USING OF CULTURE FILTRATES IN THE SELECTION OF *BOTRYODIPLODIA THEOBROMAE* RESISTANT DATE PALM PLANTS VIA TISSUE CULTURE

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Abstract

This investigation was planned to select and develop date palm plants from selected resistant callus variants to wilt disease caused by *Botryodiplodia theobromae*. The chemical analysis of selected date palm resistant plants was also investigated.

Virulent strains of *B. theobromae* fungus was tested as a selective agent for use *in vitro* selection for resistance by measuring the response of explants (callus) of two date palm genotypes (Bartamoda and Samani). It was obviously clear that the higher concentrations of culture filtrate (CF) *B. theobromae* showed destructive effect on the callus growth. Bartamoda cv. recorded the highest survival percentage of callus growth (74.99%) comparing with Samani cv. (41.66%).

Calli culturing on culture filtrate of causal fungus increased reducing sugars content with Samani cv. and reduced those sugars with Bartamoda cv. While pathogen culture filtrate mostly decreased non-reducing sugars into treated callus of both Samani and Bartamoda compared with control. Increasable rates of *B.theobromae* culture filtrate than 5 % decreased total sugars into treated callus of both cultivars. Increasable rates of pathogenic fungus culture filtrate increased amino acids content and total phenol contents. Different concentrations of culture filtrate decreased indoles content in treated callus of the two cultivars.

Use of descending rates of *B.theobromae* culture filtrate treatments resulted in reasonable resistance at the rates of 5 and 7.5% v/v. Higher rate (10%) highly decreased growing individuals. Whereas lowest rate (2.5 %) resulted in complete growth of treated callus.

Key words: disease-resistance, *in vitro*-selection, date palm, plant-pathology, fungi.

INTRODUCTION

Date palm is restricted to the semi temperate region in the world especially near the deserts of Africa and Asia. The knowledge about diseases affecting this plant is somewhat limited. However, some serious diseases were reported in certain countries to cause great losses. The fungus cause shoot dry rot, shoot tip soft rot and seedling damping of date palm off shoot (Abdel-Megid and Gafar, 1966), and it is a wide host

range pathogen, Narendra, *et al.,* 1979 declared that heart-rot of date palm was associated with it.

In vitro selection with toxins or culture filtrates of plant pathogenic microorganisms has in many cases improved the efficiency of plant breeding. The use of toxins should be preferred to that of culture filtrates because they are pure substances which do not cause artifacts and do not falsify (Crino, *et al.*, 1996). The use of tissue culture and *in vitro* mutagenesis for the improvement resistance to *Alternaria solani* in Irish Potato (*Solanum tuberosum* L.) var. Desiree was investigated (Veitia, *et al.*, 2001).

This investigation was planned to select and develop date palm plants from selected resistant callus variants to wilt disease caused by *Botryodiplodia theobromae*. The chemical analysis of selected date palm resistant plants was also investigated.

MATERIALS AND METHODS

The present sutdy was performed during the years of 2002 to 2007 by the cooperation between Botany Department Faculty of Agriculture Al-Azhar University and The Central Laboratory for Date Plam Researches and Development, Agriculture Research Center, at Giza, Egypt. This investigation is aimed to select the resistant date palm lines to infection with *Botryodiplodia thiobromae* via tissue culture techniques

Plant material

The propagation process was started with the selection of healthy offshoots from mother date palm trees of dry cultivar Bartamoda grown at Aswan Governorate and soft cultivar Samani grown at El-Badrasheen, Giza, Egypt. The young offshoots were of 2 - 4 years, ranging in weight from 5 - 7 kg and about 50 - 80 cm in length.

The shoot tip explants were surface sterilized under aseptic conditions by soaked in 70% ethanol alcohol solution for 30 seconds, followed by immersion in (1.0 g/l) of mercuric chloride for 5 min and thoroughly washed with sterilized distilled water for one-time. After that additional leaf primordial were removed from sterilized explants and then these explants were sterilized in 50%(v/v) commercial blanch (Clorox) 5.25% w/v, sodium hypochlorite NaOCl) plus 1 drop Tween 20 for 15 min with rotary agitation, rinsed three times with sterilized distilled water (Zaid, 2003).

Callus induction

Shoot apex was sliced longitudinally into 4 pieces and then cultured on Murashige and Skoog (MS) basal nutrient medium (1962) supplemented with 170 mg $NaH_2Po_4.2H_2O$, 200 mg glutamine, 40 mg adenine sulfate, 0.4 mg Thiamine HCL, 3g

activated charcool (AC), 30g sucrose, 6 g agar and 100 mg 2,4-D +3 mg 2ip / liter as described by Mater (1986).

The pH of all culture media were adjusted to 5.8±0.1 prior to the addition of agar, and then 35 ml of medium was dispensed into small jars (150 ml),the jars were autoclaved at 121°C and 1.1 kg/cm² for 20 min.

The cultures were maintained at the growth room under full darkness at 27±2 °C. After 6 month the calli were re-cultivated for mass production for further work. Some somatic embryos were differentiated from calli on MS medium (free-hormone medium) to use as explant during our study

Pathogen and subculturing

Botryodiplodia thiobromae was isolated from wilted date palm plants. The fungus was cultured on two kinds of Potato dextrose agar (PDA), the solidified media wer used for interval sub-culturing of the fungus. While the liquid medium (PD) was used for producing the fungus culture filtrate. The pH of these media was adjusted to 4.0.

The fungus was grown on PDA medium and transferred to the liquid medium (PD) for 3 weeks of culture in the dark at 25-28 °C. The collected culture filtrate was sterilized using filter membrane (white man paper 11.0cm) and conserved in deep freezer for further use.

The selection

Various concentrations of culture filtrate were added into MS medium to give concentrations of 0.0 (control), 5.0, 7.5 and 10.0% of selective agent (CF) measured to the medium volume. The explants (callus) were cultured on different concentrations of CF for 4 weeks. Each treatment contains 9 replicates each contains 3 jars (150 ml), every on contained about 0.5 g of callus. The percentages of tolerant callus were calculated after 12 weeks from culturing .The selection callus, which showed stable resistance during the selection process were transferred onto MS medium only or supplemented with 0.1 mg/ NAA for regeneration resistant plants

Chemical analysis

There are enhancement of defense responses against fungus disease by treatment of date palm seedling with pathogen infection. Thus, the date palm plants were evaluated for resistance of *Botryodiplodia theobromae* by chemical analysis such as reducing sugar, non reducing sugar, total free amino acids, total soluble phenols and total indoles.

Determination of sugars

Sugars were determined by picric acid method discoed by Thomas and Ducher (1924) .

Determination of free amino acids

according to Moore and Stein (1954).

Determination of phenolic compounds

Phenols were determined by the colorimetric method described by Snell and Snell (1953).

B-Determination.

Total indoles were determined according to (Larsen *et al.*, 1962).

Statistical analysis

The randomized factorial design was used and data were subjected to analysis of variance. Separation of means among treatments was determined using L.S.D test at 5% according to Snedecor and Cochran (1972).

RESULTS AND DISCUSSION

Culture filtrate of virulent strains of fungus was tested as a selective agent for use *in vitro* selection for resistance by measuring the response of explants (callus) of two date palm genotypes (Bartamoda and Samani), are,

Table1. Tested and selected of different culture filtrate (CF) concentrations of *Botryodiplodia theobromae* of two date palm genotype after two months of selections

(A) CF%	(B)Genotype		
	Bartamoda	Samani	Mean(A)
0.0 (control)	100	100	100a
5.0	100	33.33	66.66b
7.5	66.66	33.33	49.99c
10.0	33.33	00.00	16.66d
Mean(B)	74.99a	41.66b	
L.S.D at 0.05%	A=2.22	B=1.57	AB=3.14

It was obviously clear from Table (1) that the higher concentrations of *B. theobrome* CF generally showed destructive effect on the callus growth. Data declared that survival percentage of callus growth was the greatest (100%) during it culture on control medium (MS medium-CF Free) for at the two cultivars while the lowest rate of survival percentage (16.66%) was recorded on 10% CF. On the other hand,

Bartamoda cv. recorded the highest survival percentage of callus growth (74.99%) comparing with Samani cv. (41.66%). Concerning the interaction between CF and cultivars data observed that, Bartamoda cv. Recorded the same and the highest significant value of survival percentage (100%) when the explants (callus) were cultured on control medium (MS medium-CF Free) for 5% CF. Samani cv. recorded the lowest significant value of survival percentage (0.0%) when the explants were cultured on 10% CF.

El-Kazzaz *et al.*, 1998 showed that resistant calli of three tomato genotypes selected *in vitro* under challenging of the culture filtrate which obtained from the wilt pathogen *Fusarium oxysporum*. Enhancement of defense responses against fungus disease by treatment of date palm seedling with pathogen hypo aggressive *Fusarium oxysporum* were isolate (El-Hassni *et al.*, 2004).

Chemical analysis

1- Reducing sugars content

The effect of *B. theobrome* on reducing sugars level in the two date palm cvs. plants were presented in Table (2), fungal culture filtrate significantly decreased levels of reducing sugars in treated callus of Bartamoda cv. at rates of 7.5 % only, while all other CF % increased reducing sugars in each two cultivars. However, 10 % treatment significantly increased reducing sugars in Samani cv (Table 2.), also its clear

Table 2. Effect of CF of *B. theobromae* and date palm genotypes on reducing sugars

CF% (A)	(B)Genotype		
	Bartamoda	Samani	Mean(A)
0.0 (control)	0.368	0.383	0.378
5.0	0.373	0.384	0.378
7.5	0.364	0.393	0.387
10.0	0.374	0.412	0.378
Mean(B)	0.368	0.393	
L.S.D. at 0.05%	A=0.002	B= 0.001	AB=0.002

On the other hand no significantly change in amounts of reducing sugars were detected with the lowest rate of fungal culture filtrate, (5%). Bartamoda in general contained less amount of reducing sugars than Samani. Kapur, 1978 reported that infection of date palm leaves with smut disease caused by *Graphiola phoenicis* decreased sugars, phenols, some macro-elements but other micro-elements were increased.

2 - Non reducing sugars content

Effect of CF of *B. theobromae* on the non reducing sugars content in different date palm genotypes were presented in Table (3). Data showed that, *B. theobromae* culture filtrate treatments of callus were significantly decreased amounts of non-reducing sugars at all rates tested except Bartamoda at 5 % compared with control (Table 3.).

Table 3. Effect of CF of *B. theobromae* and date palm genotypes on non reducing sugars

CF% (A)	(B)Genotype		
	Bartamoda	Samani	Mean(A)
0.0 (control)	2.702	2.775	2.738
5.0	2.699	2.723	2.711
7.5	2.235	2.556	2.395
10.0	0.885	1.477	2.362
Mean(B)	2.130	2.283	2.738
L.S.D. at 0.05%	A=0.019	B= 0.009	AB=0.014

The reductions each culture filtrate rate significantly decreased in Bartamoda than Samani. Bartamoda-generally was lower in non-reducing sugars than Samani cv. El-shehaby, (1982), found that sugars and phenols content were lower in the less susceptible onion cultivars to smut disease (Giza 6) than others of higher levels of susceptibly to the disease.

3 – Total soluble sugars content

Fungal culture filtrate treatments at 7.5 and 10 % dilutions significantly on under the total sugars for the two genotypes than 5 % (Table 4.).

Table 4. Effect of CF of *B. theobromae* and date palm genotypes on total sugars

(A) CF%	(B)Genotypes		
	Bartamoda	Samani	Mean(A)
0.0 (control)	3.076	3.158	3.117
5.0	3.072	3.107	3.089
7.5	2.599	2.949	2.774
10.0	1.248	1.889	1.567
Mean(B)	2.498	2.775	
L.S.D. at 0.05%	A=0.056	B= 0.027	AB=0.039

The control medium recorded the highest treatment in reducing the content total sugars. The rate of 5 % culture filtrate was not significantly effective on change amounts of total sugars. Total sugars were generally lower in Bartamoda than Samani cv.

4 - Total amino acids content

The CF of *B. theobromae* effect on date palm content from amino acids was obtained and presented in Table (5).

Amino acids content of callus were significantly increased with 7.5 % culture filtrate treatments for both Bartamoda and Samani, however amino acids were increased in Bartamoda than decreased in Samani with 10 % culture filtrate treatment. No change in amino acid content was detected in embryogeniec callus treated with 5 % culture filtrate. Amino acids content was significantly higher in Bartamoda than Samani control treatments.

Table 5. Effect of CF of *B. theobromae* and date palm genotypes on amino acids content.

(A) CF%	(B)Genotypes		
	Bartamoda	Samani	Mean(A)
0.0 (control)	0.374	0.383	0.478
5.0	0.378	0.384	0.381
7.5	0.397	0.431	0.414
10.0	0.485	0.353	0.420
Mean	0.408	0.388	
L.S.D. at 0.05%	A=0.005	B= 0.003	AB=0.004

Mandavia, and parameswaran, (1993),Reported that resistant Lima bean plants to infection with *M.phaseolina* contained higher amounts of amino acids cystine and arginine + histidine. Catechol and chlorogenic acid were higher in resistant plants subjected by fungal infection.

5 – Free phenols content

CF of *B. theobromae* were found to be higher increase at 5% rate on free phenol of Bartamoda cv. only. While the fungal culture filtrate treatments on callus significantly increased free phenols content at the rate of 7.5 and 10 % only, (Table, 6), for each of two genotypes.

However, the increase in these bio-chemicals was significantly higher in Bartamoda than Samani at any of the two rates. Control treatments significantly performed higher amount of free phenols in Bartamoda than Samani.

Table 6. Effect of CF of B. theobromae and date palm genotypes on free phenols

(A) CF%	(B)Genotype		
	Bartamoda	Samani	Mean(A)
0.0 (control)	0.165	0.077	0.121
5.0	0.188	0.079	0.133
7.5	0.209	0.132	0.170
10.0	0.391	0.212	0.301
Mean(B)	0.238	0.135	
L.S.D. at 0.05%	A=0.029	B= 0.014	AB=0.029

6 - conjugated phenols content

Conjugated phenols of callus grown on B.theobromae culture filtrate amended MS media were determined it was found that there was a significant decrease with Bartamoda cv. (less susceptible) and increased with Samani cv.(more susceptible) compared with controls (Table, 7).

Table 7. Effect of CF of *B. theobromae* and date palm genotypes on conjugated phenols

CF %(A)	(B)Genotype		
	Bartamoda	Samani	Mean(A)
0.0 (control)	0.069	0.005	0.037
5.0	0.071	0.006	0.038
7.5	0.065	0.040	0.052
10.0	0.040	0.040	0.040
Mean(B)	0.061	0.030	
L.S.D. at 0.05%	A=0.001	B= 0.004	AB=0.009

The decrease in the phenols of growing Bartamodacv. callus was the less at 10 % v/v culture filtrate. While the increase of these compounds in Samani cv.(more susceptible) was detected at the rate of 10, 7.5 % v/v fungal culture filtrate. Conjugated phenols in control treatment were also higher with Bartamoda cv. than Samani cv. callus. The increase in conjugated phenols content increased as rate of fungal culture filtrate added into MS medium increased in Samani cv..

7 – Total phenols content

Addition of B.theobromae culture filtrate into Ms Medium mostly increased total phenols content in Samani and Bartamoda growing callus compared with plain

Table 8. Effect of CF of *B. theobromae* and date palm genotypes on total phenols

(A)CF %	(B)Genotype		
	Bartamoda	Samani	Mean(A)
0.0 (control)	0.084	0.234	0.159
5.0	0.085	0.266	0.175
7.5	0.186	0.244	0.230
10.0	0.252	0.432	0.342
Mean(B)	0.151	0.301	
L.S.D. at 0.05%	A=0.009	B= 0.006	AB=0.013

The increase in total phenols amounts was higher in Bartamoda cv. (less susceptible) than Samani cv. (more susceptible) at similar rates of fungal culture filtrate. The increase in total phenols increased as rate of culture filtrate increased. Ramos *et al,* (1980)ported that phenolic and phenolamidic compounds were detected into date palm tissues as a response to infection with *Fusarium oxysprorum* f.sp *albidinis.* They added that there were three of detected hydroxycinnamoylamids were isolated and found highly inhibitors to spore germination of the pathogen.

8 – Total indols content

Callus treated with *B.theobromae* culture filtrate significantly reduced indoles at all tested rates except Samani cv. at 5 % dilution (Table, 9).

Table 9. Effect of CF of *B. theobromae* and date palm genotypes on indoles content.

(A) CF %	(B)Genotype		
	Bartamoda	Samani	Mean(A)
0.0 (control)	0.660	0.258	0.459
5.0	0.653	0.204	0.428
7.5	0.599	0.132	0.365
10.0	0.570	0.046	0.308
Mean(B)	0.620	0.160	
L.S.D. at 0.05%	A=0.029	B= 0.014	AB=0.021

Indoles reductions in Samani cv. were significantly lower than Bartamoda cv. at each rate. Indoles reduction was also increased as are rate of fungal culture filtrate increased. generally Samani cv. Had higher in indoles content than Bartamoda cv.

Induction of resistant somatic embryos

The effect of NAA rates added into MS medium on counts and fresh weight of resulted embryos of genotypes Bartamda (Table, 10) and Samani (Table, 11) were studied compared with plain MS medium.

Counts of Bartamoda cv. somatic embryos differentiated on NAA amended MS salts which previously derived from disease resistant calli selected on MS medium amended with rates of pathogen culture filtrate.

Culture filtrate of virulent strains of fungus were tested as a selective agent for use *in vitro* selection for resistance by measuring the response of callus date palm genotype Bartamoda .

Table 10. Counts of Bartamoda cv. somatic embryos differentiated on NAA amended MS salts which previously derived from disease resistant calli selected on MS medium amended with rates of pathogen culture filtrate.

(A) CF %	(B) Treatment mg/l					
	Fresh weight (g)			No. of embryo		
	0.0	0.1 NAA	Mean (A)	0.0	0.1 NAA	Mean (A)
5.0	2.0	2.5	2.25	9.33	14.66	11.99
7.5	2.0	3.2	2.60	9.33	11.33	10.33
10.0	1.7	1.7	1.70	7.00	9.00	8.00
Mean (B)	1.90	2.46		8.55	11.66	
L.S.D at 0.05% (A)			0.18			0.99
(B)			0.15			0.81
(AB)			0.12			0.98

Data obtained were presented in Table (10), Bartamoda cv. callus resisted fungal culture filtrate at rates of 5 and 7.5 % v/v and grown on MS medium produced counts of embryos with fresh weights significantly higher than those derived from callus resistant to 10 % fungal culture filtrate, (Table, 10). Counts of embryos differentiated on 0.1 mg NAA MS Media except fresh weight of 10 % culture filtrate treatment of callus produced counts of embryos on axon amended MS media significantly higher but less in fresh weight than 7.5 % culture filtrate

Counts and fresh weight of Samani cv. somatic embryos derived from fungal disease resistant callus and differentiated on MS and NAA MS media

Counts of somatic embryos differentiated on NAA free - MS medium were significantly higher at 5 % culture filtrate callus treatment than 10 % (Table 11). Fresh weight of Samani cv. somatic embryos was not significantly affected by the rates of culture filtrate. NAA amended MS Medium significantly increased counts of embryos only with 10 % culture filtrate callus treatment. The auxin increased the fresh weight of somatic embryos at only 7.5 % culture filtrate callus treatment.

Table 11. Counts and fresh weight of Samani cv. somatic embryos derived from fungal disease resistant callus and differentiated on MS and NAA MS media

(A) CF %	(B) Treatment mg/l					
	Fresh weight			No. of embryo		
	0.0	0.1 NAA	Mean (B)	0.0	0.1 NAA	Mean (B)
5.0	1.5	3.3	2.90	11.00	14.66	12.16
7.5	1.6	2.9	2.25	9.33	11.33	10.49
10.0	1.6	2.3	1.95	7.00	9.00	8.50
Mean (B)	1.53	2.83		9.11	11.66	
L.S.D at 0.05% (A)			2.42			0.15
(B)			1.96			0.15
(AB)			2.43			0.12

El-Kazzaz *et al.*, 1998 declared that the regeneration of callus to plantlets could occur under stress of 5%, 10% and 25% CF.

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إستخدام الراشح الفطرى لأختيار نباتات نخيل البلح مقاومة للأمراض خلال تكنيك زراعة الأنسجة

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٢: المعمل المركزى للأبحاث وتطوير نخيل البلح – مركز البحوث الزراعية – الجيزة

أجريت هذه الدراسة للحصول على نباتات نخيل البلح مقاومة لفطر بتزودبلوديا ثيروما وكذلك إجراء التحاليل الكيمائية مثل السكريات المختزلة والغير مختزلة والكلية للأجزاء النباتيـــة موضـــوع الدراسة. وقد استخدم فى هذه الدراسة صنفان من أصناف نخيل البلح هم برتمودا (صنف جـــاف) و سمانى (صنف رطب).

أوضحت النتائج أن معاملة الكالس بالتركيزات العالية من الراشح الفطرى لفطر بترودبلوديـــا ثيروما سبب أذى وضعف للكالس وقد سجل صنف البرتمودا اعلى نسبة حيوية(٧٤,٩٩%) مقارنـــة بالصنف السمانى (٤١,٦٦%). أدت زراعة الكالس على التركيزات المختلفة للراشح الفطـــرى آلـــى زيادة محتوى الكالس من السكريات المختزلة و انخفاض محتوى السكريات الغير مختزلـــة للصـــنف البرتمودا وكذلك السمانى. انخفض محتوى السكريات الكلية عند زيادة تركيز الراشح الفطرى عن ٥ %. زاد محتوى الأحماض الأمينية وكذلك المحتوى الكلى للفينولات بزيادة تركيز الراشح الفطـــرى. كما انخفض محتوى الأندولات بالعينات بزيادة تركيز الراشح الفطرى. إضـــافة الراشـــح الفطـــرى بتركيزاته المختلفة الى بيئة زراعة الكالس احدث استجابة لنمو الكالس المقاوم.