DIAGNOSIS OF CITRUS GREENING (HUANGLONGBING) DISEASE BY PCR AND *Citrus tresteza* VIRUS BY DAS-ELISA TECHNIQUES FROM DIFFERENT PARTS OF NEPAL

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RECOMMENDATION

This is to certify that the dissertation work entitled, **"Diagnosis of Citrus Greening (Huanglongbing) Disease by PCR and *Citrus tristeza* Virus by DAS-ELISA techniques from different parts of Nepal"** submitted by Ms Bal Kumari Oliya for the partial fulfillment of Master's Degree in Botany, has been carried out under our supervision and guidance. The result of this work has not yet been submitted for any other Degree. We are pleased to recommend her work for approval and acceptance.

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ABSTRACT

Citrus species are highly nutritious sub-tropical fruits of Rutaceae Family. The climatic and geographical conditions of Nepal are suitable for citrus cultivation. However, the productivity of citrus in Nepal is only (10-11mt/ha) which is very low compared to that of developed countries. In present scenario there has been a growing awareness on the occurrence of virus and virus like disease in crops of Nepal. Plant viruses damage almost all parts of plant. Most of the citrus farms are affected by citrus greening disease (Huanglongbing) and *Citrus tresteza* virus (CTV) causing citrus decline. Due to this cause, farmers face huge economic loss and search for preventive and curative actions. There are more than 30 virus and virus like disease of citrus known in the world of which Huanglongbing and CTV are the most destructive.

The PCR reaction specific for Asian Citrus psyllid was done by using 703bp long fragment of ribosomal protein genes (rpl-PCR) in the rplKAJL-rpOBC (β-operon) using primer A2 and J5 (Hocquelle*t et al*., 1999). Among 9 samples tested 7 samples gave PCR positive reaction detecting Huanglongbing (Asian *Citrus psyllid),* from Lamjung and Kathmandu District. .

In vivo leaves samples of Junar (*C. sinensis)*, kagati, (*C.aurantifolia*), mandarin orange (*C. reticulata* nibuwa (*C. limon*), vogate, [*C. grandi*s), and trifoliate orange(*Poncirus trifoliata)* collected from four districts Lamjung, Gorkha, Dolakha and Kathmandu and in vitro shoot tip and node cultured samples of infected mandarin orange and trifoliate orange were tested for CTV by DAS-ELISA technique. All cultivars except *C. grandis*, in vivo were infected by CTV. Similarly, seedling grown samples tested from screen house nursery and tissue cultured samples showed negative reaction to CTV.

Virus free citrus plant production technique is only the effective method for saving citrus trees and to get optimum production. There are numerous disease-transmitting vectors in open or uncontrolled environment that can easily attack the immature young plants. Pathogens of Huanglongbing and CTV are graft transmissible and vector borne. So they can easily transmit from infected scion and in open environment. Grafting with virus free trifoliate rootstock with virus free scion of different citrus cultivars of seedling plant in controlled environment (screen- house) to produce certified citrus plant is the most effective technique to control the declining condition of citrus production in Nepal. Citriculture of mandarin orange (up to 60% of total citrus plants) and Junar (20%) can enhance productivityup to 20Mt/ha in Nepal. This can be achieved by completely removing the old plants of problematic farms with disease-free indexed plants.

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ABBREVIATIONS

ARS	=	Agriculture research station
CGD	=	Citrus Greening Disease
CTAB	=	Cetyltrimethyl ammonium bromide
CTV	=	Citrus Tresteza Virus
CFD	=	Citrus Fruit Disease
DAO	=	District Agriculture office
DAS-ELISA	=	Double Antibody Sandwich Enzyme Linked Immuno-sorbent assay
DNA	=	Deoxyribo Nucleic Acid
dsRNA	=	Double stranded Ribose-Nucleic Acid
EDTA	=	Ethylenediamine terta acetic acid
ELISA	=	Enzyme Linked Immuno-sorbent Assay
GPS	=	Global Positioning System
HDP	=	Horticulture Development Program
FAO	=	Food and Agricultural Organization
HLB	=	Huanglongbing
MMM	=	Multiple molecular Marker
NARC	=	Nepal Agricultural Research council
NAST	=	Nepal Academy of Science and Technology
NCDP	=	National Citrus Development Program
PBST	=	Phosphate Buffered Saline with Tween-20
PCR	=	Polymerase Chain Reaction
RFLP	=	Restriction fragment Length Polymorphism
RNA	=	RiboNucleic Acid
SAARC	=	South Asian Association for Regional Cooperation
Sd	=	standard deviation
sPAGE	=	sequential Polyacrylamide Gel Electrophoresis
SSCP	=	Single-stranded Conformation Polymorphism
STG	=	Shoot-tip Micro grafting

USSR	=	Union of Soviet Socialist Republics
US	=	United State
VDC	=	Village Development committee
WDR	=	Western Development Region
IgG	=	Immune globulin G
rDNA	=	ribosomal DNA
-ve	=	negative
+ve	=	Positive
M±SD	=	Mean plus minus standard deviation

Measurements

Å	Angstrom
Bp	base pair
Cm	Centimeter
g/cm3	Gram per Cubic centimeter
g/ml	Gram per milliliter
Kb	Kilo Base
m	meter
M	Molar
Mg	Milligram
Mm	Millimeter
Ml	Milliliter
Mt	Metric ton
Nm	Nano meter
oC	Degree Centigrade
rpm	Rotation per minute
t/ha	ton per hectare
µl	microliter

1. INTRODUCTION

1.1. General Background

Citrus is long lived perennial shrubs and trees belonging to Rutaceae family and consist of many species. Citrus species are among the most important fruit crops in the world. They are widely grown in the tropical, subtropical, and borderline subtropical/temperate areas of the world, which can be propagated by seedling, root stock, layering, grafting, and can give product within temperature ranges of 14^{o}C to 40 ^{o}C but the optimum growth temperature is 30^{o}C (Whiteside *et al*., 1993). Citrus is grown in 49 countries of the world and is one of the highly demanded fruit having high consumers performance both as fresh fruit and refreshing processed juice. Citrus and citrus products are rich source of vitamins, minerals and dietary fiber (non-starch polysaccharides) that are essential for normal growth and development and overall nutritional well-being. Citrus has long been regarded as a food and also as a medicinal plant. It has high nutritive value and antioxidant activity. The antioxidant activities of citrus species are in accordance with the presence of phenolic compounds. Citrus species containing antioxidant activities are *Citrus hystrix, C. aurantifolia C. microcarpa, C. sinensis*. Citrus is adoptable to many subtropical and tropical environments and soil in home gardens together with other important species zinger, millet, cardamom, papaya, coconut, breadfruits and numerous others. Different nutrition reported in citrus species are listed in (Annex I).

The climatic condition of midhill of Nepal favours the cultivation of high quality Citrus fruits. Mandarin (*C. reticulata* Blanco), rough lemon (*C. jambhiri* Lush.), sweet orange [*Citrus sinensis* (L.) Osbeck], lime (*C. aurantifolia* Swingle), lemon [*C. limon*(L.) Burm. f], shaddock (*C. grandis* Osbeck), citron (*C. medica* L.*)*, sour orange (*C. aurantium* L.) are the main species cultivated in these regions (Roistacher, 1996).Because of excellent qualities and superior commercial values, mandarin, lime and junar cultivation have been rapidly expanded in last few years. The terrain lying between the 900m & 1400m above sea level have suitable temperature range throughout the year and ample of distribution of rainfall between the months of June through September. The pocket area with deep sandy

loamy soil pH range of 5.0 to 6.5 is most suitable for cultivation of citrus fruits (Ranjit & Ghartichhetri, 1997). Of all these citrus species, mandarin ranks first both in terms of production and acreage.

Plant virus and virus like disease damage almost all parts of plant causing huge economic loss, due to reduction in quality and quantity of products. Severity of virus disease varies with locality, crop variety and season and therefore accurate figure of crop loss due to virus disease are not available for Nepal or even south Asia. Citrus disease are mainly caused by virus, fungi, bacteria and nutritional disorders, numbers of them are described as virus like diseases because the symptoms resemble those caused by viruses but the causal agent are not clearly determined. More than 30 virus diseases, two phytoplasma diseases, one spiroplasma disease, three viroid disease, 11 fungal, three bacterial and two nematodes diseases are known to occur in Citrus throughout the world along with some diseases of uncertain etiology like Citrus (Ahlawat & Pant, 2003). Some of these diseases are of high economic significance as they can wipe out the whole citrus industry if not managed in time. These diseases affect tree health and production. The recognized virus disease of Nepal is very numerous. They all are transmitted by budding or grafting, some can also be transmitted by insect vectors, and a few can be mechanically transmitted via sap of infected plants. The main virus and virus like disease of Nepal are Citrus Greening Disease and *Citrus tresteza* Virus.

Many disease transmitted by psyllid are probably similar to stubborn and greening. Pathogenic fungi and bacteria can attack citrus tree causing severe troubles on the roots, trunk, branches, fruits, new shoots and flowers. The type of climate for citrus plant is also suitable for many pests. Scale insects, white flies nematodes and mites are important groups of Citrus pest found worldwide.

Citrus Greening Disease

Citrus greening disease is one of the most destructive maladies of Citrus affecting citrus production world-wide (Halbert & Manjunath, (2004),Teixeira *et al*., (2005), Bove´ (2006), Wang *et al*., (2006), Batool *et al.,* (2007), Manjunath *et al*., 2008). Greening disease was first reported in 1947 from South Africa, although the disease has been

known since 1929. Until 1970, it was considered to be a virus disease, but it is now known to be caused by a phloem limited non- cultured bacteria *Candidatus liberibacter* spp belonging to alpha-proteobacterial subdivision. The disease probably originated in China and was common there in the early 1900s (Zhao, 1981). It seriously affects citrus production in Indian subcontinent and in Asia, south east Asia, the Arabian peninsula and Africa. Recently disease has been reported from USA. Thousands of trees were reported to have decline due to greening disease worldwide. The disease is known by several other names in different countries. In Taiwan, likubin, drooping disease (Ôtake, 1990), Philippines mottle leaf disease (Lee, 1921), India citrus die-back, (Raychaudhuri *et al*., 1974). Vein phloem degradation in Indonesia (Aubert *et al*., 1996) and greening in south Africa (Van der Merwe, 1937). It is one of the major causal factors of Citrus decline in areas where greening and its vectors are common. The official name of the disease changed from "greening" to 'huanglongbing' since it was first shown to be graft transmissible in China in 1956. According to Zhao, (1981), "huanglong" means the yellowing of some new shoots in the green canopy, and "bing" means disease. Specifically, "huanglong" means "yellow dragon" because as symptoms progress, they appear "draped" over the tree almost like a "yellow dragon" (CAB International, 2000). Among the commercial cultivars, group of sweet orange is more susceptible to greening than the group of lime and lemon.

Citrus tresteza **Virus**

Citrus tresteza Virus (CTV) apparently originated in Asia where it existed for centuries. CTV is the most destructive viral pathogen of citrus trees (Bar-Joseph et al., 1989). It belongs to the closterovirus and not transmitted by seed and soil and only transmitted by Aphid vectors and during grafting. It has cause huge economic loss worldwide. CTV is the causal agent of "tristeza" (also known as "quick decline"), which has destroyed over 50 million citrus trees growing on sour orange rootstocks globally. CTV is also responsible for another viral disease known as stem pitting which reduces fruit quality and negatively impacts the production of limes, grapefruits, and sweet oranges. Numerous strains of CTV exist. Mild strains that infect sweet orange, mandarins, and

many other cultivars sometimes do not cause symptoms and result in less severe crop losses (Nelson *et al*., 2011). CTV disease has been the most extensively studied among the entire citrus virus and virus-like diseases throughout the world. There are more than 30 virus and virus like disease of Citrus known in the world, of which CTV is arguably most destructive. It is safe to predict that *tristeza* is present in all citrus growing counties. However, in some countries, serious spread as yet has not occurred.

CTV has many synonyms including Citrus quick decline virus (Fawcett & Wallace, 1946). Citrus seedling yellows virus (Fraser, 1952). Grapefruit stem pitting virus (Oberholzer *et al*., 1949). Lime die-back virus (Hughes & Lister, 1949). Taxonomically it is Closterovirus, tentative species (Candresse & Martelli, 1995). In English, the disease is called by different name as tristeza, seedling yellows, and quick decline similarly in French and Spanish it is called tristeza.

Symptoms vary depending on the Citrus species, the root stock used, and the strain of virus prevalent. Symptoms resemble those produced by severe girdling of tree or those of advanced stages of certain root rots. The most economically important symptom is the quick decline or death of the tree. In area where the aphid *Toxoptera citricida* is present, some CTV strains cause stem pitting which may give bumpy appearance to the trunk of limbs of the tree. The virus may also have latent form which gives no symptoms. The tree show general decline, sudden wilting , lack of new growth during a normal flush period, gradual drop of the leaves, reduced fruit size and phloem sieve tube necrosis which hinders transport of carbohydrates from the top to the roots, resulting in the starvation of the root. The main symptoms of CTV are: vein clearing, leaf cupping, vein corking and stem pitting.

The general aim of this thesis is to show the distribution pattern of CGD and CTV in the tested Districts and to assess the condition of severity in affected areas. PCR can be conducted for detection of CGD and DAS-ELISA can be used for detection of the virus. Shoot tip and node culture can be applied for the reduction/ elimination of virus from the CTV infected sample. Tendency of screen-house nursery to produce disease CTV free Citrus plant can be tested.

1.2. Statement of Problems and Justification

Mid hill of Nepal is good for Citriculture and it can hold local demands as well government can export to the foreign countries. Citrus cultivation has been practiced since long times ago for homely consumption and till now some farmers are attracted for commercial production. Though they are getting good production rate, most of the citrus farm has been suffered with disease of amazing different symptoms causing Citrus production declination. On the basis of acreages and production, Citrus fruit stand first and the Commercial citrus tree are grown from seedling and still now in practice in most of the places of Nepal. Seedling grown plants have numerous inherent disadvantages, including juvenile nature of the trees resulting into late fruiting period (five years) and susceptibility to various diseases (eg. Grafted sapling and production of resistant rootstock can overcome the problem of some diseases such as *Phytophthora* and Gummosis. However due to careless during grafting, and introduction of uncertified grafted plants in Citrus orchards, graft transmissible diseases like HLB and CTV destroy most of the citrus farms of the world including Nepal, due to which most of the citrus industries are in huge economic loss. In Nepal, In 1999/2000, Citrus cultivation occupied 11277 ha of land with a total annual production of 115067tons giving an average production of 10.20 t/ha (Agriculture Department of Nepal, 2012) But in 2011/2012, Citrus cultivation occupied 24089 ha of land with a total annual production of 240739 tons giving an average production of 10.0 mt/ha. This data is not satisfactory as the area under citrus production is increasing resulting less average production. CTV and CGD are the most serious problems appeared in the world (China, Asia, South Africa, India, Florida etc), where these disease has caused huge economic loss. CGD was first reported in Nepal from *Pokhara* valley, since then disease started expanding to other parts of the country, leading to decline and poor quality citrus fruit. Citrus tresteza virus is another important and most devastating disease appeared in Citriculture. Both HLB and CTV are serious disease and graft transmissible diseases and once its initiation in Citrus family destroy the whole Citrus family and transmit to the whole pocket areas. Farmers are getting frustration due to lack of preventive and curative action against these problems.

Sometimes this problematic farm is sprinkled by pesticides with suggestions of concerned Agriculture Office, but long term solutions has still not found. These kinds of sprinkling pesticides are spoiling soil than getting solution from actual problem. Short term solution may tackle the problem at meantime but this will ball up soil fertility in a minute gained from scores of centuries. If this still continues to more 3-5 years then our land will be converted into desert. Most of the arable lands now need to be conserved with much care so that we can grow and convert our land to diamond farm.

DAS-ELISA is the confirmative test for CTV and by this technique large number of samples can be analyzed qualitatively as well as quantitatively and among several detection technique of HLB detection PCR based analysis is the only one most reliable and robust technique to perform rapid, sensitive and specific diagnosis of HLB. Timely diagnosis of HLB and CTV in citrus mother plants in the nurseries and orchards can prevent further spread of this disease in Nepal. Therefore, PCR diagnosis of HLB and DAS- ELISA technique for CTV detection are highly justifiable and holds great promise for the integrated management of these diseases in Nepal.

Recent problem appeared in citrus orchards can be overcome by tissue culture of infected plants, and nursery should insect proof. Production of certified grafted plant and destruction of infected garden plants may be the solution for citrus species declination.

1.3. Research objectives

The broad objective of this research is to report the present status of HLB and CTV disease in different parts of countryand to find out the long term solution of disease infection incitrus family.

Specific objectives are:

- To survey different districtsfor *Citrus tresteza* Virus (CTV) and Huanglongbing(HLB) along with other associated diseases.
- To detectof CTV in different Citrus species of Screen house nursery, open orchards, mother plant and of tissue cultured by DAS-ELISA technique.

- To conduct PCR based diagnosis of HLB in field collected samples of Mandarin orange.

1.4. Limitations

- Due to time and budget limitations, the survey to all citrus orchards of different districts could not be conducted.
- Due to technical and load shedding problem, further optimization experiments of PCR assays could not be carried out.
- Due to technical problem and time limitation, the efficacy of ELISA kit and PCR assays in different growing periods of citrus family could not be carried out.

2. LITERATURE REVIEW

2.1. Origin and History of Citrus fruits

Citrus species and many related genera of the subfamily Aurantioideae, family Rutaceae, are indigenous to southern Asia (Eastern India, Indonesia, Southern China,and Phillipines) (Kochhar, 1998). It is widely grown in most areas with suitable climates tropical, subtropical, and borderline subtropical/temperate (Kahn *et al*., 2001).Theseare native to parts of India, china, northern Australia and New Caledonia. The origin of Citrus species has been considered in north-eastern India and its neighboring areas (Tanaka, (1954), Zieler & Herbert, 1961)**.** According to (Hooker, (1872), Bhattacharya & Dutta, 1956). It is reported that about 78 species of citrus under Rutaceae family have their origin in India North West of India is the place of origin of citron (*C. medica* L.), lemon (*C. aurantifolia* Swingle.) and lime [*C. limon*(L.) Burm. f.] and are indigenous to India and Malaya. Sweet orange (*C. sinensis* Osbeck) originated in Southern China from where it was spread and introduced in India. Pummelo (*C. grandis* Osbeck) originated in FijiIsland and in China. Grapefruit (*C. paradise*) is a native of West Indies. Thus except grapefruit, most of the important species in genus *Citrus* and related genera originated in the old world. Some taxonomist (Tanaka) believed that the modern species which was originated in this region and had spread into China which later on diffused into Southern China, Indo-China and eventually extended to Malay and nearby regions (Datta, 1958). Citrus fruit has been cultivated for thousands of year. Its cultivation entered a period of rapid expansion around the start of the twentieth century, in response to the growing market demand and improvement of market quality.

2.2.Citrus production in the world

The world production of citrus was about 88 million mt. annually. Out of this, 30 million mt. was produced in Asia and about 6 million mt. was produced in SAARC countries[1]. The top ten citrus producing countries of the world were Brazil, China, United State,

[1]FAOSTAT, 1998; http://faostat3.fao.org/faostat-gateway/go/to/download/Q/QC/ENovember-10-2013

Maxico, India, Spain, Iran, Italy, Nigeria and Turkey respectively[2] (FAO, 2007).Recently, in season 2010/2011, world total citrus production was 115525.2 thousand tones. China is the largestproducer of Citrus fruits followed by Brazil, US, Mexico, India, Spain and Turkey which are listed in Annex(III).Mexico rank first in lemon, Brazil rank first in Oranges and China rank first in grapefruit. In total citrus oranges and tangerines export, Spain rank first, however Turkey take first in lemon export and Former USSR imports maximum citrus fruits (FAO, 2012). The bulk of global citrus production is dominated by oranges, followed by lemons, then grapefruit and soft citrus. Citrus production declined by 6 % in the 2011/2012 season because of a reduction in orange production. Brazil is by far the largest producer of oranges, accounting for 37 % of global orange production in the 2011/2012 season. The bulk of oranges produced in Brazil are processed into juice and only small quantities are exported as fresh produce due to safety and quality issues. The second largest producer of oranges is the United State of America with a global production share of 16 %, followed by China with 13 %, the EU-27 with 11 % and Mexico with a 6 % global production share. Interestingly, although South Africa is not a major global producer of oranges, it is the largest exporter of oranges in the world.

Mexico is the biggest producer of lemons, accounting for 27 % of global lemon production in the 2011/2012 season. The lemon volumes from Mexico have been declining over the last five years, coming down from 2.2 million in 2007 to 1.7 million tons in 2012. Other leading producers include EU-27 with a 21 % share, Argentina with a 19 % share, Turkey with a 14 % share and the USA with a 12 % share in the 2011/2012 season. South Africa is the 6th largest producer of lemons, with a global production share of 4 % in 2012 season.

Global orange production for 2013/14 is forecast to rise 5 percent from the previous year to 51.8 million metric tons as increases in Brazil, China, and the European Union (EU) more than offset the continued drop in the United States. Trade is forecast to reach a record level on stronger demand and increases in available supplies. (USDA, 2014).

[2]FAOSTAT, 2007;http://faostat3.fao.org/faostat-gateway/go/to/download/Q/QC/ENovember-10-2013

2.3. Citrus cultivation and Production in Nepal

The area, production, and productivity of citrus in Nepal are increasing year by year. During year 1990-1998 total area under citrus fruit cultivation was about 22,423.37ha with an estimated yield of 10.23mt/ha. However, during 1998-2008 the yield of citrus increased from 10.23mt/ha to 11.36mt/ha (Agriculture Department of Nepal, 2012)resulting in an annual productivity of about 227070.62 mt. After the year 2007/2008, Citrus productivity starts to decline resulting the productivity decreased to 11.30 mt/ha from 11.36 mt/ha of previous year, in 2009/2010. In fiscal year 2010/2011 it decreased to 8.46 mt/ha. In recent year 2011/2012 it slightly rises up to 10 mt /ha from 8.46 mt/ha. Among them productivity carried by mandarin orange was estimated at 10.89 mt /ha from the total productive area of 15304ha with production of 166612 mt. Mandarin is estimated to cover about 60% of total citrus area of the country. Western development region produce 61814mt mandarin orange from the total productive area of 5472, after mandarin orange second largest productive citrus fruit is sweet orange (Agriculture Department of Nepal, 2012). Citrus species production, potential areas and productivity is listed below (Table –1). Major citrus pocket areas are listed in annex (II, a)

Table 1 Citrus species production, potential areas, and Productivity in Nepal during 2011/12

Year	Potential area (hector)	Production(metric ton)	Productivity(mt/ha)
1998/99	10592	10725	10.13
1999/00	11277	115067	10.20
2000/01	11891.6	121665.3	10.23
2001/02	12615.5	130927.74	10.38
2002/03	13311.86	139109.55	10.45

2003/04	13930.86	148010.22	10.62
2004/05	14605.98	156955.9	10.75
2005/06	15206	163877.07	10.78
2006/07	15831.9	171874.5	10.86
2007/08	19979.73	227070.67	11.37
2008/09	22482.5	253765.7	11.29
2009/10	22903	259191	11.32
2010/11	23607	263,710	8.46
2011/12	24089	240,793	10.00

Source- Annual Report of HDP 2012

2.4. Citrus species grown in Nepal

Nepal has suitable climate, geography, and soil type for Citrus cultivation and many species of Citrus are cultivated here, for commercial as well as household purposes. Lama & Kayastha, 1999, reported fourteen species of citrus from *Pokhara* and its surrounding areas. Twelve species of citrus are available in National Citrus Development program *Kirtipur*, Namely *Citrus reticulata, C.unshinu, C. sinensis, C. grandis, C. paradise, C.lemon, C.aurantifolia, Fortunella, Japonica, Poncirus trifoliate, Citrange etc.* Mandarin *(C.reticulata),* sweet orange *(C.sinensis)*, acid lime (*C. aurantifolia*) and lemon (*C. lemon*) are cultivated commercially in Nepal. Mandarin orange grown in Nepal is excellent in terms of size and quality. Mandarin orange contributes as food, improvement of nutrition, generation of employment, income and in maintaining good environment (Shah, (1992), Gurung, 1998). Tomiyashu *et al*., (1998), has listed in priority to mandarin orange as cash generating source for mid hill farmers. Citrus species grown in Nepal are listed in Annex (II,b).

2.5. Citrus Diseases

Among the Horticultural Crops, Fruits and Vegetables (FV) are of primary importance as the key source of essential components in an adequate and balanced human diet. People used fruit and vegetable from ancient time as their food to eat. Fruit and vegetables are susceptible to a large number of diseases caused by plant pathogens. When a tree shows a new peculiar symptom, or suddenly dies prematurely, there is usually a cause. World Citrus fruit has been affected with many diseases. This disease are mainly caused by virus, bacteria, fungi, nematode and nutritional disorders . Major Citrus diseases present in world are Huanglongbing (Citrus greening), Tresteza, Greasy spot, *Alternaria* brown spot, *Phytophthora* induced diseases, Melanose, Scab, Canker, and Post bloom fruit drop (Teixeria et al., 2004) Graft transmitted virus reported from India are Rubbery wood, Citrus mosaic, a new Rhabdo virus, a new vein yellowing virus, impietratura, greening, infectious variegation, leathery leaf, vein enation, porosis and bud union (Roistacher, 1996). *Citrus tresteza* virus and Citrus greening disease is one of the serious problems for citrus fruit production in many African and Asian countries including Nepal (Teixeria et al., 2004). Major citrus diseases currently present in Nepal include *Citrus tristeza* virus, Huanglongbing, blight, greasy spot, *Alternaria* brown spot, *Phytophthora*-induced Diseases, melanose, scab, canker, post bloom fruit drop (PFD), Powdery mild, rubbery wood, citrus mosaic woody gall, and Gummosis. which are some of the serious problems in citriculture. These diseases are either transmitted by grafting vectors or dodders.

Various citrus species are susceptible to a large number of diseases caused by different plant pathogens. During 1970s, an extensive survey was conducted in the citrus growing areas of Nepal and presence of HLB, CTV, Xyloporosis and bud union crease were reported (Knorr & Shah, 1971). Among these diseases, HLB was considered to be the most important contributing factor for citrus decline in Nepal. Regmi, (1982), had also reported that HLB infected trees gave five times fewer yields than the apparently healthy ones.In 2002, twenty different diseases have been reported from Nepal, several of which are very serious either locally or world- wide. These diseases are listed in annex (II, c).

2.6. Citrus Greening Disease

2.6.1. Origin history, Geographical Distribution and Economic Impact

Greening disease was first reported in 1947 from South Africa, although the disease has been known since 1929. Until 1970, it was considered to be a virus disease, but it is now known to be caused by a phloem limited non-cultured bacteria *Candidatus liberibacter spp* belonging to alpha-proteobacterial subdivision. The disease probably originated in China and was common there in the early 1900 (Zhao, 1981).

Globally, HLB has been regarded as one of the most important threats to commercial and sustainable citrus production. (Da Graça, 1991), lists 24 countries and territories in east, south-east, south, and western Asia and in eastern and southern Africa, where HLB had been reported. Since then, its presence has been confirmed in four additional south-east Asian nations, namely Vietnam (Garnier & Bové, 1996)Myanmar, Laos and Cambodia (Garnier & Bové, 2000). For other major citrus production regions such as North and South America, Australia and the Mediterranean countries, HLB remains a major threat if introduced. For instance, HLB has resulted in the destruction of 30 million trees in Indonesia (Tirtawidjaja *et al.*, 1965). On the Indonesian island of Bali four million trees were eradicated during 1986-88, although these trees were replaced with mandarins in 1991, 40% were infected in 1993, and 90 % in 1996 (Aubert, (1993), Da Graça & Korsten, 2004). In the early 1960's, nearly 25,000 ha were planted to citrus, but 10 years later five million (i.e., 60% of the plantings) were lostdue to HLB. In Thailand, many trees are dying and going out of production five to six years after planting. Such losses are significant, since profits are only attainable 10 years after planting resulting in losses of over US$8,000/ha (Roistacher, 1996). In south-western Saudi Arabia, all sweet orange and mandarin trees had declined by 1986 leaving only limes (Aubert, 1993, Bové, 1986).

Crop losses of 30-100% have been reported in South Africa during the 1932- 1936 and 1939-1946 periods (Oberholzer *et al*., (1965), Schwarz, 1964). By 1958, the disease affected 100 000 sweet orange trees (Oberholzer *et al*., 1965) and by the mid 1970's, it was estimated that four of the eleven million trees planted in South Africa (36%) were

affected with HLB (Buitendag & von Broembsen, 1993). By then, major citrus production areas, which represented 20% of the industry, were eliminated, making it the most serious disease in South Africa. Of even greater concern was that areas previously regarded as HLB-free were showing tree symptoms (Green & Catling, 1971). By the mid 1990's the disease were reported in the Cape which, with its Mediterranean type climate, was regarded as "not likely" to get HLB. The use of a national quarantine barrier and restriction on sales of citrus trees from the northern regions were the disease is endemic to the coastal areas where psylla are endemic and not controlled, proved ineffective (McClean *et al*., 1969). Although losses resulting from HLB has been more extensively documented in Asia than in Africa, it is estimated that globally more than 60 million trees had been destroyed by the disease by the early 1990's (Aubert, 1993).During 2007-2008 seasons total economic loss reported by CGD in Florida Citrus industry was $ 9.1 billion.

Citrus huanglongbing (HLB) was first reported in Nepal in 1967 in the *PokharaValley* (Calavan, 1968) (Knorr *et al*., 1970) (Thrower, 1968) since then the disease has spread to all citrus growing districts (Lama & Amatya, 1993). Asiatic greening is present throughout Nepal (Regmi, 1994) (Regmi *et al.*, 1996). Greening and its vectors most probably introduced from India (Knorr & Shah, 1971) During the spring flush of 1986, (Lama *et al*., 1988) reported the presence of vector in the abundant numbers in the districts of *Terai, Morang, sunsari, Sapturi, Siraha, Dhanusa, Mahotari, Sarlahi, Rauthat, Sindhuli, Chitwan, Dang, Pyuthan, Salyan, Bara, Parsa, and in Kathmandu*. The vector was not found in *Dhankuta, Gorkha* and *Lamjung* districts. All species of Citrus appear susceptible, but the sweet orange, mandarin and Tangelo are most affected (Regmi & Lama, 1988a). Lemon, small fruited lime, and *Murrayapeniculata* (a citrus relative) are superior host for *D. citri* in Nepal.

2.6.2. Symptoms of HLB

A. Symptoms on whole tree

Depending on the age of a tree and time and stage of infection, the first symptoms of HLB usually start with the appearance of a yellow shoot. If infection occurs soon after

propagation, yellowing progresses over the entire canopy. However, if infection occurs at a later stage of growth, the symptoms and the causal organism remain confined to the sector initially infected. If a sector of a tree is affected, then only those parts will show typical symptoms. While the rest of the tree exhibits normal growth and produces normal healthy fruit of good quality (Oberholzer *et al*., 1965). A range of symptoms can be observed on infected trees and branches, which include heavy leaf and fruit drop, followed by out of season flushing and blossoming (Catlong, 1969 &Martinez, 1972). Severely infected trees often appear stunted, usually are sparsely foliated, and can die back. Chronically infected trees are sparsely foliated and show extensive twig die-back symptoms. Infected trees produce reduced crops of low quality fruit.

B. Symptoms on leaf

Initial foliar symptoms of African HLB are vein yellowing and a variegated type of chlorosis (blotchy mottle), which appear on fully mature leaves (Schneider, 1968, Manicom & van-Vuuren, 1990). Blotchy mottle, pattern on the leaves are asymmetrical using the mid rib as a symmetric line while with nutrient deficient plant and other disorder the patterns seen would be symmetrical. HLB plants will usually show the nutrient deficiency symptoms in addition to blotchy mottle Secondary symptoms include small, upright leaves ("rabbit's ears") with a variety of chlorotic patterns resembling those induced by zinc, iron, manganese, calcium, sulphur and/or boron deficiencies (Schneider, 1968.) (McClean & Schwarz, 1970) Many of the latter may be almost entirely devoid of chlorophyll, except for occasional circular green spots ("green islands") distributed at random on the leaves. The Asian form of the disease induces similar symptoms, but with more extensive yellowing, die-back and decline (Martinez & Wallace, 1968, Zhao, 1981) and in some cases death of small trees (1-2 years) (Lin, 1963). It is also more tolerant to heat, and thus is found in lower lying, hotter areas. In South Africa, leaf symptoms are more pronounced in the cool areas, compared to the lower lying hotter areas, and are more pronounced in winter (Schwarz, 1968). African HLB can also be eliminated by exposure to extended periods of heat (Labuschagne & Kotzé, 1980). Both forms of greening have only been found in Reunion and Mauritius,

usually separated by the temperature preferences, although both forms were detected in some trees using molecular probes (Garnier & Bové, 1996)

C. Symptoms on fruit

The most reliable diagnostic symptom of HLB represent the fruits which when infected, are small, oblong shape, lopsided with a curved columella or central core and seed, if present, is mostly aborted. A bitter, salty taste is also characteristic of affected fruit. With infected trees there is a continuous and premature shedding of greened fruit while those remaining on the tree do not color properly (McClean & Schwarz, 1970), hence the former name "greening disease". Symptoms can be exacerbated by the presence of other pathogens. Some varieties, such as sour orange are more tolerant i.e. the symptoms are not as severe as on other varieties.

D. Symptoms on root

Root systems are usually poorly developed in severely affected trees, with relatively few fibrous roots (Oberholzer *et al*., 1965), possibly due to root starvation. New root growth is suppressed and the roots often start decaying from the rootlets (Zhao, 1981).

Co-infection with Citrus tristeza virus (CTV) is common, and there are reports from several Asian countries that such trees have more severe symptoms (Martinez, 1972) (Bhagabati & Nariana, 1980) (Huang *et al*., 1980). Of interest is that some isolates of CTV apparently protect trees from HLB infection (Van Vuuren *et al*., 2000). Blotchy mottle, the most characteristic leaf symptom, can be confused with other diseases such as stubborn (*Spiroplasmacitri*), severe forms of CTV, *Phytophthora* root rot and water logging (Calavan, 1968) (McClean & Oberholzer, 1965) (Schneider, 1968.)). Due to the non-specific nature of leaf symptoms, HLB can often be confused with mineral deficiency or other stress related leaf symptoms (Korsten et al., 1993). Symptoms of zinc deficiency are also associated with the early stages of citrus blight.

2.6.3. HLB pathogen

In (Laflèche & Bové, 1970)reported mycoplasma-type bodies in sieve tubes of sweet orange infected with HLB. The observation of cellular organisms in the phloem of HLB-

infected citrus and their absence in healthy material indicated that a prokaryotic organism was responsible (Laflèche & Bové, 1970). Two distinct forms of greening were recognized based on the wider geographical spread (Capoor *et al.*, 1967)Electron microscope studies suggested that the organism was a true bacterium, belonging to the Gracilicute division, (Garnier & Bové, 1978) Molecular diagnosis suggest that, HLB pathogen is member of sub division Proteobacteriacea Subsequently the Asian species was designated "*Candidatus Liberobacter asiaticum*", and the African species "*Candidatus L. africanum*". These names have since been corrected to*"Candidatus Liberibacte rasiaticus*" and "*Candidatus L. africanus*" (Garnier et al., 2000b). However on the basis of molecular phylogenic study (Teixeira *et al*., 2005)reported three HLB pathogens as:

- *Candidatus Liberibacter africanus*, heat sensitive, found at higher elevation (900m above sea level) transmitted by *Trioza erytreae* vector (African strain),
- *Candidatus Liberibacter asiaticus*", more severe, heat tolerant, found at lower elevation (360m abov Sea level), and higher temperature (30-35^{o}C) transmitted by by *Diaphorina citri* vector (kuwayama) (Asian strain)
- *Candidatus L. americanus,* heat- tolerant, by *D. citri*vector *(*American strain)

The Asian citrus psyllid (ACP), *Diaphorina citri*, Kuwayama, (Homoptera: Psyllidae) is a well-established pest of citrus and close relatives of citrus. Asian citrus psyllid damages plant through its feeding activities. Adult psyllids are 3 to 4 mm in length with mottled wings held "roof-like" over the body (Mead, 1977)

2.6.4. Host range

All species of citrus appear to be susceptible, irrespective of the rootstock used (Aubert, 1993&Da Graça, 1991). However, symptoms are often severe on sweet orange, mandarin and their hybrids; moderate on grapefruit, lemon and sour orange; while lime, pummel and trifoliate orange are regarded as being more "tolerant" (Manicom & van-Vuuren, 1990). Both species of *liberibacter* have been transmitted to periwinkle (*Catharanthu sroseus*) via dodder inducing marked foliar yellowing (Garnier & Bové, 1983, Ke *et al*., 1988) the dodder itself also appears to support HLB multiplication.

The psylla species which transmit HLB from citrus to citrus, feed on many other rutaceous species. *D. citri* has a preference for *Murraya spp*. (Chakrabarty *et al*., 1976), and it has been suggested that *Trioza*. erytreae's original hosts include *Veprisundulata, Clausena anisata* and *Zanthoxylum capense* (Moran, 1978)(Su *et al*., 1992), has reported the detection of Asian HLB by DNA-hybridization in *Severinia buxifolia* and *Limonia acidissima*, and African HLB was detected in *Toddalia lanceolata* (Veprisundulata). The Cape chestnut (*Calodendrum capense*), an ornamental rutaceous tree in South Africa, has been shown to be infected with HLB, subsequently this organism was shown to be a subspecies of the African form of greening.(Garnier *et al*., 2000b).

2.6.5. Transmission and Epidemiology

A. Transmission by grafting

HLB was first transmitted experimentally by grafting (Chen, 1943) there by establishing the causal agent as a pathogen. Natural spread was demonstrated by exposing healthy seedlings in an infected citrus orchard (Schwarz, 1964). The variability in graft transmission of *Candidatus*. *Liberibacter* depends upon the plant part used for grafting, the amount of tissue used and the pathogen isolate with single buds (Batool et al., 2007). Transmission of HLB by grafting was first reported in china by Lin in the late 1940s. (Zhao, 1981). Kinds of tissue used (buds, side grafts or left pieces), age of the tissue, and the season of the year when inoculums collected are the major factors that led to success the graft transmission of pathogen. African greening is most rarely transmitted by grafting than Asian greening (Roistacher & Bar-Joseph, 1987).

B. Transmission by vector

Transmission of HLB by Asian Citrus psyllid vector *Diaphorina citri*, was reportd in 1967 in India (Capoor et al., 1967)and in the Phillippines (Martinez & Wallace, 1968).*Diaphorina citri* Kuwayama (Homoptera:Psyllidae) is a Hemipterian insect brown in color measuring 3-4 mm in length with piercing- sucking mouth part that allows this insect to feed on the phloem of Citrus species and other related Rutaceous plant While eating their bodies are lifted at the angle of 45^{o} on the surface of leaf. Both the nymph (fourth and fifth instar and adult can transmit the greening agent in 15 min acquisition

feeding time. Orange jasmine (*Murraya paniculata*) has been found to be the host of *Ca. Liberibacter spp* and can serve as a potential source of inocula (Brlansky *et al*., 2011) .

Another insect vector is *Trioza erytreae*. This vector is responsible to transmit African form of greening bacterium. It has been shown experimentally that *T. erytreae* is also able to transmit the Asian form (McClean & Oberholzer, 1965).

C. Transmission by Dodder

Greening is also transmitted by dodder (cuscuta spp., Cuscutaceae) to non rutaceous plant such as *Catharanthusroseus* L. G. Don (Periwinkle- apocynaceae) and *Nicotiana tobacum* L. Cv. 'Xanthii' (tobacco, Solanaceae) (Garnier & Bové, 1996), '*Ca. L. asiaticus'* can multiply and spread within infected *Cusutaceanothi* Behr (Syn. Cu. Subinclusa Dur, and Hilg."), *Cuscutacam pestris* and *Cu*. *australis.* (Ghosh et al., 1977), observed that the pathogen multiplies more favorably in dodder in diseased sweet orange.

2.6.6. Methods for Detection of HLB

Correct identification of the Greeninig disease under field condition is important for disease management. Greenining disease can tentatively be identified in the field by foliage and fruit symptoms. Irregular distribution of the disease within the tree and slow disease development make visual detection difficult. Moreover diagnosis of Citrus greening based on symptomatology is very often difficult as it can be confused with mineral deficiency, root rot or other stress related leaf symptoms and it needs to be confirmed by other diagnostic tests. For many years the only way to detect the pathogen was by biological indexing on sweet orange seedlings, observation of the bacterium in the phloem sieve tubes of leaf mid vein or columella by electron microscopy or using fluorescent Chromatographic technique. Fluorescent chromatographic technique however sometimes produces false negative/positive, so it cannot be used alone for definitive diagnosis. More recently, several molecular detection methods have been developed.

Detection by polymerase chain reaction using specific primers in the 16s rDNA (OI1/ OA1/OI2C) or in the ribosomal protein genes (A2/ J5) is the easiest and most sensitive method for *libribacte*r detection and identification. Indeed, these techniques allow distinction between the two *Liberibacter* species. Nucleic acid hybridization using radio-active or non-radioactive probes to detect the greening bacterium in plans as well as psylla vector have been reported. The two *Liberibacter spp* can be detected in plants and insect by DNA/DNA hybridization with probes In-2.6 for *candidatus Liberobacter asiaticus* and AS-1.7 for *Candidatus Liberibacter africanus*. These probes contain genes of the β-operon encoding, in particular ribosomal proteins. Monoclonal antibodies are also available to distinguish serotypes within species but are too strain specific for general diagnostic purposes. Real time PCR based detection of Greening pathogen both in plant and insect vectors has become very popular in developed countries (FAO Nepal, 2012)

2.6.7. Control measures

A. Chemical Control

The evidence that a procaryote was the causal organism led to research on the use of tree injections with antibiotics to eliminate the bacteria. Tetracycline hydrochloride had some beneficial effects but proved to be phytotoxic and attention turned to a more soluble less toxic derivative N-pyrrolidinomethyl tetracycline. Several insecticides against psylla are available, and the development of a trunk application technique has proved effective (Buitendag, 1988). Another way is the placing of sticky yellow traps in orchards which could detect a population threshold but this method has not been widely adopted and scouting is often used. Due to the potential for re-infection, and high costs, attention turned to vector control (Buitendag & von Broembsen, 1993).

B. Biological Control

The use of these parasites (vector), combined with the establishment of disease-free foundation blocks and nurseries, resulted in a dramatic reduction in the incidence of HLB - in 1995, 20 years after the launching of this strategy, only 0.5% of trees surveyed had symptoms (Aubert *et al.*, 1996). *Tamaraxia. radiata*, and another wasp species,

Diaphorencyrtusa ligarhensis, are evaluated in Florida for potential use (Hoy & Nguyen, 2000). In addition, *Diaphorina. citri* appears to be an excellent food source for several ladybeetle species (Michaud *et al*., 2002) and used to feed on psylla, aphids, citrus leaf miner and other foliage pests. Similarly, the use of versatile chryospid predator *Mallada boninensis* @30 larva per plant and growing marigold as border crop has been reported very effective to suppress psylla population in the orchards.

C. Integrated Control measures

The only way to grow citrus productively in countries where the disease has become endemic such as in South Africa and Asia is by managing the disease using sound integrated pest management. In South Africa, (Buitendag & von Broembsen, 1993) recommend a strategy of providing growers with disease-free nursery trees, focusing on reducing the inoculums by removing infected trees or branches, and following an effective psylla control program. In China, there are reports of successful management by eradicating infected trees and non-citrus psylla hosts, planting HLB-free trees, and controlling psylla populations. (Bové *et al*., 2000), conducted program in Indonesia, and showed that if citrus is eradicated before replanting, only HLB-free bud-wood is used for replanting and the control *D. citri* using insecticide sprays is effective, rehabilitation of a citrus industry could be possible. Eradication of alternate hosts in close proximity (5 km) to nurseries or commercial plantings of citrus, have been suggested and shown to be effective in Asia where *Murraya spp* are the principal alternate hosts (Aubert, 1993) (Aubert & Xia, 1990). Soil application of micro nutrient viz zincs sulphate, iron sulphate and manganese sulphate in the rate of 150- 200 g each per plant improves the general health condition of plant. Spraying Neem oil in the rate of 5ml/lit of water is another effective way to control psylla and other insect.

An intensive and integrated management approach has been recommended as the most effective against greening disease in any part of the world. This includes quarantine measures use of disease free planting material, removal of alternative host of both the psylla and bacteria, reduction of inoculums by pruning of infected branches, disinfection of pruning tolls with 1-2 % sodium hypochlorite, removal of healthy infected trees and chemicals as well as biological control of insect vector Psylla.

2.7. *Citrus tresteza* Virus (CTV)

2.7.1 Origin history, Geographical Distribution and Economic Impact

Citrus tristeza virus (CTV) has been officially reported from Turkey since 1963. (Baloglu & Birisik, 2009). Farmers in Brazil and other South American countries gave it the name tresteza meaning sadness. In late 1984, some growers were used bud wood of a very early maturing Satsuma mandarin of uncertain origin, for top working citrus orchards near Valencia. Indexing of this material showed that it was infected with a very severe strain of citrus tresteza virus (CTV). There is some evidence indicating that the original satsuma was illegally introduced from Japan. Tristeza may be present in these countries symptomless in varieties such as mandarins or sweet orange on tolerant rootstocks. The spread and movement of tristeza depends upon the distribution of infected bud wood, the vectors present, the strains of the virus present, and temperatures. China is probably the home of many species of citrus and also of the origin of the *Citrus tristeza* virus. The probable routes of tristeza through movement of infected plants and vector was from China to Japan, to the Philippines, India, Australia, and South Africa. CTV and its principal vector *Toxopteracitricida* became endemic in these countries.

Tristeza was present in California and Florida in the 1880's with the importation of satsuma mandarin trees from Japan (Tanaka, 1952). (Fraser & Broadbent, 1979)In reviewing the history of tristeza in Australia indicated the presence of the disease and its most efficient vector well before 1890 and perhaps earlier than 1870. They also indicated that there is evidence that in 1933, large shipments of citrus trees were sent from Australia to estates being developed in Argentina. There has been a direct relationship between the great Phytophthora epidemics of the 19th century and the ensuing tristeza epidemics, beginning in the 1930's and directly related to the sour orange as a rootstock.

The spread of Phytophthora species detrimental to citrus was epidemic between 1836 and 1916. The destruction of seedling citrus trees was devastating. The worldwide epidemics by Phytophthora induced a change in citrus culture from primarily growing trees as seedlings to budded scions on sour orange rootstock. The sour orange was found to be tolerant to citrus *Phytophthora* species and also was found to be a superb rootstock. The sour orange was tried as a rootstock in Australia prior to 1870 (Fraser & Broadbent,

1979)and in South Africa about 1895. Its failure in both countries was thought to be due to incompatibility, but was in fact due to tristeza. It is apparent that tristeza and its prime vector *T. citricida* were well established in Australia and South Africa at that time. In South Africa, when sweet orange was put on sour orange rootstock, the trees did not grow and ultimately died. Dead trees killed by tristeza in Argentina were piled up and the wood carted away in the mid 1930's and the 1940's.

The destruction of sweet orange on sour orange rootstock in California began in 1939. It was called 'quick decline' because the trees declined very rapidly during the springtime.Death of trees by the new 'quick decline' of citrus was related to the death or necrosis of the phloem cells in the cambium tissue of the sour orange (Schneider, 1954). This effectively girdled the tree at the bud union. With death of these phloem cells, the starch produced in the leaves of the canopy was prevented from being transported to the roots; the roots died and when the weather warmed up in the spring the trees quickly declined. Thus the name 'quick decline' this virus killed infects nearly all the citrus species and their relatives and hybrid in India (Ahlawat et al., 1992, (Buitendag & von-Broembsen, 1993, Ahlawat & Pant, 2003 &Biswas et al., 2004). Several orchards of Darjeeling mandarin are being wiped out gradually due to severe attack of CTV (Biswas et al., 2004) (Mukhopadhyay, 1985). Disease caused by CTV in mandarin orchards in Darjeeling hill region has been reported earlier based on visual observation of decline symptoms, inconsistent biological indexing and limited serological tests of field samples (Mukhopadhyay, 1985, Biswas, 2008). Citrus virus and virus-like diseases were unknown in Nepal prior to the importation of grafted citrus from Saharanpur (Uttar Pradesh) in India in the 1960's (Knorr & Shah, 1971). Subsequent surveys have shown CTV to be wide spread causing a decline in acid limes (Lama & Amatya, 1993) Since then, other virus and virus-like diseases have been observed The aphid *Toxopteracitricida* is also wide spread in Nepal and was observed. Thepossibilities of tresteza stem pitting are mentioned by (Roistacher, 1996). One possibly serious discovery was made at Kirtipur Agricultural Research Section. In Severely affected tree stem pitting was observed. Mandarin is usually symptomless carrier of severe strain of CTV (Yamada & Tanaka, 1969, Roistacher, 1988). According to Roistacher, 1996, various mandarins were introduced from Japan as a scion bud woods for the JICA project and these mandarins

contained severe strain of CTV. These strains could have been spread to the sweet orange trees by *T. citricida* and could be responsible for the decline observed.

2.7.2 Disease caused by CTV and Symptoms

There are three principal diseases caused by CTV in citrus trees: tristeza, stem pitting, and seedling yellow (Nelson et al., 2011). Other Various types of symptoms caused by CTV in different citrus species include decline, Vein clearing and flecking,vein corking depending on the scion root stock combinations, virus stain, time of infection, environmental condition etc. tristeza is a decline of different scion cultivars grafted onto sour orange rootstocks. This decline can occur over a period of several years, or in only a few months (this rapid form of the disease is also known as "quick decline"). Trees with tristeza initially appear water stressed; this stage is followed by defoliation and death. Stem pitting is a disease most commonly seen in grapefruits, sweet oranges, and some lime cultivars.

Trees with severe stem pitting appear stunted, with chlorotic leaves that often display "vein-clearing" symptoms Twigs and small branches on these trees are brittle and can be snapped with little effort. When the bark is removed from twigs or branches, the wood will have small pinhole-like pits, or long grooves that give it a rope-like texture. Stem pitting is diagnostic and suggestive of severe strain of CTV (Roistacher, 1996).

Seedling yellow is a disease of sour orange, lemon, and grapefruit seedlings. Susceptible seedlings infected with these CTV strains become stunted and have small, chlorotic leaves. Seedling yellows is most devastating in nursery operations (Nelson *et al*., 2011).

Trees grafted on sour orange rootstocks usually show dieback and defoliation, stunting, phloem necrosis and in many cases complete decline. This symptomatology is caused by the starvation of the roots as a consequence of sieve-tube necrosis induced by the virus below the bud union. Cells of the medullary rays of the wood become lignified in this area and produce the symptom called inverse pitting, honey combing or pin holing. However, this symptom is not specific to tristeza and can also be induced by

Spiroplasmacitri (stubborn disease). The fruit sizes on infected trees were small, stunted, and unproductive.

2.7.3 The CTV Virus

CTV is a member of the virus family Closteroviridae, genus *Closterovirus*, member of mono-particle of which Beet yellows virus (BYV) is the type member. The closteroviruses are positive single stranded RNA viruses with long, flexuous particles with a size of approximately 6.5 x 106. A typical CTV particle is approximately 2000 nm in length and 12 nm in diameter. The capsid protein has a molecular weight of 27 000-28 000 Dalton. (Biswas, (2008), Bar-Joseph *et al*., (1989) &Lee & Bar-Joseph, 2000)

2.7.4 Host range

CTV is limited to the phloem tissues of infected hosts. The natural host range for CTV is confined to members of the plant family Rutaceae, of which citrus species are members. Some *Passiflora* species are the only known non-rutaceous hosts (Moreno & Garnsey, 2010) (Roistacher & Bar-Joseph, 1987). CTV infects all species, cultivars, and hybrids of citrus. It also infects some citrus relatives such as *Aeglopsis, Afraegle, Fortunella* and *Pamburus* and some *intergeneric* hybrids. Species of *Passiflora* have been infected experimentally (but not naturally) and are the only non-rutaceous experimental hosts. Numerous strains of CTV have been described that vary in virulence and the severity of the diseases they cause. Some strains of CTV cause tristeza, but not stem pitting. Similarly, some strains cause stem pitting in oranges, but not in grapefruit. The most reliable and effective method for differentiating the CTV strains is through the use of indicator plant. Mexican lime, sour orange, 'Duncan' grapefruit, 'Madam Vinous' sweet orange, and navel sweet orange on a sour orange rootstock was used as indicator plant. Symptom development in these indicator plants following inoculation with an unknown CTV strain helps to determine whether the strain can cause quick decline or stem pitting, as well as indicating the severity of symptoms. Severe strains of CTV will cause tristeza and/or stem-pitting symptoms, whereas milder strains cause less severe disease or may infect citrus plants asymptomatically. Some strains of CTV may induce symptoms differentially in one host but not in another.

2.7.5 Transmission

The virus is predominantly transmitted through grafting and insect vector brown citrus aphid (BrCA) insemi permanent. *Toxoptera citricidus* is the most efficient vector. Aphis *citricola* and *Toxopteraaurantii* are less efficient vectors, but are more abundant on citrus followed by the melon aphid (*Aphis gossypii*) The black citrus aphid (*T. aurantii*) and spirea (or citrus) aphid (*A. spiraecola=A. citricola*) are inefficient vectors of CTV, or are only able to transmit certain strains of the virus. These aphids, however, can build up large populations in citrus groves and therefore may contribute to the spread of CTV in some circumstances.A single aphid *of T. citricida* could transmit the tristeza disease (Costa & Grant, 1951).

Although CTV is not seed-transmissible, it is readily transmitted through grafting. Since most citrus species are vegetatively propagated, dissemination of infected bud-wood has greatly increased the distribution of CTV world-wide. CTV can also be transmitted by parasitic plants called dodder (*Cuscuta sp.*), but this means of transmission is not thought to be important in the spread of the virus.

2.7.6 Methods for Detection of CTV

CTV can be detected by biological indexing and various non-biological methods including light and electron microscopy, serology and a variety of molecular-based techniques. The latter include several types of reverse transcription polymerase chain reaction (RT-PCR), including an immune capture PCR with multiple molecular markers (MMM) (Hilf *et al.*, 2005) and real time PCR (Ruiz-Ruiz *et al*., 2009) (Ruiz-Ruiz *et al*., 2007). SSCP analysis (Rubio *et al*., 1996)oligo-probes (Narvaez *et al*., 2000)and RFLP analysis (Gillings *et al*., 1993)

A. Field diagnosis

If the rootstock is sour orange and the scion sweet orange, grapefruit or mandarin, a sudden quick decline and wilting, followed by defoliation, especially during the first warm weather of spring, would suggest possible infection by tristeza.

B. The iodine test

A simple field test can be carried out to detect starch depletion in the roots or rootstock below the bud union. The disappearance of starch from the roots is a result of girdling owing to killing of the phloem cells at the bud-union. Tristeza-induced starch depletion generally proceeds from the outer tips of the roots back toward the trunk of the tree. The application of iodine to the exposed cut surface of a root is a rapid method of testing for starch. (Roistacher, 1991)

C. Biological Indexing

A new system of biological indexing based on the use of indicator cuttings instead of seedlings was developed for the detection of the main citrus virus and viroids. The discovery of the small fruited lime (*C. aurantifolia*) as an index plant for the citrus tristeza virus (CTV) integrated many separate diseases as just one disease 'tristeza' all caused by CTV. Biological indexing is still required for detectionof the severe seedling yellows and stems pitting isolates of CTV. Most tristeza will usually induce typical vein clearing and cupping in leaves of the small fruited lime (*Citrus aurantifolia*). In addition to vein clearing, the cupping of the leaf in Mexican lime is helpful for diagnosis in greenhouse indicator plants grown under relatively cool conditions. However, the vein enation virus may also induce a leaf cupping reaction in Mexican lime indicator seedlings.

D. The detection of severe CTV isolates by ELISA

The use of ELISA for detection and diagnosis of tristeza is now a well-tested and proven technique and should be incorporated into any indexing program. It is an excellent technique for surveys and large-scale testing, for obtaining very rapid results and for verifying the presence or absence of CTV isolates that are mild reacting in indicator

plants. The time of year for ELISA testing is critical. In a certification program, both biological procedures and ELISA should be used for indexing. The acceptance and use of ELISA for determining the presence and spread of CTV is now worldwide and universal. In Israel, one million citrus trees for CTV by ELISA in the three years following the initial development of the ELISA technique (Bar-joseph *et al*., 1983). ELISA has been used for epidemiology studies throughout the world.

E. Microscopic detection of inclusion bodies

Inclusion bodies of CTV may be seen in sectioned tissue by light microscopy. This can permit avery rapid means of tristeza identification and have been reviewed by.CTV detection by dsRNA analysis. A specific band in stained electrophoretic gel is diagnostic for tristeza and there is evidence that certain seedling yellows isolates are distinguished from tristeza isolates by specific band migration locations. CTV virus particles can be detected and identified within minutes by dicing small quantities of tissue in a specific buffer, placing it on grids and observing it in an electron microscope. This is the most rapid method of detecting and diagnosing CTV (Garnsey *et al*., 1980)

F. CTV detection by dsRNA analysis

A specific band in stained electrophoretic gel is diagnostic for tristeza and there is evidence that certain seedling yellows isolates are distinguished from tristeza isolates by specific band migration locations. (Dodds & Bar Joseph, 1983), reported on double stranded Ribonucleic acid (dsRNA) from plants infected with closteroviruses. The band at 0.05 of typical DsRNA profiles has been found associated with the more exotic, severe CTV isolates. Isolation of dsRNA from citrus Complicated by variables such as virus strain, host species/variety, and time of year, which often alter the results (Dodds *et al.*, 1987)

G. Detection of CTV by electron microscopy

CTV virus particles can be detected and identified within minutes by dicing small quantities of tissue in a specific buffer, placing it on grids andobserving it in an electron microscope. This is the most rapid method of detecting and diagnosing CTV.

H. Direct tissue blot immunoassay

Garnsey *et al.,* 1993, introduced a direct tissue blot immunoassay, which is rapid, requires little sample preparation and the membranes can be stored for as long as 30 days prior to assay. Also, blotted membranes can be sent to another location for testing.

I. Reverse transcriptase-polymerase chain reaction (RT-PCR)

For CTV detection in plant and aphid tissues; although more technically challenging than ELISA, it has unparalleled sensitivity and can detect CTV when ELISA cannot. The CTV genome is a positive sense, single-stranded RNA composed of 19,296 nucleotides, and it contains the potential for encoding of 17 proteins.

2.7.7 Elimination

A. Methods for clean- up

If it shows that the variety is infected, it must be subjected to therapy procedures that can eliminate the disease or diseases from the bud-line. There are two methods of therapy, thermal therapy, and shoot-tip-micro-grafting.

B. Thermaltherapy

It is also called heat treatment, which is performed by grafting infected buds into Citrange seedlings. The newly grafted infected bud is tightly and completely wrapped with budding tape. The citrange seedlings, each with an infected bud grafted on it are placed into a hot greenhouse kept at 28-40^{o}C daytime and 25^{o}C nighttime for preconditioning to high temperatures for 30 days. Following preconditioning the seedlings are placed in a controlled temperature chamber set for 16 hours days at 40^{o}C and 8 hours night set at 30^{o}C for a period of three months. Upon removal of the plants from the temperature chamber, the buds are unwrapped. The rootstocks seedlings are

lopped over and the top of the seedlings is pushed into the potting soil such that the grafted bud will become the terminal bud. The plants are then placed in the greenhouse until sufficient bud wood growth is produced from the grafted bud for further indexing.

C. Shoot-tip-micro grafting

Some pathogens, particularly the citrus viroids (exocortis/cachexia), are difficult or impossible eliminate by thermal therapy, and are more readily eliminated by shoot-tip-micro-grafting (STG). STG is a procedure where several new growth tips slightly less than 1cm in length are taken from one of the original infected import propagations. Then with the aid of a microscope, a very small portion of the shoot tip, about 0.15 mm is removed from the infected growth tips and grafted onto the rootstock seedling growing in the test tubes. If the shoot tip that is removed is small enough the disease agent will not present in the micro- grafted propagation. STG propagation are returned to glass tubes and placed under light in a culture chamber. When the scion of the micro-grafted propagation reaches about 2 cm, it is re-grafted onto the clean rough lemon seedling

2.7.8 Control measures

- It is possible to eradicate the disease once an area becomes infected because tropical Aphid has wide spread distribution. However the manifestation of the disease can be minimized by raising future Citrus plantings on tresteza tolerant root-stocks.
- Isolation of infected plant and destruction of them is most important general precaution. Complete eradication of the source of virus is particularly impossible. The simplest method for avoiding virus infection is to grow disease resistant varieties.
- The disease occurs only when sweet orange scions are grafted on to sour orange stocks and the tree apparently dies from root rot, without showing any external symptoms.

- Time and effort has been spent on trying to find chemical that will directly eliminate or restrict virus multiplication in crop plants. To date, however, for reasons of in effeteness, phyto-toxicity or economy, no such chemical have been found. Therefore infection prevention restrictive measures must be taken. Sometimes a combination of control measures is applied known as integrated control.

3. MATERIALS AND METHODS

3.1. Map of Study areas

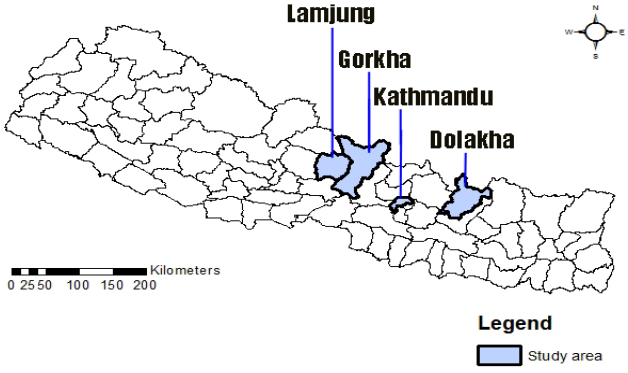

Figure 1 Map of Nepal showing study areas

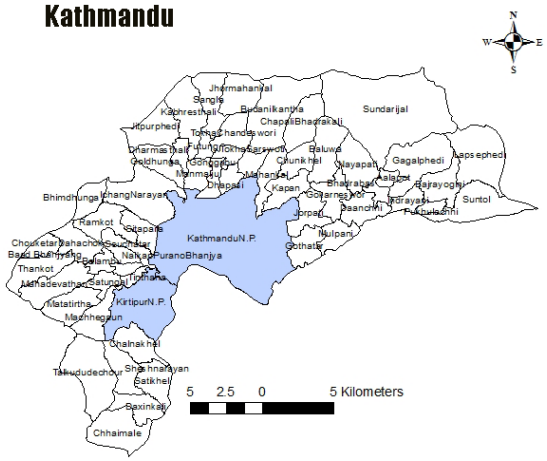

Figure 2 Map of Kathmandu with sample collected places

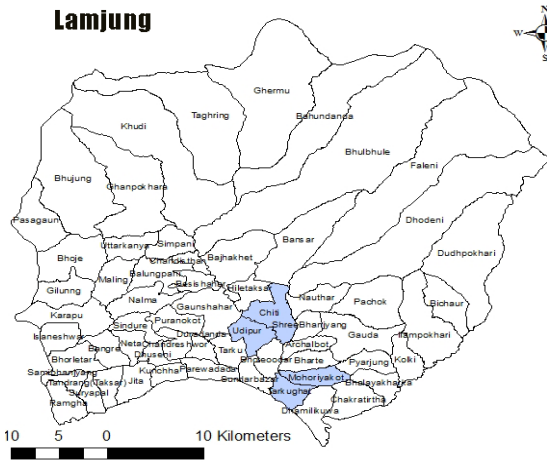

Figure 3 Map of Lamjung District with sample collected VDCs

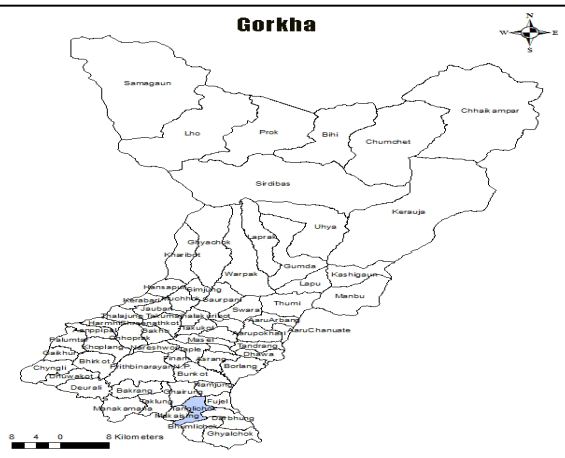

Figure 4 Map of Gorkha District with sample collected VDC

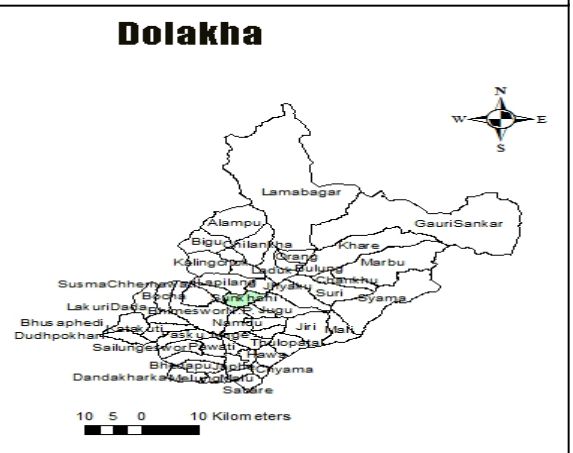

Figure 5 Map of Dolakha District with sample collected VDC

3.2. Field survey for HLB test

HLB test was done in two different districts *Lamjung*, and Kathmandu. Sample were collected from four VDCs, *Mohoriyakot*, *Tarkughat*, *Udipur* and *Chiti* of *Lamjung* district, and different places of *Kathmandu*, *Lazimpat*, *Kirtipur* and *Gairi*dhara, *SANN* college. During collection HLB positive suspected mother plants having leaves with blotchy mottle like symptoms were selected .and collection were done during month of October 2013 and samples were kept at -4^{o}c at SANN molecular biology lab before DNA extraction.

3.3.Field survey for Citrus tresteza Virus test

All the species of citrus grown in the selected orchards were considered for study. Sweet orange, mandarin, pumello(grapefruit), Junar, Hill lime, and Trifoliate Orange were the major species inspected. Observation was made on aspects such as the overall appearance of the orchards and status of the individual trees of the orchards. Samples were collected from *Lamjung*, *Gorkha Dolakha* and *Kathmandu* districts. Field survey was carried out in the year 2012.

3.4. Procedure followed during sample collect ion

- For Shoot tip and node culture- rapidly growing parts of Mandarin orange and Trifoliate orange were collected from suspected mother plant in zip lock polythene bag. Polythene bag were labeled clearly and sample collected orchard tree also numbered.
- For CTV- Leaves samples were collected in zip locked plastic bags from *Lamjung, Gorkha,Dolakha* and *Kathmandu* Districts and kept at 4^{o}c at GREAT lab until CTV test. During collection -
- *Lamjung*- Leavesamples were collected randomly from the Citrus species- including *Citrus reticulata*(mandarin-(nursery plant, grafted and seedling), *C. sinensis* (junar), *C. pseudolimon* (nibuwa), *C. aurantifolia* (kagati) and *C.grandis* (Bhogate).

- *Gorkha*- during first visit, sample was collected randomly from screen house and open orchards. Leaves samples from twelve plants were collected among them one is of sweet orange and remaining all are of mandarin orange. Labeling was done in field trees as well as zip locked polythene bags. Second visit was focused with the sample of screen house and open orchards. Trifoliate orange from infected mother plant was grown both in screen house and open orchard so leaves sample of both places were collected randomly and well labeled.
- *Dolakha*- Random sampling of leaves samples of mandarin orange was done from sunkhani VDC from three different locations.
- *Kathmandu*- Random sampling of leaves samples of Trifoliate orange was done from HDP, *kirtipur*. All collected samples were kept at 4oc in GREAT lab until CTV test.
- To test the citrus greening disease, *Candidatus Libribacter asiaticus*, altogether nine mandarin orange (*C.reticulata)* leaves with blotchy mottle symptoms were collected from two Districts, *Lamjung,* and *Kathmandu* in zip locked plastic bag. From Lamjung, five samples from four different VDCs was collected, similarly four samples from three different locality of Kathmandu was selected. All collected sample was kept at SANN molecular lab at -4^{o}C prior to Lab test.

CTV test was done two times. First test was done on 28 and 29 January 2012.At that time Mandarin orange and Junar collected from Tanglichowak, *Gorkha* District was tested. Second test was done for the collected samples of *Lamjung, Gorkha* and *Dolakha* Districts. At that time, Mandarin orange, sweet orange, Trifoliate orange, Acid lime, Hill lemon and Pummelo was tested. CTV test was performed in 2012/12/15 and 2012/12/16 with the help of DAS-ELISA.

3.5.Polymerase Chain Reaction for Detection of Citrus greening Disease

3.5.1 DNA Extraction

DNA of suspected leaf samples was extracted by following Graham DNA extraction protocol. (Graham et al., 1994). Approximately 0.2 gm of mid rib of suspected leaf

samples was taken in a disposable plastic Petri-dish(small) .Those sample was chopped into small pieces with the help of sterilized razor blade. Those sample then ground to fine powder using mortar and pestle in liquid nitrogen. 1 ml of CTAB buffer (DNA extraction buffer) was added and grinded to the fine paste and transferred into clean sterilizes eppendrof tube (vol. 1.5). Sample was incubated at 55^{o}C for 15 minutes, after incubation, samples were centrifuged at 11,000 rpm for 15 minutes at 4^{o}C. Then supernatants were transferred to clean sterile microfuge tubes and equal volume of Chloroform: isoamyl alcohol in the ratio of 24:1 was added and mixed gently by inversion for several times (5-8 minutes.) It was centrifuged for 2 minutes in 11,000 rpm at 4^{o}C and upper aqueous phase was transferred to clean eppendrof tube and re-extracted with chloroform: isoamyl alcohol and centrifugation was repeated for 1 minute. Upper layer was transferred to new tubes and 1/10th vol. (approximately 50µlof ammonium acetate (7.5M)) was added to each sample followed by addition of equal volume (500µl) of chilled ice cold absolute ethanol and stored at -20^{o}C for overnight for DNA precipitation. After overnight incubation, DNA was centrifuged at11,000 rpm for 10-15 minutesinto pellet. Supernatant was discarded and pellet was washed with ice cold 70 percent ethanol (500 µl volumes). Then it was centrifuged at 11000 rpm for 1 minute to get rid of salt. After that ethanol was pipette out and the pallet was dry for 15 minutes. Pellet was left to dry for 15 minutes and it was re-suspended in T.E. buffer 300µl and stored at 4^{o}C.

3.5.2 PCR amplification

PCR amplification based on 703bp long fragment of ribosomal protein genes (rpl-PCR) in the rplKAJL-rpOBC (β-operon) using primer A2 and J5 (Hocquellet et al., 2000)was done. PCR reaction was performed in 50 µl reaction volume containing 1µM of each of the primers, A2 (5'-TATAAAGGTTGACCTTTCGACTTT-3'), J5 (5'ACAAAAGCA GAAATAGCACGAACAA-3'), 0.4 µL each of four dNTPs with $MgCl_2$, 10µL of buffer (1x) and 1U Taq polymerase (0.5 µL) (FiNNZYMES). 10 µl of 'DNA extract' of various samples was used as template DNA in the PCR. The amplification was carried out in thermal cycler (Long Gene Scientific instrument co. ltd.), with following programs. Cycling condition for rpl-PCR was 35cycles each at 92˚C for 1 min (denaturation step),

62˚C for 1 min (Annealing of primers) and 72˚C for 2 min (strand elongation), and 72˚C for 10 minutes (final extension).

3.5.3 Gel electrophoresis

The amplification product of 16S-PCR and rpl-PCR were analyzed using 1% agarose gel in TBE (1x) buffer at 100V for half an hour using EMBI TEC (Santiago, CA), gel tank. Following electrophoresis, the gel was stained in gel tray containing TBE buffer (ca. 200ml) and 5 µl of Ethidium bromide (10mg/ml) for 45 minutes. The gel was then visualized on an UV trans-illuminator (UVITEC, Japan) and photographed using a Polaroid camera system (Geleam, UK).

3.6.In-vitro Node and Shoot tip culture

Tissue culture work was performed in Biochemistry and biotechnology lab, Central department of Botany, *Kirtipur*, Nepal.

3.6.1 Sterilization of Plant materials

Already collected shoot tips and node of mandarin orange and trifoliate orange were washed in mild detergent (Teepol) 3 times. They were kept under continuously flow using tap water for 10 minutes taking in a beaker and covered with muslin cloth. Shoot tips and node of different species were kept in 70% ethanol for 1 minute in different beakers. And immediately transferred to the beaker with 1% sodium hypochlorite and left for 7 minutes. After that, they were washed with sterile distilled water for three times.The surface sterilization was done inside laminar air flow cabinet. Shoot tips and node were cut in required size (2mm for shoot tip and single node for node culture) with the help of sterile blade. Explants were kept to soak water on whatmans number 1 filter paper in Petri dish.

3.6.2 Culture of shoot tip and node

Shoot tip of size about 2mm was inoculated in MS media containing 1BAP and 0.1 NAA` under laminar air flow cabinet in culture jar. 4 replicates were kept in one jar, and each species contained 2 jars. Similarly node was cultured in culture tubes with 5 replicates each. After that culture tubes and culture jar were kept in culture room

3.7. DAS-ELISA for detection of CTV in different Citrus species

Double antibody Sandwich enzyme Linked Immunosorbent Assay (DAS-ELISA) was used for detection of CTV titer in collected samples. The ELISA procedure using CTV positive specific antibody was performed as described by Ranjit et al 1998. Absorbance value were measured at 405 nm using Humareader (Microplate reaeder).The experiment was repeated three times.The following steps of DAS- ELISA were followed.

3.7.1 Coating the plant with antibody

The coating buffer was prepared as shown in annex (IV b,). The antibody was mixed with the coating buffer at 1:1000 ratios. 0.2 ml or 200 µl was poured into each well of the ELISA plate. with the help of a micropipette and allowed to incubate at 37^{o}C 4 hours. The coated plate was then washed very carefully with washing buffer three times.

3.7.2 Loading the virus sample

The extraction buffer was prepared as shown in (Annex IV, b). After extraction, each sample was loaded into each well with different micropipette tip. 0.2 ml or 200 µl was poured into each well. The plate was then incubated at 4-6^{o}C for 12 hours. After incubation the plate was washed three times with washing buffer.

3.7.3 Conjugate Loading

After preparation of conjugate buffer which was shown in (Annex IV, b), the conjugate antibody was mixed with the buffer at 1:1000 ratios. 200 µl was poured into each well and the plate was the incubated at 37^{o}C for 3-6 hours. After the allotted incubation period the plate was washed three times with a washing buffer.

3.7.4 Subatrate Loading

The final step of DAS- ELISA is to add substrate which is alkaline phosphate. The substrate solution is freshly prepared in substrate buffer at the rate of 1 mg/ml The substrate was prepared as shown in Annex (IV, b). The substrate solution was dispensed into the plate at the rate of 200 µl per well. The plate was incubated for 30-60 minutes for color reaction to take place. Strong yellow color was observed in the positive control and CTV positive samples.

3.7.5 Result Reading

After the yellow color formation, the optical density of each well was calculated with the help of an ELISA plate reader at 405 nm wavelength. This gives the quantitative result.

3.7.6 Reaction Stop

The color reaction was stopped after plate reading. This was done by adding 5 µl of 5% NaOH solution in each well.

4. RESULTS

4.1. Detection of HLB on Citrus samples collected from Lamjung and Kathmandu by PCR

Altogether 9 samples were tested for PCR reaction. CTAB-DNA extracted samples were preserved at -20^{o}C and were quantified by spectrophotometer before PCR amplification and only samples with reciprocal OD reading (of A260/A280), in between 1.4 and 2.0 were analyzed by PCR amplification. Samples from different districts were tested for Citrus Greening Disease. The PCR reaction was carried outusing 703bp long fragment of ribosomal protein genes (rpl-PCR) in the rplKAJL-rpOBC (β-operon) using primer A2 and J5 (Hocquellet et al., 2000). Among 9 samples tested 7 samples gave PCR positive reaction by PCR.

Table 2PCR detection of HLB on tested samples of Lamjung and Kathmandu

SN	Sample collected District	Place of sample collection VDCs	Total sample analyzed	Symbol used	CGD organism
1	Lamjung	Mohoriya kot	2	1M	+ve
				2M	+ve
		Tarkughat	1	3M	-ve
		Udipur	1	4M	+ve
		Chiti	1	5M	-ve
2	Kathmandu	Kirtipur	2	427 mada	+ve
				3272	+ve
		Lajimpat	1	A7	+ve
		Gairi dhara	1	A8	+ve

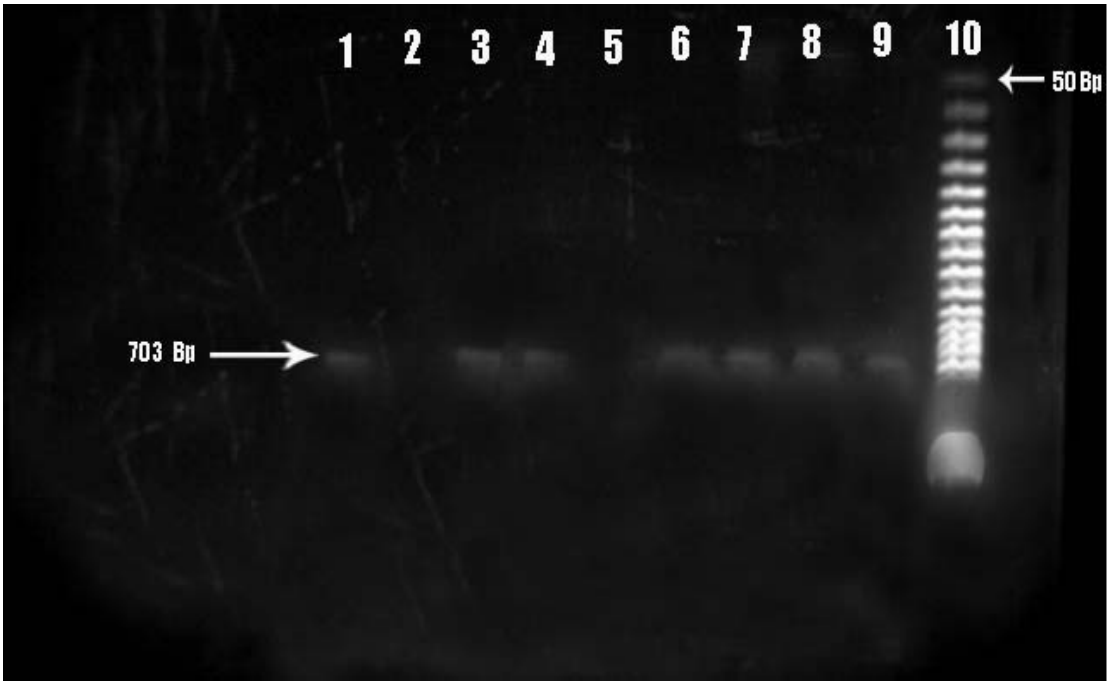

Figure 6 Electrophoresis on 1.5 % agarose gel of duplex PCR products amplified from DNA of symptomatic Citrus leaves using primer A2/J5 Lane 10, 50bp DNA ladder (Promega, USA); lane 1,3,4,6,7,8,9, DNA from *Ca. L. asiaticus*- infected leaves

4.1.1 Detection of Huanglongbing (HLB) from Lamjung

All collected species are of *C. reticulata*leaf sample with blotchy mottles were collected from suspected mother plants. From *Lamjung* District, among tested five samples three out of five were detected with HLB. Thus rate of infection in this district is 60%. From all samples collected VDCs except *Tarkughat*, were analyzed HLB positive.

4.1.2 Detection of Huanglongbing (HLB) from Kathmandu

C. reticulata samples from three different places, *Kirtipur*, *Lazimpat*, and *Gairidhara* SANN college, were analyzed by PCR reaction and all samples gave Positive PCR reaction with detection of HLB. Thus it is concluded that rate of infection by citrus greening from *Kathmandu* is 100%.

4.2. Result from Tissue culture

4.2.1 Shoot multiplication from shoot tip explants of *C. reticulata* and *Ponicirus trifoliate*

Explants were cultured on MS supplemented with combination of BAP 1mg/l and NAA 0.1 mg/l. *Ponicirus trifoliata* explants produced maximum 4 number (individual) of shoots with multiple leaves and initiation of several new shoot buds from single shoot tip in 5 weeks and *C. reticulata* showed maximum 3 number (individual) of shoots from single shoot tip in same time period.

4.2.2 Shoot multiplication from nodal explants of *C. reticulata* and *Ponicirus trifoliate*

Explants were cultured on MS supplemented with combination of BAP 1mg/l and NAA 0.1 mg/l. Trifoliate orange explants produced maximum 6 number (individual) of shoots with development of several leaves, and initiation of numerous new shoot buds was observed from single node in 4 weeks period. Similarly, *C. reticulata* showed maximum 5 numbers (individual) of shoot buds with initiation of shoots from single node in the same time period. Multiple leaves were developed from each individual buds after 6

weeks period and in some replica callus like substances appeared and cover the whole plant part.

4.3. Results of *Citrus tresteza* Virus

All in-vivo collected samples and tissue cultured samples were tested for *Citrus tresteza* virus test in ELISA, and optical density (OD) reading at 405 nm wave-length with the help of Humareader or ELISA- kit reader or spectrophotometer was recorded. CTV test was done two times for different samples. First test was done on 28 and 29 January 2012, with Test plate-1 and Second test was done on 15thand 16thOctober2012. In Test plate-2. Both tests were done with the help of DAS-ELISA protocol.

4.3.1. CTV Results from Test plate -1

A. CTV results of Gorkha District

From *Gorkha* district samples were collected randomly both from screen house as well as open orchard and all twelve samples were from grafted plants. The result was presented in figure 1 below. Sample 1g, 3g, 4g, 5g, 6g and 7g were from screen-house C. *reticulate* plant. All screen-house plants were nursery plants. These plants were grafted, around 4-5 month old and were transplanted into screen-house from open orchards. Other samples, namely 2g,8g, 9g, 10g, 11g and 12g are from open orchard *C. reticulata* plant. All open orchard plants were matured and grafted. They were around 5-6 years old. These plants gave good annual productions. Reference positive sample has the mean OD reading of 0.896 and that of negative reference has the mean OD reading of 0.295. OD readings of tested samples equal to or greater than the value of positive reference is considered as highly CTV infected, OD reading equal to or less than the OD value of negative reference is considered as CTV negative and OD value in between positive reference and negative reference is considered as mildly CTV positive. Sample named 2g, 3g, 4g and 6g were highly CTV infected, samples named 1g,7g,8g,and 9g were mildly CTV positive and sample named 5g, 10g,11g, and 12g are not infected with CTV.

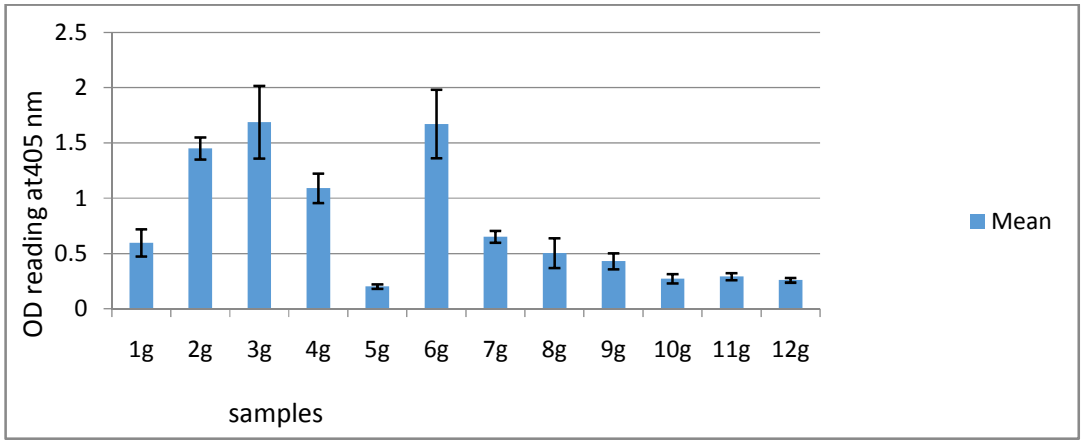

Figure 7.Condition of CTV in samples collected from Thapa Nursery Tanglichowk, Gorkha, (M±SD).

4.3.2. CTV Result of test plate -2

Result of test plate -2 was analyzed in different ways. Sample from different places and of different cultivars in different conditions were tested together in one ELISA kit. Positive reference had the mean OD of 0.573 and negative reference had mean OD of 0.206.

A. Trifoliate orange samples from mother plant

Among the five leaf samples of Trifoliate orange mother plant collected from HDP *Kirtipur* two samples Ti4 and Ti5 had mean OD value in between positive control and negative control and two samples were mildly CTV infected.

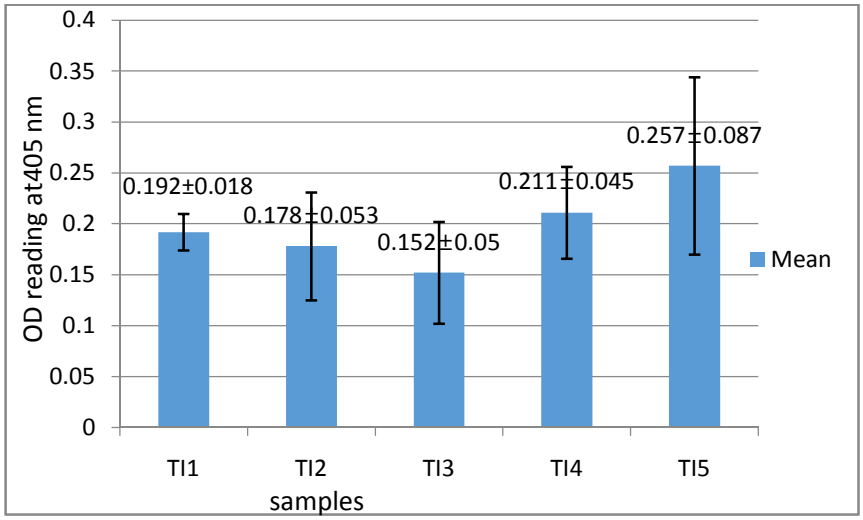

Figure 8. Optical Density reading at 405 nm wavelength of Trifoliate orange samples collected from mother Plant

B. Trifoliate orange samples from open orchards

Among the six samples that were collected from *Thapa-Nursery Yangdi*,*Gorkha* and open nursery only one sample, TO6(OD value 0.209) was mildly CTV infected and all other samples had mean OD values below negative control (OD value 0.206). Samples with OD value above positive reference (0.573) were not detected.

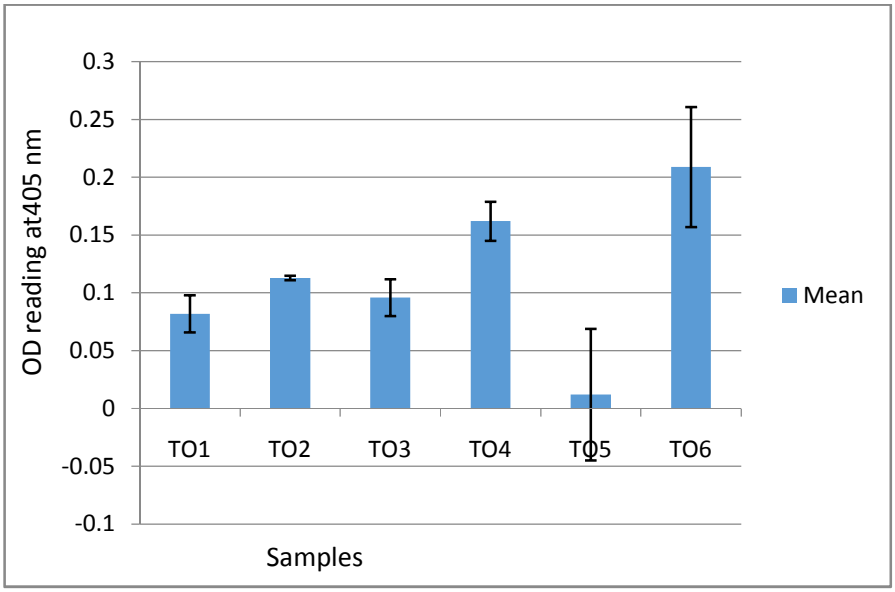

Figure 9. Optical Density reading at 405 nm wavelength of Trifoliate orange samples collected from open orchards. (M±SD)

C. Trifoliate orange samples from screen-house nursery

Among six samples tested, CTV infected samples were not detected. All samples had mean OD reading below negative control.

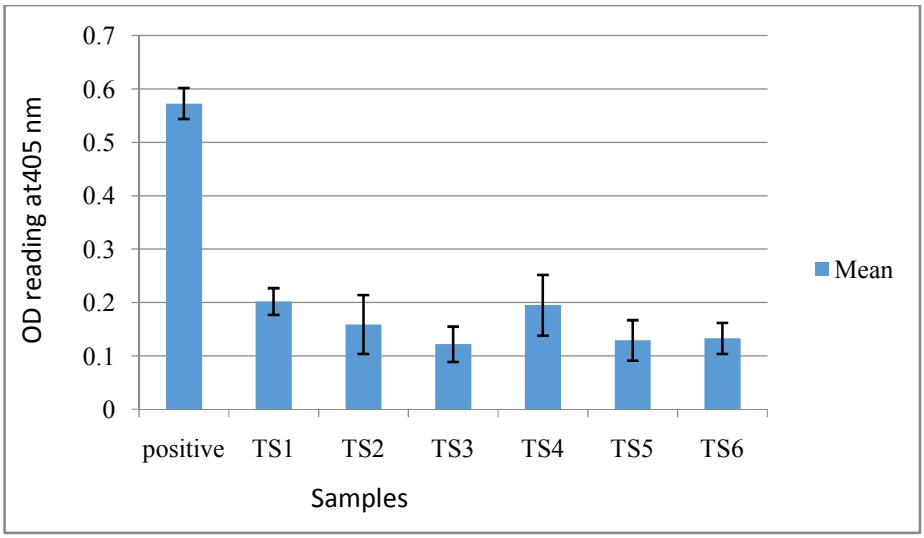

Figure 10. Optical Density reading at 405 nm wavelength of Trifoliate orange sample collected from screen house nursery, (M±SD).

D. CTV result from tissue cultured samples

Three samples were tested from tissue culture and all samples has mean OD value less than negative control so resulted as CTV infection negative. Among three samples two samples were Node culture and shoot tip culture of Trifoliate orange whose mean OD value was 0.107 and 0.068 respectively. And one was shoot tip culture of suspected infected *C. reticulata* mother plant, and its mean OD value was detected as 0.205.

The highest mean OD reading, the test result was obtained from the mother plants. has The seedling grown in open orchards and the seedling grown from screen house had less OD reading was obtained from tissue cultured sample (shoot tip culture).

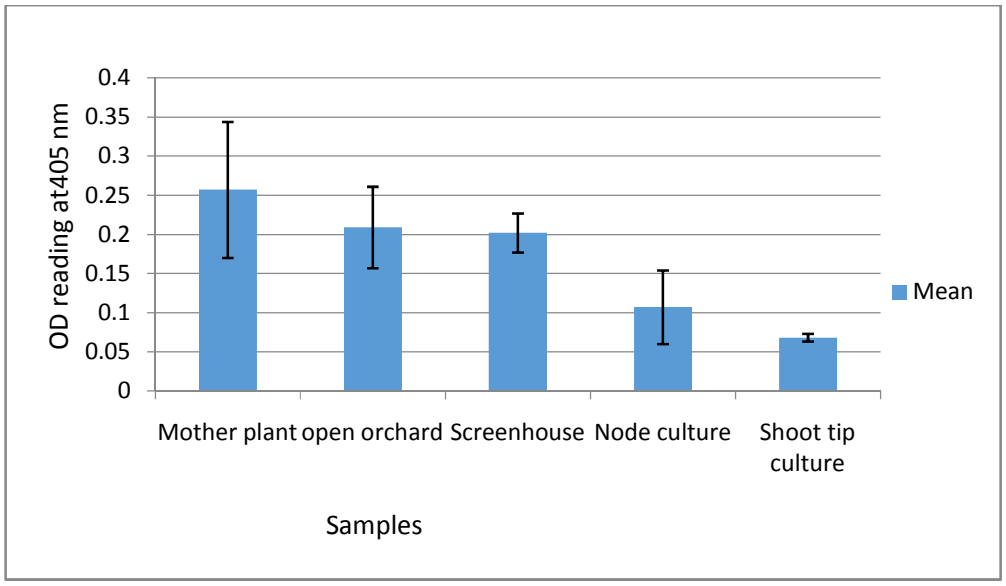

Figure 11. Maximum OD values from four different conditions of tested Trifoliate orange samples, (M±SD)

E. CTV results of Lamjung District

Altogether eight samples were tested for CTV reaction. Among them seven samples were from in-vivo and of different Citrus cultivars of same orchards and one sample was in-vitro shoot tip cultured of suspected infected mother plant. All tested samples except *C. grandis*and shoot tip culture got positive CTV reaction. Three citrus cultivars, Junar[*C. sinensis* (LMj)], kagati, [*C.aurantifolia* (LMl)], and mandarin orange seedling in origin [*C. reticulate* (LMs)] had OD reading greater than the value of positive control and

resulted as highly CTV infected. Similarly three cultivars, mandarin orange[*C. reticulata* grafted(LMg)], nibuwa [*C.pseudolemimon* (Lmp)], and mandarin orange [*C. Reticulata* (LMn) nursery plant] had the OD values in between positive control and negative control and resulted as mildly CTV infected. Negative CTV infection was obtained from in-vivo leaf sample of vogate, [*C. grandi* (LMv)] and shoot tip cultured sample of infected mandarin orange (LMt).

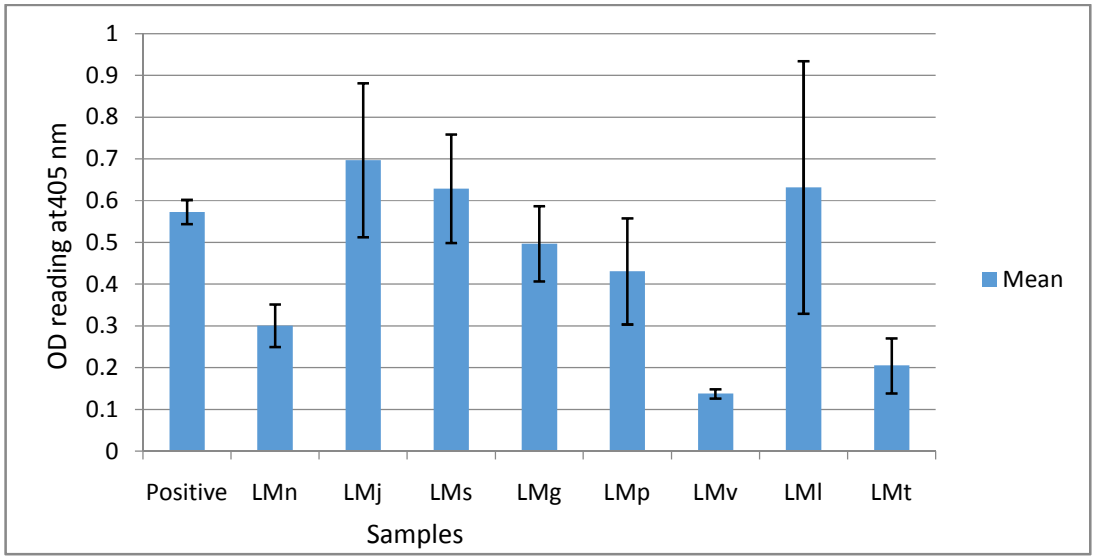

Figure 12. Condition of CTV from Lamjung District, (M±SD)

4.3.3. Distribution of CTV in samples collected Different districts

Samples were collected from four districts, *Mohoriyakot* VDC- *Lamjung*, *Tanglichowk.*- *Gorkha*, *Sunkhani- Dolakha* and HDP *Kirtipur*, *Kathmandu*. Samples of different citrus cultivars, mandarin, junar, kagati, vogote and nibuwa were collected from *Lamjung* district. Leaf samples of junar(1), mandarin(11) and Trifoliate orange (12) were taken for study from *Gorkha* district. Similarly, mandarin orange (5) from *Dolakha* and trifoliate orange (5) were collected from HDP *Kirtipur*. Samples were collected according to the research objective. By analyzing the graph below, it was concluded all samples from *Gorkha* district, 3 samples were highly positive, 5 samples were mildly positive and 14 samples were CTV negative. More negative result was obtained from Trifoliate orange 3-4 month old plant.

Similarly among 7 samples analyzed from *Lamjung* 3 were highly CTV infected, 3 were mildly CTV infected, and 1 was not infected by CTV. Similarly in Dolakha, 3 were mildly CTV positive and 2 were negative and from similar result for *Dolakha* was 3 for mildly CTV positive and 2 for negative and from Kathmandu 2 samples out of 3 were mildly CTV positive and 3 were negative to CTV reaction. All tested samples from shoot tip and node cultured were free of CTV infection.

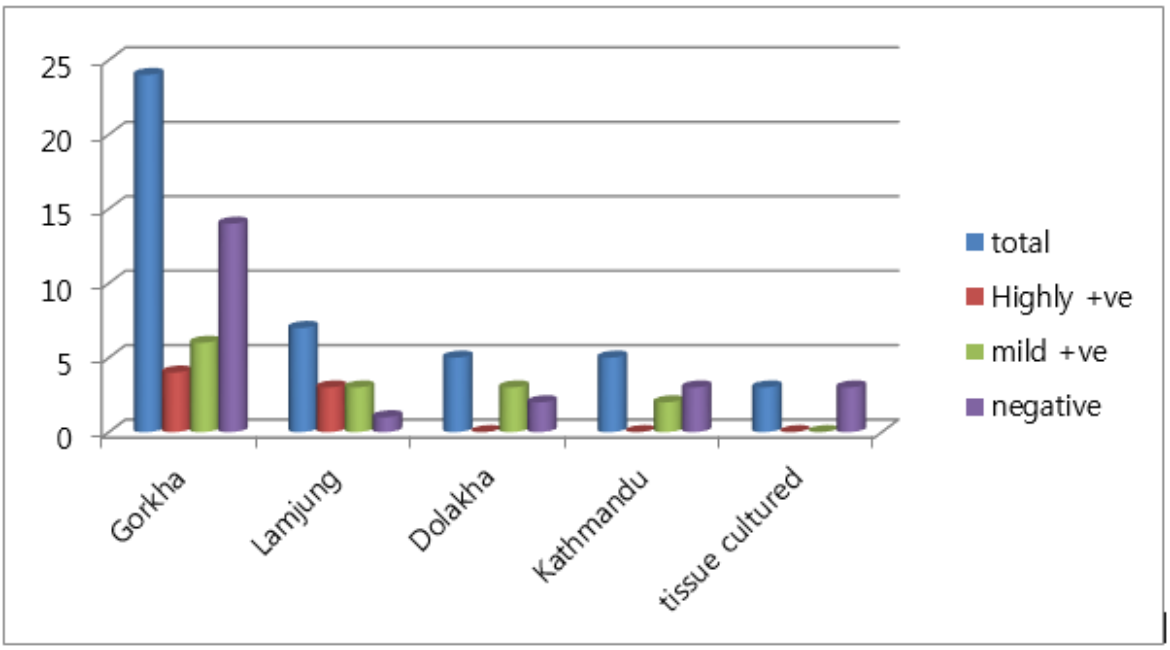

Figure 13. Distribution of CTV according to district, tissue cultured samples were negative.

4.3.4. CTV results of total analyzed samples

44 leaf samples were analyzed, Among them 7 samples were highly CTV infected, 14 samples were mildly CTV infected and 23 samples were free of CTV. Among the negative samples 3 were from tissue culture, 1 was of vogote, 1 of junar 3 of mandarin orange and 15 samples were of Trifoliate orange.

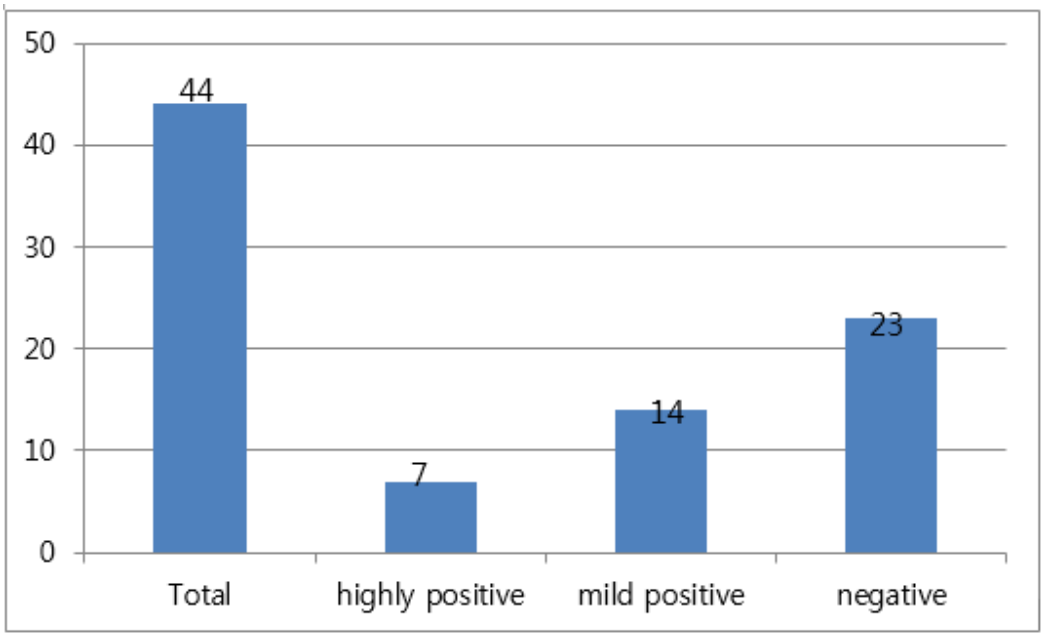

Figure 14. Graph of CTV distribution on the tested Citrus samples.

4.3.5. Percentage of CTV infection according to District in total analyzed samples

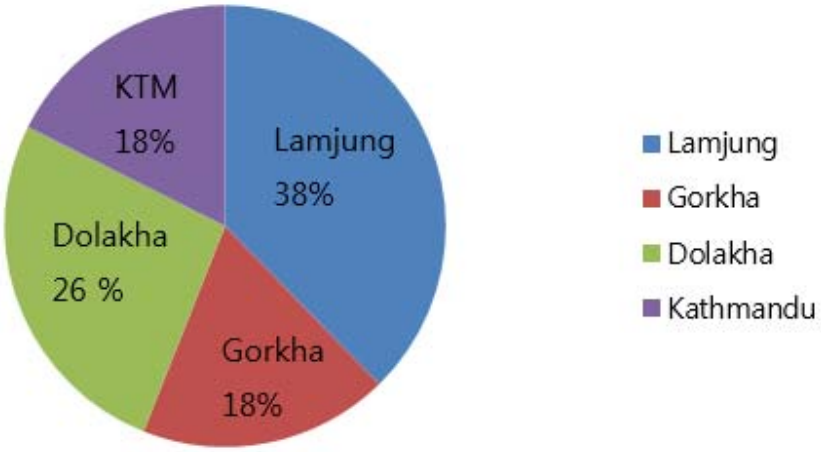

Figure 15. Percentage of CTV infection in different districts.

5. DISCUSSION

Citrus fruits have been grown for millennia in their area of origin. They have high nutritious values and are important in an economical perspective. The mid-hill of Nepal are suitable for cultivation of Citrus fruit. Mandarin (*Citrus reticulata*), Sweet orange (*C. sinensis*), lime (*C. aurantifolia*) and lemon (*C. limon*) are the most routinely cultivated citrus fruit and popular in this regions. The area lying between 900m and 1400m with sandy loam soil of PH range 5.0 to 6.5 is most suitable for its cultivation. About 60% of productive area is covered by mandarin while 20 % is by Junar and rest by Lime and lemon (Ranjit & Ghartichhetri, 1997). The Citrus Greening Disease and *Citrus tresteza* virus are the leading disease of Citrus responsible for decline of yield of fruits.

More than 30 virus and virus like disease of Citrus have been described by Klotz et al 1972. All of these have been spread into tropical and sub-tropical world with infected bud wood. Some of them, particularly tresteza and greening which are the most dangerous disease of citrus, can also be spread by insects (Trung, 1991).

5.1. Polymerase chain Reaction for Citrus Huanglongbing (Citrus greening)

Citrus Greening Disease (CGD) is perhaps the most serious and devastating of all the disease which affect citrus. It is one of the limiting factors for Citrus fruit production in many of the Asian countries including Nepal and in many European countries. The disease spread by the importation of both the organism and the vectors. In Nepal, more than 40-70% trees are infected with CGD (Knorr & Shah, 1971) (Schwarz, 1970). The Disease has already spread throughout the country causing severe damage to the citrus plant. The Citrus decline was reported in Nepal for the first time in by FAO export (Thrower, 1968). The detection of disease on the basis of visual symptoms was in practice. Knorr & Shah, 1971, used thin layer chromatography and confirm the disease to be greening. However, the technique to use TLC in detection of CGD is not reliable due to presence of poly-phenolic compounds that may also give positive result (Ranjit, 2003).

Citrus greening disease caused by fastidious bacteria that infect sieve cells is the most serious problem in tropical and subtropical Asia and Africa. The greening organism are transmitted by vectors *Diaphorina citri* in Asia and *Trioza erytreae* in Africa as well as by grafting or layering. There is a great diversity in susceptibility among Citrus cultivars. Transmission in field is enhanced by strong wind due to the ability of vector to move long distances.

Molecular biological techniques have recently been introduced in Nepal for diagnosis of CGD (Tamot & Gresshoff, 1999) (Ranjit, 2003). Ranjit detected CGD from Nepalese Citrus fruit in France using PCR technology with the help of DNA extraction kit (Ranjit, 2003). (Korsten et al., 1996)reported that this method is safest and easiest for diagnosis of CGD. Several forms of PCR Randomly amplified Polymorphic DNAs (RAPD) and DNA amplified fingerprinting (DAF) have been successfully used for the diagnosis of CGD (Hocquellet et al., 2000) (Tamot & Gresshoff, 1999) (Hocquellet *et al*., 1999). Use of PCR based analysis is widely adopted for detection of CGD in many countries including India, Thailand etc. Although some work has been done by using PCR based method in Nepal for detection of CGD, much relevant paper has not been published in Nepal by the use of PCR. In this research DNA was extracted by using CTAB method (Graham et al., 1994)and specific PCR using primer A2 and J5, to amplify the rpl β operon gene. This method is specific for Asian Citrus psyllid. PCR reactions were analyzed after spectrophotometric quantification OD reading within the range of 1.4-2 and concentration of DNA is calculated in the test samples.

Detection of CGD by PCR technology solely depends on the nucleic acid quality and purity of the causal organism that is extracted from the citrus leaves. The CTAB method is appropriate for the extraction and purification of DNA from plants and plant derived food stuff as it is suitable to eliminate polysaccharide and polyphenolic compounds, which otherwise affect the DNA purity and therefore quality.

Another PCR based method for detection of Greening bacteria is amplification of 16S ribosomal RNA (16S rDNA). This method is not specific and both species *Ca. L. asiaticus* and *Ca. L. africanus* can be detected, yielding the same size of 1160 bp of 16S rDNA fragments (Korsten *et al.*, 1996). A time-consuming enzyme digestion of the 1160

bp PCR products with XbaI is needed to distinguish the two *Liberibacte*r species. In China, one specific primer set was developed based for detection of "*Ca. L. asiaticus*" (Tian et al., 1996). The primer pair OI1/OI2c is able to amplify the rDNA of both Liberibacter species, while the pair OA1/OI2 camplifies preferentially the African liberibacter DNA (Thrower, 1968). (Thapa, 2011), detected CGD from Palpa, Tanahun and Lamjung Districts by using PCR based analysis with the help of "Wizard DNA extract", by using both method.

Prokaryotic rpl β operon DNA amplification with the help of PCR using primer A2 and J5 indicated that out of nine sample (*C. reticulate*) tested seven samples contained approximately 703 base pair DNA (Asian greening) and was found to be infected with CGD. It can be concluded that almost all Citrus farms of Nepal are infected by Citrus greening Disease leading to huge economic loss in Nepalese Citrus industries, and making frustration to the farmer. There is only least published work by using PCR based diagnosis of CGD from Nepalese Citrus samples. Ranjit, 2003 and Korsten et al., 1996, reported CGD from Nepalese citrus sample using PCR based diagnosis. Almost all samples collected from suspected mother plant with blotchy mottle symptoms were detected CGD positive; this result could compare satisfactory with the previous research is more satisfactory. The bands were not so distinct in ladders which may be due to problems either in Ladder and primers used and optimization of PCR machine.

All species of the Citrus appear to be susceptible, but the sweet orange, mandarin and tangelo are most affected. Regmi & Lama, 1988a, showed that lemon, small fruited lime and *murrya paniculata* (a citrus relative) is superior host for *D. citri* in Nepal. Lemon is symptomless carrier of the greening bacteria and lime shows no symptoms or only a diffused mottle (Roistacher, 1996). Mandarin orange with blotchy mottle symptoms in leaves is a good samples to detect greening bacteria. The greening organism *L. asiaticus* can be detected by DNA-DNA probes (Garnier & Bové, 1983) (Bove *et al*., 1993). As symptoms of Greening organism is confusing with Zinc deficiency and other nutrient deficiency symptoms, only visual observation is not the means to detect disease, that's why Nucleic acid based detection technique using CTAB DNA extract is the best and confirmative test for identification of greening organism. PCR method permitted much

more rapid detection of Greening bacteria as compared to more conventional biological indexing.

5.2.Citrus Tresteza Virus indexing

Tresteza is one of main disease that occurs in Citrus mainly leading to decline of yield of the fruit. It has been found in all Citrus growing areas of the world. The virus causes stem pitting, seedling yellow reaction, vein clearing and cupping of leaves. Citrus tresteza virus test was performd by DAS-ELISA for in vitro as well as invivo samples. DAS-ELISA is an acronym for double antibody sandwich enzyme linked immune-sorbent assay. It is used for the detection of several plant pathogens, especially viruses. It is very sensitive, accurate, and rapid detection method, which is effective where large number of samples must be tested, where result are needed rapidly, and where suitable indicator plant and or greenhouse facilities are not available (Bar-Joseph *et al*., (1979) (Cambra *et al.*, 1979). It has higher sensitivity than other serological methods for the identification It acts on the basis of antigen and antibody reaction; The virus-specific antibodies (gammaglobulin: lgG) are adsorbed to the surface of each well in the micro titer plate, when the sample to be tested is added to the plate, if the plate contains particles of the specific virus (antigen) for the antibodies coated, the particles will adhere to the antibodies and will not be eliminated during rinsing after adding the enzyme-gamma globulin conjugate (lgG– AP). These antibodies adhere only to the virus particles. Finally, development of yellow color after adding the substrate will indicate that the plant is infected by CTV. The intensity of the yellow color that develops is proportional to the quantity of virus in the sample.

The classic identification procedure for CTV is to graft-inoculate indicator seedlings of Mexican lime (Wallace & Drake, 1951) and observe them for vein clearing, leaf cupping, and stem pitting. Electron and light microscopy can be used to identify CTV particles and inclusions, but DAS-ELISA (Bar-Joseph *et al.*, 1979) (Cambra *et al.*, 1979) revolutionized diagnosis, making it feasible to test many samples during surveys of large citrus areas, for CTV control in nurseries and for epidemiological studies.

CTV test was done in two ELISA plates in different periods. In test plate -1, twelve randomly collected samples both from screen house and open orchards were tested. All were grafted and among them one was *C. sinensis* and the remaining all were *C. reticulata*. Second test was done for the in-vivo collected samples of *Lamjung*, *Gorkha*, *Kathmandu*, and *Dolakha* district and tissue cultured samples of trifoliate orange and mandarin orange, this was named as ELISA test plate 2. CTV test was performed on 2012/12/15 and 2012/12/16 with the help of DAS-ELISA.

Standard positive reference was kept to detect samples whether they were positive or negative. Due to the chemical and climatic differences, each ELISA plate had different readings for the standard positive and negative indicators for CTV. Thus each plate was categorized according to its specific condition. All tested in-vitro samples, cultured from suspected infected mother plant were found Citrus Tresteza virus negative. Although meristem culture is the best method for virus elimination, virus can be eliminated through shoot tip culture and node culture.

From the result of test plate 1, screen house sample with CTV infected plants was due to careless while grafting as the plants were grafted in open orchards and planted to screen house nurseries. All screen house sample were around 7-8 months grafted plants and Disease may be transmitted either my aphid vector or from infected scions during grafting. CTV is easily graft transmissible because phloem tissue contains viruses (Rocha-Pena *et al*…1991). Many research indicated that CTV is graft transmissible disease and the virus is predominantly transmitted through grafting and insect vector brown citrus aphid (BrCA) in semi-permanent manner (Bar-Joseph *et al*., 1989) (Rocha-Pena *et al*., 1995).

Similarly, result of test plate 2 was categorized in different parts and analyzed. Samples from different districts, different cultivars, and different conditions were tested in one ELISA KIT. Test results of four condition samples of Trifoliate orange concluded that tissue culture was the best method for virus elimination and reduction after that application of screen-house nursery. This approach was proved from the present study where, seed collected from infected trifoliate orange were grown in open orchard and within screen house simultaneously. Among the six/six samples tested from both

condition, no samples with CTV positive were obtained from screen house nursery and OD reading obtained was less compare to open orchards and mother plant samples. One sample with mildly CTV positive was detected from open orchards. Both samples were from three month old plant, and the samples collected from mother plant found mildly infected.

Micro-budding technique and thermotherapy combined with, tissue culture and screen-house nurseries are the best means to protect Citrus species and to control the declining condition of citrus. Virus indexing based on ELISA may significantly reduce the time to develop CTV-free Citrus plants. This approach may also facilitate and enhance citrus bud wood certification program (Abbas *et al*., 2008).

Different Citrus cultivars of same orchard of *Lamjung* District were tested for CTV reaction. All species except *C.grandis* was CTV reaction positive. Among the positive samples, three citrus cultivars, Junar[*C. sinensis* (LMj)], kagati, [*C.aurantifolia* (LMl)], and mandarin orange seedling in origin [*C. reticulate*(LMs)] were highly CTV positive with mean OD reading greater than that of positive control. Similarly three cultivars , mandarin orange[*C. reticulata* grafted(LMg)],nibuwa [*C.pseudolemimon*(Lmp)], and mandarin orange [*C. Reticulate* (LMn) nursery plant] were mildly CTV infected Negative CTV infection was obtained from invivo leaf sample of vogate, [*C. grandi*(LMv)] and shoot tip cultured sample of infected mandarin orange (LMt).

As nursery plant are weak by immunological process, they are more prone to virus attack in open environment. So nurseries should be established within screen house. More expression of virus in seedling plant than grafted plant can be analyzed as aphids vectors are the main agents of virus transmission in open environment and in the time of grafting, disease has transmitted to the new grafted varieties. Plantation of diseased varieties in the adjacent area, can transmit virus to the healthy plants Although CTV is not seed-transmissible, it is readily transmitted through grafting. Since most citrus species are vegetatively propagated, dissemination of infected bud-wood has greatly increased the distribution of CTV worldwide. CTV can also be transmitted by parasitic plants called dodder (*Cuscuta sp.),* but this means of transmission is not thought to be important in the spread of the virus.

From the test result of CTV from four different districts of Central Nepal, regional percentage of CTV infection in total analyzed samples was obtained as *Lamjung* (38%), *Dolakha* (26%), *Gorkha* (18%) and *Kathmandu* (18%). But in *Dolakha* the severity of infection and citrus production declination was not so high. Sample collected from *Lamjung* district was highly declining area where commercial citrus production was totally destroyed during last three years period. This variation in percentage of CTV infection is due to variation in collected samples and species. In this study random sampling was performed during collection. By observing the percentage of infection it is hard to categorize the district with maximum CTV infection.. If the sample was collected from same Citrus cultivar then the accurate rate of CTV distribution could be analyzed. It was observed that *Lamjung*, *Kathmandu* and *Gorkha* districts were severely infected by CTV with declination of Citrus fruit production.

Previously a study of the CTV infection in *Gorkha*, *Lamjung* and *Kathmandu* was done by (Ranjit et al., 1998), and it was seen that the infection percentage of CTV had *Gorkha* (62%), *Lamjung* (96%) and *Kathmandu* (56%). At that time total number of 140,156 and 25 leaf samples were taken from *Gorkha*, *Lamjung* and *Kathmandu* respectively and among them positive infection number for CTV were 87, 151 and 14 respectively. All collected samples were from mandarin orange. In the present study total 7, 12 and 5 leaf samples (by excluding Trifoliate orange sample in this case) were analyzed for CTV infection for *Lamjung*, *Gorkha*, and *Dolakha* distris. Among them, 6, 8 and 3 samples were CTV infected. So the percentage of CTV infection was 85.71%, 66.67%, and 60% for the respected Districts. Though the sample number for *Gorkha* and *Lamjung* district was considerably less, it shows a similar distribution of Disease.

Among the citrus cultivars less infection was obtained from trifoliate orange. It can be concluded that Trifoliate orange is more resistant to viral disease like CTV than other types. This result is supported by many researches. Garnsey et al., 1987, reported that Trifoliate orange (*Poncirus trifoliata*), a root stock had a high degree of resistance to Citrus Tresteza virus. Tresteza can cause mild and severe both vein-clearing and stem-pitting symptoms in 'Mexican' lime *(C. aurantifolia*, and can cause seedling yellows in 'Eureka' lemon (*C. limon*). The large numbers of uninfected grapefruit trees on sour

orange in Florida appear vulnerable to further and more rapid spread of tristeza isolates capable of causing decline (Garnsey *et al*., 1987).

By analyzing this result it can be concluded that, rate of disease transmission is higher in adjacent area than farther areas. Disease vector may transmit by introduction of uncertified grafting plantlets or transmission of dphid vector by different means. CTV is the major cause of Citrus declination in Nepal and in Asian countries. The aphid *Toxoptera citricida* is also wide spread in Nepal and was observed (Roistacher, 1996). Mandarin is symptomless carrier of severe strains of CTV (Yamada & Tanaka, 1969); (Roistacher, 1988). However there are recent reports of severe stem pitting and decline of mandarins with certain new and severe strains of the virus (Tsai *et al*., 1993). Only the visual observation is not sufficient and good method to detect whether the plant is healthy or not but suitable experimental and laboratory work are required. In this experiment CTV is detected from all types of varieties except *C. grandis*,

These results shows that the sample in various regions and various conditions has different pattern of distribution. Some areas were affected more than other regions. CTV spreads into new regions (long spread) via movement of infected propagating materials such as seedlings and bud woods. Aphid dispersion of virus is important within a citrus growing area (short spread). The principal vectors of CTV are *Toxoptera citricida* (Kirkaldy), the brown citrus aphid (BrCA), *Aphis gossypii* (Glover), the melon cotton aphid, and *A. spiraecola* (Patch), and the spirea or green citrus aphid (Roistacher & Bar-Joseph, 1987). The other aphid species with limited transmission reported are: *Toxoptera aurantii* (Fonscolombe), black citrus aphid, in Florida. *Myzus persicae* (Sulzer), green peach aphid, Aphis *craccivora* (Koch), groundnut aphid, and *Dactynotus jacae* (Linnaeus) from India.Efficiency of CTV transmissibility is affected by the species of aphid, by the source plant at acquisition feeding and the CTV isolate. The BrCA is the most efficient aphid vector of CTV. In areas where BrCA is not present, *A. gossypii* is the most efficient vector implicated (Bar-Joseph *et al*., 1989) (Bar-joseph *et al*., 1983).
In 1952, Morel and Martin were able to show that certain viruses could be eliminated from potato and dahlia by asceptic culture of meristem tips, Since that time tissue culture has been widely used to recover virus free plant initially from herbaceous species and

more recently from woody plants. Shoot tip grafting was developed by Murashige and was used for eliminating much virus infection. This technique has been proved in different ways such as root stock, scion, environment and indexing .

There are not sufficient research work done from Nepal on this disease although the disease is very serious problem in reduction of citrus production and destruction of citrus industries from the country. One serious research done by Mr Tomiyasu, horticultural specialist in charge of the citrus plantings, reported that serious decline of mandarin tree at horticulture research station *Kirtipur* was due to severe strain of CTV not by greening. The decline observed was due to introduction of various mandarin scion bud woods from Japan for the JICA project in this area and it is possible that these mandarin contained severe strain of CTV and could have been spread to the sweet orange tree by *T. citricida* and could be responsible for the decline observed. Such evidence were reported in Peru where Satsuma mandarin imported from Japan and planted throughout the country The plant showed severe stem pitting symptoms with low fruit set, the novel citrus industry of the country was virtually destroyed (Roistacher, 1988). Similar destruction was reported in Capao Bonito districts of Brazil.

During early part of the 20thcentury, epidemics of tree losses on sour orange rootstock were reported from South Africa and in Argentina and Brazil in the 1930s following the importation of CTV-infected plants and the efficient aphid vector *Toxoptera citricida*. More than 80 million trees grafted on sour orange (*Citrus aurantium*) rootstock have been killed or rendered unproductive by CTV-induced decline. The losses caused in Argentina (more than 10 million trees), Brazil (more than 6 million trees) and USA (more than 3 million trees) have been reported by (Bar-Joseph et al., 1989). Only in Spain more than 40 million trees, mainly sweet orange (Citrus sinensis) and mandarin (*Citrus reticulata*) grafted on sour orange, have declined progressively. This virus killed infect nearly all the citrus species and their relatives and hybrid in India (Ahlawat et al., (1992) Ahlawat & Pant, (2003), Biswas *et al*., 2004). Recently in the year 2006-7 CTV has been determined to cause citrus decline in Iran.

6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

Citrus is the most popular and income generative fruits for mid hills farmers, as geography, climate and rainfall all is suitable for citriculture. Although the area under Citrus cultivation is increasing year by year, production and productivity of the citrus fruit is low compare to increased area under cultivation. This fact is supported by annual report of (Agriculture Department of Nepal, 2012) according to which productivity of Citrus start to decline from the fiscal year 2010/2011, resulting the total productivity of 8.46 mt/ha from the productive area of 23607, which was 11.32 mt/ha in total productive area of 22903 in fiscal year 2009/2010 which is very low annual production compared to world production rate per hector[3].

From the present study it was found that both central and western development region are affected by CTV and CGD. Both CTV and CGD are graft transmittable diseases and they both are responsible for citrus species declination as well as reduction of Citrus production from Nepal. Thus proper method to reduce infection must be applied. Visual observation is not the mean of disease identification as symptoms of both disease are almost similar, yellowing of shoot, lopseeded fruits, reduction of fruit size, quick decline and etc. So lab based experiment for their identification is necessary. PCR based analysis for CGD is most reliable and confirmative test to detect disease infection, and the PCR reaction with the help of A2/J5 is specific for *Candidatus L. asiaticus* (Asian *Citrus psyllid*) and detect disease by making DNA band in 703bp long fragment of ribosomal protein genes (rpl-PCR) in the rplKAJL-rpOBC (β-operon). DAS- ELISA based analysis to detect CTV is most reliable and suitable to detect large samples at a time and to establish quarantine nurseries.

[3] http://faostat3.fao.org/faostat-gateway/go/to/download/Q/QC/E accessed on November-23-2013

PCR result of different places samples tells that CGD is the major cause of citrus infection and Psyllid vectors are the main cause of CGD. Seven samples with CGD among 9 samples tested resulted that Asian Citrus psyllid is the major agent of greening disease in Nepal and is spreading to almost all citrus pocket areas of Nepal leading to less production and destruction of Citrus farms and Citrus industries.

All tested samples of the same orchards except *C. grandis,* positive to CTV reaction indicate that CTV is epidemic and can spread by grafting as well as aphid vectors and winds, animals, water etc. may transmit the disease from place to place. Another main reason is the introduction of uncertified grafted varieties. Trifoliate orange being more resistant to CTV and its root is also resistant to *phytophora* induced diseases root stock should be used of trifoliate orange and grafting should be done in vectors free zones like screen-house of certified root stock and scions. Similarly, by analyzing the OD reading at 405nm it can be concluded that tissue culture is the best method for virus elimination and reduction and thus produced plantlets should acclimatized in insect proof Screen house nurseries

Thus it can be concluded that both Citrus tresteza Virus and Citrus Greening (Huanglongbing) Disease are responsible for the present destructive condition of Citrus orchards to carry on. Both are graft transmittable disease and Greening is vectorized by Citrus psyllid and CTV is vectorized by brown Citrus aphids in open environment. Both disease can attack in the same orchards and same plants. Once the disease attack the Citrus plant it is hard to control. So care should be given while making nurseries and at the time of grafting. Only the best method to control the Diseases is destruction of old infected all Citrus varieties and plantation of new certified grafted varieties, which give good productivity from fewer areas in short time period.

6.2. Recommendations

- Research on Citrus Disease must be done to the whole Citrus pocket areas of the country.
- CTV and CGD being very destructive disease and symptoms of the disease are being very confused with other diseases, hence experts must be involved in field diagnosis of the disease during sample collection.
- Serological facilities as ELISA are being established in few laboratories in Nepal. The number of laboratories should be established at least in regional level and their routine use for virus identification must be carried out.
- PCR diagnosis of HLB must be made mandatory for the certification of Citrus planting materials and highly sensitive PCR like real time PCR should be used in Nepal (RTi-PCR).
- Both methods are being very expensive government must give priority and economic help to the researcher who interest to do research on this disease.
- Government must give facility to each commercial citrus farmer to establish Screen house nurseries and strict rules must be done to the farmers who produce nursery in open orchards.
- Certified bud woods must be distributed to the farmers and training and awareness programmerelated to Citrus Diseases like Greening and tresteza virus must be organized by DAO in time to time and strict quarantine regulation must be implemented to restrict the movement of disease and its vector.

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PHOTOPLATES

Photoplates 1

Photos collected from Field Visit

Orchards with symptomatic trees

Tree infected by Brown Citrus aphid

Leaves with blotchy mottle symptoms, yellow patterns are unsymmetrical on

Fruit showing greening symptoms

Collection of shoot tip sample for tissue culture

Cutting down of infected mandarin plant

Leaves with psyllid vectors

Asian Citrus psyllid

Screen house nursery

Plants with in screen house nursery

Fruit with lop-seeded seeds

Grafted plants in open nursery

Photoplate -2

Photographs from laboratory work of ELISA

Grinding 200mg leaf sample for CTV

Loading virus sample

ELISA kit before addition of substrate

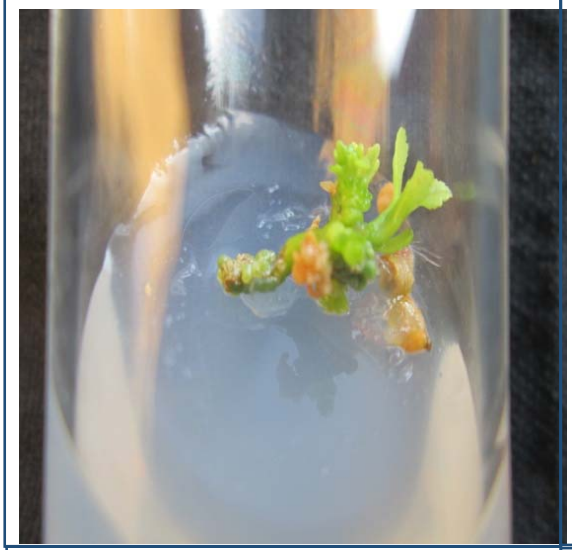
ELISA kit after addition of substrate, Development of yellow colour Detect CTV positive reaction

Micropipette with pipette tips

ELISA plate reader or spectrophotometer

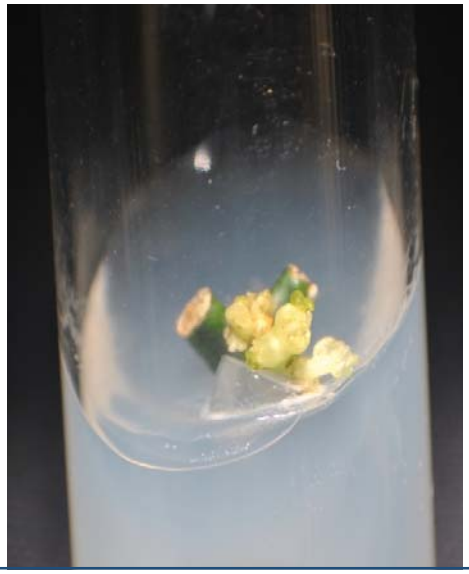
Photoplate 3

Photograph from tissue culture



Development of multiple buds from shoot tip culture of Trifoliate orange after 4 weeks

Development of multiple buds and leaves from node culture of Trifoliate orange after 5 weeks



Node culture of mandarin orange after four week

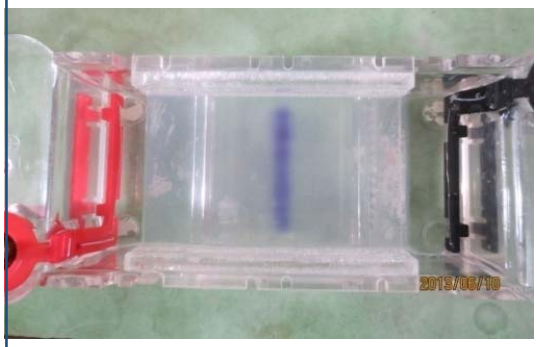
shoot tip culture of Mandarin orange after 8 weeks.

Photoplate 4

Photograph from Laboratory work of Citrus greening disease

Centrifuge machine

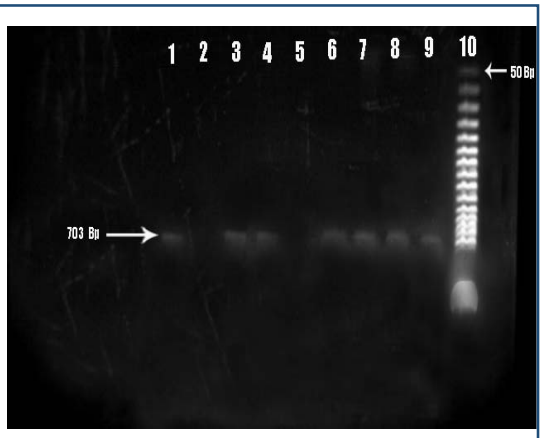
Cooling micro centrifuge



Movement of DNA particle in agarose gel

leaf samples after extraction and centrifugation in ependroff tubes

PCR machine



Electrophoresis on 1.5 % agarose gel of duplex PCR products amplified from DNA of symptomatic leaves primed with A2/J5 Lane 50bp DNA ladder (Promega, USA); lane 1,3,4,6,7,8,9, DNA from Ca. L. asiaticus-infected leaves

ANNEXES

Annex I

Amount of principal, minerals, and vitamins in citrus species.

SN	Principal nutrient value	Value per 100 gm
1	Energy	47kcl
2	Carbohydrate	11.75g
3	Protein	0.94g
4	Fat	0.12g
5	Cholesterol	0g
6	Calcium	40g
7	Copper	39mg
8	Thiamin	0.100mg
9	Magnesium	10mg
10	Manganese	0.24mg
11	Pyridoxine	0.060mg
12	Riboflavin	0.04mg
13	Vitamin C	53.2mg
14	Vitamin A	125IU
15	Vitamin E	0.18mg

(Source: USDA National Nutrient data base)

AnnexII

a) Major citrus pocket areas of Nepal

Eastern Development Region		
SN	**District**	**Pocket area**
1	Taplejung	Dokhu, Nidhuradin, Change
2	Dhankuta	Telia, khoku, chinntang, Dhankuta,Belhara, khwafok, Maunabudhuk.
3	Panchthar	Amarpur, Nagi, Panchami, Ranigaun, Kurumba, Luwamfu
4	Bhojpur	Gupteshwor, Annapurna, Kota, Ranibas, Aamtep, Rangpang, Mulpani, Baikuntha
5	Sankhuwasabha	Chainpur, Mamling, Siddhapokhari, Sitalpati, Khandbari
6	Illam	Barbote, Soyang, Namsaling, Jirmale, Goduk, Kanyam, Sumbek, Pashupati nagar.
7	Okhaldhunga	Manebhanjyang, Dhulachap, Rumjatar, Taluwa, Moli.
8	Khotang	Simpani, Temba/Damkha, Mangaltar, Lamidada
9	Udyapur	Lekhani, Limpatar, Mayenkhu, Okhale, Aaptar, Khanbu, Pokhari, Mainamaini, Katunjebawla, Beltar, Hadiya, Rampur, Nepaltar, Katari
CENTRAL DEVELOPMENT REGION		
SN	**District**	**Pocket area**
1	Sindhuli	Tinkanya, Ratanchura, Baseshwor, Nirmanadhin, Rajmarg side, Bhimeshwor, Jalkanya, Majhuwa, Sitalpati, Purano, Jhangajholi, Ratmata Jhangajholi, Baseshwor.

2	Makwanpur	Namtar, Kalikatar.
3	Ramechhap	Ramechhap, Bhaluajor, Okhareni, Salu, Dadhuwa, Phulasi
4	Kavre	Sharda Batase, Panauti N.P., Patalekhet, Kushadevi, Sankhu, Balthali
5	Chitwan	Darechowk-1,4,5, Chandi Bhanjyang-5,
6	Dhading	Jogimara, Sayardul, Kallari, Nalang, Katunje
WESTERN DEVELOPMENT REGION		
SN	**District**	**Pocket area**
1	Myagdi	Dana, Okharbot, Ghatan, Darwang, Niskot, Singa, Arthunge, Pipale, Beem, Devisthan, Bhagawati,Arman, Jyamrukot
2	Palpa	Chhahara, Palung Mainadi, Mujhung(namuna), Deurali, Khasyaoli, Ringeraha, Jalpa
3	Baglung	Tityang, Malika, Damek, Sarkuwa, Jaedi, Bhakunde, Sisakhani, Hatiyachetra,
4	Lamjung	Chiti, Udipur, Kunchha, Duradada, Bhorletar, Ishaneshwor, Mohoriyakot, Tarkughat, Simpani, Bhulbhule.
5	Arghakhanchi	Khan, Khanadaha, Hansapur, Pokharathok, Padeni, Khidim, Pathauli, Maidan, Mareng, Bhagwati, Arghatos.
6	Gulmi	Nayagaun, Pipaldhara, Hadahade, Bhanbhane, Bhurtung, Gaidakot, Arkhale, Purkot, Shringa, Bletaksar.
7	Kaski	Bharat Pokhari, Nirmalpokhari, Pumdibhumdi, Thumki, Kalika, Hansapur, Salyan, Rupakot, Bumakodado
8	Gorkha	Manakamna, Tanglichok, Bunkot, Bhirkot, Ghayampesal,

		Palungtar, Tara Nagar.
9	Parbat	Banskharka, Majhphant, Deupurkot, Deurali, Kusi, Nilahar, Limithana, Thana Maulo, Pangrang
10	Navalparasi	Babkaraiekot
11	Tanahu	Baidi, Chandrawati. Chok, Rupakot, Basantapur, Purkot, Jamune, Chhang, Manpagn, Keshavtar, Arunodaya, Dharampani, Kyamin, Dhorfirdi, Bhirkot, Aanwu, Sepa, Bagaicha.
12	Sayangja	Setidobhan, Pauwegaude, Biruwa, Rangmang, Arjun chaupari, Dahathum walling N.P., Galayang, Pidikhola, Putalibazar-12,13
MID-WESTERN DEVELOPMENT REGION		
SN	**District**	**Pocket area**
1	Salyan	Marke, Tharmore, Kotmala, Dhorchaur, Bhotechaur, Bhalchar, Rangechaur
2	Rukum	Syalapakha
3	Jajarkot	Dhime
4	Mugu	Haryanju
5	Dailekh	Dullu, Chiudi, Lakuri.
6	Pyuthan	Swargadwari, Dhuwang, Maranthana, Dhuwang, Dangwang
7	Kalikot	Mehalmudi
8	Surkhet	Malarani, Dharapani, Kafalkot.

9	Rolpa	Dhawang, Kotgaun, Liwang, Ghartigaun, Eriwang.
FAR-WESTERN DEVELOPMENT REGION		
SN	**District**	**Pocket area**
1	Acham	Mangalsen, Marku, Tosi
2	Bajhang	Thalara, Chirchetra, Bugalchetra.
3	Kailali	Nigali, Sahajpur.
4	Doti	Aagar Bhadisain Mahadevsthan, Bhudbhara, Wayel, Durgamandau.
5	Bajura	Jugada, Barhabise, Kailashmandu, Jayabageshwori, Kolti, Kotila
6	Darehula	Bhrahamadev

(Source-Annual Report of HDP 2010/11)

B) Citrus species grown in Nepal

S.N	Local name	Common name	Scientific name
1	Kagati	Acid lime	*Citrus aurantifolia* Swingle.
2	Junar/Mausami	Sweet orange	*C. sinensis* Osbeck
3	Nibuwa/Chasme kagati Eureka	Hill lemon/Nepali oblong lemon/ Eurekha lemon	*C. pseudolimon* Tanka./*C. limon* (L.) Burn. f.

4	Kalo jyamir	Sour orange	*C. aurantium* L
5	Keep	Bitter orange	*C. aurantium* L
6	Bhogate	Pummelo	*C. grandis* Osbeck/*C. maxima (*Burm.) Merrill
7	Seto jyamir	Rough lemon	*C. jambhiri* Lush.
8	Suntala/Kamala	Mandarin/tangerine	*C.reticulate*Blanco/*C. tangerine*
9	Bimiro	Citron	*C. medica* L
10	Chaksi	Sweet lime	*C. limettioides* Tanaka.
11	Sankhatro	Possible hybrid of shaddock or pummelo	
12	Chaku paw	Possible hybrid of grapefruit	
13	Tinpate suntala	Trifoliate orange	*Poncirus trifoliate* L
14	Muntala	Kumquat	*Fortunella japonica* Swingle/ *F. margarita*
15	Kinnow suntala	Kinnow mandarin	*C. nobilis x C. deliciosa* Hybrid
16	Satsuma suntala	Satsuma orange	*C. Unshiu* M

Source: *Source: Annual Report of HDP 2012*

C.) Citrus diseases reported from Nepal

S.N	Name of disease	Causal organism	Microbe
1	Foot rot	*Phytophthora nicotianae* var. parasitica Dart.	Fungal
2	Root rot	*Phytophthora citropthora* (Sm. and Sen.) Leaon	Fungal
3	Citrus melanose	*Diplodia natalensis* Pole Evans	Fungal
4	Citrus black melanose (greasy spot)	*Mycospharella citri* Whiteside / *Cercospora citrigrisea* Fisher	Fungal
5	Powder mildew	*Ascosporium(Oidium)tingtanium* Carter	Fungal
6	Citrus scab	*Elsione fawcetti* Bitancourt and Jeklins	Fungal
7	Green mould of citrus	*Penicillium digitatum* Sall.	Bacterial
8	Felt disease	*Septobasidium pseudopidicellatum* Burt.	Bacterial
9	Pink disease	*Pilicularia(Corticium) salmonicolor*	Fungal
10	Anthracnose/Wither tip	*Colletotrichum gleosporiodes* (Penz).	Fungal
11	Brown rot Gummosti's	*Phytophthora citophthora* (Sm. and Sen.) Leaon.	Fungal

12	Styler-end-rot	*Alternaria citri*	Fungal
13	Leaf spot	*Pestalotia citri*	Fungal
14	Sooty mould	*Capnodium citri*	Fungal
15	Citrus canker	*Xanthomonas citri* (Hasse) Dawson.	Bacterial
16	Huanglongbing (Citrus greening)	*Candidatus*Liberibacter asiaticus	Bacterial
17	Tristeza	CTV mild /virulent strain	Viral
18	Damping off	*Rizoctonia solani* Kuhn.	Bacterial
19	Twing blight	*Sclerotina sclerotiroum* (lig) Cabbage group, Stalk rot	Fungal
20	Dilodia Gummosis	*Phytophthora palmivora* Butl / *P.parasitica*	Fungal

Source: Annual Report of HDP 2012

Annex III

a.) World's top ten countries producing fresh citrus fruit up to 2012 (in 1000 tons)

SN	Countries	2004/05	2005/06	2006/07	2007/08	2008/09	2009/10	2010/11
1	China	14 910.0	14 500.0	15845.0	18877.05	21397.5	23850.0	22940.0
2	Brazil	18 896.1	20 365.4	20778.3	18966.1	19147.8	17483.2	22704.5
3	U S	10 280.5	10 624.8	9 496.8	11645.8	10 40.2	9 978.7	10445.2
4	Mexico	6 427.7	6 688.9	6 685.9	7 401.0	7 033.1	6 793.0	6 744.0
5	India	4 495.1	5 754.4	6 755.1	7 549.0	7 966.7	7 966.5	8267.2
6	Spain	6 181.3	5 377.6	7 036.2	5 579.4	6 614.1	5 347.6	6 627.0
7	Turkey	2 316.8	2 779.0	3 162.5	2 639.5	2 820.0	2 780.0	3 077.5
8	Italy	3 320.9	3 495.7	3 278.1	3 240.9	2 691.2	3 671.6	3 203.5
9	Argentina	2 670.0	2 630.0	2 700.0	2 950.0	2 887.0	2 270.0	2 490.0
10	Iran	2 922.2	3 174.0	3 839.0	3 942.3	2 541.4	2 541.4	2 344.6

	World	97 446.6	102605 .7	10555 60	10957 0.0	10965 6.0	10933 8.9	115525 .2

Source-Statistics division FAO, 2012

b.) Worlds Fresh Citrus fruit forecast during 2011/2012 (in thousand tons)

SN	Countries	Total citrus (Th tones)	orange	Tangerines	Lemons and limes	Grapefruit
1	United States	10 246.8	7 954.4	572.4	699.8	1 020.2
2	China	11 280.0	6 600.0	1 380.0	300.0	3 000.0
3	Mexico	4 120.0	3 200.0	450.0	170.0	300.0
4	Japan	1 029.0	4.0	1 017.0	8.0	
5	Greece	1 081.0	910.0	120.0	45.0	6.0
6	Italy	3 569.0	2 260.0	781.0	520.0	8.0
7	Spain	5 527.6	2 684.2	2 072.5	722.9	48.0
8	Israe	567.4	131.0	169.4	62.0	205.0
9	Algeria	571.0	415.0	111.0	44.0	1.0
10	Morocco	1 866.8	948.5	763.9	154.4	
11	Tunisia	359.1	209.0	47.8	49.0	53.3
12	Cyprus	260.7	113.9	77.6	19.7	49.5
13	Egypt	3 461.0	2 350.0	731.0	320.0	60.0
14	Turkey	3 384.6	1 403.7	584.4	1 032.0	364.5
15	Argentina	2 530.0	750.0	350.0	1 300.0	130.0
16	Brazil		18 155.0			
17	South Africa	2 160.0	1 400.0	160.0	240.0	360.0

Source- Trade and Market division FAO, 2012

c.) World Top Five Countries and Orange fresh fruit production in 1000 metric tons

SN	Countries	2008/2009	2009/10	2010/2011	2011/2012	2012/2013	2013/2014
1	Brazil	17,014	15,830	22,603	20,563	16,361	17,750
2	China	6,000	6,500	5,900	6,900	7,000	7,600
3	United State	8,281	7,478	8,078	8,166	7,574	6,707
3	European Union	6,530	6,530	6,198	6,023	5,878	6,600
4	Mexico	4,193	4,051	4,080	3,666	4,000	3,900
5	Egypt	2,372	2,401	2,430	2,350	2,450	2,570

Source- Statistics Division FAOSTAT, 2014Division

AnnexIV

a. Chemical Composition for Stock Solutions of MS-Media

1. Stock A (Macronutrients)	mg/L	(10 ×) g/L
Potassium Nitrate (KNO3)	1900	19
Ammonium Nitrate (NH4NO3)	1650	16.50
Magnesium Sulphate (MgSO4.7H2O)	970	9.70
Calcium Chloride (CaCl2.2H2O)	440	4.40
Potassium Dihydrogen Phosphate (KH2PO4)	170	1.70

2. Stock B (Micronutrients) mg/L (100 ×) mg/ 100mL

Boric Acid (H3BO3)	6.2	620
Manganese Sulphate 9MnSO4.4H2O)	22.3	2230
Zinc Sulphate (ZnSO4.7H2O)	8.6	860
Sodium Molibdate (Na2MoO4.2H2O)	0.25	25
Copper Sulphate (CuSO4.5H2O)	0.025	2.5
Cobalt Chloride (CoCl2.6H2O)	0.025	2.5
Potassium Iodide (KI)	0.83	83

3. Stock C (10 ×) mg/100mL

Sodium Ethylene Dimine Tetra Acetate (Na2EDTA)	373
Ferrous Sulphate (FeSO4.7H2O)	278

4. Stock D (100 ×) mg/100 mL

Glycine	200
Nicotinic Acid	50
Pyridoxin HCl	50
Thiamine HCl	10

Myoinositol was added fresh in the medium in the concentration of 100 mg/L

5. Others Carbon source/ Solidifying agent

Sucrose	30 g/L
Agar	8 g/L

Hormone used for shoot tip and node culture.

a. Auxin

NAA

b. Cytokinin

BAP

b. ELISA buffer and solutions

1.) Coating buffer(for 1 liter)- pH= 9.6

Na2CO3	1.59g
NaHCO3	2.93g

2.) Phosphate buffer saline(PBS). (for 1 liter) pH = 7.4

NaCl	8g
K_2HPO_4	0.2g
$NaHPO_4$12H2O	2.9g
KCl	0.9g

III) Washing buffer pH= 7.4

PBS	1 liter
Tween 20	0.5 ml

IV) Extraction buffer(1 Liter) pH =7.4

PBS	1 liter
Polyvinylpyrrolidone 40 (PVP 40)	20g

V). Conjugate buffer- pH =7.4

PBS	1 liter
PVP 40	20g
Ovalbumin (OVA)	2g

VI). Substrate buffer (1 liter) pH= 9.8

Diethanol amine	97ml
Distilled water	800ml

VII) Reaction stopping solution

NaOH	120g (3M)

The antibody and conjugated antibody for CTV and the substrate were received from BIO-RAD laboratories France (ϭ company, USA)

c. CTAB Isolation buffer preparation

2% Hexacecyltrimethylammoniumbromide (CTAB), 0.4M Nacl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100mM Tris- HCL, pH-8.0