

MOLECULAR DIAGNOSIS OF BYMV IN GLADIOLUS PLANTS

MOLEKULÁRNÍ DIAGNOSTIKA BYMV U GLADIOL

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ABSTRACT

A one step reverse-transcription polymerase chain reaction (RT-PCR) method was used to detect *Bean yellow mosaic virus* (BYMV) in gladiolus plants. One step RT-PCR analysis, employing primers specific to BYMV with different gladiolus cultivars. Total RNA was extracted from leaves and corms of gladiolus plants. Total RNA from leaves and corm were used to amplify specific primer sequence of BYMV. The one step RT-PCR method determined BYMV in all leaf samples and in corms it revealed very weak amplification. The aim of this paper is to discuss the diagnosis method from one step RT-PCR in leaves and corms of gladiolus plants.

Key words: Gladiolus, corms and leaves, BYMV detection, RT-PCR

INTRODUCTION

Gladiolus sp of Iridaceae family is an important ornamental plant grown for beautiful flowers, bouquets, floral baskets and cut flowers. The floriculture industry is being affected due to infection of various pathogens, resulting in drastic reduction in quality and quantity of flowers. Among various pathogens, viruses play an important role in the deterioration of ornamental quality of flowers, which ultimately affects the floriculture trade. The use of infected propagating material (corms) is considered as the main source for the virus dissemination and spreading of diseases (Raj *et al.*, 2002).

Bean yellow mosaic virus (BYMV), is readily detected in leaf tissue of gladioli plants (Zettler and El-Nil, 1977), but cannot be readily detected by ELISA, Serum Immunospecific Electron Microscopy (SSEM) or by biological test in corm tissue obtained from infected plants (Stein *et al.*, 1979; Stein *et al.*, 1986). Test for viral RNA in such corms using dot-spot hybridization with a BYMV –cDNA radioactive marked riboprobe were successful (R. Vunush, unpublished data). Nevertheless, leaves containing high concentration of BYMV can grow from corms in which virus can not be detected (Stein *et al.*, 1979). Thus, it seems that the amount of BYMV in infected corms is below the detection limit of the above mentioned methods. We have investigated whether other, more sensitive; diagnosis methods can be developed to detect BYMV in gladioli corms.

Methods based on the polymerase chain reaction (PCR) have been developed for the *in vitro* amplification of nucleic acid sequences (Doherty *et al.*, 1989; Wong *et al.*, 1987). These methods proved to be highly sensitive and capable of detecting single copies of DNA molecules (Wong *et al.*, 1987). In order to identify viral RNA, usually modified PCR method (RT-PCR) is used. RT-PCR could be used to detect the RNA genome of an encapsidated plant virus, BYMV, in gladiolus leaves (Vunsh *et al.*, 1990). Using a combined amplification and hybridization procedure were able to increase the sensitivity of this diagnostic method by a factor of up to 10^5 more than the commonly used ELISA and dot-spot nucleic acid hybridization methods, because the concentration of BYMV in gladiolus corm tissue is probably very low and detection in such tissue therefore poses a greater challenge than the virus detection in leaves. Furthermore virus detection in corms is more important work aimed at obtaining virus –free stocks. In this paper we report the method used to detect BYMV in gladiolus using one step RT-PCR.

MATERIAL AND METHODS

Virus isolates

1. Isolates were selected based on the previous result of ELISA test (Duraisamy and Pokorný – in preparation). The gladioli plants infected by selected isolates were planted in the green house, MZLU, Brno from corms collected from infected plants. The leaves were collected and tested for the presence of BYMV by ELISA before the collection of isolates. All plants were infected by this virus.

2. Gladiolus leaves and corms were collected from the greenhouse. Then they were tested for the presence of BYMV by one step PCR as follows.

RNA extraction from leaf and corms

Preparations of virus particle and total RNA extracts from leaf (100 mg) and corm tissues (100mg) were made similarly as described by (Vunsh *et al.*, 1990). RNA was extracted as per the manufacture's instruction (Qiagen and Ambion kit). The obtained RNA was quantificated by spectrophotometer and in one reaction we used 100ng of nucleic acid (Qiagen) and about 60ng (Ambion). The presence of virus specific sequences was detected by one Step RT-PCR. The set of primers that were used for amplification are described in Table 1

Table 1: The primers used for BYMV detection in gladiolus leaves and corms

Primers	Sequences	Authors
P1 P2	5' TTGAATCTGAACTGAAGTATT 3' 5' CTCCTTTCTACAAAATGGACA 3'	Vunsh <i>et al.</i> , 1990
BYF BYR	5' TGAATCTGAACTGAAGTATT 3' 5' CTCCTTTCTACAAAATGGACA 3'	Vunsh <i>et al.</i> , 1990
BYMV1 BYMV2	5' CAGTTTATTATGCAGCGG 3' 5' GTTATCATCAATCTTCCTGC 3'	Hiroyuki Uga, 2005
S1 S2	5' GCCTTATGGTGTGGTGCATAG 3' 5' CAAGCATGGTGTGCATATCACG 3'	Hammond and Hammond, 1989
F2 R2	5' GGTGTCAAGCAAGCGTATGA 3' 5' TCACCTGTTCTCTCCATCC 3'	*

* These primers were designed by authors, based on the coat protein region of BYMV by using the programme P3

The choosed primers belong to the C-terminal region of coat protein of BYMV (Hammond and Hammond, 1989) and amplify only BYMV. Same region was utilized by most of workers for designing of BYMV specific primers (Castro *et al.*, 1993; Rosner *et al.*, 1994). The procedure for one step RT-PCR consisted of a 50 °C for 30min, incubation at 95 °C (15 min for the first cycle), 94 °C for 1 min, 53 °C for 1.30 min and 72 °C for 1 min (35th cycle for annealing temperature) 72 °C for 10 min and 4°C for hold. These conditions were used to run the cycle for all the primers except F2R2. We slightly modified only the annealing temperature into 56 °C for 30 min and remaining condition is same as others Followed RT-PCR reaction product 25 ml was analyzed on 1.5% aggrose gel. Electrophoresis was done with TBE buffer and the gel was stained with ethidium bromide. DNA was visualized using an UV transilluminator and 100 bp ladder used as a size standard.

RESULTS

The ability of the standard treatments for PCR amplification of total RNA extracted from leaves and corms tissues were tested to detect BYMV in gladiolus plants. The different primers were used in RT-PCR experiments with RNA extracts of leaf tissues infected with BYMV. RT-PCR followed by gel electrophoresis revealed specific amplification product approximately 800bp with P1/P2 (Fig.1) and BYF/BYR (Fig.2) primers whereas in BYMV1/BYMV2 it revealed amplification around 700bp and in sample no.5 (J95) revealed two different products with this primer (Fig.3), this may be due to different virus isolate infection. In case of S1/S2 it amplified around 500bp (Fig4) and in F2/R2 is 200bp (Fig.5). Similarly, the RNA extract from corm tissues were also tested in one step RT-PCR with different primer specificity. In case P1/P2 and BYMV1/BYMV2 primer, we found one product around 800bp (Fig. 6) and 700bp (Fig.7) whereas in remaining product we couldn't find. But in the BYF/BYR primer we found specific product (800bp) in three samples (Fig8) whereas other we couldn't find. In F2/R2 primer; we noticed weak product (200bp) in four samples (Fig9).

DISCUSSION

The maintenance and rapid propagation of disease free nuclear stock has become a prominent procedure in the bulb industry (Brunt, 1985). This detection of viruses in bulb and corm planting material is essential for building such nuclear disease – free stocks. In some cases, however, virus cannot easily be detected directly in the organ itself.

BYMV is very ubiquitous plant virus and have been found infecting gladiolus in several regions of the world (Katoch *et al.*, 2003) and also in the Czech Republic (Duraismy and Pokorný - in proceeding). During the last decade, PCR has been widely used with varying degree of modification for detection for viral genome in infected plants. RT-PCR followed by gel electrophoresis revealed amplification of approximately 800 bp, 700bp, 500bp and 200bp DNA fragments using specific primers in all of our leaf samples of gladiolus. We haven't any healthy plants for comparison as negative controls Amplification confirms the presence of BYMV infection in this stock. Like the present observations, Vunsh *et al.* (1990) also detected specific sequence of BYMV in RNA extracts of infected plants by PCR. Bojra and Ponz,1992; Dovas *et al.*, 2001 also reported that the crude extract, obtained from leaf tissues showed positive results of all tested samples for BYMV. However, methods developed for leaf tissues, were used to detect virus in corms tissues was not successful, this is due to either very low concentration of virus in the corms (below the threshold that cannot detect by PCR method), or that extracts of corm tissues contain a substance(s), which interferes with the PCR reaction. Extraction of corm tissues was shown to inhibit the amplification of viral sequences when added to a PCR reaction with specific hybridization with radioactive labeled probe (Vunsh *et al.*, 1990). Stein *et al.* (1986) reported that BYMV could not be detected in the majority of corms tissues, even after following different procedures of purification RT-PCR could detect BYMV only in leaf and failed to detect in bulb samples. This could be due to reason that corm tissues have some inhibitors (Saiki *et al.*, 1988; Wong *et al.*, 1987; Fenby *et*

al., 1995). In the representing experiment, the BYMV from corms was trapped by the low responding. This might be due to either very low concentration of virus in corms or corm tissue contains the substance interfering with the PCR reaction. This single polymerase promoter of RNA from leaves served as better template for particular orientation than a template containing RNA from corms. The result indicated that one step RT-PCR is useful as a diagnostic tool for virus detection only in leaves but not in corms tissues.

SUMMARY

The one step - polymerase chain reaction (PCR) method readily detected BYMV in gladiolus leaves but it fails to detect in corms tissues. In order to rectify this problem, an additional purification step is required for RNA extracts from corm tissues in order to eliminate the inhibitory effect and enables PCR to amplify to detect viral RNA in corm tissue preparation.

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FIGURES

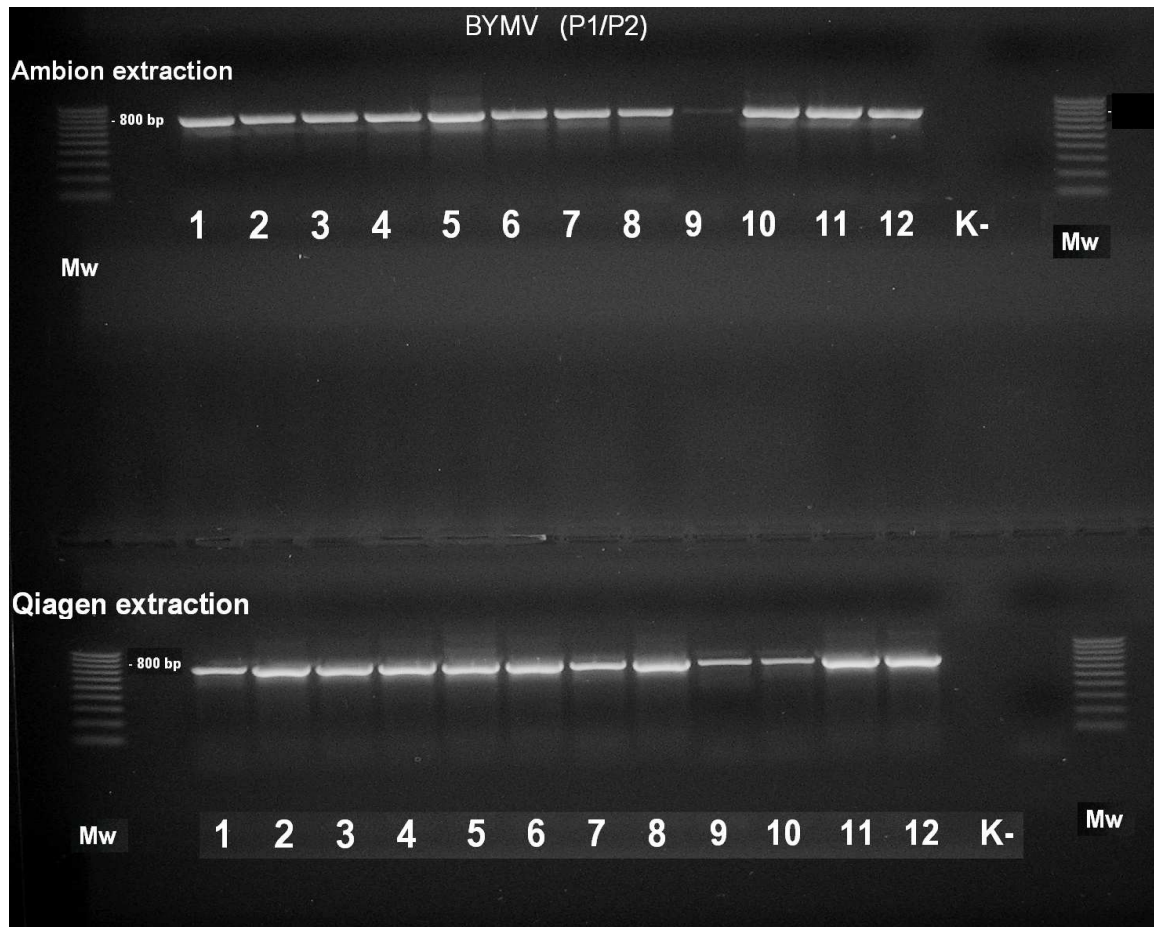


Fig.1. Detection of BYMV specific RNA in gladiolus leaves based on One Step RT PCR reactions with primers P1/P2. There are compared two different RNA extraction methods (upper: RNAqueousTM extraction kit from Ambion and lower: RNeasy Plant Mini Kit from Qiagen)

The individual virus isolates are marked by numbers 1 -12 (1-J16, 2-J34, 3-J49, 4-J50, 5-J95, 6-M63, 7-M71, 8-M78, 9-M103, 10-N4, 11-N7 and 12-N14). The amplified products (about 800bp) were fractionated by agarose (1%) gel electrophoresis and stained with ethidium bromide. Mw – DNA size marker, K- is a negative control (a sample without nucleic acids)

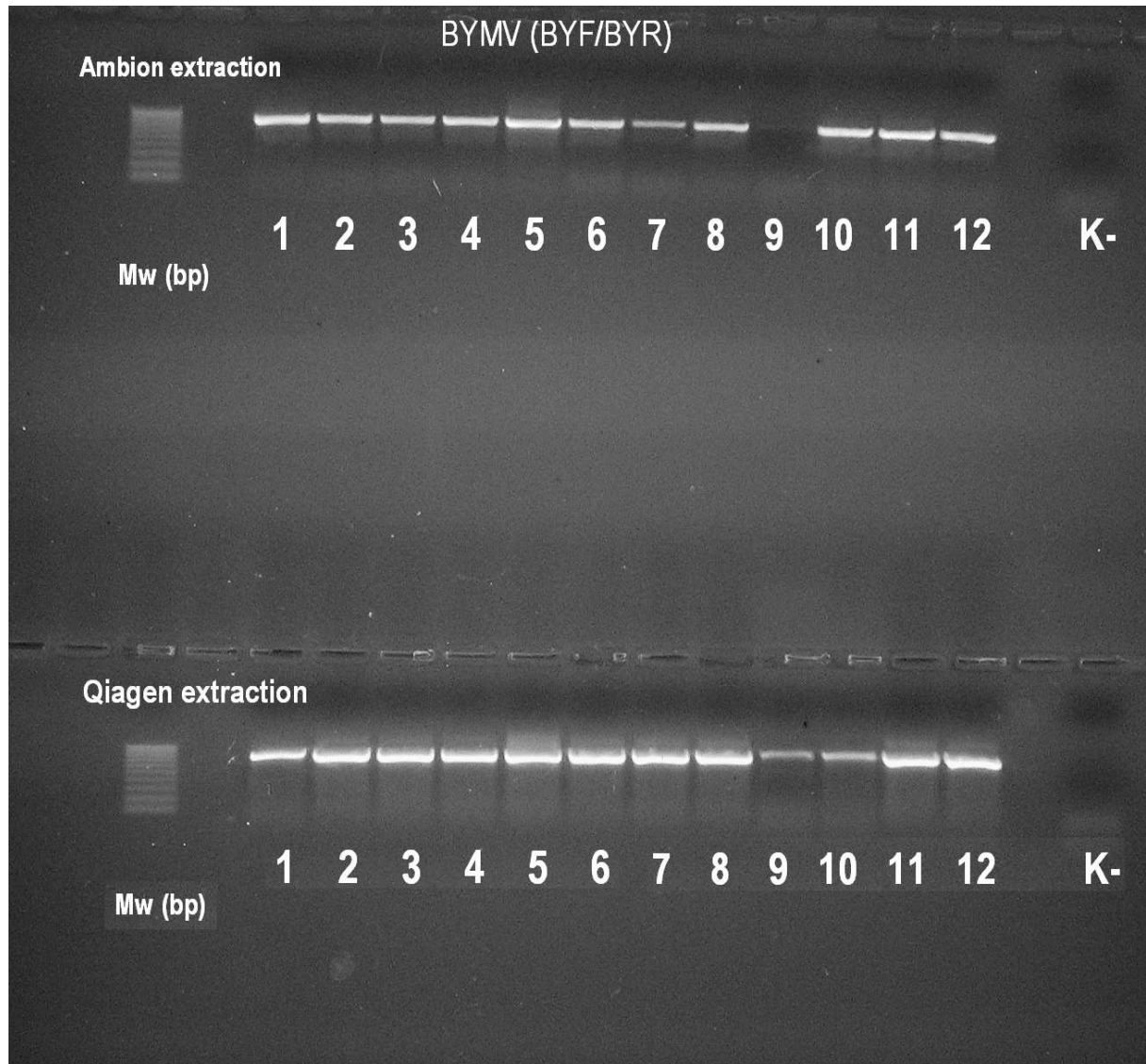


Fig.2. Detection of BYMV specific RNA in gladiolus leaves based on One Step RT PCR reactions with primers BYF/BYR. There are compared two different RNA extraction methods (upper: RNAqueous™ extraction kit from Ambion and lower: RNeasy Plant Mini Kit from Qiagen). The individual isolates mentioned are same as Fig.1.

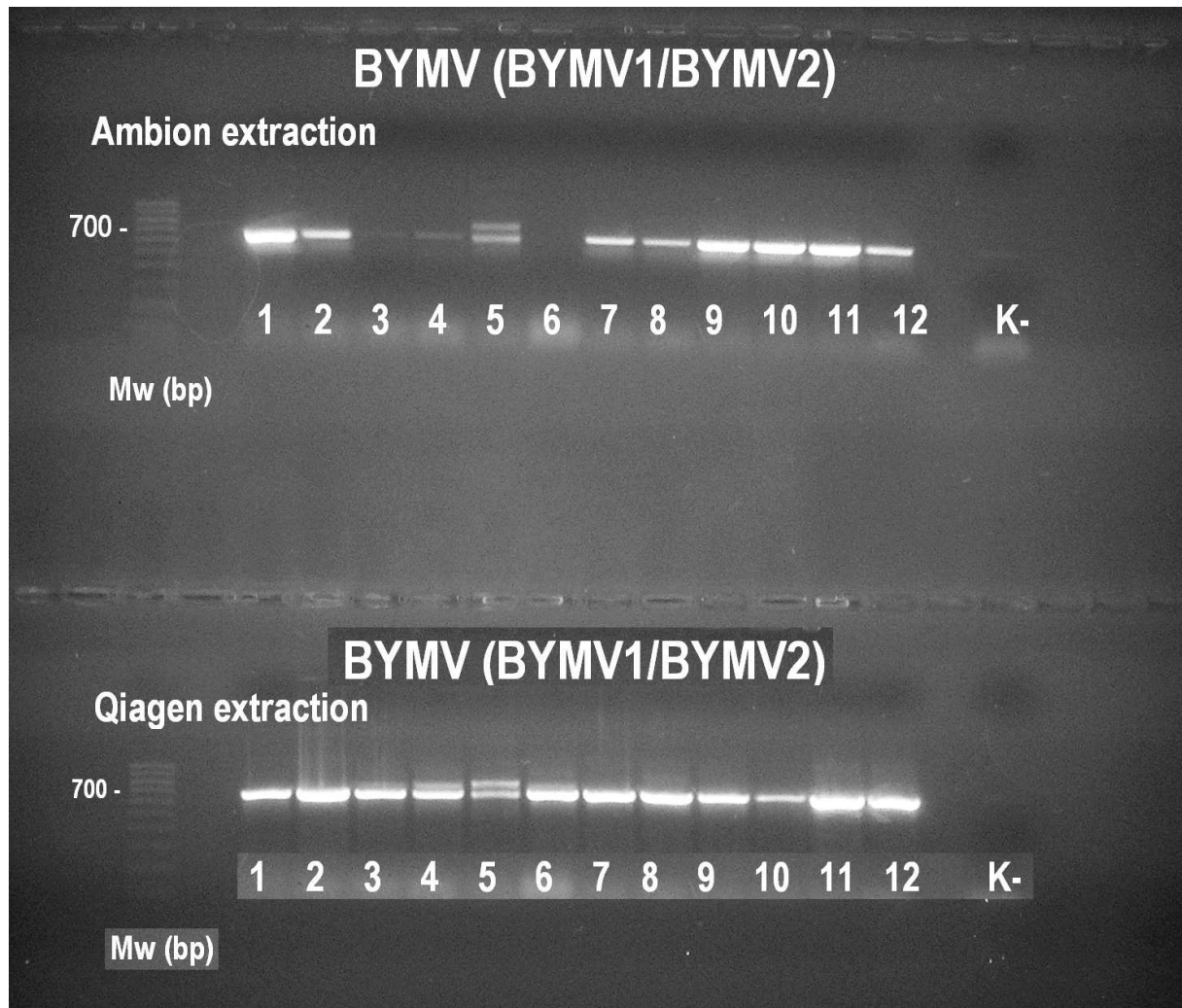


Fig.3. Detection of BYMV specific RNA in gladiolus leaves based on One Step RT PCR reactions with primers BYMV1/BYMV2 There are compared two different RNA extraction methods (upper: RNAqueousTM extraction kit from Ambion and lower: RNeasy Plant Mini Kit from Qiagen). The individual virus The individual isolates mentioned are same as Fig.1

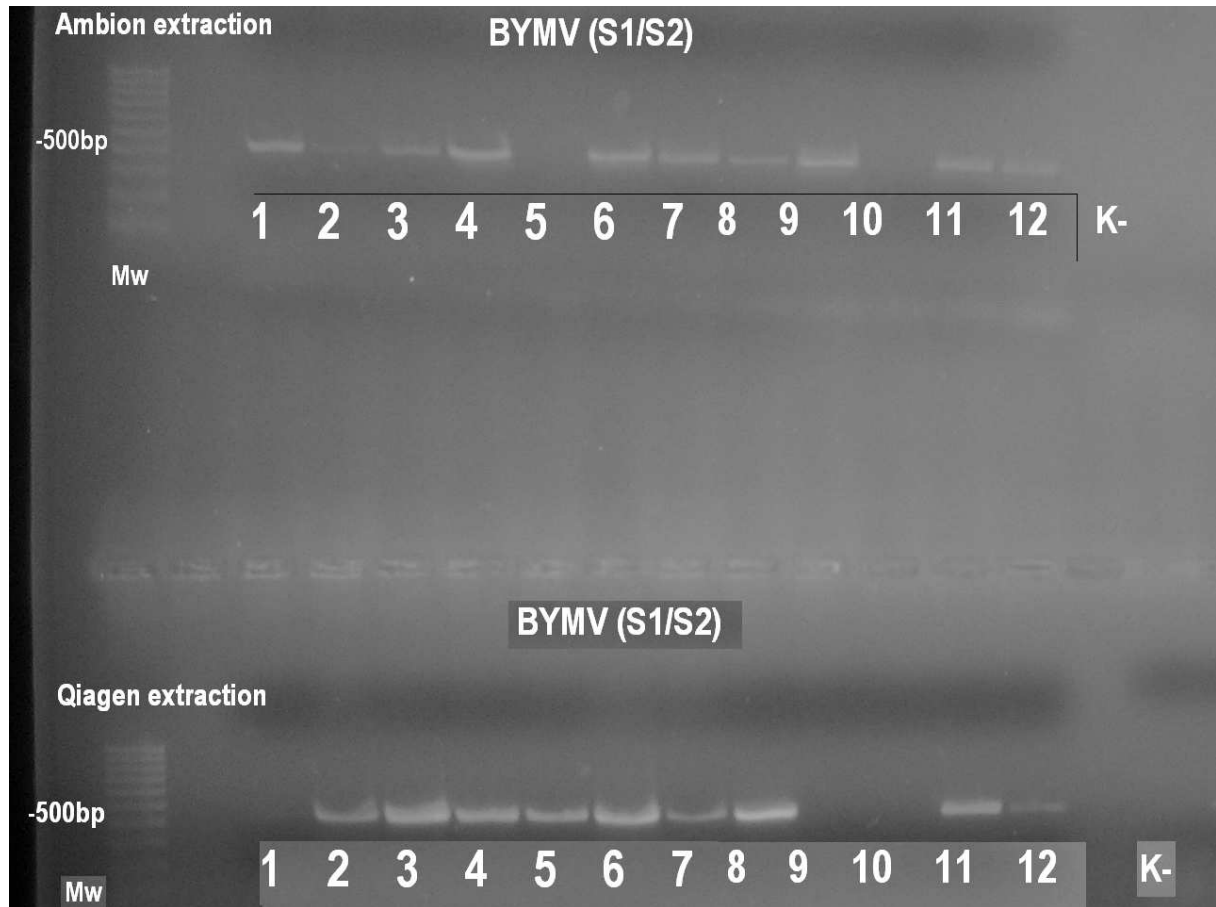


Fig.4. Detection of BYMV specific RNA in gladiolus leaves based on One Step RT PCR reactions with primers S1/S2. There are compared two different RNA extraction methods (upper: RNAqueousTM extraction kit from Ambion and lower: RNeasy Plant Mini Kit from Qiagen). The individual isolates mentioned are same as Fig.

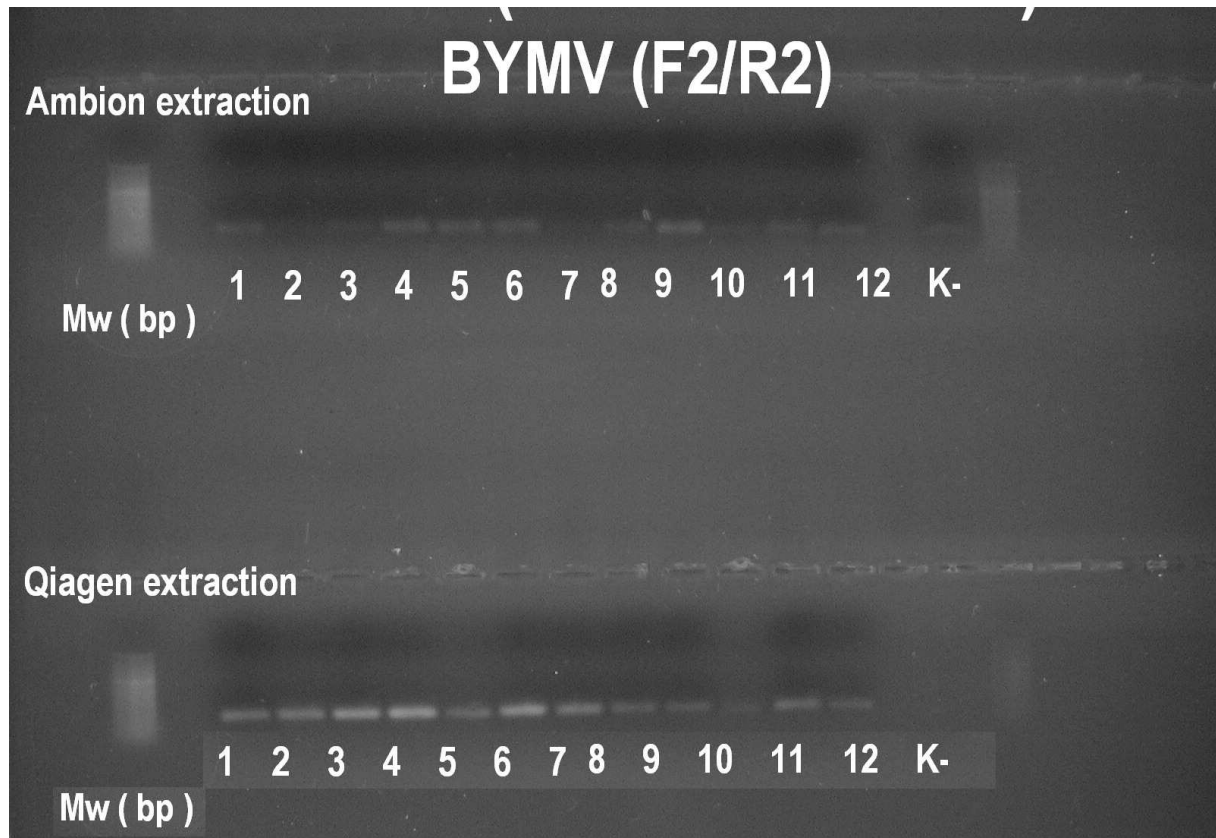


Fig.5. Detection of BYMV specific RNA in gladiolus leaves based on One Step RT PCR reactions with primers F2/R2 There are compared two different RNA extraction methods (upper: RNAqueous™ extraction kit from Ambion and lower: RNeasy Plant Mini Kit from Qiagen). The individual isolates mentioned are same as Fig. 1

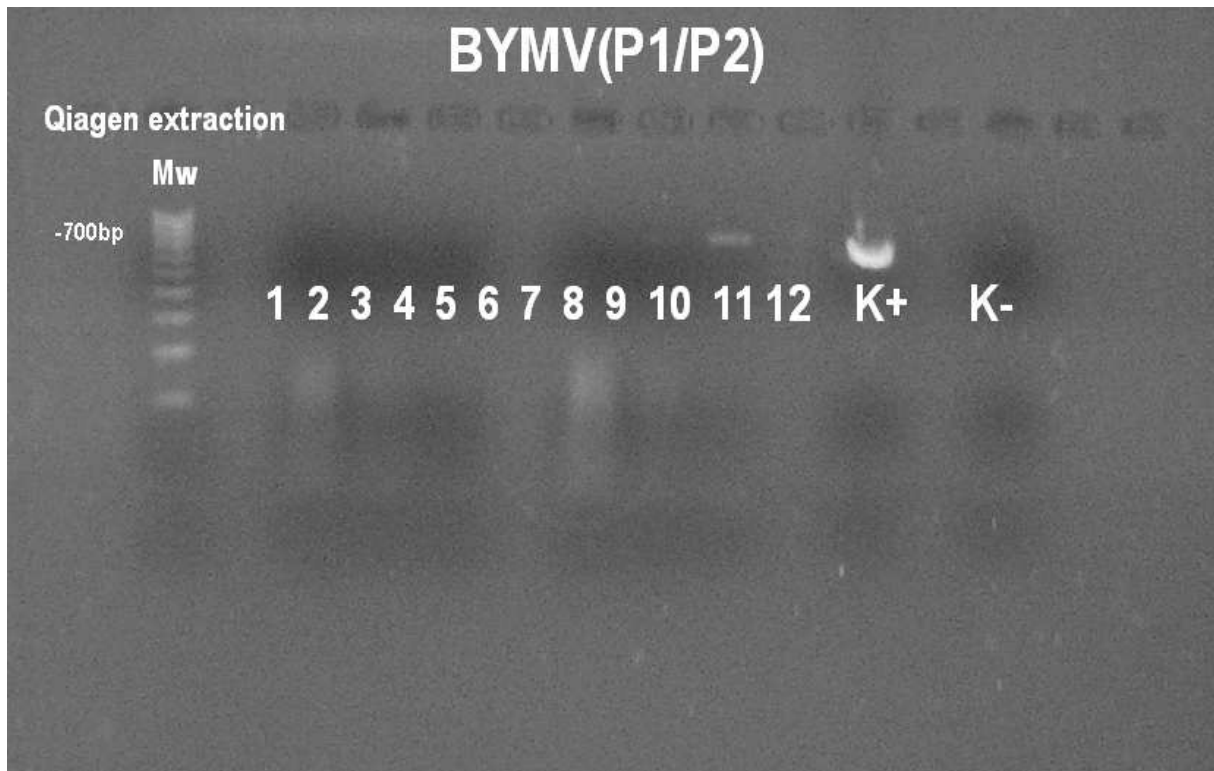


Fig.6. Detection of BYMV specific RNA in gladiolus leaves based on One Step RT PCR reactions with primers P1/P2. There are compared two different RNA extraction methods (upper: RNAqueous™ extraction kit from Ambion and lower: RNeasy Plant Mini Kit from Qiagen). The individual isolates mentioned are same as Fig.1



Fig.7. Detection of BYMV specific RNA in gladiolus leaves based on One Step RT PCR reactions with primers BYMV1/BYMV2. There are compared two different RNA extraction methods (upper: RNAqueous™ extraction kit from Ambion and lower: RNeasy Plant Mini Kit from Qiagen). The individual isolates mentioned are same as Fig.1

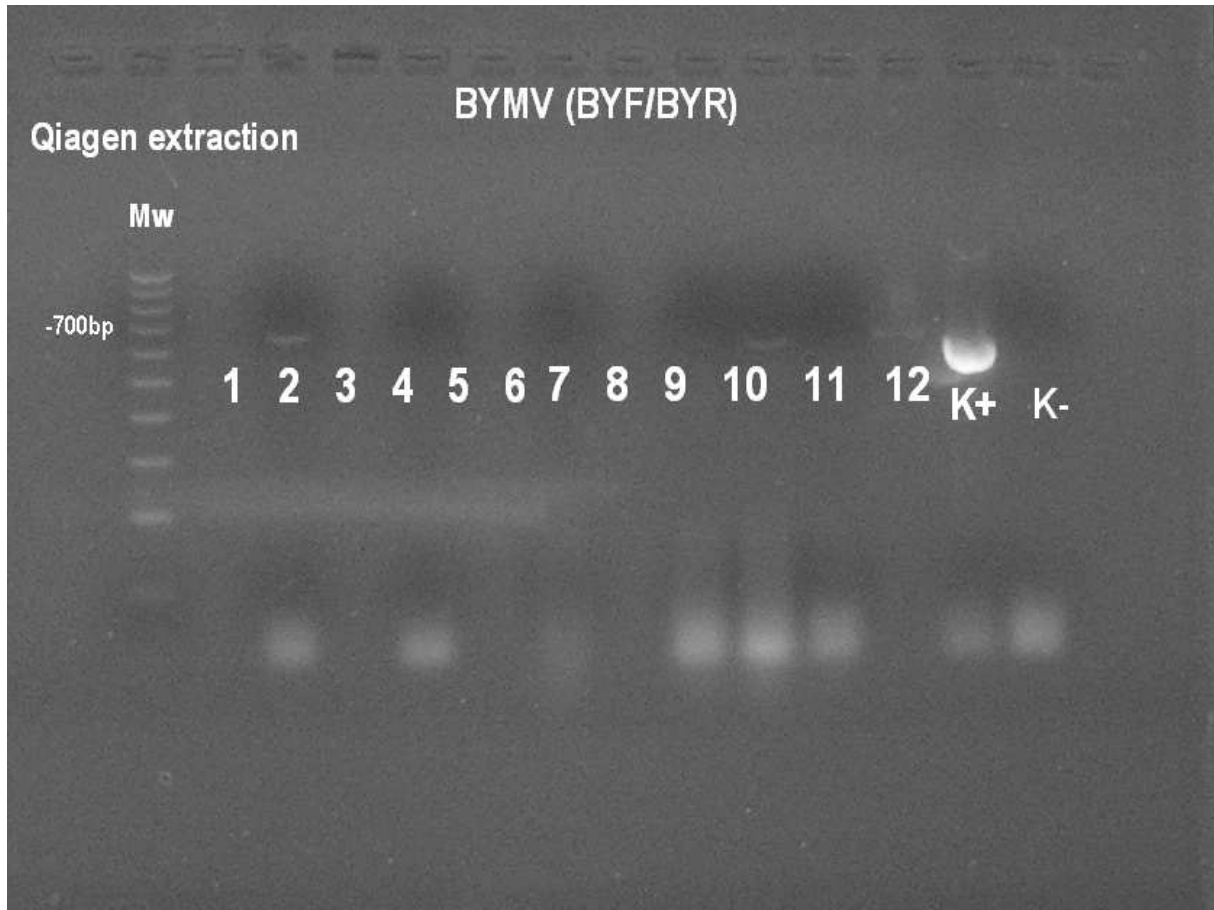


Fig.8. Detection of BYMV specific RNA in gladiolus leaves based on One Step RT PCR reactions with primers BYF/BYR. There are compared two different RNA extraction methods (upper: RNAqueous™ extraction kit from Ambion and lower: RNeasy Plant Mini Kit from Qiagen). The individual isolates mentioned are same as Fig.1

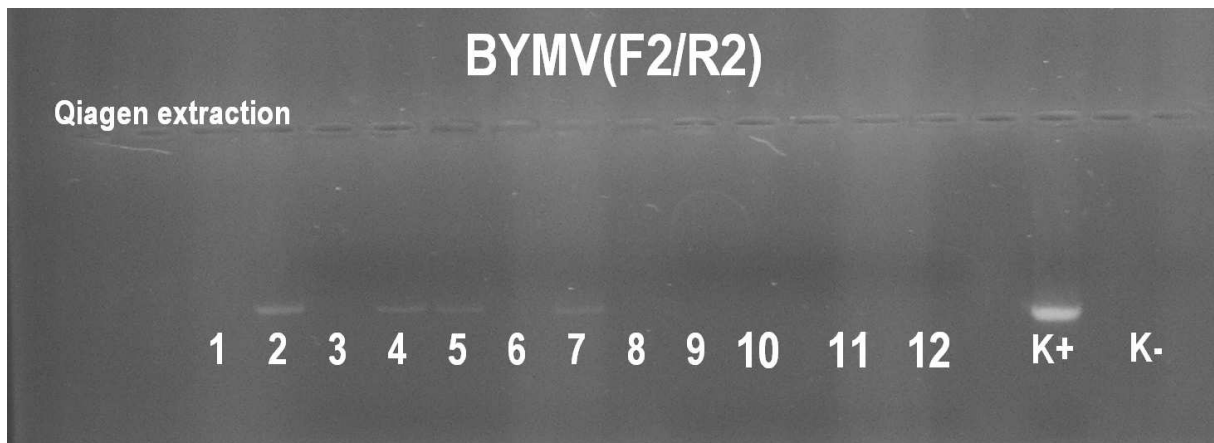


Fig.9. Detection of BYMV specific RNA in gladiolus leaves based on One Step RT PCR reactions with primers P1/P2. There are compared two different RNA extraction methods (upper: RNAqueous™ extraction kit from Ambion and lower: RNeasy Plant Mini Kit from Qiagen). The individual isolates mentioned are same as Fig.1