Presence of Virus like Particles in Human Pathogenic Fungi: *Chrysosporium* sps and *Candida albicans*

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Abstract Out of eight isolates of different human pathogenic fungi, four were found to be infected with virus like particles (VLP): *Chrysosporium pseudomerderium*, *C. xerophilum*, *C. keratinophilum* and *Candida albicans* through electron microscopy. Frozen mycelium without abrasive was seemingly better disruption method for VLP extraction in *Chrysosporium* extracts but in *Candida albicans* disruption with abrasive revealed the presence of considerable amount of VLP. The growth of five isolates of *Chrysosporium* species associated with VLP was significantly affected by different media tested. Absorption spectra revealed the nucleoprotein nature of VLP in *C. albicans*. Virus like particles in *Chrysosporium* species reported for the first time and in *C. albicans*, it is reported for the first time in India.

Keywords Mycoviruses · VLP · *Candida albicans* · *Chrysosporium* sps

Introduction

Mycoviruses are widespread in all major groups of plant pathogenic and industrially important fungi. Typically, mycoviruses have double-stranded (ds) RNA although single stranded (ss) RNA genome is also reported. Viruses of fungi might be found by screening procedures which involve purely physical methods, i.e. partial purification

and electron microscopy. A number of laboratories have screened fungal isolates for the presence of virus and met with considerable success. The term VLP has been used in reference to particles that have not been characterized completely but have one or more of the physical characteristics of viruses.

Occurrence of mycoviruses or VLP have been implicated with increased or decreased pathogenicity of the host fungi. Mycoviruses mediated hypovirulence is a phenomenon in which virulence of fungal pathogen is reduced or completely lost as a consequence of virus infection. There are varying reports related to increased or decreased pathogenicity of fungi by viruses. Classic example of successful utilization of hypovirulence for biological control of chestnut blight fungus, *Cryphonectria parasitica*, has led to discovery of hypovirulent strains in other fungi. In other pathogenic fungus, *Botrytis cinerea*, presence of ss and ds RNA mycoviruses is reported but hypovirulence was not always associated with occurrence of mycoviruses [9]. However in another strain of the same fungus, mycoviruses infected strains were less aggressive than the mycovirus free strain [4].

Mycoviruses have also been held responsible for a disease of national importance i.e., mango malformation. Causal agent of this disease, remained a question mark for a number of years. Etiology of disease was attributed to *Fusarium moniliforme* var. *subglutinans* but the fungus could not give the infection everytime [23]. Later it was reported that presence of mycovirus in *F. moniliforme* var. *subglutinans* might be responsible for the disease as VLP infected strains of fungus produced malformations in the pathogenicity tests conducted and VLP free isolates resulted in healthy mango seedlings [5, 6]. Exploitation of mycoviruses for biological control is a promising method and a milestone yet to be achieved in disease control.

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Limited evidence is available on the direct influence of viruses on fungal host metabolism particularly production of secondary metabolites. Diverse reports related to toxin production (galactosamine), [3] associated with the presence or absence of mycoviruses of *Penicillium stonloniferum* are there. Mycoviruses have also been implicated in RNA silencing [7, 10].

Current mycovirus publications primarily concern economically important fungi such as cultivated mushrooms, yeast, and fungal pathogen of plants. However, work related to mycoviruses in India and on human pathogenic fungi is limited. Therefore, present investigations were undertaken to detect presence of VLP in human pathogenic fungi and food spoiling fungi, and to make an attempt to correlate presence of VLP to fungal pathogenicity at a later stage.

Materials and Methods

Evaluation of Economically Important Fungi for the Presence of Virus like Particles (VLP)

Human pathogenic ascomycetous fungi such as *Chrysosporium indicum*, *Chrysosporium lobatum*, *C. pseudomerdarium*, *C. xerophilum*, *Chrysosporium keratinophilum*, *Candida albicans*, *Trichophyton mentagrophytes* and food spoiling fungi such as *Aspergillus fumigatus* were screened for the presence of VLP.

Maintenance of Fungal Culture

Culture isolates of *C. indicum*, *C. keratinophilum*, *C. lobatum*, *C. pseudomerdarium*, *C. xerophilum*, *C. albicans*, *T. mentagrophytes* and *A. fumigatus* were maintained on SDA medium.

Mass Propagation and Harvest of Mycelia

For obtaining mycelial mats of different fungal isolates mass production in liquid culture medium was carried out. Autoclaved flasks were inoculated with a 4–5 mm piece of agar culture under aseptic conditions. Inoculated flasks were incubated for 15–20 days at 27°C in incubator.

Harvest of Mycelia

Mycelial mats were harvested by filtration through cheese cloth and washed twice with distilled water to remove media. Mycelial mats were squeezed dry in double folds of blotting paper and fresh weight of fungal mycelium was recorded.

Disruption of Frozen Mycelium

Harvested mycelial mats were blotted dry in double folds of blotting paper and wrapped in cellophane sheets. These were kept in deep freeze –20°C for 48 h. Frozen mycelium was powdered in a sterile mortar with pestle.

Mycelial disruption was achieved through two methods:

With Abrasive

5 ml of 0.1 M phosphate buffer, pH 7.0 per gram weight of mycelium was added. After thorough grinding with carborundum (abrasive), material was filtered and squeezed through double layered cheese cloth. Re extraction of mycelium was done. Homogenate was centrifuged at 5,000 rpm for 45 min to remove cell debris.

Without Abrasive

Similar procedure was followed without carborundum.

Detection of VLP in mycelial extract

Supernatant obtained after low speed (5,000 rpm for 45 min) centrifugation of homogenate was applied to carbon coated grids, stained with uranyl acetate and rinsed twice with distilled water. Presence of VLP was checked by examining grids under electron microscope (Model: JEOL-100 CXII) at an instrument magnification of $\times 20,000$.

Determining the Effect of Different Carbon and Nitrogen Sources on Growth of VLP Infected and VLP Free Fungal Isolates

On the basis of electron microscopy, *Chrysosporium* sps. were selected to study effect of various nutrient media. Effect of different carbon (maltose, sucrose or starch) and nitrogen (peptone, malt) sources was studied to differentiate between VLP free (*C. lobatum*, *C. indicum*) and infected (*C. pseudomerdarium*, *C. xerophilum*, *C. keratinophilum*) fungal isolates, as also to select a suitable medium to support optimum growth of selected *Chrysosporium* isolates. Fungal isolates were inoculated on four different agar media viz Potato dextrose agar (PDA), Malt agar (MA), Czapek agar (CPA) and Sabouraud's agar medium (SDA).

Inoculation on sterilized slants was done with VLP infected and free *Chrysosporium* species under aseptic conditions. All treatments were replicated three times. Inoculated tubes were incubated at 25°C. Linear growth was measured at 2 days interval for 5 days. The data obtained was analysed by measuring linear growth.

Absorbance of the Fungal Samples

For quantitative estimation, the absorbance of the sample was measured spectrophotometrically. UV absorption spectra was measured in SHIMADZU. UV–Vis spectrophotometer which was calibrated with phosphate buffer. Another cuvette with virus preparation was then placed in the chamber and absorbance was recorded. Sample was scanned over the wavelength ranging from 200 to 700 nm.

Results

Evaluating Economically Important Fungi for the Presence of Virus like Particles (VLP)

The eight fungal isolates screened for the presence of VLP, mostly had isometric VLP which could be distinguished into three categories based on their size and stain penetration.

In *C. pseudomerdarium*, only small groups of isometric VLP (20 nm) were observed. Approximately, 100 VLP were detected per grid square (Fig. 1a).

Mycelial extracts of *C. keratinophilum*, contained isometric VLP which were of two types i.e., small (20 nm) and medium (33–40 nm). Stain penetrated with core giving ring like appearance to both types of VLP. In this isolate some rod shaped VLP (approximately 7) were also observed. The number of isometric VLP ranged from 20 to 25 in each grid square (Fig. 1b).

Isolates of *C. xerophilum* were associated only with isometric VLP. These were of three types i.e., small (20 nm), medium (33–40 nm) and large (66–70 nm) giving thick ring like appearance, number of VLP ranged from 2 to 10 approximately per grid square (Fig. 3c). In *C. albicans*, mostly the isolate contained medium isometric VLP (33–40 nm) in scattered form. Number of VLP per grid square was more than 100 (Fig. 1d).

VLP were not detected in crude extracts of *C. indicum*, *C. lobatum*, *A. fumigatus*, *T. mentagrophytes* through Electron Microscopy.

Fig. 1 Electron micrographs of virus like particles in *Chrysosporium* sps (a) *C. pseudomerdarium*; (b) *C. keratinophilum*; (c) *C. xerophilum* and *Candida albicans* (d)

Effect of Different Carbon and Nitrogen Sources on Growth of VLP Infected and VLP Free Fungal Isolates of *Chrysosporium* spp

The four media tested showed significant differences in growth. In *C. lobatum*, the highest growth was supported by SDA medium followed by PDA and lowest growth in MA and CPA. In *C. indicum*, the highest growth was supported by SDA followed by CPA and PDA and no growth in MA. In *C. pseudomerdarium*, the highest growth was supported by SDA followed by PDA and lowest growth in CPA and MA. In *C. xerophilum*, the highest growth was supported by SDA followed by MA and PDA and no growth in CPA. In *C. keratinophilum*, the highest growth was supported by SDA followed by MA and lowest growth in PDA while CPA exhibited no growth. Therefore, the maximum growth of *Chrysosporium* isolates was supported by SDA (Fig. 2a, b, c, d).

Quantitative Assay of the Virus

For quantitative estimation, the absorbance of the samples was measured spectrophotometrically. All the mycelial extracts recorded absorbance in the range 200–400 nm except for *C. albicans* which recorded a characteristic peak at 262 nm (Fig. 3a). All other extracts exhibited a broader peak irrespective of the occurrence of VLP detected through electron microscopy. The absorbance spectra of all the fungal extracts tested are given below (Fig. 3b, c, d).

Discussion

Mycoviruses are known to infect and multiply in all major taxa belonging to the kingdom, fungi. For the present investigations fungal strains belonging to Division Deuteromycotina were taken. Majority of the information on

Fig. 2 Mean linear growth of virus like particles (VLP) infected and VLP free fungal cultures of *Chrysosporium* spp. in four different media. **(a)** VLP infected (*C. pseudomerdarium*, *C. keratinophilum*, *C. xerophilum*) and VLP free (*C. indicum*, *C. lobatum*) fungal cultures in potato dextrose agar (PDA). **(b)** VLP infected (*C. pseudomerdarium*, *C. keratinophilum*, *C. xerophilum*) and VLP free (*C. indicum*, *C. lobatum*) fungal cultures in Czapek agar (CPA). **(c)** VLP infected (*C. pseudomerdarium*, *C. keratinophilum*, *C. xerophilum*) and VLP free (*C. indicum*, *C. lobatum*) fungal cultures in malt agar (MA). **(d)** VLP infected (*C. pseudomerdarium*, *C. keratinophilum*, *C. xerophilum*) and VLP free (*C. indicum*, *C. lobatum*) fungal cultures in Sabouraud's dextrose agar (SDA)

Fig. 3 Absorbance spectra of virus like particles infected fungal cultures of *Candida albicans* (a) and different species of *Chrysosporium* (b) *C. pseudomerdarium*, (c) *C. keratinophilum*, (d) *C. xerophilum*

mycoviruses is revealed through studies on plant pathogenic fungi. Not much information is available on mycoviruses of human pathogenic fungi. ds RNA isometric virus 44–50 nm in diameter is reported from human pathogenic fungus, *Blastomyces dermatitidis* [11].

Present investigations were focussed on screening VLP in Human pathogenic fungi viz., *Chrysosporium* spp.; *Trichophyton* spp.; *C. albicans*; *A. fumigatus*. The present literature survey did not reveal any report on occurrence of VLP in *Chrysosporium* spp and *T. mentagrophytes*. However, large isometric VLP 50–60 nm have been reported in *Candida utilis* [12]. Also, in *Candida tropicalis* unusually large isometric particles (100–170 nm) were reported [18]. Isometric VLP (35 nm) in diameter have been isolated and characterized from *Candida curvata* [20]. *C. albicans* also revealed the presence of spherical VLP 12, 18 and 28 to 30 nm in size [21]. There are few reports on occurrence of isometric VLP (25–30 nm) in *Aspergillus flavus* [17]; *A. glaucus* [8]; *A. foetidus* and *A. niger* [22]; and ds RNA virus in *A. niger* [1]. *C. keratinophilum*; *C. xerophilum*; *C. pseudomerdarium*; and *C. albicans* were indexed for the presence of VLP after screening of all the fungal isolates through electron microscopy. Two types of VLP (i.e. isometric and rod shaped) were found associated with *C. keratinophilum*. Most common were the isometric VLP which were of three types i.e., large, medium and small. The first category was of small (20 nm) VLP giving thick ring like

structure, due to stain penetration. The second category was of medium isometric VLP measuring (33–40 nm). In some of these VLP, stain penetrated more strongly forming fine rings. The third category of large isometric VLP measured (66–70 nm) and were again of two types as in some the stain penetrated giving slightly irregular ring like structure and in others stain did not penetrate and their sizes varied within a particular range. Crude extract of *C. albicans* revealed maximum amount of medium isometric VLP through electron microscopy whereas minimum concentration of VLP was observed in *C. xerophilum*.

There are reports on occurrence of various morphological types ranging from isometric to rod, club shaped, filamentous and some other anomalous shapes. However, most common shape observed is isometric. Two kinds of double-stranded RNA (dsRNA), estimated to be 1.9 and 1.7 kb in size, from 30 nm isometric viruses were detected in the plant pathogenic fungus, *Fusarium solani* f. sp. *robinia* [19]. Virus like particles from *Metarhizium anisopliae* were isolated [15, 2]. Icosahedral VLP (ca. 33 nm in diameter) were observed by transmission electron microscopy in extracts of these isolates. Isometric mycovirus (40 nm) in size were reported in *Pseudocercospora griseola*, the causal agent of angular leaf spot in common bean [16].

Present investigations provide the evidence for presence of VLP in *Chrysosporium* species and *C. albicans* which are human pathogenic fungi. Occurrence of VLP in

Chrysosporium species reported for the first time and in *C. albicans*, it is reported for the first time in India.

Morphological type identified is isometric which is one of the most common for mycoviruses; though multiple infection was seen in *C. keratinophilum*.

Observations on VLP free and VLP infected isolates of *A. bisporus* revealed that these isolates could be clearly differentiated by their growth on eleven different media, as VLP free isolates grew better on all the media tested [13, 14]. Similar observations were recorded in PDA medium where there was a clear differentiation in growth between VLP infected and free isolates. The latter showing better growth.

VLP free isolates of *Chrysosporium* showed better growth and VLP infected showed comparatively lesser growth in PDA.

Czapek medium supported the linear growth of *C. indicum* only. Absolute nil growth was recorded in two VLP infected strains, *C. xerophilum* and *C. keratinophilum* whereas scanty growth of *C. lobatum* VLP free isolate was comparable to *C. pseudomerdarium*. Owing to the lesser growth in Czapek medium, this medium cannot be used as supporting medium for these fungal cultures.

In Malt agar, except for *C. pseudomerdarium* growth of rest of the two VLP infected isolates, *C. xerophilum* and *C. keratinophilum* was better than the VLP free isolate.

Sabouraud's dextrose agar supported the linear growth of all the *Chrysosporium* cultures irrespective of the presence or absence of VLP. Malt agar can be more suitable medium for growth of VLP infected isolate of *Chrysosporium* but overall growth of even VLP infected strains was best in Sabouraud's Dextrose Agar. Therefore, this medium can be used as propagating or supporting medium for *Chrysosporium* cultures.

Absorption spectra of crude extracts revealed the nucleoprotein nature of VLP in *C. albicans*. For rest of the crude extracts of other fungal isolates, the UV-absorption spectra followed a similar pattern by absorbing light in the range of 200–360 nm. The pattern of absorption was similar for fungal isolates indexed for the presence of VLP and those in which VLP were not detected by electron microscopy.

Owing to this anomaly, it can be said that VLP might be present in other fungal isolates that could not be detected through electron microscopy. Probably, concentrating the VLP through partial purification procedures might give a clear picture and nature of VLP in these fungal isolates.

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