management. In addition, meetings have been held with local stakeholders, which were successful and have led to the formation of the CCC with representation from all stakeholders in the landscape.

Our preliminary conversation with local stakeholders indicates that people and local institutions are willing to support such a restoration initiative in this area. In addition, the CCC will ensure monitoring and compliance with their own regulations regarding conservation of the wildlife corridor. Along with researchers, forest managers and local institutions as well as local community participation, this will emerge as one of the successful, action-oriented and policy-driven projects in this landscape. Moreover, such initiatives bring confidence among local stakeholders regarding conservation. Since nature conservation is often labelled as crisis science, associated with the socio-political dimensions[12], the interventions made through this line of research will not only improve wildlife corridors, but also maintain the ecological processes, and reduce HWC in the area. At the time of unprecedented habitat destruction, such interventions will contribute not only towards reduced emissions from deforestation and forest degradation, but also enhance carbon stocks in degraded forests[13].

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Characterization of marine actinomycete having antiviral activity against cucumber mosaic virus

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Metabolite of 28 marine actinomycete isolates was assessed for antiviral activities against cucumber mosaic virus (CMV). Metabolite of one isolate (no. 21) was found most effective in controlling the CMV infection under glasshouse conditions by dual inoculation, seed treatment and spray treatment, individually. Under field conditions, treatment of cucumber seeds with the metabolite of actinomycete isolate no. 21 followed by four foliar sprays of the metabolite on cucumber plants, effectively controlled CMV infection. The morphological structures of the isolate were identical to *Streptomyces* sp. It was identified as *Streptomyces olivaceus* on the basis of 16-S ribosomal RNA gene sequencing which was in conformity with morphological, physiological and biochemical characteristics of the isolate.

Keywords: Actinomycetes, antiviral activity, characterization, CMV, *Streptomyces olivaceus*.

CROP losses due to plant infecting viruses are second only to fungal diseases, but viruses are much more difficult to control. In case of emerging infectious diseases of plants, about 47% of the diseases have been reported to be caused by viruses[1]. Some virus outbreaks have been severe enough to destroy entire target plants in specific areas. Various strategies are being employed to control plant

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viruses. Majority of these methods are designed to prohibit vector activity, transferring the plant virus rather than inhibiting the virus itself. However, the phytotoxicity of insecticides used against viral vectors often results in prolonged recovery time and poor survival of plants. Plant virus control has its own drawbacks, at the root of which is the acellular nature of the virus. In many cases, once a crop is affected, uprooting the plant remains the only choice to combat further spread of the pathogen. Considering all these limitations and the current interest in environment and human health, development of new alternative plant virus control methods has been intensified.

Actinomycetes are known for their ability to produce a wide range of bioactive secondary metabolites having great structural and functional diversity. They include antibacterial, antifungal, antiviral, anticancer and immunosuppressants, vitamins, enzymes, amino acids, organic acids, pigments, insecticides, herbicides and other metabolites[2]. Discovery of most of the antibiotics stemmed from decades of research on soil dwelling actinomycetes. Terrestrial forms have been widely exploited and reported to produce hundreds of antibiotics. However, aquatic forms, particularly their estuarine and marine counterparts, have largely been ignored and remain underexploited for useful metabolites. Marine environment is a potential source for new actinomycetes and novel antibiotics. Recent studies have shown that novel compounds are being frequently reported from marine sources, e.g. the new antibiotics like arenicolides A, B and C from marine actinomycete *Salinispora arenicola*, salinosporamides D–J from *Salinispora tropica*, piericidins C7 and C8, the cytotoxic antibiotic produced from marine *Streptomyces* sp.[3,4]. The present study was therefore undertaken to identify marine actinomycetes having antiviral capability against plant virus.

Seawater samples were collected from Arabian Sea in the Konkan coastal region for isolating marine actinomycetes. The actinomycetes were isolated by serial dilution technique using starch casein agar (SCA) medium[5]. Over 28 isolates were obtained. The colonies were purified by streak plate method and the pure isolates were maintained on SCA slants. The isolates were mass multiplied on starch casein broth. For this, loop full inoculum of each isolate was inoculated in a 100 ml flask containing 30 ml starch casein broth and incubated at 28°C for 15 days with intermittent shaking. After incubation, actinomycetes mats were separated by straining through muslin cloth. The broth was then centrifuged at 15,000 *g* for 15 min. The supernatant was collected, filtered through Whatman No.1 filter paper and subsequently through G-5 sintered glass bacteriological filter assembly. The cell-free metabolite of each isolate thus obtained was further used for antiviral assay.

The muskmelon leaves showing typical virus symptoms were collected from the field. Based on host range studies and symptomatology the virus was confirmed as cucumber mosaic virus (CMV). The virus was maintained on cucumber plants under glasshouse conditions throughout the study and the actinomycetes metabolites were assessed for their antiviral activity against CMV using cucumber as systemic host of the virus.

The virus inoculum was prepared by grinding the virus infected cucumber leaves and the metabolite of each of the actinomycete isolate was mixed individually with the virus inoculum i.e. sap in 1 : 1 proportion. The mixtures were kept for 1 h at room temperature and each of the mixtures was then inoculated on to cucumber plants (10 each) at cotyledon stage. The symptoms produced on cucumber plants were recorded up to 30 days after inoculation.

The five actinomycete isolates capable of inhibiting the virus symptoms, identified by dual inoculation treatment, were further evaluated during seed and spray treatments.

Seeds of cucumber var. Pune khira were first surface sterilized with 0.1% $HgCl_2$ for 30 sec followed by three subsequent washings with sterilized distilled water. The sterilized seeds were dipped in the respective actinomycetes metabolite solutions for 8 h at room temperature. Seeds dipped in distilled water served as a control. After 8 h, the treated seeds were sown in the pots filled with sterilized soil in glasshouse. Ten plants for each treatment were maintained. The plants were inoculated with the virus sap at cotyledon stage and the symptoms produced were recorded up to 30 days after inoculation.

In the spray treatment, the cucumber seeds were sown in pots filled with sterilized soil in glasshouse. When the plants were at virus inoculation stage, i.e. cotyledon stage. They were sprayed with the respective actinomycete metabolite (10 plants/metabolite), a day prior to inoculation. Next day the plants were mechanically inoculated with the virus sap. The metabolite spray was repeated after 15 days. The systemic symptoms were again recorded up to 30 days after inoculation.

The metabolite of one actinomycete isolate that exhibited stronger antiviral activity in glasshouse experiments was further evaluated under field conditions against systemic infection of the test virus.

The cucumber seeds were dipped in actinomycete metabolite for 8 h and sown at 1.0×0.5 m spacing in the field with ridges and furrows. The 15-day-old plants were inoculated artificially using an insect vector of the virus (aphids-*Myzuspersicae*). A day prior to the release of aphids, these plants were sprayed with actinomycete metabolite. An insecticide was sprayed for 24 h after the release of aphids to destroy them. Subsequent sprays of actinomycete metabolite were given at 15-day interval (from the day of first spray) until the crop was 60 days old. Plants raised from the seeds that were neither dipped nor sprayed with actinomycete metabolite served as control. The systemic symptoms produced were recorded seven days after each spray and the per cent inhibition was worked out on the basis of number of healthy

Table 1. Efficacy of actinomycetes metabolites against CMV infection (dual inoculation treatment)

Test virus with metabolite of actinomycete isolate no.	Virus inoculated plants	Diseased plants	Incubation period (days)	Ratio of healthy to virus infected plants
4	10	3	22	7 : 3
13	10	4	22	6 : 4
16	10	3	20	7 : 3
20	10	3	25	7 : 3
21	10	0	–	10 : 0
Without actinomycete metabolite (control)	10	10	10	0 : 10
Un-inoculated control	10	0	10	10 : 0

Table 2. Efficacy of actinomycetes metabolites as seed treatment against CMV infection

Seed treatment with metabolite of actinomycete isolate no.	Virus inoculated plants	Diseased plants	Incubation period (days)	Ratio of healthy to virus infected plants
4	10	8	14	2 : 8
13	10	8	16	2 : 8
16	10	9	13	1 : 9
20	10	6	20	4 : 6
21	10	2	22	8 : 2
No. of actinomycete metabolite (control)	10	10	10	0 : 10
Un-inoculated control	10	0	–	10 : 0

plants/diseased plants. Morphological, physiological, biochemical and molecular characterization was done for the effective actinomycete isolate with strong antiviral activity.

The morphological characteristics of actinomycete isolate were studied by cover slip method[6]. Observations on spore bearing hypha, spore chain and number of spores were recorded. Colony characteristics of the isolate were studied as per the method by International Streptomyces Project (ISP)[7]. The physiological characteristics, viz. sodium chloride tolerance, ability to grow at different pH and utilization of different carbon sources were studied. Sodium chloride solution of different concentrations (0%, 5%, 10%, 15%, 20% and 25%) was added to ISP-2 medium to test the isolate's sodium chloride tolerance capacity. Ability of the isolate to grow at different pH was tested by using SCA medium, whereas the ability to use nine different carbon sources for energy and growth was examined by using carbon utilization medium. Ten per cent solution of each carbon source was prepared and filter-sterilized. These solutions were added separately to the basal mineral salt agar medium to give a final 1% concentration of carbon source. The isolate was then streak-inoculated onto the medium plates having different carbon sources. Plates with no carbon source served as negative control, whereas, with D-glucose, they served as positive control. The growth on each plate was compared with these two controls and observations were recorded.

To study the biochemical characteristics, molecular characterization of the isolate was done to identify the isolate up to species level. Microbial identification services of Geneombio Technologies (Pune), based on 16-S ribosomal RNA gene sequencing were used to identify the test isolate. The genomic DNA of the isolate having 1314 base pairs was aligned by matching with the reported gene sequences using BLASTN 2.2.28+ programme.

In dual inoculation (mixture of virus sap and actinomycete metabolite) treatment, highest virus control was obtained with metabolite of isolate no. 21 and the ratio of healthy to virus infected plants was 10 : 0 (Table 1). The metabolite of four other isolates, viz. isolate no. 4, 13, 16 and 20 also showed promising virus control. In the treatment of isolate nos 4, 16 and 20 the ratio of healthy to virus infected plants was 7 : 3; whereas, it was 6 : 4 in case of isolate no. 13. Further, incubation period of the virus was also prolonged up to 20–25 days with these isolates. These five isolates were further evaluated during seed and spray treatments against the virus. The remaining 23 isolates were found ineffective to inhibit the virus symptoms and the symptoms on plants appeared within 10–18 days after dual inoculation.

Virus symptom expression on cucumber plants was delayed with seed treatment of all the five metabolites (Table 2). The maximum virus infection control was observed for metabolite of isolate no. 21 with 8 : 2 ratio of healthy to virus infected plants. The symptoms on these two virus infected plants appeared 22 days after virus inoculation. The seed treatment of metabolites of isolate nos. 4, 13, 16 and 20 though delayed the symptom expression, could not control virus infection. In the spray treatment, metabolite of isolate no. 21 gave maximum virus infection inhibition and the ratio of healthy to virus infected plants was 7 : 3 (Table 3). The systemic symptoms

Table 3. Efficacy of actinomycetes metabolites as spray treatment against CMV infection

Spray of metabolite of actinomycete isolate no.	Virus inoculated plants	Diseased plants	Incubation period (days)	Ratio of healthy to virus infected plants
4	10	9	15	1 : 9
13	10	10	16	0 : 10
16	10	10	11	0 : 10
20	10	8	15	2 : 8
21	10	3	26	7 : 3
No. of metabolite spray (control)	10	10	10	0 : 10
Un-inoculated control	10	0	–	10 : 0

Table 4. Efficacy of metabolite of actinomycete isolate no. 21 under *in vivo* conditions

	Percentage of diseased plants after			
Treatment	1st spray	2nd spray	3rd spray	4th spray
Metabolite of isolate no. 21	–	5.26 (1/19)	10.52 (2/19)	21.05 (4/19)
Control	–	65.00 (13/20)	90.00 (18/20)	100.00 (20/20)

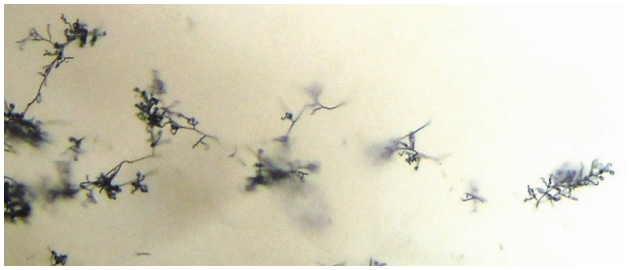

Figure 1. Spiral spore arrangement of actinomycete isolate no. 21.

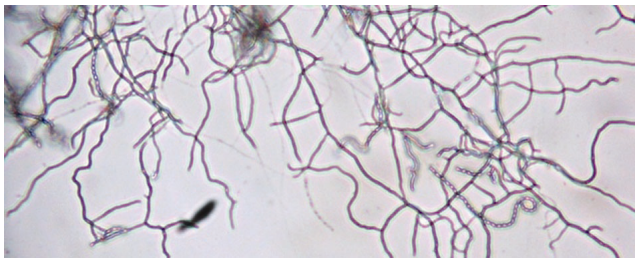

Figure 2. Spore chains of actinomycete isolate no. 21 (40×).

on these three virus infected plants appeared 26 days after virus inoculation. Thus, dual inoculation, seed and spray treatments of metabolite of actinomycete isolate no. 21 were found most effective in controlling the systemic infection of CMV over inoculated control.

Under field conditions, seed and spray treatments of isolate no. 21 effectively controlled the systemic virus infection (Table 4). There were only 21.05% diseased plants under field conditions compared to 100% in the control treatment where no seed or spray treatment was given.

The characterization of the effective actinomycete isolate no. 21 was studied following standard procedures. Microscopic observations showed spiral spore arrangement (Figure 1). The actinomycete mycelium produced long chains of sporophores containing more than 35–40 spores which were cylindrical to oval in shape (Figure 2). Spore arrangement and long chains of conidia were typical as that of *Streptomyces* spp. The other characteristics of the effective isolate are listed in Table 5. The isolate grew well on all the culture media, i.e. ISP 2, ISP 3, ISP 4, ISP 5 and SCA. Aerial mycelium colour of the colony was in different shades of grey in all the media. The reverse side/substrate mycelium was grey in almost all media tested except SCA, wherein it was brown. The isolate did not produce diffusible colour on any media. Regarding the physiological characteristics, the isolate tolerated sodium chloride up to 10% and a good growth was recorded. At higher concentrations of sodium chloride (15%, 20% and 25%) the isolate did not show any growth. It was able to grow on the medium having pH 7–10, whereas it did not show any growth at pH 5. The isolate utilized all the carbon sources as evident from its growth and showed a strong positive utilization of glucose and sucrose, positive utilization of L-arabinose, D-xylose and I-inositol, whereas weak utilization of the other four carbon sources. It was positive for lipolytic and urease activity, gelatin liquefaction and nitrate reduction, but was negative for starch hydrolysis, protease and cellulase activity, casein hydrolysis, indole production, oxidase test and catalase test.

On basis of these results, the effective isolate was tentatively identified as *Streptomyces* sp. Further study on the basic 16-S ribosomal RNA gene sequencing revealed that the genomic DNA of the isolate had 100% similarity to *Streptomyces* sp. The phylogenetic tree generated by using MEGA 5.1 software showed closest match of the test isolate (BI-14) with *Streptomyces olivaceus* (JN 204723) (Figure 3) which was in conformity with its morphological and physiological characteristics[8].

Table 5. Characteristics of actinomycete isolate no. 21

Character	On medium				
	ISP 2	ISP 3	ISP 4	ISP 5	SCA
Colony characteristics					
Aerial mycelium colour	Light grey	Light grey	Dark grey	Dark grey	Grey
Reverse side/substrate mycelium colour	Grey	Grey	Grey	Grey	Brown
Diffusible colour other than melonid pigmentation	–	–	–	–	–
Growth	Good	Good	Good	Good	Good

Physiological characteristics

Sodium chloride tolerance limit (%)		Utilization of carbon sources*	
0	Less growth	Glucose	++
5	Moderate growth	L-Arabinose	+
10	Good growth	Sucrose	++
15	No growth	D-Xylose	+
20	No growth	I-Inositol	+
25	No growth	D-Mannitol	±
		D-Fructose	±
5	No growth	Raffinose	±
6	Less/poor growth	Cellulose	±
7	Very good growth	Control (no carbon)	–
8	Very good growth		
9	Good growth		
10	Moderate growth		

Biochemical characteristics

Character/test	Reaction	Character/test	Reaction
Starch hydrolysis	Negative	Gelatin liquefaction	Positive
Lipolytic activity	Positive	Nitrate reduction	Positive
Urease activity	Positive	Indole production	Negative
Protease activity	Negative	Oxidase test	Negative
Cellulase activity	Negative	Catalase test	Negative
Casein hydrolysis	Negative		

Carbon utilization*

Strongly positive utilization (++)	Vibrant growth
Positive utilization (+)	Good growth but less than on glucose
Weak utilization (±)	Weak growth but better than on control, i.e. without carbon source
Utilization negative (–)	No growth

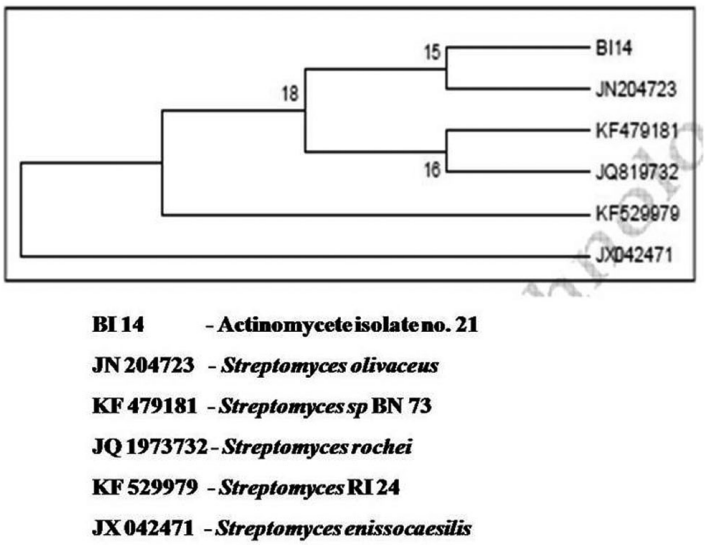

Figure 3. Phylogenetic tree of actinomycete isolate no. 21 (40×).

Organisms growing in marine environments are metabolically and physiologically different from terrestrial organisms and marine actinomycetes are recognized as a potential source for many novel bioactive metabolites[9]. The present study reveals that the metabolite of marine actinomycete isolate has antiviral activity against CMV as it effectively controlled its systemic infection in cucumber under glasshouse and field conditions. Through characterization, the isolate was identified as *Streptomyces olivaceus* 21. Earlier, Mohamed and Galal[10] reported that dual inoculation of culture filtrates of *Streptomyces* isolates and virus sap mixture reduced the number of necrotic local lesions produced by tobacco mosaic virus (TMV) and potato virus Y on *Nicotiana glutinosa* and *Chenopodium quinoa* respectively. Similarly, Xing *et al.*[11] reported that mixing of actinomycetes fermentation broth with TMV sap for 10, 30 and 60 min displayed significant virus inactivation in *Nicotiana glutinosa* and *N. tabacum*. Antiviral activity of *S. rimosus* and *S. qouqerotti* against TMV[12] and *S. caeseorhomyces* against potato virus X[13] have also been reported. Similar results were obtained in the present study with dual inoculation of metabolite of actinomycete *S. olivaceus* 21 and virus sap. systemic infection of CMV was not observed on cucumber plants treated by dual inoculation treatment.

Ghaly *et al.*[14] reported 95–100% reduction in symptoms of zucchini yellow mosaic virus in *Cucumis sativus* by foliage treatment with culture filtrate of *S. albovianaceaus* and *S. sparasogenes* respectively. Further, they observed that these culture filtrates showed more virus inhibition activity when applied before virus inoculation than after inoculation. Similar results were obtained by Galal[15] against CMV in *Cucumis sativus* who also reported that soaking of seeds for 2 h in actinomycetes filtrates resulted in highest virus inhibition. In our study, under glasshouse conditions, it was observed that seed treatment (dipping for 8 h) with metabolite of actinomycete *S. olivaceus* (isolate no. 21) effectively controlled CMV infection. In spray treatment experiment, foliar application of the actinomycete metabolite prior to inoculation of virus was effective in controlling CMV. Under field conditions, seed treatment with metabolite *S. olivaceus* followed by spraying of the metabolite at 15-day interval on foliage resulted in significant control of CMV infection in *Cucumis sativus*.

Severe economic losses occur worldwide due to viral plant diseases. CMV has an extremely wide host range including more than 1200 plant species in 500 genera of 100 families[16] and is one of the most important viruses that affects the economy of cucurbitaceous crops. Exploitation of antiviral properties of the metabolite of *S. olivaceus* 21 can certainly help in the control of plant virus disease.

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