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INVESTIGATION ON SEED TRANSMISSION OF CUCUMBER MOSAIC VIRUS IN COWPEA

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ABSTRACT

Cowpea breeding lines were infected with cucumber mosaic virus (CMV) by mechanical inoculation to investigate seed transmission rates for this virus. Transmission rates ranging from 0% to 6% were scored by symptom assessment. However, when cowpeas grown from seeds of infected mother plants were tested by ELISA, up to 30% of the plants were infected with CMV. Evaluating ELISA for CMV detection in cowpea revealed that virus concentration was highest in flowers and lowest in primary leaves. To compare the capacity of ELISA for CMV detection, the serological test was compared with RT-PCR; ELISA proved sensitive and reliable for detection of CMV in infected tissue. For seed analysis, a non-destructive assay for CMV detection based on tissue blotting of hypocotyls tissue was developed.

Key Words: *Bromoviridae*, DAS-ELISA, RT-PCR, tissue printing, *Vigna unguiculata*

RÉSUMÉ

Des varietés sélectionnées du niebé étaient infectées par inoculation mécanique avec le virus de la mosaíque de concombre (CMV) afin d'étudier la transmission par semence de ce virus. Les taux de transmission de l'ordre de 0 % à 6 % étaient enregistrés après l'évaluation des symptômes. Cependant, lorsque les plantes de niébé issues de semences provenant des parents infectés ont été testéés par ELISA, jusqu'á 30 % des plantes étaient infectées par le CMV. L'évaluation de L'ELISA pour la détection du CMV chez le niébé a révélé que la concentration du virus était la plus élevée dans les fleurs et le plus faible dans les feuilles primaires. Afin d'évaluer la capacité d'ELISA de détecter CMV, le test sérologique était comparé á la RT-PCR, où l'ELISA s'est montré sensible et fiable pour la détection du CMV dans des tissus infectés. Pour l'analyse de semence, une méthode non-destructive de détection de CMV basée sur le blotting de tissus d'hypocotyle était développé.

Mots Clés: *Bromoviridae*, DAS-ELISA, RT-PCR, tissue printing, *Vigna unguiculata*

INTRODUCTION

Cucumber mosaic virus (CMV) is the type member of the genus Cucumovirus, family Bromoviridae, and was first found in cucumber in the USA (Price, 1934). CMV is an ubiquitous pathogen and is regarded as one of the five most important viruses worldwide in field grown vegetables (Tomlinson, 1987). It has the largest host range of any plant virus infecting more than 800 species

(Palukaitis *et al.*, 1992) including banana (Bouhida and Lockhart, 1990) and cowpea (Robertson, (1966). A large number of isolates have been described showing different host plant reactions and pathogenicities (Kaper and Waterworth, 1981; Srivastava *et al.*, 1995; Garcia-Arenal *et al.*, 2000), which are often moderated by the existence of satellite RNA (Raj *et al.*, 2000).

In the field, aphids spread CMV in a non-persistent manner (Palukaitis *et al.*, 1992; Samad *et al.*, 2000) and more than 60 aphid species have been reported to transmit this virus. Infected ruderal plants and weeds can act as a primary source of inoculum, but seed infection also frequently occurs. Despite the generally low seed transmission rates reported for CMV (Bashir and Hampton, 1996), a few infected seedlings coupled with an efficient virus spread in the field through aphid vectors, can explain severe disease outbreaks (Bos, 1999).

CMV was first reported in cowpea by Robertson (1966) who described two isolates from naturally infected cowpea in Nigeria. CMV infections in cowpea are usually characterised by mild mosaic symptoms and crop losses due to CMV are generally minor. However, CMV can contribute to serious cowpea diseases when other cowpea viruses are present in mixed infections (Piero-Ribeiro *et al.*, 1978). In Africa, the seed-borne blackeye cowpea mosaic (BlCMV) and cowpea aphid-borne mosaic (CabMV) viruses are major diseases of cowpea (Thottappilly and Rossel, 1992), occurring wherever cowpea is grown (Huguenot *et al.*, 1993). Mixed infections of these viruses together with CMV lead to severe disease symptoms and virus synergism causes the decline of cowpea plants and serious crop losses (Gillaspie *et al.*, 1998).

The rate of seed transmission of CMV in cowpea has so far not been studied extensively. Yet several seed-borne viruses have been introduced into new geographical regions due to increased seed exchange and movement. Despite its significance, only very limited data is available on seed transmission of viruses, especially since seed health testing for seed-borne viruses often is not a straight forward task particularly in the developing countries of the tropics where adequate facilities and techniques are often lacking.

In this paper, we report on the usefulness of serological assays, double antibody sandwich-enzyme linked immunosorbent assay, DAS-ELISA, and tissue printing ELISA, for CMV detection in cowpea plants and, especially for indexing of seeds. For seed health testing, a technique was envisioned by which virus could be tested without destroying the germination capacity of the seed and plant development.

MATERIALS AND METHODS

Virus strains and propagation of test plants. All plant materials were grown in sterile topsoil mixed with shredded cocoa fibre and maintained under the protective conditions of the screen house. *Nicotiana glutinosa* L. was used for CMV propagation and inoculum production.

Since a cowpea plant naturally infected with CMV was not available, a banana isolate of CMV infectious to cowpea was used for the virus investigations. This was obtained by mechanical transmission of plant homogenates from CMV infected banana leaves to *N. glutinosa*. A symptomatic leaf of banana, showing mild mosaic and diffuse streaking along with chlorotic line pattern (Fischer and Lockhart, 1976) was collected and homogenised (1:10 w/v) in ice cold inoculation buffer, containing 0.02 M phosphate buffer, pH 7.0, 0.05 M sodium diethyl dithiocarbamaminate, using a pestle and mortar. The virus homogenate was rubbed onto 3 fully expanded leaves of *N. glutinosa*. After inoculation, excess inoculum was washed off with distilled water. Inoculated plants were kept at moderate temperature (24-27°C) under screen house conditions. For virus inoculum preparation, systemically infected *N. glutinosa* leaves were harvested 21 days post inoculation and homogenised in ice cold inoculation buffer as stated above.

Cowpea (*Vigna unguiculata* (L.) Walp) seeds were obtained from the Germplasm Resource Unit of the International Institute of Tropical Agriculture, IITA. Thirteen cowpea lines, Ife Brown (susceptible to and good host for CMV), TVU 2657, TVU 76, IT86D-719, IT81D-994, IT86D-1010, IT83D-442, IT81D-985, IT86D-715, IT845-22464, TVX3236, IT90K-59, IT90K-76, were used in the study.

Virus inoculation was done on the fully expanded primary leaves of cowpea, just at the time when the first true leaves started to emerge. Infected cowpea plants and healthy, mock-inoculated controls were kept under screen house conditions until harvest of seeds. Seeds from CMV infected plants were harvested and replanted for virus examinations and ELISA tests.

Virus detection using serological assays. Antiserum for CMV detection was obtained from DSMZ (AS-0473), purified by ammonium sulphate precipitation (Jensen *et al.*, 1985) and DEAE-ion exchange chromatography (Bruck *et al.*, 1982). CMV was detected by double antibody sandwich ELISA (DAS-ELISA), essentially as described by Clark and Adams (1977). Three weeks after inoculation, flowers, primary and trifoliate leaves and stems of cowpea plants were ground 1:20 (w/v) in extraction buffer and subjected to ELISA.

For virus detection in seeds, 400 cowpea seeds were randomly taken from lots of the CMV infected cowpea breeding line IT83D-442 and from a respective lot of virus free seeds serving as a control. Seeds were soaked overnight in distilled water then carefully cut transversely using a sterile blade, ensuring that no damage was done to the embryo. One half of the cotyledon bearing the embryo was sown in sterile soil for germination while the other section was tested for presence of CMV, using a modification of the tissue printing procedure of Banttarri and Goodwin (1985).

The freshly cut surface of the seed section was firmly pressed onto a nitrocellulose membrane (Schleicher & Schuell, BA 85, 0.45 um). The membrane was soaked in PBS-Tween (PBS-T, 0.05 %) and subsequently blocked with PBS-T, 2 % BSA. The blocking solution was replaced with CMV antibodies, applied at 1µg/ml in PBS-T, and incubated for 2-4 hours. Following this incubation and three washing steps in PBS-T, the membrane was incubated in a species specific antibody conjugate, goat anti-rabbit alkaline phosphatase conjugate, diluted to 1: 2000 in PBS-T and incubated for a further 2 hours. After three washings in PBS-T, the membrane was immersed in substrate buffer (0.1M Tris, 0.1 M NaCL, 5 M $MgCl_2$, 5 mM EDTA, pH 9.5) for 10 minutes. The membrane was then incubated in substrate solution (8 mM 5-Bromo-4-Chloro-3-Indonylphosphate, 9 mM Nitroblue tetrazolium in substrate buffer) until the development of a purple colour in positive reactions. Following the substrate development, the membrane was washed in distilled water, dried and photographed.

Seed transmission. One hundred seeds of each of the 13 cowpea lines were randomly selected and sown in trays. Germinating seeds and developing plants were maintained in screen house and assayed for symptom development. Twenty trifoliate leaf samples were randomly selected from each line when the plants were at the flowering stage and subjected to DAS-ELISA.

Virus detection by RT-PCR. To evaluate the detection capacity of ELISA and to prove its reliability, a reverse transcription PCR assay, RT-PCR, was performed for comparison. Total RNA of cowpea plants infected with CMV and total RNA of two CMV strains - Kaper S, (DSMZ- PV 0242) and Deggendorf (DSMZ-PV 0187) - used as standards, were isolated using a guanidium isothiocyanate protocol as described by Chomczynsky and Sacchi (1987). One hundred mg of leaf samples were shock frozen in liquid nitrogen and ground in extraction buffer (0.42 M Guanidinium isothiocynate, 1 M sodium citrate, 10 % sodium lauryl sarcosine, 1 % PVP, 0.4 % mercaptoethanol). Phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v)) was then added (1:1 v/v) to remove proteins. The mixture was vortexed and centrifuged at 10 000 rpm for phase separation. The aqueous phase was removed and adjusted to 0.3 M sodium acetate, pH 5.3, and 2.5 vol EtOH to precipitate the RNA. Total RNA was recovered by centrifugation at 14000 rpm for 20 min and the resultant pellet was washed in 70 % ethanol, dried and re-dissolved in 50 µl distilled water.

RT-PCR was performed using the CMV specific primers described by Rizos *et al.* (1992). One micro litre of the total plant RNA extracts prepared were subjected to cDNA synthesis prior to PCR. Reverse transcription was performed in a 20 µl reaction mixture containing 1 µl RNA, 1 µl downstream primer (5'GGC GAA TTC GAG CTC GCC GTA AGC TGG ATG GAC 3'), 5 µl first strand reaction buffer, 2µl 0.1M DTT, 1.25 µl dNTPs (25 mM), 2.5 U RNasin, 100 U M-MLV

reverse transcriptase, and 9 µl distilled water. The reaction was incubated at 43 °C for 40 min after which a 5 min denaturation step followed. During this heat denaturation, a master mix (10 µl 10 x Taq polymerase buffer, 4 µl $MgCl_2$ (50 mM), 2.5 U Taq polymerase, 1 µl upstream primer (5'CTC GAA TTC GGA TCC GCT TCT CCG CGA G 3') and 64.5 µl distilled water) was added directly to the cDNA reaction. PCR was then performed for 35 cycles using thermal settings of 95 °C for 1 min, 45 °C for 1 min, 72 °C for 1 min and a final incubation at 72 °C for 5 min. All enzymes used were purchased from Gibco BRL, Karlsruhe, Germany. The PCR reaction products were separated on 1.5 % agarose gel, subsequently stained with ethidium bromide, visualised in UV light and photographed.

RESULTS

Distribution of CMV in infected cowpea plants. All 13 cowpea lines inoculated were susceptible to CMV, although virus replication varied in infected plants. Highest concentration of virus was recorded in the flowers and trifoliate leaves (Fig. 1). Stem and primary leaves contained least amounts of detectable virus. Symptoms of CMV infections in cowpea were generally mild mosaic and included chlorotic mottling with blistering of young leaves.

Seed transmission of CMV in cowpea. Most cowpea plants grown from seeds harvested from CMV inoculated plants did not show any symptom of virus infection. By symptom assessment, 4 % of the seeds of Ife Brown, 1 % of IT86D-994 and 6 % of IT83D-442 developed into plants infected with CMV.

When trifoliate leaf samples of cowpea plants were subjected to DAS-ELISA, much higher CMV infection rates were recorded. These ranged from 10 % (IT86D-1010, IT845-22464, IT90K-59) to 30 % (IT83D-442, IT81D-985) of CMV infected plants grown from seeds obtained from virus-infected plants (Fig. 2). In several breeding lines (TVU 2657, IT 86D-715, TVX 3236, IT 90-76) infection of seeds was not detected and despite the mother plants being infected with CMV, offspring grown from these plants remained virus-free.

From 400 cowpea plants grown from randomly sampled and sectioned seeds of the cowpea breeding line IT83D-442, eight plants tested positive indicating a seed infection rate of 2 %.

Non-destructive virus detection assay for seed health testing. When cowpea seed was carefully

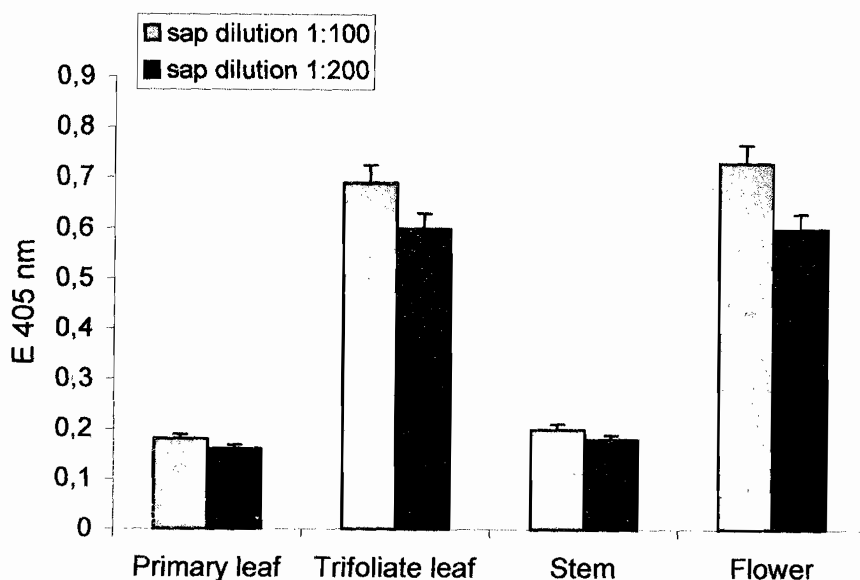

Figure 1. Determination of cucumber mosaic virus (CMV) concentrations in different parts of cowpea "Ife brown" plants by DAS ELISA.

cut and the embryo-bearing half sown, 100 % germination was observed. When the remaining sections were subjected to a tissue blotting ELISA procedure both healthy and infected seeds resulted in colour reactions indicating false positive signals (Fig. 3a). However, when the CMV antibodies were diluted in antibody buffer containing 10 % healthy plant sap, the crossed-adsorbed antibodies revealed unambiguous signals in tissue print ELISA (Fig. 3b). From the randomly taken 400 seeds of the breeding line IT83D-442 only five tested positive for presence of CMV in this test. Virus infections verified by tissue printing ELISA coincided with the CMV infected plants tested using DAS-ELISA in the growing on test. However, three CMV infections found by ELISA during the growing on test remained undetected by tissue print ELISA of the corresponding cowpea seed sections.

Virus detection by RT-PCR. In PCR, a single fragment of about 920 bp was amplified from total RNA extracts of cowpea plants infected with CMV (Fig. 4, lanes 4 - 9). The two CMV strains used for reference also produced fragments of similar size (Fig. 4, lane 1 and 3). Plants testing positive by ELISA also tested positive in RT-PCR and CMV infections resulting in negative ELISA reactions were not detected. Thus RT-PCR confirmed the findings of ELISA.

DISCUSSION

Several viruses have been described as seed-borne in cowpea (Bashir and Hampton, 1996) among which the potyviruses blackeye cowpea mosaic virus (BlCMV) and cowpea aphid borne mosaic virus (CaBMV) are frequently found. However CMV is also often found in cowpea plants grown from presumably infected seeds. Since CMV infections often result in only very mild symptoms in infected plants, with strong virus symptoms becoming visible often only as a result of mixed infections with other viruses (Piero-Ribeiro *et al.*, 1978) the detection of this virus in plants can present a challenging task.

This study showed that a DAS-ELISA could be used for detection of CMV in infected plants. Despite differences in concentration of CMV in different plant parts, as expected, CMV was most reliably detected in trifoliate leaves of systemically infected cowpea plants. All cowpea plants that revealed negative DAS-ELISA results, also tested negative by the more sensitive RT-PCR indicating the reliability of the DAS-ELISA for CMV detection in cowpea.

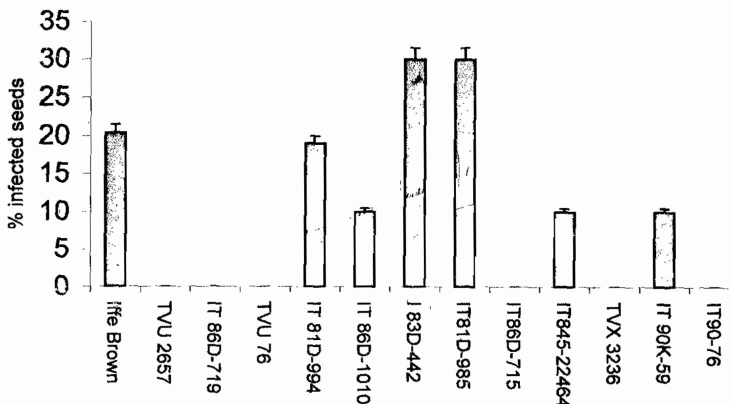

Figure 2. CMV infection of cowpea breeding lines grown from seeds obtained from CMV infected plants.

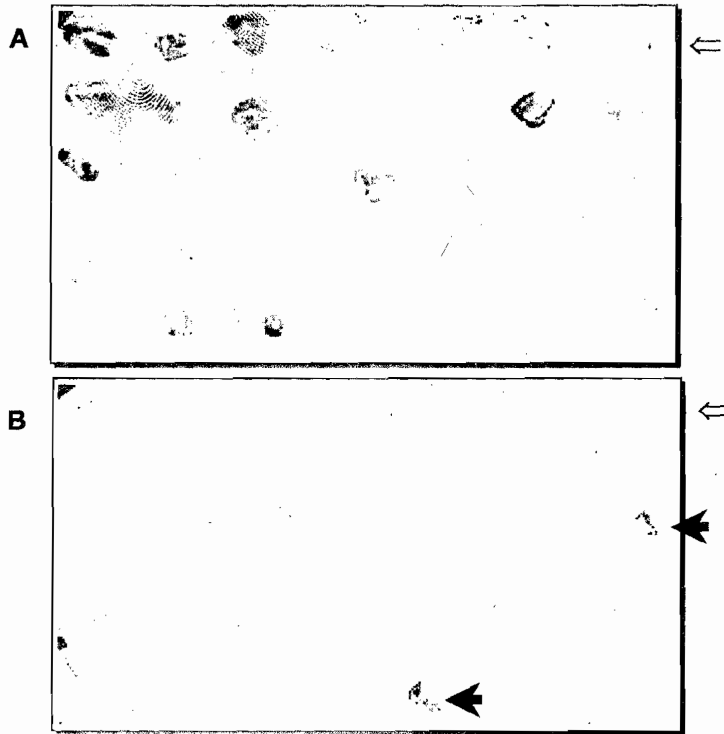

Figure 3. Tissue printing of cowpea seeds on nitrocellulose membrane and subsequent detection of CMV using CMV specific antibodies. (A) Antibodies not cross absorbed. (B) Antibodies cross absorbed with healthy plant sap. Open arrow indicates healthy control lane. Bold arrows indicate positive reaction for CMV.

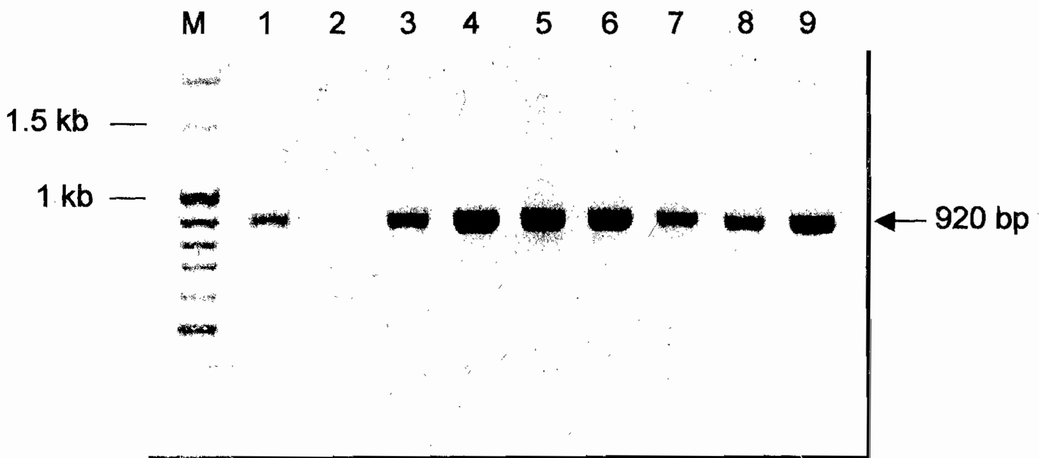

Figure 4. Detection of cucumber mosaic virus (CMV) in cowpeas by RT-PCR. M=100 bp molecular size marker (NEB). Lane 1 = isolate Deggendorf, lane 2 = healthy control, lane 3 = isolate Kaper, lane 4 to 9 = CMV infected cowpea plants.

All breeding lines and the land race "Ife Brown" used in the experiments were susceptible to CMV and sustained virus replication and systemic infections so that differences in the levels of CMV accumulation were negligible. However, when seeds of CMV infected lines were tested for seed-transmission of CMV, remarkable differences in seed transmissibility were noted, with 6 breeding lines apparently not supporting transmission of virus through seeds.

The seed transmission rate recorded for the breeding lines supporting seed transmission varied between 10 % and 30 % of CMV infected plants developing from infected seed. This is very high compared with a natural CMV infection found by Bashir and Hampton (1996) of 0-2 % of all cowpeas tested. However, these relatively high numbers of infected plants developing from infected seeds is most probably due to the artificial infection of CMV by mechanical inoculation of CMV onto cowpea cotyledons. Hence, at a very early stage in cowpea development, at the most susceptible stage (Bos, 1999), virus infection was initiated with long period of virus replication. These conditions are of course not sustained under natural conditions, explaining the much lower seed transmission rates of about 5 % found in the seed lot of the susceptible cowpea breeding line IT83D-442 as compared to a 30 % seed transmission of CMV from mechanically inoculated cowpea (Fig. 2). Still the rate of seed transmission recorded in this experiment is even relatively high compared to the observation of Rossel and Huttinga (1982) whose findings were not based on ELISA test results.

The use of ELISA for CMV indexing proved highly sensitive since some cowpea plants with no obvious symptoms of CMV infection were positive in ELISA. Thus higher virus transmission rates were observed by using ELISA indexing than with visual examination of symptoms.

In seed health testing, screening for viruses is problematic since most of the tests are performed as growing-on tests with plants grown from a representative seed sample (400 seeds per lot). Multiple factors contribute to the success of virus indexing methods, but labour and time considerations and the complex problems of maintaining plant material under protective environments especially in developing countries can be serious constraints. A seed health test, which guarantees reliable detection of CMV in infected seeds without the necessity of a large scale growing on test, would be most advantageous. The assay format described here presents a test, which can be performed with seeds that simultaneously can be grown into cowpea plants. The seed that was tested negative by tissue printing ELISA can be planted for multiplication of virus free cowpea. However, the growing-on test using ELISA is more reliable than tests performed directly with seeds. As described above, in tissue printing ELISA, several CMV infections in seeds remained undetected by this method. This is explained by the often extremely low concentrations of virus in infected seeds hence the limited detection possibilities. In the leaf, the virus replicates rapidly, because the tissue is growing, hence the differences in the detection level in comparison with dormant seeds. Tissue printing ELISA for seed health testing can provide very valuable data on virus infection pressure in seeds already at a very early stage.

In addition, multiple tissue prints performed with the seed section can be developed for detection of BlCMV and CabMV, hence providing a full picture of viruses present in cowpea seeds. For seed health testing in quarantine stations of developing countries, this will be a very valuable addition to the possibilities of virus testing procedures.

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