Pak. J. Bot., 51(6): 2283-2289, 2019. DOI: http://dx.doi.org/10.30848/PJB2019-6(44)

EVALUATION OF BIOCONTROL AND PLANT GROWTH PROMOTING POTENTIAL OF ENDOPHYTIC YEASTS ISOLATED FROM HEALTHY PLANTS

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Abstract

Yeasts have been used as an industrial microorganism from thousands of years. Little attention was given to yeasts as biocontrol agents against plant pathogens as compared to other microorganisms in the past. In this study, endophytic yeasts (n=22) isolated from healthy plants and biocontrol efficiency of selected yeasts isolates was investigated. They inhibited the mycelial growth of *Fusarium solani, F.oxysporum* and *Macrophomina phaseolina*, but they were ineffective against the *Rhizoctonia solani*. Yeast isolates KUAY-1, KUAY-9, KUAY-34, and KUAY-62, used in screen-house experiment in soil amended with neem cake, significantly suppressed the root rot of sunflower and enhanced plant growth. Endophytic yeast were found more effective in soil amended with neem cake in improving plant growth and suppression of fungal infection as compared to each treatment alone.

Key words: Endophytic, Yeasts, Root rot, Biocontrol, Soil amendment, Sunflower.

Introduction

Treating fruits and vegetable crops with fungicides is the primary method for controlling the pre-harvest and post-harvest diseases. Although these pesticides often work well and maintain the high quality of produce and increase crop yield, but they create health hazed for humans. Agrochemical treatments are mostly non-specific *i.e*. they not only affect the target pathogens but also other beneficial microorganisms (Ranganathswamy *et al*., 2013). The continuous application of chemical fungicides results in the contamination of environment, that affects beneficial micobes, human and animals (Sparks, 2013; Yoom *et al*., 2013; Tupe *et al*., 2014).

The use of biological antagonists is now becoming the best alternative to agrochemical treatments (Nguyen *et al*., 2011; Dawoud *et al*., 2012). Besides, other microorganisms, yeast are emerging as competitive antagonists of postharvest fungi (Droby *et al*., 2002; El-Ghaouth *et al.,* 2003). *Pichia membranefacians* has been reported to suppres *Aspergillus* and *Rhizopus* (Paster *et al.*, 1993; Fan & Tian, 2000), while *Cryptococcus albidus* suppressed *Penicillium expansum* (Tian *et al*., 2002). Inhibitory effects of *Cryptococcus*, *Rhodotorula* and *Saccharomyces* on the growth of *Fusarium sporotrichioides* have also been reported (Wachowska *et al*., 2013).

Endophytic microorganisms such as bacteria or fungi live inside plant without causing any negative effect on their host (Schulz & Boyle, 2006). The potential of endophytes to improve plant growth with the suppression of several diseases is making them more valuable in scientific and commercial interest (Korejo *et al.,* 2014, 2017; Shafique *et al*., 2015; Urooj *et al.,* 2018). However, use of endophytic yeast as biocontrol agent for controlling plant root diseases has been largely neglected (Mohamed *et al.,* 2013).

Organic amendment is a method being used to produce healthier plants with improved crop yield (Shafique *et al.*, 2016). It is also reported to have negative impact on soil borne pests (Sultana *et al.,* 2011; 2018; Rahman *et al.,* 2016). Among various organic matters, neem cake used as an organic fertilizer with both fungicidal (Ehteshamul-Haque *et al.,* 1995) and nematicidal (Singh & Singh, 1997) effects. Several reports have described neem cake efficacy against plant pathogens (Rahman *et al.,* 2016; Shafique *et al.,* 2016). Isolation and identification of endophytic yeast from healthy plants and their effect in protecting sunflower roots from root rotting fungi alone or in soil amended with neem cake, have been investigated in this study.

Materials and Methods

Plant material: In this study, 30 healthy plant samples belonging to 6 plant species viz *Azadirachta indica* A. Juss., *Carica papaya* L., *Chenopodium* sp., *Cucumis sativus* L., *Lycopersicon esculentum* Mill., and *Tagetes erecta* L. grown at the Karachi University campus were collected, and the isolation of yeast was made within 24 h.

Isolation of endophytic yeasts: Stems, roots and leaves samples (2cm long) from each plant were cut, wash separately with sterile water, then with the solution of 1% sodium hypochlorite, followed by 70% ethanol and sterile distilled water. After surface sterilization these tissues were macerated with homogenizers under a-septic condition. Macerated tissue solution was diluted upto 10^{-4} with sterile distilled water and 0.1 ml from final dilutions was spread on Yeast extract-malt extract-peptone-glucose (YM) agar medium plates. The plates were incubated for 5-7 days at 25±1ºC. Morphologically similar colonies of yeast were purified.

Morphological and physiological characterization of yeasts: Selected yeast isolates were initially examined for their morphological characteristics based on colony colour and texture (Kurtzman *et al.*, 2011). Each isolate was grown on the plates containing YM agar medium for 3 days at 25ºC. Examination of pseudohyphae was done by applying Dalmau Test on Corn Meal Agar Medium (CMA) (Beech, 1972). The yeast isolates were further subjected to physiological and biochemical assays for preliminary identification. These assays included the fermentation of sugars in semi-anaerobic environment and the assimilation of various carbon compounds aerobically. Hydrolysis of urea, growth at 37ºC, and tolerance of 1% acetic acid were also included for the characterization of yeast (Kurtzman & Fell, 2005; Kurtzman *et al.*, 2011).

Molecular identification of yeast isolates: The selected yeast isolates (KUAY-34, KUAY-38, KUAY-62 and KUAY-67) were further identified by polymerase chain reaction (PCR) and restriction endonuclease analysis (RFLP) of internal transcribed spacer region (ITS1-5.8S-ITS2) of ribosomal DNA (rDNA) as described by Esteve-Zarzoso *et al.*, (1999) and Mohammadi *et al*., (2013) using the primers set ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) as described by Karimi *et al*., (2015). *Saccharomyces boulardii* and *Saccharomyces cerevisiae* were used as positive control. The restriction patterns were analyzed by GelClust v1.0 and phylogenetic tree was constructed by the UPGMA-dice coefficient method applied on the distance matrix.

In vitro antifungal activity of yeast: A loop full of yeast culture streaked onto the malt-yeast-glucose-peptone agar (YM) and a disc of *F.solani*, *F. oxysporum*, *R. solani* and *M. phaseolina* was placed 70 mm away from the streak. Three Petri plates of each pathogen were inoculated as control (i.e. without streak of yeast isolates). Each test was replicated thrice and experiment was repeated twice. Observations were made from 3rd to 9th day of inoculation, growth of fungal colonies was measured and inhibition zone was recorded.

Screen house experiment: Potential isolates of yeast viz., KUAY-1, KUAY-9, KUAY34 and KUAY-62 were separately grown in YM broth medium at 25±1ºC with shaking (at 50 rpm per min.) for 72 h. The broth was diluted with water to obtain 10^8 cfu mL^{-1} concentration. For the experimentation, non-sterilized sandy loam soil with pH 8.0 was used having natural infestation of root infecting fungi, *M. phaseolina* (3-6 sclerotia/g of soil), *Fusarium* spp., (3000 cfu/g) and *Rhizoctonia soalni* (5-10% colonization of sorghum seeds) were determined using methods described by Sheikh & Ghaffar (1975), Nash & Synder, (1962) and Wilhelm (1955). Soil was amended with neem cake at 1% w/w and transferred to 12 cm diameter earthen pots 1 Kg per pot. After one week of watering 25 mL yeast suspension (10^8 cells/ml) was drenched into each pot and seeds (6 seeds per pot) of sunflower (*Helianthus annuus)* were sown. Carbendazim suspension (200 ppm) at 25 mL per pot was kept for comparison, while plants not receiving carbendazim or yeast were kept as control. Each treatment was randomized with 4 replicates. Four seedlings per pot maintained after germination and the observations were recorded after 6 weeks. Data on plant growth was recorded, whereas suppressive effect of yeast on root infecting fungi was determined as described by Habiba *et al.,* (2016).

Data analysis: Data on plant growth and fungal infection was analyzed using software, CoStat. Means were separated and significant level at (p<0.05) were calculated.

Results

Isolation of endophytic yeasts: Twenty two isolates of endophytic yeasts were recovered from roots, stems and leaves of *Azadirachta indica*, *Carica papaya*, *Chenopodium* sp., *Cucumis sativus*, *Lycopersicon esculentum* and *Tagetes erecta* (Table 1).

Identification of yeast: On the basis of morphological, physiological and biochemical characters, five yeast genera were identified as *Saccharomyces*, *Debryomyces*, *Kluyveromyces*, *Torulaspora* and *Rhodotorula* (Table 2).

Molecular identification of yeast isolates: Amplification of the fungus specific internal transcribed spacer region (ITS1 and ITS4) of ribosomal DNA (rDNA) led the identification at genus level Fig. 1(a-c). The ITS region of endophytic yeast (Table 3) were amplified in the range of approximately 550-650bp, while *S.boulardii* and *S. cerevisiae* showed ITS products of 830 and 1000bp, respectively.

***In vitro* antifungal activity of endophytic yeasts**: Twenty two yeast isolates were selected for testing *in vitro* antifungal activity against four root rotting fungi. Growth of the three test fungi viz., *F. usarium solani*, *F. oxysporum*, *M. phaseolina* was inhibited by all the endophytic yeasts as indicated by zone of inhibition of varying degrees. Maximum zones of inhibition were produced by the yeast isolates KUAY-1, KUAY-9, KUAY-34, and KUAY-62. Lysis of fungal hyphae was also caused by some yeast isolates (Table 1; Fig. 2).

Screen-house experiment: Yeast isolates effectively suppressed the root rotting fungi on sunflower roots. Yeast isolates KUAY-9, KUAY-34, and KUAY-62 applied individually or in neem cake amended soil effectively suppressed *F. solani*, *F. oxysporum,* and *M. phaseolina* than control plants. Maximum reduction in root disease was found in plants received yeast isolate KUAY-34 in neem cake amended soil when compared to the control and carbendazim treatment (Table 4). *Rhizoctonia solani* was completely suppressed when the yeast isolates KUAY-34 and KUAY-62 were applied individually.

Maximum plant height was observed in the plants treated with yeast isolate KUAY-62 alone and in soil amended with neem cake. The KUAY-9 also increased the shoot length when applied individually and in combination with KUAY-62 and neem cake. Combined treatment of yeast isolates KUAY-9, KUAY-62 with neem cake significantly increased the shoot weight. Plants treated with KUAY-62 and grown in neem caked amended soil showed maximum root length as compared to other plants (Table 5).

Table 1. ***In vitro*** **growth inhibition of** ***Fuasrium solani, F. oxysporum, Macrophomina phaseolina*** **and** ***Rhizoctonia solani*** **by endophytic yeast isolates.**

Yeast strains	Source	*F. solani*	*F. oxysporum*	*M. phaseolina*
		Zone of inhibition (mm)		
KUAY-1	*Tagetes erecta*	17	20	18
KUAY-2	*T. erecta*	4	11	15
KUAY-4	*T. erecta*	7	5	15
KUAY-5	*T. erecta*	10	15	25
KUAY-6	*T. erecta*	15	10	12
KUAY-9	*Azadirachta indica*	13	25	22
KUAY-10	*A. indica*	14	9	11
KUAY-12	*Cucumis sativus*	11	10	20
KUAY-13	*C. sativus*	12	7	15
KUAY-23	*T. erecta*	11	6	9
KUAY-25	*T. erecta*	16	27	12
KUAY-26	*Chenopodium* sp.	5	9	15
KUAY-27	*Chenopodium* sp.	4	11	12
KUAY-34	*A. indica*	22	28	24
KUAY-38	*A. indica*	14	22	25
KUAY-52	*A. indica*	15	3	20
KUAY-54	*A. indica*	18	21	22
KUAY-62	*Carica papaya*	28	24	26
KUAY-67	*C. papaya*	22	19	20
KUAY-70	*C. papaya*	10	15	19
KUAY-72	*C. papaya*	11	20	14
KUAY-90	*Lycopersicon esculentum*	11	05	07

Table 2. Morphological and biochemical/physiological characteristics of yeasts.

Yeast isolate	Colony color	Colony texture	Pseudohyphae	Germtube	Glucose fermentation	Sucrose fermentation	D-Xylose Growth	D-Mannitol Growth	L- Rhamnose Growth	Sucrose Growth	Cellobiose Growth	Growth at 37ºC	1% Acetic acid growth	Urea Hydrolysis	Genus
KUAY-1	Cream	Smooth	-	-	±	±	±	±	-	±	±	v	-	-	*Saccharomyces*
KUAY-2	Cream	Smooth	-	-	±	±	±	±	-	±	±	±	-	-	*Saccharomyces*
KUAY-4	Cream	Smooth	-	-	±	±	±	±	-	±	±	-	-	-	*Saccharomyces*
KUAY-5	White	Butyrous	-	-	±	±	±	±	-	±	±	-	-	±	*Torulaspora*
KUAY-6	White	Butyrous	-	-	±	±	±	±	-	±	±	-	-	±	*Torulaspora*
KUAY-9	White	Smooth	-	-	±	±	±	±	-	±	-	-	-	±	*Debryomyces*
KUAY-10	White	Smooth	-	-	±	±	±	±	-	±	-	-	-	-	*Debryomyces*
KUAY-17	White	Smooth	-	-	±	±	±	±	-	±	-	-	-	±	*Debryomyces*
KUAY-20	White	Butyrous	-	-	w	v	-	±	-	-	v	v	-	-	*Kluyveromyces*
KUAY-23	White	Butyrous	-	-	w	v	-	±	-	-	v	v	-	-	*Kluyveromyces*
KUAY-25	White	Butyrous	-	-	w	v	-	±	-	-	v	v	-	-	*Kluyveromyces*
KUAY-31	Cream	Smooth	-	-	±	±	±	±	-	±	±	-	-	-	*Saccharomyces*
KUAY-34	Cream	Smooth	-	-	±	±	±	±	-	±	±	-	-	-	*Saccharomyces*
KUAY-38	Cream	Smooth	-	-	±	±	±	±	-	±	v	v	-		*Saccharomyces*
KUAY-52	Pink	Mucoid	-	-	-	-	v	±	-	±	-	±	-	±	*Rhodotorula*
KUAY-54	Pink	Mucoid	-	-	-	-	v	±	±	±	±	±	-	±	*Rhodotorula*
KUAY-62	Pale white	Butyrous	-	-	±	±	w	±	w	±	w	±	-	w	*Torulaspora*
KUAY-67	Pale white	Butyrous	-	-	±	±	w	±	w	±	w	±	-	w	*Torulaspora*
KUAY-70	Cream	Glossy	-	-	w	v	-	±	-	-	v	v	-	-	*Kluyveromyces*
KUAY-72	Cream	Glossy	-	-	w	v	-	±	-	-	v	v	-	-	*Kluyveromyces*

Symbols: ± (positive); - (negative); w (weak); v (variable)

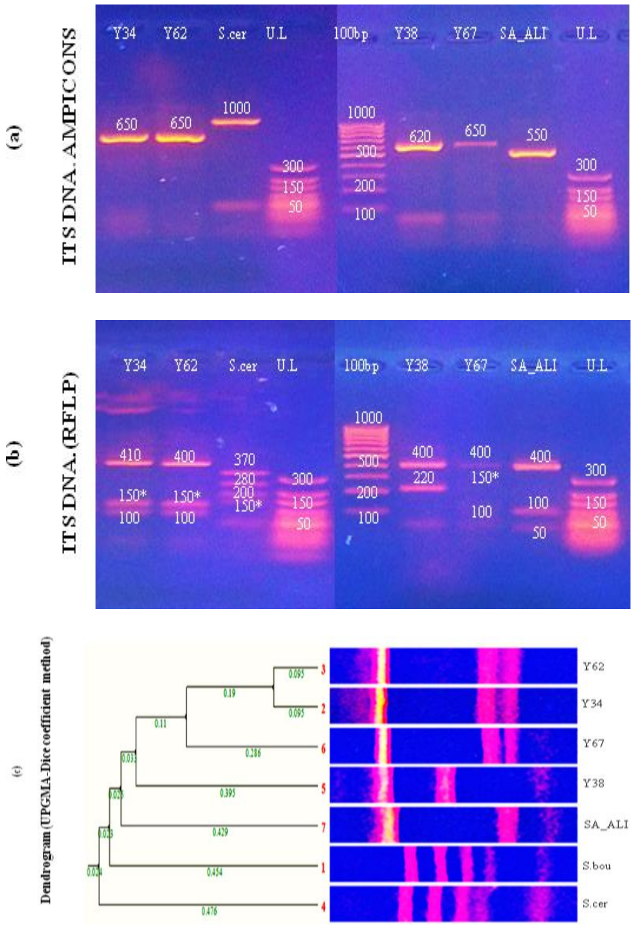

Fig. 1. Molecular identification of endophytic yeast. (a) PCR amplification of internal transcribed spacer sequences (ITS DNA) used as a molecular marker of yeast identity (b) RFLP analysis of ITS, obtain by the restriction enzyme Hae-III (Fermentas, USA) (c) Phylogenetic tree constructed by UPGMA-dice coefficient method.The reaction products were analyzed on, 2% (for a) and 2.5% (for b), agarose gels containing 0.5 µg/mL ethidium bromide, respectively. S. bou, S. cer, and SA_ALI are the control strains of *S. boulardii, S. cerevisiae*and *Saccharomyces.* sp. respectively, while Y numbers represents endophytic yeast isolates (KUAY-34, 38, 62 and 67). 100 bp=100 bp and U.L=ultra-low range DNA ladders respectively (Fermentas, USA).

Discussion

Use of biological antagonists to manage fungal diseases of crop plants has become a positive approach in comparison to using synthetic fungicides. Many microbial agents including bacteria and mycelial fungi have been reported by a number of workers as the best biological antagonists to a variety of crop pathogens. In this study, all the selected twenty two endophytic yeast isolates from different sources inhibited the growth of *F. solani*, *F. oxysporum* and *M. phaseolina* to a varying degree. Yeasts have the ability to secrete compounds or peptides with antimicrobial activity (Pérez-Montaño *et al.*, 2014; Schulz *et al.*, 2013). These antibiotics inhibit the growth of target organism by affecting their membrane permeability (Avis & Be´langer, 2001). The activity of these effective yeasts was further assessed in soil amended with neem cake for the growth of sunflower. Organic amendments are known to increase the agricultural yield with the suppression of soil-borne diseases (Stone *et al.*, 2003; Sultana *et al.*, 2011). Oil seed cakes have been reported for the significant suppression of soil-borne pathogens (Ehteshamul-Haque *et al.*, 1995; Urooj *et al.*, 2018). Significant increase in plant height and decrease in fungal infection was observed by the application of yeast isolates. Fungal infection was greatly suppressed and better plant growth was obtained when the yeast isolates were applied in soil amended with neem cake as compared to the neem cake alone. The reduced fungal pathogenecity and increased plant growth by the selected yeast bio-agents might be due to the production of plant growth promoting substances such as IAA, gibberellins, siderophores and phosphate solubilizing activity (Ignatova *et al.*, 2015; Kamel *et al.*, 2013). It has been reported that neem seed cake possesses fungicidal property and it improves the quality and yield of crops by supplying gradual nourishment to the plant (Gaur *et al.*, 1992; Ehteshamul-Haque *et al.*, 1995; Urooj *et al.*, 2018). This study has revealed that yeasts are not only a part of soil environment but they also live as endophyte and compete with other microorganisms for their survival and establishment in particular niches.

Table 3. RFLP analysis of *ITS* amplicons of endophytic yeast isolates.

Strain ID	ITS Amplicons Base Pairs (bp)	Hae III (BsuRI) digest Base Pairs (bp)
Saccharomyces cerevisiae (GenBank: AY247400.1)	754	In-silico digest 311 ± 172 ± 172 ± 99
Saccharomyces boulardii (GenBank: AY428861.1)	837	In-silico digest 311 ± 230 ± 172 ± 124
KUAY-34	660	41 0± 150 ± 100
KUAY-62	650	400 ± 150 ± 100
KUAY-38	620	400 ± 220
KUAY-67	650	400 ± 150 ± 100
SA_ALI	550	400 ± 100 ± 50
Saccharomyces boulardii Probiotic (Enflor sachet)	830	300 ± 270 ± 170 ± 130
Saccharomyces cerevisiae Baker’s yeast (Resmor sachet)	1000	370 ± 280 ± 200 ± 150

Table 4. Effect of endophytic yeasts on root infection by, *Fusarium solani, F. oxysporum, Macrophomina phaseolina* and *Rhizoctonia solani* on sunflower roots in screen house experiment.

Treatment	Infection %			
	F. solani	*F. oxysporum*	*M. phaseolina*	*R. solani*
CONTROL	93.7	25	50	43.7
Carbendazim	75	12.5	43.7	18.7
Neem cake (NC) @ 1%	68.7	50	37.5	25
Carbendazim± Neem cake	68.7	18.7	25	31.2
Yeast isolate (KUAY-1)	87.5	6.2	12.5	25
KUAY-9	81.2	25	12.5	18.7
KUAY-34	31.2	37.5	18.7	0
KUAY-62	75	25	18.7	0
KUAY-1 ± NC	81.2	18.7	25	0
KUAY-9 ± NC	31.2	12.5	31.2	0
KUAY-34 ± NC	43.7	6.2	25	0
KUAY-62 ± NC	31.2	18.7	6.2	6.2
KUAY-1 ± KUAY-9 ± NC	37.5	6.2	18.7	12.5
KUAY-1 ± KUAY-62 ± NC	62.5	12.5	6.2	6.2
KUAY-9 ± KUAY-62 ± NC	43.7	18.7	12.5	0
$LSD_{0.05}$	**Treatments = 13.8[1], Pathogens = 7.1[2]**			

[1]Mean values in column showing differences greater than LSD values are significantly different at $p<0.05$

[2]Mean values in rows showing differences greater than LSD values are significantly different at $p<0.05$

Table 5. Effect of soil drench with endophytic yeasts on growth of sunflower plants in screen house experiment.

Treatment	Root length (cm)	Shoot length (cm)	Root weight (g)	Shoot weight (g)
CONTROL	12.63	37.8	0.62	4.52
Carbendazim	14.19	40.44	0.81	4.87
Neem cake (NC) @ 1%	12.35	32.6	0.83	5.3
Carbendazim± NC	13.16	30.57	0.9	5.16
Yeast isolate (KUAY-1)	14.53	38.81	0.72	4.18
KUAY-9	14.16	41.19	0.73	5.9
KUAY-34	12.69	42.47	0.76	5.05
KUAY-62	12.28	50.76	0.63	6.02
KUAY-1 ± N	12.63	31.72	0.81	5.7
KUAY-9 ± N	15	36.19	1.09	6.06
KUAY-34 ± N	14.69	50	2.11	6.92
KUAY-62 ± N	16.75	52.58	2.4	7.52
KUAY-1 ± KUAY-9 ± NC	13.28	39.6	1.01	6.77
KUAY-1 ± KUAY-62 ± NC	16.5	45.78	2.9	7.47
KUAY-9 ± KUAY-62 ± NC	12.19	43.09	2.5	8.66
$LSD_{0.05}$	**ns**	**9.83[1]**	**0.36[1]**	**2.52[1]**

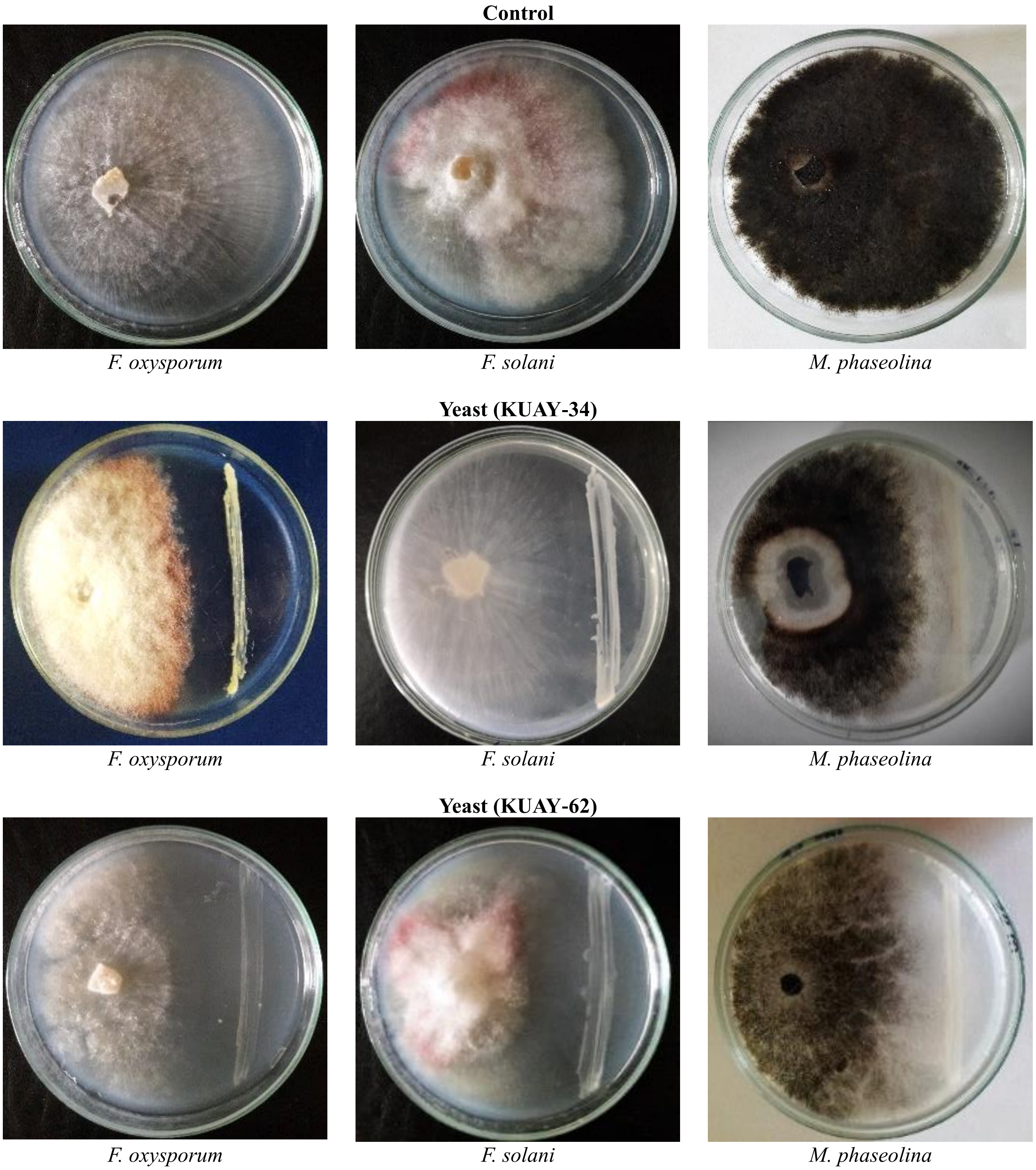

Fig. 2. *In vitro* growth inhibition of root rotting fungi by the endophytic yeasts in dual culture plate assay.

Acknowledgement

Financial assistance provided by the Dean, Faculty of Science, University of Karachi is acknowledged.

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(Received for publication 5 May 2018)