SUMMARY AND CONCLUSIONS
To fulfill the aim of work ‘Development of a diagnostic kit against gladiolus virus’ studies like standardization of diagnostics, incidence of viruses infecting gladiolus, maintenance of culture, transmission studies, purification of virus, characterization of virus/antigen and different isolates of BYMV, development of diagnostic reagents and their field testing, utilization of kit for certification of virus-free plants were done.

During the survey of the field of Division of Floriculture, Institute of Himalayan Bioresource Technology, Palampur, in the year 2000-2001 and 2001-2002, gladiolus was found to be infected, based on visual symptoms, with many viral diseases. They revealed symptoms like flower or leaf colour break, leaf mosaic, overall stunting and reduced flower and cormel production. The percent visual infection was ranged between 0-100 percent in 1819 plants of 32 cvs of Gladiolus. The maximum infected plants were observed in Princess Mary, Summer Sunshine Yellow, Bonier and American Beauty cvs and minimum in Bright Eye, Peter Pears, White Friendship, Top Brass, Apple Blossom, Friendship Pink, Gold Field, Jessica and Jester where percent infection was below 10%.

As per the plan of the work, emphasis was given on the diagnosis of the viruses. Using ELISA, gladiolus was found to be infected with BYMV, TSWV, TRV and TRSV. The percent infection ranged between 3-100%. The percent infection of these viruses was found to be for BYMV 100%, TSWV 15%, TRV 9% and TRSV 3%. All the 32 gladiolus cvs of were found infected with BYMV, whereas White Friendship, Yellow Supreme, Video, Oscar and Her Majesty cvs were found to be infected with TSWV also. TSWV was also recorded from the plants with chlorotic
degeneration symptom and without symptoms, TRV infection was found on Top Brass, Aldebaran and Oscar cvs, whereas Yellow Supreme cv was found to be infected with TRSV.

Using RT-PCR with specific primers, gladiolus was found to be infected with BYMV and PNRSV. Gladiolus cvs viz. Victor Borg, Jessica, Gold Field, Red Beauty, Oscar, Yellow Supreme, Hunting Song, Aldebaran, and Friendship were found to be infected with BYMV, whereas Jessica, Aldebaran, and Friendship were found to be infected with PNRSV. BYMV was also diagnosed by IC-RT-PCR.

*Cucumber mosaic virus*, another important virus infecting gladiolus was not found during present investigation using RT-PCR as well as by IC-RT-PCR. Using ‘Leaf Soak’ of infected *C. sativus* in RT-PCR, CMV was found positive but not in ‘Leaf Soak’ of infected gladiolus.

Diagnostic methods like ELISA, RT-PCR and IC-RT-PCR were evaluated for the detection of BYMV in different parts of gladiolus plants viz, leaves, stems, roots and corms. ELISA showed presence of BYMV in leaves and stems. RT-PCR using specific primer could amplify approximately 750-bp long fragment from leaves, stems and roots but not in corms, whereas IC-RT-PCR detected the BYMV in all parts of gladiolus.

Therefore, detection of virus from leaves and stems could be done by any of the used diagnostic method, whereas RT-PCR could able to detect the virus from roots, which was not detected by ELISA due to low concentration of virus. Detection from corms by ELISA and RT-PCR was failed either due to low concentration of virus or presence of some interfering substances in tissue, whereas IC-RT-PCR
could detect the virus from roots and corms, probably because trapping of antigen
by antibodies provides a template for amplification and washing steps removes the
interfering substance present in corm sample automatically. Therefore, IC-RT-PCR
was found to be the most suitable and sensitive test to detect the BYMV from
different parts of gladiolus plant in comparison to ELISA and RT-PCR.

For the characterization of virus at biochemical and molecular level, pure
culture of BYMV was developed on V. faba. Based on transmission studies, it was
concluded that virus could be transmitted mechanically as well as through aphids.

In host range studies, the virus developed systemic infection on P. sativum,
N. benthamiana, C. ambrisoides and P. vulgaris, whereas it showed vein clearing
on inoculated Raphanus spp., D. barbatus, L. sativa and mild mosaic on Petunia,
Nicotiana sp. and Trifolium sp.. On S. oleracea, mottling and stunting was observed
after inoculation while local lesions were developed on B. vulgaris, C. amaranticolor
and C. quinoa. C. sativus, Dhatura, L. esculentum, Vigna spp. and C. pepo were
found to be unsusceptible hosts along with Phlox spp., Valliriana spp. and Solanum
spp.

For the purification of virus, different parameters were standardised. Out of
two hosts, V. faba was found to be best suitable host to propagate the virus
because it produced high titre of virus after 3 weeks of inoculation. The best buffer
to extract maximum stable virus particles from the propagative host was found to be
0.5 M potassium phosphate of pH-7.5 along with 0.02 M sodium sulfite whereas
combination of carbon tetra chloride and chloroform was found to be the best
clarification solvent. The virus was successfully purified.
Purified virus was characterised through UV Absorption spectrum, Agar Gel Diffusion Test, Electron Microscopy, ISEM, DAS-ELISA, Dot ELISA. Molecular weight determination of coat protein, Western blotting, Determination of size and nature of nucleic acid, RT-PCR and through Cloning and sequencing of RT-PCR amplified product.

The purified virus showed absorption maxima at 260 nm wavelength and absorption ratio at $A_{260}/A_{280}$ was recorded to be 1.2. A sharpened defined precipitation line in Agar Gel Diffusion Test confirmed the specific reaction of antigen and antibody.

Electron Microscopy using purified virus preparation showed presence of flexuous virus particles measuring 750x 12 nm while excellent clumping of virion was clearly discernible with BYMV specific antibody in Immunosorbent Electron Microscopy.

Further Ultrastructural studies of V. faba (containing pure culture of virus) and gladiolus leaves (having infection of BYMV only) showed presence of cylindrical inclusions which comprised of pinwheels, laminated aggregates and proteinaceous crystals. In DAS-ELISA and Dot-ELISA, the purified virus reacted upto 1:500 and 1:640 dilutions, respectively, using BYMV specific antibodies. On analysing the purified virus on SDS-PAGE it showed the protein subunit of 34Kd as major viral band. When the protein was transferred to PVDF membrane and probed with BYMV specific antibodies, it showed the band of 34 KD.

Nucleic acid isolated from purified virus preparation, was found to be 10 Kb size and monopartite, on agarose gel electrophoresis. The nature of nucleic acid
was determined by treating with enzyme RNase, DNase and S1 Nuclease. It was found to be single stranded RNA. RT-PCR was conducted using this nucleic acid and BYMV specific primers. It amplified approximately 750 bp band of expected size. On cloning and sequencing of the amplified product, it revealed 98% homology to BYMV MB4 strain, 94% to BYMV S strain, 66% to Clover yellow vein virus, 62% to Lettuce mosaic virus, 60% to Plum pox virus, 59% to Papaya ringspot virus, 58% to Peanut stripe virus, 57% to Tobacco vein mottling virus and 56% to Johnson grass mosaic virus.

Variability studies were also conducted to find out the variable isolates of BYMV infecting gladiolus. When RT-PCR amplified product of different gladiolus cvs were digested with restriction enzyme Pvu II and Hind III separately as well as in combination, they produced different kind of restriction digestion pattern on agarose gel electrophoresis.

On digestion with Hind III, Victor Borg, Jessica, Gold Field, Red Beauty, Yellow Supreme, Hunting Song, Friendship and Aldebaran cvs produced three bands ie approximately 750bp, 530bp and 220bp, whereas Oscar showed only one undigested band.

On digestion with Pvu II, Victor Borg, Jessica, Red Beauty, Oscar, Yellow Supreme and Hunting Song cvs showed only two bands ie approximately 530bp and 220bp, while Gold Field, Friendship, Aldebaran showed 3 bands of approximatey 750bp, 530bp and 220bp indicating the variation of BYMV isolates with in gladiolus.
Summary and Conclusions

On digestion with Puv II and Hind III, Gold Field and Friendship cvs produced three major bands of 750bp, 530bp and 430 bp, while Victor Borg and Jessica produced only one major band of 430bp. Oscar produced only band of 530bp whereas Red Beauty, Yellow Supreme, Hunting Song and Aldebaran produced two major bands of 530bp and 430bp. This indicated four kinds of isolates, one without Puv II and Hind III sites, one with both the sites and others two having either Puv II site or Hind III site.

For confirming the two last isolates, cloning of PCR product was carried out in suitable vector and analysed by same set of restriction enzymes. It showed three types of clones-one without Puv II and Hind III sites, one with both the sites and one having only Puv II site. On sequencing of these clones, same pattern of restriction sites occurred. Therefore it revealed the presence of three types of isolates infecting gladiolus and there was a mixed infection of BYMV isolates with in a gladiolus cvs.

Characterization at biochemical and molecular level confirmed the presence of BYMV in purified preparation, so it was used to develop the diagnostic kit.

Antiserum was raised against BYMV in healthy New Zealand White rabbits by injecting antigen along with Freund’s complete or incomplete adjuvant through subcutaneous and intramuscular route, separately. On evaluation by TAS-ELISA, the titre of antibodies raised through subcutaneous route was found to be more (1:500-1000) in comparison to intramuscular route (1:500). Conjugate was prepared by linking the alkaline phosphatase enzyme with purified IgG, which reacted upto 1:1000-1500 in DAS-ELISA.
Summary and Conclusions

To check the activity of kit components and it was compared with standard kit, field trials were conducted using test kit and standard kit. Since kit was based on Polyclonal antibodies, field trials were conducted using indigenous (procured from different states of country) and imported gladiolus along with other ornamental (Iris, Crocus) and vegetable crops which may be infected by BYMV. Using test kit, 69% plants were found infected with BYMV whereas using standard kit, 64% plants were found infected. When the 5% plants, which were found negative by standard kit, subjected to RT-PCR, they were found positive for BYMV. Thus, kit prepared in present study was found to be more sensitive as compared to standard kit. Probably it has antibodies against the Indian isolates of gladiolus which makes more sensitive as compared to commercially available kit.

On analysing the components of kit at different temperature, it revealed that the activity remains same throughout the year at 4-10°C similar to the shelf-life of commercial kit, whereas at room temperature (20-37°C), they revealed positive result up to 2-3 months.

Purified antibodies were also conjugated with Biotin. Its titre was found to be 1:500 but it was found to be more specific than alkaline phosphatase.

For the use of diagnostic kit, virus-free plants were produced using tissue culture techniques. Small segments of cormels from gladiolus cvs viz. Frienship, Gold Field, Priscilla, Aldebaran, White Prosperity, Victor Borg, Peter Pears and Jessica were cultured in M&S medium containing 0.1mg/L NAA, 2.5 mg/L of Kinetin, 30g/L sucrose, 7g/L agar and antiviral chemicals viz, Ascien, Virazole and Zidovudine at conc. of 10 and 20mg/L separately. Explants of Victor Borg cv
responded better in the medium in comparison to other gladiolus cvs but number of
shoots/explant were developed more in Gold Field. The plantlets found positive for
BYMV by ELISA and RT-PCR, were further subcultured in the medium containing
antiviral chemicals.

Both Aescin and Virazole were found effective in virus elimination at the
concentration of 10 mg/L. After five subculturings, 85% plantlets were found
serologically negative in both the medium, whereas only 32% elimination occurred
in case of Zidovudine. Growth of plantlets was normal in the medium containing
Virazole (10mg/L), whereas Aescin in the medium (10mg/L) promoted the growth of
plantlets in terms of time, high chlorophyll content and thickness of plant parts. High
concentration of antiviral compounds (20mg/L) was found phytotoxic. Percentage of
virus-free plantlets was different in different gladiolus cvs. It was observed to be the
highest in Gold Field and lowest in Priscilla.

On transplantation in soil containing a mixture of soilrite: soil: FYM in the ratio
of 1:1:1, 80% virus-free plantlets were survived and they were hardened gradually
in the culture room followed by poly house.

These virus-free plantlets were also checked using test kit for the
certification of plants free of BYMV. These plantlets were found negative for BYMV
using the component of the kit.

CONCLUSIONS

Thus, the following conclusions could be achieved from the present study:

1) ELISA based indigenous diagnostic kit was developed for the detection of BYMV.
The kit prepared in present study was found to be more sensitive as compared to
standard kit. Probably it has antibodies against the Indian isolates of gladiolus, which makes it more sensitive as compared to commercially available kit.

2) Three isolates of BYMV were detected in gladiolus, which were found different at genomic level from each other as well as from other isolates reported in gene bank.

3) Different diagnostics were standardized to detect gladiolus viruses like
   - ELISA for the detection of BYMV, TRSV, TRV and TSWV.
   - PCR for the detection of BYMV, CMV and PNRSV.
   - IC-RT-PCR for the detection of BYMV.

4) Comparative evaluation of different diagnostics like ELISA, RT-PCR and IC-RT-PCR for detecting BYMV suggested that the IC-RT-PCR is the best suitable method to diagnose BYMV from all parts of gladiolus tissue.

5) BYMV-free plants were produced using tissue culture techniques with the use of diagnostic kit, which certified the plants free from BYMV.

6) For the development of BYMV-free plants, a plant extract, Aescin was used which was found better than other used synthetic compounds viz. Virazole Zidovudine.