondary metabolites, which are extensively dispersed in most of the plants and show a significant function in plant resistance versus many diseases(Derbalah *et al.*, 2011) . In this study, the antifungal activity of four plant extracts from different families at concentrations of 1,000 and 5,000 ppm was evaluated in the laboratory using the poisoned food technique. Inhibiting the mycelial growth of the pathogen, the tested plant extracts differed

significantly in their effectiveness. Pomegranate extract demonstrated the highest level of growth inhibition (55.76%). Tannins and alkaloids are the chief active components of pomegranate extract. The principal phytochemical components of pomegranate peel are gallotannins, ellagic acid derivatives, catechins, procyanidins, and flavonols (Heber *et al.*, 2006) . Due to concerns regarding cost, health, and the environment, the use of chemical pesticides cannot be a long-term solution for the management of disease. Therefore, bio-control treatments of *T. harzianum*, *B. subtilius*, and *P. fluorescens* could be considered long-term solutions for the management of plant diseases, as they have been shown to be environmentally friendly and effective against numerous plant pathogens. (Raut *et al.*, 2020). Microorganisms with antagonizing properties control crop diseases biologically. (Raut *et al.*, 2020). Integrated Pest Management (IPM) programs for the future management of tomato foliar diseases may therefore include biological treatment in addition to chemical control. (Whipps *et al.*, 2001). *T. harzianum* exhibited the highest percent growth inhibition of all the biocontrol agents examined in the current study. (76.5%). *Trichoderma inhibits* the pathogen's growth due to its rapid growth rate and competition for food and space. (Devi *et al.*, 2012). It was followed by *B. subtilis* and *P. fluorescens* and has antipathogenic effects. (Muriungi *et al.*, 2013). Due to their ability to form endospores and their antibiotics' broad-spectrum activity, *Bacillus* species as a group offer several advantages over other bacteria for protection against pathogens. *(Abdalla et al., 2014)*. By observing zones of inhibition on Petri plates, bacterial biocontrol agents belonging to the genera Agrobacterium, *Bacillus*, *Pseudomonas*, and Streptomyces were identified. (Larkin and Fravel, 1998).

It is recommended that botanical extracts and bioa-gents be incorporated into the disease management program along with the most effective fungicides. All of the tested fungicides were found to be effective against the pathogen, but their efficacy varied depending on their chemical composition. Pyraclostrobin was the best resident of *A. solani* due to its broad spectrum of action and excellent spore germination inhibitory properties. (Jiang *et al.*, 2009). By destroying the pathogen's cell membrane or its permeability or by inhibiting the pathogen's metabolic processes, azoles are the most effective pathogen inhibitors (Fisher *et al.*, 2004). Moreover, strobilurins inhibit mitochondrial respiration by preventing the transfer of electrons at the cytochrome complex. (Jiang *et al.*, 2009). Foliogold has the third-highest inhibition ratio (56.9%), whereas Zoxi has the lowest (40%) Zoxi and Foliogold were notably distinct from one another (figure 5).

6.Conclusions

In conclusion, plant extracts like pomegranate peel extract, which is a cheap and available source, have a potential effect on the inhibition of plant pathogenic fungus, *A. solani*. *T. harzianum* could be the most efficient bioagent to combat the pathogen. However, in severe disease states, Pristine and Difecor (both containing two different active ingredients) could be used alone or in integration with other methods.

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