COLORIMETRIC DETECTION OF POTATO VIRUS Y (PVY) BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION OF NUCLEIC ACIDS (LAMP) AS AN EXAMPLE OF A DEVELOPMENT POINT-OF-CARE TEST FOR POTATO VIRUSES

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INTRODUCTION

To develop field tests for detection of potato virus Y, we investigated the colorimetric RT-LAMP, in which virus detection involves visual observation of the color change of the reaction for samples from virus-infected plants. Among tested dyes, only the hydroxy naphthalene blue (HNB) specifically changed color in the presence of PVY virus. Field detection requires a fast sample preparation. We have shown that virus Y can be detected in the sap without RNA isolation through fluorescent real-time RT-LAMP. However, for the colorimetric RT-LAMP with HNB dye, the addition of even heavily diluted juice induced a change in color for both samples from diseased and healthy plants. Therefore, we tested several organic solvents, among which chloroform proved useful. After extraction of the juices with this reagent, we have observed a PVY-specific color change in the LAMP reaction. Summarizing, we have shown that it is possible to visually detect PVY using a colorimetric RT-LAMP test with HNB dye without RNA isolation from the tested samples. The sensitivity of visual detection was the same as with the fluorescence RT-LAMP test.

MATERIAL AND METHODS

Leaves from plants infected with PVY virus and leaves from healthy plants were used in the experiments. The juice from the obtained leaves was extracted with three organic reagents: chloroform, acetone, ethyl acetate. Sample buffer was used to dilute the juice obtained from the leaves. In the next stage of the experiment, a thermoblock reaching 90 degrees Celsius was used to heat the samples. Then, three additions to the sample buffer were used: sodium sulfite, beef albumin and alpha casein, to check their effect on the intensity of the color obtained. Hydroxy naphthalene blue (HNB) was used as a dye in all stages of the experiment. At the end of the study, the sensitivity of the colorimetric RT - LAMP test was compared with the RT - LAMP fluorescent test. In both cases, RNA isolation was not used, only potato leaf juice was directly used.

RESULTS AND DISCUSSION

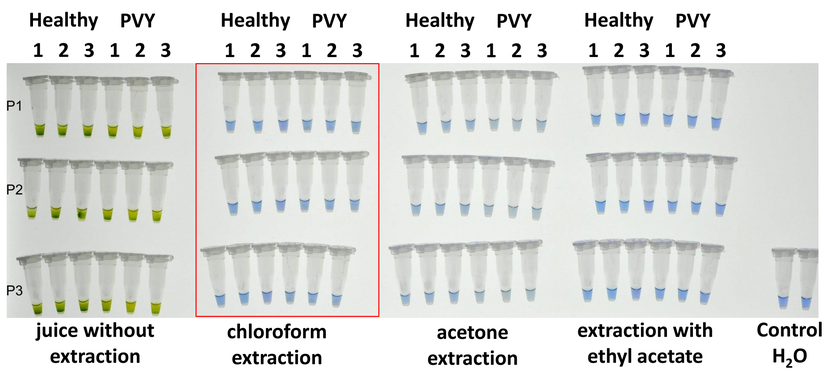

Fig. 1. Visual detection of the PVY virus by RT-LAMP directly on juice and extracts with the addition of HNB. Healthy 1,2,3 - samples obtained from healthy plants in three biological replications. PVY 1,2,3 - samples obtained from plants infected with virus in three biological replications. P1, P2, P3 - technical repetitions from the same tests. H_2O control - water was added to the reaction instead of juice or extract.

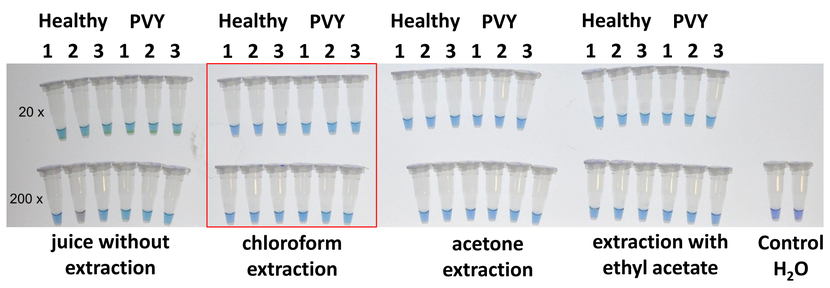

Fig. 2A. Visual detection of PVY virus by RT-LAMP directly on juice and extracts with the addition of HNB. Healthy 1,2,3 - samples obtained from healthy plants in three biological replications. PVY 1,2,3 - samples obtained from plants infected with virus in three biological replications. 20x and 200x - dilution of juice and extracts. H_2O control - water was added to the reaction instead of juice or extract.

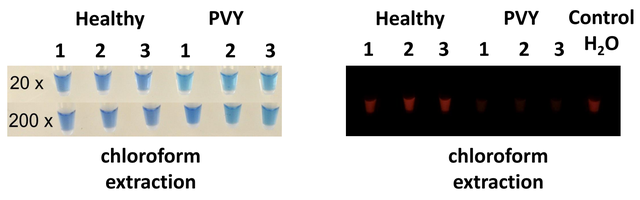

Fig. 2B. Visual detection of PVY virus by RT-LAMP directly on juice and extracts with the addition of HNB. Healthy 1,2,3 - samples obtained from healthy plants in three biological replications. PVY 1,2,3 - samples obtained from plants infected with virus in three biological replications. 20x and 200x - dilution of juice and extracts. And the same samples diluted 200 x plus control with water, highlighted on a with blue light.

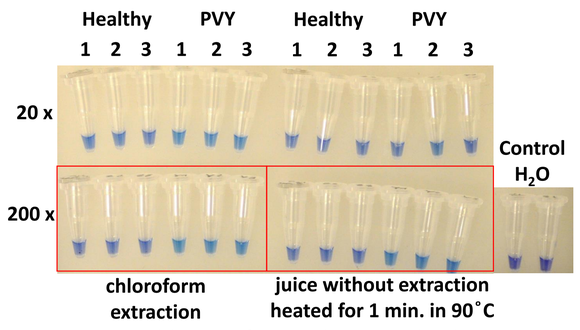

Fig. 3A. Visual detection of PVY virus by RT-LAMP directly on juice and extracts with the addition of HNB. Healthy 1,2,3 - samples obtained from healthy plants in three biological replications. PVY 1,2,3 - samples obtained from plants infected with virus in three biological replications. 20x and 200x - dilution of juice and extracts. H_2O control - water was added to the reaction instead of juice or extract.

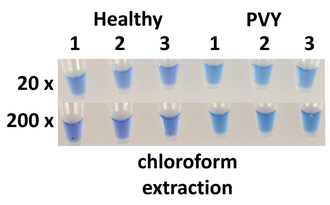

Fig. 3B. Visual detection of PVY virus by RT-LAMP directly on juice and extracts with the addition of HNB. Healthy 1,2,3 - samples obtained from healthy plants in three biological replications. PVY 1,2,3 - samples obtained from plants infected with virus in three biological replications. 20x and 200x - dilution of juice and extracts.

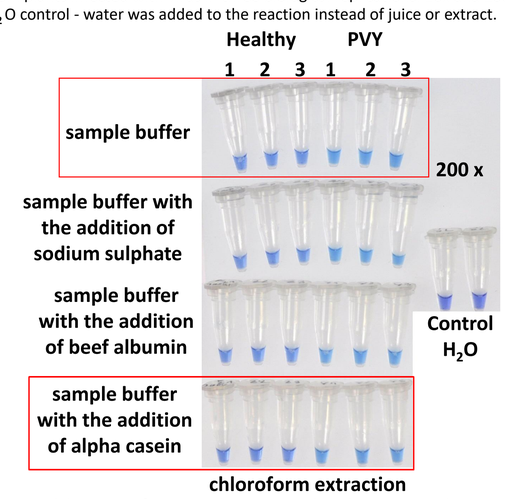

Fig. 4A. Visual detection of PVY virus by RT-LAMP directly on juice and extracts with the addition of HNB. Healthy 1,2,3 - samples obtained from healthy plants in three biological replications. PVY 1,2,3 - samples obtained from plants infected with virus in three biological replications. H2O control - water was added to the reaction instead of juice or extract. Samples were diluted 200x with assay buffer; sample buffer with the addition of sodium sulfite, beef albumin and alpha-casein.

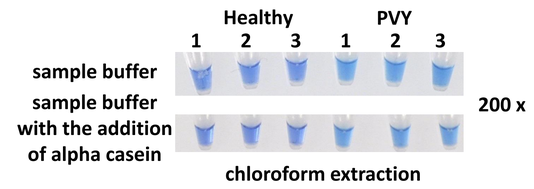

Fig. 4B. Visual detection of PVY virus by RT-LAMP directly on juice and extracts with the addition of HNB. Healthy 1,2,3 - samples obtained from healthy plants in three biological replications. PVY 1,2,3 - samples obtained from plants infected with virus in three biological replications. Samples were diluted 200x with assay buffer; sample buffer with the addition of alpha-casein.

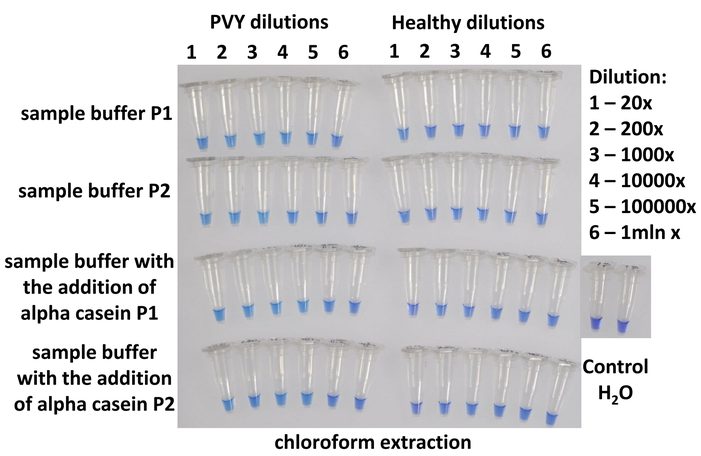

Fig. 5A. Visual detection of PVY virus by RT-LAMP directly on juice and extracts with the addition of HNB. Healthy 1,2,3 - samples obtained from healthy plants in three biological replications. PVY 1,2,3 - samples obtained from plants infected with virus in three biological replications. Samples were diluted 20x, 200x, 1000x, 10000x, 100000x, 1 million x: sample buffer; sample buffer with the addition of alpha-casein. P1, P2 - technical repetitions.

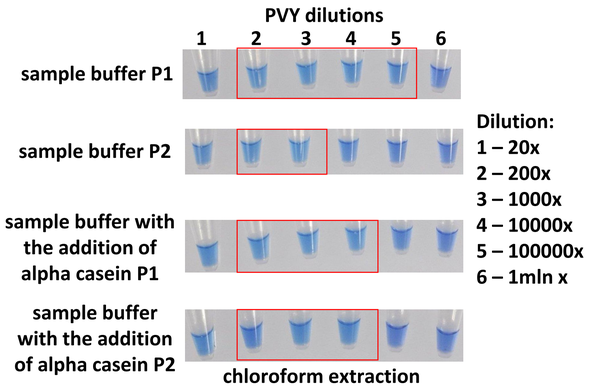

Fig. 5B. Visual detection of PVY virus by RT-LAMP directly on juice and extracts with the addition of HNB. PVY 1,2,3 - samples obtained from plants infected with virus in three biological replications. Samples were diluted 20x, 200x, 1000x, 10000x, 100000x, 1 million x: sample buffer; sample buffer with the addition of alpha-casein. P1, P2 – technical repetitions.

Dilutions	Sample buffer repeat 1				Sample buffer repeat 2				Sample buffer with the addition of alpha casein repeat 1				Sample buffer with the addition of alpha casein repeat 2			
	Healthy		PVY		Healthy		PVY		Healthy		PVY		Healthy		PVY	
	Melt temp	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp	Cq
20x	None	13,00	None	4,57	None	12,87	None	3,16	None	13,94	None	4,28	None	14,47	None	6,28
200x	None		83,50	14,37	None		83,50	14,33	None		83,50	15,20	None		83,50	16,12
1000x	None		83,50	15,58	None		83,50	15,14	None		83,50	16,31	None		84,00	16,24
10000x	None		83,50	16,81	None		84,00	18,26	None		84,00	22,20	None		84,00	17,69
100000x	None		84,00	45,13	None		84,50		None		84,00	24,83	None		83,50	22,87
1 mln x	None		None		None		None		None		None		None		None	

Table 1. Sensitivity of PVY detection with fluorescent RT-LAMP. Dilution of juice from healthy plants and those infected with the PVY virus, diluted with the duplicate buffer and the duplicate sample buffer with the addition of alpha-casein. Cq - cycle in which amplification was obtained. The specificity of the product was assessed by determining the melting point (Melt temp).

In samples extracted with chloroform from plants infected with PVY virus, a slight change in the shade of the dye to light blue is visible. Acetone extraction causes dye degradation, which causes the disappearance of the visible color. When using ethyl acetate extraction, no color change is observed (Fig. 1). The results obtained in the second stage of the experiment show that the change the HNB color to light blue was observed only when chloroform extraction was used both at a dilution of 20 and 200 times (Figures 2A and 2B). In the third stage, the diluted juice was heated for 1 min. at 90 ° C. The obtained results show that the heating of the samples had the effect only at a dilution of 200x, but it was not as pronounced as in the case of samples subjected to chloroform extraction (Figures 3A and 3B). The fourth stage of the experiment consisted in the use of test buffer additives, which were diluted with extracts: sodium sulfite (0.4%), beef albumin (0.2%) and alpha-casein (1mg / 1µl). On the basis of the results obtained, that the best results are obtained by the addition of alpha-casein as well as the lack of any addition to the sample buffer (Figs. 4A and 4B). The goal of the fifth stage of the experiment was to check the sensitivity of detection of PVY virus in samples extracted with chloroform and diluted with sample buffer and sample buffer with the addition of alpha-casein. Based on the results obtained (Figs. 5A and 5B), it was found that the test sensitivity allows the detection of PVY virus in extracts diluted with sample buffer: P1 at 100,000 x, P2 at 1000 x. In the case of extracts diluted with sample buffer with alpha addition - casein: P1 at 10,000 x, P2 at 10,000 x. Therefore, we can conclude that in both cases the sensitivity is 10,000 x. For 20 x diluted samples, the dye color change to light blue was weaker. This may be due to the fact that at such a small dilution there are still substances that inhibit HNB color change to a significant extent. The effectiveness of the test in the fluorescent version allows the detection of PVY virus at a dilution of 100,000x, both when diluting the samples with sample buffer and sample buffer with the addition of alpha-casein (Table 1).

CONCLUSION

A method of preparing juices for RT-LAMP has been developed, eliminating from the juice the factors causing HNB color change in samples from healthy plants.
The addition of alpha-casein to the sample buffer has been shown to accelerate amplification at the highest dilution that gives a positive result.
The RT-LAMP colorimetric test reduces the time from sampling to obtaining a result compared to the fluorescent RT-LAMP test.
Carrying out the test directly on juices allows you to reduce the costs of the test by skipping the isolation step.

ACKNOWLEDGEMENTS

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