

**DEVELOPMENT OF IMMUNO-DIAGNOSTIC KIT FOR
THE DETECTION OF BANANA BUNCHY TOP
VIRUS (BBTV)**

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**DEVELOPMENT OF IMMUNO-DIAGNOSTIC KIT FOR
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VIRUS (BBTV)**

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BY

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CERTIFICATE

This is to certify that the thesis entitled “DEVELOPMENT OF IMMUNO-DIAGNOSTIC KIT FOR THE DETECTION OF BANANA BUNCHY TOP VIRUS (BBTV)” submitted by Ms. NAMRATHA S. D., for the degree of MASTER OF SCIENCE (AGRICULTURE) in MOLECULAR BIOLOGY AND BIOTECHNOLOGY, to the University of Agricultural Sciences, Dharwad is a record of research work done by her during the period of her study in this university under my guidance and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

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LIST OF ABBREVIATIONS

Abbreviation	Description
ScFv	: Single chain fragment variable
PBS	: Phosphate buffered saline
PBST	: 0.05% (v/v) Tween- 20 in phosphate buffer saline
PLL	: Poly L lysine
APS	: Ammonium per sulphate
pNPP	: Para-Nitrophenylphosphate
SDS	: Sodium dodecyl sulphate
BSA	: Bovine serum albumin
DTT	: Dithiothritol
PEG	: Polyethylene glycol
TEMED	: Tri ethyl methyl diammine
X- gal	: 5-Bromo-4chloro-3- indo -D-galactopyranoside
IPTG	: Isopropyl B-D thiogalactopyranoside
MAb	: Monoclonal antibody
TMB	: Tetramethyl benzidine
ELISA	: Enzyme linked immunosorbent assay
pVIII	: M13 Phage major coat protein VIII
pIII	: M13 Phage minor coat protein III
PVDF	: polyvinylidene fluoride
NC	: Nitro Cellulose
TBS	: Tris Buffer Saline

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1. INTRODUCTION

Banana (*Musa* spp.) belongs to the family *Musaceae* and is one of the globally important fruit crops grown in more than 125 countries with production of 99.99 MT (faostat.fao.org). India is the largest producer of banana with total annual production of 28.45 MT (nhb.gov.in/area, production, html). Due to intensification of banana cultivation in the recent years, an increased number of pests and diseases causing significant reduction in yield have been reported. Diseases are caused by various agents like fungi, virus, bacteria and nematodes. The viral diseases have been causing considerable damage to banana production due to their systemic invasion and common spread through vegetative propagated planting materials and their subsequent transmission in nature by insect vectors (Geering., 2007). Banana productivity is generally reduced by viral diseases; the most deleterious disease which lemmatizes banana production is banana bunchy top nanovirus (BBTV), the causal agent of Banana Bunchy Top Disease (BBTD) (Roba *et al.*, 2011). Banana Bunchy Top Virus (BBTV) is the most economically important virus infecting banana the world over (Dale, 1987). Banana viruses are primarily transmitted through vegetative planting materials such as suckers and have direct effect on production by decreasing plant growth and yield (Vishnoi *et al.*, 2009).

In order to manage the disease, virus free suckers should be planted to establish new orchard, aphids should be suitably managed and quarantine regulatory measures, screening and certification of suckers should be strictly followed. To achieve this, early disease diagnosis of BBTV in planting material is required. The molecular approaches such as Polymerase Chain Reaction (PCR) and DNA hybridization have been used to diagnose the disease but immunological methods are very convenient and have the potential to be developed into a routine method for the detection of viral diseases in plants and animals and also economically. Immuno based diagnostic methods against any pathogen is simple, rapid and user friendly and also does not require sophisticated instruments.

The immunological method includes the production of monoclonal antibody against an antigen that can be done by hybridoma technology which involves immunization and sacrificing animal. The isolation of spleen cells and subsequent mixing with myeloma cells for the continuous production of antibodies. The spleen cell isolation is time consuming, laborious and each time requires immunizing an animal. Therefore, the emerging techniques called phage display techniques was developed (George Smith., 1985). Phage circumvents the need for production of large scale cell culture by generating large natural display library from V gene repertoires. It is even possible to completely bypass both immunization and hybridoma development and isolate antibodies with high affinity against any antigens (Marks *et al.*, 1991). This technique produces antibody quickly in bacterial culture and facilitate genetic manipulation of their structure and improve their performance. Since the antibodies can be generated *de novo*, phage display techniques does not require sacrificing any animals.

A phage display technique mainly employs filamentous phages such as M13, fd and f1. The M13 filamentous phage is cylindrical rod shaped and circular single stranded DNA about 6407 nucleotides long consists of eleven genes. Among these, gene VIII is major coat protein (2700 copies) and gene III is minor coat protein (3-5 copies). The technology employs fusion of desired gene with one of the coat protein gene. The technology makes use of comprehensive library of amplified immunoglobulin genes or gene fragments that were cloned into phage expression vector, where expressed antibody was fused to M13 capsid protein (gIIIp), so that the antibody is displayed on phage surface. This phage library is screened for its ability to bind to an antigen of interest, which is artificially mounted on solid support. Phage expressing specific antibody against given antigen is then selected and enriched by several rounds of biopanning. These engineered antibodies make them suitable for developing diagnostic kits.

The ELISA is a highly specific and sensitive serological techniques introduced for identification of plant viruses (Hoes *et al.*, 1994). The ELISA technique is based on principle in which the viral antigens are recognized by their specific antibodies (IgG) in association with colorimetric properties. The principle of ELISA technique consists of detecting the antigen-antibody interactions by enzyme induced colour reaction rather than by observing their precipitation. A technique called Immune Capture Polymerase Chain Reaction (IC-PCR), which combines the technical advantages of PCR with the practical advantages of serology was developed for the detection of several different plant viruses (Nolasco *et al.*, 1993).

Enzyme-Linked ImmunoSorbent Assay (ELISA) tests with monoclonal antibodies (mAbs) are commonly used for the accurate detection of ELISA is convenient but limited in detecting sensitivity, especially with very low concentrations of BBTv. Therefore, more sensitive assay based on western blot and Dot Blotting Immuno Assays has been developed. In addition to the detection of BBTv, the PCR based assay had been used for the identification of various BBTv strains (Abdelkader *et al.*, 2004). Prokaryotic (*E. coli*) expression of plant viral antigen provides several advantages in antigen preparation over the traditional methods and the recombinant antigen has utilized to develop immunodiagnostics of several plant viruses (Vijayanandraj *et al.*, 2013).

The recombinant single-chain variable fragment (scFv) antibodies offer several advantages over mouse monoclonal antibodies (mAbs) for use in biotechnological applications due to their availability for genetic manipulation, which allows fusion of effectors protein such as fluorescent proteins or toxins. Antibody libraries, with their wide range of antigen-binding specificities, have increasingly been a source of specific recombinant antibody fragments via phage display technology. However, there remains great interest in cloning and sequencing the functional VH and VL domains of a hybridoma derived monoclonal antibody (mAb) in order to determine their contribution to antigen binding. Isolation of functional variable domains is also valuable for the rescue of unstable and/or valuable hybridoma cell lines that otherwise could be lost during long-term storage.

Unfortunately, genetic PCR amplification of functional VH and VL genes from hybridoma cell lines is somewhat complicated due to the presence of aberrant mRNAs from rearranged and nonfunctional heavy and light chain genes. It is highly likely that these nonfunctional VH and VL genes are more abundantly amplified than their functional counterparts due to the use of degenerate PCR primer sets, or further by mismatches and PCR errors that generate nonsense point mutations and out-of-frame clones. Therefore, it is critical to confirm the binding specificity of the recombinant antibody protein, whose gene is cloned into an expression vector, to determine how it compares with that of the parental monoclonal antibody. This was accomplished by utilizing an experimental approach with high selection power for the identification of *E. coli* clones that express functional scFv or Fab fragments. So far, phage display technology and colony lift assays have been successfully applied. Nonetheless, the phage display technology suffers from technical complications that constrain its routine use, whereas colony lift assays are somewhat problematic due to a high frequency of false positives. To develop a simple, straight forward methodology that quickly identifies functional antibody variable genes from hybridomas, utilization of scFv-phoA construct generates an immuno-detective tool (Lee *et al.*, 2009)

Although based on the Western blot principle, the Rapid Dot Immunoblotting Assay (DIBA) requires a simple and easier method to prepare and apply the samples on nitrocellulose or nylon membranes (Almeida *et al.*, 2001, Astier *et al.*, 2007). Based on this principle the capture immunoassay is one of the robotics, simple, rapid and user friendly field level diagnostic assays.

The resistance to the BBTv can be produced by scFv genes which produce antibodies against the crude protein of an antigen. Through Phage display techniques several scFv antibody clones were produced against BBTv. The monoclonal ELISA reveals that the two clones pBSNMAB5 and pBSNMAB40 had shown highest affinity for BBTv-CP (Shilpa *et al.*, 2013).

The present investigation has been initiated to detect BBTv by utilizing scFv antibodies in RapiDot Immuno Assays.

Objectives

1. To express and characterize scFvbbtv antibodies (pBSNMAB5 and pBSNMAB40)
2. Standardization and development of immunoassays for Banana Bunchy Top Virus.
3. Validation of RapiDot Immuno assays against field infected samples from different locations

2. REVIEW OF LITERATURE

Banana is one of the oldest tropical fruit crops cultivated by man from prehistoric times with great socio-economic significance. Globally, the banana (*Musa* spp.) ranks fourth after rice, wheat and maize in consumption. Banana is grown in about 121 countries (Anon., 2011) providing major source of carbohydrates for over 400 million people in tropical countries and the most important fruit crops in world in terms of production and consumption (Bairu *et al.*, 2006). Over 97 MT were produced globally in 2009 from extent of 4.92 million ha (Anon., 2011).

Banana is one of the most important fruit crops in India and major source of income for small holders. India ranked world first producer with total annual production of 26.99 million tonnes with 27.72 per cent of global production from 0.75 million ha (Anon., 2011), it contributed 31 per cent of the total food production in India. The major banana growing states of India are Maharashtra, Tamil Nadu, Karnataka, Andhra Pradesh, Assam, Bihar and Uttar Pradesh. Though banana cultivating area is being increased continuously due to the high returns, banana viral diseases are also increased to be the main constraint of banana cultivation.

2.1 Banana bunchy top virus

BBTV is an important pathogen of banana causing significant losses to banana industry in Australia, South Pacific, Egypt and Asian countries (Sadik *et al.*, 1997). The disease was first recognized in Fiji in 1889 (Stover, 1972), subsequently in Taiwan in 1890, in Egypt in 1901 (Fahmy *et al.*, 1927) and in Australia in 1913. The spread of disease caused devastating problems in many countries including Fiji, India, Egypt, The Philippines, Taiwan and China (Amin *et al.*, 2008). In USA, the BBTV was first detected on the island of Oahu, Hawaii, which is major constraint to banana production in the Philippines (Molina *et al.*, 2007) and Iran (Bananej *et al.*, 2007). The banana bunchy top viral disease has established itself in half of the banana-growing regions of sub-Saharan Africa worsening food insecurity for 30 million people on the continent whose primary source of food is banana.

In India, BBTV was first reported in Kerala in 1943, later it has been reported from many banana growing regions. Now the disease has been found even in the deep forest of North Eastern hill regions. The hill banana of lower plane hills has been wiped out due to BBTV (Das, 2007).

2.1.1 Molecular properties and genomic organization of BBTV

BBTV especially has multi-component genome consisting of at least six ssDNA components each about 1 kb size (Burns *et al.*, 1995; Karan *et al.*, 1997). Harding *et al.* (1993) has cloned and sequenced first DNA component from an Australian isolate of BBTV (DNA-Aus1) and Burns *et al.* (1994) has subsequently reported that the genome of BBTV contain more than one component. Later on Burns *et al.* (1995) demonstrated that BBTV has

multi-component genomes consisting of at least six components of circular ssDNA size ranging from 1018 and 1111 nucleotide encapsulated as single-stranded coding sense DNA which is mainly originating from Pacific region. These components (DNA-1 to DNA-6) are proposed to be integral part of the BBTV genome (Karan *et al.*, 1994; Hafner *et al.*, 1997; Karan *et al.*, 1997) as they have been consistently associated with all viral isolates tested (Bell *et al.*, 2002). Each component contains a single ORF in the virion sense except for DNA-1 which encodes two virion sense gene products; the master replication protein (Rep), capable of initiating replication of integral BBTV components (Horser *et al.*, 2001) with an unknown regulatory function (Beetham *et al.*, 1997). These components are known as DNA-R (encoding a rolling-circle replication initiator protein [Rep]), DNA-S (encoding the coat protein [CP]), DNA-C (encoding the cell-cycle link protein [Clink]), DNA-M (encoding the movement protein [MP]), DNA-N (encoding the nuclear shuttle protein [NSP]) and DNA-U3 (the function of which remains to be determined) (Burns *et al.*, 1995; Karan *et al.*, 1997). With the exception of DNA-R, all these components encode only a single gene in the virion-sense that is transcribed during infection and these are responsible for its replication, multiplication and virulence (Beetham *et al.*, 1999).

Two genes associated with BBTV DNA-1 have been shown through RNA transcript analysis. Two mRNAs are transcribed from DNA-R, one mRNA is mapping to the Rep and the other to a small open reading frame (ORF) internal to the major ORF (Beetham *et al.*, 1997). The major gene of BBTV is DNA-1 encodes a putative replication protein (Rep) based on a dNTP binding motif GGEGKT (Harding *et al.*, 1993). The second gene is within and in the +2 coding frame of the major gene. The function of the product of this internal ORF (ORF I) has not been determined. The BBTV DNA-3, 4, 5 and 6 have major ORFs in the virion sense that are associated with conserved TATA boxes and polyadenylation signals. These four ORFs potentially encode proteins of 20.11 kDa, 13.74 kDa, 18.97 kDa and 17.4 kDa respectively (Burns *et al.*, 1995). Wanitchakorn *et al.* (1997) has demonstrated that the BBTV DNA-3 encodes the viral coat protein of 19.6 kDa. Although BBTV DNA-2 has nine ORFs, none of have appropriately located TATA boxes and polyadenylation signal (Burns *et al.*, 1995).

Shimaa *et al.* (2012) showed that the component 1 of BBTV-DNA-R consists of 1108 nts and contains a sequence of 69 nts representing the CR-SL of 31 nts. A CR-M (90 nts) at the position (972–1062) characterized with GC-rich sequence from nts 76 to 90 (average of 80% G +C) was found. Large ORF of 861 nts at position 102 to 962 in the virion sense were detected. The predicted protein of this ORF consisted of 286 amino acids and had a molecular weight of 33.8 kDa. The DNA-phylogenetic analysis showed a percent identity of 98.0 and 97.9 between BBTV DNA-R and isolates of Pakistan (isolate TJ1) and Australia (isolate V1), respectively.

2.2 Molecular characterization of BBTV

The genome of BBTV comprises of six circular single stranded DNA components of approximately 1.1kb in size referred to as DNA-R (encoding the replication initiation protein), DNA-U3 (the function of potentially encoded protein remains unknown), DNA-S (encoding the capsid protein), DNA-M (encoding movement protein), DNA-C (encoding cell cycle link protein) and DNA-N (encoding nuclear shuttle protein); Vetten *et al.*, 2005). These DNAs encode single open reading frame (ORF) in virion sense except DNA-R which has an additional small internal ORF. The function of this internal ORF has not been determined. Each component of BBTV shares common genome organization and has a conserved major common region (CR-M), a stem-loop common region (CR-SL), a potential TATA box and a polyadenylation signal associated with each ORF (Burns *et al.*, 1995; Beetham *et al.*, 1997).

Vishnoi *et al.* (2009) cloned, sequenced and characterized the full length of six genomic DNA components of Indian (Lucknow) isolate of BBTV. Analysis of sequence data revealed that the presence of six full-length components of BBTV: DNA-R (1111 bp), DNA-U3 (1060 bp), DNA-S (1075 bp), DNA-M (1048 bp), DNA-C (1018 bp) and DNA-N (1096 bp). The comparisons of sequence data of the six DNA components of the BBTV Lucknow isolate revealed the highest identities with sequences of other BBTV isolates from the South Pacific group: [DNA-R (98%), DNA-U3 (93%), DNA-S (100%), DNA-M (98%), DNA-C (97%) and DNA-N (99%)]. A phylogenetic analysis revealed a close relationship of the Lucknow isolate with BBTV isolates of South Pacific group rather than those of the Asian group. Similarly, Amin *et al.* (2008) characterized the Pakistan (Sindh) isolate of BBTV by analyzing the sequences of five DNA components and indicated that the BBTV present in Pakistan belongs to the South Pacific group of isolates. The virus showed the highest levels of sequence identity to BBTV isolates originating from Egypt, India and Australia.

Burns *et al.* (1995) cloned, sequenced and analysed DNA-2, 3, 4, 5 and 6 components of Australian (South Queensland) isolate of BBTV. They reported that all the five components were present in all BBTV infections tested and identified one large ORF in the virion sense located 3' of a stem-loop structure in components 3, 4, 5 and 6. These ORFs had a potential TATA box and one or two potential polyadenylation signals associated with it and each polyadenylation signal had an associated GC-rich region containing the trinucleotides sequence TTG. Even though, a number of ORFs were identified in component 2, none of these had appropriately located potential TATA boxes and polyadenylation signals associated with them. An Indonesian BBTV isolate was characterized based on DNA1 and DNA3 components (Furuya *et al.*, 2005). CR-M of DNA1 showed higher homology with isolates from Japan, Taiwan, Philipines, China and Vietnam than with isolates from Australia and Fiji (93 to 95 per cent and 63 to 65 per cent respectively).

Bell *et al.* (2002) first characterized an additional Rep like component from BBTv isolates from Vietnam. This component designated BBTv-S3 shared less than 70 per cent similarity at the nucleotide level with the other previously described Rep-like components BBTv-S1, S2 and Y1 from Taiwan indicating BBTv-S3 was a newly described Rep-like component associated with some isolates of BBTv in Vietnam. Similarly, three additional Rep-like components have been associated with BBTv isolates in Vietnam (Horser *et al.*, 2001b; Wu *et al.*, 1994; Yeh *et al.*, 1994). The detection of BBTv-S3 in only half of the BBTv infected samples were analyzed and suggested that it was not an integral component of the BBTv genome with a characteristic of other *nanovirus* Rep-like components described previously (Boevink *et al.*, 1995; Timchenko *et al.*, 1999). Further, all BBTv-infected plants collected and displayed characteristic BBTv disease symptoms irrespective of whether BBTv S3 was detected in them and suggesting that the BBTv S3 did not modulate symptom expression.

2.3 Molecular and serological detection of BBTv

Several methods were successfully used for detection of BBTv including serological and molecular techniques (Soweha, 2005). Enzyme-Linked Immunosorbent Assay (ELISA) tests with monoclonal antibodies (MAbs) were commonly used for the accurate detection of BBTv (Wu and Su, 1990; Dietzgen and Thomas 1991; Geering and Thomas, 1996). The BBTv was detected by using Double Antibody Sandwich Enzyme Linked ImmunoSorbent Assay (DAS-ELISA) and Dot-Blot ImmunoAssay (Geering and Thomas, 1996). The virus was successfully detected in BBTv infected and symptomless banana plants. ELISA is convenient but limited in detecting sensitivity especially with very low concentrations of BBTv. Therefore, more sensitive assay based on Polymerase Chain Reaction (PCR) has been developed (Xie and Hu, 1995; Hafner *et al.*, 1997). Generally, PCR tests are more sensitive than ELISA tests (Sadik *et al.*, 1997) and better suited to plant tissue that is likely to have lower numbers of pathogens such as that developed during tissue culture (Abdel-Hamid *et al.*, 2003). The PCR was successfully used for detecting several plant viruses (Wetzel *et al.*, 1992) and BBTv (Xie and Hu, 1995; Harding *et al.*, 2000). One of the most sensitive molecular methods for pathogen detection currently available is PCR (Abdel-Hamid *et al.*, 2003). Two oligo nucleotides were used for amplification of fragment with size of about 1000 bp from the BBTv DNA-1 (Harding *et al.*, 2000; Abdel-Hamid *et al.*, 2003). In addition to the detection of BBTv, the PCR based assay had used for the identification of various BBTv strains (Abdelkader *et al.*, 2004). The BBTv was detected in DNA extracted from the viruliferous banana aphid (*Pentalonia nigronervosa coquerelli*) as low as 10^{-1} and 10^{-2} dilutions through PCR (Abdel-Hamid *et al.*, 2003). Wetzel *et al.* (1992) was reported that the ImmunoCapture-PCR (IC-PCR) technique is combination of ELISA and PCR techniques that allows for the detection of low concentrations of viruses in oxidizing plant extracts. Shamloul *et al.* (1999) were utilized three pairs of primers for IC-PCR and PCR for detection of BBTv in BBTv infected banana leaves or tissue culture or viruliferous aphids. Two different methods such as Indirect-Enzyme Linked ImmunoSorbent Assay (I-ELISA) and IC-PCR were used for studying the presence of BBTv in viral-infected and non-infected banana plants (Soweha, 2005). In addition, the

presence of BBTv in different parts of banana plant was also studied. IC-PCR technology was found to be more sensitive than I-ELISA for BBTv detection. IC-PCR using Agdia® coating antibody appeared as the most sensitive assay detecting 0.003 mg of BBTv particles in semi-purified preparations while ELISA and PCR detection limits were 2 mg and 0.03 mg respectively (Anceau *et al.*, 1996).

A new protocol was developed by Busogoro *et al.* (2007) for the molecular detection of BBTv in banana. PhytoPASS abrasive membrane was tissue printed with BBTv infected sample. BBTv specific DNA fragment was amplified using BBTv specific primers without purified DNA or the immunocapture step preceding the PCR reaction.

2.3.1 Rapid PCR based molecular methods for detection of BBTv

Sensitive and reliable methods are needed in certification programs for detection and characterization of plant pathogens. Serological methods (Enzyme-Linked ImmunoSorbent Assay) are routinely employed for these purposes because they allow sensitive, specific and simultaneous analysis of many samples in a single micro plate or membrane (Garnsey and Cambra, 1991). However, molecular methods such as Polymerase Chain Reaction is more sensitive compared to serological techniques and facilitate the analysis of sequence data. Different molecular methods based on PCR amplification of the nucleic acids enable greater sensitivity (Bariana *et al.*, 1994; Olmos *et al.*, 1996; Wetzel *et al.*, 1992). Availability of rapid, sensitive virus indexing techniques at cheaper rate encourage tissue culture production units as well as commercial banana growers for evaluation of their banana plants on periodic basis as precautionary measures against transmission of virus through planting materials and re-infection in the field. Highly sensitive detection technique is required as BBTv is phloem-limited virus and used to occur in low concentrations in infected banana plants (Harding *et al.*, 1991). Further, the early detection of BBTv in the nonsymptom young plants plays an important role in its control (Harding *et al.*, 2000). Soweha (2005) demonstrated the PCR as suitable technology to detect BBTv in both infected plants showing characteristics symptoms and in nonsymptom plants of Egyptian banana cultivars.

A simple, reliable, and high-throughput method for detecting BBTv in plants and aphids has been developed, (Chen *et al.*, 2013) which involves tissues disruption from banana plants and viruliferous aphids followed by real-time TaqMan®PCR. Extraction of BBTv single-stranded DNA using this method is simple and less prone to contamination than using the CTAB (hexadecyl trimethyl ammonium bromide) method. The high throughput TaqMan®PCR system was highly sensitive, detecting as few as 2.76 copies of BBTv genomic DNA or 1.0 ng–1.0 mg of infected banana leaves. The entire assay could be completed within 2 h. Regression analysis showed that the quantitative results of TaqMan®PCR and copies of the virus have good correlation for plasmids ($R^2 = 0.966$) and for infected leaves ($R^2 = 0.979$). This quantifies BBTv in aphids and plants, even before the appearance of symptoms of banana bunchy top disease.

2.3.1.1 Effect of inhibitors in template DNA on PCR amplification

PCR inhibitors in DNA preparations (templates) hinder the sensitivity and reliability of the PCR technique leading to false negatives (Bertolini *et al.*, 2003). Factors that inhibit the amplification of nucleic acids by PCR are present with target DNAs from many sources. The inhibitors generally can inhibit nucleic acid capture and polymerase activity for amplification of target DNA. The PCR reaction inhibition may be total or partial and can manifest itself as complete reaction failure or as reduced sensitivity of detection (Su *et al.*, 2003). In some cases, inhibition may be the cause of false-negative reactions. Thus, Incorporation of internal controls into each tube can monitor the success of PCR reaction. Although wide range of inhibitors was reported, the identities and modes of action of many remain unclear. A 1:10 dilution of the processed specimen has been used by some investigators. The study found that the dilution was reduced inhibition of PCR for cervical specimens from 19 per cent to 9.3 per cent (Verkooyen *et al.*, 1996). Thus, more sensitive detection can be achieved by dilution of DNA which in turn dilutes the inhibitors to the desirable level to facilitate the amplification. Further, high concentration of non-target DNA could inhibit PCR. In case of detection of plant viruses through PCR, host plant genomic DNA act as nontarget DNA which is available in high concentration in DNA preparations compared to the target viral DNA. Sadik *et al.* (1999) reported that the PCR was useful technique in detecting the BBTv in different purification steps.

The sensitivity test in PCR (Ramasamy *et al.*, 2015) showed that this assay could detect 0.1 pg/μl plasmid DNA which is equivalent to 1×10^4 copies. Virus could be detected using SEP from freeze dried as well as CaCl₂ dried samples of BBTv infected banana leaves. This methodology provides quality PCR product for direct sequencing suitable for identification and characterization of BBTv.

2.4 Protein preparation

Protein expression is subcomponent of gene expression, *E. coli* is one of the most widely used hosts for the production of heterologous protein and its genetics are well understood than those of any other microorganism. The Gram-negative bacterium remains one of the most attractive because of its ability to grow rapidly and at high density on inexpensive substrates, simple life cycle, well-characterized genetics and the availability of an increasingly large number of cloning vectors and mutant host strains. Although, there is no guarantee that recombinant gene product will accumulate in *E. coli* at high levels in full-length and biologically active form. Expression of recombinant protein can be approached in general by constructing a plasmid that encodes the desired protein, introducing the plasmid into the required host cell, growing the host cells and inducing protein expression and then lysing the cells, purifying the protein and performing Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) to verify the presence of the protein. This technique used to resolve the proteins on Polyacrylamide gel. It is powerful technique which exploits differences in molecular size to resolve proteins differing by as little as 1% in their electrophoretic mobility through the gel matrix (John and Mike, 2003).

The recombinant gene technology offers powerful tools for expressing the foreign gene in unrelated organisms. From many years *E. coli* was being used as host for the expression of target gene. Nowadays, pET expression systems gaining lot of importance in expressing target gene in host cells by positioning the target gene under suitable promoter. That is under the control of *lac* derived regulatory elements (Grossman *et al.*, 1998). The development of expression vectors designed to provide various fusion tags to the expressed protein have made recombinant protein purification much easier. The expression vectors that provide polyhistidine tags are widely used for rapid purification of the expressed protein of interest.

Metal chelate or immobilized metal affinity chromatography (IMAC) is powerful technique for isolating recombinant fusion proteins under mild conditions (Linder *et al.*, 1992; Porath *et al.*, 1992). A metal chelating group immobilized on chromatographic medium binds multivalent metal ion (usually Cu^{2+} , Ni^{2+} , Zn^{2+} , or Co^{2+}) in a way that leaves some coordination sites free for selective interaction with proteins. Typically, 5-6 histidine residues are added to the C or N terminus of target protein using recombinant techniques. The tag specifically interacts with the chelated metal ions, thereby holding these proteins on the medium other components bind weakly or not at all. Elution of the fusion protein is usually performed by increasing the concentration of competitive eluting agent such as imidazole. This enables rapid purification of poly histidine tag fusion protein from bacterial lysate.

2.5 Production of monoclonal antibodies

The use of monoclonal antibodies in immunodiagnostic kits and assays has already been well established. However, the development of highly sensitive and specific monoclonal antibody-based detection systems can be achieved by two main techniques *viz.*, Hybridoma technology and phage display technology. These techniques could be used for the production of monoclonal antibodies against any antigens.

2.5.1 Hybridoma technology

This technology involves immunizing the mice with specific antigen and then spleens cells are collected from hosts with the highest titers. The separated spleen cells cannot produce immunoglobulin on their own. These are mixed with myeloma cells which does not interfere the production of antibody at the ratio of 10:1 in the presence of Poly Ethylene Glycol (PEG) to change the membrane permeability and allow the cell fusion. The cell fusion is random; the cell culture contains mixture of myeloma-spleen cell fusion, myeloma-myeloma fusion, spleen-spleen cell fusion, single myeloma cells and single spleen cells. Selection for only myeloma-spleen cell fusion is accomplished by culturing the cell mixture in hypoxanthineaminopterin-thymidine (HAT) medium. Aminopterin is folic acid analog that blocks the *de novo* biosynthesis of purines and pyrimidines vital for DNA synthesis. Myeloma cells used in hybridoma production lack the enzyme hypoxanthine-guanine phosphoribosyl

transferase (HGPRT2), so they cannot use the exogenous hypoxanthine to synthesize purines by the salvage pathway. Aminopterin also blocks their endogenous synthesis of purines and pyrimidines. In HAT medium, the myeloma–myeloma cells and free myeloma cells die in the first week. Although single spleen cell and spleen–spleen cell fusion express HGPRT. Myeloma cells (HGPRT2) that have fused with HGPRT+ spleen cells, now have the enzyme then grow in HAT medium. About 500 hybrids are formed per mouse spleen and 20 to 30 of them produce specific antibodies. The correct antibody producing hybridomas are then identified by enzyme assay method (Kohler and Milstein, 1975). However, this hybridoma technology has its own limitation such as, time consuming, labour intensive, need to sacrifice the animals, clone produced by the hybridomas are genetically unstable and sometimes they result in less or no expression of antibody gene. It does not provide the facility to create the mutation in monoclonal antibody (mAb) for the improvement in binding efficiency. These problems were overcome by phage display techniques.

2.5.2 Phage display technology

The word itself stands for display of foreign peptides on phage surface; it was first discovered by Smith in 1985. Recently, the use of display vector and *in vitro* selection technologies has transformed the way in which to generate ligands. Using these techniques, now able to design repertoires of ligands and use the power of phage display techniques to select those ligands having the desired (biological) properties. The phage display, tailor made antibodies may be synthesized and selected to acquire the desired affinity of binding and specificity for *in vitro* and *in vivo* diagnosis or for immunotherapy of diseases (Smith *et al.*, 1985). The gene encoding protein is cloned into the genome of phage along with one of the coat protein which allows the display of foreign peptide on surface of the phage and target to any specific protein. By use of this technique, one can construct the phage library of protein which allows the easy screening of library than any other methods. Mainly phage display method practiced by filamentous phage (M13, f1 and fd), which has single stranded DNA. They infect only male specific *E. coli* strains that contain F' factor (Marvin *et al.*, 1969). The genome of M13 (or fd) is closed circular DNA molecule of 6.4kb and 90 per cent of which codes for the ten genes essential for the completion of life cycle (Van *et al.*, 1980). The phage particles look like long flexible tube (Berkowitz and Day, 1976) with 1.5-2 nm thick shell and diameter of about 6.5 nm (Glucksman *et al.*, 1992). The major coat proteins, gVIIIp (product of the gene VIII) is present about 2,700 copies per phage particle. Each phage particle also contains four minor proteins, the products of genes are pIII, pVI, pVII and pIX present in 3-5 copies each. Recently, vector based filamentous phages have been developed for displaying the proteins and peptides fused to phage coat proteins on the phage surface and are being used as genetic tools for selecting high affinity variants of engineered proteins / peptides (Van Antwerp and Wittrup, 2000).

According to Larocca and Andrew (2001) four types of selection have been evaluated over the past few years: 1. Affinity selection against simple targets such as immobilized proteins, 2. Affinity selection against complex targets such as the cell surface, 3. Selection after phage processing such as their internalization by cells and 4. Functional selection such as phage mediated gene transfer. Phage display technology involves the display of proteins or peptides as coat protein fusions on the surface of phage or phagemid particles with helper phage, which is essential for the replication and assembly of phagemid particles during library production and biopanning. 'Fusion phages are filamentous Bacteriophage vectors in which foreign DNA sequences are cloned with phage gene III and displayed along with the gene III protein (gIIIp) at one tip of the virion. Fusion phage, where displayed protein binds to ligands can be selected from nonbinding phage by the process called biopanning. A filamentous phage library displaying vast repertoire of synthetic single chain fragment variable (scFv) antibody was subjected to affinity selection on purified BlueTongue Virus (BTV) particles. After four rounds of selection and amplification, 73 out of 90 fusion phage clones tested were found to bind purified BTV in ELISA (Van Wyngardt and Du Plessis, 1998). This recently emerged technology has played an important role in enzymology mainly in creating variation in enzyme and improving their efficiency and selection. Wirsching *et al.* (2003) has reported on generated variants of hirudin, a thrombin specific inhibitor, which increased the protease resistance that may be useful for haematological disorder.

2.5.2.1 Antibody structure and function

The structural unit of most mammalian antibodies are glycoprotein (MW~150,000 daltons) composed of four polypeptide chains secreted by specialized B lymphocytes known as plasma cells. The basic structural molecule of an antibody consists of "Y" shaped structure and composed of two light chains and two heavy chains, which are connected by disulfide bonds. Each light chain has molecular weight of 25,000 daltons and composed of two domains are variable domain (VL) and constant domain (CL). There are two types of light chains: lambda (l) and kappa (k). In humans, 60 per cent of the light chains are k and 40 per cent are l chains, whereas in mice 95 per cent of the light chains are k and only 5 per cent are l. A single antibody molecule contains either k light chains or l light chains, but never both. Each heavy chain has molecular weight of 50,000 daltons, they have constant and variable region. The heavy and light chain contains a number of homologous regions consisting of similar amino acid sequences but not identical groups. These homologous region of about 110 amino acids long is called as called immunoglobulin domains. The heavy chain contains one variable domain (VH) and either three or four constant domains such as CH1, CH2, CH3, and CH4 depending on the antibody class or isotype. The region between the CH1 and CH2 domains are called the hinge region and permits flexibility between the two Fab arms of the Y-shaped antibody molecule which allows them to open and close to accommodate binding to two antigenic determinants separated by fixed distance. The heavy chain also serves to determine the functional activity of the antibody molecule. In mammals, there are five antibody classes -IgG, IgA, IgM, IgE and IgD. And are distinguished by their heavy chains g,

a, m, e and d respectively. The IgD, IgE and IgG antibody classes are made up of single structural unit, whereas IgA antibodies may contain either one or two units and IgM antibodies consist of five disulfide-linked structural units. IgG antibodies are further divided into four subclasses often referred to as isotypes due to polymorphism in the Fc region within the C domain (Janeway *et al.*, 2001).

The chemical structure of antibodies explains three functions of antibodies: 1. Binding versatility 2. Binding specificity and 3. Biological activity. A mammal is capable of responding to more than 100 million antigenic determinants and can even respond to artificial antigens that do not exist in nature. Because the amino acid sequence differs in the arms of various antibody molecules, each different antibody can bind specifically to one unique epitope. Thus, the arms of an antibody molecule confer the versatility and specificity of responses that the host can mount against antigens. The stem region of an antibody molecule decides its biological activity and defines whether the response against particular antigen will lead to complement-mediated lysis, enhanced phagocytosis or allergy. The antigen binding activity of the antibody molecule is determined by the conformation of its amino acids in the Complementarity Determining Regions (CDR), which is present in the variable regions of both the light and heavy chains of the antibody (Smith *et al.*, 2005). The ability of antibodies to selectively bind to specific epitope present on chemical, carbohydrate and protein or nucleic acid has been thoroughly exploited as therapeutic tool to treat diseases as well as to modulate physiological responses.

2.5.2.2 Phage display libraries

Smith (1985) originally described the phage display technique. He presented the use of the nonlytic filamentous bacteriophages for the display of specific binding peptides on the phage coat. This powerful methodology was further enhanced by Winter *et al.* (1994), who demonstrated the display of functional folded antibody fragments on the phage surface. Clackson *et al.* (1991) used fd phage to display antibody fragments fused to minor coat protein using a random combinatorial library of the rearranged heavy (VH) and Kappa (VK) light chains from mice immune to the Hapten 2-phenyloxazole-5-one (phox). They have displayed diverse libraries of antibody fragments on the surface of fd phage.

Vaughan *et al.* (1996) constructed large non-immunized phage display library to generate high affinity human antibodies to any given antigen. They used functional Vgene segments from 43 nonimmunized human donors to construct a repertoire of 1.4×10^{10} single chain Fv fragments displayed on the surface of phage. They cloned the fragments into phagemid vector, enabling both phage displayed and soluble scFv to be produced without sub cloning. They also incorporated hexahistidine tag for rapid purification of scFv by nickel chelate chromatography. Hyland *et al.* (2003) generated scFv antibody library from cDNA derived from the spleens of mice immunized with recombinant human Epidermal Growth Factor Receptor (EGFR) kinase domain. ScFvs in this library consist of an N-terminal light chain variable domain and C-terminal heavy chain variable domain linked by 7 or 15 amino

acid flexible linker. The diversity of this library was more than 2×10^8 independent clones. Hashiguchi *et al.* (2003) constructed phage antibody libraries from peripheral blood lymphocyte cDNA from 20 healthy volunteers. The V genes for the r, k or l chain was separately amplified by PCR using appropriate V gene family, based on reverse and forward primers. The PCR products of VH and VL gene segments were assembled by PCR, with a linker DNA that encoded (Gly4Ser3) peptide to construct scFv gene. The purified scFv gene repertoire was cloned into phagemid pCANTAB5E vector. They constructed four phagemid libraries and named as Vr-Vk, Vr-VI, Vm-Vk and Vm-VI. They contained 1.1×10^8 , 8.4×10^7 and 5.3×10^7 members respectively. Screening of phage display library

Phage display is an extremely powerful tool for selecting peptides or proteins with specific binding properties from vast numbers of variants. Its utility lies principally in generating molecular probes against specific targets and for the analysis and manipulation of proteins/ligands interactions. Put at its most simple, phage display is the expression of peptides, proteins or antibody fragments at the surface of phage particles as originally described by Smith (1985).

'Fusion phages' are filamentous bacteriophages vectors in which foreign DNA sequences are cloned into phage gene III and displayed as part of the gene III protein (pIII) at one tip of the virion. Fusion phage where displayed protein binds to ligands can be selected from vast background of non-binding phage by affinity purification. The idea of using fusion phage to develop an 'epitope library' was inspired by the synthetic 'mimotope' strategy by Geysen *et al.* (1986).

Jalal *et al.* (2013) successfully employed antibody phage display (PD) to produce high affinity single chain variable fragment (scFv) against tumour necrosis factor- α (TNF- α), which is potent pro-inflammatory cytokine and play roles in various inflammatory diseases and malignancies. To develop scFv antibody fragments against human TNF- α , they performed five rounds of biopanning using stepwise decreased amount of TNF- α (1 to 0.1 mg), a semi-synthetic phage antibody library (Tomlinson I + J) and TG1 cells. Antibody clones were isolated and selected through Enzyme-Linked ImmunoSorbent Assay (ELISA) screening. The selected scFv antibody fragments were further characterized by means of ELISA, PCR, Restriction Fragment Length Polymorphism (RFLP) and Western blot analyses as well as fluorescence microscopy and flow cytometry. Based upon binding affinity to TNF- α , 15 clones were selected out of 50 positive clones enriched from PD *in vitro* selection. The selected scFvs displayed high specificity and binding affinity with Kd values at nm range to human TNF- α . The immunofluorescence analysis revealed that significant binding of the selected scFv antibody fragments to the Raji B lymphoblasts. The effectiveness of the selected scFv fragments were further validated by flow cytometry analysis in the lipopolysaccharide (LPS) treated mouse fibroblast L929 cells. Based upon these findings, they proposed that selected fully human anti-TNF- α scFv antibody fragments are potential immunotherapy agents that may be translated into preclinical/clinical applications.

Single chain variable fragment antibody (scFv) is capable of binding its target antigens and one of the most popular recombinant antibodies format for many applications. In a study conducted by Xiao Zhang *et al.* (2012), the large human synthetic phage displayed library (Tomlinson J) was employed to generate scFvs against Cry1B toxin by affinity panning. After four rounds of panning, six monoclonal phage particles capable of binding with the Cry1B were isolated, sequenced and characterized by Enzyme-Linked Immunosorbent Assay (ELISA). Two of the identified novel anti- Cry1B scFvs, namely H9 and B12 were expressed in *E. coli* HB2151 and purified by Ni metal ion affinity chromatography. Sodium Dodecyl Sulphate Polyacrylamine Gel Electrophoresis (SDS-PAGE) indicated that the relative molecular mass of scFv was estimated at 30kDa. The purified scFv-H9 was used to develop an indirect competitive Enzyme-Linked ImmunoSorbent Assay (ic-ELISA) for Cry1B toxin. The linear range of detection for standards in this ic-ELISA was approximately 0.19–1.1 mg/ml and 50 per cent inhibition of control (IC₅₀) was 0.84 mg mL⁻¹ for Cry1B. The affinity of scfv-H9 was $(1.95 \pm 0.12) \times 10^7$ M⁻¹ and showed cross-reactivity with Cry1Ab toxin and Cry1Ac toxin (8.53 per cent and 7.58%, respectively) and the higher cross-reactivity (12.8%) with Cry1C toxin. The average recoveries of Cry1B toxin from spiked leaf and rice samples were in the range of 89.5–96.4 per cent and 88.5–95.6 per cent respectively, with coefficient of variation (C.V) less than 6.0%. These results showed promising applications of scfv-H9 for detecting Cry1B toxin in agricultural and environmental samples

Phage display library was screened with recombinant *Mycoplasma hyorhinitis* P37. The positive phage clones were identified by ELISA and were sequenced and the amino acid sequences of the polypeptides displayed on phage were deduced. After GST-polypeptides fusion protein was constructed and expressed, its binding to P37 was determined by GST-pull down and Western blot. After 4 rounds of bio-panning, the enriched positive phage clones were identified by ELISA and eighteen positive phage clones were sequenced. The results from GST-pull down and Western blot showed that the peptides could interact with P37 (Yang *et al.*, 2011).

A study was conducted by Monzo *et al.*, 2012 to raise recombinant antibodies against the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), one of the most important pests in agriculture worldwide. This is for the first time to produce recombinant antibodies from a commercial phage display library of human scFv antibodies. The specificity and sensitivity of the selected recombinant antibodies were compared with that of a rabbit polyclonal serum raised in parallel using a wide range of arthropod species as controls. The selected recombinant monoclonal antibodies had a similar or greater specificity when compared with classical monoclonal antibodies. The selected recombinant antibodies were successfully used to detect the target antigen in the gut of predators and the scFv antibodies were sequenced and compared. These results suggested the potential for recombinant scFv antibodies to be used as an alternative to the classical monoclonal antibodies or even molecular probes in the post-mortem analysis studies of generalist predators.

Recently application of phage display technology in plant pathogens diagnosis in India has gained importance. At Indian Institute of Horticultural Research (IIHR), Bangalore, A. Vani's group have explored Griffin-1 library (H. Griffin, MRC, Cambridge, UK) to develop diagnostic dipstick kits for several plant pathogens using scFv antibody gene transcriptionally fused to bacterial alkaline phosphatase gene (PhoA). Chandrashekara *et al.* (2006) produced monoclonal antibodies (MAbs) scFv transcriptionally fused to ALP specific to detect field infected samples of *Ralstonia solanacearum* virulent and avirulent strains, Krishnamurthy (2005) had generated scFv specific to Citrus tristeza virus coat protein for disease diagnosis using phage display technique, Jagadish *et al.* (2008) developed a dipstick diagnostic kit for detection of downy mildew in grapes caused by *Plasmopara viticola* using Griffin-1 phage display library. Vijaya Mahanthesha (2010) produced monoclonal scFv antibodies against *Alternaria solani* causing early blight of tomato; Pradeep (2010) using Griffith phage display antibody library developed the recombinant scFv antibodies against the *Colletotrichum musae* causing anthracnose disease of banana and he reported that recombinant scFv antibodies highly effective in diagnosis of the banana anthracnose disease and Satish (2004) developed anti-M13ScFv-ALP conjugate monoclonal antibody using phage display technology. Similarly, N. Moger at the Central Institute of Fishery Science, Mumbai developed diagnostic test for White Spot Syndrome Virus (WSSV) using Tomlinson library. Also at Institute of Agricultural Biotechnology, Dharwad N. Moger group have developed scFv monoclonal antibody against different antigens. Varma. S (2009) developed scFv monoclonal antibody against *Sclerotium rolfsii* lectin protein and Chidanand (2010) using Tomlinson I library produced monoclonal scFv antibodies against bacterially expressed Cry2B protein and Polyclonal scFv against Cry1Ac protein. Further Shashikumara (2012) developed immunodiagnostic assay for detection of Cry2B protein. Shilpa (2013) developed two monoclonal antibodies against BBTV using Tomlinson I library.

2.6 Transcriptional fusion of monoclonal antibody to ALP gene

Expression of enzymatically active protein fusions in *E. coli* (where single-chain Fv fragments are fused to the N-terminus of *E. coli* alkaline phosphatase) could facilitate the analysis of proteins and even replace some reagents frequently used in immunology such as chemically produced antibody-enzyme conjugates. pQUANTabody vector is convenient positive cloning recombinant system for the generation of foreign proteins fused to highly active bacterial alkaline phosphate (PhoA) in *E. coli*. The presence of the PhoA signal peptide directs the fusion protein to the periplasm where the PhoA dimerises to its active form. The fusion partner derived from any source of nucleotide sequence e.g. PCR products, cDNAs and scFv genes restores the frame of the PhoA gene in the vector. Recombinants are therefore, identified directly as blue colonies by plating the transformants onto an agar plate containing chromogenic substrate for PhoA like pNPP (p-Nitrophenyl phosphate), BCIP (5-bromo- 4chloro-3 indolyl phosphate).

Bruno *et al.* (1999) recloned anti-AAF ScFv gene into fusion vector for the production of hybrid protein comprising the antibody fragment fused to the potent bacterial alkaline phosphatase variant possessed both antigen binding capacity and PhoA activity. This showed that genetic fusion of scFv with PhoAV provides a new tool for immunodetection which presents easier and quicker production and use with the same sensitivity and specificity.

Rau *et al.* (2002) constructed a system for the production of alkaline phosphatase (ALP)-labelled antibody single-chain Fv (scFv) fragments in *E. coli*. The expression vector pASK75 was modified by sequentially inserting the *E. coli* ALP coding region and the scFv cloning cassette. Engineering the cloning sites *SfiI* and *NotI* located at the 5' and 3' end of the scFv gene provides an easy means to insert scFv fragments. These cloning sites are widely used in recombinant antibody technology and thus, enable the one-step cloning of scFv fragments derived from corresponding antibody phage libraries into the expression vector.

Kohl *et al.* (1991) have screened single-chain Fv fragment representing the variable domain of the human monoclonal antibody 3D6 binding specifically to HIV-1 gp41 and fused to the coding region of *E. coli* alkaline phosphatase (*E. coli* PhoA) and expressed in *E. coli*. The *E. coli* PhoA signal peptide was used to direct the recombinant fusion protein to the periplasmic space of the bacteria, from where it was purified by hydrophobic interaction chromatography and gel filtration followed by antigen-affinity chromatography using synthetic HIV-1 peptide as ligands. The purified fusion protein was bifunctional, showing both phosphatase activity as well as antigen binding specificity identical to that of the original antibody. DNA encoding the single chain Fv antibody fragment (scFv) obtained from synthetic phage-antibody library after four rounds of selection against purified preparations of potato leaf roll luteovirus (PLRV) was sub-cloned into pDAP2 by Harper *et al.* (1997) such that the scFv -alkaline phosphatase fusion protein was produced by transformed bacteria following induction by isopropyl-beta-Dthiogalactopyranoside (IPTG). The fusion protein was obtained at concentrations of 10 mg/ml of *E. coli* culture medium and these fusion protein preparations were used directly in ELISA to detect PLRV in sap extracts from infected plants. The results demonstrate the potential of scFv and enzyme- scFv fusion proteins in routine testing for plant viral infection.

Suzuki *et al.* (1997) have designed and constructed bacterial expression vector to produce a fusion protein of hapten-specific single chain Fv (scFv) and alkaline phosphatase (PhoA) in *E. coli*. The scFv gene was assembled using genes encoding the heavy and light chain variable domains of anti-NP (4-hydroxy-3-nitrophenyl acetyl) mouse monoclonal antibody. The scFv gene was then fused to the 5' terminus of the *E. coli* PhoA coding region. The expressed fusion protein scFv (NP)-PhoA was purified using an NP affinity column and gel-filtration. ScFv (NP)-PhoA can be used to detect nanogram concentrations of NP-BSA in ELISA without the use of chemically conjugated secondary antibodies.

Griep *et al.* (1999) constructed the vector pSKAP/S to enable over expression of single-chain variable fragment antibody (scFv) alkaline phosphatase fusion proteins. In pSKAP/S, the scFv were genetically fused to the mutated *E. coli* PhoA/S gene that encodes an alkaline phosphatase with increased specific activity. The restriction sites incorporated into pSKAP/S allowed the scFv genes to be easily transferred from pUC119-derived phagemid vectors that are used frequently in phage display antibody library technology. The pSKAP/S vector was successfully used to obtain scFv fragments from preparation of phage-antibody clones after sub cloning and expression of individual clones as scFv -alkaline phosphatase fusions. ScFv specific to PLRV were sub cloned into pC(L) and pSKAP/S for expression as fusion proteins with human kappa constant region (scFv -C(L)) or they were used as soluble scFv (scFv-c-myc) for detection in ELISA by Harper *et al.* (1999). Expression of these constructs in *E. coli* yielded 0.2-1 mg protein per liter of bacterial culture. Absorbance values obtained with the fusion proteins were greater than those obtained with the scFv-c-myc and were similar to those obtained in assays done using monoclonal or polyclonal antibodies.

A generic strategy was established for subcloning scFv variable regions from pCANTAB 5E into the plasmid pASK85 for the expression of F(ab) fragments. The F(ab) fragment was purified from the periplasmic extract in single step by Immobilized Metal Affinity Chromatography (IMAC). A yield of 100µg/lx OD (550) purified F(ab) fragment was obtained employing a standard fermentation scheme. The sensitivity and cross-reactivity of the F(ab) was comparable to the parent scFv when assayed by enzyme immunoassay. However, the F(ab) fragment exhibited significantly improved long-term stability (Kramer *et al.*, 2002).

An expressed herbicide-specific scFv ALP fusion protein retained both, analyte binding and enzymatic activity determined by ELISA. Therefore, this system permits the production of scFv-ALP conjugates in *E. coli*, which can replace conventionally prepared ALP-labelled antibodies in immunoassays. These fusion proteins are designed to accelerate the immunochemical detection of analytes, since the assay duration is essentially reduced by omitting the use of enzyme labelled secondary antibodies (Rau *et al.*, 2002).

Kerschbaumer *et al.* (1997) developed A double antibody sandwich ELISA for the detection of beet necrotic yellow vein virus, the causal agent of sugar beet rhizomania using fusion proteins obtained by means of pZIP1 and pDAP2/S into which scFv have been cloned. The plant pathogen was detected with sensitivity higher than that reached in conventional ELISA employing polyclonal antisera. Alvi *et al.* (1999) expressed the scFv clone that specifically binds to Venezuelan equine encephalitis (VEE) virus in *E. coli* strain TG-1 and 5B4D-6-6 as a approximately 30kDa scFv protein and higher molecular weight fusion products which were functional in recognizing VEE virus by Enzyme-Linked Immunosorbent Assay (ELISA). Results were reproduced in *E. coli* strain HB2151, where clone D66 was expressed mainly as soluble periplasmic protein. ELISA and DIBA were highly sensitive in detecting about 5ng and 0.1ng of expressed protein (Vijayanandraj *et al.*, 2013).

2.7 ScFv antibody based detection test for BBTv

Western blotting showed of 1/5000 was used for the secondary antiserum-conjugated alkaline the binding of 6x-His tag fusion coat protein of BBTv with 1-1000 dilution of Ni-NTA alkaline phosphatase conjugate. In Dot Blotting ImmunoAssays (DBIA) 0.5µg of BBTv-CP expressed in *E. coli* reacted with two tested primary antisera (BBTv-AS and BBTv-AS6xHis) at a dilution of 1-1000, with a dilution phosphatase (Abdelkader *et al.*, 2004)

Lee *et al.* (2009) showed that Specific binding of the scFv-AP fusion proteins to the branched chain keto acid dehydrogenase complex-E2 (BCKD-E2) antigen was tested by western blot using total cell lysates of the BCKD-E2-expressing *E. coli* clone. It was found that clones #21, #46 and #47 were reactive to recombinant BCKD-E2 protein (~56 kDa in size) only and not to any other bacterial proteins, similar to the binding reactivity of parental 2H5A12 anti-BCKD-E2 IgG mAb

Reactivity and specificity of the J591 ScFv to prostate specific membrane antigen PSMA was conducted through an immunoblot analysis where J591 ScFv was used as the primary antibody. The immunoblot analysis was conducted on the cell lysate of PC3-PIP (PSMA+) and PC3-Flu (PSMA-) cells. The J591 ScFv detected a protein at 100 kDa in the PC3-PIP lysate, which corresponds to the molecular weight of PSMA. The J591 ScFv identified a 100 kDa band in the PC3-PIP lysate but not from the PSMA-negative PC3-Flu lysate. The positive detection in comparison to the negative control cell line establishes qualitatively the capability of the J591 ScFv to detect and bind to PSMA (Stephanie *et al.*, 2013)

Vijayanandraj *et al.* (2013) showed that the LCCV antiserum at 1:5,000 dilutions showed strong positive signal with 1.0–0.1ng of expressed protein. With the reduction of protein quantity the detecting signal reduced, protein as low as 1.0–0.1ng could be detected by DIBA. Efficient detection was obtained in the crude sap of infected large cardamom leaf at a dilution of 1:5–1:10. The intensity of the developed spot gradually decreased as the dilution of sap increased. The virus was detectable in the sap dilution up to 1:100. The LCCV antiserum showed very weak detection signal with CdMV leaf extract in small cardamom at only 1:5 dilution. Sixty three symptomatic large cardamom samples from different cultivars and places in Darjeeling district of West Bengal and Sikkim, those were positive in ELISA, also showed strong positive reaction in DIBA

2.8 Dipstick assay

Attallah *et al.* (1991) performed Enzyme ImmunoAssays (EIAs) to detect keratin in urine samples using CK1K10 and anti-mouse IgG. A fast dot-ELISA was developed in which urine is applied onto nitrocellulose (NC) sheets or dipsticks. A high positive correlation was found between the fast dot-ELISA and other EIAs. The assay does not require sophisticated equipment nor highly trained technical staff and can be performed in less than 30 min. The keratin dot-ELISA could be reliable screening test for the early detection of bladder cancer.

Allan *et al.* (1993) developed dipstick dot ELISA for detection of *Taenia* specific coproantigens of the 41 cases diagnosed by the three diagnostic techniques (dipstick, micro and dot ELISA). 31 were detected by the dipstick assay making it the most sensitive technique employed. The specificity of the dipstick assay was 99.9% with positive predictive value of 88.6%. The combined diagnostic approach did not, however, diagnose all cases. The tests conducted with 75 serum samples from patients with clinically suspected acute DEN infections, the IgM dipstick ELISA showed high sensitivity of 97.9% and specificity of 100% compared to those of an IgM antibody capture microplate ELISA (Wu *et al.*, 1997). Monoclonal antibodies generated against *Phytophthora nicotianae*, causing root rots in citrus reacted with the surface of *P. nicotianae* cysts but not with cysts of any other species of *Phytophthora* or *Pythium* tested by Gautam *et al.* (1999). The antibodies used in dipstick assay detected *P. nicotianae* spores at densities as low as 10 spores per ml. An immunostick test was developed for detection of karnal bunts using crude and DE-52 purified anti-teliospore antibodies. The intact teliospores and solubilised teliosporic antigen were coated on to nitrocellulose pad of dipsticks. The positive results after immunoprocessing indicated that the antibodies are specific to the antigen (Ravikesari and Anil Kumar, 2002).

Shashikumara *et al.*, 2012 developed immunodiagnostic kit to detect cry2B protein in which the antigen coated on NC membrane at different concentration was allowed to react with antibody-ALP conjugates. When antigen was spotted on NC membrane it is revealed that at concentration of 0.8 µg/ml, the antibody conjugate was able to detect. In case of ELISA the antibody conjugate was able to detect antigen at the concentration of 4 µg/ml. At this concentration, there was significant color development, the ELISA reading was 0.071 and control value was 0.055.

3. MATERIAL AND METHODS

The previously developed scFv monoclones were used for the present study. Development of Immuno-Diagnostic kit for the detection of Banana Bunchy Top Virus . The materials used and methodologies followed are presented in this chapter.

3.1 Source of antigen

Production of scFv antibody against BBTv protein (antigen), construct pBSNMCP30 expressed in *E. coli* M15 was taken from IABT, UAS, Dharwad and the crude protein was used for purification by using 6x his-tag purification kit.

3.2 Confirmation of pBSNMCP30

3.2.1 Colony PCR of *E. coli* M15 containing pBSNMCP30

Polymerase chain reaction (PCR) was conducted to The BBTv-CP gene using gene specific primers by using following components for 20µl reaction mixture.

Components	Quantity
10x <i>Taq</i> assay buffer	2.0 µl
dNTPs (2 mM)	1.0 µl
Forward primer (5 pM)	1.0 µl
Reverse primer (5 pM)	1.0 µl
<i>Taq</i> polymerase (5 U/µl)	0.2 µl
Template (100 ng/µl)	1.0 µl
Double distilled water	13.8 µl

Note: Template refers to BBTv DNA sample.

The above mentioned components were mixed in 0.5 ml micro centrifuge tube and given short spin and then distributed to 0.2 ml tubes into which the template DNA was added. The entire process was carried out in cooler box in the laminar air flow chamber with autoclaved materials.

The following thermal profile was used for the amplification of BBTv-CP

Steps	Temperature	Time
Initial denaturation	94.0 °C	5.0 min
Denaturation	94.0 °C	1.0 min
Annealing	59.0 °C	1.0 min
Extension	72.0 °C	45 sec
Number of cycles- 30		
Final extension	72.0 °C	10.0 min &
Hold at	4.0°C	

Amplified products were loaded on 1.2 per cent agarose gel and electrophoresed. The obtained amplicon size 531bp confirmed the presence of *bbtv* gene.

3.2.2 Confirmation by restriction digestion

The *bbtv* gene carrying pQE30 vector was isolated from *E. coli* M15 clone and subsequently the clone was subjected to restriction analysis using *Bam*HI and *Hind*III for the confirmation of presence of gene *bbtv* in pQE30 vector.

3.3 Plasmid isolation

The alkaline lysis protocol (Sambrook *et al.*, 1989) with certain modifications was used for isolation of plasmid. The colonies were inoculated in 10 ml LB with ampicillin (100µg/ml) and incubated for overnight at 37°C with 200 rpm. The overnight grown culture was centrifuged at 5,000 rpm for 2 min in 2.0 ml micro centrifuge tubes then the supernatant was removed and pellet was washed with STET buffer (0.25 volume of original culture) and again it was centrifuged at 5,000 rpm for 2 min. The pellet was resuspended in 200µl of ice-cold alkaline lysis solution-I by vigorous vortexing and later 400µl of freshly prepared alkaline lysis solution-II was added to each tube then contents were mixed by inverting the tubes for 5 times and kept in ice for about 5 min. To this suspension, 300µl of alkaline lysis solution-III was added and again mixed thoroughly by gently inverting the tubes for 5 times. The tubes were stored on ice for 5 min and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was transferred to fresh tubes and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to precipitate and mixed well, then centrifuged at 13,000 rpm for 10 min at 4°C. The aqueous layer was transferred to fresh tube and two volumes of isopropanol were added. The contents were mixed and allowed to stand for 5 min at room temperature. The solution was later centrifuged at 13,000 rpm for 5 min then the supernatant was discarded and the pellet was washed with 70 per cent ethanol and spun for 1 min at 13,000 rpm to recover the plasmid. The supernatant was discarded then kept for drying and dispensed into 30µl of T₁₀E₁ (pH 8.0) containing 3µl of RNase A (10 mg/ml). The solution was kept at 50 °C for 15 min and then stored at - 20 °C.

3.4 Restriction digestion of pBSNMbbtv

The plasmid was subjected to restriction analysis. The extracted plasmids from *E. coli* M15 were double digested in 20µl reaction mixture using *Hind*III and *Bam*HI (New England Bio-labs). Reaction mixture for double digestion is mentioned below.

Components	Volume
Nuclease free water	29 µl
Buffer 3 (10X)	6 µl
BSA	1 µl
pBSNM13/pQE30	20 µl (10 µg)
<i>Hind</i> III (5U/ µl)	2 µl
<i>Bam</i> HI (5U/ µl)	2 µl
Total volume	60 ul

The two double digestion reactions were carried out in 500µl micro-centrifuge tube. The reaction components were mixed gently by tapping and brief spun for a few seconds. The tube was incubated at 37°C water bath for 1hr. Restriction enzymes were then heat inactivated by heating the reaction mixture at 65°C for 10min in the thermocycler. The digested product was checked on 1 percent agarose gel electrophoresis for the release of BBTv-CP gene fragment.

3.5 BBTv/CP confirmation through SDS-PAGE

The overnight grown recombinant colonies were separately inoculated into tube containing 5 ml of LB broth containing ampicillin at 100µg/ml. The cells were grown until they reached 0.6-0.8 OD at 37 °C at 180 rpm in a shaker incubator. After reaching the OD the cells were induced with IPTG at the concentration of 1mM and uninduced samples were kept separately. All tubes with different recombinant colonies were incubated for another 5 hr to find out the desired level of expression of protein. Then tubes without IPTG were considered as uninduced treatments. A total of 20µl from each cell extract was denatured at 98 °C with SDS loading dye and fractionated by SDS-PAGE (Sambrook *et al.*, 1989) on a 12 per cent resolving gel. The differences of protein profile of induced and uninduced transformed bacterial cells were compared. The molecular weight of expressed protein was determined by comparing with standard protein marker.

3.6 His-tag fusion protein purification

The expressed protein was subjected to purification under native conditions. The protein was purified using Genei His-tag purification kit. The bacterial cell pellet was dissolved in binding buffer (1:2) that is 1g of cell pellet was dissolved in 2ml of buffer, to this lysozyme was added at the concentration of 1mg/ml. Then it was kept in ice for 30 min without shaking. After 30 min the cells were sonicated at 10 bursts with 10 sec interval for six times. Further the cells were spun at 10,000 rpm for 30 min at 4 °C. Then the cell lysate was collected in fresh tube. Then clarified lysate was applied to the equilibrated Ni⁺ column and it was allowed to flow through the column. Then the column was washed with wash buffer and the bounded protein was eluted with elution buffer. The eluted his-tag fusion protein was analyzed using 12 per cent SDS-PAGE. The purified protein was used as antigen for production of scFv monoclonal antibodies.

3.7 Antibody Phagemid isolation

Phagemid of clones pBSNMAB5 and pBSNMAB40 were isolated as similar to plasmid isolation.

Amplification of pBSNMAB5 and pBSNMAB40 fragments

LMB3 (Forward primer)	5'CAGGAAACAGCTATGAC3'
pHEN seq (Reverse primer)	5'CTATGCGGCCCCATTCA3'

The polymerase chain reaction of clones pBSNMAB5 and pBSNMAB40 were performed using following components for 20 µl reaction mixture.

Components	Quantity
10x <i>Taq</i> assay buffer	2.0 µl
2 mM dNTPs	1.0 µl
Forward primer (5pM)	1.0 µl
Reverse primer (5pM)	1.0 µl
<i>Taq</i> polymerase (5U/ µl)	0.2 µl
Template (70 ng/ µl) (pIT2 Phagemid vector)	1.0 µl
Double distilled water	13.8 µl

Note: Template refers to pBSNMAB5 and pBSNMAB40 antibody fragment in pIT2 phagemid Vector

The above mentioned compounds were mixed in 0.5 ml tube and given short Spin then distributed to 0.2 ml tubes where the template DNA was added. The entire process was carried out in cooler box in the laminar airflow chamber with autoclaved materials.

The following thermal profile was used for the amplification of scFv of both monoclonal genes.

pBSNMAB5	and	pBSNMAB40		
Steps	Temp.	Time	Temp.	Time
Initial denaturation	94.0 °C	5.0 min	94.0 °C	5.0 min
Denaturation	94.0 °C	1.0 min	94.0 °C	1.0 min
Annealing	55.0 °C	1.0 min	55.0 °C	1.0 min
Extension	72.0 °C	2.0 min	72.0 °C	2.0 min
Number of cycles- 30				
Final extension	72.0 °C	10.0 min	72.0 °C	10.0 min
Hold at	4.0 °C			

Amplified products were loaded on 1.2% agarose gel and electrophoresed.

3.8 Restriction digestion of pBSNMAB5 and pBSNMAB40 along with pQUANTabody plasmid DNA with *Not I* and *Sfi I*

In the Tomlinson scFv library, scFv genes were cloned into the phagemid vector between *NotI* and *SfiI* restriction sites. Therefore scFv gene can be eluted by digesting the phagemid DNA with *NotI* and *SfiI* restriction enzymes. pQUANTabody vector is convenient positive cloning recombinant system for the generation of foreign proteins fused to highly active bacterial alkaline phosphatase (PhoA) in *E. coli*. The presence of the PhoA signal peptide directs the fusion protein to the periplasm where the PhoA dimerises to its active form. The fusion partner derived from any source of nucleotide sequence e.g., PCR products, cDNAs, scFv genes restores the frame of the PhoA gene in the vector. Recombinants are therefore, identified directly as dark purple color colonies by plating the transformants onto an agar plate containing chromogenic substrate for PhoA like BCIP (5-bromo-4chloro-3 indolyl phosphate) and NBT (nitro-blue tetrazolium chloride).

Not-I Restriction Digestion

In three micro centrifuge tubes the followings were added separately. 44 µl of pBSNMAB5, pBSNMAB40 and pQUANTabody vector DNA into 3 separate tubes. Five µl of 10x *Not-I* restriction enzyme buffer, 0.5 µl of purified BSA, 1 µl of *NotI* enzyme were added into each tube separately and mixed. These tubes were incubated at 37 °C for 2 h in water bath. Five µl of 3M Sodium acetate and 130 µl of 70% ethanol were added to precipitate the DNA. The tubes were incubated on ice for 1 h and spun at 5,000 rpm for 30 min. The pellet was dissolved in 25 µl sterile T₁₀E_{0.1}.

Sfi-I Restriction Digestion

Twenty five µl of *Not-I* restriction digested pQUANTabody plasmid DNA, monoclones pBSNMAB5 and pBSNMAB40 DNA were taken in three tubes. Three µl of 10x *SfiI* restriction digestion buffer, 1 µl of *SfiI* restriction enzyme and sterile distilled water was added to final volume of 30 µl. The tubes were incubated in water bath at 50°C for 2 h and the digested samples were stored at -20 °C till further use.

3.9 Gel Elution of restricted product using Qiagen mini-elute gel extraction kit

The DNA fragment was excised in the agarose gel with clean and sharp scalpel. The size of the gel slice was minimized by removing extra agarose. The gel was purified according to manufacturer's instructions.

3.9.1 Ligation of vector and insert DNA fragments

One µg of each double digested pBSNMAB5, pBSNMAB40 and pQUANTabody vector DNA was taken in sterile test tube then the volume was made upto 17 µl with sterile distilled water. 2 µl of 10x T4-DNA ligase buffer was added to the tube and 1 µl of T4-DNA ligase (5 U/µl) enzyme was added. The tubes were incubated in water bath at 16 °C for 4 h. The ligated sample was stored at -20 °C till further use (fig 1).

3.9.2 Transformation of *E. coli* DH5 α with Ligated mixture competent cell preparation by calcium chloride method

Overnight grown *E. coli* DH5 α culture (100 μ l) was inoculated into 100 ml LB broth and incubated at 37 °C at 210 rpm till OD at 600 nm was 0.5-0.6. The exponentially grown cells were incubated on ice for 30 min. The *E. coli* DH5 α cells were pelleted by centrifuging at 6000 rpm for 8 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 0.1M (volume 12.5ml) of calcium chloride solution and incubated on ice for 30 min. The cells were again pelleted by spinning at 4000 rpm for 5 min at 4 °C. The supernatant was discarded and the cells were resuspended in 1 ml 0.1M CaCl₂. The cells were aliquoted into sterile micro centrifuge tubes (100 μ l/tube) and the tubes were stored at –80°C until further use.

3.9.3 Transformation

Competent *E. coli* DH5 α cells were thawed on ice for 10 min. Using 8 μ l of ligated mixture (pQUANTabody + pBSNMAB5 and pQUANTabody + pBSNMAB40) was added to the thawed cells and incubated on ice for 30 min. The cells were given heat shock exactly for 2 min at 42 °C and immediately quenched on ice for 15 min to which 900 μ l of sterile LB broth was added and incubated at 37 °C at 210 rpm for 60 min. Meanwhile LB agar plates with ampicillin (100 μ g/ml) were prepared and 20 μ l of BCIP (20mg/ml) and 20 μ l of NBT (20 mg/ml) were spread and incubated at 37 °C at least for 1h. Transformed cells were spun at 5000 rpm for 5 min to pellet the cells. Cells were resuspended in little amount of supernatant and plated on above medium, then the plates were then incubated overnight at 37°C. The following controls were also included in the experiment. Competent cells with pQUANTabody vector DNA (2 μ l) were used as positive control. Competent cells which did not receive plasmid DNA was kept as negative control (on ampicillin 100 μ g/ml).

3.10 Screening of the transformants

3.10.1 PCR amplification of anti-BBTV-ALP clones

The colonies which were blue in color on plates containing ampicillin, BCIP and NBT were transformed colonies which contained inserts in the pQUANTabody vector. Those in white color were the vector without the insert. Two transformants are obtained and which are designated as pBSNMALP5 and pBSNMALP40. These were further revived on LB agar plate containing ampicillin and substrates (NBT and BCIP). Plasmid was isolated from transformants and it was confirmed by using following ALP primers

PhoA forward	GCACTGGCACTCTTACCGTTAC
phoA reverse	CAGTCTGATCACCCGTTAAAC

The following thermal profile was used for the amplification of anti-BBTV-ALP clones.

Steps	Temperature	Time
Initial denaturation	94.0 °C	5.0 min
Denaturation	94.0 °C	1.0 min
Annealing	56.0 °C	1.0 min
Extension	72.0 °C	2.0 min
Number of cycles- 30		
Final extension	72.0 °C	10 min

3.10.2 Restriction digestion of pBSNMALP5 and pBSNMALP40

The transformants were confirmed by restriction digestion using *Sfi*I and *Not*I as described in 3.8

3.11 Screening of the transformants by protein expression analysis

The transformants were further tested for the presence of scFv gene by expressing the protein in *E. coli* and using that protein for detecting BBTV coated on the ELISA plate. In order to determine the clone that has full length functional antibody gene. Blue clones were picked and tested for their ability to bind BBTV in an ELISA experiment.

3.11.1 Protein Expression from pBSNMALP5 and pBSNMALP40 clones

Each putative transformed colony was picked from the LB plate and grown overnight at 37 °C in 5 ml LB broth containing ampicillin at 100 µg/ml. From the overnight culture small aliquot was inoculated into 10 ml LB broth containing ampicillin 100 µg/ml and grown under gentle agitation at 37 °C till OD reached 0.6 at 600 nm. The culture was induced by adding IPTG to final concentration of 0.5 mM. Growth of the culture was continued further for 3-4 h with gentle agitation at 37 °C.

3.11.2 Extraction of periplasmic proteins by lysozyme shock

The bacterial cell pellet was resuspended in 1/10 volume Lysis buffer. Lysozyme was added to final concentration of 0.1 mg/ml. The suspension was incubated for 20 min at 4 °C with agitation. The cells were sonicated 10 times for 10sec interval. The suspension was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was stored at 4 °C and used within 1 week. The extracted protein was analysed on 10 % SDS- PAGE.

3.11.3 One step detection of BBTv using fusion antibodies

The extracted protein, which contained anti-BBTv-ALP conjugate, was used to detect the BBTv by ELISA. It gives a confirmation that the extracted protein contains anti-BBTv-ALP conjugate. The protocol followed was similar as that of 3.2.6.

3.12 Development of scFv antibody based detection test for BBTv

3.12.1 Determining virus protein specificity of scFvbbtv by western blot:

Purified BBTv-CP proteins were separated on 12.5% SDS-PAGE (Laemmli *et al.*, 1970). The protein samples were mixed with loading dye and loaded into the gel along with the molecular weight marker. The sample was electrophoresed along with two reference lanes of the gel (molecular weight marker and protein) separated and stained with Coomassie blue. The remaining portion of the gel was transferred to PVDF membrane. Protein lanes on the PVDF membrane was cut and reacted with blocking solution (3% BSA in PBS). The blot was then exposed to scFv monoclonal antibodies for 2h. After washing three times with PBS-Tween-20, the substrate was diluted ten times and then reacted with the membrane. The developed bands were then compared with molecular weight marker

3.12.2 Determining virus protein specificity of scFvbbtv by RapiDot Immuno Assay

Antigen (3µl) from purified sample BBTv-CP and other antigen (for comparison) were dotted on pre-washed and dried PVDF membrane. Immunodot test will be performed by adding 3 µl of antibody protein. The specific reaction of the scFv with antigen was then recorded.

3.13 Standardization of immunoassay for BBTv-CP.

The extracted protein (BBTv) concentration was estimated according to Lowry *et al.*, (1951). Serial dilution (10^{-8}) of protein was made and 2µl volume from each dilution was dotted onto PVDF strip. A negative control without antibody was used for comparison. The PVDF strip was then reacted with scFv antibodies which later lead to development of visible colour dots. Intensity of color development against different concentration of antibody protein was then recorded.

3.14 Development of antibody based detection kit for BBTv

The phages obtained from pBSNMAB5 and pBSNMAB40 were coated onto nitrocellulose (NC) membrane cassettes and also on NC membrane strips in carbonate buffer for overnight. Next day morning the unbound sites were blocked with 3% BSA in 1XPBS for 90 min. These cassettes were stored at 4°C until further use. The BBTv protein was coated with the capture antibody (scFv) for 90 min at room temperature. After washing the unbound antigen in 1XPBS, it was blocked with 3% BSA in 1X PBS. The captured antigen was detected with second antibody (single chain monoclonal scFv-BBTv-ALP) conjugated to alkaline phosphatase. NBT-BCIP was used as substrate and color was allowed to develop for 30 min.

3.14.1 Determination of sensitivity of anti-BBTV-ALP clones

The purified BBTV protein was dotted onto nitrocellulose strip at different concentrations like 1µg to 10µg. A negative control without antigen was used for comparison. The NC strip was then reacted with scFv antibodies. The visible purple dots developed with lowest concentration of the BBTV protein were recorded. ELISA was performed to check the sensitivity of anti-BBTV-ALP clones as described in 3.2.7.1.

3.14.2 Validation of scFvbbtv antibodies by protein expression analysis

The different infected or on farm field samples from different locations from parts of Uttarakannada, Dharwad, Belgaum, Shivamogga, Chikmagalur and Hassan were collected and was tested for the presence of BBTV. The expressed scFvbbtv (antibody) protein in *E. coli* was used for detecting BBTV (antigen) coated on the ELISA plate and tested for their ability to bind to BBTV in an ELISA plate and as well as dot immunoassay for different samples.

4. EXPERIMENTAL RESULTS

The experimental results of the present study on Development of Immuno-Diagnostic kit for the detection of Banana Bunchy Top Virus are presented in this chapter.

4.1 Confirmation of antigen clones

4.1.1 PCR confirmation

The BBTV-CP gene was PCR amplified using BBTVCP-F and BBTVCP-R primers in which the correct 531bp PCR product was observed on 1 per cent agarose gel. Therefore, it has been confirmed the presence of BBTV-CP gene (Plate 1).

4.1.2 Restriction confirmation

The release of 513bp fragment when restricted with *Bam*HI and *Hind*III confirmed the presence of *bbtv* gene in pBSNMCP30 construct in *E. coli* M15 (Plate 2).

4.1.3 His-tag purification of BBTV

The induced protein was purified using genei his-tag purification kit. After purification it was analyzed on 12 percent SDS-PAGE. The expected band size of 21KDa was observed in BBTV purified sample (Plate 3).

4.2 Confirmation of antibody clones

4.2.1 PCR amplification of pBSNMAB5 and pBSNMAB40

Two monoclones pBSNMAB5 and pBSNMAB40 which showed highest affinity than any other clones in monoclonal ELISA were taken for further studies. The target region of pBSNMAB5 and pBSNMAB40 was amplified using LMB3 forward and pHEN reverse primers. The size of amplicon in both cases was approximately 990 bp on a 0.8 per cent agarose gel stained with ethidium bromide and visualized under UV transilluminator (Plate 4).

4.2.2 Restriction confirmation of pBSNMAB5 and pBSNMAB40

Phagmids of pBSNMAB5 and pBSNMAB40 were subjected to restriction digestion. In both the cases fragment of approximately 795bp was released. This fragment is responsible for antibody production of the clones (Plate 5).

4.3 Cloning of scFv from pBSNMAB5 and pBSNMAB40 into pQUANTabody vector raised against BBTV

The pBSNMAB5 and pBSNMAB40 phagmids were subjected to restriction digestion. In both the cases fragment of approximately 795 bp was released. These restricted products along with pQUANTabody vector were ligated, transformed and screened via blue white selection method on specific medium. The initial screening of pBSNMALP40 and pBSNMALP5 clones were done by PCR using *phoA* forward and *phoA* reverse primers. The clones were confirmed by restriction digestion with *Sfi*I and *Not*I enzymes. A fragment of 795 bp was released (Plate 6). In both the cases an amplicon of 990 bp was observed. The isolated antibody conjugate from these clones was subjected to color development using NBT-BCIP substrate along with protein isolated from pQUANTabody which indicated that scFv antibody is fused with bacterial ALP enzyme as fusion (fig1).

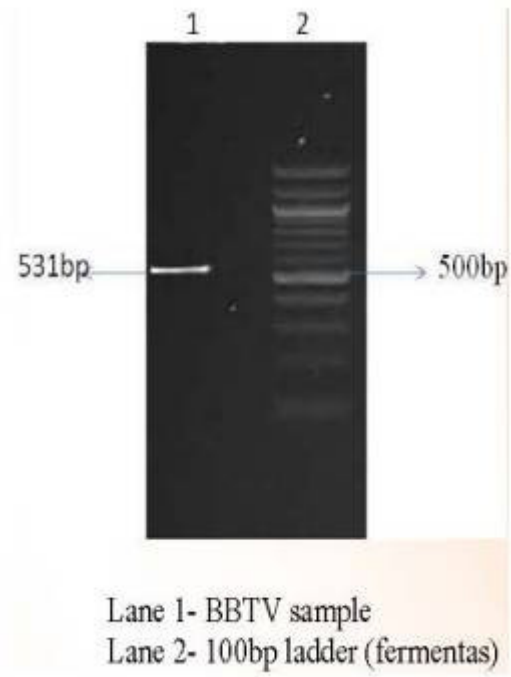


Plate 1: PCR confirmation of *bbtv* gene

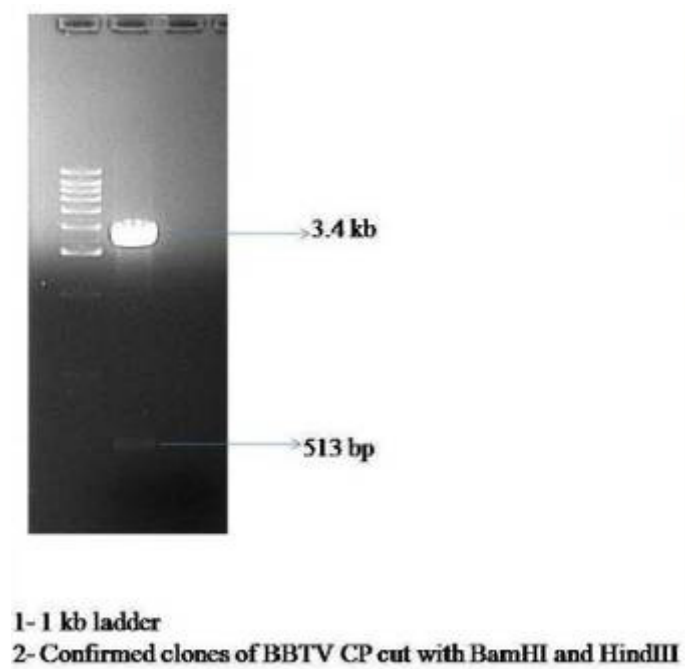


Plate 2: Restriction confirmation of *bbtv* gene in pBSNMCP30 construct in *E. coli* M15

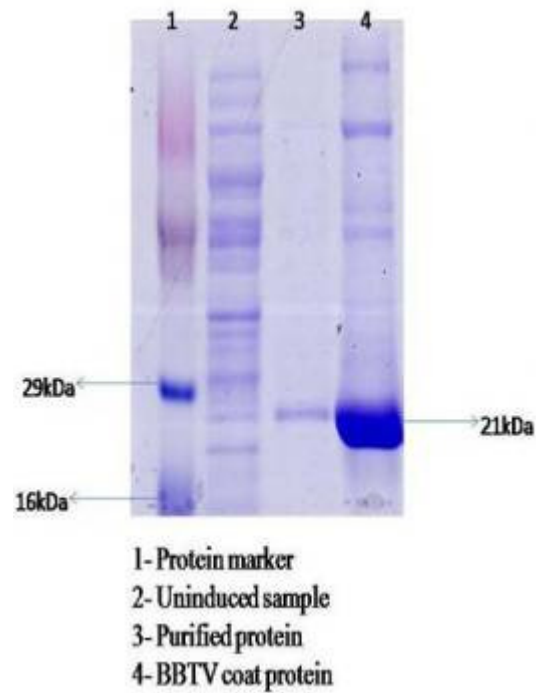


Plate 3: SDS PAGE analysis of BBTV protein

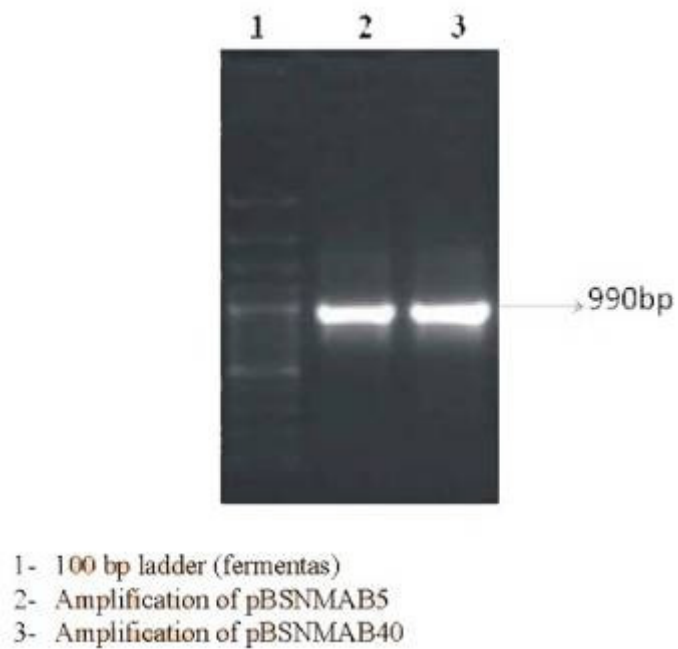


Plate 4: PCR confirmation of antibody clones

4.4 scFv sequence result

The pBSNMAB5 and pBSNMAB40 sequence results of both primers were assembled in Bio Edit software. It was found that the sequences of both clones are hundred per cent similar. The assembled sequence was found to have *Sfi*I and *Not*I restriction sites; the sequence between these two restriction sites is responsible for production of scFv for BBTv coat protein. The homology search through BLASTn programme for similar nucleotide and BLASTx (<http://www.ncbi.nlm.nih.gov>) programme for similar amino acid was carried out. Results are depicted in Table 1 and (fig 2).

4.5 Protein Expression from pSNMALP5 and pSNMALP40 clones

Periplasmic protein was extracted from the antibody clones by lysozyme shock using lysis buffer. The extracted protein when analysed on 10% SDS PAGE showed the protein band size of approximately 34KDa (Plate 8).

4.6 One step detection of BBTv using fusion antibodies

The Direct ELISA was carried out for the above antibody conjugated clones such as pBSNMALP5 and pBSNMALP40. In this experiment, it was noticed that all the crude conjugate protein detected BBTv antigen efficiently (1.35 by pBSNMALP5 and 1.76 by pBSNMALP40). Almost 22 fold increase in absorbance value when compared to control value (0.061). The antibody conjugated with ALP reduced the cost of the diagnosis and time (Table 2 and Plate 7).

4.7 Determining virus protein specificity of scFvbbtv by western blot and Dot Blot Immuno Assay

In Western blot, the antiserum could recognize the expressed protein in crude bacterial lysate and also in purified preparation showing hybridization at 21kDa, whereas no reactivity was observed in the uninduced bacterial lysate with 1:1000 dilution of antiserum. In DBIA, the BBTv antiserum at 1:1000 dilutions showed strong positive signal with 50ng of expressed protein (Plate 9).

4.8 RapiDot Diagnostic assay for BBTv

pBSNMAB5 and pBSNMAB40 phages were used as capture scFv on nitrocellulose membrane. The pBSNMALP5 and pBSNMALP40 clones were used as antibody conjugate for the detection. There was visible color observed on both cassettes and NC membrane strips except in negative control. The antibodies produced were used for cross testing with other protein like Cry2B protein sample. But pBSNMALP5 and pBSNMALP40 antibody conjugates are reacted only with BBTv protein samples and not with other.

4.9 Sensitivity of RapiDot diagnostic assay

The antigen coated on NC membrane at different concentration was allowed to react with antibody-ALP conjugates. When antigen was spotted on NC membrane it is revealed that at concentration of 0.9µg/ml, the antibody conjugate was able to detect. In case of ELISA the antibody conjugate was able to detect antigen at the concentration of 3µg/ml. At this concentration, there was significant color development, the ELISA reading was 0.080 and control value was 0.052 (Table 3 and Plate 10).

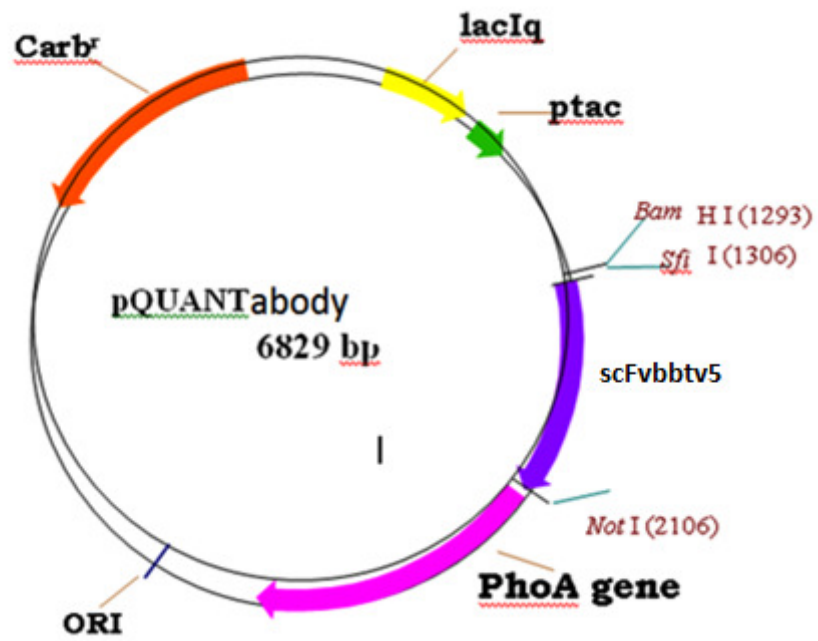


Fig 1(a): pBSNMALP5 vector map

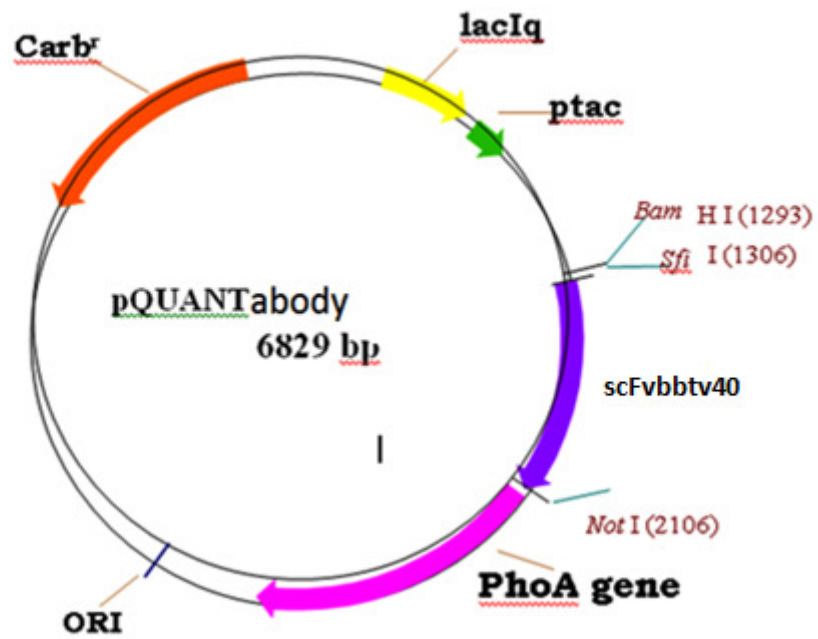


Fig 1(b): pBSNMALP40 vector map

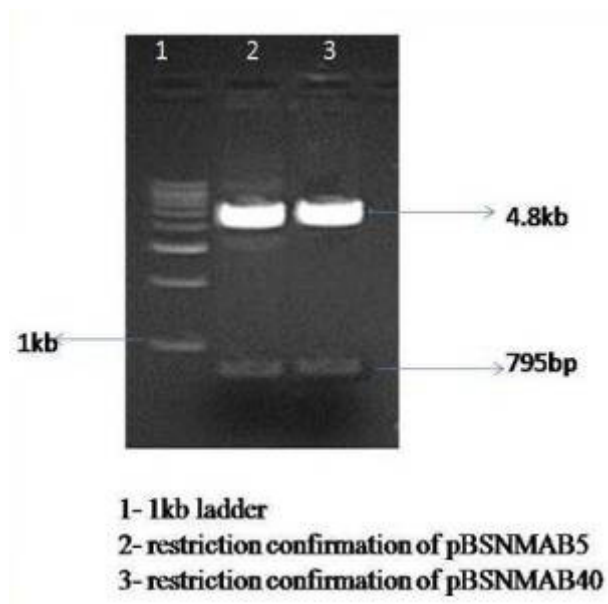


Plate 5: Restriction confirmation of antibody clones

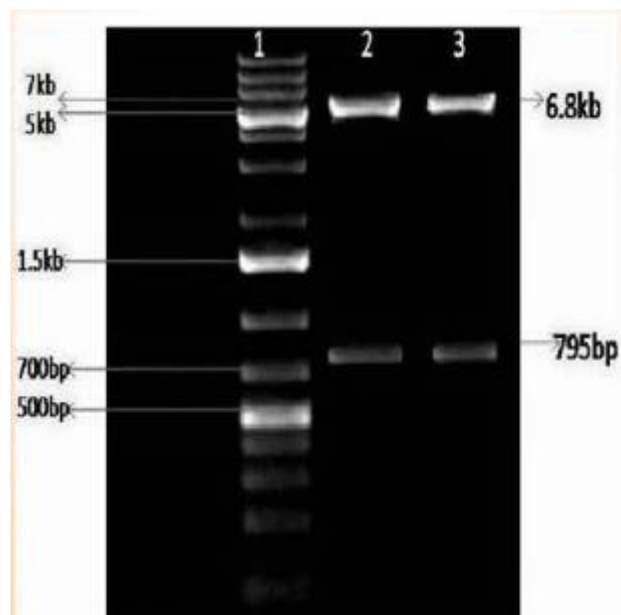


Plate 6: Confirmation of cloned pAUANTabody releasing scFv fragment

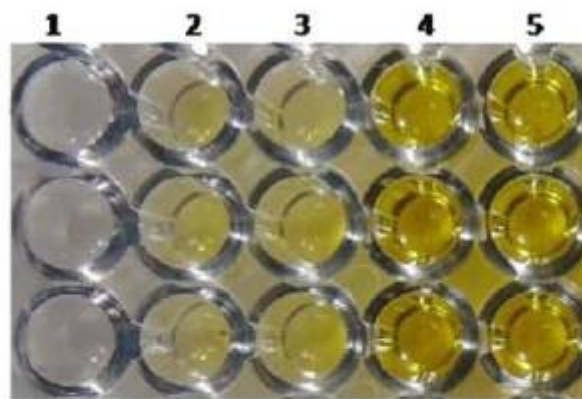
1	ggc	cca	gcc	ggc	cat	ggc	cgt	acg	tag	cac	act	ggt	gca	gca	gta	45
1	Gly	Pro	Ala	Gly	His	Gly	Arg	Thr	End	His	Thr	Gly	Ala	Ala	Val	15
46	gta	agt	ttg	caa	aag	gct	tca	gga	cac	aga	ctg	cgg	atc	cgt	gag	90
16	Val	Ser	Leu	Gln	Lys	Ala	Ser	Gly	His	Arg	Leu	Arg	Ile	Arg	Glu	30
91	act	gaa	atc	tgt	cag	caa	atc	caa	tga	cac	tga	aca	gtg	atg	gga	135
31	Thr	Glu	Ile	Cys	Gln	Gln	Ile	Gln	End	His	End	Thr	Val	Met	Gly	45
136	cca	tca	cac	ttt	gca	aac	agg	atg	cac	cga	aga	tca	gga	gag	tta	180
46	Pro	Ser	His	Phe	Ala	Asn	Arg	Met	His	Arg	Arg	Ser	Gly	Glu	Leu	60
181	agg	tgt	ctt	tcc	ctg	gtt	tct	gct	gga	tac	caa	aca	aat	tac	gca	225
61	Arg	Cys	Leu	Ser	Leu	Val	Ser	Ala	Gly	Tyr	Gln	Thr	Asn	Tyr	Ala	75
226	gct	aat	gct	caa	gac	gtg	ccc	ggc	aca	gtg	atg	ggg	gac	taa	gtc	270
76	Ala	Asn	Ala	Gln	Asp	Val	Pro	Gly	Thr	Val	Met	Gly	Asp	End	Val	90
271	taa	cac	tat	tga	tgt	cgt	aca	ggg	aag	atg	gag	act	ggg	tca	tcg	315
91	End	His	Tyr	End	Cys	Arg	Thr	Gly	Lys	Met	Glu	Thr	Gly	Ser	Ser	105
316	gga	tct	ccg	ccg	tac	acg	cca	ccg	ccg	aag	aca	cct	ccg	cct	gaa	360
106	Gly	Ser	Pro	Pro	Tyr	Thr	Pro	Pro	Pro	Lys	Thr	Pro	Pro	Pro	Glu	120
361	ccg	cct	cca	ccg	ctc	ggg	aga	cgc	tga	cca	cgg	gct	cca	tgg	ccc	405
121	Pro	Pro	Pro	Pro	Leu	Gly	Arg	Arg	End	Pro	Arg	Ala	Pro	Trp	Pro	135
406	cag	tag	tca	aaa	gaa	gca	taa	gca	gtc	gca	cag	gaa	tat	acg	gcc	450
136	Gln	End	Ser	Lys	Glu	Ala	End	Ala	Val	Ala	Gln	Glu	Tyr	Thr	Ala	150
451	gcg	tcc	tcg	gct	ctc	agg	ctg	ttc	att	tgc	aga	tac	agc	gtg	tac	495
151	Ala	Ser	Ser	Ala	Leu	Arg	Leu	Phe	Ile	Cys	Arg	Tyr	Ser	Val	Tyr	165
496	ttg	aga	cag	agt	cac	cgg	aga	cgg	tga	acc	ggc	aac	ttt	cag	cgg	540
166	Leu	Arg	Gln	Ser	His	Arg	Arg	Arg	End	Thr	Gly	Asn	Phe	Gln	Arg	180
541	agt	gcg	gcg	taa	ctg	aag	cag	cac	cag	cag	aat	aaa	tag	aag	aga	585
181	Ser	Ala	Ala	End	Leu	Lys	Gln	His	Gln	Gln	Asn	Lys	End	Lys	Arg	195
586	ccc	aat	cca	gcc	cct	tcc	cgg	gag	ccc	ggc	gga	ccc	agc	tca	tgg	630
196	Pro	Asn	Pro	Ala	Pro	Ser	Arg	Glu	Pro	Gly	Gly	Pro	Ser	Ser	Trp	210
631	cat	agc	tgc	taa	agg	tga	atc	cag	agt	gct	gca	cag	gag	agt	ctc	675
211	His	Ser	Cys	End	Arg	End	Ile	Gln	Ser	Ala	Ala	Gln	Glu	Ser	Leu	225
676	agg	gac	ccc	cac	agg	ctg	tac	caa	gcc	tcc	ccc	aga	ctc	caa	cag	720
226	Arg	Asp	Pro	His	Arg	Leu	Tyr	Gln	Ala	Ser	Pro	Arg	Leu	Gln	Gln	240
721	tct	gca	ccg	tcg	gaa	cca	taa	ccg	gat	ggg	ccg	tcg	agc	aag	ggc	765
241	Ser	Ala	Pro	Ser	Glu	Pro	End	Pro	Asp	Gly	Pro	Ser	Ser	Lys	Gly	255
766	aac	aat	cca	gcg	gaa	atg	aag	tgc	ggc	cgc						795
256	Asn	Asn	Pro	Ala	Glu	Met	Lys	Cys	Gly	Arg						

Fig 2 : scFv sequence result (pBSNMALP5 and pBSNMALP40)

Nucleotide and amino acid sequence of pBSNMALP5 and pBSNMALP40 clones encoding scFv monoclonal antibody against BBTv coat protein

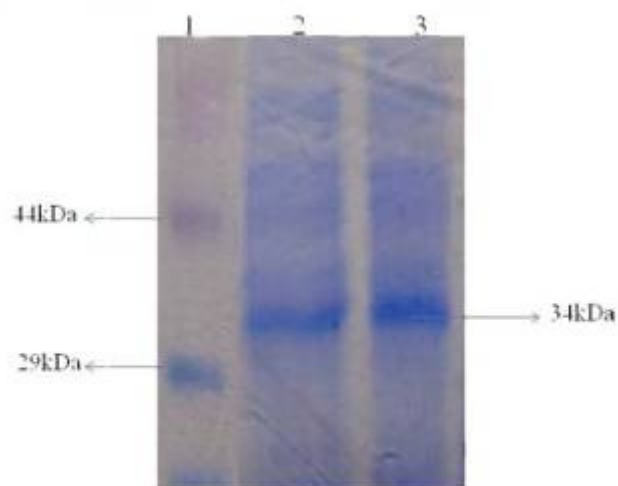
Table 1. Blast n results

BLAST Type	Accession No.	E value	Match	Per cent identity
BLASTn	JN887438.1	0.00	Synthetic construct anti-TNF alpha single chain Fv antibody gene, partial cds	85.00
BLASTn	DQ375454.1	0.00	Synthetic construct clone D1 anti-TREM-like transcript-1 antibody gene, complete cds	85.00
BLASTn	DQ375453.1	0.00	Synthetic construct clone C10 anti-TREM-like transcript-1 antibody gene, complete cds	85.00
BLASTp	BAJ52218.1	5e – 20	Immunoglobulin gamma heavy chain (Homo sapiens)	86.00
BLASTp	ABF83223.1	1e – 19	Circulating B cell antibody heavy chain variable region (Homo sapiens)	86.00



- 1- Only buffer
 2- Only antigen
 3- Only antibody
 1- pBSNMALP5
 2- pBSNMALP40

Plate 7: ELISA reading of expressed scFv fragment in pQUANTabody vector

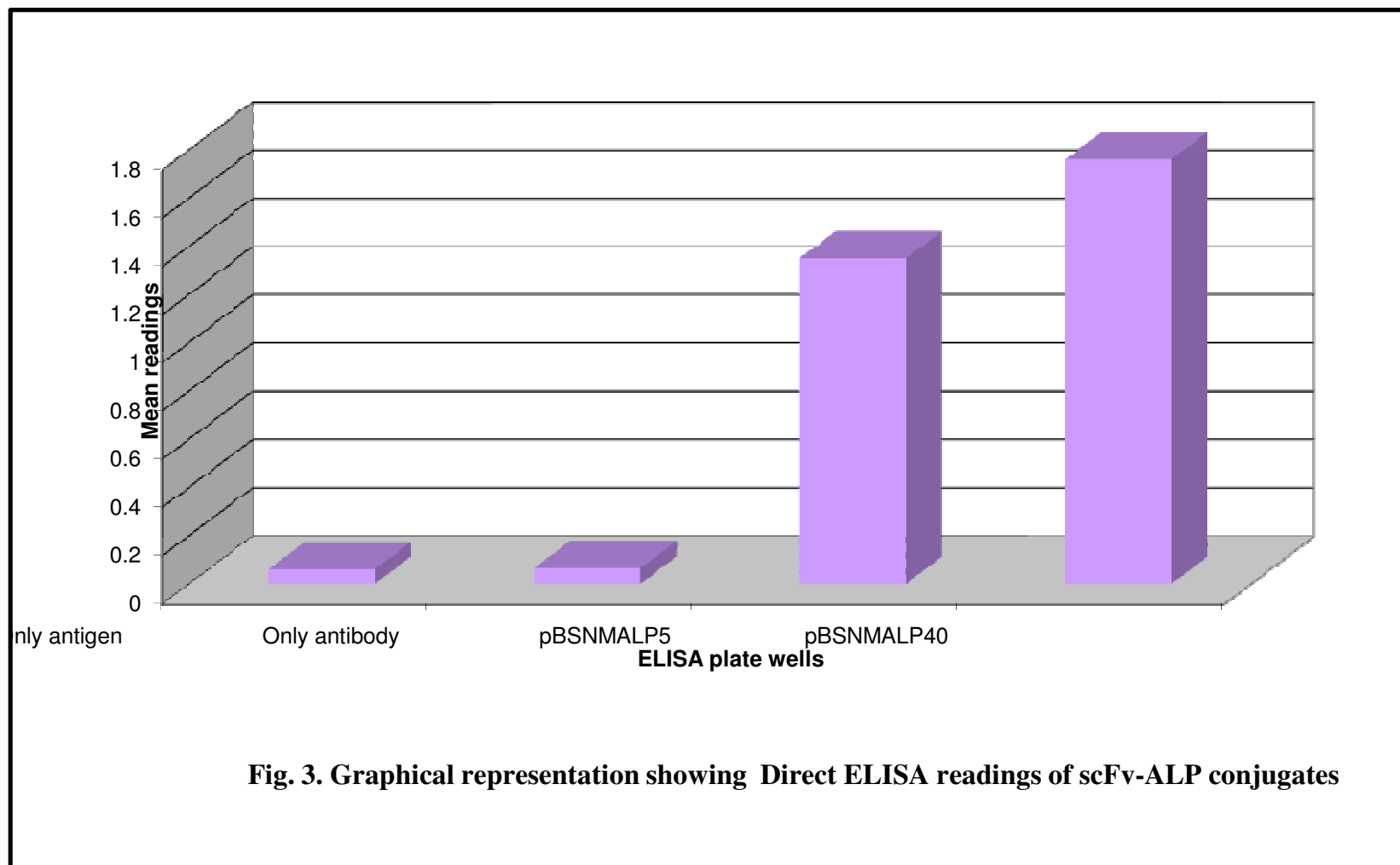


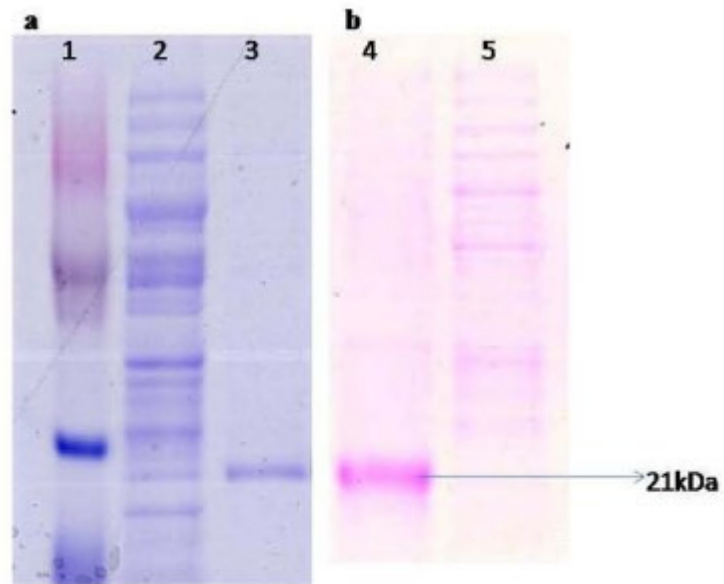
- 1- Protein marker
 2- pBSNMALP5
 3- pBSNMALP40

Plate 8: SDS PAGE analysis of BBTv antibody clones

Table 2. Direct ELISA readings of scFv-ALP conjugated antibodies

ELISA plate wells	Mean readings
Only antigen	0.061
Only antibody	0.065
pBSNMALP5	1.35
pBSNMALP40	1.76

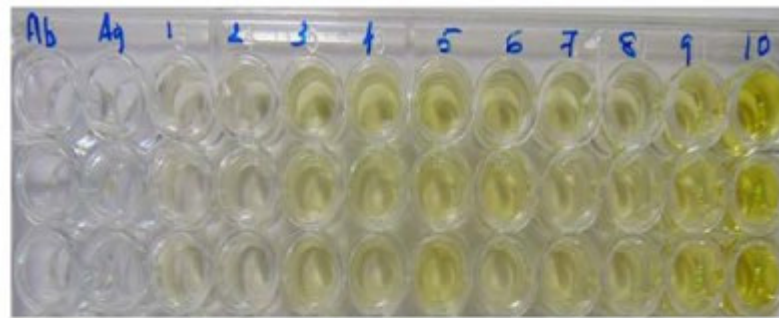




a- SDS PAGE
1- Protein marker
2- Healthy sample
3- Purified protein

b- Western blot
4- Purified protein
5- Healthy sample

Plate 9 : Western blot analysis of BBTV purified protein with BBTV antibody



Ab – only antibody	5- 5µg antigen
Ag- only antigen	6- 6µg antigen
1- 1µg antigen	7- 7µg antigen
2- 2µg antigen	8- 8µg antigen
3- 3µg antigen	9- 9µg antigen
4- 4µg antigen	10- 10µg antigen

Plate 10a: Standardization of antigen concentration in ELISA



pBSNMALP5

1- 0.8 µg/ml of BBTv antigen
 2- 0.9 µg/ml
 3- 1.0 µg/ml

pBSNMALP40

4- 1.1 µg/ml
 5- 1.2 µg/ml
 6- 1.3 µg/ml

Plate 10b: Standardization of antigen concentration in Dot Blotting Immunouno Assay

Table 3. Standardization of antigen concentration with ELISA

ELISA plate well	Mean readings
A1B1C1	0.052
A2B2C2	0.048
A3B3C3- 1µg	0.071
A4B4C4- 2µg	0.078
A5B5C5- 3µg	0.080
A6B6C6- 4µg	0.089
A7B7C7- 5µg	0.092
A8B8C8- 6µg	0.098
A9B9C9- 7µg	0.101
A10B10C10- 8µg	0.118
A11B11C11- 9µg	0.123
A12B12C12- 10µg	0.135

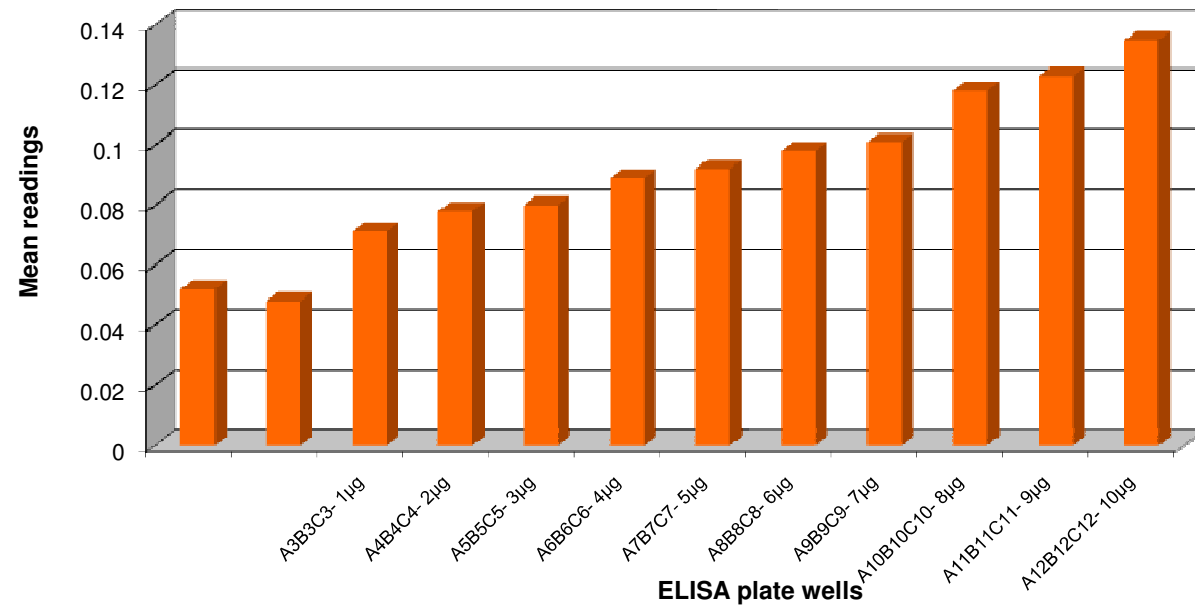
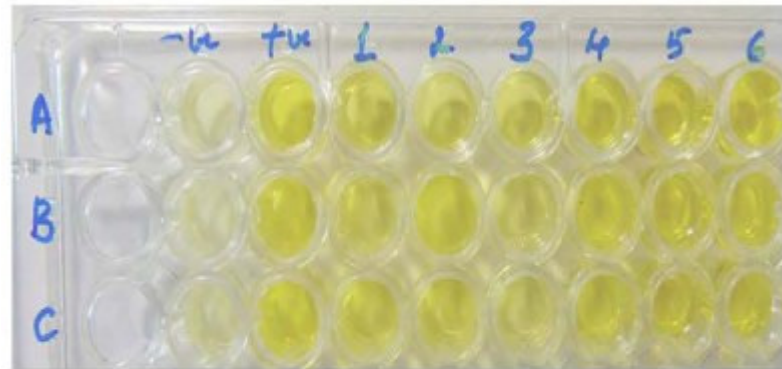


Fig. 4. Graphical representation showing Standardization with ELISA

A1B1C1
A2B2C2



-ve control - Healthy sample	A3B3C3- Chickmagalur
+ve control - Purified protien	A4B4C4- Shimoga
A1B1C1- Hassan	A5B5C5- Belgaum
A2B2C2- Dharwad	A6B6C6- Uttara Kannada

Plate 11a: Validation of different on farm field samples using ELISA



- 1- Infected field sample from Uttara Kannada
- 2- Infected field sample from Dharwad
- 3- Infected field sample from Belgaum
- 4- Infected field sample from Hassan
- 5- Infected field sample from Shivamogga
- 6- Infected field sample from Chickmagalur

Plate 11b: Validation of different on farm field samples using NC strip

Table 4. ELISA readings depicting Validation of infected on farm field samples

ELISA plate well	Mean readings
Only buffer	0.048
Healthy sample (-ve control)	0.359
Purified protein (+ve control)	1.23
A1B1C1 (Hassan)	0.652
A2B2C2 (Dharwad)	0.665
A3B3C3 (Chickmagalur)	0.552
A4B4C4 (Shimoga)	0.992
A5B5C5 (Belgaum)	1.031
A6B6C6 (Uttara Kannada)	1.106

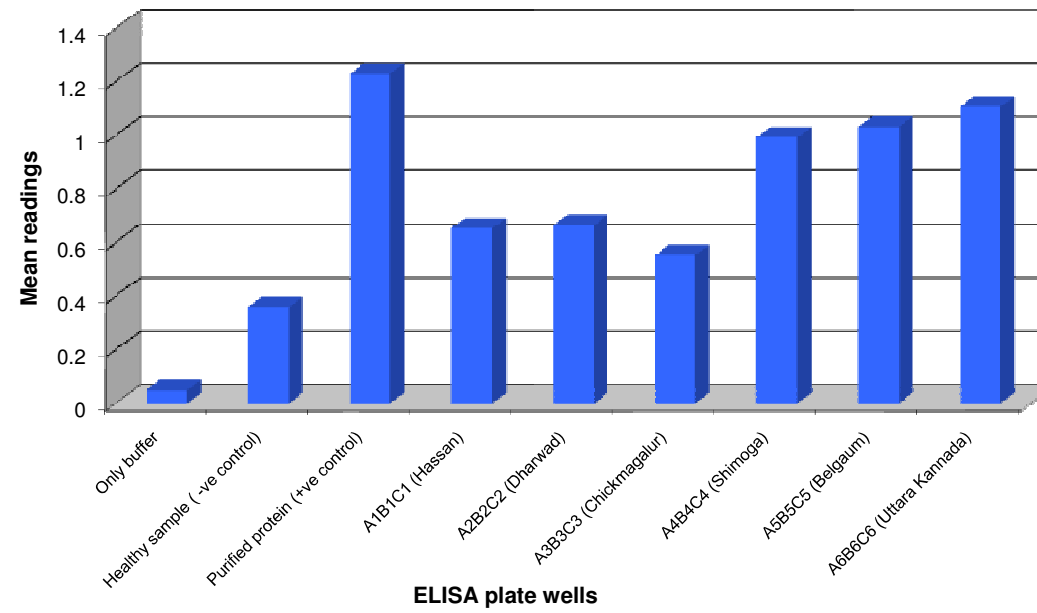


Fig. 5. Graphical representation showing Validation of infected on farm field samples in ELISA



Plate 12a: Immunodiagnostic kit to detect BBTV

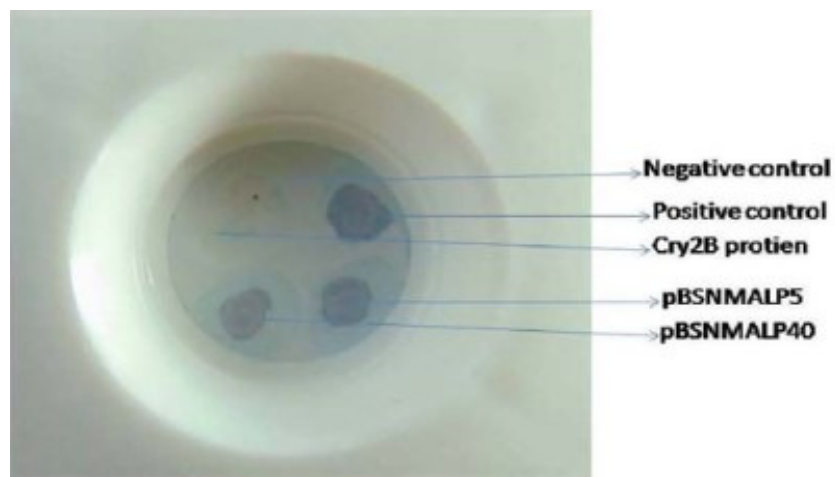


Plate 12b: Detection of BBTV samples in Rapidot cassette using kit

BBTV infected on farm field samples from 6 different places were reacted with antibody-ALP conjugates by both ELISA and DBIA on NC strip. Field sample from Uttara Kannada showed OD value of 1.176 and color development which was highest among other field samples and Chickmagalur (OD value of 0.552) showed color development which was lower than other samples (Table 4 and Plate 11).

5. DISCUSSION

Banana bunchy top virus (BBTV), a complex circular single stranded DNA virus with multiple genomic components and destructive pathogen in banana-cultivating areas worldwide. The symptoms induced by BBTV are similar to those caused by abiotic factors and other vascular diseases. This lack of unambiguous symptomatology necessitates production of reagents for the reliable diagnosis of BBTV infections. The most commonly used diagnostic tool for BBTV is Enzyme-Linked ImmunoSorbent Assay, which is dependent on the availability of highly specific antisera to differentiate the viruses. While some BBTV can be purified easily, others remain difficult to extract sufficient quantities for antiserum production. To test the ability of producing antiserum to BBTV by expressing the coat protein in *E. coli* and then immunizing the animal, here a technique called phage display technology which avoids scarifying the animal and even produces the stable monoclones was used. It has capacity to produce monoclonal antibody against any antigen (Griffiths *et al.*, 1994).

Phage display technique has been successfully attempted in producing monoclonal antibodies against toxins, hormones, growth factors, chemical, protein, enzyme, viruses, fungi and bacteria (Geuijen *et al.*, 2005). The success rate of monoclonal antibodies production is hundred per cent by using phage display technique depicted in many research reports (Griffiths *et al.*, 1994; Francoise *et al.*, 2005 and Anderson *et al.*, 2007). In this study, single chain fragment variable antibodies which were produced against BBTV (pBSNMAB5 and pBSNMAB40) present in pIT2 vector were used to develop Immuno-Diagnostic kit for the detection of BBTV.

5.1 Expression and characterization of pBSNMAB5 and pBSNMAB40 antibodies

scFv gene in pIT2 vector as pBSNMAB5 and pBSNMAB40 is present in fused form with gIII gene. The antibody protein produced consists of both scFv and gIII. So the scFv fragment when restricted from pIT2 vector and cloned into expression vector like pQUANTabody will produce highly expressed and specific antibody protein. The pQUANTabody vector contains alkaline phosphatase gene which acts as secondary antibody in determining its specificity to antigen in less time. The present study starts with the confirmation of antigen and antibody in PCR and restriction digestion, and extracting the antigen protein through IPTG induction method.

One of the important applications of gene technology is the over production of different proteins that can be utilized as pharmaceutical agents and as antigen for the production of antibodies or as tools for structural and functional analyses (Janknecht *et al.*, 1991). The construct pBSNMCP30 in *E. coli* M15 was induced by IPTG. The expected band size of 21kDa was observed in BBTV induced sample and in uninduced sample; the corresponding band was very less intense. This confirmed the presence of protein of interest for further purification of induced protein.

The induced protein was purified using nickel affiliated columns. The columns follow the principle of metal chelate or immobilized metal affinity chromatography (IMAC). Typically, 5-6 histidine residues ("tag") were added to the C or N terminus of target protein using recombinant techniques. This tag is responsible for purification of target protein because it interacts with the chelated metal ions present in the column, thereby holding this protein in the column itself and remaining other proteins to wash out. The strong hold protein was eluted with competitive eluting agent like imidazole. The purified protein was subjected to SDS-PAGE and expected single band of 21kDa was observed. This purified protein was used as antigen for production of monoclonal antibodies. The reason for purification of induced protein is in order to avoid the development of antibodies for other host cell proteins (*E. coli* M15). So that developed antibody should be specific to protein of our interest (BBTV).

Single chain antibody fragments (scFv) genes of pBSNMAB5 and pBSNMAB40 were subcloned into pQUANTabody expression vector which enabled to transcriptionally fuse with scFv monoclonal antibody genes and the gene coding for bacterial alkaline phosphatase (PhoA) enzyme. Upon expression of clone, it has produced 34kDa fusion protein of ALP along with pBSNMAB5 and pBSNMAB40 monoclonal antibody. After digesting the pQUANTabody vector, the pBSNMAB5 and pBSNMAB40 clones with *Sfi-I* and *Not-I* enzymes, the digested sample was ligated and transformed into competent *E. coli* DH5 α . The transformed colonies grown on selection medium containing ampicillin 100 μ g/ml and NBT-BCIP, the blue colonies were picked and tested by PCR using phoA forward and phoA reverse primers for the insert. The clones with the right amplicon were picked based on gel electrophoresis. This type of work that monoclonal scFv antibody-ALP conjugate produced against Cry2B protein was first time reported by Shashikumara *et al.* (2012). This is similar to the finding of other workers (Mousli *et al.*, 1998) who used crude preparations containing the conjugate in rapid visual immunoassay for the specific detection of *A. australis* hemocyanin.

The clones were sequenced and characterised, the clone pBSNMAB5 was of 795bp long and clone pBSNMAB40 had same length base pairs. When assembled the sequences of both the clones using Bio Edit software, it is revealed that both the clones were hundred per cent similar in their sequence. The assembled sequence of both clones was subjected to BLASTn and BLASTp analysis. The results indicated 85 per cent homology with synthetic construct anti-TNF alpha single chain Fv antibody gene, partial cds and further the BLASTp analysis showed that the 86 per cent homology with circulating B cell antibody heavy chain variable region [*Homo sapiens*]. The analyses of conserved domains of both proteins were showed the existence of similar domains and the sequence homology to Ig superfamily was evident. The amino acid sequence of both proteins was showed almost similar amino acid sequences. In this back ground we can predict that the both scFv antibodies bind to single epitope.

5.2 Standardization and development of immunoassays for Banana Bunchy Top Virus

The low volume of the sample required for Dot Immuno-Binding Assays is an important advantage over ELISA in analyzing samples without dilution. One advantage of the immune-dot blot technique is that the membranes used to bind the proteins also allow their retention of antigenicity and accessibility to antibody (Zein *et al.*, 2007). SDS-PAGE and western blot analysis of the purified recombinant CP revealed the presence of a major protein band with an estimated molecular mass of 21 KDa. Antibody serum at a dilution of 1:1000 showed the binding of 6x- His tag fusion coat protein of BBTV.

The advantage of using the transcriptionally fused antibody is to save time and cost. We have developed anti-BBTV-ALP conjugate antibody for BBTV. Single chain antibodies developed here are advantageous over traditional monoclones because the production costs are less and stability of single chain antibodies during storage is high due to its small size. The monoclonal antibody conjugates prepared as crude extract readily detected BBTV even at low concentrations (0.9 µg/ml) in NC membrane and at 3µg/ml in ELISA. This was similar to the finding of other workers (Mousli *et al.*, 1998) who used crude preparations containing the conjugate in rapid visual immunoassay for the specific detection of *A. australis* hemocyanin.

The RapiDot immuno assay have developed using monoclonal antibody (pBSNMAB5 and pBSNMAB40 as capture and pBSNMALP5 and pBSNMALP40 conjugates as detection) proved to be efficient and reliable for BBTV detection. The antibody conjugates able to detect the antigen BBTV in the span of 20-25 minutes. Similar results were obtained (Gautam *et al.*, 1999) against *Phytophthora nicotianae* spores and (Shashikumara *et al.*, 2012) against Cry2B. The assay does not require any sophisticated equipment nor can highly trained technical staff for carrying out this assay. It was used to validate different infected field samples to detect the presence of BBTV infection.

5.3 Validation of RapiDot Immuno assays against field infected samples from different locations

Antibody serum at a dilution 1:1000 showed binding of BBTV coat protein. Antibody serum was prepared at 1:1000 dilution with 1x PBS buffer. Immune-Diagnostic kit was developed for the detection of BBTV infected samples. This developed kit was used to validate different on-farm field infected samples from different location to detect presence of BBTV infection. Infected leaf samples were collected from six different places viz. Uttara Kannada, Dharwad, Belgaum, Shivamogga, Hassan and Chikmagalur. These samples were tested in Diagnostic kit using both ELISA and NC strip. Among the six different isolates from different places selected for the study, the isolates from Uttar Kannada district was showed higher intense of color development. Dharwad, Belgaum and Shivamogga isolates were showed detectable color development when compared to Uttar Kannada isolates. Whereas Hassan and Chickmagalur isolates were showed low intense of color development. The difference in color intensity was due to varying viral loads in the collected infected samples.

The cost of production of RapiDot immuno assay is reasonable. The components of the RapiDot immuno assay are wash buffer (solution-1), conjugate antibody solution (solution-2), substrate buffer (solution-3) and blocking solution provided with both NC strips and membrane cassettes. The overall cost of RapiDot immuno assay for 50 reactions is about Rs.2000.

6. SUMMARY AND CONCLUSIONS

Developments in molecular detection techniques led to the development of highly convenient and specific assays and permitted the use of these tests for detecting the plant pathogens, including viruses. Banana bunchy top virus (BBTV) is one of the serious diseases in banana plantation and need to be detected in early stage to manage the disease. Development of specific assays like immunological assays to detect BBTV, will help the growers, agronomists and plant health professionals not to rely exclusively on symptomology and permits early detection of viral infection. Phage Display Technology was used in the present study, Development of Immuno-Diagnostic kit for the detection of Banana Bunchy Top Virus with high specificity.

The antigen protein was isolated by inducing the *bbtv* gene pQE30 vector and it was purified using His-tag purification kit. The purified BBTV protein was used as antigen at 100 µg/ml concentration.

Two scFv monoclonal antibody clones, viz. pBSNMAB5 and pBSNMAB40 which showed strong binding specificity to the target antigen (BBTV) than the other monoclonal antibodies were used. In pBSNMAB5 and pBSNMAB40, gene responsible for scFv was amplified by the specific primers (LMB3 forward and pHEN reverse primer) showed 990bp.

The scFv genes of both clones were restricted using restriction enzymes *Sfi*I and *Not*I showed 795bp long. These fragments were then cloned into pQUANTabody expression vector and transformed into *E. coli* DH5α and they were found to have similar sequence. The scFv genes of both clones were 795 bp long and they found to share similar homology sequence. BLASTn and BLASTx analysis results indicated 85 per cent homology with Synthetic construct anti-TNF alpha single chain Fv antibody gene, partial cds and further the BLASTx analysis showed that the 86 per cent homology with circulating B cell antibody heavy chain variable region [*Homo sapiens*].

Protein was isolated from pBSNMALP5 and pBSNMALP40 and used to detect antigen protein in western blot. Antibody protein detected antigen protein and formed hybridization at 21kDa on polyvinylidene fluoride (PVDF) membrane.

The selected scFv gene was used in Rapidot Immunodiagnostic kit with fusion antibodies through expressing in expression vector. This Rapidot Immunodiagnostic kit detects the BBTV as low as 0.9 µg/ml concentration in both NC membrane and membrane cassette. The Rapidot Immunodiagnostic kit is rapid, simple, economical and farm friendly.

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Appendix I: Media, broth and buffers for phage isolation and amplification

1. 2xTY: pH 7.2

Composition	Grams / liter
Tryptone I	16.0
Yeast extract	10.0
Sodium chloride	5.0
Water	Make up to 1 lt

2. TYE: pH 7.2

Composition	Grams / liter
Tryptone I	10.0
Yeast extract	5.0
Sodium chloride	8.0
Agar Agar Type I	15.0

3. Top agar: pH 7.2

Composition	Grams / liter
Tryptone I	16.0
Yeast extract	10.0
Sodium chloride	5.0
Agar Agar Type I	7.5

4. Phosphate buffer saline (PBS), pH 7.4: for 1x

Composition	Recipe
Sodium chloride	137mM
Potassium dihydrogen phosphate	2mM
Disodium hydrogen phosphate	10mM
Potassium chloride	2.7mM

5. PEG/NaCl

Composition	Grams/ 100 ml
Polyethylene glycol	20.0
Sodium chloride	14.6.

6. Carbonate buffer: pH 9.6

Composition	Grams/100ml
Disodium carbonate	1.59
Sodium bicarbonate	2.93

7. PBS-Tween 20

Composition	For 100ml
1x PBS	99.95 ml
Tween 20 (0.05%)	0.05 ml

8. Substrate solution

Substrate	Working concentration
NBT	50 μ l/10 ml substrate buffer
BCIP	33 μ l/10 mL substrate buffer
X-GAL	20 mg/ml substrate buffer
TMB	1 ml/20 ml dilution buffer

9. Substrate buffer (PH 9.8)

Diethanolamine 97ml, add distilled water to 800ml, adjust pH to 9.8 with con. HCl and make up the volume to 1L with distilled water.

10. Substrate solution

Add 66 μ l of NBT (stock solution: 20 mg/ml in distilled water) and 33 μ l BCIP (stock solution: 20 mg/ml in dimethyl formamide) into 10ml Substrate buffer (pH: 9.8).

Appendix II: Media and broth for *E. coli* strains

1. Luria Agar: pH 7.2

Composition	Grams/liter
Tryptone I	10.0
Yeast extract	5.0
Sodium chloride	5.0
Agar Agar Type I	18.0
Water	Make up to 1 lt

2. Luria broth: pH 7.2

Composition	Grams/liter
Tryptone I	10.0
Yeast extract	5.0
Sodium chloride	5.0
Water	Make up to 1 lt

Appendix III: Plasmid isolation solution

1. STET

Composition	Recipe
Tris-cl (pH-8.0)	10 mM
Sodium chloride	0.1 M
EDTA (pH-1mM)	1 mM

2. Alkali lysis solution I

Composition	Recipe
Glucose	50 mM
Tris-cl (pH-8.0)	25 mM
EDTA (pH-8.0)	25 mM

3. Alkali lysis solution II

Composition	Recipe
Sodium hydroxide	0.2 N
Sodium lauryl sulphate	1% (w/v)

4. Alkali lysis solution III

Composition	Recipe
5M potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Double distilled water	28.5 ml

Appendix IV: Agarose gel

1. Recipe for 1% of agarose gel (100ml)

Composition	Recipe
Agarose	1 gm
1x TAE buffer	100 ml
EtBr (10mg/ml)	4 μ l

2. TAE Buffer: pH 8.0

Composition	50x for liter
Tris base	242.0 gm
Glacial acetic acid	57.0 ml
0.5M EDTA (pH 8.0)	100.0 ml

Appendix V: SDS PAGE Solution preparation

1. Polyacrylamide solution

Composition	Grams / 100 ml
Acrylamide	29.0
Bisacrylamide	1.0
Double distilled water	Make upto 100 ml

2. SDS loading dye (1x)

Composition	Recipe
Tris-cl (pH: 6.8)	50 mM
DTT	100 mM
SDS (w/v)	2%
BPB	0.1%
Glycerol	10% (v/v)

3. Staining solution

Composition	Recipe for 1 lt
Methanol	500 ml
Acetic Acid	100 ml
Coomassie brilliant blue R-250	2.5 gm

Bacterial strain

Bacterial strains	Source
<i>E. coli</i> DH5 α	Genei

Appendix VI : Western blot preparation solution

1. Transfer buffer

Composition	Recipe for 1lt
Tris	25 mM
Glycine	190 mM
Methanol	20%
Ph	8.3

2. TBS

Composition	Recipe
TBS	20mM
Nacl	150mM

DEVELOPMENT OF IMMUNODIAGNOSTIC KIT FOR THE DETECTION OF BANANA BUNCHY TOP VIRUS (BBTV)

NAMRATHA S. D.

2016

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ABSTRACT

Banana Bunchy Top Virus (BBTV) was used as an antigen to produce single chain fragment variable (scFv) antibody using phage display technology. Two clones showing highest reading in monoclonal ELISA were selected viz., pBSNMAB5 and pBSNMAB40. Single chain antibody fragments of pBSNMAB5 and pBSNMAB40 were subcloned into pQUANTabody expression vector which enabled to transcriptionally fuse scFv monoclonal antibody fragment and the gene coding for bacterial alkaline phosphatase (PhoA) enzyme. The clones were sequenced with LMB3 forward and pHEN reverse primer and characterized. The scFv genes of both clones were 795 bp long. BLASTn and BLASTx analysis results indicated 85 per cent homology with Synthetic construct anti-TNF alpha single chain Fv antibody gene, partial cds and 86 per cent homology with circulating B cell antibody heavy chain variable region [*Homo sapiens*]. Upon expression of clone, it has produced fusion protein of ALP along with pBSNMAB5 and pBSNMAB40 monoclonal antibody against BBTV protein.

In Western blot, the antiserum could recognize the expressed protein showing hybridization at 21kDa and in DBIA the BBTV antiserum at 1:1000 dilutions showed strong positive signal with 50ng of expressed protein. Using this antibody conjugate, a detection kit was developed. This Rapidot Immunodiagnostic kit detects the BBTV as low as 0.9 µg/ml concentration in both NC membrane and membrane cassette and it was also cross checked with other proteins to evaluate the specificity of monoclonal antibodies raised against BBTV. Validation of the detection kit was done using BBTV infected on farm field samples from 6 different places, they were reacted with antibody-ALP conjugates by both ELISA and DBIA on NC strip. Colour development with varying OD values were observed.