

Production of polyclonal antibodies against the BYMV isolate infecting gladiolus crop naturally and development of polyclonal antibodies based serological diagnostic system

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ARTICLE INFO	ABSTRACT
Original Research article Received on March 18, 2017 Accepted on April 26, 2017 Article Authors B. R. Singh, Javed Musarrat, Aminuddin Corresponding Author Email brajviro@gmail.com	In this study a bean yellow mosaic virus isolate infecting gladiolus crop with leaf mosaic and color breaking symptoms on flower was characterized based on mechanical transmission, symptomatology on hosts, and viron particle structure and capsid protein molecular weight determination. The virus isolate was successfully purified from the propagating host and purified viron particles were used for the production of polyclonal antibodies for the development of a cost effective and sensitive serological diagnostic system. The produced polyclonal antibodies would be useful for the assessment of BYMV infection in gladiolus crop and seed materials.
PUBLICATION INFO International Journal of Agricultural Invention (IJAI) RNI: UPENG/2016/70091 ISSN: 2456-1797 (P) Vol.: 2, Issue: 1, Pages: 5-9 Journal Homepage URL http://agriinventionjournal.com/ DOI: 10.46492/IJAI/2017.2.1.2	KEYWORDS Gladiolus, Bean Yellow Mosaic Virus, Polyclonal Antibodies, Electron Microscopy

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The rapid mechanization and international expansion of trade, commercial cultivation of Gladiolus crop has become a global industry in India and abroad (Selvaraj *et al.*, 2009). This crop is asexually propagated and a few major propagators supply most of the plants to growers over a very large geographic area. This makes the crop production industry conducive to the proliferation and distribution of diseases (Beute, 1970 and Singh *et al.*, 2007). Indeed, gladiolus crop production has frequently been affected by various kinds of plant pathogens including viruses. Not only the viral diseases cause severe losses to the Gladiolus cut flower industry per se, but the infected crop plants and plant materials also serve as a source of transmitting viruses into other crops and zones/countries (Srivastava *et al.*, 1983 and Singh *et al.*, 2007). Several viral associated diseases have been reported in the Gladiolus crop, among them Bean yellow mosaic virus (BYMV) has been found the most prevalent and economically important. BYMV is a monopartite strand of positive-sense, single-stranded RNA surrounded by a capsid made for a single viral encoded protein. It is now well established that the BYMV is a major factor responsible for the significant yield losses in Gladiolus crop in the world wide including in India (Bridgmon and Walker, 1952; Fry, 1953; Zaidi *et al.*, 1993; Katoch *et al.*, 2002; Singh *et al.*, 2007 and Selvaraj *et al.*, 2009). In India, BYMV

was first reported by (Srivastava *et al.*, 1983) and (Singh *et al.*, 2007) from Uttar Pradesh and (Katoch *et al.*, 2002) from Himachal Pradesh. BYMV infection in Gladiolus crop was reported to cause mosaic and white-break symptoms on leaves, color break symptoms in flowers and also reduced vigor of the cultivar (Bridgmon and Walker, 1952, Raizada *et al.*, 1989, Katoch *et al.*, 2002, Singh *et al.*, 2007). Although some time latent infection (without causing any symptom) was reported. Therefore, control and management of the BYMV in Gladiolus crop is very necessary to minimize the losses through the improvement of the quality, yield and productivity of produce. In order to improve the crop quality yield and productivity, virus free seed material to growers the BYMV infection selective and sensitive diagnosis is on prime importance. Considering the importance of the BYMV diagnosis, the present study was aimed to develop a serological diagnostic system using polyclonal antibodies against indigenous BYMV isolate infecting gladiolus crop.

MATERIALS AND METHODS

Sample Collection of BYMV Infecting Gladiolus

Virus infected Gladiolus plants exhibiting visible mosaic symptoms were collected from the NBRI experimental field. The sample was then

desiccated at 4°C over anhydrous CaCl₂ and preserved in ultra-deep freezer at -80°C.

Mechanical Inoculation and Propagation of BYMV Strain Infecting Gladiolus

The infected plant material was inoculated on to diagnostic (*Chenopodium amaranticolor*) and propagating (*Vicia faba*) hosts by using phosphate buffer. The plants pre dusted with carborundum at 4-5 leaf stage were inoculated with extracts from infected plants. The inoculated plants were observed for symptom development over a period of three weeks after inoculation. Mechanically inoculated *Vicia faba* plants were grown for 20-25 days in a green house at 25°C. The leaves were harvested, washed with MQ water, air dried and frozen at -80°C until use. Virus was purified from infected leaf tissues as described by (Dijkstra and Jager 1998). Briefly, systemically infected 200g leaves were ground in liquid nitrogen and homogenized in a blender with extraction buffer [0.05 mM KH₂PO₄, 0.01 mM EDTA, 1% (w/v) Na₂SO₃, 5% (v/v) Ethanol, pH 7.6] in the ratio 1: 3 (w/v), then filtered through three layers of cheesecloth. The filtrate was centrifuged (10,000 rpm, 4°C, 20 min, in GSA rotor), Triton X-100 was added into the supernatant to a final concentration of 3% and stirred for 1 h at 4°C. The supernatant was layered over a cushion of 20% (w/v) sucrose and centrifuged for 3 h at 26,000 rpm in Ti 45 rotor. The pellet was dissolved in 20ml resuspension buffer (Same as extraction buffer excluding ethanol), stirred on ice for 4 h, and then centrifuged for 10 min at 8,000 rpm. The supernatant was collected and resuspended in 10ml of resuspension buffer. The collected supernatant containing virus suspension was subjected to cesium chloride density gradient centrifugation in vertical rotor SW-55 for 16-20 h at 36,000 rpm. The virus particles zone was collected and dialyzed in storage buffer (100 mM NaH₂PO₄, pH 7.2) at 4°C with three successive changes. Dialyzed purified virus was collected and OD at 260 nm was measured by spectrophotometer. Purified virus was stored in 50% glycerol at -80°C and infectivity of purified preparation was tested on *Vicia faba* plants by mechanical inoculation. Aliquots were taken for electron microscopy (EM), SDS-PAGE and EIBA analysis.

Electron Microscopy

The electron microscopy of purified virus preparation was carried out using standard method as described by Aminuddin *et al.* (1999). A carbon coated grid was floated on a drop of purified virus suspension for 10 min and washed with 1 ml of phosphate buffer (pH 7.0 containing 1 mM EDTA). The virus particles were negatively stained by floating the grid on a drop of uranyl acetate (2%, pH 4.2) for 50 sec. The excess stain was soaked away on a piece of filter paper and the grid was air dried. The grid was observed in transmission electron microscope (TEM) and photographed. The electron microscopy of purified virus preparation was carried out by using negative staining with uranyl acetate (2 %, pH 4.2).

Determination of Molecular Weight of Capsid Protein Subunits

Molecular weight of coat protein subunits were determined by SDS-PAGE. Purified virus was disrupted in equal volume of SDS gel-loading buffer (50 mM Tris. HCl, pH 6.8, 100 mM DTT; 2% SDS, 0.1% bromophenol blue, 10% glycerol) boiled for 5 min for denaturation and immediately chilled on ice. The denatured virus coat protein was separated by SDS-PAGE in 12% polyacrylamide gels at pH 8.8 using Tris-glycine electrophoresis buffer (25 mM Tris, 250 mM Glycine, 0.1% SDS, pH 8.3) along with medium range prestained protein markers for comparison of viral capsid protein in a Bio-Rad minigel system and stained with Coomassie Brilliant Blue R-250 and destained (5% methanol, 7% acetic acid).

Production of Antiserum

Rabbit was injected with ~0.5mg of purified virus mixed with an equal volume of Freund's complete adjuvant. After one week, rabbit was immunized with the same amount of the purified virus mixed with Freund's incomplete adjuvant. The third dose was administered similar to the second dose. One week after the last booster dose the rabbit was bled and the titer of serum was determined. IgG from the antiserum was purified by (NH₄)₂SO₄ precipitation using standard method and evaluated for reactivity (Jager and Dijkstra, 2000). Total protein of 100mg infected leaves (naturally infected)

was extracted by grinding them in a mortar and pestle with liquid nitrogen followed by adding equal

volume of protein extraction buffer (100 mM potassium phosphate buffer, pH 7.8, 1mM EDTA,

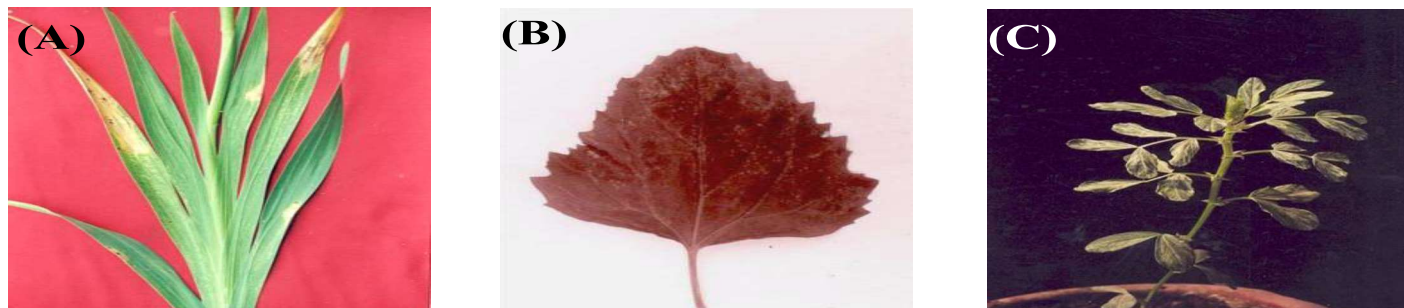


Fig. 1. (A) Gladiolus plant showing the typical mosaic symptoms on leaf; (B) BYMV isolate induced necrotic local lesions on *C. amaranticolor* and (C) Mechanical transmission of BYMV isolate on propagating host *Vicia faba*.

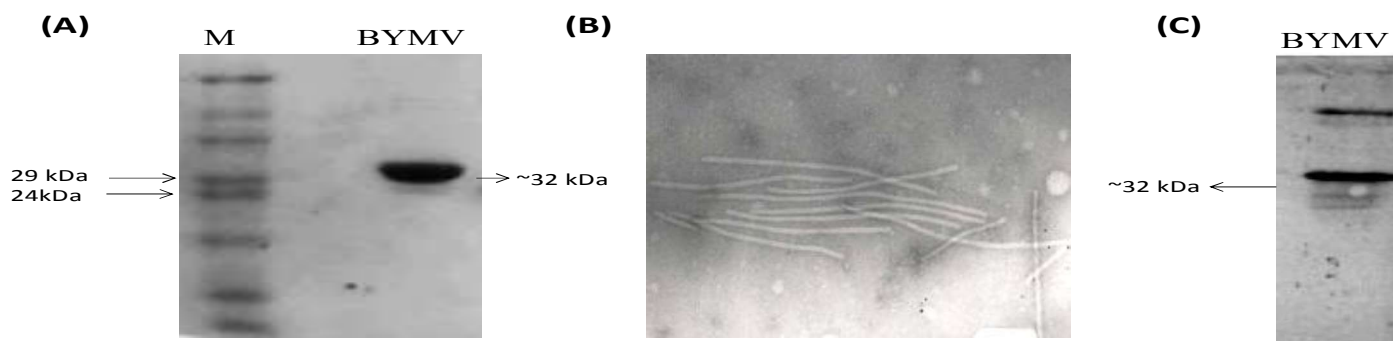


Fig. 2. (A) Electron micrograph of purified BYMV isolate from propagating host (*Vicia faba*); (B) Determination capsid protein molecular weight of purified BYMV isolate using SDS gel electrophoresis and (C) Detection of BYMV infection in gladiolus leaf sample using polyclonal antibodies produced against the maintained BYMV isolate.

1% Triton X- 100; 10% Glycerol, 1 mM DTT). The leaf extracts was separated from the remaining leaf debris following centrifugation at 13,000 g for 5 min at 4°C. On the other hand, purified virus was directly subjected to denaturation. The extracted plant proteins were disrupted in equal volume of SDS gel-loading buffer. The samples were placed in boiling water for 5 min for denaturation and immediately chilled on ice. The separation of the proteins was carried out on a SDS 12% polyacrylamide gel in a Bio-Rad minigel system. The proteins were transferred from the gel to a NC membrane using electroblotting with a transfer buffer (0.02 M Tris, 0.15 M glycine, 0.01% SDS, 20% methanol). The membrane was incubated in blocking buffer (1% dry milk, 0.5% BSA in TBS) for 90 min, washed once in TBS-T and incubated at 4°C for 2 h with produced antibodies against the BYMV infecting gladiolus crop. The blot was then washed four times in TBS-T before being incubated in a 13,000 dilution of alkaline phosphatase-labeled goat anti-rabbit antibody for 2 h, followed by three washes of 10min each in TBS-T and a 10 min incubation in substrate

buffer (0.1M Tris, pH 9.5; 0.1M NaCl; 5mM MgCl₂) containing 175 mg/ml BCIP and 350 mg/ml NBT.

RESULTS AND DISCUSSION

Naturally infected gladiolus leave with typical symptoms of BYMV was collected from NBRI garden experimental plot, Lucknow, India. The higher concentration of these virus particles has been reported in the leave parts as compared to corms (Stein *et al.*, 1988). The naturally infected plants showed severe mosaic, dark green stripes on leaves, color-breaking of petals and stunting symptoms along with poor blooming was also noticed in affected plants (Fig. 1A). The mechanical inoculation was performed to transmit the virus isolate from gladiolus to diagnostic (*Chenopodium amaranticolor*) and propagating (*Vicia faba*) hosts (Figs. 1B and C) (Srivastava *et al.*, 1983, Mokra and Gotzová, 1994, Katoch *et al.*, 2002 and Singh *et al.*, 2007). The BYMV isolate infecting gladiolus was purified from propagating host and UV-visible spectrophotometry was used to determine the

characteristics of the purified BYMV particles. The data showed the characteristic absorbance spectrum of nucleoprotein under UV range and A 260/280 and Amax/min ratios were calculated 1.37 and 1.22, respectively. The BYMV yield basis of an extinction coefficient of 2.4 was estimated ~4 mg/ 100 g of infected leave of propagating. The high yield BYMV from the *Vicia faba* leave tissues suggests the simplicity of mechanical transmission of the virus (Khattab and Abdelkader, 2015). The purified BYMV virus was found infectious when tested on propagating host, SDS-PAGE revealed the Mw of gladiolus isolate capsid protein as ~32 kDa (Fig. 2 A). The size of the capsid protein was corroborate with findings reported by the other researchers (Srivastava *et al.*, 1983, Singh *et al.*, 2007 and Selvaraj *et al.*, 2009). The purified BYMV preparation obtained from propagating host showed plenty of rod shaped virion particles visualized under transmission electron microscope (Fig. 2B). The similar viron particle structures of BYMV have been reported in the past years (Francki *et al.*, 1985 and Katoch *et al.*, 2002). The antiserum production of Gladiolus infecting BYMV isolate was obtained by immunization of New Zealand white rabbits with a total of 1.5 mg purified virus preparations. The titer and reactivity of the purified polyclonal antibodies from the produced antiserum were determined with similar BYMV isolate.

The produced polyclonal antibodies sensitivity and selectivity was tested against the BYMV isolate naturally infecting gladiolus crop. The EIBA assay revealed the successful detection of BYMV from the natural samples and produced polyclonal antibodies based diagnostic system was found cost effective as compared with the commercially available antibodies (Khattab and Abdelkader, 2015). Polyclonal antibodies derived from animal immunized with purified BYMV viral proteins are particularly valuable for sero-diagnostic study at the laboratory, field and quarantine places. The sero-diagnostic detection systems like dot blot, tissue, western blots immunostaining or EIBA, and ELISA and immunoprecipitation complement fixation assays have been commonly used for the detection of BYMV infecting Gladiolus crop (Nagel *et al.*, 1983, Selvarajan *et al.*, 1998, Katoch *et al.*, 2002 and Singh *et al.*, 2007).

CONCLUSION

In conclusion, the produced cost effective BYMV polyclonal antibodies would be suitable for many diagnostic applications, like dot blot, tissue, western blots immunostaining, and immunoprecipitation complement fixation assays for detection of BYMV infections in field, floricultural and horticultural crops. The diagnostic screening of BYMV associated diseases using produced polyclonal antibodies against the BYMV isolate infecting Gladiolus is needed because polyclonal antibodies could be able to recognize multiple epitopes (native and denatured forms) on the specific and similar group of virus belongs to *Potyvirus* genus.

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