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Application of *Saccharomy*ces *cerevisiae* as a biocontrol agent against some diseases of Solanaceae caused by *Macrophomina phaseolina* and *Fusarium solani*

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ABSTRACT

Local isolate of *Saccharomy*ces *cerevisiae* had a reduction potential against radial growth of pathogenic fungi *Macrophomina phaseolina* and *Fusarium solani*, the cause of root rot diseases in tomatoes and eggplants. Maximum growth reduction was obtained on Sabauroud and Richard agar media at 28°C. Scanning electron microscopy revealed interaction between *S.cerevisiae* and both fungi. It was observed that the biocontrol agent reduced, lysed and separated host hyphae of both fungi. Application of biocontrol agent reduced disease percentage, improved root lengths and fresh and dry weights of tomato and eggplants seedlings in both laboratory and pot experiments, increased the naturally produced content of NPK elements. When the electrophoretic pattern of the two fungal sub-cultures with and without the bicontrol agent were studied, it was found that inoculation with biocontrol agent led to the production of an altered immune protein and on increase in total soluble protein, total carbohydrate, peroxidase and acid phosphatase isozyme content compared with controls.

KEYWORDS: Root rot disease, *Saccharomy*ces *cerevisiae, Macrophomina phaseolina, Fusarium solani*, pathogenic fungi.

INTRODUCTION

Since the end of the Second World War man has gradually used more hazardous pesticides to solve problems with plant diseases or insects. Misuse of these highly toxic substances led to a great disturbance of natural biological balance. On the other hand, using chemical fertilizers also led to changes in soil microflora, plant physiology, soil texture and soil chemistry. All these factors resulted in highly susceptible plants and the appearance of new aggressive races of pathogen, and new pests due to the great reduction in their natural enemies. The most serious result was the great increase in concentrations of toxic substances in the human food chain.

Biological control is promoting plant growth and resistance of plant pathogens by means of microbial agents or rhizospheric microorganisms may involve direct or indirect effects on the pathogen. Direct mechanisms include competition with the pathogen for nutrients, whereas indirect influences could involve altered plant defense responses or induction of systemic resistance. Biological control when used properly is much cheaper in cost, more durable and safer for application and user (Abd-EL Moity 1998). For all these reasons we have been investigating biological control as an effective mean to control plant disease since 1969.

When biological control was used microbial agents are introduced into the Rhizosphere and improve plant growth by suppressing root pathogens are currently laboratory studied. *Saccharomyces cerevisiae* was used as biocontrol agent and systemic resistance mechanisms which are associated with increasing certain enzymes such as peroxidase, chitinase and pathogensis-related protein which are often located in intercellular spaces and are acid soluble, were activated by using an isolate of *Saccharomy*ces *cerevisiae,* (El-Sayed 2000).

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In our work, root rot disease caused by *Fusarium solani* and *Macrophomina phaseolina* is one of the most important causes of disease in Solonaceae especially in tomatoes and eggplants. Heavy crop losses due to pre- and post- emergence killing of seedlings, were biologically controlled using a local strain of *Saccharomy*ces *cerevisiae* to explore the possibility of using this microbial model system in biological suppression of both pathogens.

MATERIAL AND METHODS

Microorganisms and Seeds Source: Phytopathogenic strains of *Macrophomina ph*aseolina, *Fusarium solani* and a local strain of *Saccharomy*ces *cerevisiae* were obtained from Agricultural research center, Ministry of Agriculture, Giza. Tomato seeds, *Lycopersicon esculentum* Mill, Cultivar, castle rock, and eggplants seeds, *solanum melongena,* cultivar, black beauty were obtained from Agriculture research center Ministry of Agriculture, Giza.

Culture Media: are Czapek-Dox's agar (Thom & Raper 1954): Sucrose, 30.0; sodium nitrate, 3.0; KH_2 PO_4, 1.0; $MgSO_4$. $7H_2O$, 0.5; potassium chloride, 0.5; $FeSO_4$. $7H_2O$, 0.01; agar, 20.0 and distilled water, 1000ml. Potato-dextrose agar (Booth 1971) (g/L): peeled potato slices, 200.0; glucose, 20.0; agar, 20.0 and distilled water, 1000 ml. Richard's agar (Riker & Riker 1936) (g/L): Sucrose 50.0; Sodium nitrate, 10.0; KH_2 PO_4, 5.0, $MgSO_4$. $7H_2O$, 2.5 agar, 20.0 and dist. H_2O 1000ml. Sabauroud (Atlas & Parks 1993): glucose 20.0, peptone 10.0, agar 20.0 and dist. H_2O 1000ml.

Growth Parameters of Tested Fungi: The effect of different culture media on the growth of *Fusarium solani* and *Macrophomina phaseolina* in the presence and absence of *Saccharomy*ces *cerevisiae* was studied. Radial growth (cm) of both fungi was measured when 0.5 cm inoculum of each were subcultured using four specified media namely Gzapek-Dox, potato dextrose, Richard and Sabauroud at $28 \pm 1\ ^oC$. The radial growth was measured for the six days, and results compared with control (absence of *S.cerevisiae*), the reduction potential percentage was recorded. Interactions between *S.cerevisiae* and both fungi were estimated and photographed after four days incubation using scanning electron microscopy when 1:25ml (V/V) of 10^3 conc. of liquid culture of the biocontrol agent was applied per petridish. Previous technique was repeated using *S. cerevisiae* compared with three fungicides in action

Laboratory experiment: Tomato seeds were infected with $1x10^6$ spores/ml suspension of *Fusarium solani* for 24 h. and eggplant seeds with $1x10^6$ spores /ml suspension of *Macrophomina phaseolina* for 24h. The seeds used were first sterilized with 3% sodium hypochlorite. After air-drying for 24h. tomato seeds and eggplant seeds were each divided into five groups and germinated by the following treatments: the first group of seeds were treated by spore suspension alone (F and M groups), the second were treated by spore suspension and then by *Saccharomy*ces *cerevisiae* liquid culture medium (SF). The third were treated (SF and SM groups) at a concentration of 10^3 liquid culture of *S. cerevisiae* twice a week, and the fourth group of seeds were left without any as a control, (C) group. All were germinated in 15cm petri dishes (20 seeds per dish) and left to grow for 3 weeks with four replicates each, then transferred into growth chamber under 12 hr Light/day at 30 ± 2^oC.

Pot experiment: Test plants were grown in plastic pots with a diameter of 25cm. The pots were sterilized with 0.5% formaline solution, dried and packed with sandy soil. The treated seeds as previously mentioned for both plants were sown in each pot, irrigated regularly and left to grow in a constant environment (temp. 30 ± 2^oC and pH= 6.5 approx.). After three weeks, observations were made of seedling length, fresh and dry weight, percentage of infection and the content of the nutrients N, P and K.

Determination of Certain Metabolites: Pathogenesis total protein content of both fungal liquid subcultures which were inoculated with the biocontrol agent was determined using Sabauroud stationary liquid medium at 28$\pm$1^{o}C, incubated for 10 days, filtered, then determined according to the method of Lowery *et al*. (1951). Fungal carbohydrate content was estimated when both fungi were grown as previously mentioned, filtered then centrifuged at 10.000 rpm. for 15 minutes. The supernatant was used to determine carbohydrate content by the method of Said and Naguib (1964). 100 ml of each fungal filtrate (both fungi previously grown in liquid sabauroud medium for 10 days at 28$\pm$1 ^{o}C) were centrifuged at 15000 rpm for 30 minutes and the supernatant used for peroxidase assay according to the method of Lee and Liu (1996) in a reaction mixture containing 50mM. potassium phosphate buffer (6.8).

SDS-PAGE electrophoresis: (50mM Tris-HCl pH 7.2, 2mM EDTA, 1% mercaptoethanol, 2% Na dodecyl sulphate (SDS) and 5% glycerol boiled for 5 minutes and centrifuged at 10.000g for 15 minutes. SDS-PAGE was done according to the method of Laemmli (1997) for protein, peroxidase and acid phosphates with 12% polyacrylamide gel, was stained with Coomassi blue R-250 and destained with 5% MeOH/acetic acid mixture. The lanes on the gel were photographed and drawn.

Visualization of Protein and isozymes: Reactions and Visualization of protein, peroxidase and acid phosphatase isozymes were performed according to (Hussien & Stegemann 1978; Scandalious 1964 and Windle & Weeden 1989).

Statistical analysis: The "F" value was calculated in all cases, significance measured at $P \leq 0.01$.

RESULTS

The results revealed that sabauroud medium followed by Richard medium were generally the best for maximum reduction potential (Tables 1&2). The two media may fail to supply *M. phaseolina* and *F. solani* with their optimum essential nutritional demands. The reduction potential was increased after 6 days by the following percentages: 77.42, 78.57, 65.00 and 74.29% using Richard and sabauroud medium for *M. phaseolina* and *F. solani* respectively.

The interaction between *S. cerevisiae* and both fungi was studied using scanning electron microscope technique after 4 days of inoculation. The spores *of S. cerevisiae* grew towards *M. phaseolina* (Fig.1C) and *F. solani* (Fig. 1D) and many of host fungal hyphae were shown to distorted and lysed.

Tables 3 and 4 illustrate the effect of different fungicides on radial growth (cm) of *M. phaseolina* and *F. solani* and reduction potential (%) after 6 days of growth on Sabauroud medium at 28$\pm$1^{5}C. Dithane (M45) caused maximum inhibitory effect on radial growth of both fungi and maximum reduction potential percentage followed by Topsin M then Rizolex T (85.71, 64.29 and 71.43%) using Sabauroud medium for *M. phaseolina* respectively, and 88.57, 78.57 and 81.43% for *F. solani* .

Tables 5 and 6 show the effect of *S. cerevisiae* on disease, growth parmeters and on NPK content of tomato and eggplant infected seedlings in the pots experiment and the lobaratory experiment. The data show a significant decrease in infection percentage when *S. cerevisiae* was applied, 35.00 and 15.00% for *F. solani* from laboratory and pots experiments respectively, compared with 95.00 and 55.67% in the absence of *Saccharomyces* biocontroller, 40.85 and 27.82% for *M. phaseolina*-comparing with 86.00 and 67.96% for them respectively .

The effect of inoculation with *S. cerevisiae* on total carbohydrates, total soluble protein and peroxidase activity in liquid culture media of both pathogenic fungi was recorded (Table 7). Maximum peroxidase activity (umole H_2O_2/ ml) was obtained by *S. cerevisie* (60.00), followed by mixing inoculation of *S. cerevisie* and *M. phaseolina* (48.80), or *S. cerevisiae* and *F. solani* (48.00).

The electrophoretic protein banding patterns were shown in the five isolates, they exhibited a maximum number of eight bands (Fig. 2), while peroxidase isozyme electrophoretic patterns exhibited three bands (Fig. 3). Furthermore, Acid phosphatase isozyme was electrophoritically patterned for the five isolates (Fig. 4), a maximum of five bands were detected, but not necessarily in all the isolates.

Table (1): Effect of different culture media on growth of *M. phaseolina* in presence and absence of the biocentroller (*S. cerevisiae)*.

Culture media		Radial growth (cm) of *M. phaseolina*/incubation period (days)					
		1	2	3	4	5	6
Czapek-Dox	Control	0.5	1.5	2.5	3.4	5.0	6.5
	Treated	0.5	1.2	2.0	2.6	3.9	5.0
	Reduction Potential (%)	0.0	20.0	20.0	23.53	22.0	23.08
	Control	1.0	1.6	2.3	3.6	4.8	7.0
Potato dextrose	Treated	1.0	1.2	1.2	1.9	2.3	3.5
	Reduction Potential (%)	0.0	25.0	47.82	50.0	52.08	57.14
	Control	0.6	0.6	2.6	3.5	4.4	7.0
	Treated	0.5	0.5	1.5	1.2	1.4	1.4
Richard	Reduction Potential (%)	16.67	16.67	42.3	65.71	68.18	77.42
	Control	1.6	1.7	2.7	3.4	4.6	7.0
	Treated	1.5	1.5	1.0	1.5	1.5	1.5
	Reduction Potential (%)	6.25	11.76	62.96	44.12	50.0	78.57
Sabauroud							

Table (2): Effect of different culture media on growth of *F. solani* in presence and absence of the biocentroller (*S. cerevisiae)*.

Culture media		Radial growth (cm) of *F. solani* /incubation period (days)					
		1	2	3	4	5	6
Czapek-Dox	Control	0.5	0.5	0.5	1.6	2.4	3.5
	Treated	0.5	0.5	0.5	1.0	1.3	1.3
	Reduction Potential (%)	0.0	0.0	37.5	37.5	45.83	62.86
	Control	1.0	1.2	1.9	2.6	3.0	3.7
Potato dextrose	Treated	0.9	0.9	1.1	1.5	1.7	1.7
	Reduction Potential (%)	10.0	25.0	42.11	42.31	43.33	54.05
	Control	0.9	1.4	2.0	2.7	3.3	4.0
	Treated	0.5	0.7	1.0	1.2	1.4	1.4
Richard	Reduction Potential (%)	44.44	50.0	50.0	55.55	57.57	65.0
	Control	0.8	1.6	2.2	3.1	4.0	7.0
	Treated	0.6	1.0	1.3	1.5	1.8	1.8
	Reduction Potential (%)	25.0	37.5	40.9	51.61	55.0	74.29
Sabauroud							

F value (treatments) = 61.4, $P \leq 0.01$ (Highly significant); F value (media) = 1.167; $P \geq 0.05$ (Non significant); F value (Fungi) = 20.3 $P \leq 0.01$ (Highly significant).

Table (3): Effect of different concentrations (ppm) of different fungicides on radial growth (cm) of *M. phoseolina* and reduction potential (%) on sabauroud medium after 6 days growth period.

	Radial growth (cm)	Reduction potential (%)	Topsin M conc. (ppm)	Radial growth (cm)	Reduction potential (%)	Rizolex-T con. (ppm)	Radial growth (cm)	Reduction potential (%)
Control	7.0	---	Control	7.0	---	Control	7.0	---
5	6.0	14.28	5	6.5	7.14	5	6.3	10.00
10	6.0	14.28	10	6.5	7.14	10	6.3	10.00
20	6.0	14.28	20	6.2	11.43	20	5.01	28.57
50	4.2	40.00	50	5.0	28.57	50	3.31	52.86
100	1.0	85.71	100	2.5	64.29	100	2.0	---

Table (4): Effect of different concentrations (ppm) of different fungicides on radial growth (cm) of *F. solani* and reduction potential (%) after 6 days growth on sabauroud medium.

Dithane (M45) conc. (ppm)	Radial growth (cm)	Reduction potential (%)	Topsin M conc. (ppm)	Radial growth (cm)	Reduction potential (%)	Rizolex-T con. (ppm)	Radial growth (cm)	Reduction potential (%)
Control	7.0	---	Control	7.0	---	Control	7.0	---
5	5.7	18.57	5	5.5	21.42	5	5.2	25.71
10	5.7	18.57	10	5.5	21.42	10	5.2	25.71
20	5.2	25.71	20	5.5	21.42	20	5.2	25.71
50	2.0	71.43	50	2.8	60.00	50	2.2	68.57
100	0.8	88.57	100	1.5	78.57	100	1.3	81.43

F value (treatments) = 87..90 , $P \leq 0.01$ (Highly significant); F value (concentrations) = 0.248, $P \leq 0.01$ (Highly significant); (Non significant); F value (Fungi) = 586.19, $P \leq 0.01$ (Highly significant).

Table (5): Effect of *S. cerevisiae* application on disease parameter growth, N, P and K contents (%) of Tomato seedling (3 weeks old), infected by 1×10^6 spores/ml suspension of *F. solani.*
F= *F. solani*; SF= *F. solani*+*S. cerevisiae*.; C = Control (untreated); *= Post experiment.

Treatments	% infection	Seedling high (cm)	Fresh wt. (g seeding-1)	Dry wt. (g seeding-1)	N (%)	P (%)	K (%)
F	95.00	10.5	0.45	0.037	3.20	0.51	1.5
SF	35.00	12.3	0.69	0.090	4.09	1.42	2.9
C*	---	12.0	0.66	0.090	3.96	1.00	2.2
F*	55.67	14.6	0.50	0.057	3.80	0.60	1.7
SF*	15.00	15.8	0.54	0.159	5.00	1.53	3.0
C*	---	15.0	0.53	0.150	1.00	1.10	2.4

Table (6): Effect of *S. cerevisiae* application on disease parameter growth, N, P and K contents (%) of Eggplants (3 weeks old), infected by 1×10^6 of spores/ml suspension of *Macrophomina phaseolina*.
M = *F. solani*; SM = *M. phaseolina*+*S. cerevisiae*; C = Control; * = Post treatments

Treatments	% infection	Seedling high (cm)	Fresh wt. (g seeding-1)	Dry wt. (g seeding-1)	N (%)	P (%)	K (%)
M	86.00	9.3	0.49	0.052	2.10	0.67	1.70
SM	40.85	12.0	0.78	0.094	2.82	1.51	3.00
C	---	11.4	0.75	0.087	2.20	1.10	2.60
M*	67.96	11.0	0.58	0.089	2.63	0.75	1.90
SM*	27.82	13.1	0.63	0.160	2.89	1.68	3.40
C*	---	13.0	0.60	0.155	2.60	1.60	2.90

All statistical analysis used are: 2-way Anova with factors of cervisiae treatment (control, treated) and fungus treatment (control, *F. solani, M phaseolina*) showed significant difference with $P \leq 0.01$.

Table (7): Effect of inoculation with *S. cerevisiae* on the activities of peroxidose (µnol H_2O_2) carbohydrates (µmol glucose) and protein (µg/ml) contents produced by *M. phaseolina* and *F. solani*.

Treatments	Total carbohydrates	Total soluble protein	Paroxidase/h 100 ml culture
M. phaseolina	1.40	50	20.00
M. phaseolina+S. cerevisiae	1.45	200	48.80
F. solani	1.37	350	36.50
F. solani + S. cervisiae	1.44	500	48.00
S. cervisiae	1.47	700	60.00

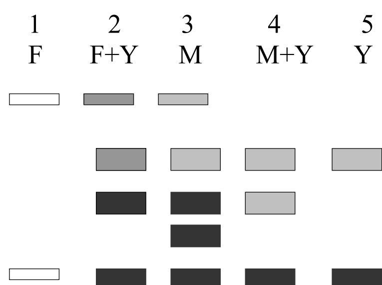

Fig. 4: Banding patterns of Acid phosphatase for the five isolates

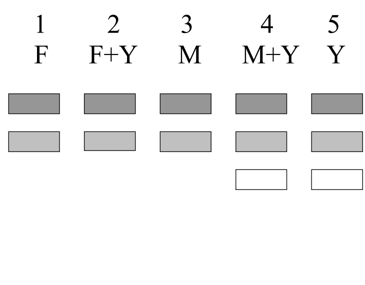

Fig. 3: Banding patterns of peroxidase for the five isolates

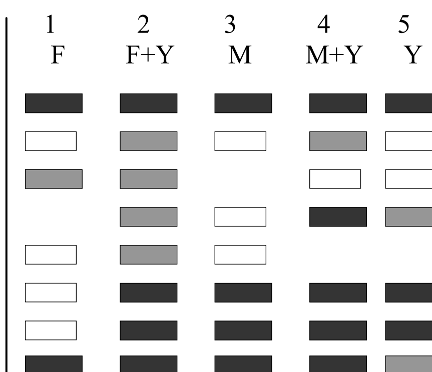

Fig. 2: SDS electrophoretic patterns of the five isolates. M= protein standard from top to bottom: 180, 150, 100, 75, 58, 35, 19 & 13 KD.

1, F= *Fusarium solani*; 2, F+Y= *Fusarium*+ yeast; 3, M= *Macrophomina*; 4, M+Y= *Macrophomina* + yeast

5, y= yeast; [dense box] = dense; [moderate box] = moderate; [faint box] = faint

DISCUSSION

Sabauroud medium gave the least reduction potential between S.*cerevisia*e strain and the two tested fungi after one day of incubation, compared with Zapek–Dox and potato dextrose (Malviya *et al.* 1994), it was found that *S. cerevisiae* was good producer of antifungal antibiotics and showed activity against *Macrphomina phaseolina* and *Fusarium* spp (Suzzi *et al.* 1995; Kalra & Sohi 1984).

Exclusive growth of the mycoparasitism towards its target is probably a chemotrophic response towards leaking or released substances from both fungal hyphae. Such response in filamentous fungi can only be seen microscopically for a single hyphae and not macroscopically for the whole colony, as demonstrated in motile microoganisms (Carlile 1975) and contact with the host hyphae (phytopathogens) by coiling.

Maxium reduction potential (%) was obtained when Dithan (M 45) fungicides was applied followed by Topsin M and Rizolex T against both fungi. By comparing these data with reduction potential percentage obtained by *S. cerevisiae,* it was found that reduction potential (%) obtained by Dithane (M45), against *M. phaseolina* and *F. solani* was directly followed by *Saccharomces* reduction percentage, 8.57 and 74.29% against both fungi respectively, followed by the other two fungicides (Topsin M and Rizolex T) values.

Application of *S. cerevisiae* showed significant improvement of seedlings high, fresh and dry weight and when NPK content was determined, it was noticed that content percentage increased when *Saccharormyces* was applied in both laboratory and pots experiments. Data were found to be 4.09,1.42 and 2.9% of N, P and K respectively for tomato seedlings infected

with *F. solani*, 2.82, 1.51 and 3.00% of N, P and K content, for tomato seedlings infected with *M. phaseolina.* Pots experiments determinations showed NPK content of 5.0, 1.53 and 3.0% for seedlings infected with *Fusarium* and 2.89, 1.68 and 3.40% for seedling infected with *Macrophomina*, similar data were obtained for eggplants treatments.

Benhamou and Lafontaine (1995) found that restriction of fungal growth into host tissues, decrease in pathogen viability and formation of numerous apposition at sites of attempted penetration were the main features of the host- pathogen interaction in tomato root tissues from treated plants infected by *Fusarium oxysporum*. Their studies revealed that pectin and phenolic compounds play an important role in this way and the key of defense mechanism in relation to the possibility that an alarm signal provided by the pathogen itself is required for the expression of resistance in these plants.

Total carbohydrate (µmole glucose/ml) was found to be 1.47, 1.45 and 1.44 for them respectively, and the increase in carbohydrates content with *Saccharomyces* inoculation was in agreement with results obtained by Zaworski & Gill (1999). Also, total soluble protein (determinate as µg protein/ml liquid culture) was found to be 700, 200 and 500 respectively.

Different protein bands were obtained when protein was electrophoretically banding, band with 180 KD was found in all the isolates with same density and intensity and it was very dense. The second band with150 KD found in isolates 1, 3 and 5 having the same density and intensity and they were very faint, while isolate 2 and 4 were denser, isolate number 2 was the densest one. The third band with 100 KD was absent from isolate 3, the band with 75 KD was absent from isolate 1 and found in isolates 2, 3, 4 and 5 with different densities and intensities. The band with 58 KD was absent from isolates 4 and 5, while it was found in isolates 1, 2 and 3 with different densities and intensities. On the other hand, bands with 35, 19 and 13 KD are present in all the isolates, mainly with same density and intensity (very dense), except isolate 1 which was very faint for both 35 and 19 KD, also isolate 5 was less dense.

In peroxidase isozyme patterns, bands number 1 and 2 appear in the five isolates with the same density and intensity (band number 1 was denser than number 2). The third band is present in isolate 4 and 5 only (very faint). It supports lignification (Fielding & Hall 1978), wound-healing (Kawashima & Vritini 1963) and disease- resistance (Johnson & Cunningham 1972) and so it is potentially of considerable importance.

Acid phosphatase electrophoretic patterns were maximum of five bands, number one was found in isolates 1, 2 and 3 with different densities and intensities, while band number 2 was absent in isolate number 1 only and found in the isolates 2, 3, 4,and 5. On the other, the third band was found in isolate 2, 3 and 4 only with different densities and intensities while band 4 was found in isolate 3 only and was absent in all the other isolates. Finally, band number 5 found in all isolates with the same densities and intensities (very dense) except isolate 1 was very faint. Phosphatase catalysis plays a vital role in regulations of the plant cell metabolism through inorganic phosphorus level (Tsuboi *et al.* 1957). It has been demonstrated that carbohydrate metabolism of plants may be indirectly controlled by availability of inorganic phosphate which in turn is regulated by acid phosphatase activity level and this affect plant response against pathogen. Murray & Collier (1987) have shown that a specific isoenzyme of acid phosphatase present in seed coats of *Pisum* seeds regulates the movement of phosphorus from seed coat to developing embryos.

Maximum activation on total carbohydrate production, total soluble protein and peroxidae was also obtained when *S. cerevisiae* was inoculated, peroxidase expressed as H_2O_2 activates defense related genes, our results were in agreement with results obtained by Graham &Graham (1999); Busch & Lelley (2000).

Finally, the protection of tomato and eggplants against plant pathogenic fungi obtained from *Saccharamyces cerevisiae* activates defense related genes and produce newly

immune type pathogenesis-related protein. Apparently the protection may be the direct action of the yeast on the pathogen (biological control) as well as by inducing resistance in the host plants (Kamida *et al.* 2000).

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Gel electrophorasis