PAPER • **OPEN ACCESS**

A novel and Rapid Method for Detecting Bio-fungicide Properties in Plant Species

To cite this article: R. M. Theer *et al* 2021 *IOP Conf. Ser.: Earth Environ. Sci.* **761** 012024

A novel and Rapid Method for Detecting Bio-fungicide Properties in Plant Species

R. M. Theer*, A. A. Alheeti and A. O. Alfalahi

Department of Plant Protection, College of Agriculture, University of Anbar, Iraq

*Corresponding author's e-mail: ag.rashid_mashref@uoanbar.edu.iq

Abstract. Searching for eco- friendly bio-fungicides have become promising strategy to confront the resistance phenomenon against synthetic fungicides in pathogenic fungi. Unlike the previously described techniques to survey the plants for their secondary toxic metabolites against fungi, a novel technique reported here. The new technique designing on using a detached leaf bioassay and inoculum of *Macrophomina phaseolina,* the charcoal rot pathogen of sesame. Of an eleven different plant species belong to different families examined with this technique only one species (Giradol, *Chrozophora tinctoria* of Euphorbiaceae) showed no response to *Macrophomina phaseolina* inoculation on detached leaf even with wounding. The aseptic aqueously extracted leaf of the Giradol inhibited the growth of the *Macrophomina phaseolina* by 90% on potato dextrose agar media. This technique is relatively easy, fast and enabling to evaluate for bio- fungicidal existence in several species of plant at the same time.

1. Introduction

Plant diseases are major constraints facing agriculture word wide and their management is very crucial to ensure both food security and safety. The reported annual losses in agriculture production in the world's return that caused by plant disease are estimated at 25,000 million US dollars. Most of such losses are caused by plant pathogenic fungi. Therefore, several methods have been recommended to control the fungal diseases. Chemical fungicides are the commonest and the most successful measures for controlling the fungal diseases on economic crops [1]; [2]. Although, several fungicides have been marketed, yet some of them of adverse side effects as hurting the environment and human health, inducing pathogen resistance and having an effect on non-target species.

The conclusion from the previous researches revealed that over 200 fungal pathogens have become resistant to fungicides [3]; [4]. Therefore, many fungicides lost their therapeutic effectiveness, but they still of an adverse effect, since they are non- gradable and of adverse effect to human and other organisms. Given these complications, it is necessary to seek for alternative control materials such as the natural eco-friendly fungicides. In this regard, the botanical fungicides are strongly recommended as a healthy and eco- friendly alternative. Many species of higher plants proved to be of antimicrobial properties [5]; [6]; [7]; [8]; [9]. The botanical antifungal components, are identified as secondary metabolites belong to broad chemical groups included phenols, phenolic acid, coumarins, pyrons, flavonids, isoflavonides, and steroidal alkaloids [10]. These biocides considered as environmentally safe and easily biodegradable [11]; [12] and most likely that their occurrence plays an important natural defence mechanism against fungi and other pests of plants [3]; [7]; [13]; [14]. The ordinary method

described for detecting the antifungal properties in plants was by obtaining the plant extracts [6]; [7]; [8]. The extracts obtained by macerating the plants either in water or in an organic solvent. The toxicity, then evaluated against fungi growth on culture media [15]; [13]. This procedure is relatively a time consuming, costly, and not granted for antifungal discovery until it evaluated against fungi on culture media. The *Macrophomina phaseolina* is reported to attack up to five hundred plant species worldwide under various environmental conditions. It causes charcoal rot, which is also known as summer or dry weather wilting or black rot [16]; [17]; [18]. The fungus is also reported to produce several phytotoxic compounds [19]; [20]. The goal of this research was to report a novel rapid, simple and reliable technique to detect the antifungal potential of higher plant species using *Macrophomina phaseolina* inoculation on a detached leaf bioassay.

2. Material and Methods:

2.1. The fungus

The fungus used in this research was *Macrophomina phaseolina*, isolated from a diseased sesame plant (*Sesamum indicum*) with symptoms of a charcoal rot disease [18] obtained from a sesame field at Al-Anbar providences, Iraq. Plant tissue pieces from the crown and tab root of the infected plant were washed and sterilized with 5% bleach solution (17% NaOCl) and washed with sterilized water. The sterilized plant parts were placed aseptically on potato dextrose agar (PDA) in Petri plate and incubated for three days at 25 + 2 ° C. The dominant growing fungus was morphologically identified according to previous references [16]; [17]. The identified fungus was a subculture on PDA and preserved in the refrigerator for further use.

2.2. Plant materials

Eleven different plant species of different families include fruit, ornamental, and weed species were examined in this research (Table1). The leaves of each species were collected relatively of same size and age from the experimental field of the Plant Protection Department, College of Agriculture, University of Anbar. The leaves were washed with tap water, surface sterilized with 5% bleach solution (17% NaOCl), thoroughly washed with sterilized water and each placed aseptically on moistened sterilized filter paper in Petri plat. Six plats, for each plant were prepared three of them were inoculated with a 5mm disc of 5 days old *M. phaseolina* culture grown on PDA medium as a detached leaf bioassay following the same procedure described before [11]. The other three plates were left without inoculation, serving as a control. All plats were randomly distributed according to completely randomized design and incubated in the growth room at 25±2 ºC and 10 hours daily light for 5 days. The positive responses as specified by the leaf tissue yellowing, discoloration, dissociation was recorded according to the previously reported method [21] by applying the following formula:

$$\text{Damaged leaf area (\%)} = \frac{\text{Width of damaged area}}{\text{Length of damaged area}} \times \frac{\text{Width of total leaf area}}{\text{Length of total leaf area}} \times 100$$

Leaves of plant species showed weak or no clear response to the *M. phaseolina* were inoculated for the second time by the same method, but the leaves of each species were wounded before the inoculation. Across wounds of 0.75 cm length were aseptically made in the leaf of each plant species with a surface-sterilized razor. Three replicates of wounded leaves of every plant species was inoculated with the culture of the fungus and three more replicates of the wounded leaves were left without inoculum. All plats were randomly distributed according to the completely randomized design and incubated in the growth room at 25±2 ºC and 10 hours daily light. The results were reported 5 days later. The positive responses as specified by the leaf tissue yellowing, discoloration, dissociation were recorded according to the previously reported method [21]. The data were subjected to ANOVA followed by LSD at 5% [22].

2.1.1. Collecting and Extraction of the Giradol leaves

Several samples of the Giradol leaves (about100 gm each) were collected, washed, put into plastic bags and held in the freezer for further analysis. The Giradol leaves were aqueously extracted following a modified method reported before [4], a sample of 100 gm of Giradol leaves was blended by electrical blender with 150 ml sterilized distilled water for 10 minutes. The extracts were filtrated through a double-layer Muslin cloth. The filtrates then passed through Whatman paper No.1. The final filtrate then sterilized by different methods either by aseptically filtration through a Millipore filter membrane (0.45 um) under vacuum pressure or by autoclaving for 10 and 20 minutes.

2.3. Evaluation of the antifungal potentiality of Giradol

The leaves extract was examined for their antifungal effect following the method described before (4), where five ml of the extract of the Giradol was aseptically added to sterilized Petri plate along with 20 ml of could melted PDA and poured in each plate. The plates were then gently agitated to ensure mixing of the filtrate with the culture media. Three plates with the leaves extract for each of the sterilized treatments and another three plats without extract serving as a control were inoculated with *M. phaseolina*. Each plate was inoculated in the center with a 5 mm disc of *M. phaseolina* culture of 5 days old grown on PDA media. All the plates were randomly distributed according to the completely randomized design and incubated for five days at 25 + 2 ° C. The fungal growth on all plats was recorded as % growth inhibition following the formula reported before [10].

$$\text{Growth inhibition of the fungal growth (\%)} = \frac{\text{dc} - \text{dt}}{\text{dc}} \times 100$$

Where dc= average of the control colony diameter of the fungus and dc = average of the treated colony diameter of the fungus. The data were subjected to ANOVA followed by LSD at 5% [22].

3. Results and Discussion:

The results of the isolation from the infected sesame plant showed dominantly association of *Macrophomina phaseolina* according to its culture morphology [16]; [17] as being a major pathogen of sesame worldwide [18]. The results of the detached leaf bioassay in this research revealed that *Macrophomina phaseolina* evidently reacted with six of the eleven examined species included the sesame. The detached leaf response of various plant species indicated by tissue discoloration, yellowing, dissociation and death. The percentage of responses in this test ranged from zero for the Giradol to 100% of the sesame (Figure 1) others plants showed moderate to week response of significant differences (Table 1).

Table (1) Response of detached leaves of various plant species to *Macrophomina phaseolina* inoculation

Plant Species	% Detached Leaf Damage *	
	Control	Treated
Oleander *Nerium oleander*	0.0	8.67
Giradol *Chrozophora tinctoria*	0.0	0.00
Eucalyptus *Eucalyptus camaldulens*	0.0	80.0
Christ's thorn jujube *Ziziphus spina-christi*	0.0	13.0
Rose *Rosa amascenad*	0.0	5.00
Hop bush *Dodonaea viscosa*	0.0	13.33
Sesame *Sesamum indicum*	0.0	100.00
Pomegranate *Punica granatum*	0.0	100.00
Olive *Olea europaea*	0.0	90.0
Holy basil *Ocimum basilicum*	0.0	85.00
Cockscomb *Celosia argentea*	0.0	100.00
LSD at 0.05	5.355	

Notes: Results taken after five days and each value is an average of three replicates.

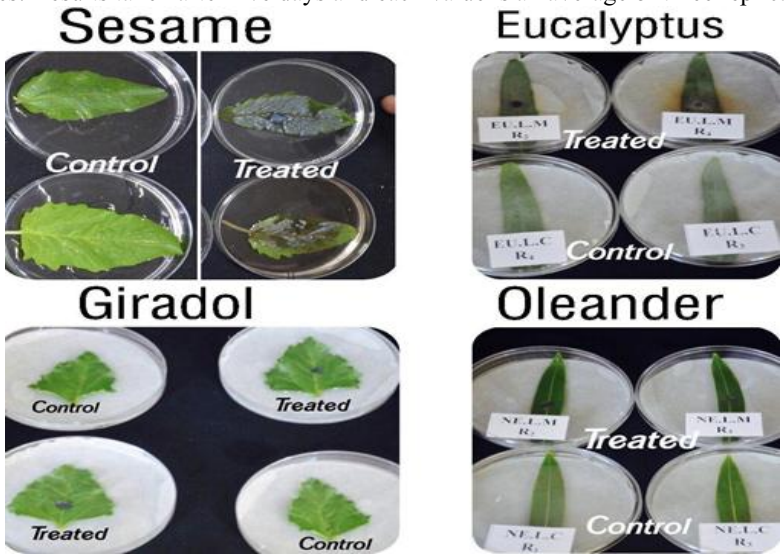

Figure (1) Response of detached leaves for different plant species to the *Macrophomina phaseolina* artificial inoculation.

The plant species that found to be of weak responses (< 14% phytotoxicity damage) on the first test such as Oleander, Rose, Christ's thorn, and Hop bush were previously reported on the antifungal activities [23]; [24];; [25]; [14]; [26]; [27]; [28]. In the second inoculation trial however, revealed higher responses of the examined species compared to the first non-wounded trail (their responses ranged from 33 to 76 % of Christ's thorn jujube and Rose respectively), (Table 2 and Figure 2).

Table (2) Response of wounded detached leaves of various plant species to the Macrophomina phaseolina inoculation

	% Detached Leaf Damage *	
Plant Species	Control*	Treated*
Giradol *Chrozophora tinctoria*	0.0	0.00
Christ's thorn jujube *Ziziphus spina-christi*	0.0	33.3
Rose *Rosa amascenad*	0.0	75.5
Oleander *Nerium oleander*	0.0	46.8
Hop bush *Dodonaea viscosa*	0.0	65.5
LSD at 0.05	11.25	

Notes: Results taken after five days each value is an average of three replicates.

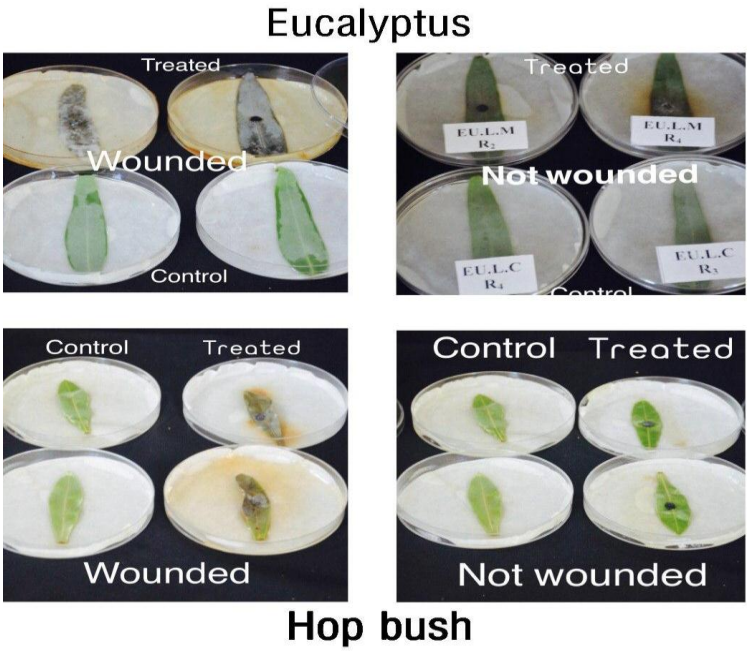

Figure (2). The effect of wounding on the response of Eucalyptus and Hop Bush detached leaf bioassay to the *Macrophomina phaseolina* inoculation.

This controversy responses might be attributed to the effect of the leaf wall and epidermis that most likely to act as a barrier against the pathogen invasion and their antifungal chemical constitutes [1]. The findings of this research however, proved the sensitivity of the applied technique to distinguish the previously reported plants of antifungal properties [23]; [24];; [25]; [14]; [26]; [27]; [28]. The Giradol leaves on the other hand was the only plant species showed no response to *M. phaseolina* even with wounding (0% leaf phytotoxic damage). This result is most likely to indicate a highly incompatibility between the Giradol and *Macrophomina phaseolina* and its toxins [19]; [20]; [29].

The incompatibility of the Giradol leaves to the *Macrophomina phaseolina* invasion might be to the possible existence of antifungal contents in this plant species. This assumption was confirmed in this research as the aqueous extract of the Giradol leaves showed highly toxicity against *Macrophomina phaseolina* growth on culture. The Giradol extract was inhibited the *Macrophomina phaseolina* growth on PDA culture by 77 and 90 % with significant differences depending on the extract sterilization methods (Table 3). The highest effectivity of the aqueous leaf extract was obtained with cold sterilization with Millipore membranes. The lowest fungal growth inhibition activity (%77) of the Giradol aqueous leaf extract was of the 20 minutes autoclaving (Figure 3).

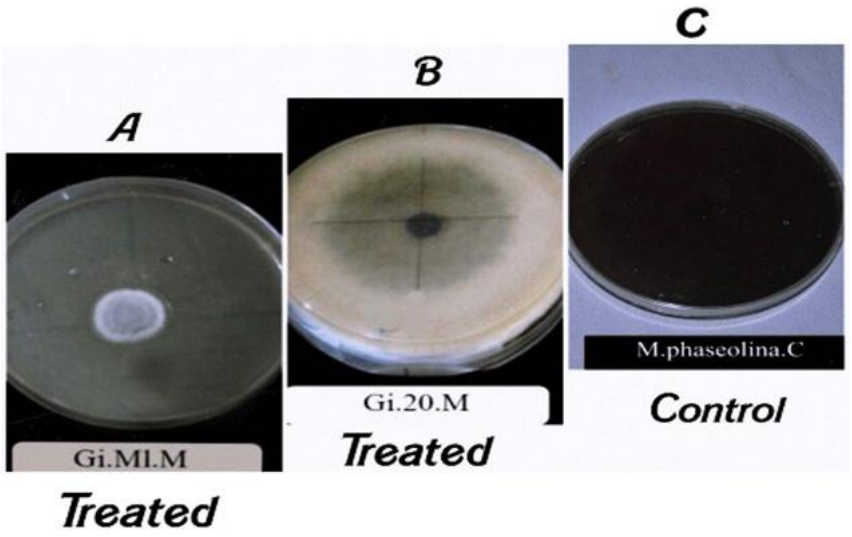

Figure (3) The effect of the Giradol aqueous leaf extract of different sterilization methods (A= Millipore filtration, B= autoclaving for 20 minutes and C= Control) on the growth of the *Macrophomina phaseolina* on PDA culture media.

Table (3) The inhibition effect of the aqueous extract of Giradol Chrozophora tinctoria leaves of different sterilization methods to the growth of *Macrophomina phaseolina* on PDA culture media

Aqueous Extract Sterilization Method	% Inhibition*
Millipore filtration	91.33
Autoclaving for 10 min	85.55
Autoclaving for 20 min	77.44
LSD at 0.05	2.625

Notes: Results taken after five days, each value is an average of three replicates.

The results also revealed that the antifungal content (s) of the Giradol leaves seemed to be relatively of heat stable (it still of effectivity to inhibit *Macrophomina phaseolina* growth up to 77% even after 20 minutes autoclaving) water soluble and produced and occurred at high concentration.

4. Conclusions

The findings of this research recommend for the first time the antifungal potential of Giradol as a source of effective bio -fungicide. Further investigation whoever, is still needed to characterize the antifungal properties of Giradol against *Macrophomina phaseolina* and might be of other fungi. The technique described in this research for detecting bio-fungicides in higher plants is relatively easy, fast and enabling to examine many plant species at the same time.

References

[1] Agrios, GN 1997, Control of plant diseases. Plant pathology, 4th edition. *California: Academic Press*.
[2] Chandler, J 2005, Cost reduction in SIT programs using expect auto-dissemination as part of area-

wide integrated pest management, *International Journal of Pest Control*, **47**, 257-260.
[3] Dellavalle, PD, Cabrera A, and Alem, D 2011, Antifungal activity of medicinal plant extracts against phytopathogenic fungus Alternaria spp., *Chil. J. Agr. Res*, **71**(2), 231.
[4] Harris, CA , Renfrew, MJ, and Woolridge, MW 2001, Assessing the risk of pesticide residues to consumers: recent and future developments. *Food additives and Contamination* **18**, 1124-1129.
[5] Carolina A, Herliyana E N, and Sulastri H.2019. Antifungal activity of castor (*Ricinus communis* L.) leaves methanolic extract on *Aspergillus niger*. International Food Research Journal 26(2): 595-598.
[6] Chaudhary, D, Mohammad SK, Shah AP, and Yadav AP 2019, Antifungal activity of three different ethanoic extract against isolates from diseased rice plant. *Journal of Analytical Techniques and Research* **1**, 047-063.
[7] Díaz-Dellavalle, P, Cabrera, A, Alem, D, Larrañaga, P ,Ferreira, F,and Dalla-Rizza M 2011, Antifungal activity of medicinal plant extracts against phytopathogenic fungus *Alternaria* spp. *Chilean J. Agric. Res.* **71**, 231–239.
[8] Gwa, VI, Nwankiti, AO, and Ekefan, EJ 2018, Antifungal effect of five aqueous plant extracts on mycelial growth of *Penicillium expansum* isolated from rotted Yam tubers in storage. *Acta Scientific Agriculture* **2**(6), 65-70.
[9] Mohana, D C, and Raveesha K A 2007, Anti-fungal evaluation of some plant extracts against some plant pathogenic field and storage fungi. *Journal of Agricultural Technology* **4**(1), 119-137.
[10] Mitra, S R, Choudhuri, A, and Aditya N 1984, Production of antifungal compounds by higher plants-a review of recent researches. Plant Physiol. *Biochem*., **11**, 53-77.
[11] Sharma, P, Sharma, SR, and Sindhu M 2004, A detached leaf technique for evaluation of resistance in cabbage and cauliflower against three major pathogens. *Indian Phytopathol*., **57**, 315-318.
[12] Shrivastava, DK, Swarnkar, K, and Chandra, T P 2018, Fungi-toxic properties of leaf extracts of Some herbaceous Wild Plants.
[13] Grover, R K, and Moore JD 1962, Toximetric studies of fungicides against brown rot organisms, *Sclerotia fructicola,* and *S. laxa*. *Phytopathol*., **52**, 876-880.
[14] Hadizadeh, PB, and Kolahi M 2009, Antifungal activity of nettle (*Urtica dioica* L.), colocynth (*Citrullus colocynthis* L. Schrad), oleander (*Nerium oleander* L.) and konar (*Ziziphus spina-christi* L.) extracts on plants pathogenic fungi. *Pak. J. Biol. Sci*., **1**, 58-63.
[15] Amadioha, AC , and Obi VI 1999, Control of anthracnose disease of cowpea by *Cymbopogon citratus* and *Ocimum gratissimum*. *Acta Phytopathologica et Entomologica Hungarica*, **34**, 85-89.
[16] Ellis, MB 1971, Dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, England.
[17] Gupta, GK, Sharma, SK, and Ramteke, R 2012, Biology, epidemiology, and management of the pathogenic fungus *Macrophomina phaseolina* (Tassi) Goid with special reference to charcoal rot of soybean (*Glycine max* (L.) Merrill). *J. Phytopathol*. **160**, 167–180.
[18] Sinclair, J 1982, Compendium of soybean diseases .St.Paul: The American Phytopathological Society .
[19] Abbas, HK, Bellaloui, N, Butler, AM, Nelson, J, Abou-Karam, M, and Shier, WT 2020, Phytotoxic Responses of Soybean (*Glycine max* L.) to Botryodiplodin, a toxin produced by the charcoal rot disease fungus, *Macrophomina phaseolina*. *Toxins*, **12**, 25.
[20] Dhar, TK, Siddiqui, KAI, and Ali, E 1982, Structure of phaseolinone, a novel phytotoxin from *Macrophomina phaseolina*. *Tetrahedron Lett*. **23**, 5459–5462.
[21] Aregbesola, E, Alejandro, O, Falade, T, Jonathan, G, Hearne, S, and Bandyopadhyay 2020, A detached leaf assay to rapidly screen for resistance of maize to Bipolaris maydis, the causal agent of southern corn leaf blight. *Eur J Plant Pathol*, **156**, 133–145.
[22] Steel, RGD, and Torrie, JA 1980, Principles and Procedures of Statistics, 2nd ed., USA: McGraw-

Hill, pp. 183-193
[23] Bidarigh, S, Massiha, A, Khoshkholgh, M, Pahlaviani, RM, Issazadeh, K, Panah, ZM, Ebrahim, A 2012, Antimicrobial (Screening) properties of various plant extracts from *Ocimum basilicum* L. and *Nerium oleander* L. against fungal common rots of potato *in vitro* bioassay. J. Basic. *Appl. Sci. Res.*, **2**(7), 6810-6815.
[24] Bokhari, FM 2009, Antifungal activity of some medicinal plants used in Jeddah, *Saudi Arabia. Mycopath*. **7**(1), 51-57.
[25] Dixit, SN, Tripathi SC, and Upadhyay RR 1976, The Antifungal Substance of Rose Flowers (Rosa indica). Economic Botany, **30**(4), 371-374.
[26] Hussain, MA, and Gorsi, MS 2004, Antimicrobial Activity of Nerium oleander Linn.. Asian *Journal of Plant Sciences*, **3**, 177-180.
[27] Mardani, M, Badiee, P, Gharibnavaz, M, Jassebi, A, Jafarian, H, and Ghassemi, F 2018, Comparison of anti-Candida activities of the ancient plants Lawsonia inermis and Ziziphus spina christi with antifungal drugs in Candida species isolated from oral cavity. *J Conserv Dent*. **21**(4), 359-362.
[28] Pirzada, AJ, Shaikh, W, Usmanghani, K, and Mohiuddin, E 2010, Antifungal activity of *Dodonaea viscosa* Jacq extract on pathogenic fungi isolated from superficial skin infection. *Pak. J. Pharm. Sci.*, **23**(3),337-340.
[29] Salhi, N, Saghir, S, Mohammed, A, Terzi, V, Brahmi, I, Ghedairi, N, and Bissati, S 2017, Antifungal activity of aqueous extracts of some dominant algerian medicinal plants. *BioMed Research International*, **1**- 6. https://doi.org/10.1155/2017/7526291.