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(RESEARCH ARTICLE)



In vitro: stem cutting a simple technique for determination aggressive potential of fungal isolates causing root rot disease of grapevine

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Abstract

Pathogenic soil borne fungi causing epidemic root rot disease on grapevine and high loss of plant growth and fruit yield. Fast detection of aggressive fungal isolates is the first tool for introduce selection effective a agent for management disease progress. *In vitro* two simple techniques based on stem cutting of grapevine i.e., mycelial growth and cell wall degrading enzymes activities were evaluated for screening of aggressive fungal isolates in comparsion with classical pathogenicity test. Fourteen isolates of fungi associated with root rot disease of grapevine plants i.e. *Fusarium oxysporum* Schlechtend, *Fusarium solani* (Mart.) Sacc, *Botryodiplodia theobromae*, *Rhizoctonia solani* Kuhn and *Macrophomina phaseolina* (Tassi) Gold. were tested and pathological action on grapevine plant (Cv.crimson) under soil artificially infested by classical method with their mycelial growth, cell wall degrading enzymes activities on stem cutting of grapevine as well as each fungal isolates of shoot and root compare the control. Fungal isolate of *Botryodiplodia theobromae* No. (7) was the most isolate recorded high and significant root rot (100%) incidence and disease severity on grapevine plants followed by *Fusarium avenacum* isolate No. (11) and *Fusarium solani* isolate No. (12). Positive correlation co-efficient was observed between mycelial growth of fungal isolates tested on stem cutting of grapevine plants and root rot disease incidence and their disease severity followed by their production pectinase and cellulase enzymes. So, mycelial of fungal growth on stem cutting of grapevine is a good and simple of opportunity technique for determination aggressive degrees of fungal isolates *in vitro* as alternative accuracy, very short time test and very shape cost than classical pathogenicity test.

Keywords: Fungi; Grapevine; Stem cutting; Pathogenicity; Technique

1. Introduction

During pathogenesis process by fungi are producing several chemical metabolites in a initial time of infection plant tissue such as cell wall degrading enzymes for degradation cell wall components. The roles of cell wall degrading enzymes and disease incidence of tomato plants were reported that higher activities of cell wall degrading enzymes and wilt disease incidence of tomato were higher in susceptible cultivars of tomato than resistant cultivars sowing in soil infested by *Fusarium oxysporum* f. sp. *lycopersici* the causal of wilt disease of tomato [1]. Over the last decade, its incidence has increased significantly in all major viticulture regions throughout the world, including South Africa, New Zealand, Italy, Australia and North America [2]; [3]; [4] and [5]. Although the disease occurs in young and mature vines, losses usually occur during the first 5 years, sometimes even during the first year after planting [6]. For assay pathogenic potential of soil borne fungi causing root-rot on grapevine. The production cell wall degrading enzymes i.e. pectinolytic and cellulytic enzymes was more obvious in the susceptible cultivars than in the resistant ones [7].

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activities of hydrolytic enzymes *i.e.* pectin methyl esterase (PME), polygalacturonase (PG) and cellulase (Cx) were higher in tissues of the susceptible strawberry cultivars infected with leaf spot pathogens than resistant cultivars. [8] *in vivo* production of pectinolytic enzymes was similar of all isolates of *F. solani* associated with wilt of cauliflower and apparently not correlated with pathogenic ability. However, the highly virulent isolate showed maximum of amylase activity and minimum of protease activity [9]. *in vitro* isolates of *F. oxysporum* causing wilt of grapevine (Cv. crimson) varied for producing lytic enzymes *i.e.* polygalacturonase (PG) and cellulase [10]. The aimed of this study comparative analysis between some morphological and physiological related pathological activities of soil borne fungi causing root rot grapevine.

2. Material and methods

2.1. Fungal isolates

Fourteen fungal isolates were isolated of diseased grapevine plant by root rot and identified in Plant Pathology Department, National Research Centre, Egypt.

2.1.1. Technique 1 Classical pathogenicity test

The pathogenic ability of fungal isolates was carried out by classical method under greenhouse conditions at Plant Pathology Department, National Research Centre, Egypt. Plastic pots (25 cm - diameter) were sterilized by dipping in a 5% formalin solution for 15 min. Loam sandy soil was sterilized with formalin solution (5%), then covered with a polyethylene sheet for 7 days to retain gas, and left to dry for 2 weeks until all traces of formaldehyde disappeared. Pots were filled with sterilized soil (3.5 Kg / pot). Each isolate of tested fungi were grown on sterilized corn meal and sand medium (75g corn meal grain +25g clean pur sand +100 ml of water) of glass bottles. Each bottle was inoculated by disk (1 cm - diameter) taken from 7 days-old of mycelial culture of each tested fungal isolates, then incubated at $27\pm 2^{\circ}\text{C}$ for 15 days. Pots were infested with each tested fungi by the rate 5% of soil weight (w/w). Pots were irrigated regularly three times a week before planting to ensure even distribution of the inoculated fungi in soil. One grapevine plant Cv. crimson (one year old) was cultivated of each pot. Ten pots were used as replicates. Ten pots free fungi infestation were cultivated as a control. Percentage of root rot disease incidence and disease severity were recorded 2.5 months after cultivation grape plants. Disease severity of grapevine plants was determined according to [10], on shoot system using two linear scale from 0 to 3 as follows: 0= healthy, 1= yellowish +1/3 plant wilted, 2= 2/3 plant wilted, 3= whole plant wilted and 4= plants dead showed sever wilt and on root on linear as scale from 0 to 3 on follows: 0 = normal color (health) of root system, 1 = slight brown discoloration cover 25% of root system, 2 = moderate brown discoloration of root cover 50% of root system, 3 = dark cover 100% of root system.

2.1.2. Technique 2 Fungal growth on stem cutting of grapevine

This technique aimed to determination of mycelial colonization of fungi on stem cutting of grapevine pieces 2 cm length of stems one year old were taken of healthy grapevine trees 5 years old, samples were thoroughly washed under running tap water then cut into small pieces (2- cm). Pieces were surface sterilized by dipping in 1% sodium hypochlorite solution for 2 min then washing several times in sterile distilled water. Pieces were dried between two layers of sterilized filter papers then transferred to Petri dishes. Four pieces were set of each plate from fungal mycelium growth after 2 days at 27°C on PDA medium. Five plates were used as a replicates for each fungal isolates and five plates free fungal growth were used as a control. All Petri dishes were incubated at $27\pm 2^{\circ}\text{C}$ for 5-7 days. Mycelial growth of each fungal isolates tested were determined according to adopted linear scale (0-3) as following

- 0 = No mycelial growth on piece
- 1 = Mycelial growth covered 1/3 stem cutting piece surface
- 2 = Mycelial growth covered 2/3 stem cutting piece surface
- 3 = Mycelial growth covered hole stem cutting piece surface

2.1.3. Technique 3 Cell wall degrading enzymes on stem cutting of grapevine

This technique aimed to determination of cell wall degrading enzymes activity of fungal isolates on stem cutting of grapevine after incubation by extraction enzymes from homogenized cutting grapevine pieces in 0.1 M sodium phosphate buffer (pH 7.1) [11] at the rate of 1/3 w/v. then centrifuged at 3000 rpm for 15 minutes. The supernatant was used to determine cellulase enzyme activity according to [12] by taken one milliliter of 1% CMC in 0.1M citrate buffer (pH 5.5) placed in a test tube and 1ml of extract was added then incubated at 50°C for 30 min and the reaction terminated by adding 1.5 ml 3,5-dinitrosalicylic acid (DNSA) reagent. The tubes were heated at 100°C in a boiling water bath for 15 min and then cooled at room temperature. The absorbance was read at 540 nm. Enzyme activity is expressed

as m mol glucose released per sec-1ml-1 of enzyme extract as enzyme solution. In addition, pectinase enzymes was assay according to [12], by add 0.5 ml of 1% pectin in 0.1M citrate buffer (pH 5.8) in a test tube and 0.5 ml of cutting grapevine pieces extract was added. The reaction mixture was incubated at 50°C for 30 min and the reaction terminated by adding 1.5 ml 3,5- dinitrosalicylic acid (DNSA) reagent. The tubes were heated at 100°C in a boiling water bath for 15 min and then cooled at room temperature. The absorbance was read at 575 nm. Enzyme activity is expressed as M mol glucose released per min-1ml-1 of enzyme solution.

2.2. Protein determination

Protein concentration was determined by measuring the absorbance at 750 nm by the method of [13].

2.3. Statistical analysis

Statistical analyses of all the previously designed experiments were carried out according to (ANOVA) procedures reported by [14].

3. Results

3.1. Comparative studies on pathogenicity test, fungal growth and cell wall degrading enzymes activities of fungal isolates on stem cutting in grapevine

Data in Table (1) indicated that all fourteen fungal isolates varied of pathological action of grapevine plant mycelial growth on stem cutting and their activities of, pectinase and cellulase enzymes. *Fusarium solani* isolate (No.12) highly and significantly activity pectinase enzyme (10 mg/ ml) followed by *Fusarium oxysporum* isolate (No.6) 7.3 mg/ ml then 7.2 mg/ml of *F.solani* isolate (No.9), *B. theobromaei* (No.1), *F. solani* isolate (No.3) and *B. theobromaei* (No.7). *F. oxysporum* isolate (No.14), *Fusarium avenacum* isolate (No.11) and *Fusarium oxysporum* isolate (No.4). Meanwhile, highly and significantly produced cellulase enzymes recorded by *Fusarium avenacum* isolate (No.11) followed by *B. theobromaei* isolate (No.13) then (No.7) and *Fusarium oxysporum* isolate (No.6 &10). Data in Table (1) also, indicated that *Fusarium avenacum* isolate (No.11) and *Fusarium solani* isolate (No.12) were recorded equal activities rate of two pectinase and cellulase enzymes (12.3mg/ml and 12.4 mg/ml respectively) followed by *B. theobromaei* (No.7) recorded (10.9 mg/ml) total activity of two enzymes. On the other hand, the lowest activities of pectinase and cellulase enzymes by *Macrophomina phaseolina* isolate (No.5). Data in Table (1) and Fig. (1) also, showed that the highly colonization of mycelial growth on stem cutting of grapevine by *Fusarium avenacum* isolate (No.11) and *B. theobromaei* isolate (No.7) followed by *B. theobromaei* isolate (No.13), *Fusarium oxysporum* isolate (No.4 & 6) and *Fusarium solani* isolate (No.12). In addition, data in Table (1) indicated that highly and significantly pathogenic activity of fungal for causing root rot disease incidence showed by *B. theobromaei* isolate (No.7) followed by *Fusarium avenacum* isolate (No.11), *Fusarium oxysporum* isolate (No.6) then *Fusarium solani* isolate (No.12) and *Rhizoctonia solani* isolate (No.8 & 9). Positive correlation between high pathogenic isolates on grapevine plants as shown in pathogenicity test with *Fusarium avenacum* isolate (No.11), *Fusarium oxysporum* isolate (No.6) then *Fusarium solani* isolate (No.12) and their activities of pectinase and cellulase enzymes as well as colonization of mycelial growth of these isolates on stem cutting of grapevine plants. On the other hand, leak correlation between another fungal isolates tested between three criteria in this investigation. Furthermore in Table (2) as shown analysis indicated that high correlation co-efficient between mycelial growth (colonization), pectinase activity on stem cutting of grapevine and root rot incidence of grapevine under artificial infested of pots.

Table 1 Pathogenicity test, fungal growth and cell wall degrading enzymes activities of fungal isolates on stem cutting of grapevine

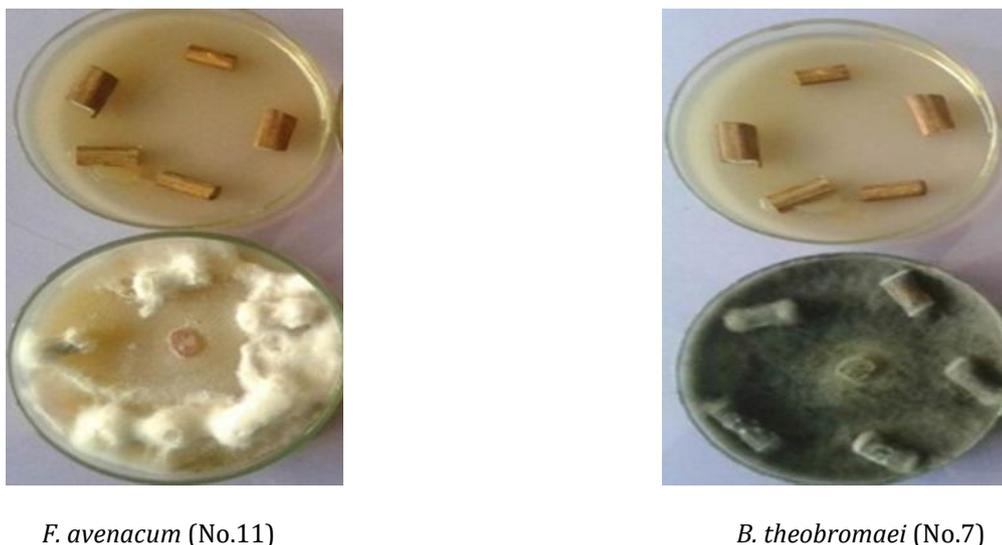
Fungal isolates Name	No	Enzymes activities (ug/ml)		Colonization rate	Root rot incidence		
		Pectinase	Cellulase		Infection %	Disease severity	
						Shoot	Root
<i>B. theobromaei</i>	1	7.2b	5.4b	1.2cd	75.0c	1.6cde	1.7c
<i>R. solani</i>	2	4.7g	3.1de	0.8de	75.0c	1.8bcde	1.9bc
<i>F. solani</i>	3	6.9bc	2.6f	0.8de	63.0d	1.4e	1.5c
<i>F. oxysporum</i>	4	6.4de	3.1de	1.4bc	50.0e	1.6cde	1.7c
<i>M. phaseolina</i>	5	1.7h	0.9g	1.0cd	62.0d	1.2e	1.5c
<i>F. oxysporum</i>	6	7.3b	3.8c	1.4bc	87.5b	2.5ab	2.6ab
<i>B. theobromaei</i>	7	6.7cd	4.2c	2.6a	100.0a	2.9a	3.2a
<i>R. solani</i>	8	4.8g	2.5f	0.5e	87.5b	2.0bcde	2.2abc
<i>F. solani</i>	9	7.2b	2.7ef	0.8de	87.5b	2.5ab	2.4abc
<i>F. oxysporum</i>	10	5.5f	3.8c	0.9de	70.0cd	2.4abc	2.5abc
<i>F. avenacum</i>	11	6.4de	5.9a	2.6a	87.5b	2.6ab	2.7ab
<i>F. solani</i>	12	10.0a	2.4f	1.4bc	87.5b	2.5ab	2.6ab
<i>B. theobromaei</i>	13	6.2e	3.2d	1.8b	75.0c	2.1abcd	2.1bc
<i>F. oxysporum</i>	14	6.5cde	3.8c	1.0cd	75.0c	2.0bcde	2.1bc
Control		0.25i	0.08h	0.0f	00.0f	0.0f	0.0d

Values within each column followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test

Table 2 Correlation co-efficient between pathological potential, cell wall enzymes activities and fungal mycelial growth on stem cutting of grapevine

		Pectinase	Cellulose	Colonization	Root rot incidence		
					infection %	Disease severity	
						shoot	root
Pectinase	-pearson Correlation sig.	1	0.61	0.55	0.74	0.58	0.55
			0.05	0.05	0.004	0.04	0.05
Cellulase	-pearson Correlation sig.	0.62	1	0.78	0.65	0.64	0.62
		0.05		0.002	0.02	0.02	0.02
Colonization	-pearson Correlation sig.	0.74	0.65	1	0.60	0.90	0.91
		0.004	0.02		0.04	0.00	0.00
D.S. Shoot	-pearson Correlation sig.	0.58	0.64	0.55	0.90	1	0.99
		0.04	0.02	0.05	0.00		0.00
D.S. Root	-pearson Correlation D.S. = disease severity -sig.	0.55	0.62	0.55	0.91	0.99	1
		0.05	0.02	0.05	0.00	0.00	

D.S. = disease severity



F. avenacum (No.11)

B. theobromaei (No.7)

Figure 1 Mycelial growth of aggressive fungal isolates causing root rot of grapevine plants grown on stem cutting of grapevine compare stem cutting free fungal infestation upper of each plate

4. Discussion

This investigation aimed to identify the alternative efficient simple method that might reflect the classical pathogenicity test which need more time 2-3 months and high cost for preparation experimental requirements and conditions in greenhouse as well waiting appearance of root rot disease syndromes are included growth retardant, chlorosis, yellowish, wilt and death of shoot systems, reduction of fruit yield as well as reduction of root system mass of feeder and secondary roots. In addition, brown discoloration and maceration of main and feeder roots as well stem base of grapevine plants were observed [15]; [10]; [16], and [17]. In this study high positive correlation of mycelial growth of fungal isolates on stem cutting of grapevine and pathogenicity test in this manner, several investigators had been evaluated pathogenicity of fungi independent on fast test growing mycelial in culture [18], [19] and [20]. Positive correlation between *in vitro* growth rate and pathogenicity of *Ophiostoma nlmii* [18], on the other hand, no correlation was found between colony growth rate and pathogenicity of Eucalyptus canker causal pathogen fungal isolate of *Chrysosporthe austroafricana*. In this manner, *in vitro* simple and effective screening technique of tuber piece inoculum of potato positively correlation with black scurf disease incidence in the field caused by *Rhizoctonia solani* it is practical used as the rapid identification of resistant potato germplasm [21] In addition, in this study also, positive correlation between aggressive potential of fungal isolates causing root rot on grapevine plants in soil artificially infested by fungal inocula in green house *i.e.* *Fusarium oxysporum* No. (6), *Fusarium avenacum* No. (11), *Fusarium solani* No. (12) and *Botryodiplodia theobromae* No. (7). These isolates were production a good amount of pectinase and cellulose at the same equal approximately of the activities of both enzymes. The roles of cell wall degrading enzymes and plant disease incidence of tomato plants were studied by [1] reported that higher activities of cell wall degrading enzymes and wilt disease incidence were higher in susceptible of tomato than resistant cultivars sowing in soil infested by *Fusarium oxysporum* f. sp. *lycopersici* the causal of wilt disease. So mycelial growth of fungal isolates (colonization) on stem cutting of grapevine is the a simple of opportunity techniques for determination aggressive fungal isolates *in vitro* alternative accuracy, very short time test and very cheap cost than classical pathogenicity test.

5. Conclusion

Due to positive correlation between mycelial growth of fungal isolates on grapevine stem cutting as a dopted technique *in vitro* and classical methods in greenhouse by artificial sterilized potted soil by soil borne fungi then recorded their effect on cultivation plants after at least 3 months for determination aggressive potential of fungal isolates tested. So, *in vitro*, stem cutting technique is alternative accuracy, very short time test, very cheap cost and very easy assay than classical pathogenicity test *in vivo*.

Compliance with ethical standards

Disclosure of conflict of interest

There is no conflict of interest among the authors.

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