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Endophytic Yeast *Rhodotorula rubra* Strain TG-1: Antagonistic and Plant Protection Activities

Nailya Akhtyamova[1*] and Rano K. Sattarova[2]

[1]*Institute of Genetics and Plant Experimental Biology of Academy of Sciences, Tashkent, Uzbekistan*

[2]*Tashkent State Agrarian University, Uzbekistan*

Abstract

Red yeast antagonist *Rhodotorula rubra* strain TG-1 was isolated from the tissue of sample rice plant in Uzbekistan. Strain TG-1 was transferred into plant vascular system by stem inoculation into rice plant. The antimicrobial activity of strain TG-1 against several pathogenic fungal and bacterial plant pathogens, including *Fusarium* genus strains and *Xanthomonas malvacearum* strains were determined. The combination of yeast strain TG-1 as a pathogens antagonist and the exfoliated vermiculite is able to increase the growth stimulating property on the cotton seed, and protect the plant from diseases. From the red pigmented TG-1 strain, was isolated non-pigmented stable mutant, which acquired yeast vigor ability. Plant tissue may emerge as a potential source for novel endophytes with a broad spectrum of inhibitory activity against pathogens.

Keywords: Red yeast; Niche; Pathogens; Antimicrobial activity; Exfoliated vermiculite; Cotton; Non-pigmented yeast

Introduction

Yeasts *Rhodotorula species* are ubiquitous saprophytic organisms that can be isolated from environmental sources: soil, fresh water, air samples, milk, salad, fruit juice, on shower curtains and toothbrushes [1,2]. Representatives of *Rhodotorula* genus may be found on plants, or within plants. The endophytic colonization of apple shoots *Rhodotorula* sp. was determined [3]. *Rhodotorula taiwanensis* sp. has been isolated from a plant in Taiwan [4]. *Rhodotorula* glutinis has been found on a medium containing coconut water [5]. Two novel endophytic yeasts strains, *Rhodotorula graminis* and *Rh. mucilaginosa* have been isolated from within the stems of poplar (Populus) tree [6].

Rhodotorula genus yeast is the common pigmented environmental inhabitants that produce carotenoid pigment. Information about non-pigment isolates of *Rhodotorula* genus strains is not numerous. Such strains can be isolated from natural sources and can be propagated under experimental conditions. Non-pigmented new species of the genus *Rhodotorula* were isolated from permafrost soils of Eastern-Siberian Arctic [7]. Twenty stable mutants with various intensity of colors and non-pigmented mutants were obtained from yeast *Rhodotorula muchilaginosa*. The authors received such mutants by using nitrosoguanidine [8].

The reports about the *Rhodotorula species* as pathogens antagonists have been described. Two strains *Rhodotorula* glutinis with antagonistic effect upon *Penicillium expansum* was isolated from delicious apples [9]. Killer activity of *Rhodotorula* genus strains against *Candida* species was determined [10]. The yeast antagonist *Rhodotorula* glutinis (Fresenins) Harrison against *Botrytis cinerea* was isolated from the surfaces of strawberries [11].

Microbial contaminations of patients and the manufacture of drug products by several pathogens, including ubiquitous saprophytic yeasts *Rhodotorula species*, phytopathogenic *Fusarium* fungus species, conditional pathogenic bacterium *Enterobacter cloacae*, human opportunistic pathogen *Pseudomonas aeruginosa*, have been discussed in the 7-the year scientific Meeting on 23rd October, 2012 [12].

Rhodotorula genus not only isolates pathogens antagonists from pathogens. Several strains that were isolated from different sources have growth stimulating effect on important agricultural crops. The effect of combination of soil yeast, *Rhodotorula* mucilaginosa, and fungus *Glomus mosseae* on growth of Cowpeas has been showed by Boby et al. [13]. The capacity of the soil *Rhodotorula* mucilaginosa exudates to increase the positive effect of fungus *Glomus mosseae* on soybean has been described [14]. Endophytic *Rh. glutinis*, some other endophytic yeasts and bacteria were isolated from grapevine. Isolated endophytes inhibited the growth of grapevine pathogenic fungi. Endophytes play its role as a plant growth promoter and protects against plant pathogens. It was also indicate that endophytes have the potential to play a future key role as biocontrol agents and biofertilizers [15].

We have studied the antagonistic and plant protection activities of red–pigmented yeast *Rhodotorula rubra* strain TG-1 and some properties of non-pigmented mutant from TG-1 strain.

Materials and Methods

Red yeast *Rhodotorula rubra* strain TG-1 and its non-pigmented mutant were isolated. Taxonomic identification of red yeast TG-1 strain was conducted by Kreger-van Rij [16]. Yeast suspension (1×10^6 cells) in a volume 0.5 ml was injected in the rice stem. Common media used for the cultivation of strain TG-1 include potato dextrose (glucose) agar, Sabouraud agar, wort agar and growth media with the plants extracts. The potential pathogenicity of yeast TG-1 strain was studied by intraperitoneal injection in mice with the doses: 1.6×10^6; 8×10^6; 40×10^6; 200×10^6 and 1×109, into a 6-8 week old mice per group. Yeast TG-1 strain culture filtrate were assessed for its antagonistic activities against 166 plant pathogens by hole-plate diffusion method. Saprophytic and phytopathogenic isolates from the museum

***Corresponding author:** Nailya Akhtyamova, Institute of Genetics and Plant Experimental Biology of Academy of Sciences, Tashkent, Uzbekistan, E-mail: team2186@gmail.com

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collection of National University of Uzbekistan (the Thallophytes and Microbiology Department of Tashkent State University, 1994), fungal and bacterial isolates from soil, potato, banana, cotton were tested. Saprophytic cultures of the *Bacillus, Sarcina, Arthrobacter genus*, fungi of *Penicillium, Trichoderma, Mucor* genus strains and phytopathogenic microorganisms: *Xanthomonas malvacearum, Erwinia genus strains, Fusarium oxysporum, F. moniliforme, F. vasinfectum strains, Verticillium dahliae* and *V. albo-atrum* strains were used. For the influence of TG-1 strain culture filtrate on the cucumber seed germination seeds (*Cucumis sativus* "Uloc") were soaked in the culture filtrate of yeast TG-1 strain for one, two and three hours. Two day-old culture filtrate of yeast *Rhodotorula rubra* strain TG-1 (initial concentration 106 cell/ml), separated from cell debris by centrifugation, and was concentrated using an Ehrist ALPHA 1-5 Freeze dryer. Effect of the TG-1 strain culture filtrates on the growth of AN-Bayayt-2 cotton (*Gossipium hirsutum*) seeds germination was studied by the Roll Towel (Rolled Towel) by treatment of seeds for two, three, five and six hours in culture filtrate of TG-1 strain. Germination of cotton seedlings was done below 10°C-12°C temperature under greenhouse conditions. Cotton seeds were soaked in sterile water (version 1), seeds were treated with Exfoliated Vermiculite (EV) that was saturated with yeast TG-1 strain (version 2), the seeds were treated by suspension culture of yeast TG-1 strain (version 3), and the cotton seeds were soaked in saturated vermiculite (version 4). Exfoliated vermiculite was received from Techservisevermiculite Ltd., Russian. Vermiculite particles size in the range from: more than 1.5; 1.0-1.5; 0.5-1.0; 0.3-0.5; 0-0.3; less than 0.01 were tested. The weight ratio of EV to cotton seeds was 1:5; 1:7; 1:10. Data of triplicate experiments were analyzed using Microsoft Excel 2010.

Results and Discussion

Yeast *Rhodotorula rubra* strain TG-1

Red pigmented yeast was isolated from the tissue of samples rice plant in Uzbekistan. From this observation, it was determined that red yeast isolate was able to be transferred in the vascular system by stem inoculation into rice plant. The living cells of red yeast culture by cytomorphological study of the tissue stem and the leaves of the rice plant were found. According to their morphological, cultural, physiological, biochemical properties, red pigmented yeast was identified as *Rhodotorula rubra* strain. Identification index is TG-1 (Institute of Genetics and Plant Experimental Biology of Academy of Sciences, Tashkent, Uzbekistan). TG-1 strain cells are round and oval, the size of yeast cell is 2.5-4.5×5.0-11.5 mm. Yeast TG-1 strain forms coral-red colonies, which are smooth and round. *Rhodotorula rubra* strain TG-1 accumulated biomass well. TG-1 strain was able to form a primitive pseudomycelium after 8-9 days incubation on the growth media with the plants extracts. *Rhodotorula rubra* strain TG-1 is not pathogenic. Intraperitoneal injection of TG-1 strain suspension 1.6×10^6; 8×10^6; 40×10^6; 200×10^6; and 1×10^9 was injected into the blood stream of mice. The persistence of TG-1 strain in the blood of mice was not observed until 10th day. After 10-14 days, all mice were live. Blood sample and tissue of mice spleen gave negative results.

Antagonistic activity of TG-1 strain

Antimicrobial activity of *Rhodotorula rubra* strain TG-1 culture filtrate was tested against 112 of museum collected saprophytic and phytopathogenic fungal and bacterial strains, by the hole-plate diffusion method on potato dextrose agar plate (Table 1). The best antifungal activity of TG-1 culture filtrate was detected against *Fusarium vasinfectum*. Among the four genus fungi, the *F. vasinfectum* strains were more susceptible. Among bacterial genus strains, the *Xanthomonas malvacearum* (nine strains) was more susceptible to the TG-1 strain culture filtrate. Antagonistic activities of TG-1 strain culture filtrate on other significant important fungal agricultural crops: *Penicillium spp., Verticillium dahlia, V. albo-atrum* and *Trichoderma spp*. strains were established. *Fusarium* genus species are among the most economically important phytopathogenic and mycotoxigenic fungi in the world. We tested *Fusarium oxysporum* (24 strains) and *F. moniliforme* (11 strains) for their susceptibility to the yeast TG-1 strain, and its culture filtrate by agar diffusion assay. 100% growth inhibition against all tested *Fusarium* genus strains, with the zone of inhibition ranging from 20-26 mm by the hole-plate diffusion method was observed. Antagonistic activity of TG-1 strain and its culture filtrate are shown in figures 1-3.

Fungicidal effect of yeast TG-1 strain on *Fusarium oxysporum* (6 strains) and *F. moniliforme* (3 strains) under two years and fungistatic effect on other thirty nine *Fusarium* genus strains were observed.

Among the 72 bacterial pathogens, the strong inhibitory effect of TG-1 strain culture filtrate against 9 museum *Xanthomonas malvacearum* strains was observed. Bacteria *Erwinia* genus that are cause of important economic species were also susceptible to the culture filtrate of TG-1 strain. Antagonistic activity of culture filtrate of TG-1 strain, with the zone inhibition ranging from 14 to 17 mm was detected.

All 19 tested *Xanthomonas malvacearum* strains that were isolated from infected cotton plants were susceptible to the yeast TG-1 strain and its culture filtrate. Fungistatic effect on *X. malvacearum* strain by the agar block diffusion method and hole-plate diffusion method was determined. Inhibition zone by hole-plate diffusion method, with the zone of inhibition ranging from 20 to 24 mm was observed. Antagonistic activities of TG-1 strain and its culture filtrate are shown in figure 4 and 5.

The antifungal and antibacterial activities of endophytic yeast strain *Rhodotorula rubra* strain TG-1 and its culture filtrate against several pathogens of agricultural crops, including *Fusarium* genus strains and

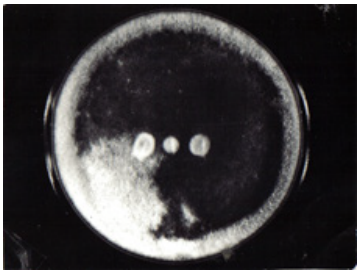


Figure 1: The inhibitory effect of yeast *Rhodotorula rubra* strain TG-1 on *Fusarium oxysporum* strain.

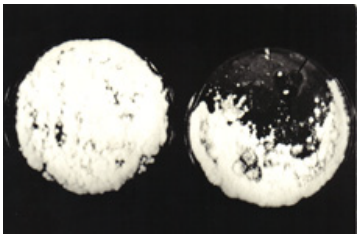


Figure 2: The antifungal activity of TG-1 strain culture filtrate on *F. oxysporum* strain.

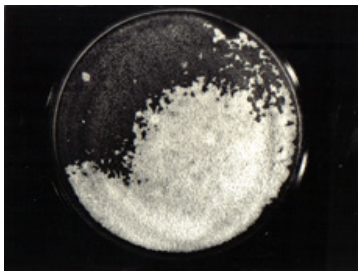


Figure 3: The inhibitory activity of TG-1 strain against *F. moniliforme* strain.

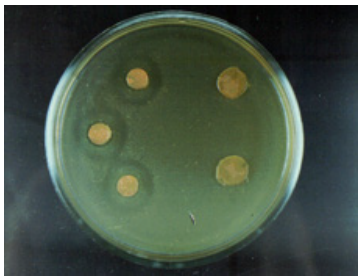


Figure 4: Antibacterial effect of yeast TG-1 strain against *Xanthomonas malvacearum* strain (on the left), (control-the block of growth media to the right).

Figure 5: Antibacterial activity of TG-1 strain culture filtrates against *Xanthomonas malvacearum* strain (to the right), control hole on the left.

Test cultures	Zone of inhibition (mm)
Fusarium vasinfectum	18.33 ± 2.41
Penicillium spp.	15.67 ± 3.05
Verticillium dahlia	13.33 ± 1.50
Verticillium albo-atrum	12.33 ± 2.08
Trichoderma spp.	12.00 ± 2.64
Xanthomobas malvacearum	17.67 ± 2.49
Erwinia spp.	15.33 ± 1.53
Artrobacter globiformis	11.33 ± 2.08
Bacillus spp.	10.67 ± 2.51
Sarcina spp.	9.67 ± 1.53

Table 1: Antagonistic activity of *Rhodotorula rubra* strain TG-1 culture filtrate on the plant pathogens.

Xanthomonas malvacearum were established [17]. Yeast TG-1 strain with the ability to reduce pathogen population can be considered as a potential biological agent.

Non-pigmented mutant *Rhodotorula rubra*

Non-pigmented mutant was received from the native red pigmented *Rhodotorula rubra* strain TG-1. We obtained such mutant without chemical treatment. The steps for receiving a non-pigmented W mutant from red TG-1 strain included seven passages.

TG-1 culture can form mixed colonies with red pigment, and colourless colonies in the 1th-4th passage under some conditions. Colonies of yeast culture on a petri dish were characterized as slime round colonies, and had the ability to accumulate a primitive pseudomycelium. Microscopic examination showed oval to monomorphic round cells. We observed stable, colorless white colonies, chiefly from the 5th passage. We received total stable non-pigmented mutant only with round cells at the 7th passage. Obtained non-pigmented yeast strain was marked as NIA-7-W. We can see native red colonies TG-1 strain and its non-pigmented mutant NIA-7-W on the petri dishes (Figure 6 and 7).

It has been determined that NIA-7-W mutant preserved antagonistic activity on two significant agricultural pathogens-*Fusarium* genus strains and *Xanthomonas malvacearum*. The inhibition ranges of native TG-1 strain and its mutant on these plant pathogens are the same. These results indicate that antimicrobial activity of native red pigmented *Rhodotorula rubra* strain TG-1 has no connection with the pigment. We didn't tested non-pigmented mutant against other plant pathogens.

Antibacterial activity of NIA-7-W mutant against *Xanthomonas malvacearum* strain is shown in figure 8 and 9.

It was important that non-pigmented mutant NIA-7-W acquire yeast vigor ability. The native red pigmented *Rhodotorula rubra* strain TG-1 had no such ability.

Yeast TG-1 strain for plant protection

For starting healthy seeds: Experimental study of the effect of TG-1 strain culture filtrate on the cucumber seeds germination showed significant positive impact on seeds. We have received the best effect

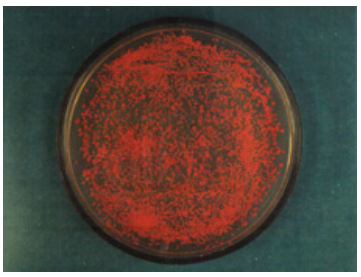


Figure 6: *Rhodotorula rubra* strain TG-1 growing on potato dextrose agar plate.

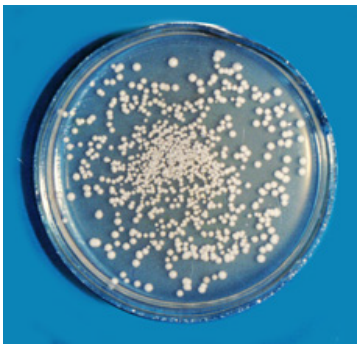


Figure 7: Non-pigmented mutant NIA-7-W growing on potato dextrose agar plate.

on the cucumber seeds with two hours of seeds treatment. 100% germination of healthy cucumbers seeds was obtained. Microscopic study of the roots seeds showed pure seeds without seed-borne pathogens. 91% of the cucumbers seeds were germinated for one hour and 94% for three hours and observed. Control seeds that were soaked in sterile water for one, two and three hours were infected by *Fusarium* spp. Culture filtrate can be used for giving jump start to seeds. The perfect germination rate of culture filtrate of yeast TG-1 strain on seeds germination is given in figure 10.

The positive outcome was obtaining one of the most economic vegetable crops.

Figure 8: Antibacterial activity of non-pigmented mutant NIA-7-W on *Xanthomonas malvacearum* strain.

Figure 9: Inhibitory activity of culture filtrate non-pigmented mutant NIA-7-W on *X. malvacearum* strain (to the right), the control hole (on the left).

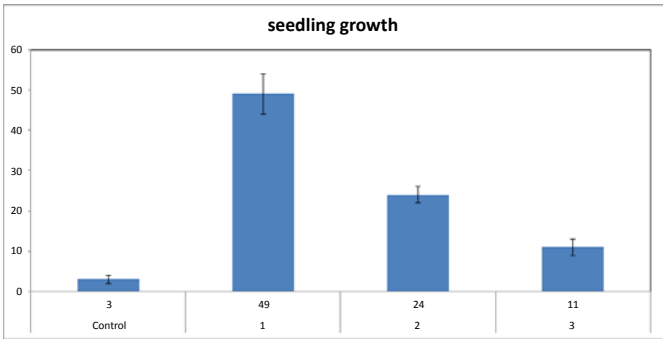
Figure 10: Effect of treating cucumber seeds with culture filtrate of TG-1 strain, (to the right). Control hole on the left.

Yeast TG-1 strain and mineral: The ability of TG-1 strain culture filtrate for the cotton seeds growth promotion by the roll towel method was determined. The plant growth stimulating effect on the seeds germination (85%) was observed with seeds treatment for five hours. Germination percentage ranged from 65%, 72% and 81% were respectively for two, three and six hours treatments. Control cotton seeds germination was 38%. These experiments were conducted at room temperature in the laboratory condition. Specialists recommend that planting cotton seed should be on soil temperature, average 25˚C-30˚C. We have developed the method of cotton seeds germination and planting cotton seeds in low temperature (below 10°C-12°C) for the region with continental climate. The cotton seeds were treated by the composition of yeast TG-1 strain and mineral vermiculite [18].

Vermiculite is a natural mineral that is used for germination, planting and sowing seeds. Exfoliated Vermiculite (EV) particles are lightweight and are capable of holding several times its own weight in water. Our method included the ability of yeast *Rhodotorula rubra* TG-1 strain to act as pathogens antagonist having useful properties of exfoliated vermiculite. Root development during the early vegetative stage by low temperature below +10˚C to +12˚C was determined. The cotton root growth during germination and seedling was established. The investigation was performed under greenhouse conditions.

Significant level of the length of cotton roots growth was observed in all three experiment versions (Graph 1). We observed very weak cotton root growth in control, 3 mm (version 1). Cotton seeds treated of TG-1 strain and vermiculite of 49.0 ± 3.0 (version 2). Seeds that were treated of TG-1 strain culture filtrate of 24 ± 2.0 (version 3). Cotton seeds soaked in saturated vermiculite of 11.0 ± 2.0 (version 4). The results showed that culture filtrate was found effective to enhance root length percentage, compared to control (soaked in water). Seedlings appeared healthy with no observable disease symptoms. Microscopic study of the shoot, roots seeds showed pure young seedlings without seed-borne pathogens. The influence of vermiculite, particularly on the treatment of seeds, indicate that 95% germinating seedlings on combining with yeast strain TG-1 can receive vermiculite particles every 0.3-0.5 mm. The composition of yeast TG-1 strain and vermiculite can increase germination ability, for obtaining a healthy stand of cotton root seedlings.

Vermiculite particle size less than 0.01-0.03 mm and more than 1.5 mm displays bad absorption properties. We observed 80% and 65% of seeds germination, accordingly. The best vermiculite: seeds weight ratio was 1: 10. The first true leaf emergence was about 11 days after seedlings were established. 90% first cotyledons and 80% true leaf



Graph 1: Significant level of the length of cotton root's growth: (version 1) Control; (version 2) Cotton seeds treated with TG-1 strain and vermiculite; (version 3) Seeds that were treated withTG-1 strain culture filtrate; (version 4) Cotton seeds soaked in saturated vermiculite.

were obtained by the combination of yeast antagonist and exfoliated vermiculite. This method promoted the initiative of leaf primordia and their development to lateral roots in the experiment. No visible molds were observed and bacterial growth on roots and seeds without any symptoms of infection was obtained. The composition yeast antagonist and natural vermiculite showed higher resistant to the pathogens and helped preventing from diseases. Germination and seedling emergence was completed and the plant begins its active vegetative growth. Such composition will be able to be used as the biological method for plant protection. Such method provides an effective way for important agricultural crops seeds planting into soil under low temperature in the region with continental climate.

Conclusion

Yeast strain *Rhodotorula rubra* strain TG-1 was isolated from the tissue of rice plant. TG-1 strain was successfully able to transfer into the plant vascular system by plant stem inoculation.

Antagonistic activity of TG-1 strain on several plant pathogens groups was determined. *Fusarium* genus strains and *Xanthomonas malvacearum* strains were most susceptible to the strain TG-1, among all tested saprophytic and phytiopathogenic fungi and bacteria.

Culture filtrate of strain T-1 showed the positive effect on cucumbers seeds. 100% germination of healthy cucumber seeds was obtained. The combination of yeast *Rhodotorula rubra* strain TG-1 and exfoliated vermiculite for cotton seed germination by low temperature below 10°C-12°C was developed. The combination allowed receiving a high percentage of cotton seed germination and significant positive effect on root length.

From native red–pigmented *Rhodotorula rubra* strain TG-1 was isolated non-pigmented mutant, which acquired yeast vigor ability. In our opinion, received non-pigmented mutant is one of yeast phase of life cycle of *Rhodotorula*. The sexual stages of strain, the red-pigmented yeast *Rhodotorula rubra* was discovered by Banno [19], and demonstrated a *Basidiomycetes* life cycle for some species of *Rhodotorula*.

Rhodotorula rubra strain TG-1 is endophytic yeast strain that had significant inhibitory effect against plant pathogens, and growth stimulating effect on some agricultural crops. The plant tissue may be the potential source of novel antagonists against fungal and bacterial pathogens.

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Conflicts of Interest

The authors have no conflict of interest.

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