Identification of Mycoviruses from Soil: A Short Study

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Abstract

Mycoviruses are fungi-infecting viruses. The first mycovirus was discovered in the Agaricus bisporus, a cultivated fungus, in 1962. The infected mushrooms had abnormal fruiting bodies, grew slowly, and matured early, which led to significant yield losses. Mycoviruses require the live cells of other species to replicate, just like viruses that affect animals and plants. Mycoviruses have several traits in common with plant and animal viruses, but they also have the following special qualities: Mycoviruses appear to lack a movement protein, which is crucial for the life cycle of animal and plant viruses. This is because most mycoviruses lack an extracellular route for infection, are only intercellularly transmitted through cell division, sporulation, and cell fusion, and lack an extracellular route for infection in some cases. So, four soil samples collected from various locations in Swat yielded eight different kinds of fungi. Pythium-like fungi with isolated, white, fluffy growth were thought to be present. A purified colony on a PDA plate that had a greenish colony was thought to be Tricoderma, hence this pure plate was moved to a PDB for extraction purposes. Also isolated from the soil samples were Fusarium with pinkish growth, Alternaria, and Phytophthora with flower-shaped colonies. Due to the growth of Aspergillus, Niger, and other slimy bacteria, some of the plates were contaminated. The samples were screened for the presence of any Mycovirus by shifting all eight clean plates to Potato dextrose agar (PDB), crushing them afterwards. Testing crushed samples for mycovirus presence: Crushed samples were extracted to determine whether mycovirus was present. In order to discover any RNA/DNA bands in those 8 samples, extraction was carried out, and the samples were then seen under a gel dog. The following outcomes were attained: In each sample, only degraded bands and no discernible DNA/RNA band were discovered. As no band formed, it is inferred that there was no mycovirus in the samples.

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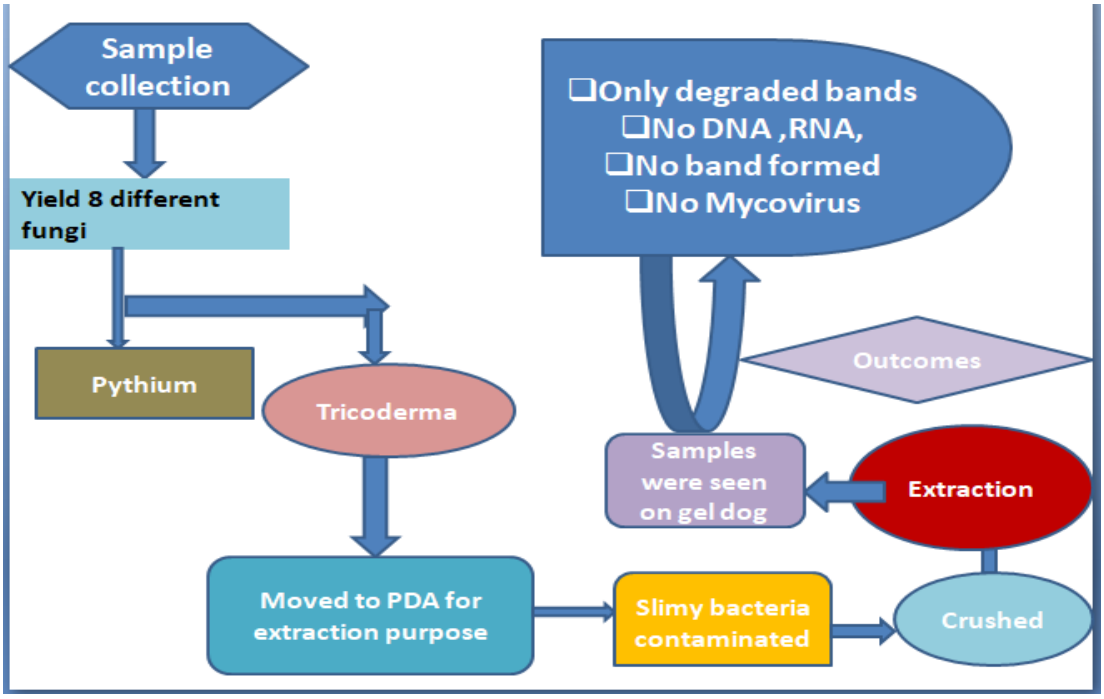


Introduction:

Mycoviruses are viruses that infect fungi. Mycoviruses are present in all major group of filamentous fungi. The first mycovirus was reported in 1962 from the cultivated mushroom, *Agaricus bisporus*; the infected mushrooms developed malformed fruiting bodies, grew slowly, and matured early, resulting in serious yield losses. Mycoviruses require the living cells of other organisms to replicate. While sharing some characteristics with animal and plant viruses, mycoviruses also have the following unique characteristics: most mycoviruses lack an extracellular route for infection; mycoviruses are transmitted intercellularly only through cell division, sporulation, and cell fusion; and mycoviruses apparently lack a movement protein, which is essential for the life cycle of animal and plant viruses.

According to the most recent report concerning virus taxonomy, the genome of most mycoviruses consists of double-stranded RNA (dsRNA), while the genome of about 30% of mycoviruses is composed of a positive, single-stranded RNA (+ssRNA). Mycoviruses have been detected in all the major phyla of fungi, including the Chytridiomycota, Zygomycota, Ascomycota, Deuteromycete, and Basidiomycota. Although many mycoviruses and their host fungi have been identified, many mycoviruses undoubtedly remain unknown. Recently developed metagenomic approaches will be useful for detecting and identifying new mycoviruses. Mostly mycovirus are dsRNA, while some are ssRNA and sometimes DNA. Those with RNA genome are now classified into 10 families of which four accommodate double stranded RNA (dsRNA) and remaining six composed of single stranded RNA (ssRNA) viruses. (Ghabiral and Suzuki, 2009). Most mycoviruses cause cryptic infection but some cause phenotypic infection alterations including hypovirulence and debilitation (Nuss, 2010). Some mycoviruses cause hypervirulence in Fungi. Most of the mycovirus particle identified were isometric, some

uncapsicated dsRNA have been reported. complete genome sequence of members of two genus chrysovirus (jamal etal., 2010) & partitivirus (Bhatti et al., in press) has been sequenced.
Phylogenetic studies have demonstrated that viruses in the same taxonomic families can infect diverse hosts, including fungi, plants, animals, and protozoa. For example, a recent taxonomic review indicated that the family *Partitiviridae* contained dsRNA viruses that infect plants, fungi, or protozoa]. In addition, the positive-strand RNA mycoviruses, which include Cryphonectria parasitica hypovirus 1–4 (CHV1–4), Fusarium graminearum virus 1 (FgV1), and Botrytis virus X, are phylogenetically related to plant viruses. Their genomic organization and expression strategy resemble those of plant potyviruses or potex-like viruses. Moreover, Sclerotinia sclerotiorum RNA virus L is closely related to the human pathogen hepatitis E virus and rubi-like viruses.

Origin:
Two major hypotheses have been proposed to explain the origin of mycoviruses. The "ancient coevolution hypothesis" states that although the origin of mycoviruses is unknown, the association between mycoviruses and fungi is ancient and reflects long-term coevolution. The "plant virus hypothesis," in contrast, suggests that mycoviruses originated relatively recently from plant viruses, i.e., the original mycovirus was a plant virus that moved from plant to fungus within the same host plant. Similar scenarios might also explain the origin of plant viruses, i.e., some plant viruses may have originated from mycoviruses that moved from fungus to plant. Because convincing data are lacking, however, the origin of mycoviruses remains a mystery.

Pathogenicity and Virulence:
Although mycoviruses are common among fungi, they usually remain latent and seldom induce symptoms. Some mycoviruses, however, cause dramatic changes in their hosts, including irregular growth, abnormal pigmentation, and altered sexual reproduction. Perhaps the most important effect is the reduced virulence—i.e., hypovirulence—of plant-pathogenic fungi. Hypovirulence has attracted much attention because it has the potential to reduce the losses of crops and forests caused by plant-pathogenic fungi. Sometimes mycovirus cause symptomless infection in their fungal host(Buck, 1998) as the host limits the virulence of the virus. Over the last 50 years, research on mycoviruses that induce hypovirulence has greatly increased our understanding of mycoviruses and their interactions with their plant-pathogenic fungal hosts. Much of the early research on mycoviruses concerned the interaction between hypovirus CHV1 and the chestnut blight fungus *Cryphonectria parasitica*. Infection by CHV1 resulted in reduced growth and abnormal pigmentation in *C. parasitica.* Most importantly, CHV1 induced hypovirulence in *C. parasitica*. Along with the CHV1, the mycoviruses that infect the important (Son, Yu1, & -Hyung Kim, 2015)plant-pathogenic fungus *F. graminearum* have also been detected and studied. Among them, FgV1 confers hypovirulence to *F. graminearum* just as CHV1 confers hypovirulence to *C. parasitica*.

As mentioned earlier, mycoviruses are transmitted intercellularly only through hyphal anastomosis or spores. Virus transmission between different strains is restricted by fungal vegetative incompatibility (vic). Vegetative incompatibility is an obstacle in the use of hypo virulent mycoviruses as biological control agents. Recent research has demonstrated that the seven *vic* genes associated with five of six vic loci in *C. parasitica* contribute to incompatibility and affect virus transmission. Recently developed analytical techniques have enabled research on mycoviruses to enter a new phase. Using genome-wide linkage analysis, for example, researchers have begun to answer the question, "How do mycoviruses affect their fungal hosts?" RNA-Seq–based, genome-wide expression analysis revealed totally distinct expression patterns of *F. graminearum* transcriptomes in response to infections by four phylogenetically different mycovirus. As obligate intracellular parasites, mycoviruses reprogram host cell metabolism to replicate within host cells and avoid antiviral responses. Identifying the crucial determinants in all steps of the viral life cycle is important for understanding the pathology caused by mycoviruses. To identify host factors important in the interaction between mycovirus and fungus, researchers have used genome-wide approaches in their studies of Cryphonectria hypoviruses, Fusarium graminearum viruses, and Sclerotinia sclerotiorum debilitation-associated RNA virus. The results revealed that the expression level of specific host genes differed not only between virus-free and virus-infected fungus isolates but also among viruses belonging to different groups and among virus strains that differed in the degree of hypovirulence that they induced. FgV1 RNA in host cells. Viral RNA accumulation is decreased in Δ*hex1* and increased in the overexpression mutant compared to the wild type.

Fungi cause devastating diseases in different host plants. Fungicides are used to control various fungal diseases, but usage of fungicide have adverse effect on environment and human. Mycoviruses are potentially used as biocontrol agents. Frist mycovirus used as biocontrol agent was against Chryphonectria parasitica (Nuss,1992). To act as a biocontrol agent the virus adopt hyphal interaction mechanism for movement between fungal colonies. Genetic compatibility may hinder the success of biocontrol caused my mycoviruses. For example, Sclerotina sclerotiorum cannot be control using SsDRV because of genetic incompatibility (Hambleton et al.,2002). The hypovirulence caused by mycovirus make the virus as a tool for biocontrol and successful application of mycovirus is determined by their spread in natural population against the pathogen. Now a days research is going on to find the interaction between viruses and fungi and the future of this study is very vast. My work focuses on the isolation of mycovirus from soil and to determine the biocontrol function of that fungi due to presence of mycovirus.

Material and methods:

Material:

For extraction of nucleic acid (DNA/RNA) of the fungal culture in PDB tubes Following materials were used:

Mortar and pestle (for grinding)

Liquid Nitrogen
2ml Eppendorf tubes/Stand
Ethanol /PDB tubes
Eppendorf tubes / Pipette

Chemicals:

Table 1 Chemicals used in extraction and their function.

Sr. No	Chemicals/reagents	Function
1	Extraction buffer	Cell lysis and extraction Removal of Phenolics
2	Phenol	Cell Lysis Cause denaturation of DNA
3	Chloroform isoamyl alcohol	Purification of DNA Removal of protein from nucleic acid solution
4	Sodium acetate	Extraction of DNA from cell DNA precipitation and analysis for the presence of nucleases.
5	Ethanol	DNA is insoluble in alcohol;its addition followed by centrifugation will cause the DNA protein to come out of solution.

Equipments:

Table 2 Appratus/ Equipments with their function

Sr. No	Appratus	Function
1.	Autoclave	Used to strelize Equipments.
2.	Centrifuge Machine	Used for separation of liquids.
3.	Electronic balance	Used for quantitative measuring of chemicals.

4.	Weight machine	Used to weight powdered chemicals in grams.
5.	Water Bath	Used for incubation samples at constant temperature for particular period of time.
6.	Vortex machine	Used to mix liquids.
7.	Refrigerator	Used for freezing of samples at -20 degree.
9.	Microwave oven	Used for heating.
10.	PCR	Used to amplify segments of DNA.
11.	Gel Doc	Used to check the confirmation of DNA and PCR product is either present or not.
12.	AFC	It filters air and spores of fungi therefore used for inoculation in sterilized condition.

Preparation of PDA Media:

1. 19.5 g of PDA dry powder was dissolved in 500ml pf distilled water.
2 The flask was kept on stirrer for 5 mint for proper and even mixing.
3 After that loosely covered the flask and kept in autoclave at 121 degree centigrade for 15 to 20 mint.
4 When the autoclave temperature reached at 60 degree centigrade keep the flask out and keep on stirrer for 2 mint for mixing.
5 Now took the media to the Air flow chamber before use disinfect the AFC with 70% ethanol and disinfect your hand.
6 poured 125 ml of media in each plate and filled around 20 plates.
7 Kept the plates in AFC to cool down and covered the lid of each plate.
8 Following cooling kept the plates in a zipper bag and stored in freezer.

Serial dilution:

5gof soil wastaken from Kala Kalay Uc sample and labelled as sample #1 .5g of composite soil from sample #2 was labelled as Bi ha Bar Sawat composite sample.50ml of distilled water.
Was filled in Flask 1 and flask 2. Set on shaker for 25 mints covered the mouth of beaker with aluminium foil. Afterthat, serial dilution was done up to 10-5 in laminar flow chamber.Took 9mlof sterilized water fill in 5 test tubes took 1ml of sample 1 from beaker 1 and drop in tube one. .50ml of dilution from test tube 4 was inoculated on PDA plate, using spreader properly spread on the plate. Labeled the 2 inoculated plates with sample 1 as S1(10-4) (10-5). Same procedure was repeated with sample 2, dilution from test tube# 4 and 5 was inoculate in2 plates named as S2 (10-4), S2(10-5). Also inoculated two plates of PDA were inoculated with undiluted samples and labeled as S1(UD), S2(UD). Plates were kept in incubator at 25-28% and observed the colony growth after 5-7 days.

Inoculation:

Sample #3 (Ningolai uc bandai river side, A) and sample #4 denoted as (Ningolai bandai rive side, B) 4 g of each sample was taken and 40ml water was added. Set the flask on shaker for 25 mint of proper mixing. By using 50 ml pipette 50 ml of mixture was inoculated on a plate containing media. Covered the plates and wrap with para film, kept in an incubator.

Purification:

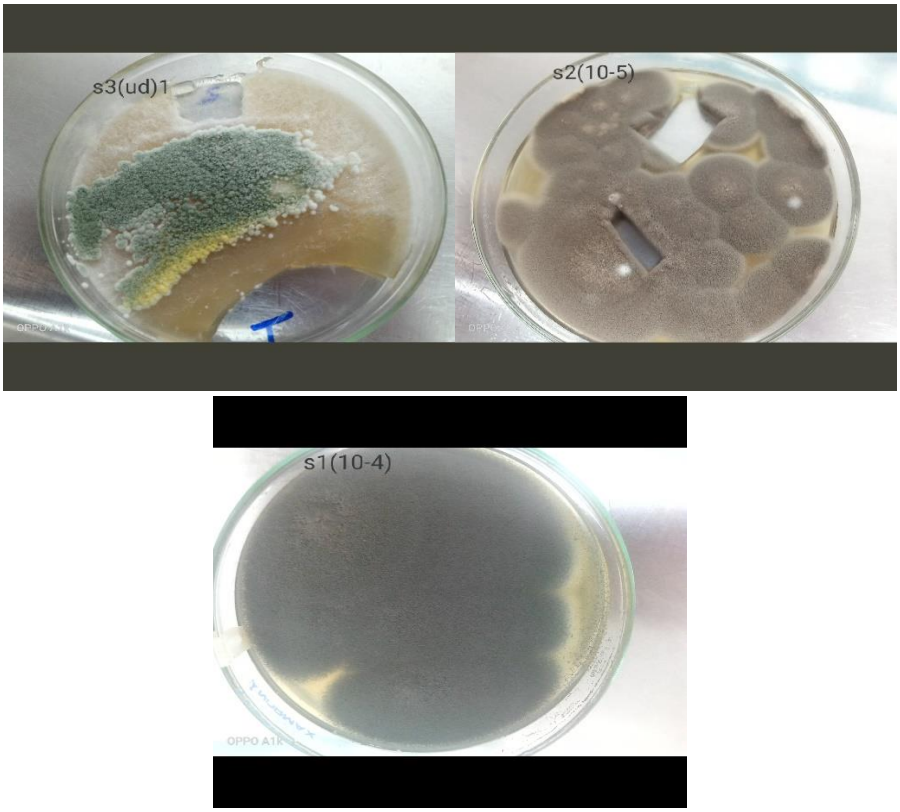
After 6 days growth appeared on the plates.

There was bulk growth of fungi in S1 and S2 UD plates. Therefore, there was need of purification of single colony. Toothpick was taken and scraped the required mycelium from the plate and shifted it to a new PDA plate. same procedure was done with both plates and purified the required colony of fungi Labeled them as S1(1)(2)(3) and S2(1)(2)(3)(4) and kept them in incubator. Moreover, in S1 (10-4 &10-5) plate there was growth of a single colony of fungi therefore, they were kept in incubator for further growth. There was a little contamination of bacteria in S2(10-4) plate, so the portion containing slimy growth was removed by cutting. There was also a single colony growth in S2(10-5) plate. Picked the plate s3(UD) there was growth of fungi,so two colonies were picked with help of toothpickand labelled as S3(UD) (1), & (2). Likewise, plate S2(3), S2(2), S1(UD2) were purified and labelled as S2(3)(1), S2(2)(1), S1(UD2) (1) (2). There was greenish growth with white margin on plate S3(UD) (1) after the process of purification the plate was named as S3(UD) (1)1. S1(UD2) (1) show two type of growth one with greenish colony and other pale whitish. these colonies were purified on plate separately and named as S1(UD2)1(1) and S1(UD2)1(2). S2(10-4) plate show growth one colony white with granules and other colony was thick whitish mycelia with pale yellow center. Were purified to new plates separately and names as S2(10-41 and S2(10-4)2.

(Fig.2) Pure colonies of different fungi

PDB shifting:

Next some purified cultures were transferred to PDB. Plate S1(10-4) had colony growth of black color. This sample on PDB tube was denoted as 12. Ablade was used to cut the pure culture into small pieces and transferred the pieces into the falcon tube containing PDB.Likewise transferred the plateS1(10-5) was transferred which had blackish growth to the PDB tube labelled as11. And transferred the plate S2(10-5) which has brownish growth to the PDB tube labelled as 13. Pure plate labelled as S3(ud)1 was shift to PDB.Plate #1 & 3 were shift to PDB previously labelled as 14 &15.



(Fig.3) PDB shifted plates

Crushing:

The PDB tubes were filtered in the vacuum filter. then the mycelium of fungiwascovered in the chess cloth and wrap in the tissue paper. After that, the samples were crushed in pestle and mortar by adding liquid nitrogen. Then the crushed samples were added in 2ml effendrop tubes and kept at 20 degree until extraction. Tubes were Denotedthe with the following code**:**

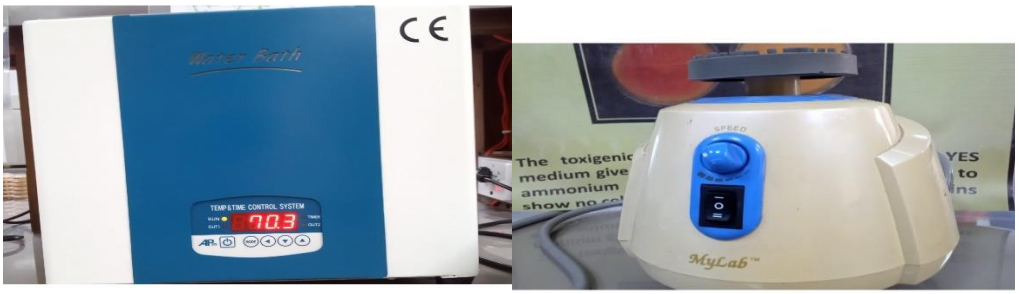
2 ,3, 11,12,13,14, 15 and 95.

(Fig.3) Crushing of samples

Screening of samples for presence of mycovirus:

Extraction:

To extract total nucleic acid content from mycelial cells 400 micro liter of extraction buffer was added following brief vertexing to homogenize the mixture.in each tube using pipette. the tubes were placed in water bath for one hour at 70-degree temperature. After incubation 400 micro liter of phenol and an equal amount of Sevag (chloroform + isoamyl) were added in centrifuge tubes containing samples followed by centrifugation at 12000 rpm. Clear supernatant was transferred to another microfuge tubes leaving the debris and other gradient layers behind. Again, top layer was decanted to fresh tube and chilled 100% ethanol was added. To facilitate precipitation, the microfuge tubes were incubated at 20 degree for 4 hours. Nucleic acid was pelleted down by centrifugation for 20 mints at 12000 rpm. Ethanol was drained and pellets were air dried. Dried nucleic acids pellet was suspended in 40 micro liter sterile distilled water and stored at- 20 degree. The samples were then analyzed on 1% agarose gel to check the presence of mycoviral genome.



Gel preparation and Gel running:

1.2 g of agarose gel was addedin120 ml of TAE buffer. It was kept in microwave until the gel became clear.

Leave the gel for 1 mint to cool down and pour in casting tray keeping the comb to make wells and let it dry. **2X** loading dye,**12X** sample of total amount **14 x** was added in each well.Also ladder of **2.5** micro liter was added. samples were placed in the well in following sequence:

Sample 95 posiitive one ,2 ,3, 11,12,13,14, 15.

10X TAE was added in the tank and run the gel by keeping the samples toward cathode and run the electricity. **100 V** of electricity was applied for time duration of 1 hour. The gel was cut and kept in ethidium bromide for **15 mint**. Visualized the gel under gel dog and observed the DNA band appeared.

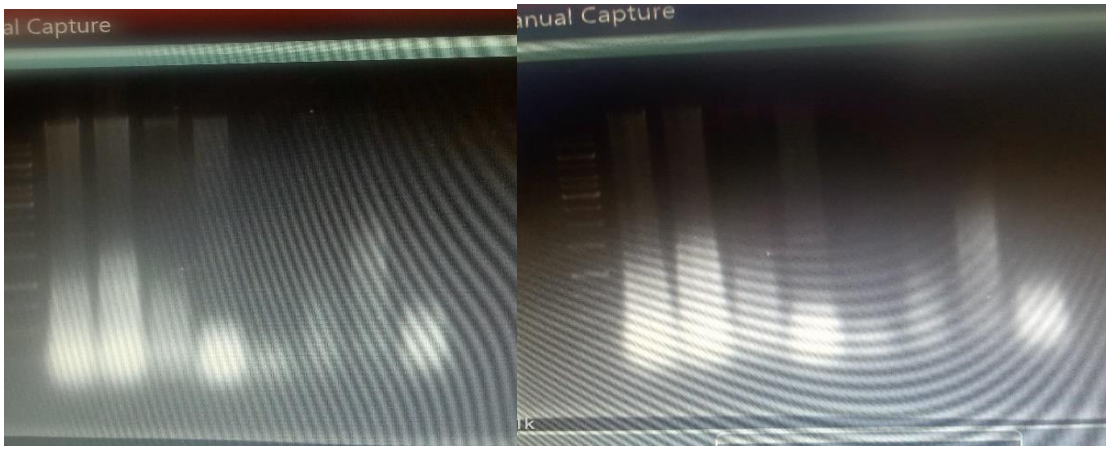
(Fig.5) casting tray

Result and Discussion:

Eight different types of fungi were isolated from four soil samples taken from different areas of Swat. Fungi with whitish fluffy growth was isolated that was suspected to be pythium. A colony was purified with greenish colony on PDA plate that was suspected to be Tricoderma, so this pure plate was shifted to PDB for extraction purpose. Moreover, Fusarium with pinkish growth, Alternaria, Phytophthora with flower shape colony, was mainly isolated from the soil samples. A few of the plates were contaminated due to the growth of Aspergillus, Niger and due to some slimy bacterial growth. All eight pure plates were shifted to Potato dextrose agar (PDB) and they were crushed later extraction was done to screen the samples for presence of any Mycovirus.

Screening of crushed samples for presence of mycovirus:

The crushed samples were subjected to extraction to identify the presence of mycovirus. Therefore, extraction was done, and the samples were visualize under gel dog to find any band of RNA/DNA in those 8 samples. Following results were obtained:



No visible band of DNA/RNA was found in any of the sample, degraded bands and no band were found.

So, it is concluded that no mycovirus was present in the samples as no band was formed.

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