10. Bhanot, V. B., Singh, V. P., Kansal, A. K. and Thakur, V. C., Early Proterozoic Rb–Sr whole-rock ages for Central Crystalline Gneiss of Higher Himalaya, Kumaun. *J. Geol. Soc. India*, 1977, **18**, 90–91.
11. Trivedi, J. R., Gopalan, K. and Valdiya, K. S., Rb–Sr ages of granitic rocks within the Lesser Himalayan Nappes, Kumaun, India. *J. Geol. Soc. India*, 1984, **25**, 641–654.
12. Krishnamurthy, M. and Krishnaswamy, V. S., Status of mineral exploration and reserves in northwest Himalaya. In Seminar on Himalayan Geology, New Delhi preprint. *Geol. Surv. India Misc. Publ.*, 1976, **41**(VI), 291–352.
13. Dissanayake, C. B., The origin of graphite of Sri Lanka. *Org. Geochem.*, 1981, **3**, 1–7.
14. Weis, P. L., Friedman, I. and Gleason, J. P., The origin of epigenetic graphite: evidence from isotopes. *Geochim. Cosmochim. Acta*, 1981, **45**, 2325–2332.
15. Katz, M. B., Graphite deposits of Sri Lanka: a consequence of granulite facies metamorphism. *Mineral Deposit*, 1987, **22**, 18–25.
16. Dissanayake, C. B., Origin of vein graphite in high-grade metamorphic terrains. *Mineral Deposit*, 1994, **29**, 57–67.
17. Hapuarachchi, D. J. A. C., Decarbonation reactions and the origin of vein-graphite in Sri Lanka. *J. Nt. Sci. Council*, 1977, **5**, 29–32.
18. Landis, C. A., Graphitization of dispersed carbonaceous material in metamorphic rocks. *Contrib. Mineral. Petrol.*, 1971, **30**, 34–45.
19. Buseck, P. R. and Bo-Jun, H., Conversion of carbonaceous material to graphite during metamorphism. *Geochim. Cosmochim. Acta*, 1985, **49**, 2003–2016.
20. Hahn-Weinheimer, P. and Hirner, A., Isotopic evidences for the origin of graphite. *Geochem. J.*, 1981, **15**, 9–15.
21. Santosh, M. and Wada, H., A carbon isotopes study of graphites from the Kerala Khondalite Belt, Southern India: evidences for CO_2 infiltration in granulites. *J. Geol.*, 1993, **101**, 643–651.
22. Radhika, U. P. and Santosh, M., Shear-zone hosted graphite in southern Kerala, India: implications for CO_2 infiltration. *J. Southeast Asian Earth Sci.*, 1996, **14**, 265–273.
23. Galimov, E. M., *Carbon Isotopes in Oil and Gas Geology*, NASA Translation-TTF, Washington, 1975.
24. Eichman, R. and Schidlowsky, M., Isotopic fractionation between coexisting organic carbon–carbonate pairs in Precambrian sediments. *Geochim. Cosmochim. Acta*, 1975, **39**, 589–595.
25. Santosh, M., Wada, H., Satish-Kumar, M. and Binu-Lal, S. S., Carbon-isotope 'Stratigraphy' in a single graphite crystal: implications for the crystal growth mechanism of fluid-deposited graphite. *Am. Mineral.*, 2003, **88**, 1689–1696.
26. Dobner, A., Graf, W., Hahn-Weinheimer, P. and Hirner, A., Stable carbon isotopes of graphite from Bogala Mine, Sri Lanka. *Lithos*, 1978, **11**, 251–255.
27. Sanyal, P., Acharya, B. C., Bhattacharya, S. K., Sarkar, A., Agrawal, S. and Bera, M. K., Origin of graphite, and temperature of metamorphism in Precambrian Eastern Ghats Mobile Belt, Orissa, India: a carbon isotope approach. *J. Asian Earth Sci.*, 2009, **36**, 252–260.
28. Rawat, R. and Sharma, R., Features and characterization of graphite in Almora crystallines and their implication for the graphite formation in Lesser Himalaya, India. *J. Asian Earth Sci*., 2011 (in press).

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Development of non-radioactive probes for specific detection of *Dasheen mosaic virus* infecting *Amorphophallus paeoniifolius*

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A cDNA fragment ca. 1.2 kb encoding the coat protein and the 3′ untranslated region (UTR) of *Dasheen mosaic virus* (DsMV) infecting *Amorphophallus paeoniifolius* was successfully amplified from infected plant tissues using specific degenerate primers. Reverse transcription-polymerase chain reaction products were sequenced and found to be derived from the expected virus. Two specific probes, cDNA probe and riboprobe were generated from the amplicon, comprising the coat protein gene and the 3′ UTR. The probes were then successfully used for the diagnosis of the DsMV infecting *A. paeoniifolius* through nucleic acid spot hybridization. The probes detected only the DsMV infecting *A. paeoniifolius*, while they did not detect the DsMV from *Colocasia esculenta* or *Xanthosoma*.

Keywords: *Amorphophallus paeoniifolius*, *Dasheen mosaic virus*, elephant foot yam, *Potyvirus*, riboprobe.

ELEPHANT foot yam (*Amorphophallus paeoniifolius*, family Araceae) is an important edible tropical tuber crop of South-east Asian origin and grown widely in Philippines, Malaysia, Indonesia and Southeastern Asian countries. Due to its high production potential (50–60 t/ha), nutritional and medicinal values and good economic returns[1], it has achieved the status of a cash crop. The tubers are popularly used as vegetables in various delicious cuisines and in the preparation of indigenous ayurvedic medicines[2]. Among diseases of *A. paeoniifolius*, the mosaic disease caused by *Dasheen mosaic virus* (DsMV) belonging to the genus *Potyvirus* of Potyviridae family is the most destructive. Infection by DsMV causes the leaves of *A. paeoniifolius* to exhibit mosaic, leaf puckering and even shoestringing-like symptoms. A major concern of the viral infection is the reduction in the tuber yield, due to perpetuation of the virus through infected planting materials.

In India, 24–88% mosaic incidence with a maximum yield loss up to 38% was reported from *Amorphophallus* growing areas in Uttar Pradesh[3]. In our survey also, viral disease incidence of 5–10% was observed in other major *A. paeoniifolius* growing states of India, viz. Kerala,

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Andhra Pradesh and Orissa (Binoy *et al*., unpublished). In addition to *A. paeoniifolius*, DsMV is found to infect a wide variety of cultivated aroids and ornamental plants worldwide. It causes serious damage to ornamentals like *Caladium*, *Dieffenbachia*, *Zantedeschia* and is ubiquitous in commercial plantings of the tropical root crops of the genera *Colocasia*, *Xanthosoma* and *Amorphophallus*. Natural infection of DsMV is mainly restricted to the plants of the aroid family[4]. The virus is transmitted mechanically via infected sap and by aphid species, viz. *Myzus persicae* and *Aphis gossypii.* Since most of the commercially cultivated hosts are vegetatively propagated, the disease spreads by the use of infected planting material.

Earlier, serological diagnostic methods such as enzyme linked immunosorbent assay (ELISA) have been successfully used for the large-scale detection and diagnosis of plant viral diseases[5,6]. But it has major limitations such as its low sensitivity during periods of low virus titre. Moreover serological diagnosis of potyviruses is often imprecise, because of frequent serological cross-reactions between species[7] and biological indexing is very cumbersome. With advances in the field of molecular biology, nucleic acid-based methods such as reverse transcription (RT) and the polymerase chain reaction (PCR) began to be used in plant virus detection[8–10]. The PCR-based methods are fast, highly sensitive and useful for accurate detection, quantification and characterization of plant pathogens. However, PCR and RT-PCR can be carried out for a limited number of samples because after PCR amplification, products need to be analysed by tedious agarose gel electophoresis. Although through real time PCR, we can get rid of gel electrophoresis, the cost of the consumables and the equipment are still not affordable by many laboratories. In this regard, nucleic acid spot hybridization (NASH) is more convenient than RT-PCR for specific and large-scale detection of the viruses[11].

To facilitate the process of large-scale diagnosis of DsMV infecting *A. paeoniifolius*, a method similar to microarrays[12–15] can be used. Hence, the present study was aimed at development of species-specific probes (cDNA probe and riboprobe) and subsequent detection of the virus through the process of nucleic acid hybridization.

DsMV isolated from *A. paeoniifolius* plants and maintained in the virology glasshouse of the Central Tuber Crops Research Institute (CTCRI) was used as the source of virus for the preparation of nucleic acid probes. Leaves with typical viral symptoms (100 mg) were harvested and ground into fine powder in liquid nitrogen, and then transferred to a 1.5 mL microfuge tube. Total RNA was extracted using the QIAGEN RNeasy® plant mini kit, according to the manufacturers' protocol and resuspended in 50 µL nuclease-free water.

One step RT-PCR was performed on the isolated total RNA from *A. paeoniifolius*, using specific primers DMV-F and DMV-R[16]. The reactions were set up as follows, containing 5 µl RNA, 1 µl of 20 pmol oligo $d(T)_{16}$, 1 µl of 20 pmol each of DsMV-specific primers designed for the amplification of partial coat protein and the 3′ untranslated region, DMV-F 5′-CCAAGCTTATGAYGARGTTGTRTTGC-3′ and DMV-R 5′-AAGGATCCGTCYGATGTAGGTGCAG-3′, 2.0 µl of 10×/5× reaction buffer, 0.5 µl of 10 mM dNTPs, 0.5 µl of 50 mM $MgCl_2$, 0.25 µl of AMV reverse transcriptase (5 U/µl) and 1.0 µl of Dynazyme DNA polymerase (1 U/µl). The RT–PCR was subjected to the following cycling conditions: 48°C for 45 min, 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 1 min and followed by 72°C for 10 min. The amplified product was analysed on 1% agarose gel, stained with ethidium bromide and photographed under UV-gel doc system (Alpha Imager, USA).

The biotinylated DNA probe containing the coat protein gene and the 3′ UTR region of the DsMV was prepared employing the technique of random primer biotin labelling of DNA using the NEBlot® Phototope kit (New England Biolabs). The process was carried out according to standard protocol: Gel eluted template DNA was diluted to a total volume of 34 µl and denatured at 100°C for 5 min and immediately chilled on ice. The solution was centrifuged briefly and the following components were added, 10 µl of 5× labelling mix, 5 µl of dNTP mix, 1 µl of Klenow fragment, incubated at 37°C for 2–3 h and the reaction terminated by adding 0.2 M EDTA (pH 8.0). The probe was precipitated by incubating at –20°C (1 h) with the addition of 4M $LiCl_2$ and 150 µl of ethanol. The mix was centrifuged briefly, washed with 70% ethanol and resuspended in 20 µl 1× TE. A serial dilution (10^{-1}–10^{-6}) of the biotinylated probe and the prebiotinylated markers in 0.1 N NaOH were analysed for checking the probe quality.

The Digoxigenin (DIG) labelled cRNA probe of the coat protein (CP) gene and the 3′ UTR region of the DsMV was prepared using the *in vitro* transcription protocol of the DIG RNA Labeling kit (Roche). The process was carried out according to the standardized protocol: the template DNA was cloned into transcription vector pSPT18 having T7 and SP6 RNA polymerase promoter region. After linearization of the template DNA at a suitable site, the following components were added as per the manufacturers' instruction: 1 µg purified template DNA, 10× NTP labelling mixture, 10× transcription buffer, RNase inhibitor, T7 RNA polymerase. The reaction was terminated by adding 0.2 M EDTA (pH 8.0). The quality of the cRNA probe at varying concentrations ranging from 10 ng/µl to 0.01 pg/µl in RNA dilution buffer (DMPC-treated double distilled water : 20× SSC : formaldehyde, 5 : 3 : 2) was analysed.

Twenty-seven samples of *A. paeoniifolius* showing varying symptoms collected from the CTCRI field (Table 1) and a non-infected healthy control, two DsMV infected *C. esculenta* plants maintained at the CTCRI glasshouse,

Table 1. *Amorphophallus paeoniifolius* and *Colocasia esculenta* leaf samples with varying symptoms

Sample no.	Symptoms	Sample no.	Symptoms
1	Severe mosaic	17	Severe mosaic
2	Mosaic and puckering	18	Mature leaves with slight mosaic
3	Puckering and leaf distortion	19	Severe puckering and mild mosaic of young leaves and mature leaves having severe mosaic and mild puckering
4	Mild puckering	20	Leaf yellowing and puckering
5	Mild mosaic	21	Puckering and leaf distortion
6	Shoestringing	22	Severe puckering
7	Severe mosaic, shoestringing and puckering	23	Leaf narrowing
8	Mild mosaic and puckering	24	Mosaic and shoestringing
9	Mature leaves showing severe dark mosaic and mild puckering	25	Mosaic
10	Leaf distortion and curling of leaf tips	26	Puckering mature leaves
11	Mature leaves showing no visible symptoms	27	Puckering and mosaic
12	Severe mosaic at leaf sides	28	Healthy *A. paeoniifolius* plant*
13	Severe puckering	29	DsMV infected taro plants
14	Mosaic and shoe stringing	30	DsMV infected taro plants
15	Leaf yellowing and puckering	31	Positive control (plasmid containing *cp* gene)
16	Mature leaves showing no visible symptoms	32	Buffer control (negative)

*Healthy plant (no symptoms) confirmed through RT–PCR.

and a positive and a negative control were used for analysis in this study. Total RNA was extracted from symptomatic and non-symptomatic leaves of *A. paeoniifolius* and DsMV-infected *C. esculenta* plants and resuspended in 30 µl nuclease-free water. 5 µl of total RNA and control sample was directly spotted on to 1 cm^2 nitrocellulose membrane pieces and UV-crosslinked. The membrane was subjected to prehybridization for 1 h at 68°C (20× SSC, 5× Denhardts reagent, 0.5% SDS, denatured salmon sperm DNA), followed by hybridization (prehybridization solution containing 20 ng/ml denatured biotin labelled DNA probe) overnight at 68°C. The membranes were washed twice with low stringency solution (2× SSC, 0.1% SDS), high stringency solution at 68°C (0.1× SSC, 0.1% SDS). The membrane was incubated in blocking solution, followed by incubation in streptavidin (final concentration of 1 mg/ml). The membrane was washed twice in wash solution 1 (blocking solution 1 : 10 dilution), incubated in biotinylated alkaline phosphatase (1 : 1000 dilution in blocking solution), followed by a single wash in wash solution 1 and twice in wash solution 2. The membrane was then subjected to detection reagent 1× CDP star reagent and exposed to X-ray film development.

Hybridization using cRNA probe was carried out using the standardized protocol of Millipore (http://www.millipore.com/immobilion). The strategy is similar to the above method, but with different hybridization buffer (0.5 M sodium phosphate, 2 mM EDTA, 7% (w/v) SDS, 0.1% sodium pyrophosphate), wash buffer 1 (1× SSPE, 0.5% (w/v) SDS) and wash buffer 11 (0.2× SSPE, 0.1% (w/v) SDS).

The membrane was washed briefly in washing buffer (maleic acid buffer [(0.1 M maleic acid, 0.15 M NaCl, pH 7.5)] + 0.3% Tween 20), and then incubated in blocking buffer (1% blocking reagent in maleic acid buffer) at room temperature for 30 min. The membrane was incubated in 20 ml diluted antibody-conjugate (Roche Applied Science, Mannheim, Germany; diluted to 1 : 5000 in maleic acid buffer) at room temperature for 30 min. After incubation, the membrane was washed twice in washing buffer at room temperature for 15 min and briefly incubated in detection buffer for 2 min and then incubated in substrate BCIP/NBT at room temperature for 15 min.

RT-PCR analysis of the total RNA isolated from symptomatic leaves of *A. paeoniifolius* using the degenerate primers DMV-F and DMV-R resulted in the amplification of 1200 bp amplicon (data not shown). The region amplified the complete coat protein and the 3′ UTR region of DsMV. The amplified product was cloned in pGEMT-Easy vector and sequenced. Sequence analysis and comparison of the 1200 bp amplicon using the BLAST revealed that the virus under study is DsMV. The sequence was then submitted to Genbank under the accession number FJ160764.

The 1200 bp amplicon of DsMV comprising the coat protein and the 3′ UTR was subsequently used for the production of species specific probe of either types – DNA and cRNA probe. The DNA probe was produced using the random primer biotin labelling procedure. Serial dilution of the DsMV probe showed that the probe could be detected up to a dilution of 10^{-2}. The results showed that the leaves of *A. paeoniifolius* infected with DsMV could be best detected using the DNA probe generated against the coat protein and 3′ UTR region (Figure 1). The probe detected the plant samples with varying symptoms from mild to severe mosaic, puckering and shoestringing. The probe did not show any typical reaction with the total RNA isolated from the *Colocasia*

infected with DsMV, showing that the probe generated from *Amorphophallus* infected with DsMV is specific for the isolate. The plasmid containing the 1200 bp gene was used as a positive control and showed good reaction with both the probes.

Analysis of the riboprobe indicated that the probe could be detected up to a concentration of 10 ng/µl. The strategy utilizes RNA–RNA hybridization, and the hybridization was detected using BCIP/NBT substrate, producing a purple colour. Analysis showed a highly specific detection of the virus in the infected leaf samples of *A. paeoniifolius* while it did not indicate the reaction in infected taro plants (Figure 2).

Among the tropical tuber crops, *A. paeoniifolius* plays an important role in the agricultural sector. It has gained widespread economic importance as a staple food as well as for its medicinal values. The viral infection of *A. paeoniifolius* caused by DsMV is very destructive, causing a high rate of reduction in the tuber yield. Although the tuber is of high economic importance, very few studies have been done to characterize the virus infecting the plant, and thereby reduce the rate of viral infection. As a first step towards development of resistant lines, the detection of the virus seems to be an important factor.

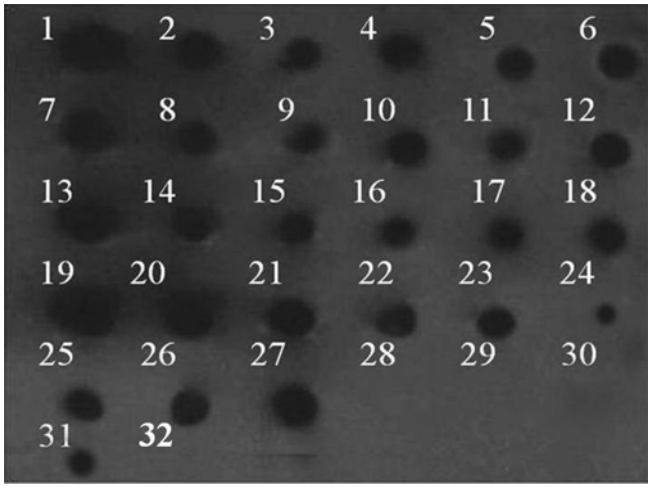

Figure 1. Detection of DsMV from *Amorphophallus paeoniifolius* leaf samples of varying symptoms (as in Table 1) using specific DNA probe.

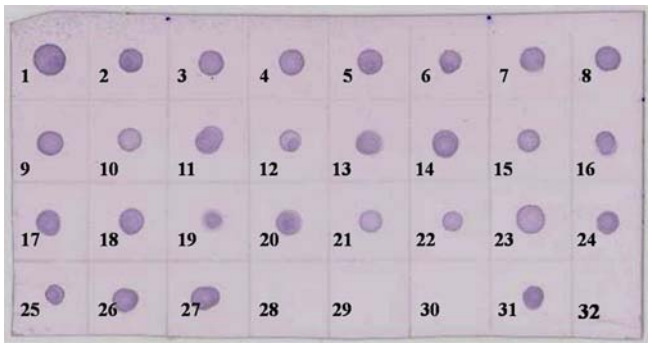

Figure 2. Detection of DsMV from *A. paeoniifolius* leaf samples of varying symptoms (as in Table 1) using specific riboprobe.

Serological techniques such as ELISA have been employed for the detection of DsMV in many ornamental plants[17]. However it has its own limitations. The process required specific antibodies for the virus, which had to be obtained through immunization strategy of purified viruses or through the expression of specific genes. Nucleic acid-based techniques such as RT–PCR have been developed for the efficient identification of the DsMV virus (Binoy *et al*., unpublished). But the method of PCR and RT–PCR has some limitations, such as analysis of the large number of samples for the viral amplicon on an agarose gel electrophoresis, and its further confirmation through sequence analysis is tedious, and secondly the difficulty in maintaining the viral nucleic acids for a longer period. But in the case of nucleic acid hybridization strategy, the samples can be spotted onto a membrane, and can be stored for a longer period of time. The method even facilitates the transport of spotted membrane from one laboratory to the other where the detection process can be carried out.

In this communication, we have developed two specific probes – DNA probe and riboprobe, which could detect DsMVs infecting *Amorphophallus* plants. Commonly used strategy for the viral detection includes development of species-specific probe, and such probes are generated based on the conserved region of the viral genome. The main disadvantage of such a probe is that it is not possible to distinguish the specific strains or isolates infecting the plant. Hence such strategy can be used in detecting viruses of the same genus or a group of viruses belonging to the same family. For instance, development of species-specific probe based on the conserved region of the potyviral genome detected a group of viral species belonging to the family Potyviridae[18]. Therefore, our strategy was to develop a probe which could detect the specific strain/isolate of the virus belonging to a species. Such a probe could specifically discriminate and characterize the viral strain infecting the plant. Based on a previous report that 3′ UTR could efficiently differentiate the potyviral strains and isolates[19,20], a strategy was employed for the generation of two specific probes which comprises the coat protein and the 3′ UTR region. For the preparation of the probes, amplification of the specific genes of the virus comprising the coat protein and the 3′ UTR was carried out using DsMV-specific primers. The probes were prepared using the entire region of the coat protein and 3′ UTR region of the virus. The coat protein region of the potyviruses seems to be similar in almost all the species, with slight variability. Therefore, the usage of coat protein alone in the preparation of the probe would facilitate the detection of almost all the potyviruses, making it impossible for strain and isolate differentiation. Analysis of the 3′ UTR region of the DsMV isolates from the NCBI

databases indicated that the region seems to be highly variable among different strains and isolates (data not shown). Therefore, incorporation of such a highly variable region in the probe, could specifically detect the infecting viral isolate.

Among the potyviruses, DsMV is the most important one affecting *A. paeoniifolius*. Even though it is most commonly found infecting *C. esculenta*, our study using RT–PCR and nucleotide sequence analysis of the partial coat protein of the virus (Binoy *et al*., unpublished) clearly shows that the DsMV isolate infecting *A. paeoniifolius* and *C. esculenta* seems to be different. Moreover very little sequence information is present in the genome database regarding the DsMV infecting *A. paeoniifolius* and *C. esculenta*. The probes generated detected the DsMV virus infecting *A. paeoniifolius*, whereas on the other hand it failed to detect the virus infecting *C. esculenta*. Probably the 3′ UTR region could be the factor that determined the variability in detection. The 3′ UTR region of the DsMV viral strains from *A. paeoniifolius* (present study) and *C. esculenta* (from NCBI database) are highly dissimilar (data not shown). The usage of coat protein alone in the probe would have probably detected both the viral strains of DsMV infecting *A. paeoniifolius* and *C. esculenta*, even though the variability in the coat protein region seems to be less among the two strains. The 3′ UTR region of DsMV infecting elephant foot yam comprises 260 nucleotides. Therefore, the presence of highly variable 260 nucleotides of the 3′ UTR region of the DsMV infecting *A. paeoniifolius*, in addition to the coat protein in the probe will not result in the hybridization between the probe and the RNA samples of *C. esculenta*. Therefore, the use of 3′ UTR region in the probe facilitated easy and specific identification of the DsMV isolate infecting *A. paeoniifolius* plants.

Even though the two methods seem to be highly efficient, analysis of the two methods seems to be important. The DNA probe is generated using the technique of random primer biotin labelling of DNA, which requires further purification involving phenol–chloroform extraction and may take up to 20–30 min. On the other hand, riboprobes are generated using the technique of *in vitro* transcription of the gene under a strong promoter. As a result, the amount of probe generated will be so high that the template DNA will be in less quantity as compared to the probe, thereby requiring no further purification strategies. Since the DNA probes are generated using biotin labels, the detection strategy employs an X-ray film development technique and requires 15–20 min. But, since the riboprobes are generated using the digoxigenin labels, addition of a suitable substrate such as BCIP/NBT and an incubation of 5 min is sufficient for detection.

A comparative analysis including the probe preparation and detection strategy shows that the technique employing riboprobe appears to be more efficient than that of DNA probe. The steps involving the prehybridization and hybridization appear to be the same, except for the buffers and wash solutions. Moreover, the DNA probe employs a DNA–RNA hybridization strategy, whereas riboprobe employs an RNA–RNA hybridization. Advantages of the techniques are that a single probe can be used for the detection of large number of samples, and the chemicals required for the detection process are also cost effective. Another advantage is that the probes can be stripped back from the membrane and can be further used for analysis, without variation in its quality.

In this study, a detection method which can detect the specific DsMV isolate infecting *A. paeoniifolius* plants has been developed. Such probes could be used for the development of diagnostic kits specific for the detection and diagnosis of DsMV strains/isolates infecting *A. paeoniifolius*. Since the potyvirus family encompasses the largest of the plant viral group and as new strains and isolates of the potyviral species are emerging, development of such strain or isolate specific probe could further lead to the development of specific complete identification chip (blot) for specific isolates in the near future. This novel method will speed up the identification procedure detecting specific strains and isolates of the viral genus. Moreover simplifying the nucleic acid isolation strategies may further make the technique more robust and efficient diagnostic technique for the large-scale detection of DsMV infecting *A. paeoniifolius*.

1. Srinivas, T. and Ramanathan, S., A study on economic analysis of elephant foot yam production in India. *Agri. Econ. Res. Rev*., 2005, **18**, 241–252.
2. Misra, R. S., Nedunchezhiyan, M., Swamy, T. M. S. and Edison, S., Mass multiplication technique for producing quality planting material of *Amorphophallus paeoniifolius*. *Trop. Sci*., 2002, **34**, 371–376.
3. NehalKhan, M., Aswathi, L. P. and Singh, P. K., Survey on symptamatology and assessment of yield losses due to viral diseases in elephant foot yam (*Amorphophallus paeoniifolius* Blume). *J. Root Crops*, 2006, **32**, 90–93.
4. Brunt, A. A., Crabtree, K., Dallwitz, M. J., Gibbs, A. J. and Watson, L., *Viruses of Plants*, CAB International, Wallingford, UK, 1996, p. 1484.
5. Clark, M. F. and Adams, A. N., Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.*, 1977, **34**, 475–483.
6. Flegg, C. L. and Clark, M. F., The detection of apple chlorotic leafspot virus by a modified procedure of enzyme-linked immunosorbent assay (ELISA). *Ann. Appl. Biol.*, 1979, **91**, 61–65.
7. Brunt, A. A., The general properties of Potyviruses. *Arch. Virol. Supplementum*., 1992, **5**, 3–16.
8. Rowhani, A., Maningas, M. A., Lile, L. S., Daubert, S. D. and Golino, D. A., Development of a detection system for viruses of woody plants based on PCR analysis of immobilized virions. *Phytopathology*, 1995, **85**, 347–352.
9. Thomson, D. and Dietzgen, R. G., Detection of DNA and RNA plant viruses by PCR and RT-PCR using a rapid virus release protocol without tissue homogenization. *J. Virol. Meth.*, 1995, **54**, 85–95.
10. Wetzel, T., Candresse, T., Ravelonandro, M. and Dunez, J., A polymerase chain reaction assay adapted to plum pox potyvirus detection. *J. Virol. Meth*., 1991, **33**, 355–365.

11. Craig, G. W., Stephen, J. W. and Michael, G. K. J., Diagnosis of plant viral pathogens. *Curr. Sci*., 2004, **86**(12), 1604–1607.
12. Gerhold, D., Rushmore, T. and Caskey, C. T., DNA chips, promising toys have become powerful tools. *Trends Biochem. Sci*., 1999, **24**, 168–173.
13. Harrington, C. A., Rosenow, C. and Retief, J., Monitoring gene expression using DNA microarrays. *Curr. Opin. Microbiol*., 2000, **3**, 285–291.
14. Kane, M. D., Jatkoe, T. A., Stumpf, C. R., Lu, J., Thomas, J. D. and Madore, S. J., Assessment of the sensitivity and specificity of oligonucleotide (50mer) microarrays. *Nucl. Acid Res.*, 2000, **28**, 4552–4557.
15. Lockhart, D. J. and Winzeler, E. A., Genomics, gene expression and DNA arrays. *Nature*, 2000, **405**, 827–836.
16. Li, Y., Xu, C. and Chen, J., Establishment of virus-free taro (*Colocasia esculenta* cv. Fenghuayunaitou) by meristem-tip culture combined with thermotherapy. *J. Pakistan Plant Path.*, 2002, **1**, 40–43.
17. Roberto, C. A., Lima, J., Albersio, A., Lima and Rubens Aguiar, J., Serological Identification of *Dasheen mosaic virus* in *Anthurium* sp. in the State of Ceará. *Fitopatol. Bras*., 2004, **29**, 1.
18. Hsu, Y. C., Yeh, T. J. and Chang, Y. C., A new combination of RT–PCR and reverse dot blot hybridization for rapid detection and identification of potyviruses. *J. Virol. Meth*., 2005, **128**, 54–60.
19. Frenkel, M. J., Ward, C. W. and Shukla, D. D., The use of 3′ noncoding nucleotide sequences in the taxonomy of *Potyviruses*: application to water melon mosaic virus 2 and soybean mosaic virus-N. *J. Gen. Virol*., 1989, **70**, 2775–2783.
20. Pappu, S. S., Brand, R., Pappu, H. R., Rybicki, E. P., Gough, K. H., Frenkel, M. J. and Niblett, C. L., A polymerase chain reaction method adapted for selective amplification and cloning of 30 sequences of potyviral genomes: application to dasheen mosaic virus. *J. Virol. Meth*., 1993, **41**, 9–20.

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Berberine and lycopene profiling during the ontogeny of *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms fruit

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Tinospora cordifolia **(Menispermaceae) fruits were studied for pigment profile, carbohydrate content, weight and water content during ontogeny. Carotenoid pigment lycopene appeared in yellow fruits and attained maximum level in matured (red) fruits whereas chlorophyll *a* and *b* disappeared after intermediate (yellow) stage. In addition, isoquinoline alkaloid berberine was more in early (green) stage than intermediate and matured stages. Carbohydrate content increased 1.3-fold on maturation, whereas weight and water content did not change significantly.**

Keywords: Berberine, fruit ontogenesis, lycopene, *Tinospora cordifolia*.

Tinospora cordifolia (Willd.) Miers ex Hook. F. & Thoms (*Tc*) (Menispermaceae) is highly exploited for pharmaceutical purposes in Ayurvedic and Homeopathic systems of medicine[1,2]. The climbing shrub is widely distributed throughout India and neighbouring countries, like Bangladesh, Pakistan and Sri Lanka, and South East Asian countries such as Malaysia, Indonesia and Thailand[3]. It is reported to bear distinct male and female flowers[3]. However, its red fruits, a forest produce, have not yet been studied. Chlorophylls, carotenoids and flavonoids, including anthocyanins, and betalains are pigments involved in leaf and fruit colouration in plants. The pigment content changes during ontogeny to adapt to the environmental conditions, and various stresses and damages[4–6]. Fruit ontogenesis is completed in two phases: fruit induction to 'maturation' which is marked by changes in carpel followed by maximum organ expansion, followed by 'ripening' during which structure and chemical composition of the organ undergo striking modifications[7]. During the maturation process, Fleancu[8] observed a negative correlation between fruit diameter and photosynthesis rate, respiration rate, fruit chlorophyll *a* or *b* content, and a positive correlation between diameter and fruit carotenoid content. Thus, quantity and ratio of pigments determine many important physiological characteristics[9,10].

Lycopene is a red carotenoid pigment synthesized exclusively by plants and microorganisms. Its functions include absorption of light during photosynthesis to protect plants from photosensitization. Sometimes the green chlorophyll pigments mask the red colour of lycopene in fresh fruits. However, as the fruit matures, chloroplasts are transformed to chromoplasts resulting in the loss of chlorophylls, increase in carotenoids, tissue softening, and alterations in the metabolism of organic acids and monosaccharide[11]. As carotenoids increase, chlorophylls disappear during ontogeny, thereby accumulating carotenoids such as lycopene in matured fruits (pineapple, orange, lemon, grapefruit, strawberry, tomato, paprika and rose hip) and many flowers (*Eschscholtzia* and *Narcissus*)[12].

Berberine (natural colour 18), a benzyl tetra isoquinoline alkaloid, is pharmaceutically important and has been used in traditional Chinese and North American medicine. It has been reported in nine botanical families, including Menispermaceae. Therapeutic potential of ber-

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