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Detection and Identification of a Biocontrol Agent, *Rhodotorula mucilaginosa*, by a Dot Blot Hybridization Technique

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Abstract: The purpose of this study was to develop a molecular technique for rapid identification of biological control agent, strain S-33 of *Rhodotorula mucilaginosa*, of diseases of greenhouse crops. DNA from yeast cultures and from diseased plant tissue was extracted, amplified by PCR using primers specific to fungi and fixed to nylon membranes. Using sequence information obtained from the GenBank database, specific oligonucleotides were designed and after labeling with digoxigenin-d-UTP, were used as probes in a dot-blot hybridization assay. After preliminary testing with 5 probes, two of these probes were selected for further study. With probe RD5, a positive reaction was obtained with strain S-33 of *R. mucilaginosa* in pure culture and with fresh gummy stem blight (*Didymella bryoniae)* diseased plant tissue sprayed with *R. mucilaginosa*. One yeast isolate each of *Rhodosporidium toruloides*, *R. fluviale*, *R. babjevae*, *R. sphaerocarpum*, *R. kratochvilovae*, *R. azoricum,* one isolate of *Pichia anomala* and some common greenhouse pathogens were also tested using this assay. All other yeasts and pathogenic fungi tested gave a negative result. Cucumber stem tissue infected with *Didymella bryoniae* also gave a negative result. Uninfected, healthy cucumber stem tissue did not yield a positive result. The other probe (RD4) reacted with two species, *Rhodotorula mucilaginosa* and *Rhodotorula dairenensis*. The present dot blot assay can be used to identify biocontrol agent stain S-33 of *Rhodotorula mucilaginosa* in pure culture and in plant tissue.

Key words: Gummy stem blight, cucumber, *Didymella bryoniae*, *Rhodosporidium diobovatum*

INTRODUCTION

Yeast strain S-33 of *Rhodotorula mucilaginosa* (Jörgensen) Harrison is a biological agent for control of stem canker of tomato caused by the pathogen, *Botrytis cinerea* Pers. Ex Fr.[1]. Lesions produced by *B. cinerea* on tomato plants were significantly shorter compared with the inoculated control[2]. Plants treated with *Rhodotorula mucilaginosa* had significantly higher total fruit yield than those with the control. The number of dead plants were significantly lower in *Rhodotorula mucilaginosa* treated plants compared with the control. Yeast strain S-33 of *Rhodotorula mucilaginosa* also reduced lesion length caused by *Didymella bryoniae* (Auersw.) Rehm, causal agent of gummy stem blight on cucumber plants[3]. These results have shown that *Rhodotorula mucilaginosa* has the potential to control Botrytis stem canker on tomato and gummy stem blight on cucumber plants under greenhouse conditions[1-3]. Strain S-33 was isolated from apple fruit and identified as *Rhodotorula mucilaginosa* through conventional identification by Dr. John Bissett of Eastern Cereal and Oilseed Research Centre, National Fungal Identification Service, William Sanders Building, Ottawa, ON, Canada.

Molecular methods of identification are being developed which reduce time requirement for identification to a day or less. Sequence information of the internal transcribed spacers and ribosomal genes has been extensively used to develop rapid tests to differentiate and detect fungal organisms. Thus, PCR amplifications have been used to distinguish at the genus level among sugar beet pathogens and to specifically detect *Aphanomyces cochlioides* in diseased tissue[4]. Arrays of specific oligonucleotides which can screen a single sample for different pathogens simultaneously has been developed to detect and identify species of *Pythium* and *Phytophthora*[5]. *Sporisorium reiliana*, which causes head smut in maize, can be detected and identified using a dot blot hybridisation procedure on genomic DNA[6]. The dot blot procedure is useful for screening many samples for a particular organism. Prior amplification of a portion of the genome greatly enhances the sensitivity of

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a dot blot test[7]. PCR followed by dot blot hybridisation has been used to distinguish between pathogenic yeast species[8].

Conventional methods of identification of causal organisms or biological agents in diseased plant tissues require isolation of organisms in pure culture followed by microscopic examination or biochemical analysis and typically requires several days to weeks. Identification of these organisms depends upon availability of their reproductive structures, which can be difficult. Rapid identification of greenhouse pathogens such as *Botrytis cinerea* or *Didymella bryoniae* with dot blot technique has been achieved[9,10]. There is no rapid detection technique developed for a biological agent, *Rhodotorula mucilaginosa.* Biological control agent to be effective for these diseases, it is essential to monitor the population dynamics of pathogen x biocontrol agent interaction quickly and accurately on diseased tissue. The objective of this study was to develop a dot blot assay as a rapid means of detecting and identifying a biological control agent S-33 of *Rhodotorula mucilaginosa* in culture and plant tissue.

MATERIALS AND METHODS

Yeast and fungal species: Biological control agent *Rhodotorula mucilaginosa* was stored in a refrigerator at 0°C at the Pacific Agri-Food Research Centre, Agassiz, British Columbia (BC), Canada. Seven other yeast species were provided by J. P. Sampaino, Centro de Recursos Microbiologicos, Secao Autonoma de Biotecnologia, Faculdade de Ciencias e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal. They are *Rhodotorula dairenensis* comb. nov. (Hasegawa and Bano) Fell, Sampaino and Gadanho, *Rhodosporidium toruloides* Banno, *Rhodosporidium fluviale* Fell, Kurtzman. Tallman and Buck, *Rodosporidium babjevae* Golubev, *Rhodosporidium sphaerocarpum* Newell and Fell, *Rhodosporidium kratochvilovae* Hamamoto, and *Rhodosporidium azoricum* Sampaio and Gadanho (Table 1). *Pichia anomala* (E. C. Hansen) Kurtzman was provided by Fellipe Rodriguez-Vico, Departamento de Quimica-Fisica, Bioquimica y Quimica Inorg ã nica, Edificio C.I.T..E. I, Universidad de Almeria. La Canada de San Urbano, S/N. Spain (Table 1). Five fungal pathogens of greenhouse crops were also included in the experiment. They were: Gummy stem blight *Didymella bryoniae* (Auersw) Rehm (syn.: *Mycosphaerella melonis* (Pass) Chiu and Walker) from cucumber, gray mould pathogen *Botrytis cinerea* Pers. ex Fr. of tomato, damping off pathogen *Rhizoctonia solani* Kuhn, vascular wilt pathogen, *Fusarium oxysporum* Schlecht. and *Fusarium subglutinans* (Wollenweber and Reinking) Nelson, Toussoun and Marasas. Pure cultures of all fungal species were grown on Potato Dextrose Broth (PDB, Difco) at room temperature with shaking (100 rpm) for seven days. The resulting mycelium mats were collected in a Buchner funnel on filter paper, washed with sterile reverse osmosis water and used immediately for DNA extraction or frozen at -70°C. All yeast species were grown on Potato Dextrose Agar (PDA) plates for 6 days, then the yeasts were scraped off from plates with water for the DNA extraction.

Fungal and yeast samples from cucumber plants: Newly isolated gummy stem blight causal agent, *Didymella bryoniae,* from diseased cucumber plants was grown on PDA petri plates for 3 weeks and then an ascospore and conidial suspension was made with a spore concentration 1×10^6 mL^{-1}.

Cucumber plants were grown in the greenhouse of Agriculture and Agri-Food Canada at Agassiz in British Columbia, Canada. Cultivar Enigma was seeded on June 6, 2003 into rockwool cubes. Each plant received the complete nutrient solution [$Ca(NO_3)_2$ - 1005 g, $MgSO_4$-405 g, KH_2PO_4 - 270 g, KNO_3 - 825 g, KSO_4 - 54 g, micro nutients-150 mL (Plant Products, Brampton, ON), iron EDTA (7%) 75 mL and 160 mL of 5 m L^{-1} H_2SO_4 1000 L^{-1}. The pH of nutrient feed was adjusted to 6.0 and the Electrical Conductance (EC) was maintained at 2.0. Plants were watered with nutrient solution as necessary. All plants were grown in the greenhouse at 17 -21°C with 290 - 2200 Joules/cm^2 light and 60 - 80% relative humidity. Pesticides and biologicals were applied as required to control white flies, thrips and other insect pests as recommended in the Greenhouse Production Guide for Commercial Growers[11].

Stem wounds were made by pruning leaves off close (10 mm) to the stem, 2 per plant.

Fifteen plants were inoculated using the ascospore and conidial suspension of *D. bryoniae* and 5 control plants were sprayed with sterilized water. Ten-day-old cultures of *D. bryoniae,* isolated from infected cucumber plants from local greenhouse and grown on potato dextrose agar, were used to prepare the inoculum. The concentration of the *D. bryoniae* inoculum was 1×10^6 spores mL^{-1} determined by dilution plating. It was prepared as a suspension of spores in sterile distilled water and applied as a spray at 1.2 mL per wound. Eighteen days later when the stem gummy blight symptoms appeared fully, 10 diseased plants were sprayed with *Rhodotorula mucilaginosa* spore suspension and 5 healthy and 5 diseased plants were sprayed with sterile water. For spore suspension,

Table 1: Strains of yeast and fungal pathogen species studied, their strain no. origin and sources or hosts

Species	Strain	Origin	Isolated from
Rhodotorula mucilaginosa	S-33	BC, Canada	Apple fruit
Rhodotorula dairenensis	PYCC4885	Portugal	Air
Rhodosporidium toruloides	PYCC4416	Sweden	wood pulp
Rhodosporidium fluviale	PYCC4701	USA	Water
Rhodosporidium babjevae	PYCC5168	Moscow, Russia	Plant
Rhodosporidium sphaerocarpum	PYCC4104	Antarctica	Water
Rhodosporidium kratochivilovae	PYCC4583	Unknown	Unknown
Rhodosporidium azoricum	PYCC5062	Azores, Portugal	Soil
Pichia anomala	local	Spain	Soil
Didymella bryoniae	ATCC 56275	BC, Canada	Cucumber
Botrytis cinerea	local	BC, Canada	Tomato
Fusarium oxysporum	local	BC, Canada	Cucumber
Fusarium subglutinans	local	BC, Canada	Pepper
Rhizoctonia solani	local	Alberta, Canada	Potato

Rhodotorula mucilaginosa was cultured on PDA plates for 6 days at room temperature and then scraped off from the plates and the yeast spore suspension was made with a spore concentration 1 x 10^7 mL^{-1}. Eighteen days after the yeast spray 4 tissue samples were collected: 1 from healthy, 1 from diseased and 2 from diseased+ *Rhodotorula mucilaginosa* sprayed plants. For DNA extraction, 200 mg tissue of each sample was used.

DNA extraction and PCR: DNA was extracted from all species and yeast-inoculated or non inoculated cucumber plant tissue by using the FastDNA kit from BIO101(Vista, CA) and the extraction procedures were carried out following the manufacturer protocol for DNA extractions from fungi and yeasts. The extracted DNA was amplified with a thermal cycler (Robocycler from Stratagene, La Jolla, CA, USA) using the specific primers UNL028S22[12] and ITS1-F[13]. The sequences of the primers, which are specific to septate fungi and yeasts and amplify the ITS1, 5.8S ribosomal gene and ITS2 portions of rDNA are: 5' AACTTGGTCATTTAGAGGAAGTAA (SF-UP 18S70) and 5' GTTTCTTTTCCTCCGCTTATTGATATGG (UN-LO28S-22). PCR reactions were performed in 20 μL volumes with final concentrations of reagents: 1 x reaction buffer, 100 μM of each d-NTP, 500 nM of each primer and 1.25 U Taq polymerase (Gold Taq, DNAmp Ltd. Mississauga, ON), overlaid with one drop of mineral oil. Twenty-nine cycles of: 45 sec at 94°C, 45 sec at 58°C and 45 sec at 72°C were followed by a final extension of 10 min at 72°C. A positive control with *Rhodotorula mucilaginosa* DNA of the pure culture and a negative control of all ingredients plus sterile distilled water were included in each run. PCR products were analysed by electrophoresis of 1 μL aliquots on 1% minigels, stained in ethidium bromide solution bath (0.5 μg ethidium bromide per mL water) and visualized under UV light. The PCR product obtained was 633 bp long.

Preparation of probes: Nucleotide sequences for the ITS and ribosomal gene region were obtained initially from GenBank for *Rhodotorula mucilaginosa* and other related yeasts or fungi. To detect significant alignment, the BLAST procedure from the National Centre for Biotechnology Information (Bethesda, MD) was used. The short sequences unique to *Rhodotorula mucilaginosa* was selected and oligonucleotides with these sequences were obtained from Genosys (Sigma-Aldrich, Co., The Woodlands, TX). The oligonucleotides were labeled at 3' end with Dig-dUTP using the DIG Oligonucleotide Tailing Kit (Roche Diagnostics: Laval, Que., Canada). The efficiency of the tailing was measured by comparison of signal intensities produced by a chemiluminescent reaction, between that given off by a labeled control oligonucleotide included with the Kit and those given off by labeled nucleotides. Two oligonucleotides (RD4, 25 bases and RD5, 21 bases) unique to the sequenced sequence were chosen and labeled. The sequences for probes are: RD4-5' GGATAGTAACTCTCGCAAGAGGGC, RD5-5' GCCTTTAGGGTCTAGCTCGT and D6 for *D. bryoniae* - 5'CGCCGATTGGACAAAACTTAAA.

Hybridization reaction: Nylon membrane blots (Roche Diagnostics, Laval, Que., Canada) were prepared using a manifold device (Schleicher and Schuell, Manifold I, Keene, NH, USA). Five microliter aliquots of PCR product were denatured in 500 μL of 0.5 M NaOH for 5 min at 37°C, mixed with 500 μL 12XSSC (saline sodium citrate) and spotted as 200 μL aliquots onto membrane through the wells of the manifold device. Each spot contained 1 μL of PCR product equivalent to approximately 50 ng of DNA. The spotted membranes were immediately dried at 120°C for 30 min in order to fix the DNA onto the membrane. After cooling, the membrane was pre-hybridized 2.5 h in hybridization buffer composed of 6XSSC containing 1% Skim Milk Powder, 0.02% SDS and 0.1 mg mL^{-1} Poly(A) at a temperature, either 42°C or

48°C. The membranes were then hybridized overnight (about 16 h) in hybridization solution containing probe. The optimum probe concentration was 0.2 pmol labeled probe mL^{-1} of hybridization buffer. The hybridized membranes were followed by washes: twice for 10 m in 2XSSC, 0.02% SDS at hybridization temperature and twice for 10 min 0.1 XSSC, 0.02% SDS at the same temperature as above. Signal detection was done through chemiluminescent reaction. The membranes were incubated at room temperature for 30 min in a 1:20000 dilution of anti-digoxigenin-AP (Roche Diagnostics, Laval, Que.,Canada) and washed in accordance with the manufacturer's instruction. The CDP* substrate (Roche Diagnostics, Laval, Que., Canada) was used to generate the signal which then was captured on scientific imaging film (X-OMAT, Kodak, Rochester, NY).

RESULTS

Probe RD5 appears to be species specific to *Rhodotorula mucilaginosa* (Fig. 1, No. 11-14). With this probe, the test was negative for DNA obtained from the other eight yeast species: *Rhodotorula dairenensis* (Fig. 1, No. 5 and 6), *Rhodosporidium toruloides* (Fig. 1, No. 1), *Rhodosporidium fluviale* (Fig. 1, No. 3), *Rodosporidium babjevae* (Fig. 1, No. 4), *Rhodosporidium sphaerocarpum* (Fig. 1, No. 7 and 8), *Rhodosporidium kratochvilovae* (Fig. 1, No. 9 and 10), *Rhodosporidium azoricum* (Fig. 1, No. 2) and *Pichia anomala* (Fig. 1, No. 15 to 20). It was also negative for five main greenhouse crop fungal disease pathogens: *Didymella bryoniae* (Fig. 2, No. 8 and 9), *Botrytis cinerea* (Fig. 2, No. 4 and 7), *Rhizoctonia solani* (Fig. 2, No. 3), *Fusarium*

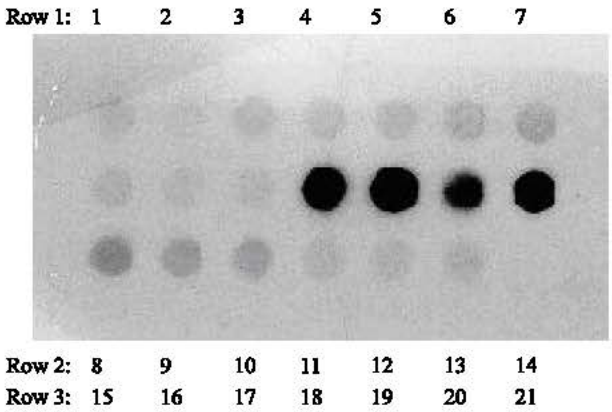

Fig. 1: Probe RD5 for *Rhodotorula mucilaginosa*. 1- *Rhodosporidium toruloides*, 2- *Rhodosporidium azoricum*, 3- *Rhodosporidium fluviale*, 4- *Rhodosporidium babjevae*, 5- *Rhodotorula dairenensis* (plate culture), 6- *Rhodotorula dairenensis* (liquid culture), 7- *Rhodosporidium sphaerocarpum* (plate culture), 8- *Rhodosporidium sphaerocarpum* (liquid culture), 9- *Rhodosporidium kratochvilovae* (plate culture), 10 - *Rhodosporidium kratochvilovae* (liquid culture), 11- *Rhodotorula mucilaginosa* (plate culture), 12- *Rhodotorula mucilaginosa* (liquid), 13- *Rhodotorula mucilaginosa* (diluted DNA 1:1), 14- *Rhodotorula mucilaginosa* (diluted DNA), 15- *Pichia anomala* (plate culture), 16- *Pichia anomala* (liquid culture), 17- *Pichia anomala*, 18- *Pichia anomala*, 19- *Pichia anomala*, 20- *Pichia anomala*, 21- water control

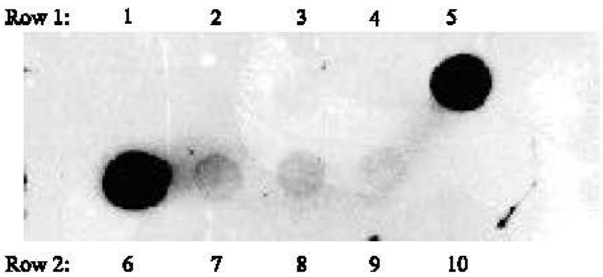

Fig. 2: Probe RD5 can differentiate *Rhodotorula mucilaginosa* from other greenhouse pathogens. 1- *Fusarium subglutinans*, 2- *Fusarium oxysporum*, 3- *Rhizoctonia solani*, 4- *Botrytis cinerea*, 5- *Rhodotorula mucilaginosa* (plate culture), 6- *Rhodotorula mucilaginosa* (broth culture), 7- *B. cinerea*, 8- *Didymella bryoniae* (plate culture), 9- *Didymella bryoniae* (broth), 10- Water control

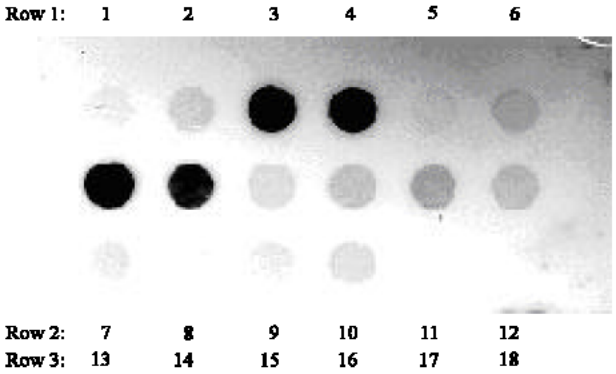

Fig. 3: Probe RD4 for *Rhodotorula mucilaginosa* and *Rhodotorula dairenenensis*. 1- *Rhodosporidium fluviale*, 2- *Rhodosporidium babjevae*, 3- *Rhodotorula dairenensis* (plate culture), 4- *Rhodotorula dairenensis* (liquid culture), 5- *Rhodosporidium sphaerocarpum* (liquid culture), 6- *Rhodosporidium kratochvilovae* (plate culture), 7- *Rhodotorula mucilaginosa* (plate culture), 8- *Rhodotorula mucilaginosa* (liquid culture), 9- *Rhodosporidium toruloides*, 10- *Rhodosporidium azoricum*, 11- *Pichia anomala* (plate culture), 12- Pichia *anomala* (liquid), 13- *Pichia anomala*, 14- *Pichia anomala*, 15- *Pichia anomala*, 16- *Pichia anomala*, 17- water control, 18- water control

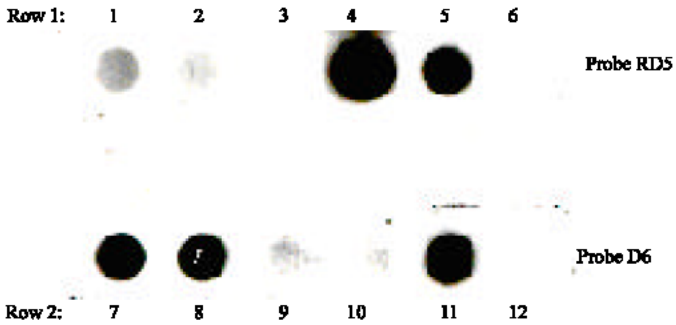

Fig. 4: Top row: Probe RD5 can detect *Rhodotorula mucilaginosa* in plant tissue. Numbers 1 and 5 - cucumber plant tissue infected with *Didymella bryoniae* that was sprayed with *Rhodotorula mucilaginosa*, 2 - cucumber plant tissue infected with *Didymella bryoniae* that was sprayed with water, 3 - healthy tissue, non inoculated, no. 4 - *Rhodotorula mucilaginosa* (plate culture), no. 6 - water control. Bottom row: Probe D6 for *Didymella bryoniae*. Numbers 7, 8, 11- cucumber plant tissue infected with *Didymella bryoniae*, 9 – cucumber pant tissue sprayed with water, 10 - *Rhodotorula mucilaginosa* (plate culture), 12 - water control

oxysporum (Fig. 2, No. 2) and *Fusarium subglutinans* (Fig. 2, No. 1) at 48°C hybridization (Fig. 2). Again, it was only *Rhodotorula mucilaginosa* that was positive on the membranes with this probe (Fig. 2, No. 5 and 6). Probe RD4 produced positive results on the membranes with both *Rhodotorula mucilaginosa* (Fig. 3, No. 7 and 8) and *Rhodosporidium dairenensis* (Fig. 3, No. 3 and 4).

RD5 effectively detected *Rhodotorula mucilaginosa* in cucumber plants infected with gummy stem blight pathogen, *D. bryoniae* and sprayed with *Rhodotorula mucilaginosa* (Fig. 4, top row Nos. 1, 4 and 5). Sample 1 and 5 were tissues from diseased lesions sprayed with *Rhodotorula mucilaginosa* and no. 4 was the pure *Rhodotorula mucilaginosa* plate culture (Fig. 4). Probe D6 that was developed for identification of *D. bryoniae* was effective to detect the cucumber gummy stem blight causal agent (Fig. 4, bottom row, No. 7, 8 and 11). Samples in no.7, 8 and 11 were *D. bryoniae* infected cucumber tissue. Samples in No. 2 (*D. bryoniae* diseased lesion without spraying *Rhodotorula mucilaginosa*), No. 3 (healthy tissue, non inoculated) and No.6 (water control) did not develop any dot blot (Fig. 4).

DISCUSSION

This technique, consisting of PCR amplification using fungal specific primers, followed by probing of the PCR product with a species-specific oligonucleotide probe in a dot-blot hybridization test, is a useful tool for the rapid identification of strain S-33 of *Rhodotorula mucilaginosa* in pure culture and in plant tissue sprayed with biocontrol agent, *Rhodotorula mucilaginosa*. Since prior isolation of pathogen from the plant tissue is not needed, the time required to obtain results from plant samples can be measured in hours rather than weeks. This assay would greatly reduce turn-around times for testing the presence of biocontrol agent in plants in the commercial greenhouses.

A dot blot assay can analyze many samples for a single organisms, as the PCR products from the samples are blotted on the membrane and hybridized to a single probe specific for the particular organism. *Sporisorium reiliana* and *Ustilago maydis,* which produce similar smut symptoms on maize were distinguished from each other by dot blot hybridisation of genomic DNA[6]. Dot blot hybridization was also a very reliable and cheaper tool for Phytosanitary Certification for virus infection in tomato plant[14]. PCR followed by dot blot hybridization has been used to distinguish between pathogenic yeast species[8].

The other probe tested, RD4, gave a positive result with *Rhodotorula dairenensis.* It is likely that this species may be misidentified. The other explanation would be that there is a 2 base pair mismatch of this oligonucleotide with *Rhodotorula mucilaginosa* at this location (all other species tested had a greater amount of mismatch). Increasing the stringency of hybridization did not remove the non-specific signal. A slightly shorter probe might improve the discrimination between these two species since the proportion of mismatch to match would increase, leading to a less stable duplex[15].

In conclusion, dot blot hybridization assay would allow rapid identification of a biocontrol agent S-33 of *Rhodotorula mucilaginosa* in pure culture and sprayed plants from growers' greenhouses and thus would help disease management to reduce economic losses. Probe RD5 identified S-33 of *Rhodotorula mucilaginosa* in cucumber plants, making it very useful for identification of this strain in this crop. It could also be used for monitoring the population of biocontrol agent on diseased plants for effective control of the disease as well as in epidemiological and etiological studies in infected plants.

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