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EFFECT OF OIL (CRUDE PETROLEUM) ON THE SURVIVAL AND GROWTH OF SOIL FUNGI

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ABSTRACT

Oil (crude petroleum) is the most common pollutant that adversely affects and deteriorates the soil environment. Our study is based on the assessment of the effects of various concentrations of oil on the survival and growth of soil fungi. The research carried out in this regard included, the selection of oil, sampling of oil- polluted soils, characterization of soil, simulated oil pollution of the soil, isolation, identification, characterization and screening of the fungal isolates. Viability tests were performed to check the survival of the fungi exposed to various concentrations of oil. Assessment of oil biodegradation was carried out by modified brown paper assay. The growth pattern and resistance potential of these isolates in varying concentrations of oil, benomyl (fungicide) and at increasing temperatures were also determined. Majority of the isolated fungi showed their growth in oil, utilizing it as a sole source of carbon. Changes in the growth pattern of these fungal isolates were also observed. It clearly indicates the potential of these fungi to be used for the bioremediation of the oil-contaminated sites without any nutrient amendment. These fungi also showed resistance against heavy metals, benomyl and high temperatures. This insinuates the importance of these stress resistant isolates in multipurpose biotechnological studies as well as in the remediation of the environment, polluted with toxicants other than oil i.e. pesticides and heavy metals etc.

Key words: Oil (crude petroleum), biodegradation, survival, growth pattern, and remediation.

INTRODUCTION

Effects of oil spills on health, environment, and economy are well documented in the literature. The quality of a particular environment is reflected by the microbial population of that area. Intimate relationship exists among physicochemical properties of soil, soil organisms and soil biological properties. The microorganisms and their activities have been extensively utilized in testing the effects of a specific chemical substance in soil, as well as in the studies of bioremediation of polluted soils (Obire and Nwaubeta, 2001). There is a big change in the balance of the bacterial community during the process of pollution (Baek *et al*., 2004) and changes also occur in characterization of microbial community during the course of the bioremediation efforts (Macnaughton *et al*., 1999). Crude oil is a complex mixture of thousands of hydrocarbons and non-hydrocarbon compounds, including heavy metals. Although the toxicity of each individual component is known, the toxicity of complex mixtures such as crude oils and refined products is extremely difficult to assess, because very limited information is available about the additive, synergistic, or antagonistic effects of various mixtures. In addition, the chemical composition of each crude oil and petroleum product varies significantly, and can have diverse effects on different organisms within the same ecosystem. These differences in toxic effects are due to qualitative compositional differences in various products, as well as concentration differences of chemical constituents (Baek *et al*., 2004). Some of the components of diesel oil are toxic for microorganisms but few may be used as nutrients, resulting into further degradation of the pollutants.

Populations of hydrocarbon-degraders normally constitute less than 1% of the total microbial communities, but when oil pollutants are present these hydrocarbon-degrading populations increase, up to 10% of the community (Atlas, 1995). If growth conditions are in accordance with the optimum requirements of the microorganisms to degrade the contaminant, faster bioremediation is achieved with increasingly active microbial population (Anderson and Anderson, 1996).

A wealth of information is available regarding the microbial utilization of hydrocarbons and crude oils by various treatments including, biremediation, bioaugmentation, biostimulation etc (Vinas *et al*., 2002; Odokuma *et al*., 2003; Pepper *et al*., 2002; Gentry *et al*. 2004). The present study aims at determining the effects of crude oil on the diversity and extent of fungi particularly challenging the indigenous soil fungi previously exposed to various metals. These isolated fungi will be used further in the forthcoming research.

MATERIALS AND METHODS

Oil (crude petroleum) was collected from Karachi oil refinery. Soil samples were collected from the local petrol Pump in Karachi. Soil samples were subjected to physicochemical analyses i.e. colour, structure, moisture content

and pH as described by Gupta, (2004). Clay pots were filled with petrol pump soil (150g/pot) and to these pots 0.5 and 1 % (w/w) oil was added. Soil without oil amendment was used as Control. Similarly slurry (oil sludge) was prepared by adding 25 ml oil into 100 g of soil in each pot. Control was also run side by side without any amendment. All the tests and controls were run in triplicate. From the first set of pots, isolation was carried out after 10 days of incubation. Standard plate count method was used as given by Pelczar *et al* (2003). Ten fold serial dilutions were made up to 10^{-5}. Sample (0.1 ml) from selected dilutions (10^{-2} and 10^{-4}) were spread on the respective culture media plates using a glass spreader. Media plates were then incubated at ambient temperature for 4– 5 days for fungi. Colonies were counted and cfu/gm was calculated by multiplying with dilution factor.

From the oily sludge, isolation was carried out after one week. Ten fold serial dilutions were made up to 10^{-5} and 0.1 ml from selected dilutions (10^{-3}, 10^{-4} and 10^{-5}) were spread on respective culture media plates by means of glass spreader. Inoculated plated were incubated at ambient temperature for 4 – 5 days for fungi. Soil extract agar (pH 5) (Rangaswami and Bagyaraj, 1996), Potato dextrose agar (pH 5) (Oxoid Manual, fifth edition), Malt extract agar (pH 5) (Difco Manual, ninth edition) were used for the Isolation of fungi. Colonies were counted and cfu/gm was calculated. Cultural and morphological characteristics were observed and the codes were assigned to these isolates. The isolates were maintained on respective media slants. Fungal isolates were identified (Barnett and Hunter, 1998; Barnett, 1960; Thom and Raper, 1945). For the screening purpose, some of the selective isolates were inoculated into modified soil extract broth (pH 5 and pH7) amended with 0.5 and 1.0 % (w/w) oil. The experiment was further divided into two batches (each run in triplicate). One batch was incubated at ambient temperature under stationary condition and the second at 37^{o}C in shaking water bath. The selective secondary isolates were again inoculated in the mineral salts medium and modified mineral salts solution containing 0.5 and 1.0 % (w/w) oil and Incubated. These isolates were inoculated in plain oil to be used as sole source of carbon and incubated at ambient temperature. From these inoculated media tests for oil biodegradation and viability were performed. In another experiment, the fungi isolated from oil and heavy metal polluted soil, were further inoculated into soil extract broth + glucose (0.1%w/v), soil extract broth + benomyl (5, 10 and 20 μg/ml), soil extract broth + oil (0.5 and 1 % w/w), soil extract broth + heavy metals (10, 25 and 50 mM).

Effects of varying temperatures on these screened isolates were also observed. These inoculated broth sets were incubated in the water bath for 20 minutes at 30 ^{o}C. Similarly each set was exposed to further elevated temperatures e.g. 35, 40, 45, 50, 55, 60 and 65 ^{o}C for 20 minutes. The isolates exposed to varying temperatures were then incubated at 30^{o}C for 4-5 days. Viability test was performed for the fungal isolates inoculated previously into modified mineral salts solution containing 0.5 and 1.0 % (w/w) oil. Loopful of samples were inoculated in the center of malt agar plates. The inoculated plates were then incubated at ambient temperature for 4-5 days. The same procedure was followed for plain oil inoculated with the fungal cultures previously. The oil biodegradation was assessed successively after 1, 4 and 8 days as method described by Williams, (1994). Cumulative data was collected, statistically analysed and graphs were plotted to represent the biodegradation. Any change in diameters greater than 2mm was taken as significant showing (degree of) biodegradation.

RESULTS AND DISCUSSION

The soil collected from petrol pump was Grayish black, silty, dry porous without aggregates, having moisture content of 2.13% (w/w) and with pH 6. Fungal cultures were identified as *Aspergillus variecolor* (RT21 and RT28), *A. luchuensis* (RT22, RT25, RT27, RT29), *A. restrictus* (RT23 and RT31), *A. micro-virido-citrinus* (RT 24 and RT33), *A. fumigatus* (RT26), *A. flavus* (RT30) and *Drechslera hawaiinsis* (RT32). Sutherland, (1992) isolated diverse species of fungi especially *Aspergillus* sp. from oil-contaminated sites which were capable of degrading oil.

All the fungi showed growth in soil extract broth amended with oil (1 and 0.5 % w/v) incubated at ambient temperature under stationary and shaking conditions. Since oil is a complex hydrocarbon, its degradation resulted in the change in consistency of oil and it was evident by the accumulation and condensation of oil in globules. Our findings are in agreement with Menn, *et al*. (1993), Guerin and Jones, (1988) that breakdown of aromatic compounds in oil produces brown/red/ chocolate brown colour. Culture RT15 was the only which didn't show any growth and was also found to be non-viable in mineral salt solution amended with 0.5 and 1% (w/v) oil. In majority of the cases, abundant growth, excessive reduction of oil and viability was observed in case of mineral salts solution amended with 0.5% oil. Although the petroleum hydrocarbons may also limit the accessibility of nutrients to microorganisms by reducing the availability of water in which nutrients are dissolved (Schwendinger, 1968; Larter *et al*., 2003; Wilhelms *et al*., 2004) but the fungi used in the present study must have utilized oil in order to satisfy their nutritional requirements. The possible reason may be gradual adaptation of fungi to increasing concentrations of oil. After 60 days of incubation, either reduction of oil was observed or accumulation of oil content in the form of viscous matter at the bottom of glass bottles was observed. In plain oil without any added carbon, minerals and

energy source, the fungal cultures were found to be viable. The consistency of oil was also changed. This implied that these fungal isolates are highly resistant ones that could survive in extremely limited nutritional condition and stressed environment so they can be used as successful candidates for the remediation of oil spills, without any amendments. Cultures RT6, RT8, RT9, RT15, RT16, RT17, RT18, RT24 and RT25 showed resistance to the oil, benomyl and higher temperature (Table 1; **Fig. 5**). The cumulative results of various treatments (oil, benomyl and varying temperature) so obtained will be discussed below.

Table 1. Effect of oil, benomyl and temperature on the growth of fungi.

Culture code	Soil extract broth + 0.1%w/v glucose	Soil extract broth + oil (%w/v)		Soil extract broth + benomyl (μg ml^{-1})			Temperature (°C)							
		0.5	1	5	10	20	30	35	40	45	50	55	60	65
Control	–	–	–	–	–	–	–	–	–	–	–	–	–	+
RT6	++	+++	+++	+	+	+	+++	+++	+++	+++	+++	+++	++	–
RT8	+++	+++	+++	+	+	+	+++	+++	+++	+++	+++	+++	+	+
RT9	++	+++	+++	+	+	+	+++	+++	+++	+++	+++	+++	+	+
RT15	+	+	–	+	+	+	+++	++	++	++	++	++	+	+
RT16	+++	+++	+++	++	++	++	+++	+++	+++	+++	+++	+++	+	+
RT17	+	++	++	+	+	+	++	++	+	+	+	+	+	+
RT18	+	+	+	+	+	+	+++	+++	+++	+++	+++	++	++	+
RT24	+	+	++	++	++	++	+++	+++	+++	+++	+++	+++	++	+
RT25	+	+++	++	++	+	+	+++	+++	+++	+++	+++	+++	+	+

- = No growth; + = Little growth; ++ = Moderate growth; +++ = Excellent growth

Fusarium solani

Culture RT6 showed resistance not only to 65ºC but also to the different concentrations of benomyl (5, 10 and 20 μg ml^{-1}). The growth of fungi observed in plain oil was heavy in comparison with the growth observed in the medium without oil (soil extract broth with 0.1 % glucose). It is elucidated that RT6 preferred to use oil as sole source of carbon instead of glucose. Culture RT6 was isolated from lead (25 mM) amended soil. Radwan, *et al.* (1995) also mentioned the potential of indigenous *Fusarium sp.* to degrade oil.

Aspergillus niger

Culture RT8 grew in the presence of oil and resisted higher concentrations of benomyl and higher temperature. It was isolated from soil amended with lead (50mM).

Cladosporium cladosporioides

Culture RT9 showed the characteristic growth as dark green to black coloured onto the surface of agar extended into the sub surface representing its microaerophilic nature. RT9 was isolated from the soil amended with silver (10 mM). The fungus showed resistance to varying concentrations of heavy metals and benomyl and the growth was also found to be present at higher temperature. In the present study, it has shown growth in the presence of oil however; *Cladosporium sp* has also been reported to degrade petroleum hydrocarbons (Frick, *et al*., 1999).

Acremonium murorum

Culture RT15 was isolated from lead amended (50 mM) soil. The growth of this fungus was very less in medium without oil, medium containing oil and benomyl. Growth was absent in increasing concentration of oil i.e. 1 % (w/v), however the observation worth noting here is the viability of the fungus in plain oil without any amendment. However the growth of RT15 at higher temperature and in benomyl amended medium (Table 1) indicated the potential utilization of this fungus in the broad field of biotechnology.

Alternateria alternata

Culture RT16 was isolated from Ag (10mM) amended soil. It has shown abundant growth in medium without oil, medium amended with oil and benomyl. The growth was also very high in mineral salt solution amended with oil. *Alternaria sp*. was also found to be thermotolerant as it has shown resistant to high temperature i.e. 65^{o}C.

Aspergillus panamensis

Aspergillus panamensis was also isolated from Ag (10 mM) amended soil. Culture RT17 has shown less growth in medium without oil when compared with the growth of other fungal cultures. However abundant growth was observed in the medium containing oil and benomyl. Temperature treatment demonstrated that increase in temperature (above 40^{o} C), adversely affected the growth of RT17 which gradually adapted to increasing temperature, hence the growth was also observed at temperatures as high as 60 and 65 ^{o}C. Growth was also observed in mineral salt solution amended with oil and after prolonged incubation of 40 days but the oil content was reduced during this period. The finding clearly revealed that RT17 gradually adapted to high temperature and also survived in the oil without any amendment and also degraded oil as evidenced by brown paper assay (**Fig. 1-4**).

A. unguis

Aspergillus unguis was isolated from Ag (25mM) amended soil. It showed growth in medium containing oil and benomyl. The growth of *A. unguis* was also found to be present at 65 ^{o}C, however there was slight reduction in the growth when the temperature increased beyond 55^{o}C. Culture RT18 has also shown growth in medium containing oil as a sole source of carbon. Oil content was accumulated on the surface of medium surrounded by the fungal growth.

A. variecolar

Culture RT21 was isolated from petrol pump soil, amended with oil (0.5 % w/w). It has shown growth in the medium containing oil. Viability was checked in 0.5 % oil as well as in plain oil. Degradation of oil was evidenced by brown paper assay (**Fig. 1-4**).

A. luchuensis

Culture RT22 was isolated from petrol pump soil, amended with oil (0.5 % w/w), which also showed growth in mineral salt solution amended with oil.

A. micro-virido-citrinus

Culture RT24 was isolated from petrol pump soil amended with oil (1 % w/w). Initially it successfully grew in mineral salt solution amended with oil but after 60 days incubation medium got exhausted and oil was found to be present in the bottom. The change in the consistency of oil was also noted. It was able to grow successfully in medium containing oil and benomyl. It was also found to resist high temperatures, 60 and 65 ^{o}C, however slight reduction in the growth was noticed at 60 ^{o}C onwards.

The isolated fungi can be successfully employed in the bioremediation of oil spills without any nutrients amendment and in the remediation of pesticide polluted lands as revealed by their potential in the laboratory set or bench scale experiment. The potential of the fungi showing resistance to high temperatures can be successfully utilized in a number of biotechnological processes where the temperature is likely to rise. In general, the rate of microbial degradation or transformation doubles for every 10ºC increase in temperature (Eweis *et al*., 1998). Oil bioremediation and degradation of hydrocarbons is approximately 72% during summer compared to 56% during winter (Wright *et al*., 1997). Furthermore resistance to various heavy metals as shown by some fungi can be utilized in the bioremediation of the polluted soils along with heavy metals since in the environment pollution is likely to be present from the multiple sources hence having combination of pollutants. Cunningham *et al*. (1996) has reported that soils from oil-contaminated sites may also be contaminated with heavy metals and/or pesticides and salts. In order to prove the efficacy of these fungi found to be successful in the bench scale experiments, field trials or pilot scale experiment are required to be performed.

Undoubtedly, we are looking forward to see yet more exciting and perhaps unusual applications of fungi in the future, which might enable us to solve the remaining or as yet unknown problems, faced by the human society in future.

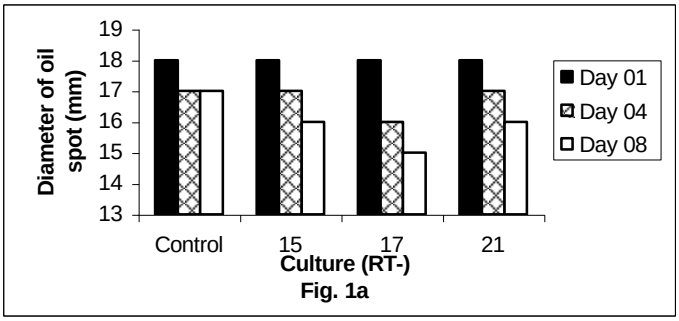

Fig. 1a

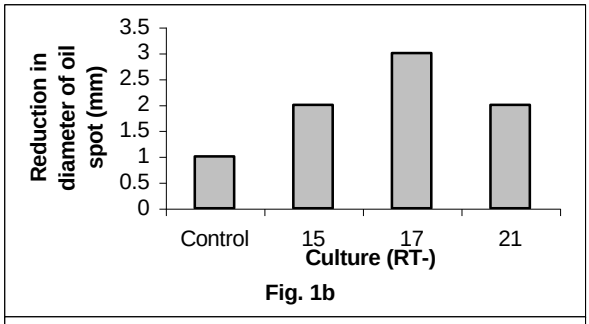

Fig. 1b

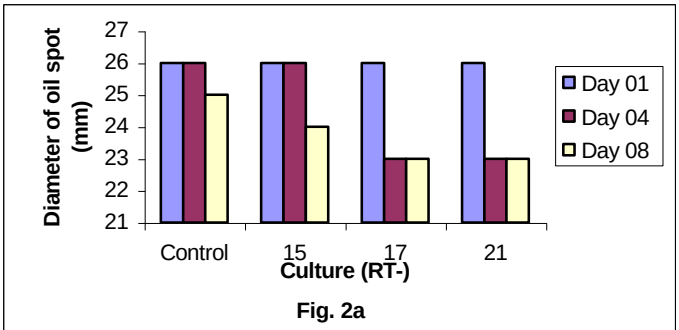

Fig. 2a

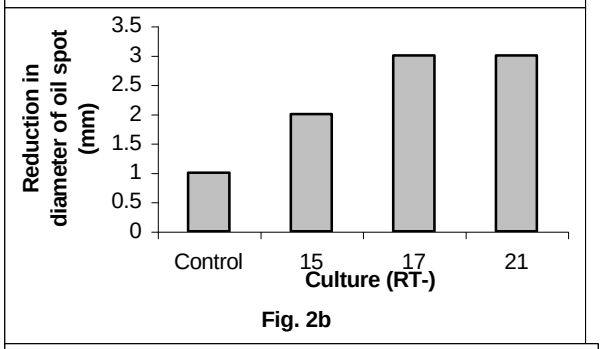

Fig. 2b

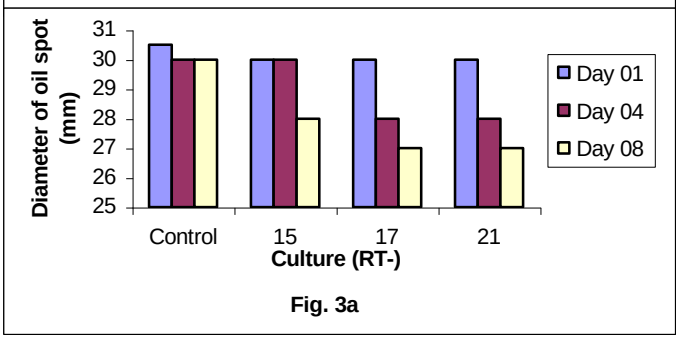

Fig. 3a

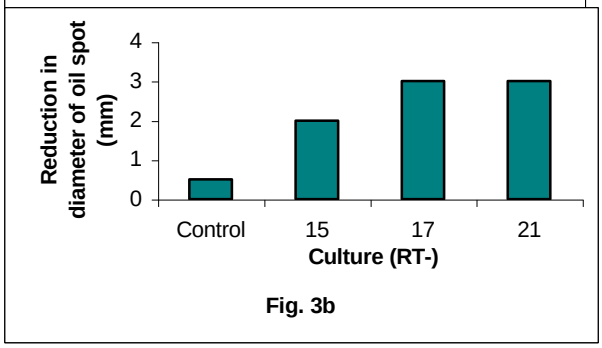

Fig. 3b

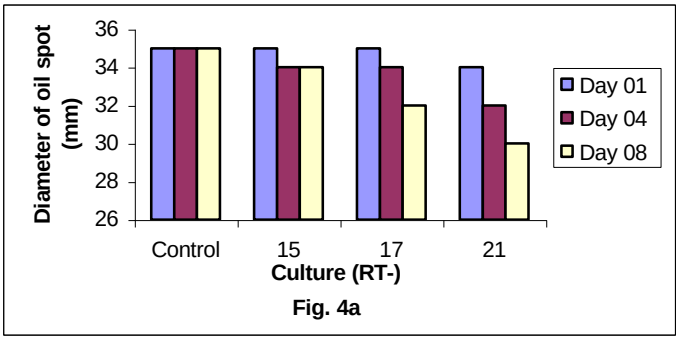

Fig. 4a

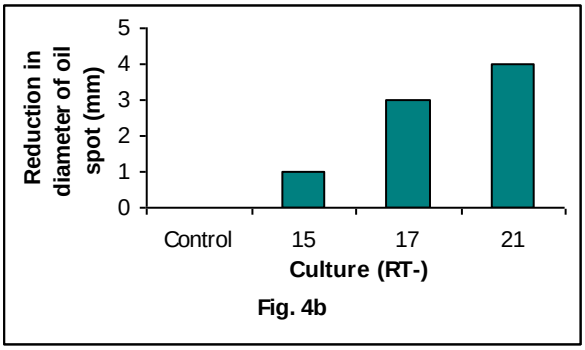

Fig. 4b

Fig. 1a and 1b: Modified brown paper assay (Wattman filter paper # 1) using 5 µl oil
Fig. 2a and 2b: Modified brown paper assay (Wattman filter paper # 1) using 10 µl oil
Fig. 3a and 3b: Brown paper assay using 5 µl oil
Fig. 4a and 4b: Brown paper assay using 10 µl oil
Key: RT15: *Acremonium murorum*, RT17: *Aspergillus panamensis*, RT21: *Aspergillus variecolor*

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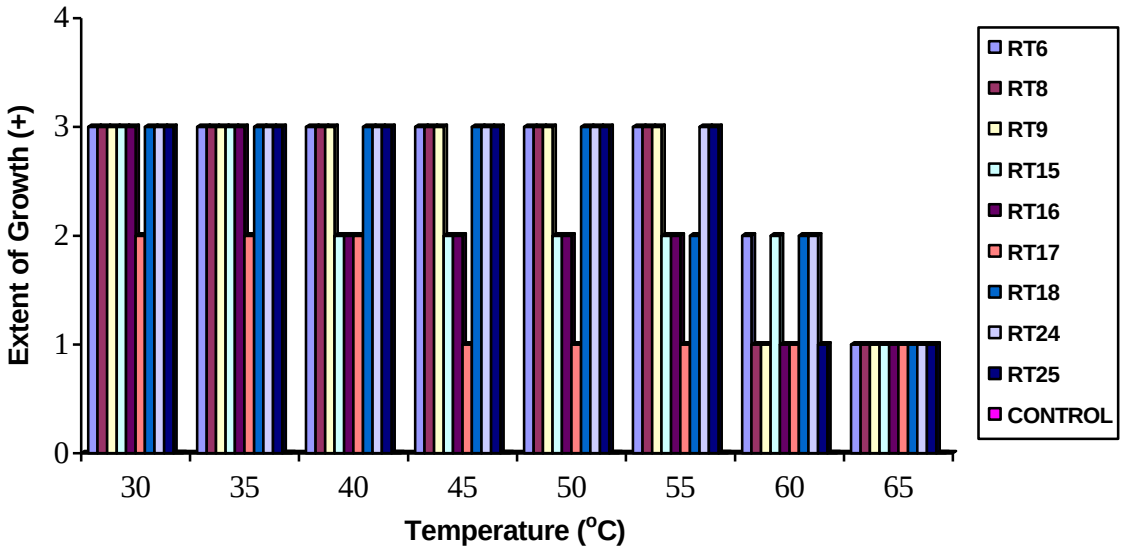

Fig. 5. Effect of different temperature on the growth of fungi.

Key: RT6: *Fusarium solani*, RT8: *Aspergillus niger*, RT9: *Cladosporium cladosporioides*, RT15: *Acremonium murorum*, RT17: *Aspergillus panamensis*, RT18: *Aspergillus unguis*, RT24: *Aspergillus micro-virido-citrinus*, RT25: *Aspergillus luchuensis*.

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