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RESEARCH ARTICLE

Potential of antagonistic yeast strains as biocontrol agents against root rot disease in tomato

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Abstract

The present study was conducted to evaluate the efficacy of the sixty plant growth promoting yeasts isolated from rhizosphere of healthy plants were tested for controlling tomato root rot disease caused by *Fusarium solani*, *Rhizoctonia solani* and *Pythium aphanidermatum* under greenhouse conditions. Their growth promoting activities on tomato were also tested. The bioagents were applied as seed dressing and as soil drenching treatments. All bioagent treatments were significantly reduced disease severity of tomato root rot relative to the infested control plants inoculated with the pathogen only. The highest survival rates in pots (82.6 %), (84.1 %) and (82.6 %) were achieved by Y1, Y2 and Y3 respectively compared to infested control. The highest root dry weights in pots (0.3657 g/plant), (0.3455 g/plant) and (0.3946 g/plant) were achieved by Y1, Y2 and Y3 respectively compared to infested control. The most potent three bioagents with high antagonistic activity against phytopathogens were identified using phenotypic and genotypic techniques as *Candida tropicalis* (Castellani) Berkhout (Y1), *Pichia caribaea* Phaff (Y2) and *Geotrichum candidum* Link (Y3). These three bioagents were tested for lytic activity and the results revealed that *Candida tropicalis*, *Pichia caribaea* and *Geotrichum candidum* showed high chitinolytic activity while only *Geotrichum candidum* showed proteolytic activity and non of them showed phospholipolytic activity. The results indicate that application of *Candida tropicalis*, *Pichia caribaea* and *Geotrichum candidum* have potential as plant promoters and may be useful for biocontrolling soil-borne fungal plant pathogens causing root rot disease of tomato in the field. The use of biological agents to control soil plant pathogenic fungi is an attractive possibility. Successful application of the antagonistic yeast bioagents could become promoting natural antimicrobial agents, may be provide a protection against root rot plant fungal pathogens and may be useful as an eco-friendly manner and sustainable agriculture.

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1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important vegetable crops all over the world. In Egypt, tomato can be cultivated in different seasons such as winter, summer and Nily; but it is subjected to the attack by many soil borne pathogens causing damping-off, root rot and wilt diseases (Nguyen *et al.*, 2011). *Fusarium* spp., *Rhizoctonia solani* and *Pythium* spp. are the most popular pathogens causing severe yield losses of tomato all over the world and disease control is difficult (Bokhari and Perveen, 2012).

Although disease resistant varieties are available, their resistance is often incomplete. Chemical control may be not suitable due to high variability of the pathogen and due to high cost and environmental risks associated with application. Today, there are strict regulations on chemical pesticides use and furthermore, new races of the

pathogen that overcome plant resistance has continued to appear (Wydra and Semrau, 2006). There is political pressure to remove the hazardous chemicals from agriculture. A promising strategy for replacement of the chemicals has been the application of biological control technology for controlling pests (Lemessa and Zeller, 2007). Biological control would be highly preferred for disease control in the future. The application of biological controls using antagonistic microorganisms has proved to be successful for controlling various plant diseases in many countries (Nguyen *et al.*, 2011; Abd El-Kader *et al.*, 2012). Various actinomycetes, fungi and bacteria such as *Bacillus subtilis*, *B. megaterium*, *B. mycoides*, *Pseudomonas putida*, *P. fluorescence*, *Enterococcus* spp., *Azospirillum* spp. and *Trichoderma* spp. have been reported as active biological control agents against phytopathogens (Nguyen *et al.*, 2011; Abd El-Kader *et al.*, 2012; Dawoud *et al.*, 2012).

A variety of yeast genera have been used extensively for the biological control of postharvest diseases of fruits and vegetables to protect moulding of stored grains (Petersson *et al.*, 1999) and to control foliar diseases such as powdery mildews (Urquhart and Punja, 1997). The three yeasts *Candida valida*, *Rhodotorula glutinis* and *Trichosporon asahii* were demonstrated to be effective biocontrol agents of post-emergence damping off of seedlings, crown and root rots of mature sugar beet, caused by *R. solani* AG-2-2 (El-Tarabily, 2004).

Various yeast species have been reported as active biological control agents. Abd El-kader *et al.* (2012) showed that some bacterial isolates and *Saccharomyces cerevisiae* as bioagents were effective against root rot pathogens under greenhouse conditions. Root rot incidence, at pre-emergence stage, significant reduction effect was observed in bioagent treatments. *Candida glabrata*, *C. maltosa*, *C. slooffii* and *Rhodotorula rubra* significantly reduced the incidence of late wilt disease when applied by seed inoculation (El-Mehalawy *et al.*, 2004). *Pichia guilliermondii* and *Candida ethanolica* gave encouraging results in the suppression of the bacterial wilt pathogen (Nguyen and Ranamukhaarachchi, 2010). Nguyen *et al.* (2011) evaluated four antagonists *Bacillus megaterium*, *Enterobacter cloacae*, *Pichia guilliermondii* and *Candida ethanolica*, and found significantly antagonistic effect to *Ralstonia solanacearum* *in vivo* and hence possess potential to control wilt disease in tomato.

Therefore, the current study was conducted to evaluate the activity of some plant growth promoting and antagonistic yeasts isolated from rhizosphere against tomato root rot incidence and for growth promoting activity when applied as seed dressing and soil drenching treatments under greenhouse conditions.

Experimental Procedures

2. Materials

2.1. Source of bioagent yeast isolates

The antagonistic yeast isolates were isolated from rhizosphere of healthy tomato, wheat, barley, onion, garlic, bean, beetroot, sunflower, fennel and alfalfa plants which collected from different agricultural locations, such as El-Fayoum, Sanoos, Abshway and Tamyia centers of El-Fayoum governorate of Egypt (Kamel *et al.*, 2013). The most active three yeast bioagents proved their antagonistic ability against pathogenic fungi were identified by phenotypic methods and by API ID32C system (bioMérieux, France) as *Candida tropicalis* (Castellani) Berkhout (Y1), *Pichia caribaea* Phaff (Y2) and *Geotrichum candidum* Link (Y3). These isolates also produced growth promoting substances (IAA, GA, HCN and siderophores) (Kamel *et al.*, 2013).

2.2. Phytopathogenic root rot fungi

The root rot fungi *Fusarium* sp., *Rhizoctonia* sp., *Pythium* sp. and *Sclerotium* sp. were the major soil-borne pathogens isolated from tomato plants showing wilt, damping-off and root rot diseases which collected from different agricultural locations in Egypt. The isolated fungi proved their ability to induce root rot disease of tomato (Kamel *et al.*, 2013).

2.3. Tomato variety

Seeds of tomato (*Lycopersicon esculentum* Mill.) cultivar Super badr were kindly provided by the Vegetable Research Institute, Agricultural Research Center, Ministry of Agriculture, Dokki, Giza, Egypt were used in this study.

2.4. Soil analysis

A representative sample of a mixture of clay and sand soils (1:1 w/w) was collected from Giza farm of Agric. Res. Center. The collected soil for pot experiment was air dried, crushed and prepared to physical and chemical properties determinations according to methods described by Piper (1950) and Page *et al.* (1982). The physical and chemical analysis of soil used in testing antagonistic activities of efficient yeast isolates against root rot fungi *in vivo* are given in Table 1.

Table 1. Some physical and chemical properties of the soil used for testing the antagonistic activities of the efficient yeast isolates.

Partical size distribution %				Texture	EC ds/m	pH	SP	Cation (meq/l)				Anion (meq/l)			
Clay	Silt	F.sand	C.sand					Ca ⁺⁺	Mg ⁺⁺	Na ⁺	K ⁺	Co ₃ ⁻⁻	HCo ₃ ⁻	Cl ⁻	So ₄ ⁻⁻
16.2	18.7	36.5	28.6	Sandy loam	1.4	7.6	40	8.5	1.1	4.0	0.21	0.0	1.5	7	5.31

2.5. Fertilizers

Ammonium sulphate, superphosphate and potassium sulphate were used as nitrogen, phosphorus and potassium fertilizers respectively.

2.6. Culture media and supplements

All media used in this study were made up in deionized water and sterilized by autoclaving at 15 psi and 121 °C for 15 minutes.

2.6.1. Potato Dextrose Agar medium (PDA) (Difco, 1984): is used for the isolation, purification and maintenance of fungal cultures.

2.6.2. Yeast Malt Agar medium (YMA) (Difco, 1984): is used for the isolation, purification and maintenance of yeast cultures.

2.6.3. Chitin Agar medium (Agrawal and Kotasthane, 2009): is used for qualitative analysis of chitinolytic activity of yeast isolates.

2.6.4. Casein Soluble Agar medium (Hameed, 1984): is used for qualitative analysis of proteolytic activity of yeast isolates.

2.6.5. Sabouraud Dextrose Agar medium (SDA) (Difco, 1984): is used for screening the antagonistic activity of yeast isolates in dual culture and for qualitative analysis of phospholipolytic activity of yeast isolates.

3. Methods

3.1. Preparation of root rot fungi; *Fusarium solani*, *Rhizoctonia solani* and *Pythium aphanidermatum* inocula for soil pre-infestation

Barley meal sand medium containing 75 g washed air-dried boiled barley grains, 25 g washed dried coarse sand, 2 g sucrose and 0.1 g yeast extract per 40 ml tap water in polyethylene transparent bags was autoclaved at 121 °C and 1.5 bars for 30 min. After cooling, the grains were inoculated aseptically with uniform two 10-mm diameter discs of potato dextrose agar colonized by 7-days-old fungal growth mycelia of *F. solani*, *R. solani* and *P. aphanidermatum* separately and incubated at 25 °C for 2 weeks to obtain sufficient growth of the fungal isolates. The bags were shaken daily to avoid lumping of the grains caused by aerial growth of the mycelium. The grains, completely colonized with the fungus were further used in soil infestation.

3.2. Preparation of biocontrol agents inoculum

Bioagent yeast isolates were separately grown on 50 ml aliquots of YM broth medium in 250 ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker at 25 °C and 150 rpm for 3 days. The counted numbers of viable cells of fresh cultures at the time of use for inoculation were adjusted to 1×10^9 cells/ml using haemocytometer slide under Light microscope.

3.3. *In situ* screening for antagonistic activities of plant growth promoting yeast isolates against root rot fungi under greenhouse conditions

Yeast isolates antagonizing pathogenic fungi *in vitro* and also producing plant growth promoting substances were evaluated for their efficiencies to control root rot disease caused by *F. solani*, *R. solani* and *P. aphanidermatum* and also to promote tomato plant growth in pot experiments under greenhouse conditions.

For root rot disease evaluation, experiments were carried out in a sandy loam soil artificially infested with root rot pathogens inocula. Soil pre-infestation was achieved by mixing inoculum of each fungus with the soil at the rate of 5% (w:w) separately, by soil weight (Abd El-Kader, 1999), to ensure equal distribution of the added pathogenic fungal inocula. Infested soil (the upper 2 cm of soil) was then filled in plastic pots (10 cm, diameter) containing 500 g of sandy-loam soil, the soil planed to a uniform level and irrigated every second day for 1 week before sowing to obtain the optimum fungal growth and distributing of the pathogenic fungal growth before sowing.

The same amount of uninoculated autoclaved barley meal sand medium was added to the soil as mentioned before to serve as negative control treatment (check pots containing non-infested soil). Each treatment was represented by 3 replicates to study the effect of tested fungi on the incidence of pre- and post emergence damping off diseases. After pre-infestation period, each pot was sown by twenty-three seeds of tested plant cultivar and watered when needed.

In this experiment, both seed dressing and soil drenching with antagonist techniques were applied. For seed coating (dressing) with antagonist technique; tomato seeds (cv. Superbadr) were surface disinfected by immersing in sodium hypochlorite solution (2%) for 2 min, and washed several times with sterilized distilled water, then dried between two sterilized layers of filter paper. Yeast inoculum containing 1×10^9 cells/ml was added to petri plates. To this, 100 mg of carboxyl methyl cellulose (CMC) was added as adhesive material. Sterilized seeds were soaked in yeast suspension to adhesion of the cells to the seed coat. Then, the yeast suspension was drained off and the seeds were air-dried at room temperature in sterile petri plates and the antagonist coated seeds were immediately sown into previously infested soil. Untreated seeds were also sown in both the soil free of the pathogen (negative control) and the infested soil (positive control). In Rizolex-T (tolclofos-methyl) fungicide treatment, tomato seeds were soaked in this fungicide and left for 1-2 h to enable the seeds absorb the fungicide and after treatment, they were spread on trays and air-dried for 30 min before sowing immediately in the soil which infested with the pathogen alone. Soil drenching with antagonist technique: Antagonists were applied regularly up to 3 times at 15-days interval from sowing. To apply antagonists, suspension at a concentration of 1×10^9 cells/ml of each of the selected antagonists as biocontrol agents was used. Pots were kept under greenhouse conditions till the end of the experiment.

3.4. Disease severity assessments

Percentages of pre and post-emergence damping-off as well as survival plants and disease severity (DS) were estimated up to 45 days from planting as described by **Arafa (1985)** as follow:

Percentage of pre-emergence damping-off was determined after 15 days as:

$$\text{Pre-emergence damping-off (\%)} = \frac{\text{No. of ungerminated seeds / pot}}{\text{No. of sown seeds / pot}} \times 100$$

Percentage of post-emergence damping off was determined after 30 days as:

$$\text{Post-emergence damping-off (\%)} = \frac{\text{No. of died seedlings / pot}}{\text{Total No. of emerged seedlings / pot}} \times 100$$

Percentage of survival plants was determined after 45 days at the end of experiment as:

$$\text{Survival plants (\%)} = \frac{\text{No. of healthy un-infected plants with root-rot / pot}^*}{\text{Total No. of plants / pot}} \times 100$$

Survival or healthy plants* = (No. of sowing seeds - pre and post emerging damping off).

Plant fresh and dry weights were estimated also at the end of the experiment. Plants of three replicates from each treatment were uprooted, washed thoroughly with running water to remove any adherent particles, blotted with tissue paper, weighed to determine fresh weights, and then oven dried at 70°C for 72 h for dry weights.

3.5. Biochemical activities of yeast bioagents

3.5.1. Chitinolytic activity

Chitin agar plate method has been used according to **Agrawal and Kotasthane (2009)**. The chitinase detection medium was autoclaved at 121°C for 15 min. After cooling the medium was poured into petri plates and allowed to solidify. The fresh culture plugs of the yeast isolates to be tested for chitinase activity were inoculated into the colloidal chitin medium containing BCP (pH 4.7) and incubated at $25 \pm 2^\circ\text{C}$ for 3-4 days and observed for the colored zone formation. Chitinolytic microorganisms resulted in breakdown of chitin into N- acetyl glucosamine causing a corresponding shift in pH towards alkalinity and change of color of pH indicator dye BCP from yellow to purple zone surrounding the inoculated fresh culture plugs in the region of chitin utilization. Chitinase activity was identified due to the formation of purple colored zone. Color intensity and diameter of the purple colored zone were taken as the criteria to determine the chitinase activity after 4 days of incubation. The isolates were grouped according to the diameter of the purple zone as A) Isolates showing no chitinase activity B) Isolates showing low

chitinase activity C) Isolates showing medium chitinase activity D) Isolates showing high chitinase activity and E) Isolates showing highest chitinase activity.

3.5.2. Proteolytic activity

For qualitative analysis of proteolytic activity, casein soluble medium was used as described by **Hameed (1984)**. Inoculated plates containing the medium were incubated at 28°C for 5-7 days. Formation of clear zones around the colonies showed protease activity. These zone formations were enhanced by flooding the plates with 10% glacial acetic acid.

3.5.3. Phospholipolytic activity

Determination of extracellular phospholipase activity was performed essentially according to **Polak (1992)** using the semiquantitative egg-yolk agar plate method. The inoculated egg-yolk plates with yeast cultures were incubated at 32°C, and readings were taken daily from day 7 to day 14. Isolates that produced extracellular phospholipase showed a distinct, white, opaque zone (precipitate) below and around the colony. The production of phospholipase (P_z index) was expressed as a ratio of colony diameter to total diameter of the colonies plus the precipitation zone. There is an inverse relationship between index and enzyme activity: the smaller the index, the higher the enzyme activity of the isolate. The phospholipase activity was classified as very high ($P_z < 0.64$), high (P_z value of ≥ 0.64 and < 1), or null ($P_z = 1$), as reported by **Coutinho and Paula (2000)**. The P_z value represents an average of the three P_z values reported.

The precipitation zone was examined and measured as follow:

$$\text{Phospholipase activity (Pz value)} = \frac{\text{Diameter of the colony}}{\text{Diameter of the colony} + \text{precipitation zone}}$$

3.6. Identification and molecular characterization of the most potent antagonistic yeast bioagents

3.6.1. Phenotypic identification

The yeast isolates showed, in vitro; good antagonistic effects against the previous pathogens were previously identified as *Candida tropicalis* (Castellani) Berkhout, *Pichia caribaea* Phaff and *Geotrichum candidum* Link (**Kamel et al., 2013**). The identification of isolates were also confirmed using the API ID32C system (bioMérieux, France) to species level based on the traditional criteria.

3.6.2. Molecular characterization of yeast isolates

DNA extraction

Yeast isolates were individually grown on YM agar medium and incubated at 28°C for 3 days. A small amount of yeast growth was scraped and suspended in 100 μ l autoclaved distilled water in 2ml sterile vials and boiled at 100°C for 15 minutes.

For extraction of genomic DNA, one milliliter of the fresh cell culture was centrifuged at 14,000 xg for 3 min in a microcentrifuge and a commercial SolGent™ Genomic DNA extraction Kit (SolGent, Daejeon, South Korea) was used according to the manufacturer's instructions. The extracted DNA was stored at -20°C for further use.

Amplification of the internally transcribed spacer 1 (ITS1)-5.8 rRNA-ITS2 region

The entire (ITS1)-5.8 rRNA-(ITS2) region of the yeasts was amplified by using two fungus-specific universal primers; forward ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and reverse ITS4 (5' - TCC TCC GCT TAT TGA TAT GC - 3') (**Moore et al., 2011**).

Polymerase chain reaction (PCR) technique was carried out in a 25 μ l reaction volume using Solgent EF-Taq consisting of: 2.5 μ l of 10X EF-Taq buffer, 0.5 μ l dNTPs-Mix (10 mM), 1 μ l forward primer (10 pM), 1 μ l reverse primer (10 pM), 0.25 μ l EF-Taq DNA polymerase (2.5U), 1 μ l of DNA template (1-5 ng) and sterile double distilled water up to 25 μ l.

The amplification reaction was carried out in a PCR thermocycler using the following parameters: initial one round of denaturation (at 95°C for 15 sec) followed by 30 cycles of denaturation (at 95°C for 20 sec), annealing (at 50°C for 40 sec), extension (at 72°C for 1 min) and a final extension step (at 72°C for 5 min). A negative control was performed with each run by replacing the template DNA with sterile water in the PCR mixture.

For the separation and detection of PCR products according to their size, horizontal electrophoresis was performed using 1 % agarose gels containing 0.5 μ g/ml of ethidiumbromide at 8.5 V/cm. The PCR products were

mixed with 1:10 volume of 10x loading buffer (50 % (w/v) glycerol, 1 mM EDTA, trace bromophenol blue). The molecular weight markers Gene Ruler™ 100bp DNA Ladder Plus (MBI Fermentas, St. Leon-Rot) were used to evaluate the size of DNA fragments. The gel was visualized using the Gel Doc™ Imager system (BioRad).

Sequencing of the PCR products

All PCR products were then eluted from gel and purified using SolGent PCR purification Kit-Ultra (SolGent, Daejeon, South Korea) according to the manufacturer's instructions. The purified PCR products were sequenced by the company MacroGen (Seoul, South Korea) on both forward and reverse strands using primers ITS1 and ITS4, respectively (Moore *et al.*, 2011). For identifying yeasts, sequences were further analyzed by searching databases using the BLAST sequence analysis tool from the National Center of Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/BLAST/>). The ITS1 or ITS2 lengths and sequence for each isolate were compared to the proposed species using nucleotide-nucleotide BLAST (blastn). Phylogenetic analysis of sequence was done with the help of MegAlign (DNA Star) software version 5.05.

3.7. Statistical analysis

Statistical analysis of the obtained data was subjected to standard analysis of variance procedure. The values of LSD were calculated at 1% level according to the method described by Gomez and Gomez (1984).

Results

1. Suppression of tomato root rot disease by antagonistic growth promoting local yeast isolates under greenhouse conditions

The efficacy of soaked tomato seeds and soil drench with sixty antagonistic yeast bioagents against root rot incidence caused by three soil-borne pathogenic fungi was evaluated under greenhouse conditions. Non treated and infested soil served as control also soil infested with pathogens and treated with Rizolex-T fungicide was tested for comparison.

Disease severity

Results presented in Figures 1-3 show that seed coat and soil drench treatments with any of the tested yeast bioagents significantly reduced root rot disease severity caused by any of the three pathogenic fungi in tomato plants compared to control. The highest disease severity reduction was observed with isolates Y1 and Y2 in the presence of *F. solani* (82.6% and 76.8%) respectively, with isolates Y2 and Y3 in the presence of *R. solani* (84.1% and 72.5%) respectively and with isolates Y3 and Y4 in the presence of *P. aphanidermatum* (82.6% and 81.2%) respectively (Figures 4-6).

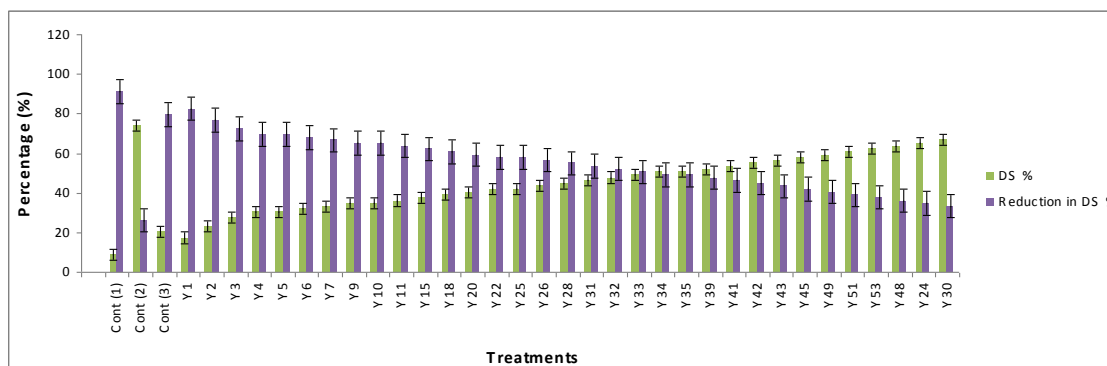


Fig. 1 Disease Severity (DS)% and Reduction in DS% of tomato seedlings as influenced by inoculation with selected yeast isolates in the presence of *F. solani*.

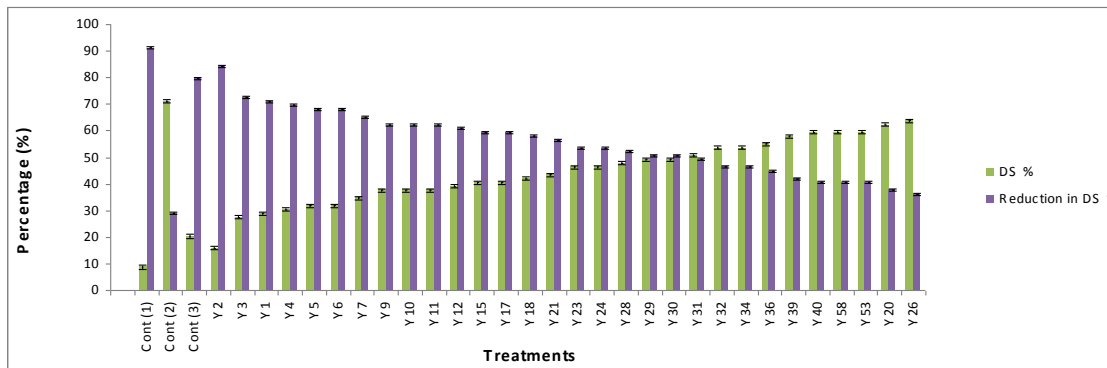


Fig. 2 Disease Severity (DS)% and Reduction in DS% of tomato seedlings as influenced by inoculation with selected yeast isolates in the presence of *R. solani*.

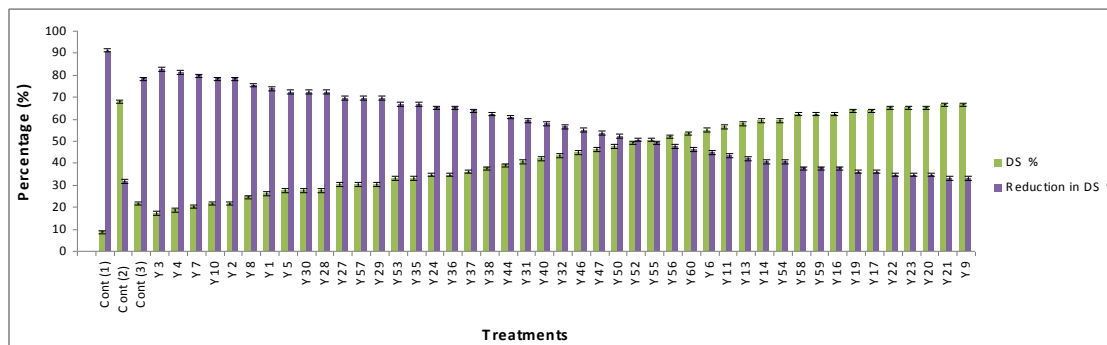


Fig. 3 Disease Severity (DS)% and Reduction in DS% of tomato seedlings as influenced by inoculation with selected yeast isolates in the presence of *P. aphanidermatum*.



Fig. 4 The efficiency of treatment with yeast isolate Y1 in the suppression of root rot disease caused by *F. solani* in tomato seedlings.
 A: Control (1): Un-inoculated soil, B: Control (2): Infested soil with *F. solani*, C: Control (3): Infested soil with *F. solani* and treated with Rizolex-T fungicide and D: Infested soil with *F. solani* and treated with yeast isolate Y1.

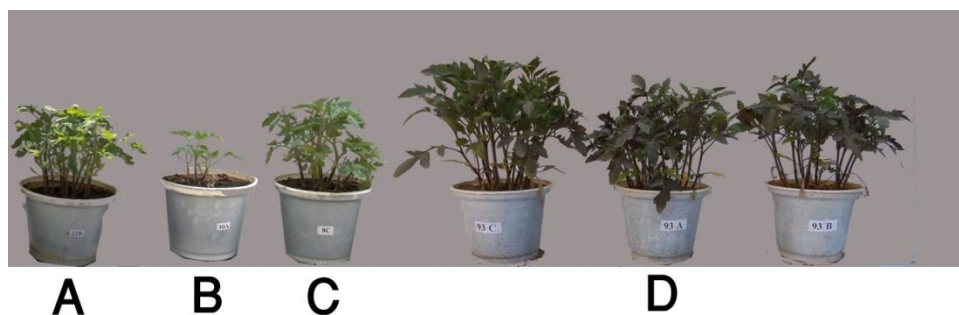


Fig. 5 The efficiency of treatment with yeast isolate Y2 in the suppression of root disease caused by *R. solani* in tomato seedlings.

A: Control (1): Un-inoculated soil, B: Control (2): Infested soil with *R. solani*, C: Control (3): Infested soil with *R. solani* and treated with Rizolex-T fungicide and D: Infested soil with *R. solani* and treated with yeast isolate Y2.

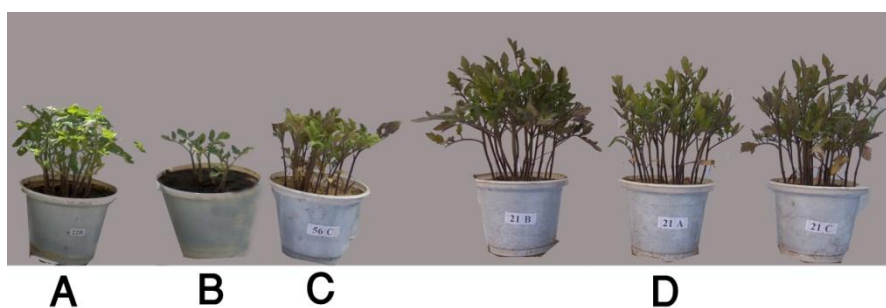


Fig. 6 The efficiency of treatment with yeast isolate Y3 in the suppression of root rot disease caused by *P. aphanidermatum* in tomato seedlings.

A: Control (1): Un-inoculated soil, B: Control (2): Infested soil with *P. aphanidermatum*, C: Control (3): Infested soil with *P. aphanidermatum* and treated with Rizolex-T fungicide and D: Infested soil with *P. aphanidermatum* and treated with yeast isolate Y3.

Pre-emergence damping-off disease

The incidence of pre and post-emergence root rot and survival rate are presented in Tables 2-4. Data show that most of the applied yeast bioagent treatments as seed coat and soil drench caused a high significant effect on root rot incidence compared with control which includes pathogen alone.

At pre-emergence stage, data in Tables 2-4 show that the recorded percentage of root rot in seed coat and soil drench treatments with yeast isolates Y1, Y2 and Y3 ranged between 11.6 – 20.3% compared with control treatment as 43.5% in soil infested with *F. solani*. The recorded percentage of pre-emergence in bioagent treatments ranged between 11.6 – 23.2% with yeast isolates Y1, Y2 and Y3 comparing with control treatment as 37.7% in soil infested with *R. solani*. The antagonistic isolate Y4 showed a superior significant effect against root rot in the presence of *P. aphanidermatum* (0.0%) which reflected in complete reduction of pre-emergence root rot incidence comparing to 42.0% in control while bioagent Y3 showed high protective effect with 5.8% root rot incidence.

Post-emergence damping-off disease

Seed coat and soil drenched with different bioagents showed more potential for reducing root rot incidence at post-emergence growth stage of tomato. Presented data in Tables 2-4 revealed that percentage of root rot incidence significantly reduced as 5.8%, 7.2% and 8.7% in treatments with bioagents Y1, Y3 and Y2 respectively compared with 30.4% in control soil infested with *F. solani* only.

Similar observations were also recorded in soil drench treatments with any of the bioagents tested and in soil infested with *R. solani*. Data in Table 3 showed that treatment with bioagent Y3 caused the highest significant reduction in root rot incidence at post-emergence stage (2.9%) followed by Y2 (4.3%) and Y1 (5.8%) compared with control treatment (33.3 %).

In soil infested with the pathogen *P. aphanidermatum*, all antagonistic treatments showed significant protective effect to reduce disease incidence at post-emergence stage. Many of the antagonistic yeast isolates showed superior significant effect against the invasion by *P. aphanidermatum*. The reduction in root rot incidence

reached 2.9% in treatments with antagonistic Y1, Y44, Y11, Y13, Y14, Y54, Y23 and Y9 compared to 26.1 % in control (Table 4).

Survival rate

Data in Tables 2-4 revealed that tomato plant treatments with yeast bioagents improved the percentage of survival plants and caused high significant increase from 26.1% in control soil infested with *F. solani* to 82.6% in treatment with bioagent Y1. Similarly the percentage of survival plants increased from 29.0% in control to 84.1% and 72.5% by treatments with bioagents Y2 and Y3 respectively in soil infested by *R. solani*. The survival rate increased from 31.9% in control soil infested by *P. aphanidermatum* to 82.6% and 81.2% in treatments with bioagents Y3 and Y4 respectively.

Table 2. Pre-, post-emergence damping-off disease (%) and survival rate (%) of tomato seedlings as influenced by inoculation with selected yeast isolates in the presence of *F. solani*.

Treatments	Pre-emergence damping-off (%)	Post-emergence damping-off (%)	Survival rate (%)
Control (1)	8.7 ^{**}	0.0	91.3
Control (2)	43.5	30.4	26.1 ^{**}
Control (3)	17.4	2.9 ^{**}	79.7 [*]
Yeast isolates + <i>F. solani</i>	Survival rate over 70%		
Y1	11.6	5.8	82.6
Y2	14.5	8.7	76.8 ^{**}
Y3	20.3	7.2	72.5 ^{**}
	Survival rate over 50% and less than 70%		
Y4	24.6	5.8	69.6 ^{**}
Y5	26.1	4.3 ^{**}	69.6 ^{**}
Y6	14.5	17.4	68.1
Y7	30.4	2.9 ^{**}	66.7 ^{**}
Y9	31.9	2.9 ^{**}	65.2 ^{**}
Y10	34.8	0.0	65.2 ^{**}
Y11	29.0	7.2	63.8 ^{**}
Y15	29.0	8.7	62.3 ^{**}
Y18	30.4	8.7	60.9 ^{**}
Y20	34.8	5.8	59.4 ^{**}
Y22	39.1	2.9 ^{**}	58.0 ^{**}
Y25	29.0	13.0	58.0 ^{**}
Y26	31.9	11.6	56.5 ^{**}
Y28	26.1	18.8	55.1 ^{**}
Y31	31.9	14.5	53.6 ^{**}
Y32	36.2	11.6	52.2 ^{**}
Y33	31.9	17.4	50.7 ^{**}
	Survival rate 50% or less		
Y34	36.2	14.5	49.3 ^{**}
Y35	34.8	15.9	49.3 ^{**}
Y39	31.9	20.3	47.8 ^{**}
Y41	39.1	14.5	46.4 ^{**}
Y42	55.1	0.0	44.9 ^{**}
Y43	43.5	13.0	43.5 ^{**}
Y45	58.0	0.0	42.0 ^{**}
Y49	50.7	8.7	40.6 ^{**}
Y51	60.9	0.0	39.1 ^{**}
Y53	58.0	4.3 ^{**}	37.7 ^{**}
Y48	60.9	2.9 ^{**}	36.2 ^{**}
Y24	44.9	20.3	34.8 ^{**}
Y30	66.7	0.0	33.3 ^{**}
L.S.D at 1%	1.4069	0.4057	3.7229

Control (1): Un-inoculated soil treatment

Control (2): Soil infested with *F. solani*

Control (3): Soil infested with *F. solani* and treated with Rizolex-T fungicide

** : Significant at level 1%

Table 3. Pre-, post-emergence damping-off disease (%) and survival rate (%) of tomato seedlings as influenced by inoculation with selected yeast isolates in the presence of *R. solani*.

Treatments	Pre-emergence damping-off (%)	Post-emergence damping-off (%)	Survival rate (%)
Control (1)	8.7**	0.0	91.3
Control (2)	37.7	33.3	29.0**
Control (3)	17.4	2.9**	79.7**
Yeast isolates + <i>R. solani</i>	Survival rate over 70% and less than 85%		
Y2	11.6	4.3	84.1
Y3	24.6	2.9**	72.5**
Y1	23.2	5.8	71.0**
	Survival rate over 50% and less than 70%		
Y4	14.5	15.9	69.6**
Y5	31.9	0.0	68.1**
Y6	23.2	8.7	68.1**
Y7	26.1	8.7	65.2**
Y9	37.7	0.0	62.3**
Y10	17.4	20.3	62.3**
Y11	17.4	20.3	62.3**
Y12	33.3	5.8	60.9**
Y15	37.7	2.9**	59.4**
Y17	37.7	2.9**	59.4**
Y18	31.9	10.1	58.0**
Y21	40.6	2.9**	56.5**
Y23	34.8	11.6	53.6**
Y24	34.8	11.6	53.6**
Y28	36.2	11.6	52.2**
Y29	49.3	0.0	50.7**
Y30	46.4	2.9**	50.7**
	Survival rate less than 50%		
Y31	49.3	Y31	49.3
Y32	47.8	Y32	47.8
Y34	47.8	Y34	47.8
Y36	55.1	Y36	55.1
Y39	55.1	Y39	55.1
Y40	56.5	Y40	56.5
Y58	59.4	Y58	59.4
Y53	50.7	Y53	50.7
Y20	53.6	Y20	53.6
Y26	43.5	Y26	43.5
L.S.D _{0.01}	0.2487	L.S.D _{0.01}	0.2487

Control (1): Un-inoculated soil treatment

Control (2): Soil infested with *R. solani*

Control (3): Soil infested with *R. solani* and treated with Rizolex-T fungicide

** : Significant at level 1%

Table 4. Pre-, post-emergence damping-off disease (%) and survival rate (%) of tomato seedlings as influenced by inoculation with selected yeast isolates in the presence of *P. aphanidermatum*.

Treatments	Pre-emergence damping-off (%)	Post-emergence damping-off (%)	Survival rate (%)
Control (1)	8.7	0.0	91.3
Control (2)	42.0	26.1	31.9**
Control (3)	8.7	13.0	78.3**
Yeast isolates + <i>P. aphanidermatum</i>	Survival rate over 80%		
Y3	5.8	11.6	82.6
Y4	0.0	18.8	81.2**
	Survival rate over 50% and less than 80%		
Y7	14.5	5.8**	79.7**
Y10	8.7	13.0	78.3**
Y2	8.7	13.0	78.3**
Y8	17.4	7.2**	75.4**
Y1	23.2	2.9**	73.9**
Y5	11.6	15.9	72.5**
Y30	11.6	15.9	72.5**
Y28	11.6	15.9	72.5**
Y27	20.3	10.1**	69.6**
Y57	20.3	10.1**	69.6**
Y29	20.3	10.1**	69.6**
Y53	23.2	10.1**	66.7**
Y35	23.2	10.1**	66.7**
Y24	20.3	14.5	65.2**
Y36	20.3	14.5	65.2**
Y37	18.8	17.4	63.8**
Y38	26.1	11.6	62.3**
Y44	36.2	2.9**	60.9**
Y31	34.8	5.8**	59.4**
Y40	36.2	5.8**	58.0**
Y32	37.7	5.8**	56.5**
Y46	39.1	5.8**	55.1**
Y47	31.9	14.5	53.6**
Y50	33.3	14.5	52.2**
Y52	34.8	14.5	50.7**
	Survival rate less than 50%		
Y55	36.2	14.5	49.3**
Y56	34.8	17.4	47.8**
Y60	34.8	18.8	46.4**
Y6	37.7	17.4	44.9**
Y11	53.6	2.9**	43.5**
Y13	55.1	2.9**	42.0**
Y14	56.5	2.9**	40.6**
Y54	56.5	2.9**	40.6**
Y58	55.1	7.2**	37.7**
Y59	55.1	7.2**	37.7**
Y16	53.6	8.7**	37.7**
Y19	58.0	5.8**	36.2**
Y17	58.0	5.8**	36.2**
Y22	60.9	4.3**	34.8**
Y23	62.3	2.9**	34.8**
Y20	58.0	7.2**	34.8**

Y21	58.0	8.7**	33.3**
Y9	63.8	2.9**	33.3**
L.S.D _{0.01}	0.2301	0.2009	0.4603

Control (1): Un-inoculated soil treatment

Control (2): Soil infested with *P. aphanidermatum*

Control (3): Soil infested with *P. aphanidermatum* and treated with Rizolex-T fungicide

** : Significant at level 1%

2. Effect of inoculation with antagonistic bioagents on growth of tomato plants

The obtained results in Figures 7-9 revealed that the three phytopathogens caused significant reductions in shoot and root dry weights of tomato plants compared with the healthy control. Compared with the control infested with either *F. solani*, *R. solani* or *P. aphanidermatum*. Inoculation treatments with tested yeast bioagents revealed significant increases in shoot and root dry weights of tomato plants. The most promising treatments that were inoculated with bioagent yeasts Y1, Y2 and Y3 in soil infested with either *F. solani* or *R. solani* whereas inoculation with either antagonistic Y3 or Y4 revealed the highest significant increases in growth in the presence of *P. aphanidermatum*.

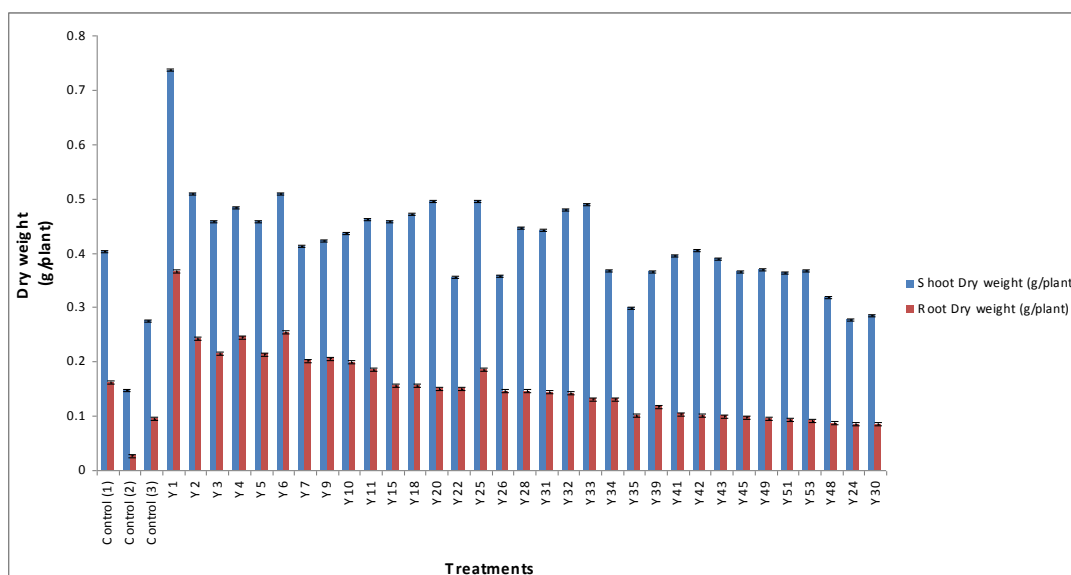


Fig. 7 Influence of inoculation with selected yeast isolates in the presence of *F. solani* on shoot and root dry weights of tomato plants.

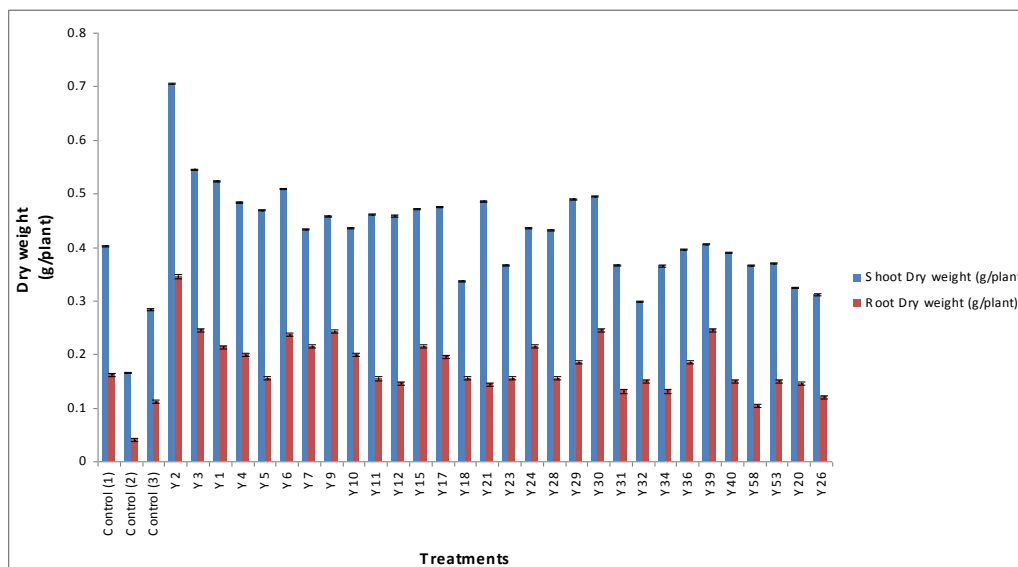


Fig. 8 Influence of inoculation with selected yeast isolates in the presence of *R. solani* on shoot and root dry weights of tomato plants.

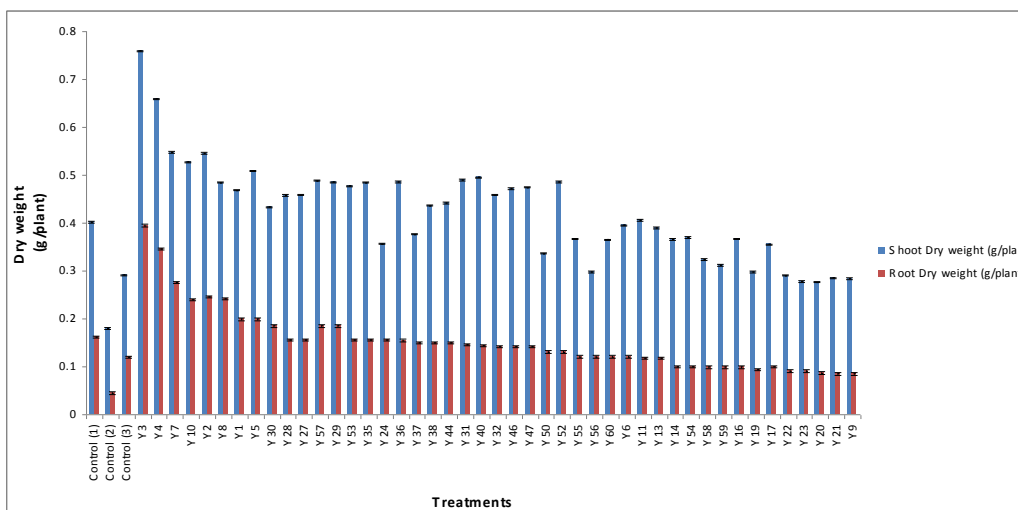


Fig. 9 Influence of inoculation with selected yeast isolates in the presence of *P. aphanidermatum* on shoot and root dry weights of tomato plants.

3. Identification and characterization of the most potent antagonistic and growth promoting yeast isolates

The selected yeast isolates Y1, Y2 and Y3 that were found to be strongly antagonistic to the three pathogens causing root rot disease of tomato plant and exhibited high chitinolytic activity were identified and characterized by phenotypic and genotypic techniques. The three isolates were previously identified using the classical morphological, biochemical and physiological methods and further confirmed by API system (Kamel *et al.*, 2013). Isolate Y1 was belonged to *Candida tropicalis* (Castellani) Berkhout, isolate Y2 was identified to be *Pichia caribaea* Phaff and isolate Y3 was suggested to be *Geotrichum candidum* Link.

Genotypic identification of yeast isolates

The entire (ITS1)-5.8 rRNA-(ITS2) regions were successfully amplified from all yeast isolates template DNA by using one fungus-specific universal primers pair, ITS1 and ITS4 (Moore *et al.*, 2011) as shown in Figure 10. The BLAST search revealed that ITS1-5.8 rRNA-ITS2 region DNA from yeast isolated had sequence similarity exceeded 99% (Table 5). Y2 isolate was *Pichia caribaea* Phaff, Y1 isolate was *Candida tropicalis* (Castellani)

Berkhout and Y3 isolate was *Geotrichum candidum* Link with maximum identity 100%, 99%, and 99% respectively.

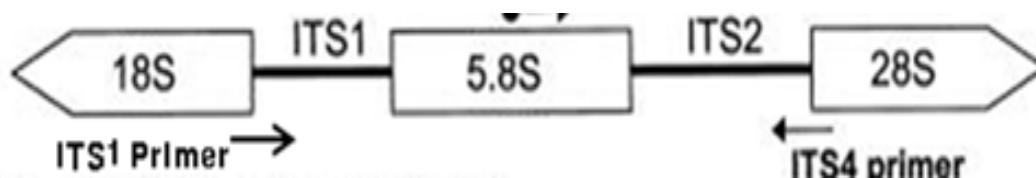


Fig. 10 Schematic representation of the fungal ribosomal rRNA gene (also referred to as rDNA) containing the primer target regions used in the amplification: the small subunit (SSU) which is the 18S region (partial sequencing), the ITS1, the 5.8S, the ITS2 (complete sequencing) and the large subunit (LSU) which is the 28S region (partial sequencing).

The lengths of ITS1, 5.8 rRNA and ITS2 regions ranged in size from 57 base pair (bp) (*Geotrichum candidum*) to 186 bp (*Pichia caribaea*) (Table 5).

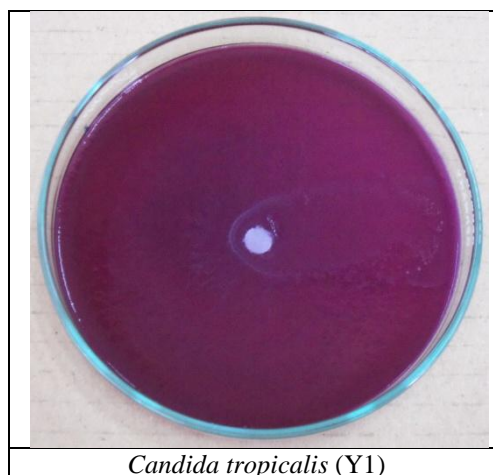
Table 5. Identification of yeast isolates by ITS1-5.8 rRNA-ITS2 sequence analysis.

Isolates number	Strain sequence analysis			Identification (maximum identity)
	ITS1 sequence	5.8 rRNA sequence	ITS2 sequence	
	Region length (bp) / identity with sequence in GeneBank (%)			
Y2	168bp/100	164bp/100	186bp/100	<i>Pichia caribaea</i> (100%)
Y1	139bp/100	157bp/100	141bp/100	<i>Candida tropicalis</i> (99%)
Y3	57bp/100	153bp/100	121bp/100	<i>Geotrichum candidum</i> (99%)

4. Lytic activity of the most potent three growth promoting bioagent yeasts

4.1. Chitinolytic activity

Isolates *Candida tropicalis*, *Pichia caribaea* and *Geotrichum candidum* showed high chitinolytic activity with 90 mm-diameter of the formed purple colored zone as shown in Figure 11.



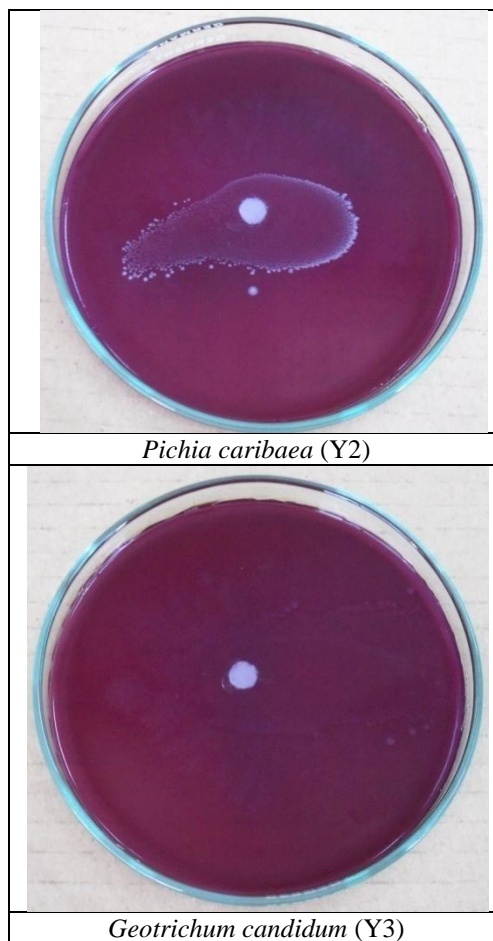
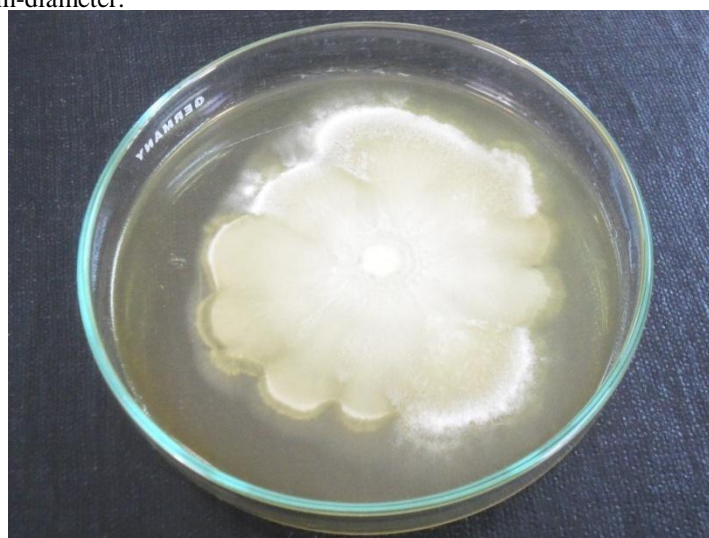


Fig. 11 Chitinolytic activity of the strongly antagonistic yeast isolates; Y1, Y2 and Y3.

4.2. Proteolytic activity

Results in Table 6 and Figure 12 indicated that only *Geotrichum candidum* showed proteolytic activity with degradation zone of 19 mm-diameter.



Geotrichum candidum (Y3)

Fig. 12 Proteolytic activity of the most potent yeast isolate *Geotrichum candidum* (Y3).

4.3. Phospholipolytic activity

Results presented in Table 6 showed that all bioagent yeast isolates gave negative results with phospholipase activity.

5. *In vitro* antagonistic activity of the most potent three selected yeast isolates against *F. solani*, *R. solani* and *P. aphanidermatum*

Pichia caribaea showed highly antagonistic activity against *R. solani* with fungal mycelium growth inhibition (77.8%) followed by *Geotrichum candidum* against *P. aphanidermatum* with mycelial growth inhibition (74.4%) and *Candida tropicalis* against *F. solani* with growth inhibition (55.6%) as shown in Table 6.

Table 6. Antagonistic activity and some lytic enzymes produced by *Candida tropicalis*, *Pichia caribaea* and *Geotrichum candidum*.

Microorganisms	Antagonistic activity MGI (%)	Lytic enzymes Zone diameter (mm)		
		Chitinase	Protease	Phospholipase
<i>Candida tropicalis</i>	55.6	90.0	0.0	0.0
<i>Pichia caribaea</i>	77.8	90.0	0.0	0.0
<i>Geotrichum candidum</i>	74.4	90.0	19	0.0

MGI % : Mycelial growth inhibition percentage

Discussion

In pot experiment, the obtained sixty antagonistic and plant growth promoting yeast isolates were evaluated for controlling root rot disease caused by *F. solani*, *R. solani* and *P. aphanidermatum* in tomato under greenhouse conditions. The tested bioagents were applied as seed dressing and soil drenching treatments. The results revealed that significant reduction in the disease incidence was observed in all the tested bioagent treatments resulting in great enhancement of germination rate of tomato seeds and reduction of pre- and post-emergence damping-off compared to the infested control. All treatments significantly reduced disease severity of tomato root rot relative to the infested control. The highest disease severity reduction was observed with isolates Y1 and Y2 in the presence of *F. solani* (82.6 and 76.8 %), with isolates Y2 and Y3 in the presence of *R. solani* (84.1 and 72.5 %) and with isolates Y3 and Y4 in the presence of *P. aphanidermatum* (82.6 and 81.2 %) respectively.

In most yeast bioagent treatments, significant effect in reducing of root rot incidence at pre- and post-emergence stages was observed. The treated seedlings showed a protective effect against the soil-borne pathogenic fungi. The obtained results also revealed that tomato plant treatments with yeast bioagents improved the survival plants and caused high significant increase in all treatments.

The most active isolates were Y1, Y2, Y3 and Y4. All tested yeast bioagents in presence or in absence of pathogen significantly improved growth of tomato plants such as shoot and root dry weights compared with positive control which infested with the pathogen alone. In accordance with these results, **El-Emery (2004)** reported that germination rate of different seeds of barley, maize, pea and bean was great enhanced by using different yeast dilutions and growth of plumules, rootlets and cotyledon enlargement was also stimulated. **EL-Mehalawy et al. (2004)** also reported that all the tested five isolates of yeast fungi; *Candida maltosa*, *C. glabrata*, *C. slooffii*, *Rhodotorula rubra* and *Trichosporon cutaneum* individually or in combination significantly increased maize plant growth measurements such as fresh and dry weights of shoot and root, length of root and shoot height as compared with the un-infested negative control and positive control which infested with the pathogen *Cephalosporium maydis* alone which causing late wilt disease of maize.

El-Wakil et al. (2009) reported that the seed coating of faba bean with a water suspension (10^9 CFU mL⁻¹) of the yeast *Saccharomyces cerevisiae* before sowing in soil artificially infested with phytopathogenic fungi and also supplemented with composted organic wastes significantly increased plant growth parameters including height, shoot and root length, number of branches/plant, number of pods/plant, pod weight/plant, fresh weight and dry weight. Also photosynthetic pigments; chlorophyll A, chlorophyll B and carotenoids were increased and total phenols content in the treated plant leaves was higher than in the control plants.

The observed reduction in the disease severity and increased vegetative growth of tomato plant by the selected yeast bioagents compared to infested control may be due to their ability to produce plant growth promoting substances such as IAA, gibberellins, siderophores and phosphate solubilizing activity as reported previously by **Kamel et al. (2013)**. Also these three bioagent yeast produce hydrolytic enzymes which are believed to play an

important role in the parasitism of phytopathogenic fungi. These results agree with those reported by **Abd El-Kader et al. (2012)** who mentioned that isolates of some antagonistic bacterial and yeast agents significantly reduced root rot disease incidence in comparison with untreated control. In this concern, reports of other investigators had attributed the improvement in growth and yield to the production of growth promoting substances such as IAA and gibberellins (**Bashan and De-Bashan, 2003**) and cytokinins, thus improving uptake of water and nutrients by inoculated plants. Similar responses were reported in tomato plants. Some workers explained the mode of action of yeast isolates. Antagonistic yeasts have been selected mainly for their capability to rapidly colonize and grow in surface and subsequently to out compete the pathogen for nutrients and space. Besides competing for nutrients and space, antagonistic yeasts parasitize major postharvest pathogens directly through strong attachment to their hyphae. This leads to partial disruption of hyphal wall structures (**El-Ghaouth et al., 1998**).

The mechanisms of yeast action towards the molds are varied as the induction of resistance in the host plants, production of cell-wall lytic enzymes capable to degrade the cell wall of phytopathogenic molds (**Urquhart and Punja, 2002**), toxic volatile compounds and killer toxins (**Bruce et al., 2004**).

Killer toxins are not restricted to *Saccharomyces*; they can also be found in other yeast genera: *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Sporidiobolus*, *Tilletiopsis*, *Zygosaccharomyces*, etc (**Santos et al., 2009**).

The most potent three yeast isolates designated Y1, Y2 and Y3 with growth promoting activity and with strong antagonistic activity against the three tested phytopathogens were selected for identification using extensive phenotypic and molecular characterization studies. The results revealed that isolate Y1 was identified as *Candida tropicalis* (Castellani) Berkhout, isolate Y2 was identified as *Pichia caribaea* Phaff while isolate Y3 was found to be *Geotrichum candidum* Link with maximum identity 99, 100 and 99 % respectively.

In the present investigation, the three yeast species; *Candida tropicalis*, *Pichia caribaea* and *Geotrichum candidum* showed high chitinolytic activity while only yeast isolate *Geotrichum candidum* showed proteolytic activity and non of them produced phospholipase enzyme. In accordance with these results, several workers reported that yeast was a producer of variety of extracellular chitinase and protease enzymes and had become an important mean of biological control of fungal diseases; **David (2004)** reported that *Saccharomyces cerevisiae* and *Kluyveromyces lactis* showed inhibitory activity against phytopathogenic fungi by producing extracellular chitinase enzymes. Also *Candida* and *Kluyveromyces* species have been shown to produce alkaline proteases (**Agrawal and Kotasthane, 2012**).

Some lytic enzymes can be involved in both antagonistic and saprophytic processes providing an evolutionary advantage to strains with both biodegrading and antagonistic potential, for the efficient colonization of different ecological niches in soil. A principal role in mycoparasitism has been attributed to chitinases (**Lorito, 1998**). However, fungal proteases may also be significantly involved in cell wall degradation, since fungal cell walls contain chitin and glucan polymers embedded in and covalently linked to a protein matrix (**Kapteyn et al., 1996**).

Cell-wall degrading enzymes such as chitinases have been implicated in the biological control of soil-borne fungal pathogens (**Singh et al., 1999**). Such enzymes are particularly important, as chitin is a major constituent of the cell walls of many plant-pathogenic fungi and the second most abundant polymer in nature after cellulose and plays a major role in fungal cell walls. As a producer of variety of chitinase enzymes yeast has become an important means of biological control of fungal diseases (**Agrawal and Kotasthane, 2012**).

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