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Review Article

Primers in Plant Pathology Bonanza for Plant Disease Diagnosis

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A B S T R A C T

In present time due to climate change new and severe disease are appearing in plants and its leading to crop loss especially in the agriculture sector. For its effective management accurate detection of pathogen not only up to genus level but up to species and strain level is indispensable. Many methods are available, but at current time molecular method are more reliable for plant pathogens such as fungi, bacteria, virus, phytoplasma and viroids. In this review, we discussed primer based detection of some important plant pathogens. These studies have been combined for better understanding and to show its significance in plant disease diagnosis. We also provided primer sequences for discussing pathogen based on the research studies from initial to current time. This review summarizes the quick understanding, a compilation of ideas and results of some significant studies and its achievement.

Keywords

Plant disease, diagnosis, molecular technique, primer based detection

Introduction

Diagnosis of any disease is a very crucial step that determines the cure of associated disease. Diagnosis can be performed *via* different methods includes identification and taxonomy. Diagnosis is based upon the "cause and effect". A plant is said to be diseased when its normal functions are disturbed and harmed (Holliday, 1989). In plant pathology disease is associated with the infectious micro-organism such as fungi, oomycetes, bacteria, viruses, viroids, virus-like organisms, phytoplasmas, protozoa, nematodes and parasitic plants (Agrios, 2005). Many laboratories are still using conventional isolation and diagnosis technique in the laboratories and some have adopted the advanced techniques. The impact of bio-technological approaches in plant disease diagnosis is essential to increase the effectiveness and precise diagnosis (Barnes, 1986). Polymerase chain reaction (PCR) provides simple method for amplification of small fragments of DNA. The specificity and accuracy of synthesis depend on the synthetic oligonucleotide primer (Henson and French, 1993).

Need of primer based PCR method over conventional method

Prior culture of the organism is not required because the technique is extremely sensitive. It can detect even a small nucleotide from a complex of mixture. Radioactive probes are not required for detection. It is quick and proficient method. It can detect single

pathogen or multiple pathogens based on primer choice. Cost is low compared to serological method (Henson and French, 1993). Amplification can be obtained even if the DNA quality is poor (Joshi and Deshpande, 2011). Primer based PCR technology is time saving, rapid and most important it is providing high quality data for accurate diagnosis. In a recent scenario Real Time- PCR data is considered to be standard in any aspect including disease diagnosis or it may be determination of nucleic acid (Bustin, 2010). Hence we can say that for accurate determination most reliable analysis can be performed using primer based technology which is intensive and time saving. Application of primer based technique in plant disease diagnosis increased our understanding and it helped us to correlate it with environmental effect as well as its progression in the population. It is also useful in distinguishing plant bacteria from phytoplasma because they both share common principles (Martin *et al.,* 2000). Primer based technique is a direct method of detection and large number of samples can be analysed in a short period of time with greater accuracy (Fang and Ramasamy 2015). Real time- PCR work in rapid cycling and produces higher number of copies and give its expression as well. In an era of genomics where many genomic sequence databases are available real time PCR is highly recommended for direct diagnosis of pathogens (Schaad and Frederick, 2002).

Primer

A primer is a short strand of RNA or DNA that serves as a starting point for DNA synthesis. The basic principle of replicating a piece of DNA using two primers had already been described by Gobind Khorana in 1971. Progress was limited by primer synthesis and polymerase purification issues (Kleppe *et al.,* 1971).

Selection criteria

Primer length

Length of the primer should be 18-20 nucleotide long (Dieffenbach *et al.,* 1995). Melting temperature and GC% are the most general criteria for primer selection (Ye *et al.,* 2012) but the length of the primer is also considerable important criteria (Wu *et al.,* 1991). Primers should maintain minimum range of length to avoid the secondary hybridization also it should not contain a single base repeatedly.

Melting Temperature (Tm)

The most important criteria for primer designing are melting temperature. In PCR, we need to deal with two primer that is forward primer and another is a reverse primer hence it is necessary that difference of Tm between this two should not exceed more than 5^0C. Much temperature difference will lead to improper functioning of primer (Ye *et al.,* 2012).

Primer3 and and Primer BLAST software are available online by which we can design primer with optimum temperature. Manually Tm can also be calculated by using the formula– Tm = 2(A+T) + 4(G+C) (Wallace *et al.,* 1979). Generally Tm range should be between 56^0C to 62^0C (Dieffenbach *et al.,* 1993).

GC Content (%)

GC content and annealing temperature is dependent on each other, such as high GC content create problem during annealing (Abd-Elsalam, 2003). The GC content percentage should vary between 40-60 % (Dieffenbach *et al.,* 1995). High GC content at 3' end makes it too sticky hence increase the chance of mis-priming.

Target specificity

Binding of primers to target, segment and its amplification is more specific criteria. Sometime primer binds to non-target sequence and amplifies it which gives variable results (Van Guilder *et al.,* 2008). It is important to select such primer designing software that can detect such mismatches. Therefore, the combination of several primer designing tool such as Autoprime and Quantprime can be combined with Primer3 and Primer BLAST software (Ye *et al.,* 2012).

Complementarity

Primers should not have sequence homology within. It will create problem in hybridization even it may lead to cause dimerization of primer (Abd-Elsalam, 2003). So primer should be selected which is having as minimum as possible homology sequence.

Considering above fact a primer can be designed. Many online tools are available to design primers. With these tools size of product, Tm of primer, GC% and maximum self-complementarity should be filled carefully. Ye *et al.,* (2012) discussed in detail about handling Primer BLAST software so one can get ease in accessing it. Primer designing guidelines are necessary for appropriate diagnosis in case of plant diseases and also to avoid irrelevant data (Bustin, 2010).

Mile stone achievements of primer based technology

Bacterial Pathogens Detection

Bacterial diseases are important plants and in agriculture field, it is necessary to diagnose exact bacterial pathogen involved. Such diagnosis became more significant and accurate with help of molecular based primer technique such achievements discussed following-

In *Pseudomonas synringae* pv. *phaseolicola, argk-tox* gene which is responsible for the phaseolotoxin has been identified by a specific toxin (Puri *et al.,* 2015). In another study similar bacterial species were studied for pathogenic and non-pathogenic strains. In this study *P. synringae* pv. *phaseolicola* which cause halo blight of bean were detected by designing site specific recombinases primers for 29 isolates of 3 different pathovar of the *P. syringae* (Cho *et al.,* 2010). They reported that the phaseplotoxin production is not pathovar specific character. This study facilitated monitoring and management of most significant bacterial disease of bean. Srinivasa *et al.,* (2012) obtained species-specific patterns of plant pathogenic bacteria *Ralstonia solanacearum, Xanthomonas axonopodis* pv. *vasicatoria* and *Xanthomonas oryzae* pv. *oryzae* by using universal primer that is *16S rRNA* in table-1.

This study showed that the 16S rRNA primer is ideal primer for distinguishing and identifying plant pathogenic bacteria. Whole genome sequence of plant pathogenic *Xyllela fastidiosa* was conducted in 2000 by Simpson *et al.,* They reported that many genes are responsible as virulence for causing disease in many plants such as a grapevine. With similar pathogen which cause oleander leaf cause specific detection and identification performed by Huang *et al.,* (2009). They developed specific primer pairs for detection of *X. fastidiosa* strain by sequence analysis using RAPD-PCR because these strains considered being different from other *Xyllela* strains. PCR primers were also developed to identify *X.*

fastidiosa strain which cause citrus variegated chlorosis by Pooler and Hartung (1995). Specific primers for *Streptomyces* spp. showed that the *nec1* gene previously considered to be responsible for virulence is actually not wholly related with pathogenicity but provides assistance (Wanner 2004, 2006).

For Ti plasmid of *Agrobacterium* two pairs of primers were deigned one is universal which is present in all pathogenic strains of *Agrobacterium* that is virD2 a highly conserved region of endonuclease domain in plasmid and another one is primer for *ipt* which is specifically for *Agrobacterium tumifaciens. ipt* is responsible for the synthesis of cytokinin (Haas *et al.,* 1995).

Primers that are designed based on internal transcribed spacer region between *16S* and *23S r RNA* region considered to be more accurate and highly specific in the diagnosis (Palacio-Bielsa *et al.,* 2009). Primers are also reported in soft rot pathogen *Pectobacterium* spp. which produces pectiolytic enzyme that is *pel* gene (Louws *et al.,* 1999). More specific primers for *Ralstoinia solanacearum* and *Clavibacter michiganensis* were suggested by re-evaluation (Arahal *et al.,* 2004), more over the result generated after protocol should be interpreted correctly (Louws *et al.,* 1999). In rice crop qualitative composition of bacterial community was tested. In this experiment they use BOX PCR fingerprinting of total genomic DNA and for this primer were designed based on the interspread repetitive sequence which was able to distinguish bacterial population at a fine level. Several bacteria's such as *Xanthomonas, Pseudomonas, Clavibacter* and *Burkholderia* were identified (Cottyn *et al.,* 2001). This study diverted the focus not only on one bacterial population, but community of bacteria interacting in a single occasion.

A newly emerging bacterial disease of rice panicle blight of rice caused by *Burkholderia glumae* were characterized by molecular based technique. Previously, no practical method was available to identify the bacterium *B. glumae,* but Sayler *et al.,* 2006 developed a method by developing specific primers based on *16S-23S rDNA* sequence.This study enables the researcher to identify and characterize a pathogen without DNA isolation as well as gel electrophoresis. Similar way for *Xanthomonas compestris* pv. *viticola* (Xcv) causing grapevine canker primes were designed based on partial sequence of *hrB* gene. Primer pairs were Xcv1F/Xcv3R and RST2/Xcv3R were able to amplify 44 pathogenic strains of Xcv (Trindade *et al.,* 2007). *Xanthomonas fragariae* causal organism of angular leaf spot strawberry for this in a recent review of Mirmaj lessi *et al.,*(2015) standard PCR protocols were discussed. In this review 22 full texts were found most useful for accuracy and sensitivity of PCR for plant pathogenic organisms. Previously study on *Streptomyces scabies* conducted and primer for gene *txtAB* were designed and correlated with phytoalexin production thaxtomin (Bignell *et al.,* 2000). For similar pathogen total genomic DNA was six from six different locations and phylogenetic analysis of DNA conducted. They developed species specific PCR method which is effective and time saving (Barrera *et al.,* 2013). *Liefsonia xyli* pv. *xyli* were detected in infected fibro-vascular fluid of sugarcane plants (Taylor *et al.,* 2003).

Fire blight pathogen *Erwinia amylovora* were detected based on the presence of plasmid *pEA29* (Barionovi *et al.,* 2006). But in recent study pathogenic strains which lack plasmid can also be identified by newly designed primers that is sequence characterized amplified region (SCAR)

primers (Obradovic *et al.,* 2007). Fire blight pathogen and necrotic pathogen of peer strains in japan were distinguish with specific primers that pathogen of peer were identified as *Erwinia pyri* pv. *foliae* (Kim *et al.,* 2001). *Acidovorax avenae* subsp. *avenae* sequence has also been sequenced by molecular method (Xie *et al.,* 2011). *Xanthomonas oryzae* pv. *oryzae* which is potential pathogen of rice causes bacterial blight disease against which resistance gene has been cloned using specific primers for this particular gene (Wang *et al.,* 1996).

Fungal Pathogens Detection

Direct detection of pathogenic fungi is not only useful for rapid and accurate diagnosis, but it is also very useful in the detection of fungi which is non-cultural biotrophic, endophytic, and mycorrhizal groups. Primer based method pave path for accurate modelling and surveillance of pathogen (Tsui *et al.,* 2011). Recently genome for *Cochliobolus* spp. and its strains were sequenced. This includes strains of *C. heterostrophus, C. sativus, C. carbonum* and *C. miyabeanus*. With reference to these genomic sequences they studied on species differentiation, secondary metabolites and effector protein molecule (Condon *et al.,* 2013). In a study by McCrtaney *et al.,* (2003) molecular methods based on PCR are developed to study the variation in plant pathogen populations such as different mating type or virulence strains. It enables more reliable and faster method for decision support system.

Heterogeneity study on *Colletotichum acutatum* were conducted from diverse host pathogen on strawberry plant.This study was based on rDNA sequence (Sreenivasaprasad *et al.,* 1992). For similar crop strawberry recent study conducted but this time not only for *C. acutatum* but also other fungal pathogen such as *Fusarium oxysporium* f.sp. *fragariae*, *Phytophthora fragariae*, *Verticillium dahliae*, *Botrytis cinerea* and *Macrophomina phaseolina*. This was achieved through specific PCR based method (Mirmaj lessi *et al.,* 2015). Quarantine important pathogen *Puccinia horiana* on *Chrysanthemum* plant highly specific primers were designed based on rDNA for selected region ITS1 and ITS2. They concluded that high amount of DNA of pathogen accumulated at the end of latent period. This helps to understand the disease cycle (Alaei *et al.,* 2009).

ITS region is most considerable aspect of designing of primer (Capote *et al.,*2012). It has been used to distinguish *Verticillium albo*-atrum to *Verticillium dahlia* (Schena *et al.,* 2004). It also distinguishes the pathogenic *Fusarium* and non-pathogenic *Fusarium* strains in tomato (Validov *et al.,* 2011). Another ITS based primers were designed to identify *Ccolletotrichum capsici* (Torres-Calzada *et al.,* 2011). A combine method using forward and reverse primer combined with ELISA has been used for detection of *Pythium*s spp. and *Phytophthora* spp. (Bailey *et al.,* 2002). In 2005 Guo *et al.,* also achieved identification based on primers in reverse transcription PCR. The pathogen was *Mycosphaerella graminicola* with primer set *E1/STSP2R*. In recent study primers were designed based on ITS1, ITS2 and TEF (Transcription Elongation Factor) detect pathogen in citrus black spot disease. These primers identified *Phyllosticta citricarpa* as causative agent of disease which earlier was confused with *Guignardia mangiferae* (Glienke *et al.,* 2011).

For seed-borne fungus detection pathogen in vegetable crop has been achieved through modified PCR protocols such as loop mediated isothermal amplification, and non-

destructive testing methods have been proposed (Mancini *et al.,* 2016). Some fungi that are associated with seed infection are *Cercospora kikuchii* (Murakishi, 2002) and *Peronospora manshurica* (Agarwal *et al.,* 2006) in soybean, peanut seeds *Cylindrocladium parasiticum* (Randall-Schadel *et al.,* 2001). For such fungi molecular detection techniques are very efficient to control the spread of pathogen. Demethylation inhibitors (DMIs), Quanoneoutside inhibitors (QoIs), and dicarboximides (DCFs) which are fungicides were studied thorough PCR primers. This study detected fungicidal resistance in related fungi (Ma and Michailides, 2005). *Fn-1/Fn-2* and *Mn-1/Mn-2*, these are two specific primer pair sequence based on ITS sequence were designed by Zhang *et al.,* (2005). These set of primers were used to detect *Fusarium oxysporum* f.sp. *niveum* and *Mycosphaerella melonis* from infected tissue and soil.

Spongospora subterranean potato scab fungi were detected by Taq man, chemistry and real time PCR method (Ward *et al.,* 2004). They detected powdery scab pathogen in several samples, even presence cystosori on tubers and it was more sensitive than conventional PCR. Compatible and incompatible interaction between host grafted melon and pathogen *F. oxysporum* were studied with help of real time PCR. In this experiment primer pairs were designed based on translation elongation factor *1-α* gene. This primer was highly specific that only bind to *F. oxysporum* species and do not show affinity with other *Fusarium* species (Haegi *et al.,* 2013). This result shows the Primer based approaches helps wave off confusion.

Rhizoctonia solani and *R. oryzae* potential pathogen of root rot disease (Cook *et al.,* 2002) was identified and quantified based on the quantitative PCR reaction. For this study primers were specific to ITS1 and ITS2 of nuclear rDNA in table-2. Phylogenetic analysis also conducted to determine the specificity of the assay (Okubara *et al.,* 2008). For same pathogen protocol for identification of 11 anastomosis group were developed (Budge *et al.,* 2009). Another group of researcher developed primer based protocol to detect *R. solani* an stomosis group *2-2 IIIB* pathogen causes of crown and rootrot disease (Abbas *et al.,* 2014). Rice blast pathogen *Pyricularia oryzae* were characterized using four primer set *Bt1a* and *Bt1b, CAL-228F* and *CAL-737R, ACT-512F* and *ACT-783R* (Chuwa *et al.,* 2015). Before this molecular and morphological characterization was attempted by Bussaban *et al.,* (2005).

RAPD 22 primers were used to identify different isolates of *Sclerotium oryzae* from the rice plant to determine genetic diversity (Kumar *et al.,* 2010). Genetic diversity of*Ascochytarabie*were determined by using RAPD primer *UBC-702, UBC-708, UBC-726, UBC-727, UBC-7, UBC-739, UBC-74* and *OP-C18*. In soybean leaves latent infection of *Cercospora kikuchii* were detected by *CKCTB6* primer which amplifies *CTB6* gene (Chanda *et al.,* 2014).

Viral Pathogens Detection

Viruses are critical to diagnose because of its living and non-living character, the complexity of etiology and often confuse with nutrient deficiency. PCR primers have laid the pathway to detect accurate virus and also multiple viruses at same time. Bunyaviridae family virus, tomato spotted wilt virus detected with sensitive RT-PCR method. RNA with 628 base pair considered as standard for further quantification of virus. Primers were designed with L and S sequence.

Table.1 Primer sequences used in studies on different bacterial crop diseases

Bacteria	Gene	Primer sequence (5' 3') →	Reference
Acidovorax avane pv. *citrulli starin* M6	BX-L1 BX-S-R2	CAGCTGGGAGCGATCTTCAT- F GCGTCAGGAGGGTGAGTAGCA-R	Bahar *et al.*, (2008)
Agrobacterium spp. (pathogenic)	*virD2* A *virD2* E	ATG CCC GAT CGA GCT CAA GT- F CCT GAC CCA AAC ATC TCG GCT GCC CA-R	Haas *et al.*, (1995)
Agrobacterium tumefaciens	ipt	GAT CG(G/C) GTC CAA TG(C/T) TGT- F GAT ATC CAT CGA TC(T/C) CTT -R	
	GALLS 2141F GALLS 2443R	TGG CCC GCT ACT CAG TTA GTT- F CTG CAG AGT CAG CGC CTT G-R	
Burkholderia glumae	ITS sequence (16S-23S r DNA)	ACGTTCAGGGATGCTGAGCAG-F AGTCTGTCTCGCTCTCCCGA-R	Sayler *et al.*, (2006)
Clavibacter michiganensis	CMR16F1	GTGATGTCAGAGCTTGCTCTGGCGGATC-F GTACGGCTACCTTGTTACGACTTAGT-R	Arahal *et al.*, (2004)
Erwinia amylovora	pEA29	CGGTTTTTAACGCTGGG-F GGGCAAATACTCGGATT-R	Kim *et al.*, (2001)
Leifsonia xyli subsp. *xyli*	pSKC2-800	CGA GTTTGGGTTTCTGCCTGC-F AGGTGGCAGCGAC AACGGTGC-R	Taylor *et al.*, (2003)
Pseudomonas syringae pv. *phaseolicola*	Phaselotoxin PHA19, PHA95	CGTCTGTAACCAGTTGATCC GAATCCTTGAATGCGAAGGC	Marques *et al.*, (2000)
	P5.1 P3.1	AGCTTCTCCTCAAAACACCTGC TGTTCGCCAGAGGCAGTCATG	Schaad *et al.*, (1995)
	HM6 HM13	CGTGTCCGTGGATAAAAGC GTTGAATTTCACTACCCG	Prosen *et al.*, (1993)
	SSRP SSRP_R	GACGTCCCGCGAATAGCAATAATC-F CAACGCCGGCGCAATGTCG-R	Cho *et al.*, (2010)
Ralstoinia solanacearum	OLI1 OLI2	GGGGGTAGCTTGCTACCTGCC CGTCATCCACTCCAGGTATTAACCGAA	Arahal *et al.*, (2004)
Streptomyces scabies	ITS sequence 16S rRNA	CCGGTAGCCCAACCCGTAAG-F GTAGTACTCACAGCCTCCGG-R	Barrera *et al.*, (2013)
Universal primer	16S rRNA	TGGTAGTCCACGCCCTAAAC-F CTGGAAAGTTCCGTGGATGT-R	Srinivasa *et al.*, (2012)
Xanthomonas spp.	XA21	GCATCGGTATTAACAGCAAAAC-F ATAGCAACTGATTGCTTGG-R	Wang *et al.*, (1996)
Xyllela fastidiosa	petC	CTGCCATTCGTTGAAGTACCT-F CGTCCTCCCAATAAGCCT-R	Elbeaino *et al.*, (2014)
	QH—OLS 08	TGTACGTCCTGAAACCATCTTG	Huang *et al.*, (2009)
	QH—OLS 05	GGGAATGAGTTTCGAAGGTCTT	

Table.2 Primer sequences used in studies on different fungal diseases

Fungi	Gene	Primer (5' 3') ⟶	References
Alternaria brassicae	ITS	CTGGTGAAAAGGTTGCGATCGT-F GTGACTTTCATGAAATGACATTGATG-R	Guillemette *et al.*, (2004)
Ascochyta rabiei	RAPD (UBC-702)	GGGAGAAGGG	Chongo *et al.*, (2004)
	(UBC-708)	GGGTTGTGGG	
	(UBC-726)	GGTGTGGGTG	
Aspergillus niger	18S rDNA	CCTGGT TGATCCTGCCAGTA-F GCTTGATCCTTCTGCA GGTT-R	Melchers *et al.*, (1994)
Bipolaris oryzae	ITS1 ITS2	TCCGTAGCTGAACCTGC CG TCCTCCGCTTATTGATATGC	Weikert-Oliveira *et al.*, 2002)
Blumeria graminis f. sp. *hordei*	GAPDH	ACGCACCCATGTTTGTCAT-F CCAATGGGGCAAGACAGTTA-R	Komínková *et al.*, 2016
Botrytis cinerea	C729	AGCTCGAGAGAGATCTCTGA-F CTGCAATGTTCTGCGTGGAA-R	Rigotti *et al.*, 2006
	BC108 BC563	ACCCGCACCTAATTCGTCAAC GGGTCTTCGATACGGGAGAA	
Cercospora kikuchii	CTB6 gene	CACCATGCTA GATGTGACGACA-F GGTCCTGGAGGCAG CCA-R	Chanda *et al.*, (2014)
Cochliobolus spp.	NPS gene (protein ID 115356)	GTCGACTGCCATCTGGAAAC/CACTGGCCGT CGTTTTACAACGTCCACTCGACAGGTCCGT AGGT-F TCATGGTCATAGCTGTTTCCTGTGGTATCCA CAAAGCCACAGCA/GACGAACCAGAGATGC ATGA- R	Condon *et al.*, (2013)
Colletotrichum gloeosporioides	MKCg	TTGCTTCGGCGGGTAGGGTC-F ACGCAAAGGAGGCTCCGGGA- R	Kamle *et al.*, 2013
Filamentous Ascomycetes	Histone 4	GCTATCCGCCGTCTCGCT-F GGTACGGCCCTGGCGCTT-R	Glass *et al.*,(2005
	Plasma membrane ATPase	TTCCTCGGTTTCTTCGTCGGTCCC-F CTAGTCAGACGAGAATGGCCGCTC-R	
	Phosphate permease	GCTGCCCTTGATGCTTGG-F CAGGGACCAGCAGGGCGA-R	
Fusarium oxysporum f.sp. *melonis*	Fef1 Fef2	TAGTCACTTTCCCTTCAATCGC-F CTCAAGTGGCGGGGTAAGT-R	Haegi *et al.*, (2013)
Phytophthora spp.	ITS4 ITS 6	TCCTCCGCTTATTGATATGC GAAGGTGAAGTCGTAACAAGG	Grünwald *et al.*, 2011
Puccinia Srtiformis f.sp. tritici	Pst gene for BAC clone	CTGGTAATGGAGGTGGAACT-F CTGGGGTAGGTAAGAAGGTC-R	Ma *et al.*, 2010
R. solani AG2-1/AG2-8	Rs2.1/8	GTTGTAGCTGGCCCATTCATTTG -F GAGCAGGTGTGAAGCTGCAAAAG-R	Okubara *et al.*, (2008)
R. oryzae genotype 1	RoGr1	CACACACAACTAGTCATTGAATG -F GTAAGACGGTTTGAAGCAAG-R	
	β- tubulin	GCA AAG AGG CTG AGG GCT GT -F CGG TCT GGG TAC TCT TCA CGA A-R	Budge *et al.*, (2009)
Sclerotium. rolfsii	RADP primer C3	CGG CTT GGG T	Rasu *et al.*, (2013)
	OPA02	TGC CGA GCT G	
	OPC20	ACTT CGC CAC	
Venturia inequalis	Beta tubulin I Beta tubulin II	GAGGAATTCCCAGACCGTATGATG GCTGGATCCTATTCTTTGGGTCGAACAT	Koenraadt *et al.*, 1992

Table.3 Primer sequences used in studies on different viral plant diseases

Virus	Gene	Primer Sequence	References
Arabis mosaic virus	Coat protein	CTGTGCCATCCTTCCCCAATGAT-F GAGATGCTCCATCCATGCCAGT-R	Osman and Rowhani, 2006
Beat necrotic yellow vein virus	Coat protein	GATCGATGGGCCCGTGTTTC-F CAGGTGTCCATGGTAACTTCAAC-R	Choi *et al.*, 2013
Citrus tristeza virus	Genomic RNA Isolate T385 X	CgCcAATTTGATCTGTGAACG-F GCGAA**a**GCAAACATCtcGACTC-R	Ruiz-Ruiz *et al.*, 2007
Cucumber mosaic virus Sub group I	Coat protein	GCCACCAAAAATAGACCG-F ATCTGCTGGCGTGGATTTCT-R	Chen *et al.*, 2011
Cucumber vein yellowing virus	Non-coding genome	CGA AGG TCT CGA ATA AGC GTT C-F GAA TAC CCC ACA CCG AAC TTC A-R	Gil-Salas *et al.*, 2007
Cucurbit yellow stunting disorder virus	Non-coding RNA 2 region.	GCT TAA TGT GGG AGA AGT TCT CCT A-F TCT GGA TAT AAC CTT CAG ACA CTC CTT-R	
Cucumber green mottle mosaic virus	Non-coding RNA 2 region.	GCATAGTGCTTTCC-F GTTTCTATGGGCAGTAATTCTGCA-R	Hongyun *et al.*, 2008
Grapevine leafroll associated virus 9 (GLRaV-9)	Coat protein	CGGCATAAGAAAAGATGGCAC-F TCATTCACCACTGCTTGAAC-R	Alkowni *et al.*, (2004)
Grapevine virus A	Coat protein	GACAAATGGCACACTACG-F AAGCCTGACCTAGTCATCTTGG-R	Minafra *et al.*, (1994)
Grapevine virus B	Coat protein	GTGCTAAGAACGTCTTCACAGC-F ATCAGCAAACACGCTTGAACCG-R	
Plum pox virus	Flanked sequence P241	CGT TTA TTTGGCTTGGATGGAA	Olmos *et al.*, 2005
	316M	GAT TAACATCAC CAG CGG TGT G-	
	P316D	GAT TCA CGT CACCAG CGG TGT G	
Potato virus X	Coat protien	AAGCCTGAGCACAAATTCGC-F GCTTCAGACGGTGGCCG-R	Mortimer-Jones *et al.*,2009
Potato leaf roll virus	Coat protein	AAAGCCGAAAGGTGATTAGGC-F CCTGGCTACACAGTCGCGT-R	Mortimer-Jones *et al.*, 2009
Prune dwarf virus	Coat protien	CCGGTATGATATCTCGTACCGAG-F TAGTGCAGGTTAACCAAAAGGAT-R	Osman *et al.*, 2012
Prunus necrotic ringspot virus	Coat protien	AGACGTCGTGACAGACGTCGAAG-F CTTGACCTGCAATATCCTACTCG-R	Hammond and Crosslin (1995)
Rice yellow mottle virus		GAAGGGCAAGAAAACCAACTC-F GCTGGCAGTATGGTGTTTACG-R	
Sugarcane yellow leaf virus	Coat protein	CCA AAC AACAAC AGG CTC CAA-F GGG CCG GGA AGA CTT TCT T-R	Korimbocus *et al.*, 2002
Tomato mosaic virus	Coat protein	CATCTGTATGGGCTGAC-F GAGGTCCARACCAAMCCAG-R	
Tomato Spotted wilt Virus	TSWV N gene	TCTGGTAGCATTCAACTTCAA-F GTTTCACTGTAATGTTCCATAG-R	Roberts *et al.*, 2000
Tomato spotted wilt virus on crysanthemum	TSWV-F3	TTGTTGGCAACGGGAAGC	Fukuta *et al.*, 2004
	TSWV-F-Loop	TGTATTGTTTTCTGCTGTCCC	
	TSWV-B-Loop	CGATCCCAACATGCCATCTG	
Tomato yellow leaf curl Sardinia virus	Genome sequence based	CGTCCGTCGATCTGGAAAGT-F ATCCGAACATTCAGGGAGCTA-R	Mortimer-Jones *et al.*, 2009
	Isolate T36	CACTAATTTGATCTGTGAACG-F GCGAAGGCAAACATCCTGACTC-R	

Primers used were species specific primer and specific primers for viruses of same sero-group (Roberts *et al.,* 2000). Family *Closteroviridae*, *Citrus tristeza virus* (CTV) was studied for different accumulation in different species of citrus cultivar using RT-PCR. Primers were designed to amplify only genomic DNA. In this study, they developed a protocol for accurate estimation of CTV in different tissue, which was earlier difficult to achieve with ELISA based method (Ruiz-Ruiz *et al.,* 2007). Primer sets were designed based on the coat protein, reason was that coat protein sequence is highly conserved region. These important pests *Beet black scorch virus, Beet necrotic yellow vein virus, Eggplant mottled dwarf virus, Pelargonium zonate spot virus* and *Rice yellow mottle virus* are of quarantine. These primer sets were designated as highly specific for the given pathogen and useful in Quarantine inspection (Choi *et al.,* 2013).

In another study species specific and sub-species specific primers were designed and successfully used to detect and distinguish *Cucumber mosaic virus, Tomato mosaic virus* and *Tobacco mosaic virus*. These primer set were also able to identify satellite RNA virus of *Cucumber mosaic virus*. Detection was carried out by using multiplex RT- PCR and useful for detecting multiple virus infection particular in tomato. A different type of infected extraction method was developed and it was cross checked by using RT- PCR for some important viruses such as grapevine leaf roll virus, *Arabic mosaic virus, Prunus necrotic ring spot virus, Prune dwarf virus* (Osman and Rowhani, 2006).

Primers were not only useful for the PCR amplification but also in new study primers were successful in the case of an immune-capture, reverse transcription loop-mediated isothermal amplification (IC/RT-LAMP). This method suggested to be more sensitive than RT- PCR when studied on *Tomatospotted wilt virus* from *Chrysanthemum* (Fukuta *et al.,* 2004). *Cucumber vein yellowing virus* and *Cucurbit yellow stunting disorder virus* was successfully detected in its vector *Bemisia tabaci* based on specific primers. Primers were designed based on non-coding region (Gil-Salas *et al.,* 2007). *Sugarcane yellow leaf* v*irus* of luteoviridae family is efficiently detected by real time fluorescent RT- PCR using specific primers designed based on conserved region of coat protein. Another set of primers was kept as an internal positive control (IPC) using mRNA sequence of *o-methyltransferase* gene.

Quantitative assay for detection and quantification was performed for the *Plum pox virus* (PPV) in table- 3. These studies gives the idea of the epidemiology of PPV virus because the virus can be detected both in infected plants as well as in its vector aphid. In this study primer used for flanked nucleotide sequence selected by universal primer P1 and P2 and then PPV specific primers *P241, P316M* and *P316D* based on 59 nucleotide sequence was designed (Olmos *et al.,* 2005). In similar way protocol for detection quantification and of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) developed. In this study TYLCSV can be detected both in infected plant as well as its vector *Bemisia tabaci* (Mortimer-Jones *et al.,* 2009). A single method through RT-PCR was developed to detect four viruses simultaneously that is *Potato leafroll virus* (PLRV), *Potato virus X* (PVX) and *Potato virus S* (PVS) in potato leaves and tubers, and *Tomato spotted wilt virus* (TSWV) in potato tubers and tomato leaves. Here again primer designed based on the coat protein (Mortimer-Jones *et al.,* 2009), this indicate that the highly conserved nature of coat protein sequence is preferred for diagnosis

and it is able to detect virus specifically in series of RT-PCR one more highly sensitive method developed using the Duo primer method. This primer designed for highly conserved region of *Cucumber green mottel mosaic virus* and are efficient in amplifying even 50 molecules of RNA (approx 0.13 pg) (Hongyun *et al.,* 2008).

Cross talk

Most important challenge in primer based technique is its specificity in many cases, non-desirable sequence get amplified. In many cases pathogenic and non-pathogenic both microbes get amplified by the same primer as in case of plasmids primers for this considered as universal, hence identification of virulence strain is difficult (Palacio-Bielsa *et al.,* 2009) and most of the time this identification limits at genus and species level not up to the strains. *Erwinia amylovora* strain pEA29 cannot be determined by universal primer for plasmid (Llop *et al.,* 2006). Primers based on the internal transcribed region which is highly variable also fail in some cases like *E. amylovora* shows less specificity with rRNA genes and there is chances that another species of *Erwina* can be amplified (Roselló *et al.,* 2007). In case of pectobacterium a soft rot pathogen recently reported that there is clusters of genes like *hrp, pth* and *vir* are involved in pathogenicity hence only a primer for *pel* gene is not enough for diagnosis therefore there is requirement of designing of new primer (Palacio-Bielsa *et al.,* 2009). It indicates that we should not solely depend on a single primer for a particular species there is always a need to improve the primers according to advanced information.

Study conducted in silico for evaluation of specificity of probes and primers by Arahal *et al.,* (2004) raised the question on accuracy available public sequence database. They checked already available primers against *Ralstoinia solanacearum* and *Clavibacter michiganensis* pv. *spedonicus* and found that most of the primers are non-specific to the organism. They also discussed the possible reason that all we should consider while designing primers. The discussed possible reasons are sequencing error in available public database, wrongly evaluated oligonucleotide sequence and small but significant is error in typing of sequence. This study suggest re-evaluation of designed primers are necessary. In other hand insufficient amount of template DNA or improper preparation of master reaction mixture is also a problem in getting errorless result and proper diagnosis (Palacio-Bielsa *et al.,* 2009). Presence of copper ion in mixture is amplification inhibitors (Hartung *et al.,* 1996).

Pre amplification treatments such as keeping sample at high temperature for few minute can result in more specificity (Lee *et al.,* 2006; Milijasevic *et al.,* 2006). Trindade *et al.,* (2007) though developed primers pairs for 44 strains of *Xcv* but it was also able to amplify *X. campestris* pv. *mangiferae indicae* and *X. axonopodis* pv. *passiflorae* hence the primer pairs were non-specific. One disadvantage of PCR method compared to immuno diagnosis assay that this method dose is not give quantification soon because starting amount of DNA will be less and if any variation in amplification product occurs it gives wrong prediction (Wittwer *et al.,* 1997). Though amplification in PCR is exponential but it changes as number of cycles increases because limited amount of substrate (McCartney *et al.,* 2003).

Drawback in PCR method is to difficult in distinguishing in vital and non-vital inoculum. Sometimes primers fail to amplify where high number of variable nucleotides

are present (Ayllón *et al.*, 2001, Vives *et al.*, 2005). Hence for effective and accurate diagnosis we need to follow the criteria for selection, designing and precise handling of the primers and its protocol. Primers based diagnosis is effective way to develop better understanding of pathogen and its interaction with host if dealt with care. Precisely conducted primer based diagnostic had tremendous result of which we some of them were discussed above. There is no doubt that in present time it is most reliable and efficient method and further has great potential.

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