EVALUATION OF THE SENSITIVITY OF NITROCELLULOSE MEMBRANE LOW-COST ALTERNATIVES USED IN TISSUE BLOT IMMUNOAASSAY (TBIA) FOR DETECTION OF SOME PLANT VIRUSES

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ABSTRACT
In an attempt of reducing the cost of tissue blot immunoassay (TBIA), some kinds of papers as alternative solid phases in place of nitrocellulose membrane (NCM), were tested for detection of three viruses: Cowpea mosaic virus (CPMV), Tomato mosaic virus (ToMV) and Zucchini yellow mosaic virus (ZYMV). Most of the examined papers developed positive reaction with all tested viruses. Canson paper (150g/m² and 300g/m²) were sensitive for detection of the tested viruses as NCM and persist in good condition during the processing. Filter paper (Whatman No. 1), local drawing paper (60g/m²), photocopy paper (80g/m²), (56g/m²) and (70g/m²) were very weak and need to taking care in handling. High glossy photo paper (200g/m²) was not effective however, it did not show any infected or healthy tissue print. Good results were obtained when both faces of NCM and Canson paper (300g/m²) were printed with all tested viruses. TBIA could detect CPMV in infected leaves using the unused face of NCM previously processed with 7 or 11 years ago. CPMV, ToMV and ZYMV were detected 2, 4, 6, 8 and 16 days after inoculation with NCM and Canson paper (300g/m²). Also, the tested viruses were detected in leaf, petiole, stem and root of infected plants. ToMV could be detected using NCM and Canson paper (300g/m²) in naturally infected tomato plant samples.

Keywords: TBIA, CPMV, ToMV, ZYMV, alternative solid phases

INTRODUCTION
Accurate detection is an essential step to control the plant viruses (Jeong et al., 2014). It is very imperative to recognize a sensitive, simple, reliable, inexpensive method for detection of the virus in the different parts of the infected plants (Aboul-Ata et al., 2011).

Tissue blot immunoassay (TBIA) is widely used for detection of plant viruses in various plant tissues. It could apply even in poorly equipped laboratories. TBIA is simple, sensitive, does not require elaborate sample preparation or extraction (Lin et al., 1990; Hsu and Lawson, 1991; Makkouk et al., 1993; Hu et al., 1997; D’Ongbia et al., 2001; Hsu, 2009; Bin et al., 2015). In TBIA, nitrocellulose membrane (NCM, 0.45 nm) is usually using as a solid support for the test.

Cowpea mosaic virus (CPMV), which a member of the Comovirus genus and Secoviridae family, is one of the most commonly virus diseases of cowpea, it decreases the yield by up to 95% (Kammen et al., 2001). Tomato mosaic virus (ToMV) in the Tobamovirus genus belongs to family Virgaviridae (Li et al., 2015) is a serious and extremely spreadable disease. The tomato fruits of plants infected with ToMV show yellowish blotches and necrotic spots and the interior of the fruit become brown, also, it causes severe reduction of the yield (Broadbent, 1976). Zucchini yellow mosaic virus (ZYMV) (genus: Potyvirus; family: Potyviridae) is a major viral pathogen of cucurbits. It causes severe stunting of plants and distortion of the fruits which led to high losses of productivity (Desbiez and Lecocq, 1997).

The objectives of the present study were to evaluate the sensitivity of different types of low-cost papers as solid phases alternative to the expensive NCM for detection of CPMV, ToMV and ZYMV, to assess the possibility of using both faces of the best papers, and to detect the sensitivity of the previously used NCM for detection of the tested viruses.

MATERIALS AND METHODS
Maintenance and inoculation of the viruses
Three viruses, namely CPMV, ToMV and ZYMV used in this study were obtained from the collection of Virology Lab., Plant Pathology Department, Faculty of Agriculture, Alexandria University. CPMV was maintained on Vigna unguiculate, while ToMV on Lycopersicon esculentum cv. Super strain B and ZYMV was maintained on Cucurbita pepo cv. Eskandarani, which served as virus source plants for further studies.

Inocula were prepared by grinding infected leaf tissues in phosphate buffer 0.02M (pH 7.0) with a mortar and pestle. Leaves of plants to be inoculated were first dusted with carbendazim (600 mesh) and then inoculated with a freshly prepared inoculum using forefinger.

Source of antisera
Antisera used in this study were locally produced in Plant virology Lab., Plant Pathology Department, Faculty of Agriculture, Alexandria University, Egypt.

Tissue blot immunoassay (TBIA)
TBIA first described by Lin et al. (1990), using the following steps and washes in between: (1) plant samples (leaves, stems, petioles or roots) from healthy and infected plants were cut by razor blade in a steady motion to obtain a single plane cut surface and blotting the exposed cut edges onto the nitrocellulose membrane (NBT, 0.45 nm, Bio- Rod Laboratories, Richmond, CA) or tested papers, (2) treated NCM or papers were then placed in blocking buffer (2% bovine serum albumin (BSA) and triton X-100 solution in Tris buffer saline (TBS) pH 7.5), (3) soaking the NCM or papers with the virus antiseraum for 2 h, (4) adding the goat anti-rabbit IgG conjugated to alkaline phosphatase, gently agitated for one hour. Finally, the NCM or papers incubated in freshly prepared 5- bromo- 4- chloro- 3- indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) substrate solution for 15-30 min. After colour development, the reaction was stopped by washing the treated membrane or paper in 0.01M Tris- HCl.
The sensitivity of different types of papers as alternative solid phases

Eight different types of papers were compared with nitrocellulose membrane (NCM, 0.45 nm, Bio-Rad Laboratories, Richmond, CA) for the ability of detection of CPMV, ToMV and ZYMV. These papers were: Canson paper (150g/m² and 300g/m²), filter paper (Whatman No. 1), high Glossy photo paper (200g/m²) made in China, local drawing paper (60g/m²), photocopy paper (80g/m²) (double A) manufactured by DA Alizay, France, photocopy paper (56g/m²) paper fect-BAHIA SUL and photocopy paper (70g/m²) (Smartist) made in Thailand.

The sensitivity of using the two faces of the selected alternative solid phases

Nitrocellulose membrane and Canson paper (300g/m²) were selected to assess the possibility of using the two sides of the paper for detection of CPMV, ToMV and ZYMV. Both faces of the certain papers were printed by healthy and infected leaves.

Testing of the unused faces of previously processed nitrocellulose membrane.

The unused faces of previously processed NCM used 7 or 11 years ago, were printed with CPMV infected leaves and healthy ones.

Detection of CPMV, ToMV and ZYMV in infected plants after different periods of inoculation

The sensitivity of NCM and Canson paper (300g/m²) to detect CPMV, ToMV and ZYMV in infected leaves after 1, 2, 4, 6, 8 and 16 days of mechanical inoculation were examined.

Detection of CPMV, ToMV and ZYMV in different parts of the infected plants

Infected leaves, petioles, stems and roots and healthy ones were printed on NCM and Canson paper (300g/m²) to detect CPMV, ToMV and ZYMV.

Detection of ToMV in naturally infected plants

NCM and Canson paper (300g/m²) were tested to detect ToMV in naturally infected tomato plants.

RESULTS

The sensitivity of different types of papers as alternative solid phases

Eight different types of papers and NCM were tested for detection of CPMV, ToMV and ZYMV by TBIA. Filter paper (Whatman No. 1), local drawing paper (60g/m²), photocopy paper (80g/m²), (56g/m²) and (70g/m²) could indicate purple colour with the tested viruses but they required high care in handling. Also, with these types of paper background was observed. High glossy photo paper (200g/m²) did not show any infected or healthy tissue print. Canson paper (150g/m² and 300g/m²) were equally sensitive with nitrocellulose membrane and persist in good condition during the assay steps (Figs. 1, 2 and 3). Therefore, Canson paper (300g/m²) was selected to use in the subsequent experiments to confirm its sensitivity as compared with NCM.
Figure 3 Detection of ZYMV by TBIA in leaves of infected squash plants blotted on different types of solid phases, 1. nitrocellulose membrane (NCM, 0.45nm), 2. Canson paper (300g/m$^2$), 3. filter paper (Whatman No.1), 4. drawing paper (60g/m$^2$), 5. photocopy paper (80g/m$^2$), 6. photocopy paper (56g/m$^2$), 7. photocopy paper (70g/m$^2$), 8. high glossy photo paper (200g/m$^2$), 9. Canson paper (150g/m$^2$).

The sensitivity of using the two faces of the selected alternative solid phases

Positive reactions were obtained on the both faces of NCM and Canson paper (300g/m$^2$) for detection of CPMV, ToMV and ZYMV in infected leaves (Fig. 4).

Figure 4 Detection of CPMV, ToMV and ZYMV in leaves of infected plants blotted on both faces of, [A]. Nitrocellulose membrane (NCM), [B]. Canson paper (300g/m$^2$). I. Infected plant tissue, H. Healthy plant tissue.

Using the unused faces of previously processed nitrocellulose membrane.

Results indicated the possibility of using the unused face of NCM even after 7 years or 11 years of processing for detection of CPMV. Pronounced purple colour with infected leaf tissues was observed (Fig. 5).

Figure 5 Detection of CPMV in leaves of infected plants blotted on the unused face of previously processed nitrocellulose membrane, [1] nitrocellulose membrane used 7 years ago; [2] nitrocellulose membrane used 11 years ago. I. Infected plant tissue. H. Healthy plant tissue.

Detection of CPMV, ToMV and ZYMV in infected plants after different periods of inoculation

Obtained results showed that CPMV, ToMV and ZYMV could be detected by TBIA in infected leaves after 2, 4, 6, 8 and 16 days of mechanical inoculation when using NCM and Canson paper (300g/m$^2$) (Fig. 6).

Figure 6 Detection of CPMV, ToMV and ZYMV in leaves of infected plants after different periods of inoculation blotted on, [A]. Nitrocellulose membrane, [B]. Canson paper (300g/m$^2$). I. Infected plant tissue. H. Healthy plant tissue.

Detection of CPMV, ToMV and ZYMV in different parts of infected plants

TBIA conducted to detect CPMV, ToMV and ZYMV in infected leaves, petioles, stem and roots gave significant results with NCM and Canson paper (300g/m$^2$) (Fig. 7).

Figure 7 Detection of CPMV, ToMV and ZYMV in different parts of infected plants (leaf, petiole, stem and root) blotted on, [A]. Nitrocellulose membrane, [B]. Canson paper (300g/m$^2$). I. Infected plant tissue. H. Healthy plant tissue.

Detection of ToMV in naturally infected plants

As shown in Figure (8), ToMV could be clearly detected using both NCM and Canson paper (300g/m$^2$) in naturally infected tomato plant samples.
DISCUSSION
The availability of sensitive and efficient detection techniques is one of the keys to successful use of viral disease control strategy. Since TBA has great benefits when compared with ELISA in terms of detection time, cost, sensitivity and convenience, it has been applied for diagnosis of many viral diseases (Jeong et al., 2014). TBA can perform mass analysis of plant samples directly in field, which can be processed later. It is proposed as a reliable alternative to ELISA in large scale survey studies (Hu et al., 1997; Djelouah et al., 2014). It has useful application for detection of naturally infected faba bean plants when compared with indirect ELISA (Kawanna and Fegla, 2015). Direct tissue blot immunoassay (DTBIA) can detect the presence of infection of Xylella fastidiosa in symptomless olive trees (Djelouah et al., 2014) and pineapple cercovirus (PCV) in symptomless pineapple plants in the field (Hu et al., 1997).

The present study resulted that, most of the tested papers recorded positive results with the tested viruses except in case of high glossy photo paper (200g/m²) which did not show any infected or healthy tissue print. Filter paper (Whatman No. 1), local drawing paper (60g/m²), photocopy paper (80g/m²), (56g/m²) and (70g/m²) were very gentle and need high caring in handling during the steps of the method. The aforementioned results are in line with Makkouk and Kumari (2002) study results where they got the same observation with filter and photocopy paper when tested to detect Alfalfa mosaic virus (AMV), Bean yellow mosaic virus (BYMV) in faba bean and Barley yellow stripe mosaic virus (BYSMV) in barley. Filter paper, local drawing paper (60g/m²), photocopy paper (80g/m²), (56g/m²) and (70g/m²) may did not persist to chemicals exposure during the assay steps. Meanwhile, NCM paper and Canson paper (150 g/m² and 300 g/m²) were persist in good condition during the process. Background in different types of paper except NCMs and Canson papers was took place. The reaction of the tested papers may be differed according to its component and structure. The NCM is characterized with the hydrophilic nature and has high affinity for binding proteins and the porous structure of it facilitates the fluid to penetrate via capillary action (Suntornsuk and Suntornsuk, 2019). High glossy photo paper used for inkjet printings, has smooth, glossy and waterproof surface. Thus, it may have not the ability to absorb the antigen or the antibody.

NCM is relatively expensive and represents 40-50% of TBA supplementary cost, which limits its use for virus detection in large scale, especially in the developing countries. Canson paper; fine art paper was more economical because it was cheaper than the NCM.

The present study concluded from the recorded data results the possibility of replacement of the nitrocellulose membrane by the Canson paper (300g/m²), in addition to the usage in its two faces. Also, using of the other side of the previously used nitrocellulose membrane in the detection which led to pronounced reduction of the cost and increase the applicability of TBA.

CONCLUSION
The present study concluded from the recorded data results the possibility of detection of Bean yellow vein clearing virus using of Canson paper (300g/m²) in naturally infected tomato samples as NCM. Thus, TBA using Canson paper could be applied in field survey studies, however large scale of samples tested with relatively low cost.

REFERENCES
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