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Antagonistic activity of epiphytic yeast against grapes mold caused by *Rhizopus* sp.

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Abstract
Post-harvest losses due to plant pathogens is a major problem in grapes which causes huge economic losses to the farmers in terms of quality and quantity. Yeast are unicellular organisms which exist on the fructosphere of crops and protects the plant from pathogens. In this study, yeast species has been exploited against *Rhizopus* sp. infecting grapes during the post-harvest stage. Thirty-three epiphytic yeasts on the surface of grapes were isolated from Madurai and Erode districts of Tamil Nadu and tested against *Rhizopus* sp. The results revealed that the yeast isolate, YCSL2 exhibited maximum inhibitory effect of 30.33% and 24.44% in dual culture and volatile compounds assay respectively over the control under *in vitro*. Similarly, wound site colonization of yeast against *Rhizopus* sp. recorded maximum inhibitory effect of 71.11% against the control under *in vivo*. Thus, the yeast species identified in this study can be used for the biological management of post-harvest pathogens in grapes.

Keywords: Grapes, post-harvest pathogen, *Rhizopus* sp., volatile compounds, wound site colonization

Introduction
Grapes is one of the important fruit crops which is cultivated throughout the world for consumption and industrial purpose. Grapes cultivation is mainly affected by various biotic and abiotic stresses among which post-harvest pathogens cause huge economic loss. Post-harvest diseases of grapes mainly occur by high water content and sugar content in the fruit. Among post-harvest pathogens, *Rhizopu*s mold caused by *Rhizopus* sp. occur at harvesting stage and storage condition. *Rhizopus* sp. quickly spread to entire areas of the fruit under favourable condition which leads to heavy economic losses. Grapes is also considered as a heavy pesticide utilizing crop and consuming the raw product will affect the human health. Based on public health concern and to avoid the fungicide resistance in pathogen, an alternative method for chemical management is the need of the day (Saravanakumar *et al*., 2008) [15].

During the last decades, beneficial microorganisms with antimicrobial properties have been isolated and utilized for the management of major diseases in horticultural crops (Kloepper *et al*., 1980) [7]. In some cases, plant and animal derived products was used as a biocontrol agent (Pal and Gardener, 2006) [12]. Biological control is a very useful approach for managing post-harvest diseases in grapes (Bleve *et al*., 2006) [3]. Yeasts are unicellular fungi that are found on/in the surface of fruits and are considered as a healthier biological control agent in post-harvest disease management, when compared to chemical fungicide (Kurtzman *et al*., 2011) [8]. Various groups throughout the world have utilized yeast strains for the management of post-harvest diseases of fruit crops (Saravanakumar *et al*., 2008) [16]. Yeast does not require luxurious components for their growth and require simple nutrient of dextrose, peptone etc. (Adel, 2004) [1]. Considerable progress has been made in understanding the action of yeast strains against pathogenic fungi (Spadaro and Droby, 2016) [17]. Other reports point to competition for space and nutrients and/or antibiosis by the protectant microorganisms as a major mechanism in disease control (Wilson and Wisniewski, 1989) [19]. In this paper, we have tested the efficacy of epiphytic yeast from the surface of grapes against *Rhizopus* sp. of grapes under laboratory condition.

Materials and Methods
Isolation of yeast from the fructosphere of grapes
For yeast suspension preparation, ten grams of grapes were transferred to a beaker containing 100 ml of distilled water and shaked vigorously for 5-10 mins. Yeast extract peptone dextrose agar (YPD) (yeast extract powder-10g/lit, peptone-20g/lit, dextrose-20/lit and agar-20g/lit) media amended with streptomycin was prepared and sterilized. Serial dilution upto 10^{-6} of the suspension was carried out and poured into the plates.

The YPD media was added to the plates and incubated at room temperature (28±2°C) for 48 hrs (Zhimo *et al*., 2016) [22]. The isolates were further maintained in slant culture.

Isolation of pathogen and pathogenicity

For isolation of pathogen, infected portion of berries were cut into small pieces using sterile scalpel, surface sterilized with 70% ethanol for few seconds and the cut portion was washed using sterile distilled water thrice. The sterilized pieces were placed in petri dishes containing Potato dextrose agar medium amended with streptomycin and incubated for 5 days at room temperature. The culture was maintained by subsequent sub-culturing. For the preparation of spore suspension, the mycelial portion was placed in 10 ml of sterile water and the spore concentration (1×10^5 spores/ml) was adjusted with a haemocytometer (Bautista-Rosales *et al*., 2015) [2].

After purification of pathogens, pathogenicity was tested for proving Koch's postulates. Grapes were surface sterilized with 70% ethanol and washed with several changes of sterile distilled water. Spore suspension (6×10^5 spores/ml) of 2 weeks old culture of *Rhizopus* sp. was prepared and artificially inoculated on the fruit in the laboratory by pin prick method. A minimum of three replications was done to check the pathogenicity of each isolate. The inoculated fruits were placed inside the polythene bag to maintain high humidity and a control was maintained by inoculating water and incubated for 7 days. The fungi from the infected fruits were reisolated and the characters were compared with the original isolate and the Koch's postulates were proved.

Dual culture assay

Yeast isolates were tested against *Rhizopus* sp. by dual plate method. In this experiment, the yeast isolates were streaked from 1cm away from edge of the plate and the mycelial disc of the pathogen is placed on the opposite side. Plates were incubated at room temperature (28±2ºC) for ten days. A plate inoculated with pathogen alone served as a control. After ten days, radial mycelial growth reduction was calculated when compared to control as follows %I (Percentage of inhibition) = (C-T/C) 100, where C - Radial growth measurement in control and T - Radial growth of the pathogen in the presence of yeast strains (Pentelides *et al*., 2015) [13].

Effects of volatile organic compounds (VOCs) from yeast against *Rhizopus* sp.

Yeast produced antifungal volatile compounds which inhibited the growth of the pathogen. Effective yeast isolates selected from dual plate assay, were streaked on the plate containing PDA medium and incubated for 48 hrs at room temperature (28±2ºC). Mycelial disc of *Rhizopus* sp. was placed on a separate plate containing PDA medium. The two plates were covered together face to face without air leakage at the edge by Parafilm, in which there is no physical contact between pathogen and the yeast. The plate without yeast culture was used as a control. The experiment was carried out in a completely randomized design with three replications. The mycelial growth reduction of the pathogen over control was measured 10 days of incubation (Nally *et al.*, 2015 and Parafati *et al*., 2015) [11, 14].

***In vivo* screening of antagonistic yeast against *Rhizopus* sp. Wound site colonization of yeast against *Rhizopus* sp. on grapes**

The antagonistic activity of yeast was tested *in vivo* by wound site colonization assay. Selected yeast isolates from dual culture assay were grown on YEPD broth for 48 hrs. The yeast population was adjusted to 10^6 CFU/ml (Cordero-Bueso *et al.*, 2017) [5]. Healthy berries of table grapes cv. Thomson seedless were collected from the market. The berries were surface disinfected with 70 percent ethanol and rinsed three times with sterile water. Wounds were made on the berries by pin prick method and the berries were immersed in the yeast suspension (10^6 CFU/ml) and incubated for 24 hrs. Spore suspension (1×10^5 spores/ml) was then inoculated into the wound and berries inoculated with pathogen alone were used as a control. The berries were incubated for 7 days and the experiments with three replications were repeated at least twice. The percentage of fungal growth inhibition was determined 7 days after pathogen inoculation, using the formula; 100- [(diameter of fungal growth on berry treated with yeast/without yeast) * 100] (Cordero –Bueso *et al*., 2017 and Pentelides *et al*., 2015) [5, 13]. The disease severity was evaluated by visual score "1-to-4" as suggested by Parafati *et al*., 2015 [13], (1- no visible symptoms, 2-soft rot, 3-formation of mycelium, 4-sporulation of mold).

Statistical analysis

Experimental datas were statistically analyzed using analysis of variance (ANOVA) and the SPSS version 17.0. The treatment means were separated at 5% significance level using Duncan's Multiple Range Test (DMRT).

Results

Isolation of pathogen and pathogenicity

Five *Rhizopus* sp. was isolated from rotted grapes from different market regions of Madurai district *viz*., Othakkadai, Simmakkal and Mattuthavani and Erode district *viz* Anthiyur and Vellithiruppur of Tamil Nadu. The pathogen was subcultured and maintained in the pure culture. Pathogenicity test by pin prick method in grapes cv. Thomson Seedless revealed that GR1 isolate was found to be more virulent (83.20 %) and GR3 was found to be least virulent (63.78 %) (Fig. 1).

Isolation of yeast from the fructosphere of grapes

Among the thirty-three yeast isolates, fifteen yeast antagonist was isolated from the samples collected from the market and eighteen yeast antagonist were isolated from the field. The yeast was isolated from different location of Tamil Nadu *viz*., Othakkadai, Mattuthavani and Simmakkal market of Madurai district and Anaimalayanpatti, Ammapatty, Gokilapuram, Cumbum, Rayappanpatti and Anaipatti fields of Theni district. One isolate was collected from Anthiyur market of Erode district. The yeast isolates were subcultured, maintained as pure culture and used for further studies.

Dual culture assay of yeast against *Rhizopus* sp.

Results of dual culture experiments revealed that none of the yeast isolates completely inhibited the growth *Rhizopus* sp., but some of the yeast isolates inhibited the mycelial growth upto 6 days. Among the 33 yeast isolates, ten isolates *viz*., YBB3, YBM2, YBAR2, YSL5, YSL3, YBB2, YBM3, YAK2, YCSL1 and YCSL2 showed maximum reduction of the mycelial growth of *Rhizopus* sp. Among them, YCSL2 recorded 30.33 percent inhibition of the mycelial growth over control followed by YCSL1 which recorded 30.00 percent inhibition over control (Table 1).

Effects of volatile organic compounds (VOCs) against growth of *Rhizopus* sp.

The volatile organic compounds of effective yeast isolates

from the dual culture were further tested against *Rhizopus* sp. Among them, YCSL2, YCSL1 and YBM3 produced volatile compounds which inhibited the growth of *Rhizopus* sp. upto 24.44%, 23.33% and 22.22% resp. ectively when compared to control. YSL3 and YSL5 doesn't inhibited the mycelial growth of *Rhizopus* sp. which indicates that these isolates do not release the volatile compounds (Table 2, Fig 2).

Wound site colonization of yeast against *Rhizopus* sp. on grapes

The effective yeast strains were tested against *Rhizopus* sp. under *in vivo* condition through wound site colonization. The result revealed that, among the ten isolates, YCSL2, YCSL1, YBM3 and YBAR2 recorded 71.1%, 55.6%, 53.3%, and 44.4% percent inhibition of *Rhizopus* sp. respectively against control (Fig 3).

Discussion

Post-harvest losses due to biotic agents are major concern in grapes cultivation which reduces the market value and cause economical loss to the farmers. Post–harvest fruit rot diseases are usually controlled by the application of chemical fungicides by the farmers. Biocontrol is the application of selected microorganism with antagonistic activity against other microorganism and large scale use of biocontrol reduces the ill-effects of chemical pesticides on human health and environment (Wilson and Wisniewski, 1989) [19]. In this paper, epiphytic yeast was isolated from grapes fruit surface and assayed for their potential ability on the biological control of *Rhizopus* sp. The usage of microbial agent isolated from fruits and vegetables is a basic approach for the biological control of plant diseases (Pantelides *et al*., 2015) [12]. Yeast was able to colonize the grapes fruit surface and competes for space and nutrition with other microorganism present on grapes and as such considered as good biocontrol agent (Nadai *et al*., 2018) [10]. In our study, thus yeast isolates *viz*., YCSL2 and YCSL1 was found to be effective in reducing the mycelial growth of *Rhizopus* sp. Saravanakumar *et al*., 2008 [15] reported that yeast strain *Metschnikowia pulcherrima* outcompetes *Botrytis cinerea*, *Penicillium expansum* and *Alternaria alternata* through iron depletion in apple. Strains belonging to the species *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Metschnikowia pulcherrima* and *Aureobasidium pullulans* isolated from different food sources were tested *in vitro* as biocontrol agents (BCAs) against the post-harvest pathogenic mold *Botrytis cinerea* (Parafati *et al*., 2015) [14]. *Kloeckera apiculata* (strain 34-9) isolated from citrus fruit, has been reported to be effective in controlling *Penicillium italicum* and *Botrytis cinerea (*Liu *et.al*, 2013) [9]. Many yeast spp. *viz*., *Pichia guilliermondii Candida musae*, *Issatchenkia orientalis* and *Candida quercitrusa* inhibited the development of *Colletotrichum capsici* in chilli pepper (Chanchaichaovivat *et al*., 2007) [4].

Volatile organic compounds from yeast were also found to play an important role in biological control. Yeast releases some of VOCs compounds *viz*., acetate and ethyl acetate during storage of cereals which was found to be effective against molds (Fredlund *et al*., 2004) [6]. Yeast also release volatile compounds *viz*., acetic acid and H_2S which inhibited the mycelial growth and spore production of mold fungus (Cordero-Bueso *et al*., 2017) [5]. In the present study, yeast strains YCSL2 produced VOCs which inhibited the mycelial growth and spore production of *Rhizopus* sp. on PDA medium. This is in accordance with Adel, 2004[1] who reported that, Yeast VOCs production possess effective antagonistic activity against pathogens. In our study, wound site colonization study revealed that yeast isolate YCSL2 colonized the surface of grapes and competes for food and nutrition. Nadai *et al*., 2018 [10] reported that yeast attains maximum growth within one day of inoculation and compete with the pathogen for food and nutrition. Yeast are also having mechanism of hyperparasitism in which the yeast cells colonize the mycelium of the pathogen and reduces the growth of mycelium (Wisniewski *et al*. 1992) [20]. In conclusion, the yeast YCSL2 strain identified in this study is an effective biocontrol agent against *Rhizopus* sp. The selected yeast possess antagonistic activity and affects the growth of the pathogen but it is necessary to test the effect under field condition. Information related to mode of action needs to be taken into account for further studies, especially with relation to formulation, large scale production and application in vineyards.

Table 1: Antifungal activity of antagonist yeast isolates against the mycelial growth of *Rhizopus* sp. (GR1) under *in vitro* condition

S. No	Treatments	Radial mycelial growth (cm)*	Inhibition over control (%)
1	YB1	7.00	22.22^{fgh}
2	YB2	7.40	17.78^{def}
3	YB3	7.17	20.33^{fgh}
4	YB4	7.93	11.89^{c}
5	YB5	6.87	23.67^{ghi}
6	YG1	7.43	17.44^{de}
7	YG2	6.90	23.33^{fgh}
8	YG3	6.83	24.11^{ghi}
9	YG4	6.90	23.33^{fgh}
10	YG5	7.40	17.78^{de}
11	YSL1	7.63	15.22^{d}
12	YSL2	6.93	23.00^{fgh}
13	YSL3	6.43	28.56^{jklmn}
14	YSL4	6.87	23.67^{ghi}
15	YSL5	6.67	25.56^{hijkl}
16	YBM1	6.40	28.89^{klmn}
17	YBM2	8.50	05.56^{b}
18	YBM3	6.33	29.67^{lmn}
19	YDJ1	7.30	18.89^{de}
20	YDJ2	7.13	20.78^{efg}
21	YDJ3	6.80	24.44^{hijk}
22	YBB1	6.87	23.67^{ghi}

23	YBB2	8.13	09.67^{c}
24	YBB3	6.70	25.89^{hijkl}
25	YAK1	6.80	24.44^{ghi}
26	YAK2	6.40	28.89^{lmn}
27	YAK3	7.00	22.22^{fgh}
28	YCSL1	6.30	30.00^{mn}
29	YCSL2	6.27	30.33^{n}
30	YCSL3	6.90	23.33^{ghi}
31	YBAR1	6.97	22.56^{ghij}
32	YBAR2	6.37	29.22^{lmn}
33	YBAR3	6.57	27.00^{ijkl}
34	Control	9.000	00.00^{a}
CD (p=0.05)			0.30

*Mean of three replications

[a]Means with same letter do not have significant difference according to Duncan's multiple range test at $p<0.05$.

Table 2: Effect of volatile compounds produced by yeast against *Rhizopus* sp. (GR 1) under *in vitro* condition

S. No	Yeast isolates	Mycelial growth (cm*)	Inhibition over control (%)
1	YAK2	8.5	5.56^{b}
2	YBAR2	7.7	14.44^{d}
3	YBAR3	8.0	11.11^{c}
4	YBB3	9.0	00.00^{a}
5	YBM1	8.3	07.78^{b}
6	YBM3	7.0	22.22^{e}
7	YCSL1	6.9	23.33^{e}
8	YCSL2	6.8	24.44^{e}
9	YSL3	9.0	0.00^{a}
10	YSL5	9.0	0.00^{a}
11	CONTROL	9.0	0.00^{a}
CD (p=0.05)			0.25

*Mean of three replication

[a]Means with same letter do not have significant difference according to Duncan's multiple range test at $p<0.05$.

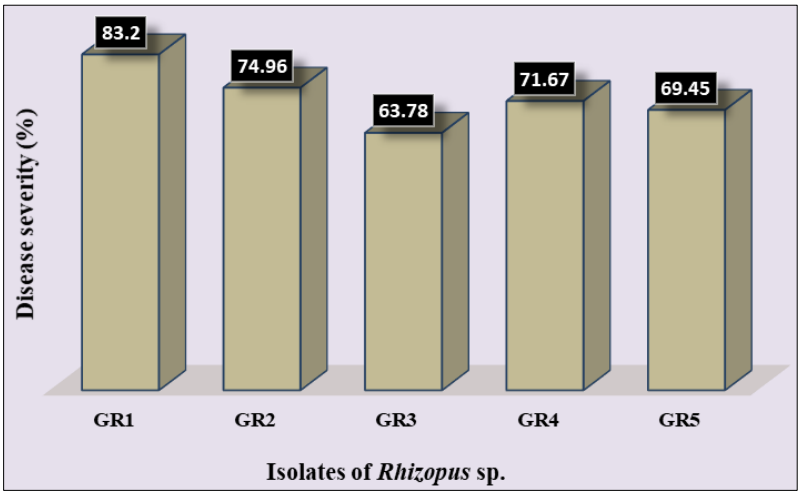

Fig 1: Virulence of different isolates of *Rhizopus* sp. in grapes cv. Thomson Seedless

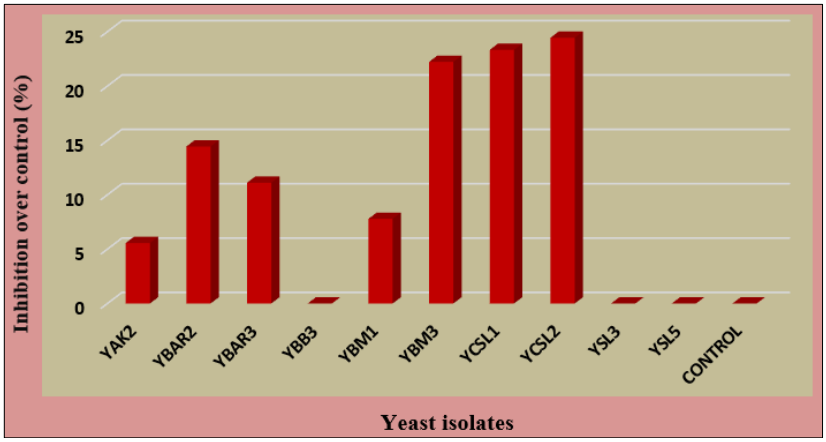

Fig 2: Effects of volatile organic compounds (VOCs) against growth of *Rhizopus* sp.

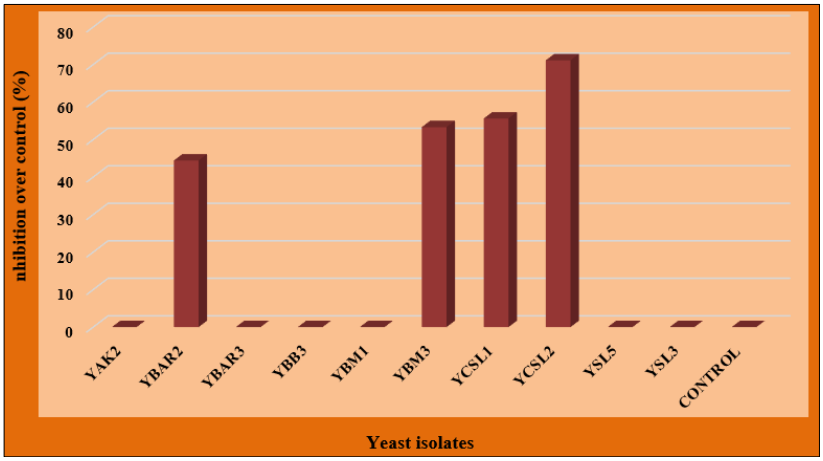

Fig 3: Wound site colonization of yeast against *Rhizopus* sp. on grapes

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