ISSN 0258-7122 (Print), 2408-8293 (Online)
Bangladesh J. Agril. Res. 46(3): 331-341, September 2021

DETECTION OF MYCOVIRUS IN *Sclerotium rolfsii*, A PHYTOPATHOGENIC FUNGUS IN BANGLADESH

M. I. FARUK[1] AND N. SUZUKI[2]

Abstract

A research project was conducted to detect presence of mycovirus, a probable virocontrol agent in *Sclerotium rolfsii*, a destructive soil-borne plant pathogenic fungus. A total of 500 isolates of the pathogen was isolated from infected wheat, bush bean, tomato, eggplant, lentil, chickpea, eggplant, chilli and okra grown Bangladesh following tissue planting methods and brought to the Virology laboratory of Okayama University, Japan. After brought to Japan, a total of 472 isolates were re-isolated and the fungal isolates were cultured on potato dextrose agar for extraction of DNA and dsRNA. The extracted nucleic acids were tested for the presence of viruses using rolling circle amplification (RCA) (for DNA viruses) and cellulose column chromatography (for RNA viruses). The detected *S. rolfsii* viruses were further characterized molecularly. For detection of DNA virus, a total of 120 isolates were tested, none were found positive for DNA viruses. For detection of dsRNA virus, a total of 472 isolates were tested, 7 isolates showed possible dsRNA positive band. The partial cDNA sequences of the dsRNA segments isolated from the strain SR336 were obtained. BlastX database search with the partial sequence from SR336 isolates showed similarities with randorna like virus. To screen the presence of randorna like virus in *S. rolfsii* isolates, 64 isolates were selected from 472 isolates of *S. rolfsii*. The isolates of *S. rolfsii* were tested by RT-PCR using randorna virus specific primers**.** Out of 64 isolates 13, (20.31%) isolates were randorna virus positive strain and rest of the isolates were absent of randorna virus.

Introduction

Mycoviruses are viruses which infect fungi. The majority of mycoviruses have double stranded RNA genomes and isomeric particles, but approximately 30% have positive sense, single-stranded RNA genomes. True mycoviruses have an ability to infect healthy plant pathogenic fungi causing mypovirulence and death. Studies in fungal and viral interaction can lead to the development of novel biological control strategies (Cho *et al*., 2013; Longkumer *et al*., 2020). The search of mycoviruses having potential virocontrol properties is focused mainly on identifying and characterizing those viral species in naturally infected fungal pathogens to be controlled (Garcia-Pdrajas *et al*., 2019). Fungal pathogens such as species of *Sclerotium rolfsii, Sclerotinia, Rhizoctonia* and *Fusarium* are ubiquitous and have broad host range enabling them to cause a severe infection resulting in huge yield losses of important crops. Various control tactics like cultural, mechanical are used to control those pathogens. However, effectiveness

[1]Principal Scientific Officer, Plant Pathology Division, Bangladesh Agricultural Research Institute (BARI), Gazipur-1701, Bangladesh, [2]Professor, Okayama University, Japan.

of those techniques may not be satisfactory. From eco-friendly view point, biological control can play vital role in infecting the virulent fungal pathogen by reducing their virulence giving to a phenomenon known as hypovirulence (Longkumer *et al*., 2020). *S. rolfsii* is a ubiquitous, widely distributed, major soil-borne fungal pathogen attacking more than 500 crop species under about 100 families including cereals, vegetables, pulses and oil seeds in the world. The fungus produces resistance spores called sclerotia, which help to survive for long time under adverse condition. It is very difficult to eradicate the fungus from infected fields (Aycock, 1966). In Bangladesh, the destructive pathogen occurs throughout the country and attacks most of the important crops causing considerable yield losses (Mondal *et al*., 1996). To control the diseases mainly cultural and fungicidal management are recommended. However, fungicides sprays often exhibit an associated risk of developing fungicide-resistant fungal isolates (Ma *et al*., 2009; Kuang *et al*., 2011) and may pose potential negative impacts on both environment and food safety (Punja *et al.*, 1982). *Trichoderma* species have long been known for their capacity to reduce plant diseases caused by soil-borne fungi (Whipps and Lumsden, 2001) and some commercial formulations of these bio-control agents are available in many countries of the World. "Virocontrol" is defined as a form of biocontrol of fungal plant pathogens using mycoviruses that infect and weaken target pathogenic fungi. It is environmentally friendly and durable. Many researchers have been inspired by the success of virocontrol of the chestnut blight disease using fungal viruses in Europe. The hypovirulence-associated mycoviruses have potential for exploitation as alternative biological control agents. Mycoviruses are viruses that selectively infect fungi and are ubiquitous in all major groups of filamentous fungi. The presence of some mycoviruses have been associated with particular phenotypic traits, including killer toxin production in *Saccharomyces cerevisiae, Ustilago maydis* and *Zygosaccharomyces bailii* (Schmitt and Neuhausen, 1994), debilitated diseases in *Helminthosporium victoriae* (Nuss and Koltin, 1990) and virulence reduction (hypovirulence) in *Sclerotinia sclerotiorum* (Boland, 1992), *Fusarium graminearum* (Chu *et al*., 2002), *Cryphonectria parasitica* (Anagnostakis 1982) and so on. Identifications of new mycoviruses from *S. rolfsii* will offer new insights into implementation of virocontrol of *S. rolfsii*. The presence piece of research was under taken to detect mycovirus, a probable virocontrol agent in *S. rolfsii* a destructive soil-borne phytopathogenic fungus.

Materials and Methods

Isolation of *S. rolfsii* isolates, their culture and preservation

Virus infected or virus-free *S. rolfsii* isolates were isolated from different infected host plants such as tomato, eggplant, wheat, cucurbits, lentil and chickpea collected from various locations of Bangladesh (Table 1) and cultured on potato dextrose agar (PDA) medium at 25°C±2°C. Growing mycelium tips were sub-

cultured for purification of isolates. The colonies were developed within 3-4 days and sclerotial formation started within a week. A total of 500 isolates of *S. rolfsii* were collected from Bangladesh and brought to the Virology laboratory of Okayama University, Japan. Out of five hundred isolates 472 isolates were re-isolated, preserved and used for further study.

Table 1. Particulars of Sclerotium *rolfsii* isolates

Name of dstrict and upazilla	Name of crops	Total number of isolates	Remarks
Gazipur, Sadar	Wheat	76	The disease infected plant samples were collected during seedling stage of the crops except bush bean, brinjal and chilli where the disease infected plant samples were collected both seedling and maturity stage of the crops
	Barley	58	
	Bush bean	18	
	Lentil	51	
	Chickpea	24	
	Chilli	16	
Jamalpur, Sadar	Brinjal	12	
	Lentil	43	
	Chickpea	06	
Jashore, Sadar	Wheat	45	
	Barley	18	
	Lentil	29	
	Chickpea	19	
Pabna, Ishurdi	Wheat	07	
	Lentil	14	
	Chickpea	11	
Madaripur, Sadar	Lentil	21	
	Chickpea	11	
Barisal, Rahmatpur	Bush bean	04	
	Lentil	12	
	Chickpea	05	
Total		500	

Morphological and molecular characterization of isolates

Initially the *S. rolfsii* isolates were identified based on the morphological characterization. Finally molecular studies were performed by PCR based partially amplification of ITS region and sequencing for identification of *S. rolfsii* isolates.

DNA isolation

Total nucleic acids were prepared from fungal mycelia as described by Suzuki *et al*. (2003). Seven days old fungal mycelia cultured in 20 ml potato dextrose broth (PDB) were harvested onto two layers sterilized Miracloth (Calbiochem). The harvested mycelia were homogenized using a mortar and pestle in the presence of liquid nitrogen and suspended in 4 ml of extraction buffer composed of 100 mM Tris-HCl (pH 8.0), 200 mM NaCl, 4 mM EDTA and 2% sodium dodecyl sulfate (SDS). The suspended solution was transferred to 15 ml Corex tubes containing 4 ml of water saturated phenol/chloroform followed by phenol/chloroform and of chloroform-isoamyl alcohol extraction. The nucleic acid solution was transferred to clean tubes containing 0.1 volumes of 3M NaOAc and 2 volumes of cold ethanol and incubated at 4 ^{0}C for 1 hr. Total nucleic acids were collected by centrifugation at 10000 rpm for 10 min, washed with 70% ethanol, dried, and re-suspended in water.

PCR amplification of ITS region

Polymerase Chain Reaction (PCR) amplification of Internal Transcribed Spacers (ITS) region of rDNA was performed using universal primers ITS-1 (5' - TCC GTAGGT GGA CCT GCG G - 3') as forward primer and ITS-4 (5' -TCC TCC GCT TAT TGA TAT GC - 3') as reverse primer (White *et al*., 1990) in eppendorf PCR master cycle. Amplification was carried out in 0.2 ml eppendorf tubes with 25 µl reaction mixture containing 2.5 µl of 10x Taq buffer, 2.5 µl of 25 mM MgCl2, 2.0 µL of ITS1 primer, 2.0 µl of ITS-4 primer, 0.5 µl of 100 mM dNTP mix, 0.125 µl of Taq polymerase and 14.37 µl of sterile PCR water and 3 µl of DNA sample. The PCR amplification was carried out by 35 cycles, of which denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1.5 min with initial denaturation at 94°C for 4 min before cycling and final extension at 72°C for 6 min after cycling. Amplified PCR products were observed in 1.0 per cent agarose gel in 0.5% TAE buffer and visualized under UV transilluminator with ethidium bromide staining. The size of the PCR product was estimated by comparison with known DNA marker of 1 kb DNA ladder. The banding profiles of ITS-PCR products were documented in gel documentation system.

dsRNA isolation and cDNA library construction

Total nucleic acid was extracted by the method as described by Sun and Suzuki (2008). For dsRNA extraction, all the fungal strains of *S. rolfsii* were cultured on cellophane membranes on potato dextrose agar (PDA) plates for 4 to 5 days at room temperature. The mycelia were collected and ground to a fine powder in liquid nitrogen. Nucleic acid fractions were obtained by treatments with phenol, phenol/chloroform and chloroform. Double-stranded RNA was further purified

by binding with CC41 cellulose. Total nucleic acids were incubated with CC41 cellulose in STE buffer containing 15% (v/v) ethanol for 1 hour. After washing the cellulose with the STE-15% ethanol for 3 times, dsRNA was eluted by STE buffer and precipitated by the addition of 2 volumes of ethanol followed by digestion with S1 nuclease and subsequently with DNase I, as described by Suzuki *et al*. (2003).

A cDNA library of total dsRNA was constructed using a classic non-PCR based method to minimize misincorporations during cDNA synthesis (Hillman *et al*., 2004). After denaturation at 65ºC in 90% dimethyl sulfoxide (DMSO), dsRNA was used as a template for cDNA synthesis with random hexamers using a Time Saver TM cDNA synthesis kit. The resulting cDNA was ligated into the cloning site of pGEM-T Easy vector in Rapid Ligation Buffer with T4 DNA ligase. The resulting ligates were transformed into competent *E. coli* strain DHα5 and incubated in a series of conditions such as on ice for 30 min followed by at 42 ^{0}C for 60 sec and then 3 min on ice. The recombinant was grown in 80 µl SOC medium at 37 ^{0}C for 60 min and spread on LB plate. The plates were incubated overnight for growing the recombinant plasmid. Selected individual plasmid clone was cultured in 3 ml LB containing 50 µg/ml ampicillin for overnight. The plasmids were then purified from *E. coli* using QIAprep Spin Miniprep Kit (QIAGEN) according to the protocol provided by the manufacturer.

Rolling circle amplification (RCA) for detection of ssDNA viruses

Amplification of small circular DNA was performed by rolling circle amplification (RCA) using a TempliPhi™ Kit following the manufacturer's protocol. Master Mix 1 was prepared by mixing 5 µl of TempliPhi Reaction Buffer and 0.2 µl of Tem-pliPhi Enzyme Mix. Five microliters of this TempliPhi premix was mixed with 1 ul of DNA template each fungal isolates then incubated at 30°C for 18 hours. After this incubation period, the enzyme was heat-inactivated at 65°C for 10 minutes. The samples were cooled and stored at 4°C. The RCA products were digested with different restriction enzyme in 10 µl final volumes for 2 h at 37°C, following the manufacturer's protocol, separated in 1 % agarose gels in 0.5 % TAE buffer and stained with 0.5 µg/ml ethidium bromides. The unit-length linear genomes purified from the agarose gels by a Wizard SV gel and PCR clean-up system kit (Promega) that was cloned in specific vector and were transformed into competent *E. coli* strain DHα5. Selected individual plasmid clone was cultured in 3 ml LB containing 50 µg/ml ampicillin for overnight. The plasmids were then purified from *E. coli* using QIAprep Spin Miniprep Kit (QIAGEN) according to the protocol provided by the manufacturer and were sequenced.

Sequencing and sequence analysis

Plasmid DNA was prepared by spin columns and sent to Institute of Plant Science and Resources (IPSR), Japan for sequencing. Sequences were analyzed using the Genetyx DNA-processing software (SDC, Tokyo). Sequence fragments were assembled with AutoAssembler™ 2.0 ABI Prism and analyzed using Genetyx Mac 10.0. Homology search was performed with the BLAST suite of programs from National Center for Biotechnology Information (NCBI) and sequence was aligned following the program CLUSTALW (Thompson *et al*., 1997).

Results and Discussion

Morphological characterization of *S. rolfsii* isolates

A total 500 isolates of *S. rolfsii* were isolated from infected plant parts grown in Bangladesh and brought to the Virology laboratory of Okayama University, Japan. After brought to the Japan, a total of 472 isolates were re-isolated and identified on the basis of morphological characterization.

Molecular characterization of *S. rolfsii* isolates by ITS-PCR

The structure of rDNA cluster and the expected amplified products with ITS-1 and ITS-4 primers are shown in Fig. 1. The primers ITS-1 and ITS-4 were used for PCR amplification of ITS region of rDNA cluster which included ITS-1 and ITS-2 regions of all 472 isolates. Both the primers produced amplified product size of 650-700 bp in all the 472 isolates. These results confirmed that all the isolates belonged to genus *Sclerotium*. Harlton *et al*. (1995) screened a worldwide collection of *S. rolfsii* which revealed variation in ITS regions of 12 sub-groups of *S. rolfsii*. Almeida *et al*. (2001) studied variability among 30 isolates of *S. rolfsii* by RAPD and were differentiated into distinct groups by ITS-PCR.

Detection of double-stranded RNA fragments/mycovirus in *S. rolfsii* isolates

All 472 *S. rolfsii* isolates were tested for the presence of putative dsRNA viruses. Extracts of dsRNA from mycelia of all 472 *S. rolfsii* isolates were treated with DNase I and S1 nuclease, and then subjected to agarose gel electrophoresis. One or two distinct dsRNA segments/band was observed in several strain of *S. rolfsii* (Fig. 2). DsRNA of selected 7 fungal strain possible virus origin of those fungal strains were reverse-transcribed and used for library construction for sequence analysis. The partial cDNA sequences of the dsRNA segments isolated from the strain SR336 were obtained (Fig. 3). BlastX database search with the partial sequence from SR336 isolates revealed that the isolated dsRNA was the partial sequence showed similarities with randorna like virus. Two pairs of primer from the partial sequence of SR336 isolates were desinged. To search the presence of

randorna like virus in *S. rolfsii* isolates, randomly 64 isolates were selected from 472 isolates of *S. rolfsii*. The selected isolates of *S. rolfsii* were tested by RT-PCR using randorna virus specific primers**.** Results showed that out of 64 isolates *S. rolfsii*, 13 (20.31%) showed randorna virus positive strain (Fig. 4). Subsequent necessary analyses were carried out by the host laboratory.

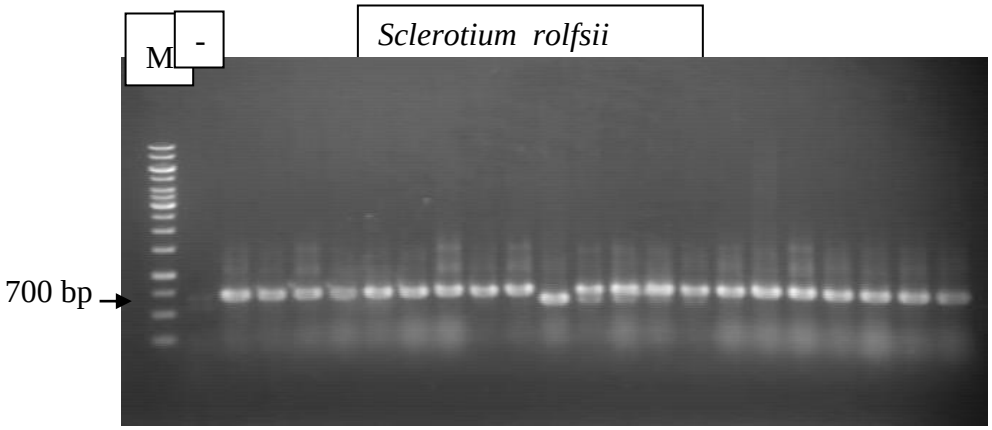

Fig 1. Amplification product of Internal Transcribed Spacer (ITS) with ITS-1 and ITS-4 ribosomal DNA primers; M=1 kb DNA ladder. All Lanes represented *S. rolfsii* isolates.

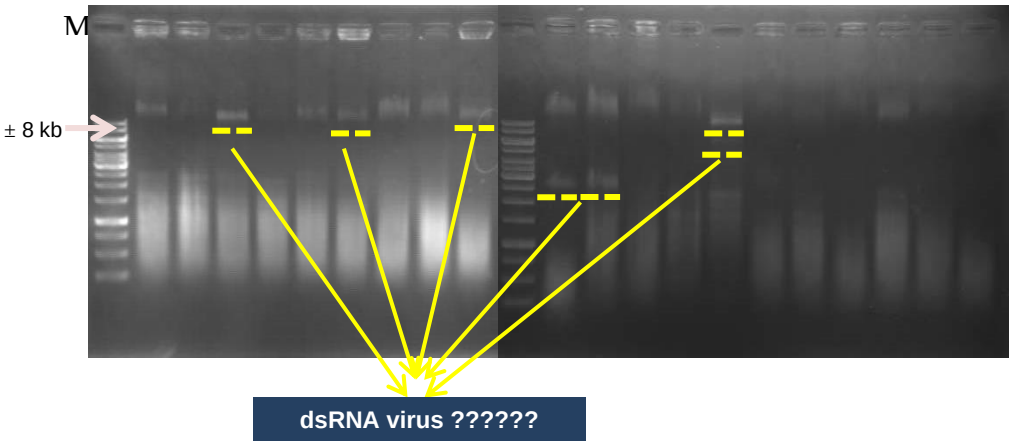

Fig. 2 Gel electrophoretic profiles of dsRNAs isolated from *S. rolfsii* isolates. DsRNAs purified from different strains of *S. rolfsii* and were analyzed in 1% agarose gel electrophoresis. A GeneRuler 1-kb DNA ladder (Thermo Scientific) was also used as a marker.

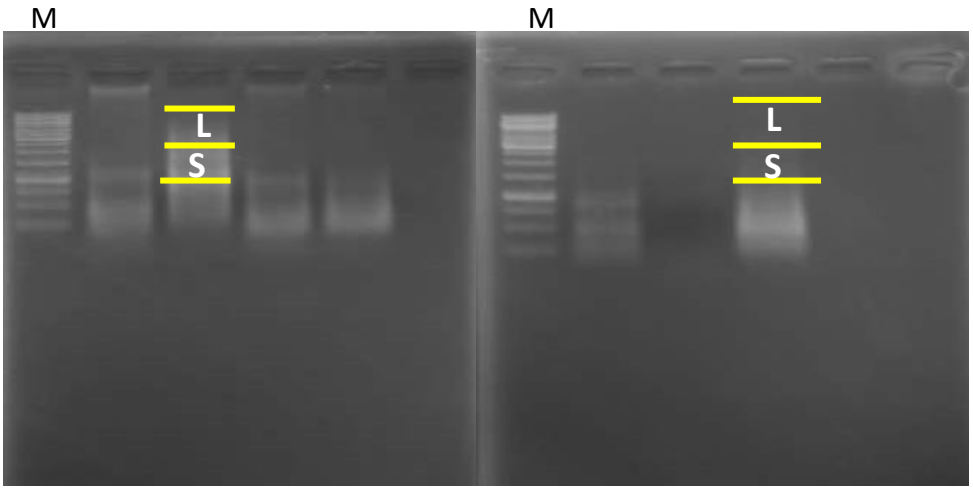

Fig. 3 Gel electrophoretic profiles of constructed cDNA of *S. rolfsii* isolates. For construction of cDNA purified dsRNA from different strains of *S. rolfsii* were used and analyzed in 1.5% agarose gel electrophoresis. A GeneRuler 1-kb DNA ladder (Thermo Scientific) was also used as a marker.

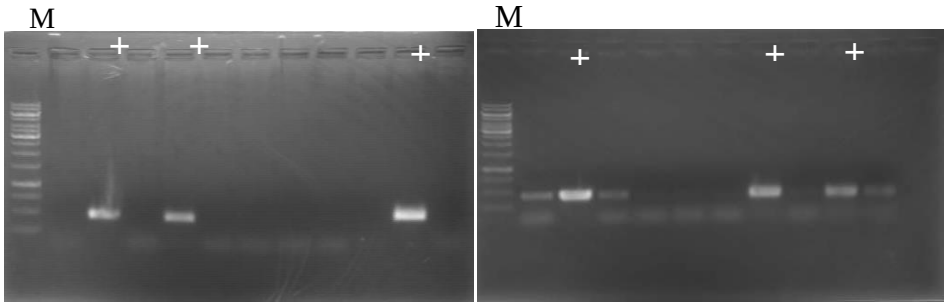

Fig. 4 Gel electrophoretic profiles of constructed cDNA of *S. rolfsii* isolates. For RT-PCR purified dsRNA from selected different strains of *S. rolfsii* were used with rendorna virus specific primers and analyzed in 1.5% agarose gel electrophoresis. A GeneRuler 1-kb DNA ladder (Thermo Scientific) was also used as a marker.

Detection of ssDNA viruses by Rolling Circle Amplification in *S. rolfsii* isolates

To detect the presence of in different strains of *S. rolfsii* isolates, rolling circle amplification (RCA) by PCR with restriction enzyme (RE) digestion was employed. A total of 35 isolates of *S. rolfsii* were randomly selected from 472 isolates of *S. rolfsii* for detection of ssDNA viruses associated with *S. rolfsii* strains. The RCA products were directly Hind III digested, and restriction products were separated by gel electrophoresis (Fig. 5). The resulted fragments were purified by a Wizard SV gel and PCR clean-up system kit (Promega). The

purified fragments were ligated into the cloning site of pGEM-T Easy vector (Promega) with T4 DNA ligase (Promega). The resulted ligates were transformed into competent *E. coli* strain DHα5 and the selected transformants were then used for colony PCR using M13F and M13R primers set to identify the positive transformants. Results showed that none of the transformants were found positive transformants which indicated that selected RCA fragments were not ssDNA virus (Fig 6). The results of the present study indicated that ssDNA virus was not associated with the tested 35 isolates of *S. rolfsii*. Therefore, more fungal strains should be tested the host laboratory for this purpose.

Conclusion

The present finding is the first evidence of mycovirus in a plant pathogenic fungus *S. rolfsii* from Bangladesh. This report might be helpful for more study of mycovirus in other plant pathogenic fungi occur in Bangladesh.

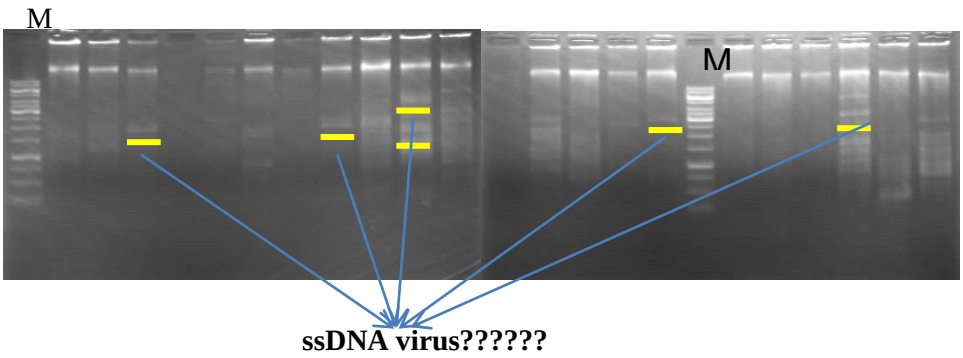

Fig. 5 Gel electrophoretic profiles of RCA products digested with Hind III. DNA purified from different strains of *S. rolfsii* used as a template for RCA reaction by TempliPhi kits and were analyzed in 1% agarose gel electrophoresis. A Gene Ruler 1-kb DNA ladder (Thermo Scientific) was also used as a marker.

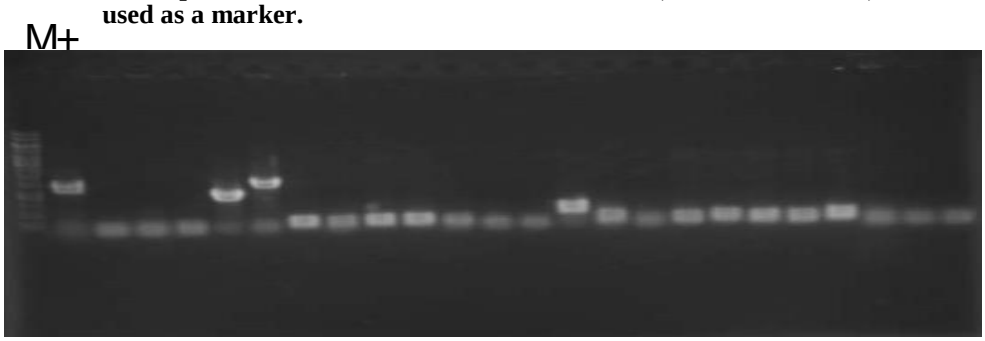

Fig. 6. Gel electrophoretic profiles of Colony PCR of *E. coli* transformants. The purified ssDNA fragment of RCA reaction were ligated into the cloning site of pGEM-T and transformed into competent *E. coli* strain DHα5. The selected colony of *E. coli* transformants were used for PCR with M13F and M13R primers and were analyzed in 1% agarose gel electrophoresis. A Gene Ruler 1-kb DNA ladder (Thermo Scientific) was also used as a marker.

References

Almeida, A.M.R., Abdelnoor, R.V., Calvo, E.S., Tessnman, D. and Yorinori, J.T. 2001. Genotypic diversity among Brazilian isolates of *Sclerotium rolfsii*. *J. Phytopathol*. **149**(9): 493-502.

Anagnostakis, S. L. 1982. Biological control of chestnut blight. *Science*. 215: 466-471.

Aycock, R. 1966. Stem rots and other diseases caused by *Sclerotium rolfsii*. North Carolina Agricultural Experiment Station Technical Bulletin No. 174, p. 202.

Boland, G. J. 1992. Hypovirulence and double-stranded RNA in *Sclerotinia sclerotiorum. Canadian Journal of Plant Pathology*. **14:** 10-17.

Cho, W. K., Kyung Lee, K. M., Yu, J., Son, M., and Kim, K. H. 2013. Advances in Virus Research. Volume 86: 273-288. Department of Agricultural Biotechnology, Center for Fungal Pathogenesis and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul, Republic of Korea. https://doi.org/10.1016/ B978-0-12-394315-6.00010-6.

Chu,Y.M., Jeon, J.J., Yea, S. J., Kim, Y. H., Yun, S. H., Lee, Y. W. and Kim, K.H. 2002. Double-stranded RNA mycovirus from *Fusarium graminearum*. *Applied and Environmental Microbiology*. **68:** 2529-2534.

Garcia-Pedrajas, M. D., Canizares, M. C., Sarmiento-Villamil, J. L., Jacquat, A. G. and Dambolena, J. S. 2019. Mycovirus in Biological Control:From Basic Research to Field Implementation. Published Online: 8 Oct 2019 https://doi.org/ 10.1094/PHYTO-05-19-0166-RVW.

Harlton, C. E., Levesque, C. A. and Punja, Z. K. 1995. Genetic diversity in *Sclerotium* (*Athelia*) *rolfsii* and related species. Phytopathology. **85:** 1269-1281.

Hillman, B. I, Supyani, S., Kondo, H. and Suzuki, N. 2004. A reovirus of the fungus *Cryphonectria parasitica* that is infectious as particles and related to the coltivirus genus of animal pathogens. J. Virol. 78:892-898.

Kuang, J., Wang, J. and Zhou, M. 2011. Monitoring on carbendazim and dimethachlon-resistance of *Sclerotinia sclerotiorum* obtained from the blight stems of rape in Jiangsu Province. Chin. Agric. Sci. Bull. **27**(15): 285–291.

Longkumer, I. Y. and Ahmad, M. A. 2020. Potential of Mycovirus in the Biological Control of Fungal Plant Pathogens: A Review. Agricultural Reviews. **41**(3): 238-247.

Ma, X. Feng, Chen, Y., Chen, C. and Zhou, M. 2009. Occurrence and characterization of dimethachlon insensitivity in *Sclerotinia sclerotiorum* in Jiangsu Province of China. Plant Dis. **93:** 36–42.

Mondal, S. N., Kageyama, K. and Hyakumachi, M. 1996. Decrease germination and virulence of oospore of *Pythium aphanidermato* in relation to loss of endogenous carbon during incubation on soil. Soil. Biol. Biochem. **28:** 545-553.

Nuss, D. L. and Koltin, Y. 1990. Significance of dsRNA genetic elements in plant pathogenic fungi. *Annu. Rev. Phytopath*. **28:** 37-58.

Punja, Z. K., Grogan, R. G. and Unruh, T. 1982. Comparative control of *S. rolfsii* on golf grass in Northern Calfornia with fungicides, inorganic salt and *Trichoderma* spp. Plant Dis. **66:** 1125-1128.

Schmitt, M. J. and Neuhausen. 1994. Killer toxin-secreting double-stranded RNA mycoviruses in the yeasts *Hanseninaspora uvarum* and *Zygosaccharomyces bailii*. *J. Virol.* **68:** 1765-1772.

Sun, L. and Suzuki, N. 2008. Intragenic rearrangements of a mycoreovirus induced by the multifunctional protein p29 encoded by the prototypic hypovirus CHV1-EP713. RNA 14:2557-2571.

Suzuki, N., Maruyama, K., Moriyama, M. and Nuss, D. L. 2003. Hypovirus papain-like protease p29 is an enhancer of viral dsRNA accumulation and vertical transmission. J. Virol. **77:** 11697-11707.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aids by quality analysis tools. Nucleic Acids Res. **25:** 4876-4882.

Whipps, J. M. and Lumsden, R.D. 2001. Commercial use of fungi as plant disease biological control agents: status and prospects. *In*: T. Butt, C. Jackson and N. Magan (eds.). *Fungal Biocontrol Agents: Progress, Problems and Potential*. CABI Publishing, Wallingford. 9-22.

White, T. J., Bruns, T. D., Lee, S. and Taylor, J. W. 1990. *In:* Innis M. A., Gelfand D. H., Sninsky J. J., White T. J., (eds.) *PCR protocols, a Guide to methods and applications Academic press, New York* pp 315- 322.