1. INTRODUCTION

1.1 General Background

Citrus fruits are members of family Rutaceae and among the cultivated species; sweet orange [*Citrus sinensis* (L.) Osbeck], grapefruit (*C. paradise* Macf), mandarin (*C. reticulate* Blanco), lemon [*C. limon* (L.) Burm. f], lime (*C. aurantifolia* Swingle) etc, constitute the key species of commercial citrus industry of Nepal (Roistacher, 1996). Of all these citrus species, mandarin ranks first both in terms of production and acreage (Hockey *et al*., 1998). The main mandarin producing districts of western Nepal are those of Kaski, Gulmi, Palpa, and Syangja, and those of eastern Nepal are Dhankuta, Tanahun, Dailekh, Ilam, Sankhuwasabha, Terathum, Okhaldhunga, Udaypur, Khotang and Bhojpur (Ghosh and Singh, 1993).

The Citrus species grow best in subtropical environment, although they have originated in the tropical region (Larry, 1999). The Citrus belt of the world covers a wide range of area varying in altitude. Citrus endures at the maximum minimum temperature range within 14˚C-40˚C and best growth occurs at 30˚C (Whiteside *et al.*, 1993).

Citrus is one of the major cash crop of Nepalese farmers in mid-hills regions of Nepal. Agro- climatic conditions being quite suitable for citriculture, it has been one of the main professions in these regions. 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. In terai belt, citrus occupies a limited area where shaddock, lemon, rough, lemon and lime are grown. Mandarin and sweet orange cultivars suitable for cultivation in the terai region have not yet been developed (Anonymous, 1990).

In Nepal, citrus is cultivated in 66 out of 75 districts. The area, production and productivity of citrus in Nepal are increasing year by year (Anonymous, 2002). 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 (HDP, 2007/2008) resulting in an annual productivity of about 227070.62 mt. Mandarin is estimated to cover about 60% of total citrus area of the country (Anonymous, 2000/2001). In fiscal year 2007/2008 the total area under mandarin is estimated to be 9,641ha having productivity of 1, 09,277mt. Similarly, sweet orange, lime, lemon and others have their total area and productivity values as 3072ha/36736mt, 2439ha/20492mt, 551ha/4343mt and 129ha/1027mt respectively (MoAC, 2007/08).

Citrus decline is the major problem of Nepalese citrus industry. It is caused by several factors including various bacterial, fungal and viral diseases such as greening, tristeza, exocortis, insect, pests and poor management practices (Chaudhary *et al.,* 1999). Of the various reported diseases of citrus in Nepal, Citrus Huanglongbing (HLB) or Citrus Greening (CGD) disease is the most important and most serious threat to Nepalese citrus industry (Singh, 1977; Roistacher, 1996).

HLB is a devastating disease of citrus. As it affects all citrus cultivars and there is no cure for this disease, the infected trees decline and die within few years. Furthermore, the fruit produced by infected trees are neither suitable as fresh fruit nor as processed juice due to significant increase in acidity and bitter taste (Internet visit, 1). During 1977, it was reported that the productivity of citrus was reduced to 80% in India due to HLB disease (Singh, 1997). During 1970s and 1980s, it had been suspected that more than 40-70% trees were infected in Thailand and Nepal (Schwartz, 1968; Knor*r et al*., 1971; Regmi, 1982). HLB was first observed in the citrus orchards of the Horticulture, Research Station Pokhara (Western Nepal), which had completely ruined the orchards (Thrower, 1968). It was suspected that the disease and its vector were introduced from India (Knorr *et al.*, 1971).

HLB is an insidious disease because it displays few symptoms in its early stages which may escape the detection with other diagnostic methods, and may be vectored over a wide area before its authentic detection (Halbert and Keremane, 2004). It is a systemic disease of citrus, and the pathogen of HLB was supposed to be a virus or mycoplasma for sometime in the past. It has now been proved that the pathogen of HLB is gram-negative, phloem restricted bacterium of the genus *Liberibacter* (Garnier *et al.*, 2000). It retards growth of the plant and causes the incomplete colouring of mature citrus fruits (da Graca, 1991). As the HLB bacteria can not be cultured in artificial media, prefix Candidatus (Ca.) is added to the name of the species. So far, three species of HLB pathogen have been reported: 1) *Candidatus* Liberibacter asiaticus, a heat-tolerant form (in which HLB symptoms can appear at temperature above 30˚C) vectored by *Diaphorina citri*, (Bove *et al*.,1994; Jagoueix *et al*., 1997; Garnier *et al.*, 2000) found in Asian countries; 2) *Ca*. L. africanus, a heat-sensitive form (in which no symptoms appear above 30˚C), vectored by *Trioza erytrea,* (Bove *et al*., 1974) found in southern Africa; and 3) *Ca.* L. americanus, another heat-tolerant form vectored by *D. citri (*Teixeira *et al.,* 2005) reported from Brazil.

Unlike other bacterial pathogen, detection of HLB pathogen is problematic. Due to its obligate nature, the bacterium cannot be cultured on any growth medium, hence making its detection difficult (Bove, 1986). Prior to the development of Polymerase Chain Reaction (PCR) based molecular tests HLB diagnosis was achieved by

biological indexing method, followed by Electron Microscopy (EM) (Schwarz, 1968; Sharma *et al.*, 1974), Monoclonal Antibodies (MAbs) (Garnier *et al.*, 1987), Enzyme Linked Immuno Sorbent Assay (ELISA), Thin Layer Chromatography (TLC) and DNA-DNA Hybridization (Bove *et al.*, 1993). HLB infestation in Nepalese samples was first diagnosed in French laboratory using DNA-DNA hybridization technique (Regmi, 1994; Regmi *et al.*, 1996). The development of monoclonal antibodies held promise for a rapid diagnostic test, but they proved to be too specific for general diagnosis. All these methods have various limitations such as high cost involvement, labour intensiveness as well as complicated nature of technologies. In this context, PCR-based method is the best available technique for quick and reliable diagnosis of HLB.

1.2 Justification

In Nepal commercial citrus trees were originally grown as seedling and is still in practice in many places. Seedling grown plants have numerous inherent disadvantages, including juvenile nature of the trees resulting into late fruiting period (five years) and susceptiblility to various diseases (eg. Phytophthora). Therefore citrus growers throughout the world prefer grafted saplings. Grafted saplings and use of resistant rootstocks can overcome the problem of some diseases such as Phytophthora and Gummosis. However, graft transmitted disease like HLB has emerged as a major constraint of citrus industry in Nepal. It has greatly reduced the productivity of citrus in Nepal. HLB being vector and graft transmissible disease, it is rapidly spreading to newer areas due to 1) the presence of vector and 2) due to lack of knowledge among the nurserymen and farmers regarding the mode of transmission and spread of this disease. It has already spread to over 33 districts (Regmi *et al*., 1996; Shrestha *et al.*, 2003) of Nepal and its spread is still continuing. Therefore, reliable diagnosis of HLB disease is crucial for the effective management of this lethal disease. On visual observation, based on symptoms alone, the diagnosis could easily escape the infected plants due to its confusion with mineral deficiency symptoms (especially of Zn). Therefore at present, PCR is the only one most reliable and robust technique to perform rapid, sensitive and specific diagnosis of HLB. Timely diagnosis of HLB in citrus mother plants in the nurseries and orchards can prevent further spread of this disease in Nepal. Therefore, PCR diagnosis of HLB disease is highly justifiable and holds great promise for the integrated management of this disease in Nepal.

1.3 Objectives

The overall objective of this research work is to know the present status of spread of Huanglongbing (HLB) disease in various citrus growing districts based on PCR-based diagnosis.

Specific objectives of the research work include:

1. To conduct epidemiological study of citrus HLB disease in 15 citrus growing districts of Nepal using PCR based diagnosis.
2. To conduct field visit to some citrus orchards of three districts (Syangja, kaski and Kathmandu) for the survey of incidence of various citrus diseases including HLB.
3. Based on PCR results, to advice citrus nurserymen and farmers regarding the necessary measures to prevent the further spread of the disease.

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, experiments to judge the efficacy of PCR assays in different growing seasons could not be carried out.

2. LITERATURE REVIEW

2.1 Center of Origin, World Cultivation and Production of Citrus

The most commercially important 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). According to Hooker (1872), Bhattacharya and Dutta (1956) about 78 species of citrus coming under this 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 Fiji Island and in China. Grapefruit (*C. paradise Macf*) is a native of West Indies (Reuther *et al.*, 1967-1989). Thus except grapefruit, most of the important species in genus *Citrus* and related genera originated in the old world. Introduction and spread of these species to new world started during beginning of Christian-era (Gmitter *et al.*, 1992; Radha and Mathew, 2007). The genera *Poncirus* and *Fortunella* are native to China.

Table 1 World's top ten citrus fruits producing countries, 2007

SN	**Country**	**Grapefruit (mt)**	**Lemons & limes (mt)**	**Oranges (mt)**	**Tangerine, etc (mt)**	**Others (mt)**	**Total (mt)**
1	Brazil	72,000	1,060,000	18,279,309	1,271,000	-	20,682,309
2	China	547,000	745,100	2,865,000	14,152,000	1,308,000	19,617,100
3	U S	1,580,000	722,000	7,357,000	328,000	30,000	10,017,000
4	Mexico	390,000	1,880,000	4,160,000	355,000	66,000	6,851,000
5	India	178,000	2,060,000	3,900,000	-	148,000	6,286,000
6	Spain	35,000	880,000	2,691,400	2,080,700	16,500	5,703,600
7	Iran	54,000	615,000	2,300,000	702,000	68,000	3,739,000
8	Italy	7,000	546,584	2,293,466	702,732	30,000	3,579,782
9	Nigeria	-	-	-	-	3,325,000	3,325,000
10	Turkey	181,923	706,652	1,472,454	738,786	2,599	3,102,414
	World	5,061,023	13,032,388	63,906,064	26,513,986	7,137,084	115,650,545

Source: FAO, 2007

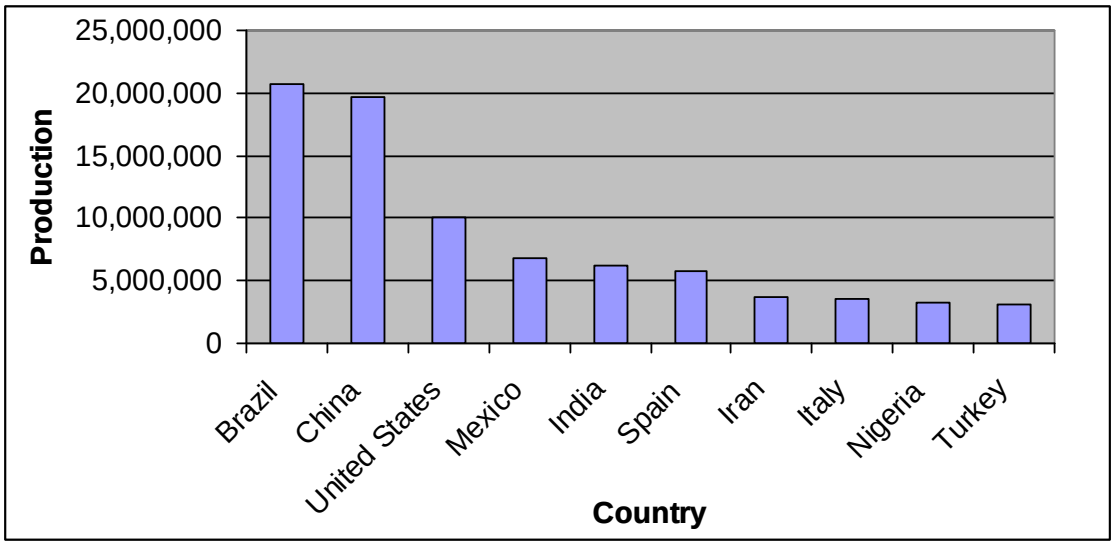

Fig 1. World total production of citrus fruit including all varieties of citrus.

Among the most common Citrus fruits; Sweet oranges occupy nearly 2/3rd of world's total area. They are grown in about 114 countries of the world (FAO, 2002). The U.S.A. is the largest producer of Citrus. The other leading centers producing citrus are Italy, Sicily, Spain, Greece, Argentina, Brazil, Mexico, Japan China Israel, India and Australia. About 90% of grapefruits of the world are produced in Florida (U.S). Italy leads in lemon, Mexico and India are main producer of acid lime, while Japan mainly grows mandarins (Sharma, 2006).

The world production of citrus is about 88 million mt. annually. Out of this, 30 million mt. is produced in Asia and about 6 million mt. is produced in SAARC countries (FAO 1998). The top ten citrus producing countries of the world are Brazil, China, United State, Meixco, India, Spain, Iran, Italy, Nigeria and Turkey respectively (FAO, 2007).

2.2 Genetics and Breeding of Citrus

The orange subfamily Aurantiodeae consists of 33 genera. The genus C*itrus,* subgenus *Eucitrus* is one of six genera of the sub tribe Citrinae, tribe Citrae of the family Rutaceae. Members of this genus are described as "True Citrus Trees" (Samson, 1986; Jackson, 1999). Among these genera, *Citrus*, *Fortunella* and *Poncirus* have been cultivated and utilized either as fruit trees or as rootstocks. Recent taxonomic studies on chemical and biochemical characteristics, and studies on chloroplast DNA and Restriction Fragment Length Polymorphism (RFLPs) based studies suggest that there are only four botanical species among the edible *Citrus* genus *viz*. 1) *Citrus grandis* Osbeck, *2) C. reticulata* Blanco, 3) *C. medica* Linn. and 4) *C. halimi.* However, *Citrus sinensis* Osbeck *and C. paradise* Macf may probably

be the hybrids between *Citrus grandis* Osbeck, *C. medica* L. and another unidentified gene source (Germana, 1997). All cultivated Citrus and related genera (*Poncirus, Fortunella*) are diploid (2n=2x=18) and have small genome (1C=0.38-0.62pg), although triploid and tetraploid citrus also exist (Soost and Cameron, 1975). Breeding and isozyme studies have shown that most *Citrus* species are rather heterozygous and the genus is highly polymorphic (Roose *et al.,* 1998).

2.3 Citrus Cultivation in Nepal

The mid-hills of Nepal are suitable for cultivation of citrus, particularly for mandarin oranges (*C. reticulata*), lime (*C. aurantifolia*) and acid limes (*C. aurantifolia*). The altitude between 900-1400m above sea level (asl) consistes a suitable temperature range for citrus cultivation. Deep sandy loam soil and soil with pH range of 5.0-6.5 is most suitable for the cultivation of citrus (Ranjit and Gharti-chhetri, 1997).

The mandarin is cultivated mostly in Eastern Nepal (Dhankuta, Ilam, Terathum, Sankhuwasabha, and Bhojpur). However, the area under sweet orange (junar) is not much at present in Eastern Nepal, but it is picking up in Sindhuli, Ramechhap, Bhojpur and Dhankuta (Ghosh and Singh, 1993). The main citrus producing districts are Kaski, Gulmi, Dhankuta, Tanahun, Dailekh, Palpa, Syangja, Ilam, Sankhuwasabha, Terathum, Okhaldhunga, Udaypur, Khotang and Bhojpur (Ghosh and Singh, 1993).

Nepal may be the only citrus producing country where the majority of citrus is grown from seed however, this trend is being replaced by grafted saplings. In Dhading district, a 300 year old seedling mandarin was observed and its fruit tested out to have excellent qualities. Also, in the Sankhuwasabha district a 125 year old mandarin seedling was observed and its fruit also had excellent qualities (Kirtipur Hort. Dev. Project 1994/95).

In Nepal mandarin, sweet orange, lime, acid lime and lemons are produced in commercial scale among which mandarin (*C. reticulata*) ranks first in terms of both in production and acerage and are most important commercially (Ghosh and Singh, 1993; Hockey *et al.,* 1998). Citrus are cultivated in the tropical and sub tropical belt between 35N and 35S of equator (Larry, 1999). In Nepal, citrus are cultivated in 66 out of 75 districts among them 34 districts are highly demanded for citrus cultivation in Nepal (NCDP, 1990) (Table 3).

Table 2. Pocket profile of main citrus producing districts 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: Horticulture Development Program, Yearly Progress Report (2007/08), Kirtipur

2.3.1 Area under Citrus Cultivation and Production in Nepal

Citrus is one of the priority crops of midhills in Nepal therefore the area, production and productivity of citrus in Nepal is increasing year by year (Anonymous, 2002). The area under citrus cultivation has been increased from 17023.18ha in year 1996/97 to 27979.8ha in year 2006/07 (HDP, 2007/08). In fiscal year 2007/08 the total area under citrus cultivation in Nepal is estimated to be 30,790.3ha resulting in an annual production of about 2, 22,070.62mt with an estimated yield of 11.36mt/ha (HDP, 2007/08). Mandarin is estimated to cover about 60% of total Citrus area of the country (Anonymous, 2001/02). In fiscal year 2007/08 the total area under mandarin, sweet orange, lemon and others are estimated to be 20167.3ha, 4866.4ha, 3720.3ha, and 2037.3ha respectively (HDP, 2007/08).

2.3.2 Citrus Species of Nepal

Nepal is rich in citrus diversity. Many of these species might have their origin in Nepal itself or have been introduced at various times in the history. Lama and Kayastha (1999) reported 14 species of citrus from Pokhara and its surrounding areas. Of these, the major citrus species grown in this area were *Citrus reticulata*, *C. sinensis*, *C. pseudolemon* and *C. aurantifolia.* Different species of Citrus have been reported from Nepal (including exotic spp.), *viz*, *Citrus reticulata* Blanco, *C. sinensis* Osbeck, *C. aurantium* Linn, *C. aurantifolia* Swingle, *C. pseudolemon* Tanaka, *C. limon* L, *C. grandis* Osbeck, *C. maxima* (Burm.*)* Merrill, *C. aurantium* Linn, *C. jambhiri* Lush, *C. medica* Linn, *C. limettoides* Tanaka, *C. nobilis* Xc, *C. unshiu* M , *Poncirus trifoliata* L, *Fortunella japonica* Swingle and possible hybrids (locally known as Chaku and Narayani) (CFDD, 2001). Citrus species mainly found in Nepal along with their local name and common names are shown (Table 3).

Table 3. List of some citrus species found 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: Nepal Citrus Development Programme, Kirtipur, Kathmandu (2002).

2.4 Economic Importance of Citrus

Citrus species are highly prized for their novel fruits having blend of sourness, sweetness and flavor. They are refreshing, delicious and rich in sugar, minerals and vitamins especially a high content of vitamin C (which varies from 25 to 100 mg/100ml). Fruit juice contains sugars (glucose and sucrose) and acids (primarily citric and a little of malic acid) (Aubert *et al.*, 1990). Beside nutritive value of fruit pulp, rind is rich in pectin, essential oils and glucosides (hesperidin in oranges and lemons, naringins in grapefruit and pummelo). A total soluble solid (TSS) in sweet group varies from 6 to 12% and acidity from 0.5 to 1.5% (Radha and Mathew, 2007). Major chemical constituents of citrus are carbohydrates, acids, vitamins, inorganic constituents, nitrogen compounds, enzymes, pigments, lipids and volatile compounds (APP, 1995. Ninth five year plan 1998; Shrestha, 1999). Per capita consumption of citrus fruit in developed countries is about 10 kg/year where as in Asian countries it is

only about 4kg/year (Aubert *et al.,* 1990). Production of concentrated frozen juice and its demand is increasing throughout the world.

Many citrus species have got medicinal values. Lime maintains good immune system and prevents from cold, scurvey and anemia. Flavonoids have a broad spectrum of biological activities including anticarcinogenic and antitumeric activities. Polymethoxylated flavonoids (PMF) such as tangeretin have anti-tumor activity (Anonymous, 2002). Iron found in citrus constitutes about 1.3mg/450g of edible part (Shah, 1992). Citrus fruit juice is given to sick peoples with high fever and jaundice and also for curing disease like dysentery and beriberi. Bitter glucoside "Naringin" provides prevention against malaria (Radha and Mathew, 2007).

2.5 Overview of Molecular Techniques

Various protein-based and DNA-based techniques have been widely employed to address various problems such as disease diagnosis and genetic diversity studies (Ollitrault, 1990).

2.5.1 Protein-Based Molecular Techniques

Protein-based techniques include the immunological, serological, electrophoretic and hybridization based techniques. In following section protein-based molecular diagnostic techniques are reviewed.

2.5.1.1 Enzyme Linked Immuno Sorbent Assay (ELISA)

Enzyme Linked Immuno Sorbent Assay (ELISA) technique has been an important landmark in serological detection and assay of plant viruses (Engvall and Perlmann, 1971). It is very accurate, sensitive and rapid detection method (Garnsey and Cambra, 1991). ELISA technique involves the use of antigen, antibody, conjugated antibody and substrate for virus detection. This is a serological assay in which the antibodies used to detect a particular substance are labeled by linkage to an enzyme. The test substance is immobilized on a plastic surface and a positive reaction i.e. antibody binding to the surface, is detected by the action of the enzyme on a colourless substrate to produce a coloured product (Lawrence, 1996). ELISA test have been used to detect the viruses in different plant parts, seeds and vectors which transmit the plant viruses even when the virus are present in very low concentration and in very early stages of disease development (Bar-joseph, *et al.,* 1979).

Roistacher (1996) used ELISA for the detection of important citrus diseases such as tristeza virus (CTV), Satsuma dwarf virus (SDV), citrus variegation virus (CVV) and

citrus mosaic virus (CMV). Prasai, (2006) used DAS-ELISA (Double antibody sandwich-ELISA) for detection of CTV in different *Citrus* species (lime, lemon and mandarin). DAS-ELISA technique has also been used for the diagnosis of citrus Tristeza virus in Nepal (Regmi *et al.,* 1997). Wells *et al.,* (1996) reported, DAS-ELISA to be routinely used for the detection of several Potato viruses namely PVA, PVM, PVS, PVX, PVY and PLRV in Nepal. Adhikari, *et al.,* (2004) also used DAS-ELISA to test three- potato viruses, viz. PVX, PVY and PLRY in two potato varieties (Malta and Pentronese) brought from Bangladesh.

2.5.1.2 Western Blotting (WB)

Western blotting consists of the transfer by a blotting technique of protein separated by electrophoresis from the gel to a medium on which they can be further analyzed by treatment with specific antibodies (Lawrence, 1996). Western blotting or immunobloting technique is commonly used to separate protein and then to identify a specific protein of interest. It is one of the most powerful methods for detecting a particular protein in a complex mixture, combines the superior resolving power of gel electrophoresis, the specificity of antibodies and the sensitivity of enzyme (Harvey, 2000).

WB has been widely used in diagnosis of plant as well as animal diseases. Proteins associated with virus infection in plants and vectors have been detected and identified using WB (Arbatosia *et al.,* 1998). Bezerra *et al.,* (1999), identified two serologically distinct tospoviruses, Chrysanthemum Stem Necrosis Virus (CSNV) and Zucchini Lethal Chlorosis Virus (ZLCV), occurring in Brazil using WB analysis. This technique can detect a specific protein in a mixture of number of protein and also provides information about the size of the protein. This method is however, dependent on the use of a high-quality antibody directed against a desired protein (Intermet visit, 2). Western blot method has also proved to be a very precise tool in the diagnosis of Wilson's disease, as inherited copper toxic disease (Chowrimootoo *et al*, 1997). WB has been used in the diagnosis of chronic infection with human immunodeficiency virus (HIV) (Interner visit, 3).

2.5.2 DNA –Based Molecular Techniques

DNA-based molecular technique can either be Hybridization-based (RFLPs/Southern Blotting) or Polymerase Chain Reaction (PCR) based. Hybridization-based molecular techniques includes restriction fragment length polymorphism (RFLP) and Southern Blotting technique.

2.5.2.1 Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) was the first technology that enabled the detection of polymorphism at the DNA sequence level (Chawala, 2003). In RFLP, DNA is digested with Restriction enzymes, which cuts the DNA at specific sequences, electrophoresed, blotted on a membrane and probed with a labeled clone. Polymorphism in the hybridization pattern is revealed and attributed to sequence difference between individuals. The DNA sequence variation detected by this method was termed as RFLP (Bostein *et al.,* 1980, cited in Chawala, 2003).

RFLP, is a combination of hybridization, southern blotting and restriction mapping forming the basis for the genetic analysis and characterization of pathogen (Bostein *et al.,* 1980). This technique have been particularly popular for detecting unidentified viruses or viroids in plants. RFLP-based technique was developed to identify members of the sooty blotch and flyspeck (SBSF) disease complex on apple because these fungi are difficult to identify using agar-plate isolation and on morphological description (Duttweiler *et al.,* 1989).

2.5.3 PCR-based Techniques

Polymerase Chain Reaction (PCR) is a versatile technique, based on the enzymatic amplification of DNA, *in vitro*. It is an extremely powerful technique that allows to make million copies of a selected DNA sequences in a genome (Weising *et al*., 1995). PCR technique has become an indispensable tool of molecular biology. In the PCR technique, DNA is amplified *in vitro* by a series of polymerization cycles consisting of three temperature–dependent steps: DNA denaturation, primer-template annealing and DNA synthesis by a thermostable DNA polymerase (Rychlik *et al.,* 1990). The PCR process was originally developed to amplify short segments of a longer DNA molecule (Saiki *et al*., 1985).

2.5.3.1 Arbitrarily-primed PCR

For arbitrarily-primed PCR assay, no prior sequence information of organism is required for primer designing. There are variants of arbitrarily primed PCR. For arbitrarily primed PCR (AP-PCR), the primers are 20 to 34 nucleotides in length in contrast to 10bp in RAPD, but low annealing stringencies are used for the first few rounds of amplification (Welsh and McClelland, 1990). AP-PCR (Welsh and McClelland, 1990), Random Amplified Polymorphic DNA (RAPDs) and DAF are variants of arbitrarily-primed PCR. Of these RAPD have been widely used in pathogen identification and diversity studies (Williams *et al.,* 1990).

2.5.3.2 Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) is an arbitrarily-primed PCR assay, no prior sequence information of organism is required for primer designing. The primers are randomly designed and may vary from 10bp (in RAPD) to 20-34bp in AP-PCR (Welsh and McClelland, 1990). RAPD is a powerful and popular technique for the investigation of genetic variation (Williams *et al.,* 1990 and Welsh and McClelland 1990). RAPD has been extensively used for plant species and varietal identification (Graham *et al.,* 1994; Golembielwski *et al,* 1997). This technique has been also used in plant disease diagnosis (Busso *et al*., 2007) and has been extensively used in genetic linkage map construction for the identification of markers closely linked to various disease resistance genes (Singh, 2005). Busso *et al.,* (2007) analyzed blast disease of wheat (*Triticum aestivum* Lam.) caused by *Pyricularia grisea* using RAPD technique. Ratanacherdchi *et al*. (2007) also used RAPD analysis of *Colletotrichum* species causing chilli anthracnose disease in Thailand. Laurie, (1996) used RAPD markers as a diagnostic tool for the identification of *Fusarium solani* isolates that cause soyabean sudden death syndrome. Identificaiton of Erwinia carotovora from Soft rot diseased plants was also done by RAPD analysis (Jean *et al.*, 1996).

RAPD, have been widely used in pathogen identification and diversity studies (Williams *et al*., 1990). RAPD is a powerful multilocus technique for the detection of polymorphism in organism. Being a fast and simple method, RAPD can be quickly and efficiently applied to identify useful polymorphism (Waugh and Powell, 1992). Guthrie *et al.,* (1992) used RAPD markers for identifying and differentiating isolates of *Colletotrichum graminicola*. A RAPD-PCR based technique was used to create a series of genetic markers that could distinguish/identify the five major weed species (*Sporobolous pyramidalis, S.natalensis, S.fertilis, S.fricans and S. jacquemonti*i) found in *Sporobolous* species (Shrestha *et al.,* 2005).

RAPD procedure works with anonymous genome, requires only small amounts of DNA and is simpler, less costly and less labor intensive than other DNA marker methodologies (Caetano-Anolles *et.al.,* 1991a, b; Hadrys *et al.,* 1992). RAPD technique is used extensively for intraspecific characterization of several plant pathogens (Mesquita *et al*., 1998; Freeman *et al.,* 2000).

2.5.3.3 Simple Sequence Repeats (SSRs)/Microsatellite

Simple Sequence Repeat (SSR) is a specifically primed PCR-based assay. Simple Sequence Repeats (SSRs) is also known as Micro-satellite (Jacob *et al.,* 1991), are polymorphic loci present in nuclear and organnellar DNA that consist of repeating

units of 1-6 base pairs in length. They are widely dispersed throughout eukaryotic genomes and are often highly polymorphic due to variation in the number of repeat units (Karp *et al.,* 1998). They are typically neutral, co-dominant and are used as molecular markers which have wide ranging applications in the field of genetics. Microsatellites can be amplified for identification by PCR, using the unique sequences of flanking regions as primer (Beyermann *et al.,* 1992).

2.5.3.4 Sequence Characterized Amplified Region (SCARs)

Sequence Characterized Amplified Region (SCAR) is another specifically primed PCR-based assay. Random Amplified Polymorphic DNA (RAPD) is most widely used molecular technique for generating molecular markers for diagnosis. However, it is very sensitive to reaction condition and cycling parameters which renders it less useful for routine analysis of large numbers of plant samples. The RAPD fragment of interest is therefore cloned and sequenced to design longer primers (24-mers) for the conversion of RAPD markers to SCAR markers (Paran and Michelmore, 1993). Paran and Michelmore (1993), cloned sequenced RAPD markers linked to downy mildew resistance in lettuce and developed longer SCAR primers. SCAR markers are robust and codominant as opposed to dominant RAPD markers. Zhang *et al.,* (1998) reported sex determination in *Silene latifolia* and have found several Y chromosomes linked RAPD markers in *S. latifolia*, he converted them to SCAR markers by cloning RAPD fragments and developing them into longer primers. Dang *et al.,* (1997) had developed and characterized SCAR markers linked to citrus tristeza virus resistance gene from *Poncirus trifoliata.*

2.5.3.5 Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) technique is based on the principle of selectivity, amplifying a subset of restriction fragments from a complex mixture of DNA fragments obtained after digestion of genomic DNA with restriction enzymes. Polymorphisms are detected by differences in their length of the amplified fragments by Polyacramyl gel electrophoresis (PAGE) (Matthes *et al.,* 1998). Vander Lee *et al.,* (1997) constructed a comprehensive genetic linkage map of the plant pathogen *Phytophthora infestans* using AFLP.

2.5.3.6 Reverse Transcriptase PCR (RT-PCR)

Many citrus viruses and viroids have been diagnosed via Reverse Transcriptase (RT-PCR) (Wang *et al.,* 2009). Here, total RNA is isolated and complementary DNA (cDNA) is synthesized by using reverse transcriptase enzyme prior to amplification

via PCR. Two most important viral diseases affecting citrus species in Brazil *viz.* tristeza and leprosies caused by CTV and citrus leprosies virus (CiLV) were simultaneously detected using RT-PCR (Freitas-Astua *et al*., 2005). Two pairs of primers were used for PCR, one that amplified 557bp within the p20 gene of CTV and the other that amplified 339bp region within the putative movement protein (MP) gene of CiLV. Total RNA was extracted from typical CiLV symptomatic areas and cDNA strand was synthesized using M-MLV reverse transcriptase and random primers.

2.5.3.7 Real Time (RTi) PCR

In molecular biology, real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (QRT-PCR) or kinetic polymerase chain reaction. It is a technique used to simultaneously quantify and amplify a specific part of a given DNA molecule. It is used to determine whether or not a specific sequence is present in the sample, and if it is present, the number of copies in the sample (Internet visit, 4). Real-time PCR has engendered wider acceptance of the PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination. The technology has been applied to areas of microbiology, virology, as well as studies of gene expression and genetics disease (Ian *et. al*, 2002). Quantitative real-time PCR is recently used for the detection and identification of *Candidatus* Liberibacter species associated with citrus HLB disease (Wenbin *et* al., 2005).

In comparison to conventional RT-PCR, real-time PCR also offers a much wider dynamic range of up to 10^7-fold (compared to 1000-fold in conventional RT-PCR). The recently introduced EZ one-step RT-PCR kit allows the use of UNG as the incubation time for reverse transcription is 60 ^{0}C. This temperature is also a better option to avoid primer dimers and non-specific bindings at 48 ^{0}C (Internet visit, 5)

2.6 Diseases of Citrus

Diseases are the main cause in reduction of citrus productivity. Numerous diseases affect citrus fruits, several of which are very serious either locally or world wide. Citrus fruits are found to be infected either by micro-organism (bacteria, virus and fungi) or nematodes (Singh, 1967). Other abiotic factors (mineral deficiency, unsuitable environmental condition, effect of insecticides, etc) can also induce diseases like symptoms.

Klotz (1978) reported 65 fungal diseases of citrus. Seventy eight citrus diseases have been reported from different countries of the world. Among them, seven are bacterial,

60 are fungal and 11 are viral diseases respectively (Internet visit, 6). Many insect and nematode also causes serious citrus diseases directly by damaging citrus plant or by acting as a vector for transmission of disease (Paudyal and Regmi, 2008). Bar-Joseph *et al* (1981) recorded 30 viruses and virus-like disease of citrus known in the world. Major citrus diseases currently present in different citrus growing areas of the world include tristeza, blight, greasy spot, *Alternaria* brown spot, *Phythophthora*-induced diseases, melanose, scab, canker, and post bloom fruit drop (PFD) (Teixeira *et al*., 2004).

In the context of Nepal, major citrus diseases reported are Huanglongbing (HLB), Citrus tristeza virus (CTV), Phytophthora, Canker, Anthracnose, Powdery mildew, Pink disease, Scab, Felt disease, Papery bark (Psorosis), Woody gall and Exocortis (Regmi *et al.,* 1996) (Table 4). Among these disease HLB, CTV, Exocortis, Woody gall and Papery bark are graft transmissible and systemic and very difficult to control (Regmi *et al.,* 1996).

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 *et al*., 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.

Nineteen different diseases were reported from Nepal (CFD, 2001). In 2002, twenty different diseases have been reported (CFD, 2002), several of which are very serious either locally or world wide. So far, twenty different citrus diseases have been recorded (Table 4).

Table 4 List of the citrus diseases reported in 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: (2001, Citrus Fruit Development Report, Citrus Fruit development section, Kirtipur).

2.7 Citrus Huanglongbing Disease

As the disease was first reported in China in 1919, the officially accepted name "Huanglongbing" has been given to the disease (da Graca and Karsten, 2004; Bove, 2006). The first symptom of HLB is usually the appearance of a yellow shoot on a tree, hence the name Huanglongbing has been given, which literally means yellow dragon disease (Halbert *et al*., 2004). The name greening was given to this disease because the fruits on infected trees remains green in colour even after maturity.

HLB disease has been reported by different names *viz*, "Likubin" in Taiwan; "Leaf Mottling" in Philippines; "Huanglonbing" in China; "Citrus greening disease" in South Africa; "Citrus Vein Phloem Degeneration" (CVPD) in Indonesia; "Quick decline" and "Citrus Greening Disease" in India, Pakistan and Nepal (Su and Hang, 1990; Bove, 2006). It has caused most serious threat to citrus industry of Asian countries like China, Thailand, Indonesia, India, Nepal, Pakistan and Bhutan. South Africa is another country that is facing this problem for many years. Recently this disease has also been found in Brazil (Bove, 2006).

HLB being the most devastating diseases of citrus it has ruined many citrus industries of the world. More than 1600 ha of citrus orchards were destroyed in China since 1958 (Broadbent, 1983). The mandarin area decreased from 19,330 ha to 4,840 ha in 14 years (from 1961 to 1974) in Philippines (Altamirano *et al.,* 1976). More than 10, 00000 trees of Novel and Valencia oranges had become commercially unprofitable in South Africa (Oberholzer *et al.,* 1965; Schwarz *et al.* 1973; Regmi, 1982). It has been reported that HLB had reduced the productivity of citrus up to 80% in different regions of India (Singh, 1977). HLB had destroyed an estimated 60 million trees in Africa and Asia (Timmer *et al*., 2000). Considering these losses, the HLB disease has been recognized as the most serious threat for citriculture in Asia and Africa.

HLB was observed in Pokhara Valley of Nepal during 1960s. After visiting citrus orchards of Nepal, renowned French scientist Prof. J. M. Bove had reported that "if nothing is done, citrus will soon disappear from Nepal, in the same way that citrus has been destroyed in India, Indonesia and the Phillippines" (Bove, 1994). Similarly Roistacher, (1996) also stated, "Greening (HLB) disease is a number one threat to citrus industry in Nepal. Unless this disease is understood and controlled, citrus will slowly but surely decline". Recently in Lamjung it has caused about 25% loss in citrus production (Nepal samacharpatra, 01/01/2009).

2.7.1 World Distribution of Huanglongbing

HLB has been reported as early as 1920s as yellow branch disease from South Africa and Yellow shoot disease from China (Oberholzer *et al*., 1965; Broadbent, 1983). At present this disease has been reported from several countries of Asia (China, Indonesia, southern islands of Japan, Malasia, Phillippines, Taiwan, Thailand and Vietnam), Africa (Burundi, Cameroon, Central Africa Republic, Ethiopia, Kenya, Malawi, Rwanda, Somalia, South Africa, Swaziland, Tanzania, and Zimbabwe) and islands of Indian Ocean (Srilanka, the Comoros Islands, Madagascar, Mauritius and Reunion Island (Toorawa, 1998) causing severe damage to citrus production. Mauritius and Reunion also have African citrus greening (Subandiyah *et al.,* 2000). Moreover, Verma and Atiri (1993) reported that over 50% of plants in some areas of Negeria show symptoms of citrus greening. Garnier and Bove (2000) added Combodia to the list of countries where citrus greening is present. Citrus greening disease was found in Papua New Guinea in 1999 (Lee, 2002). HLB disease was also reported in Brazil (Anon., 2004).

Before 2004, HLB was known to occur in Asia, from Japan in the east, through Southern China, Southeast Asia and the Indian subcontinent to Pakistan. It also exists in the Arabian Peninsula, but not in Iran. In Africa, it can be found throughout eastern, central and Southern Africa. The vector, *Diaphorina citri*, has been present in

Brazil for over 60 years (Halbert and Nunez, 2004), and since then has spread into other South and Central American countries, the Caribbean and Florida (Knapp *et al.,* 1998; Halbert and Nunez, 2004) and Texas (French *et al.,* 2001) in the United States. HLB was found in Florida during 2005 and it is also known to occur in Cuba. However, has not been reported from California as yet (Cardwell, 2008). Details on the world distribution of HLB is shown (Table 5).

Table 5 The world distribution of Huanglongbing disease and its vector.

S. N	**Country**	**Disease with synonyms**	**Vector species**	**Author reporting the disease / vector**
1	Bangladesh	Greening	*Diaphorina citri*	Catling, 1978
2	China	Yellow Shoot (Huanglongbing)	*D. citri*	Lin, 1956; Su *et al.*, 1972
3	India	Citrus decline (Quick dieback)	*D. citri*	Fraser *et a*l., 1966; Capoor *et al.*, 1967
4	Indonesia	Citrus Vein Phloem degeneration	*D. citri*	Tirtawidjaja, 1965
5	Malaysia	Greening	*D. citri*	Catling, 1968
6	Nepal	Greening	*D. citri*	Thrower, 1968; Knorr *et al.*, 1971
7	Pakistan	Greening	*D. citri*	Cochran *et al.*, 1976
8	Philippines	Leaf mottling	*D. citri*	Salibe *et al.*, 1966; Martinez *et al.*, 1967
9	Saudi Arabia	Greening	*D. citri*	Bove, 1986
10	Yemen Arabic Republic	Greening	*D. citri/ Trioza erytreae*	Bove,1986
11	Taiwan	Likubin	*D. citri*	Catling, 1970; Su *et al.*, 1972
12	Thailand	Greening	*D. citri*	Schwartz *et al.*, 1973
13	Angola	-	*T. erytreae*	Aubert *et al.*, 1988
14	Comeroon	-	*T. erytreae*	Aubert *et al.*, 1988
15	Ethiopia	Greening	*T. erytreae*	Aubert *et al.*, 1988
16	Kenya	Greening	*T. erytreae*	Aubert, 1985
17	Ruanda	Greening	*T. erytreae*	Aubert *et al.*, 1988

18	South Africa	Greening	*T. erytreae*	Oberholzer *et al.*, 1965
19	Sudan	-	*T. erytreae*	Aubert, 1985
20	Swaziland	-	*T. erytreae*	Aubert, 1985
21	Tanzania	Greening	*T. erytreae*	Aubert, 1985
22	Uganda	-	*T. erytreae*	Aubert, 1985
23	Zaire	-	*T. erytreae*	Aubert, 1985
24	Zimbabwe	Greening	*T. erytreae*	Aubert, 1985
25	Madagascar	Greening	*D. citri/ T. erytreae*	Aubert, 1984
26	Mauritius	Greening	*D. citri/ T. erytreae*	Aubert, 1984
27	Reunion	Greening	*D. citri/ T. erytreae*	Lafleche and Bove, 1970
28	Scyhelles	Greening	*D. citri/ T. erytreae*	Aubert, 1984

Source: Garnier and Bove, 1996.

2.7.2 Symptoms

Symptoms of HLB are varied, and can resemble other disorders such as Zn-deficiency. The initial symptoms are frequently the appearance of yellow shoots or mottled leaves on a tree. As the bacteria move within the tree, the entire canopy progressively develops a yellow color, retarded growth and tip necrosis (Timmer *et al.*, 2000; da Graca and Korsten, 2004; Halbert and Nunez, 2004; Bove, 2006). HLB symptoms are mainly seen on leaves and fruits.

2.7.2.1 Symptoms on Leaves

Visual leaf symptoms appear from the apical part of the tree. At the initial stage leaf mottling and leaf drop is observed only on the upper branch which gradually develops to other branches and the slightly affected trees develop severe symptoms within 2-3 years. The affected leaves develop a pattern of yellow and green areas lacking clear limits between the colors, giving a “blotchy mottle” appearance (McClean and Schwarz, 1970). This is the most characteristic foliar symptoms and the patterns are asymmetrical on the two halves of the leaf (Bove, 2006). Leaves can also become thicker, with veins enlarged and corky in appearance. In later stages, Zinc deficiency like symptoms can develop, followed by leaf drop and twig dieback. Ohtsu *et al.*, (1998), identified seven typical leaf symptoms most commonly seen in HLB infected

leaves *viz.* 1) mottling, 2) chlorosis with green netlike veins, 3) severe chlorosis with green vein, 4) pale green colour on young leaves, 5) vein yellowing, 6) vein corking and 7) yellow blotching.

2.7.2.2 Symptoms on Fruits

Symptomatic fruits are small, lopsided with aborted seeds (Regmi *et al.,* 1998) and as they mature and ripen, the stylar end remains green. In addition, the vascular bundles within the fruit axis at the peduncular end have a strong brownish stain. When the peduncle of a fruit with colour inversion is carefully removed, the resulting circular scar is stained orange, while on a normal fruit the scar is pale green. Sometimes, when one presses such fruit with the thumb, a silvery "finger mark" results. Besides, fruit from diseased trees are small, often irregularly shaped, and typically some green color remains on ripened fruit (Internet visit, 7). There is excessive fruit drop in HLB-infected trees (McClean and Schwarz, 1970). As in stubborn-affected fruit, the albedo is sometimes thicker at the peduncular end than at the stylar end (Bove, 2006).

2.7.3 Pathogen

The infectious nature of causal organism of HLB was first confirmed by Schwartz, (1968). At first the pathogen of HLB was supposed to be a virus because it was graft and vector transmissible and could not be cultured *in vitro*. However, this view was accepted only till 1970 (Martinez and Wallace, 1967). During 1970s, many scientists studied the pathogen under electron microscope and thought that HLB organism was a Mycoplasma-like Organism (MLO) (Lafleche and Bove, 1970). During the same period, Saglio *et al.,* (1971) found that the organism was enclosed by a 25nm thick envelope, which was much thicker than the unit membrane envelope of MLOs (thickness, 7-10 nm). All these properties suggested that HLB organism is a walled bacterium and does not resemble mycoplasmas. By analogy with MLOs, the HLB organism has been designated as Bacteria-Like Organism (BLOs) (Moll and Martin, 1974). While studying the HLB organism under electron microscope, Garnier *et al.,* (1984) reported Peptodoglycan (PG) layer in the envelope of HLB organism which was similar to *E. coli*, a gram negative bacterium, suggesting that organism must be a gram negative bacterium. To date, all efforts to isolate the bacterium in pure culture have been unsuccessful (Garnier and Bove, 1993), but a combination of EM and enzymatic treatments showed the cell wall to be of the Gram negative type.

Three species or forms of phloem-limited bacteria have been identified so far based on the causal agents of HLB (Bove, 2006): 1) HLB caused by *Candidatus* Liberibacter asiaticus, a heat-tolerant form (in which HLB symptoms can appear at temperature above 30˚C) vectored by *Diaphorina citri*, the Asian citrus psyllid (Bove

et al.,1974; Jagoueix *et al*., 1997; Garnier *et al.*, 2000) and prevalent in Asian countries (Asian strain); 2) HLB causesd by *Ca*. L .africanus, a heat-sensitive form (in which no symptoms appear above 30˚C, vectored by *Trioza erytrea,* the African citrus psyllids (Bove *et al*., 1974) found in southern Africa (African strain) and 3) HLB caused by *Ca*. L. americanus (American strain). This is the latest reported American species based on molecular phylogenetic study of 16S rRNA gene sequences and sequences of 16S/23S intergenic regions, another heat tolerant form vectored by *D. citri (*Teixseira *et al.* 2005), found in Brazil.

HLB is caused by fastidious bacteria (FB) that exists in sieve tubes of phloem. FB bodies are pleomorphic, and produce flexible elongated rods (100-25 nm x 500-2,500 nm) which grow in new organisms, while when they are old they form spherical bodies 700-800 nm (in the diameter) with a thin cytoplasm (Su and Hang, 2001).

2.7.3.1 Classification and Nomenclature of HLB Organism

The organism contained two separate membranes (cell wall and cytoplasmic membrane) in it's envelop. On the basis of this fact, Moll and Martin (1974) proposed the term "Bacterium like organism (BLO)" other than Greening Organism (GO). On the basis of the presence of PG layer, Bove *et al,* (1980) followed the classification of Prokaryotes proposed by Gibbonsons and Murray and used the term "Gracilicute like organism" for GO. Later on Garnier *et al,* (1984) and Daniel and Bove, (1984) proposed the GO to be gram negative bacteria belonging to the division Gracilicutes.

With the development of PCR and DNA sequencing, it became possible to characterize the organisms at the molecular phylogenetic level. On the basis of such considerations, Murray and Schleifer (1994) proposed the "*Candidatus*" designation as an interim taxonomic status, to provide a proper allocation of sequence-based potential new taxa at the genus and species level. The 16S rDNA sequence comparisons showed that HLB organism belongs to be the first member of a new subgroup of α sub-division of the *Proteobacteria* (Garnier and Bove, 1993). The trivial name, liberobacter (Jagoueix *et al*., 1994), later replaced by liberibacter (Garnier *et al*., 2000) [from the Latin (liber =bark) and (bacter =bacterium)], was given to organisms in this new sub-group. Teixeira *et al*. (2005) detected a different liberibacter isolate associated with HLB diseased plants from Sao Paulo (Brazil) and designated as "*Ca.* L. americanus", which is predominant in the state of Sao Paulo.

2.7.4 Transmission

The transmission of HLB disease takes place mainly by three different means *viz*: grafted budwood, by insect vector and also by dodder.

2.7.4.1 Vector Transmission

HLB is mainly transmitted by inscet vector commonly known as citrus psylla. Psyllid transmission is the primary means of spread in the field. Acquisition times of 30 min for Asian psyllids (Roistacher, 1991) and 24 hrs for African psyllids (Buitendag and von Broembsen, 2003) have been reported. Adults and fourth and fifth instar Asian citrus psyllids are able to transmit the pathogen (Capoor *et al.,* 1967) after a latent period as short as one day or as long as 25 days (Xu *et al.,* 1988; Roistacher, 1991).

The transmission of the pathogen by the psyllid occurs through the salivary secretion (Aubert, 1987). Capoor *et al.,* (1974) reported transmission of HLB by *Diaphorina citri* in India. He reported that single Asian psyllid which previously fed on infected sweet orange can transmits the disease to 41 to 60 plants. According to Raychaudhuri *et al.* (1972) and Capoor, *et al.,* (1974) a single psylla could rapidly transmit the Asian greening disease throughout India. They showed the period of 8 to 12 days between acquisition and transmission. Xu *et al.,* (1988) in China showed that a single psylla of *D.citri* would readily transmit the HLB organism. They confirmed the study of Capoor *et al.,* (1974) that psyllids of the I^{st} to 3^{rd} instars would not transmit, but that psyllids of 4^{th} and 5^{th} instars would readily transmit the HLB organism. They further reported variability in the latent period within the insect ranging from as little as one day to 25 days.

Koizumi *et al.,* (1994) showed the presence of many viruliferous psyllid vectors in the field in Thailand and obtained 41% transmission after a 2 days infection feeding. They also indicated a latent period of about three weeks and a minimum acquisition feeding period of about one week. Aubert (1986) reported that the psyllid cannot spread the disease more than three kilometers because of its low flying capacity. However Regmi (1990) suggested that this distance may be much less in the mountainous regions of Nepal due to obstacles of slopes and zigzag relief. Natural spread of the disease is greatest in late spring and perhaps other periods when new flush is available and psyllid populations are high (Catling, 1970; Aubert, 1987). Psyllid vectors are also attracted to yellow wavelengths of light, and thus preferentially to foliage expressing HLB symptoms.

2.7.4.2 Graft Transmission

Graft transmission of HLB disease was first reported in China during 1950s (Lin, 1956). The HLB pathogens are graft transmitted however, graft transmission of *Candidatus* Liberibacter spp. is variable, depending upon the plant parts used for grafting, the amount of tissue used, and the pathogen isolate (Van Vurren, 1993; Bove *et al.,* 1996). Transmission of HLB has been reported by buds, stem grafts, root grafts

and leaf grafts (Bove, 2006). Side grafts with twigs were even more efficient at transmitting the pathogen, whereas fruit stems and bark strips were not effective (Van Vurren, 1993). In Nepal propagation with infected buds is the main way of disease transmission. Bove, (2006) reported long distances transmission of HLB by grafted budwood. Schwarz reported that percentage of transmission was generally low by buds and higher by stem grafts in South Africa. Zhao *et al.,* (1982) reported that in China transmission of disease was very low during May-June, than other season and transmissibility was higher from orange to ponkan than from Satsuma mandarin.

2.7.4.3 Transmission by Dodder

Greening can also be transmitted by dodder (*Cuscuta* sp., family-Cuscutaceae) to non-Rutaceous plants such as *Catharanthus roseus* L. G. Don (periwinkle-Apocynaceae) (Tirtawidjaja *et al.,* 1981) and *Nicotiana tobacum* L. cv. 'Xanthii' (tobacco-Solanaceae) (Garnier and Bove, 1993) suggesting a wide physiological host range of the HLB pathogen. Garnier and Bove (1993) also reported transmission of HLB from sweet orange to Periwinkle (*Catharanthus roseus*) by dodder-*Cuscuta compestris*. The pathogen even multiplied in the dodder itself (Ghosh *et al.,* 1997; Su and Huang, 1990). They succeeded to transfer both HLB forms (Indian and African) from Madame vinous and sweet orange to periwinkle.

2.7.5 Host Range

All citrus plants are potential hosts of HLB. Historically, the most susceptible hosts are sweet oranges, tangelos, and mandarins (Bove, 2006). Moderately susceptible hosts are those of grapefruits, lemons, Rangpur lime, calamondins, and pummelos. Mexican limes and trifoliate orange have been more tolerant. Non-citrus species, such as *Murraya paniculata,* may also serve as hosts of the HLB pathogens (Timmer *et al.,* 2000; da Graca and Korsten, 2004; Halbert and Nunez, 2004; Bove, 2006).

2.7.6 Diagnosis of HLB

Positive diagnosis of any disease is very crucial for its effective management. Positive identification of HLB disease under field condition is often very difficult because it can be easily confused with mineral deficiency, root rot or other stress related leaf symptoms. Furthermore, the irregular distribution of the disease within the tree and slow disease development make both visual detection and bioassays difficult. Historically, a number of techniques have been employed for the diagnosis of HLB. These techniques will be reviewed in the following section.

2.7.6.1 Indicator Plant Method

This is also called Biological indexing and is the most primitive method commonly used for the diagnosis of HLB disease. This involves the use of indicator plants and is the most easy and cheapest way to detect greening infection. Indicator plants give specific reaction in developing symptoms with the specific pathogen. Best indicator plants for HLB is Sweet orange (Madame vinous, Pineapple, Hamlin and tangelo varieties) (Clavan *et. al.*, 1967). Recommended indicator plants are seedlings of sweet orange and Orlando tangelo for African greening, and Sweet orange or Ponkan mandarin for Asian greening (Bove, 2006). In this technique, seedling of indicator plant are inoculated with side grafts/ buds of a given source tree. After inoculation, the indicator seedlings, inoculated with the African liberibacter or American liberibacter, should be kept at cool temperature conditions (20^{0}C for 8 hours in the dark, and 24^{0} to 27^{0}C for 16 hours in the light). For the temperature-tolerant Asian liberibacter, temperatures can be higher (25^{0}C for 8 hours in the dark, and 30^{0} to 32^{0}C for 16 hours in the light) (Aubert, 1990). Typical symptoms of disease generally appear with the first emerging shoots within 3-4 months after inoculation. Although, indicator plant method is an effective method for greening detection it is highly time consuming and difficult for routine diagnosis.

2.7.6.2 Thin Layer Chromatography (TLC) Test

Thin Layer Chromatography (TLC) technique is based on the presence of fluorescence greening marker substance (gentisoyl glucose) developed by Schwarz, (1968) and also had been used for diagnosis of HLB in Nepal. This method was further modified and used by Sharma *et al.*, (1974). However, this technique is not reliable due to the presence of polyphenolic compounds that may also give positive reactions. Therefore, although chromatographic technique is relatively quicker laboratory test for HLB detection, is not absolutely specific for greening and hence not a reliable technique. The test can be carried out at all seasons of the year, and is recommended for surveying and rapid confirmation of field symptoms but not recommended for certification work (Schwarz, 1968).

2.7.6.3 Electron Microscopy (EM)

Electron Microscopy (EM) is very quick and easier method which gives the picture of size, shape and structure of pathogen (Lafieche *et al*., 1970; Catling *et al*., 1978). EM being highly sophisticated and costly equipments, is not available in Nepal. From 1970 to 1990, transmission electron microscopy (TEM) had been the first and only laboratory technique for indisputable identification and confirmation of HLB, and had been widely used (Moll and Martin, 1973; Garnier and Bove, 1996). The reliability

and specificity of EM is based on two properties of the HLB bacterium, firstly its exclusive location in the sieve tubes, and secondly the presence of a cell wall. In citrus, no bacteria other than the HLB has these properties. For EM detection of the HLB bacterium, leaf midribs are used. If the number of bacteria per sieve tube is low, it is recommended to use longitudinal sections of sieve tube cells. Several years of experience with EM detection of Asian and African HLB have shown that the number of bacteria in sieve tubes is higher in leaves with severe mottling than in those with mild mottling symptoms. Therefore, leaves with strong mottle are preferred for EM detection of HLB, while symptomless leaves are not suitable. For indisputable identification of HLB by EM, it is necessary that at least one bacterium in one section should show the electron dense cell wall layer surrounding the cell. Most often, the layer is seen only in certain parts of the cell. Sometimes, several sections have to be examined before a bacterium with a "good" cell wall is seen. EM is a heavy and time-consuming technique, and cannot distinguish between African, Asian and American liberibacters (Bove, 2006).

2.7.6.4 Monoclonal Antibody (MAb) Technique

Monoclonal Antibodies (MAbs) are antibodies produced by a single clone of β-cells and thus consists of a population of identical antibody molecules all specific for a single antigenic determinants. MAb is produced from cultured hybridoma cell lines for research and commercial purposes (Lawrence, 1996). Antibodies work by binding to the foreign substance to mark it as foreign. The substance that the antibody binds to is called an antigen. All monoclonal antibodies of a particular type bind to the same antigen (Internet visit. 8).

This technique is based on the production of hybridoma clones secreting specific monoclonal antibodies (MAb) against the Greening Organism. Thirteen MAbs specific for African and Asian *liberibacters* have been produced (Garnier *et al.*, 1991; Gao *et al.*, 1993). The first ten MAb was raised using as immunogen homogenates of phloem tissue from HLB-affected Periwinkle plants. Of these MAbs, two (including MAb 0A6) were against the Indian Poona strain, five against a strain from China (Fujian), and three against the South African Nelspruit strain. The use of these MAb for the detection of HLB-liberibacters by immuno-fluorescence on thin sections has shown that MAbs is very specific for the strain used for immunization and, therefore, they cannot be used for generalized diagnosis of HLB (Garnier *et al*., 1991). Serological techniques using monoclonal antibodies (MAb) have been developed but these MAbs can recognize only the bacterial strains from which these were prepared. No polyclonal antibodies could be developed because of its pleomorphic nature and inability to culture in synthetic media. Garnier *et.al*, (1987, 1991) have been successful to obtain four monoclonal antibodies. These MAbs are used to detect GO

in citrus and Periwinkle plants by double antibody sandwich ELISA and immunofourescence (IF) method.

2.7.6.5 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction technique (PCR) is the latest developed technique for the HLB organism diagnosis. So far, four main PCR-assay have been designed, one based on 16S-rDNA, another is ribosomal protein gene sequences and third one is multiplex PCR for the detection of Asian and American strains of HLB and last one is real time PCR (RTi-PCR). By the use of these PCR techniques, the presence of HLB has been clearly established in several African and Asian countries (Bove *et al*., 1993; Garnier and Bove, 1996; Korsten *et al*., 1996; Bove *et al*., 2000; Garnier and Bove, 2000*;* Garnier *et al*., 2000).

2.7.6.5.1 PCR based on 16S rDNA (16S-PCR)

16S rDNA PCR is based on the amplification of an 1160 bp fragment of liberibacter 16S rDNA with primer pair f-OAI/r-OI2C for *Ca*. L. africanus and f-OI1/r-OI2C for *Ca*. L. asiaticus (Jagoueix *et al.*, 1996). In countries where the two liberibacter species are known or suspected to be present, it is advised to use the two forward primers, OI1 + OA1, and the common reverse OI2C primer in the same PCR mixture (Hocquellet *et al*., 1999).

2.7.6.5.2 PCR based on ribosomal protein genes (rpl-PCR)

The rpl PCR is based on the sequence of the β-operon (*rpl*KAJL-*rpo*BC) of ribosomal protein genes, which is slightly different from one liberibacter species to the other. In particular, the intergenic region between genes *rpl*A and *rpl*J is 34 bp longer in the Asian than in the African *liberibacter*. With forward primer f-*rpl*A2, designed from the *rpl*A gene, and reverse primer r-*rpl*J5 from the *rpl*J gene, a 703 bp DNA is amplified from the Asian liberibacter. On agarose gel electrophoresis, 703 bp DNA band is seen, (Hocquellet *et al.*, 1999)**.**

2.7.6.5.3 Multiplex PCR (mPCR)

Multiplex PCR recipe has been introduced for reliable, sensitive and simultaneous detection of Asian and American strains of citrus huanglongbing disease (Bove J. M, 2007, personal communication). In this assay in addition to A2 and J5 primers specific for Asian HLB, primers GB1 (5'-AAG, TCG, AGC, GAG, TAC, CCA, AGT, ACT-3') and GB3 (5'-CCA, ACT, TAA, TGA, TGG, CAA,ATA,TAG-3') have been used. Using this assay citrus samples collected from Kaski, Lamjung and Kavre had

been analyzed for HLB detection at NAST laboratory (Shrestha, S. 2009, Personal communication). Wang *et al*. (2009), had developed a multiplex PCR (mPCR) detection system using a ribosomal protein gene-based primer set and another unique protein gene-based primer specific for *Cadidatus* Liberibacter asiaticus and *Xanthomonas axonopodis pv. Citri* (Xac), respectively.

2.7.6.5.3 Real Time PCR (RTi-PCR)

Recently, real time PCR (RTi-PCR) or quantitative real time PCR (q-PCR) have been applied to the detection and quantification of liberibacters in plants and insect vectors (Li *et al.*, 2006 and 2007; Wang *et al.*, 2006; Teixeira *et al.*, 2008). RTi- PCR has recently been shown capable of detecting the Asian liberibacter and the American liberibacter in symptomless trees of affected orchards (Irey *et al*., 2006; Teixeira *et al.*, 2008).

2.8 Control

There are no absolute curative methods for the control of HLB. The general control strategy has been to eradicate all existing sources of HLB within on area, then replant with HLB-free certified planting materials. It is important to avoid bringing propagation materials from HLB-infected area. Psyllid population must also be reduced as much as possible. With the finding that prokaryotic organisms are associated with HLB, efforts were made to control the disease by injecting trees with antibiotics (Aubert, and Bove, 1980), but only partial success was achieved and their use was therefore abandoned (Buitendag and von Broembsen, 2003).

2.8.1 Biological Control

Biological control of HLB via control of vectors (*Diaphorina citri* and *Trioza erytrae*) had been attempted. The nymphs of both psyllid (*Diaphorina citri* and *Trioza erytrae*) species are parasitized by hymenopterous ectoparasites *Tamarixia dryi* Waterston and *T. radiatus* Waterston. Therefore these have been used as biological control agents of vector populations in Reunion Island (Catling, 1969; Etienne and Aubert, 1980; Aubert and Quilici, 1984). Chiu *et al.*, (1979) attempted biocontrol of HLB disease by introduction of parasites but very limited success was reported. Similarly, psyllid parasite biocontrol attempted in Taiwan, was also not very effective. This is believed to be due to the presence of indigenous populations of hyperparasites that attack the hymenopterous ectoparasite biocontrol agents. Another internal parasitoid, *Diaphorencyrtus aligarhensis*, has also been found to attack *D. citri* (Aubert and Quilici, 1984). In the Mekong delta region of Vietnam, farmers have found that the presence of guava trees close to citrus trees prevents or at least retards huanglongbing.

It has recently been shown that the volatile compounds produced by guava trees repel *D. citri* (Noronha *et al.*, 2008).

2.8.2 Chemical Control

Immediately after the discovery, that HLB was associated with a bacterium in 1970, and not a virus, tetracycline injections into the trunks of HLB-affected citrus trees were tried in South Africa, and found to reduce significantly the incidence of symptomatic fruit (Schwarz and Van Vuuren, 1971; Schwarz *et al.*, 1974 and Moll *et al.*, 1980).The treatment of tetracycline was not precisely environment friendly. Tetracycline injections were also used in Taiwan (Su and Chang, 1976; Chiu *et al.*, 1979) and Indonesia (supriyanto and Whittle, 1991). Experimentally, penicillin was shown to give reduction of HLB symptoms, and this result supported the bacterial nature of the HLB agent (Aubert and Bove, 1980; Bove *et al.*, 1980).

3. MATERIALS AND METHODS

3.1 Study Sites

Altogether fifteen (Far-western region: Baitadi, Dadeldhura Doti and Kailali; Mid-western region: Dailekh, Rukum and Salyan; Western region: Kaski, Lamjung and Syangja; Central region: Dhading, Kathmandu and Sindhupalchowk; Eastern region: Dhankuta and Okhaldhunga) different districts were selected as study sites for HLB diagnosis. Among them in three (Kaski, Syangja and Kathmandu) districts, field visit was carried out and in rest of twelve districts, samples were received for HLB detection. Besides, information on geographical locations (altitude) of various sites were either collected during the field visit itself or collected later based on secondary information.

3.2 Field Survey

For the present investigation, field survey to certain citrus nurseries and orchards were carried out during 2007/08. Major citrus pocket areas of three districts i.e. Kaski, Syangja, and Kathmandu were visited in the supervision of NAST experts. In Kaski and Syangja the survey was carried out during 10th July to 17th July 2008. Similarly in Kathmandu the field survey was conducted on 12th August 2008. Site selection was based on the problematic pocket areas of citrus growing districts. Field survey was conducted only in the orchards that have more than 50 trees, (with few exception of the visit to homestead. gardens of Taudaha area of Kathmandu). Altogether sixteen citrus orchards from different VDC of Syangja, Kaski and Kathmandu were surveyed.

All the species of citrus grown in the selected orchards were considered for study. Sweet orange, mandarin, pumello, junar and grapefruit 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 for HLB PCR diagnosis were collected only from the suspected trees with HLB symptoms. The orchards were inspected for visual symptoms of different diseases such as Phytophthora, Canker, Sooty mould, Citrus Tristeza Virus (CTV), Exocortis, Powery mildew, Leaf miner, Felt disease, Huanglongbign (HLB) and other citrus diseases.

Semi structured questionnaire (Annex II) was also developed and surveyed with citrus growers to collect the necessary information regarding disease incidence, severity and the management practices usually carried out by farmers in order to protect their citrus orchard from various diseases. The questionnaires were filled via interviews and discussion with respondents. At least 5-10 questionnaires were filled by citrus growing farmers of each surveyed districts except Kathmandu district.

3.3 Sample collection

For PCR diagnosis, samples were either collected during field visits or were received from various sources at NAST.

3.3.1 Field collection

Samples for HLB detection were collected randomly from the trees with suspected symptoms HLB. Twigs of last flux (six months to one year old) were collected as samples from different height and directions of the tree. Suspected samples were placed in zip lock polythene bag with proper labeling and kept in icebox with ice to keep them fresh during transportation. These were brought to the NAST Biotechnology laboratory for subsequent analysis. Record of various samples collected during field visits is given (Annex IV).

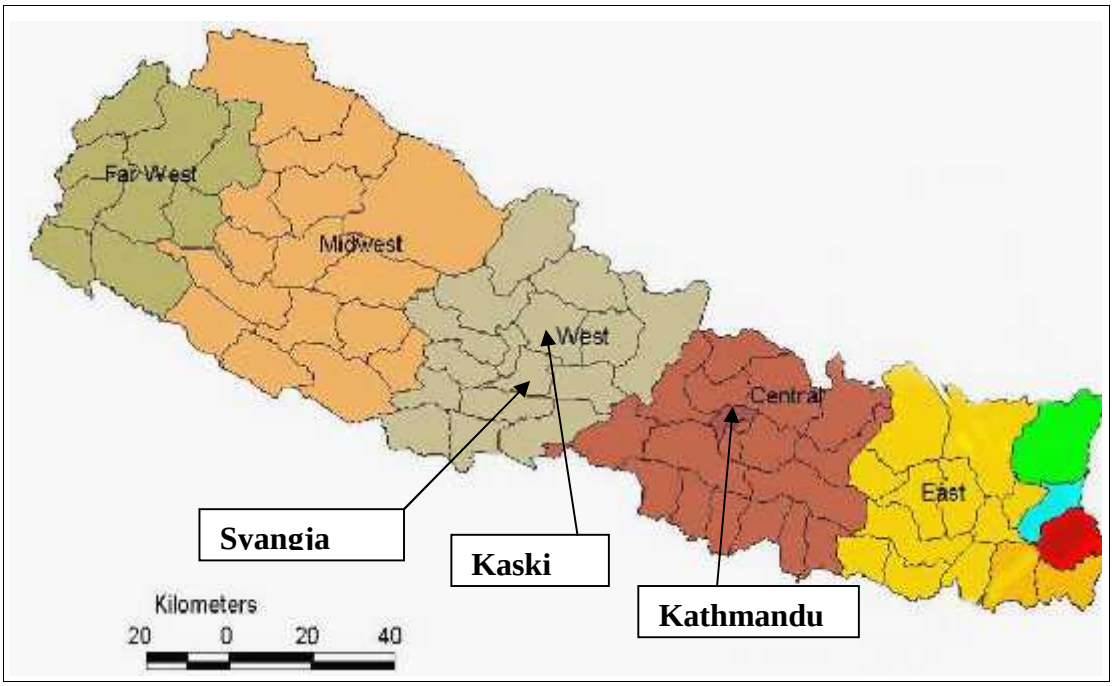

Fig 2. Map showing the survey sites.

3.3.2 Received Samples

Suspected leaf samples were received from citrus development division of MOAC (Ministry of Agriculture and co-operatives) and NARC from 12 different citrus growing districts, *viz.* Dailekh, Rukum, Salyan (Mid-western region), Dhading, Sindhupalchawk (Central region), Dhankuta, Okhaldhunga (Eastern region) , Kailali, , Baitadi, Doti, Dadeldhura (Far-western region), and Lamjung (Western region)

(Annex-III). PCR analysis of the samples was carried out at NAST, Biotechnology laboratory, Khumaltar, Lalitpur, Nepal.

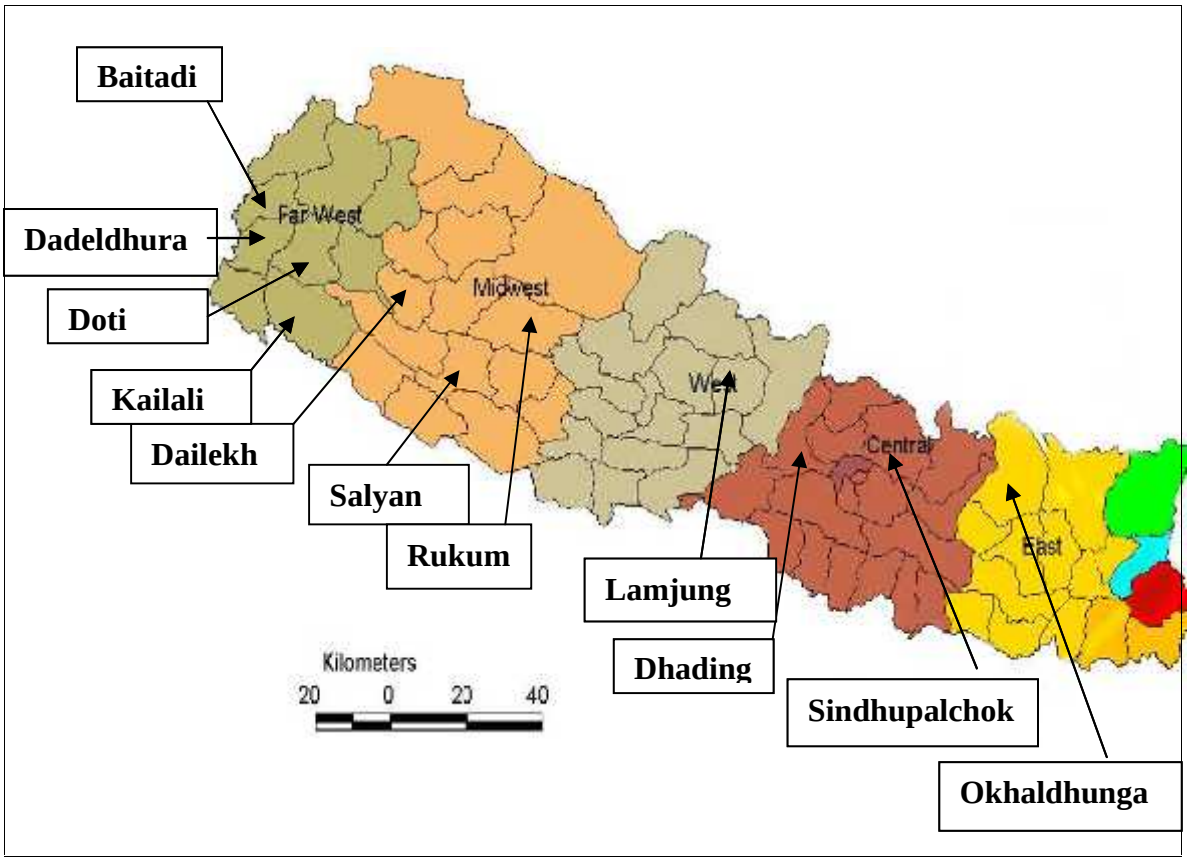

Fig 3. Map showing the districts from where samples were received for PCR detection.

3.4 DNA Extraction

DNA extraction was carried out from the suspected leaf midribs using Wizard DNA extraction technique of Jagoueix *et al*., (1996). Approximately 0.1-0.3g of leaf midrib tissue from suspected samples were chopped to a fine mince with a sterilized razor blades in disposable plastic petri plates containing 1ml of DNA extraction buffer (Tris 0.01M, pH 0.8; EDTA 0.4M, pH 0.8; SDS 1% and proteinase K 0.25 mg/µl). The homogenates were transferred into eppendorf tubes (1.5 ml) and incubated for 2 hours at 65˚C in water bath (Jalaba, TW12). Following incubation, samples were centrifuged for 15 min at 12,000 rpm at room temperature and the supernatants were transferred in fresh eppendorf tubes. Thereafter 1ml of Wizard miniprep DNA purification resin (Promega Company, USA) was added to each sample and mixed by gentle inversion. The mix was then transferred into a syringe fixed on wizard minicolumn (Promega, Madison WI, USA) and electrical vaccum pump apparatus (Promega, Madison WI, USA) and allowed to filter. After sometime when all of the suspension gets filtered, 2ml of 80% isopropanol (Qualigens Fine Chemcials, India)

was added to each column (1ml at a time). The columns were briefly centrifuged for 20 sec to remove excess isopropanol. The spin columns were then transferred into fresh eppendorf tubes and 50µl of sterile double distilled water heated at 80˚C in water bath were added to each tube. After 1 minute, the columns were centrifuged for 25 sec at 12,000 rpm. This step was repeated, yielding 100µl of DNA extracts (wizard extract) and these extracts were properly labeled and stored at 4˚C. Two microliter of this wizard extract was used in PCR.

3.5 PCR Amplification

Two different PCR assays were used for the detection of HLB organism (Jagouix *et al.,* 1996; Hocquellet *et al.,* 1999). First PCR assay (16S-PCR) was based on the amplification of 1160bp long fragment of 16S rDNA of HLB organism using primer OII, OAI and OI2C (Jagouix *et al.,* 1996). The second PCR was based on the amplification of 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). PCR reaction was performed in 50 µl reaction volume containing 1µM of each of the primers OII (5'-GCGCGTATGCAATACGAGCGGCA-3'),OAI (5'-GCGCGTATTTTATACGAGCGG-CA-3'), and OI2C (5'-ACAAAAGCAGAAATAGCACGAACAA-3') in case of 16S-PCR and primers A2 (5'-TATAAAGGTTGACCTTTCGACTTT-3') and J5 (5'-ACAAAAGC-AGAAATAGCACGAACAA-3') in case of rpl-PCR, 200 µM each of four dNTPs, 2 mM $MgCl_2$, 5µL of buffer (10x) with KCl (100 mM Tris-HCl, 500 mM KCl, 0.8% Nonidet P40) and 2.5 U Taq polymerase (Fermentas Life Science, MBI, America). Two microlitres of 'wizard DNA extract' of various samples were used as template DNA in the PCR. The amplification was carried out in thermal cycler (Eppendorf, Germany) with following programs for two different PCR. Cycling condition for rpl-PCR was 35cycles each at 92˚C for 20 sec (denaturation step), 62˚C for 20 sec (Annealing of primers) and 72˚C for 45 sec (strand elongation), whereas for 16S-PCR, it was 35 cycles at 92˚C for 45 sec (denaturation) and 72˚C for 90 sec (Annealing and strand elongation).

3.6 Gel Electrophoresis

The amplification product of 16S-PCR and rpl-PCR were analyzed using 1% agarose gel in TAE (1x) buffer at 100V for half an hour using EMBI TEC (Santiago, CA), gel tank. Following electrophoresis, the gels were stained in gel tray containing TAE/TBE buffer (ca. 200ml) and 35 µl of Ethidium bromide (10mg/ml) for 45 minutes and de-stained for 15 minutes in water. The gels were then visualized on an UV transilluminator (UVITEC, Japan) and photographed using a Polaroid camera system (Geleam, UK).

4. RESULTS

4.1 Survey Results

Almost all farmers (except Kathmandu) under survey had more than 50 trees in their orchards. Existing orchards are predominantly seedling origin. It was reported that most of the farmers prefer seedlings than grafted plant, due to cheaper in price and easy availability. Farmers were still used locally collected planting materials (seedlings) below 1m of height for the establishment of new orchard. During survey it was observed that most of the citrus plants are planted at the edge of terraces and the trees were suffering from mineral deficiency. In all the surveyed sites HLB symptom was mostly observed on mandarin than that of others citrus species.

4.1.1 Syangja District

Most of the surveyed orchards of Syangja district were of seedling origin. In addition to HLB disease, number of other diseases were also observed (Table 6). During survey root/foot rot, canker, Zn-deficiency, scale, sooty mould and HLB was found to be the common disease of cultivated citrus species in Syangja districts. Among all the cultivated species of citrus, in Syangja district mandarin (*Citrus reticulata*) was found to be the most susceptible to various diseases.

Table 6. Different diseases observed in Syangja district during field survey

S.N	**Disease/insect observed**	**Citrus species**			
		C. reticulata	*C. sinensis*	*C. limon*	Others
1	Foot/root rot	+	-	-	-
2	Powdery mildew	+	-	-	-
3	Canker	+	+	-	-
4	Scale	+	-	-	-
5	Leaf miner	+	-	-	-
6	HLB	+	+	-	-
7	Zn deficiency	+	-	-	-
8	Sooty mould	+	-	-	-
10	Other diseases	+	+	-	-

Of the ten HLB suspected leaf samples collected from surveyed orchards, eight were of mandarin (*Citrus reticulata*) and two were of sweet orange (*C. sinensis*). All of the

sampled trees were seedling established. Of ten samples subjected to PCR HLB was found positive in only two samples (*Citrus reticulata,* seedling) (Table 7).

Table 7. PCR result of Syangja district

S.N.	Citrus species	Total sample analyzed	Origin		(+) PCR reaction	
			Seedling	Grafted	Seedling	Grafted
1	*Citrus reticulata*	8	8	0	2	0
2	*C. sinensis*	2	2	0	0	0

4.1.2 Kaski District

During survey of citrus orchards of Kaski district (Hansapur and Horitculture Research Station, Malepatan) very few trees in the orchards were found with HLB symptoms. Complex of diseases (Table 8) were also reported in this district which includes HLB, canker, phytophthora, sooty mould, leaf miner, felt and many other citrus diseases (Table 8). In Horitculture Research Station, Malepatan, it was noticed that most of the orchards were destroyed by HLB and other diseases. HLB symptoms were also observed in farmers orchards at Hansapur VDC.

Table 8 Different diseases observed in Kaski district during field survey

S.N	Disease/insect observed	Citrus species			
		C. reticulata	*C. sinensis*	*C. limon*	Others
1	Foot/root rot	+	-	-	-
2	Powdery mildew	-	-	-	-
3	Canker	+	+	-	-
4	Scale	+	-	-	-
5	Leaf miner	+	-	-	+
6	HLB	+	+	-	-
7	Zn deficiency	+	+	-	-
8	Sooty mould	+	-	-	-
10	Other diseases	+	-	-	-

All the orchards of Hansapur VDC were seedling originated whereas that of Horticulture research Station, Malepatan were graft originated. Ten samples (nine *Citrus reticulata* and one *C. sinensis*) were collected from Horticulture Research Station, Malepatan, and Hansapur VDC of Kaski and were confirmed by PCR tests in the NAST Biotechnology laboratory. Out of ten samples collected eight were from

grafted and two were from seedling originated trees, among them only one sample (*C. reticulate, grafted*) showed HLB positive, PCR reaction (Table 9).

Table 9. PCR result of Kaski district

S.N.	Citrus species	Total sample analyzed	Origin		(+) PCR reaction	
			Seedling	Grafted	Seedling	Grafted
1	*Citrus reticulata*	9	2	7	-	1
2	*C. sinensis*	1	0	1	-	-

4.1.3 Kathmandu District

Field survey to Kathmandu district (Kirtipur, Horticulture Research Station and Tau daha area) was conducted during September 2008. During survey it was observed that in Tau daha area, all citrus trees were seedling originated, however, the orchard of Horticulture Research Station, Kirtipur was both seedling and graft originated as well as mother plant originated (*Citrus reticulate*, *C. sinensis*, *Fortunella japonica*). Including HlB disease various other citrus diseases were reported from orchard of Kathmandu valley. *Citrus reticulata* and *C. sinensis* trees were found to be more susceptible to various citrus diseases than other cultivars (Table 10).

Table 10. Different diseases observed in Kathmandu district during field survey

S.N	Disease/insect observed	Citrus species			
		C. reticulata	*C. sinensis*	*C. limon*	Others
1	Foot/root rot	-	-	-	-
2	Powdery mildew	-	-	-	-
3	Canker	+	+	-	-
4	Scale	+	-	-	-
5	Leaf miner	-	-	-	+
6	HLB	+	+	-	-
7	Zn deficiency	+	+	-	-
8	Sooty mould	+	-	-	-
10	Other diseases	+	+	-	-

Eighteen samples were collected, out of eighteen samples (representing various species) subjected to PCR six were detected positive for HLB (33.3%). Of the six infected samples two were from seedling grown trees while four were from grafted trees (Table 11).

Table 11. PCR result of Kathmandu district

S.N	Citrus species	Total sample analyzed	Origin			(+) PCR reaction	
			Seedling	Grafted	Mother plant	Seedling	Grafted
1	*Citrus reticulata*	7	2	4	1	1	0
2	*C. sinensis*	8	2	5	1	1	3
3	*C. grandis*	1	1	0	0	0	0
4	*C. paradise*	1	0	1	0	0	1
5	*Fortunella japonica*	1	0	0	1	0	0

4.2 PCR Results of Requested Samples

Altogether 145 samples were sent to NAST biotechnology laboratory, for PCR diagnosis of HLB. Details of the samples received from various districts of Central, Western, Mid-western, Far-western and Eastern development regions are shown (Annex IV). Of the 145 samples, received from central (Dhading and Sindhupalchowk), Western (Lamjung), Mid-western (Dailekh, Salyan and Rukum), Far-Western (Baitadi, Dadeldhura, Doti and Kailali) and Eastern (Okhaldhunga and Dhankuta) development region under study, nine samples were detected positive by PCR. Among the 12 districts, HLB was found prevalent in 5 districts indicating the rapid spread of HLB in these districts. HLB was found to be prevalent in Salyan (20.0%) and Baitadi (20.0%) followed by Lamjung (10.0%), Doti (5.9%) and Dhading (5.6%) (Table 12). Whereas, HLB was not detected in samples from Far-western region (Kailali), Central region (Sindhupalchowk), Eastern region (Okhaldhunga and Dhankuta), Western region (Rukum) and Mid-western region (Dailekh) (Fig 6).

Table 12. PCR results of 15 districts under study.

S.N.	Name of district/approx. Altitudinal range (masl)	No. 0f samples	+ PCR reaction	% infestation
1	Dailekh/ 1200-1400	10	0	0.0
2	Kaski/ 900-1450	10	1	10.0
3	Doti/ 1300-1500	17	1	5.9
4	Baitadi/ 1400-1500	10	2	20.0
5	Salyan/ 1200-1400	20	4	20.0
6	Dhankuta/ 1300-1400	10	0	0.0
7	Dhading/ 1300-1500	18	1	5.6
8	Syangja/ 1200-1300	10	2	20.0
9	Sindhupalchawk/ 1400-1500	10	0	0.0
10	Okhaldhunga/ 900-1400	10	0	0.0
11	Dadeldhura/ 1500-1600	10	0	0.0
12	Lamjung/ 1100-1200	10	1	10.0
13	Rukum/ 1300-1400	10	0	0.0
14	Kathmandu/ 1300-1350	18	6	33.3
15	Kailali/ 700-1100	10	0	0.0
	Total	**183**	**18**	

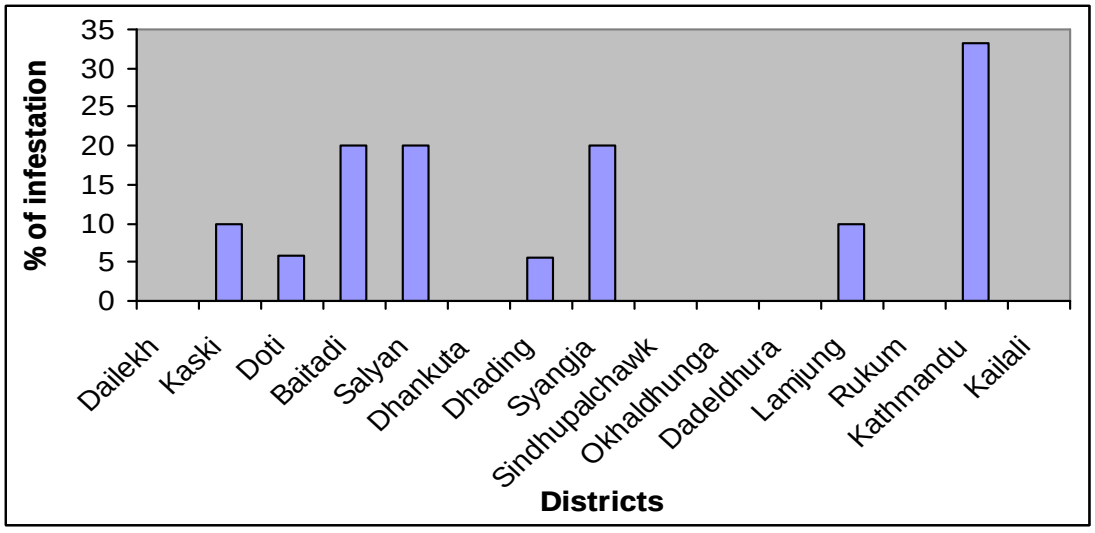

Fig 4 Percentage of HLB infestation in 15 different districts under study.

Table 13. PCR Results of HLB in samples received from 12 different districts.

DAILEKH DISTRICT

S.N.	Citrus species	Total sample	Origin		PCR reaction	
			Seedling	Grafted	Seedling	Grafted
1	*Citrus reticulata*	7	6	1	0	0
2	*C. sinensis*	3	3	0	0	0

Doti district

S.N.	Citrus species	Total sample	Origin		PCR reaction	
			Seedling	Grafted	Seedling	Grafted
1	*Citrus reticulata*	10	8	2	0	1
2	*C. sinensis*	7	7	0	0	0

Baitadi district

S.N.	Citrus species	Total sample	Origin		PCR reaction	
			Seedling	Grafted	Seedling	Grafted
1	*Citrus reticulata*	7	6	1	1	1
2	*C. sinensis*	3	3	0	0	0

Salyan district

S.N.	Citrus species	Total sample	Origin		PCR reaction	
			Seedling	Grafted	Seedling	Grafted
1	*Citrus reticulata*	17	16	1	4	0
2	*C. sinensis*	3	3	0	0	0

Dhankuta district

S.N.	Citrus species	Total sample	Origin		PCR reaction	
			Seedling	Grafted	Seedling	Grafted
1	*Citrus reticulata*	9	7	2	0	0
2	*C. sinensis*	1	1	0	0	0

Dhading district

S.N.	Citrus species	Total sample	Origin		PCR reaction	
			Seedling	Grafted	Seedling	Grafted
1	*Citrus reticulata*	16	14	2	1	0
2	*C. sinensis*	2	2	0	0	0

Sindhupalchowk district

S.N.	Citrus species	Total sample	Origin		PCR reaction	
			Seedling	Grafted	Seedling	Grafted
1	*Citrus reticulata*	9	4	5	0	0
2	*C. sinensis*	1	1	0	0	0

Okhaldhunga district

S.N.	Citrus species	Total sample	Origin		PCR reaction	
			Seedling	Grafted	Seedling	Grafted
1	*Citrus reticulata*	7	7	0	0	0
2	*C. sinensis*	3	3	0	0	0

Dadeldhura district

S.N.	Citrus species	Total sample	Origin		PCR reaction	
			Seedling	Grafted	Seedling	Grafted
1	*Citrus reticulata*	9	7	2	0	0
2	*C. sinensis*	1	1	0	0	0

Lamjung district

S.N.	Citrus species	Total sample	Origin		PCR reaction	
			Seedling	Grafted	Seedling	Grafted
1	*Citrus reticulata*	9	7	2	1	0
2	*C. sinensis*	1	0	1	0	0

Rukum district

S.N.	Citrus species	Total sample	Origin		PCR reaction	
			Seedling	Grafted	Seedling	Grafted

1	*Citrus reticulata*	8	8	0	0	0
2	*C. sinensis*	2	2	0	0	0
Kailali district						
S.N.	**Citrus species**	**Total sample**	**Origin**		**PCR reaction**	
			Seedling	**Grafted**	**Seedling**	**Grafted**
1	*Citrus reticulata*	9	9	0	0	0
2	*C. sinensis*	1	1	0	0	0

5. DISCUSSION

Various citrus fruits are delicious food items of Nepalese diet and also various citrus cultivars (mankamana suntala and Sindhuli junar) produces marvelous fruits with an excellent blend of sourness, sweetness and typical flavors. Although many midhill districts of Nepal are potential citrus producers (eg. Sutala, junar, kagati), Nepal is not yet self sufficient in citrus production. This reflects the lack of sufficient effort from all concerned authorities towards establishment of healthy citrus industry in Nepal. If taken care of all the diseases most importantly HLB and management problems, Nepal can be one of the exporter countries of citrus.

In Nepal most of the citrus saplings are supplied by private nurseries in the country which produces seedlings as well as grafted nursery trees. The abundance of viral, bacterial and fungal diseases of citrus such as Huanglongbing (HLB), tristeza, xyloporosis, phytophthora, etc. and the lack of a proper mechanism for compulsory indexing of mother plants are accelerating the spread of many citrus diseases in new areas. Among them HLB is found to be most destructive and is spreading rapidly in most of the part of the country resulting in the destruction of many citrus orchards. Recently in Lamjung it has caused about 25% loss in citrus production (Nepalsamacharpatra, 01/01/2009).

Incedence of Greening disease in Nepal can be traced back since mid 1960s (Thrower, 1968). Knorr *et al.,* (1971) observed greening disease in many areas of Nepal during 1969. They reported that most trees found growing on rootstocks were imported from Saharanpur, Uttar Pradesh, India, where they were detected positive for HLB by the Schwarz chromatographic tests (Gupta *et al*., 1972 cited in Roistacher 1996). However, with the unavailability of appropriate and reliable diagnostic technique for HLB in Nepal, this disease remained a mysterious disease till 1994, when this was diagnosed for the first time in French laboratory using DNA-DNA hybridization technique (Regmi, 1994; Regmi *et al*., 1996). Later, both 16S-PCR and rpl-PCR were used to characterize Nepalese strain of HLB (Jagoueix *et al.*, 1996; Hocquelle*t et al*., 1999).

So far, including this research work, the incidence of HLB disease has been reported in 33 districts of Nepal (Regmi, 1994; Regmi *et al.,* 1996, Shrestha *et al.,* 2003 and Regmi *et al.,* 2004). Of the 33 districts, in 10 districts the disease was diagnosed and confirmed on the visual basis only, and in rest of the 23 districts the prevalence of HLB was confirmed by applying DNA-DNA hybridization technique and PCR-based diagnostic technique (Regmi *et al.,* 1996; Shrestha *et al.,* 2003 and Regmi *et al.,* 2004). In the present study, HLB was found to be prevalent in 8 (Kaski, Doti, Baitadi, Salyan, Dhading, Syangja, Lamjung and Kathmandu) out of 15 districts. In some of

these districts (Kaski, Doti, Baitadi, Salyan, Kathmandu and Syangja) HLB has been already reported (Regmi, 1994; Regmi *et al.*, 1996a, Regmi *et al.*, 1996b, Shrestha *et al.*, 2003a and Shrestha *et al.*, 2004). According to present investigation out of 15 districts HLB was found to be most prevalent in Kathmandu (33.3%) district followed by Syangja, Salyan and Doti (20% each) (Table 12; Fig 6). HLB was detected both from seedling grown trees as well from grafts and was detected positive in suntala (*Citrus reticulata*) and Junar (*C. sinensis*) mother plant, grafted grapefruit, and grafted mandarin received at NAST lab and collected during field survey from various districts *viz*. Syangja, Kaski and Kathmandu (Table 7, 9, 11 and 13).

Huanglognbing (HLB) disease affects all the cultivars of citrus (Singh, 1977). Various citrus species subjected for HLB infestation included those of maindarin, sweet orange (junar), pummelo, grapefruit and muntala (kumquat) species of citrus. Altogether one hundred forty one-mandarin (77.04%), thirtynine-sweet orange (21.31%), one each pumello (0.55%), grapefruit (0.55%) and muntala species (0.55%) were studied and it was found that mandarine showed the highest percentage of HLB infestation in comparision to other citrus species. Among one hundred eighty three samples of different citrus species studied, thirteen-mandarin, four-sweet orange and one grapefruit show positive HLB infestation.

5.1 Field Survey of Kaski, Syangja and Kathmandu Districts

Field survey to Syangja, Kaski and Kathmandu districts were carried out in order to investigate the status of various citrus orchards with respect to infestation by different citrus diseases including HLB, mode of orchard establishment and management as well as farmers awerness regarding harmful consequences of HLB.

Syangja district is situated at (28°4'60N, 83°52'0E) an altitude of 1088m, favours citrus cultivation as well as the vector (*Diaphorina citri*) population. From the field survey it was found that most trees were of seedling origin and some were found infested with HLB (Table 7). This may be attributed to the presence of vector which could have transferred the disease from adjoining areas. While talking with farmers, it was found that most of the farmers were unaware of HLB and other diseases of citrus, they even didn’t know the importance of grafted citrus plants and the place from where they could get disease-free grafted citrus plants. Under visual observation all referred HLB symptoms *viz.* leaf mottling, Zn-deficiency symptoms, were observed and found to be confusing, when samples collected on these symptomatological basis were subjected to PCR, the result was quite surprising because out of ten samples collected on the visual basis of HLB symptoms from Syangja district only two were found to be HLB positive. Therefore, the visual diagnosis of HLB alone can not be considered. Further more, in this district, *Citrus reticulata* trees were found to be the

most susceptible to various other diseases besides HLB (Table 6). In orchads of Syangja the symptoms of HLB (on leaves and branches) were more clear than in Kaski and Kathmandu district citrus orchards, it may be due to the agro ecological condition of this region is suitable for the development of HLB disease. Regmi *et al.*, (1996) had also reported many other citrus diseases from citrus orchards of this district.

The prevalence of HLB disease in Syangja district had also been reported previously (Regmi *et al.,* 1996), however, Rangkhola VDC of Syangja district was previously found to be free from HLB (Regmi *et al.,* 1996) but the current study revealed the presence of the HLB also in this VDC. This may be attributed to presence of vector in this area or might be use of diseased planting materials by farmers.

Citrus is one of the important cash crop of Kaski district. It is situated at (84.1°E, 28.1°N) an altitude ranging of 668 to 1206 m. However, HLB infestation has ruined citrus industry of Kaski district. HLB was first reported during 1960s in this district and since then many disease management strategies had been employed to eradicate this disease from this region but every effort has been unsuccessful. Regmi, (1997), also reported that citrus disease was increased by 12% every year in Pokhara valley. From the present study, it has been clear that till now, the disease is this district is spreading at a very fast rate.

Kathmandu is situated at an altitude at (85.22°E, 27.43°N) approximately 1400 m. Field survey to Kathmandu disitrict (Kirtipur and Taudaha) has also revealed the presence of HLB along with other diseases (Table 10). Regmi *et al.*, (1996) and Regmi (1997) had also reported many other citrus diseases from citrus orchards of these districts.

From the study it was found that the incidence of HLB is very high in the Kirtipur, Horticulture Research Station, Kathmandu. The infestation of the trees in orchard of Kirtipur Horticulture Research Station may be attributed to either plantation of diseased planting material or transmission by vector. Out of 18 samples tested by PCR six, (33.3%) samples were detected positive for HLB. It might be rapidly spreading throughout the orchard because the climatic condition and altitudinal range of this region is quite suitable for the disease development and as well as for the vector population (*Diaphorina citri*). Beside, Rutaceous (*Murraya paniculata*) and non-Rutaceous host plants are also widely distributed in this region. Lama *et al* (1988) also reported the presence of the vector in Kathmandu valley at 1350 elevations, which showed the adaptation of the insect in cooler areas and higher altitudes and if the spread of disease increase in such a rapid rate, then the day is not far when all the citrus orchards of this region will vanish. Although prior studies (Regmi *et al*., 1996;

Shrestha *et al.*, 2003 and Regmi *et al.*, 2004) had also reported HLB from Kathmandu district, repeated observation of HLB from present investigation has proved that till now no control strategies have been undertaken by concerned authorities. In this district, grafted trees were found to be highly infected with HLB disease in comparison to seedling grown plants, indicating that the grafted plants used to establish the orchard were produced from unhealthy mother plants. Another possibility is that, the area might have a good psyllid population (*D. citri*) as the climatic condition is quite suitable for its growth.

5.2 Status of HLB in Twelve Districts of Nepal

Beside field survey, the samples were also received from 12 different (Dhading, Sindupalchowk: Central development region; Dailekh, Rukum, Salyan: Mid-western development region; Baitadi, Dadeldhura, Doti, Kailali: Far-western development region; Lamjung: Western development region; Okhaldhunga, Dhankuta: Eastern development region) district for the PCR diagnosis. Among the samples received from 12 different districts HLB has been reported in five district *viz.* Dhading, Doti, Baitadi, Salyan and Lamjung. However, this investigation reported the presence of HLB disease from high altitude districts (Doti, Baitadi and Dhading districts-whose altitude range from 1300-1500m asl), indicating that due to climatic change, and change in vegetation pattern, HLB vector might have adapted at higher altitude and cooler temperatures. Whereas, Dailekh, Dhankuta, Rukum, Kailali, Sindhupalchowk, Okhaldhunga and Dadeldhura were found to be free from citrus HLB disease, although, the altitude of these districts also ranged from 1200-1400m asl which is quite suitable for disease as well as vector. There may be multiple reasons for this situation viz. 1) the area may still be virgin with regards to disease invasion; 2) absence of vector; 3) citrus farmers may be aware of the HLB disease; 4) absence of alternate host plants. Sindhupalchowk, Okhaldhunga and Dadeldhura districts were also found to be free from HLB. This might be due to the altitudinal range of these districts being very high and the climatic condition not being suitable for the development of HLB disease and also for insect vector.

Prior to this investigation HLB was found to be prevalent in Dailekh, Doti, Baitadi, Dhankuta, Kailai, Dhading and Salyan. However, according to the present investigation HLB had been reported from Dailekh, Dhankuta and Kailai districts. Although, the climatic condition and altitudinal range (700m-1400m asl) of these districts are most suitable for the development of HLB disease and vector. This might be due to the absence of vector, or the farmers of these areas were using disease-free grafted plant materials which lower down the chances of causing disease. Previously, Lamjung district was found to be free from HLB infestation, however, the present study indicates the presence of HLB in this district. There are may reasons behind this

result, 1) the climatic condition favours the development of disease as well as vector which transmit the disease; 2) there must be importing of diseased plant materials in this area; 3) the farmers of this area are still using seedling plant instead of grafted.

5.3 Management of Citrus HLB Disease

Citrus is one of the prioritized fruit crops in midhills of Nepal as envisaged in Agricultural Perspective Plan (1995). However, every effort to boost up citrus production in Nepal has been unsuccessful. The main reason behind this failure is infestation of our citrus industry by citrus HLB disease. Until and unless this disease is properly understood at grass root level by citrus nurserymen and farmers, this will continue to spread in newer areas. Therefore, some key strategies need to be followed for the successful management of this disease, which include: 1) production of disease free planting materials, Shoot Tip Grafting (STG) *in vitro* technique developed by Navarro (1981) can be modified and adapted (Regmi and Shrestha, 1992); 2) certification of planting materials (STG produced plants and citrus mother plants from nurseries) and inspection of existing citrus orchards for the presence of HLB using PCR-based diagnosis (Shrestha *et al.,* 2003); 3) vector control- chemical and biological control techniques (Schwarz *et al.,* 1974; Aubert and Bove, 1980; Aubert and Quilici, 1984) can be adapted and practiced; 4) strengthening inter country and intra-country quarantine systems; 5) Awareness raising among citrus growers and others.

Therefore it is necessary to implement these key strategies to control citrus HLB disease before they completely ruin the citrus orchards. Since this disease is mainly spread by grafting and by insect vector, Shoot Tip Grafting (STG) has been found to be very useful to eliminate the HLB (CGD) (Navarro, 1981). HLB could also be controlled by controlling the vector population, in this context biological control of vector has been successfully implemented in some countries, such as Mauritius, Reunion Island and Taiwan by using their natural enemies (antiparisites) (Aubert, 1985; Etienne *et al.,* 1980; Jooyame *et al.,* 1986 and Shiu-chan, 1988). *Tamarexia radiatus* act as an antiparasite for *Diaphorina citri* (Regmi and Lama, 1988), these antiparasites could be used to check the vector population.

6. CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Citrus is one of major fruit crop in terms of nutritional and economic values. Its cultivation must be promoted to increase yield to fulfill the National and International demand. Many citrus diseases are prevalent in Nepal among them HLB is perhaps the most destructive one and it is number one threat to citrus production. It is spreading very rapidly in almost all citrus orchards, if no any control strategies are carried in time, it will definitely destroy the citrus orchard and its industry of Nepal. From the present investigation it is clear that HLB has already been spread in different parts of the country. Therefore, a pilot study has to be undertaken at the National level to check the further spread of disease. "If nothing is done, citrus will soon disappear from Nepal, in the same way that citrus has been destroyed in India, Indonesia and the Phillippines" (Bove, 1994 and 2002). Although HLB can also be detected using DNA hybridization - based technique, PCR-based technique is the most reliable and robust technique and it is been in place in research laboratories in Nepal. It was found that seedling grown, saplings are more susceptible to HLB than grafted one. In order to maintain a healthy citrus industry in Nepal, integrated disease management strategies have to be followed and the disease should be thoroughly understood and nurserymen and farmers needs to be sensitized and awared about disease and its management practices.

6.2 Recommendation

Based of the results the following recommendation can be drawn**:**

- Since HLB is very destructive disease of citrus it is number one threat to Nepalese citrus industry therefore special programs should be implemented by the governmental and non-governmental sectors for the development of the citrus industry of Nepal.
- This disease has no effective biological and chemical control measures, removal of infected trees has to be carried out after HLB detection by PCR.
- NGOs working on the promotion of citrus must be controlled by government. They should be permitted to work on these crops only with the consent of National Citrus Development Division or National Citrus Research Programme.
- Research and development activities should focus on healthy citrus plantlet production using biotechnological tools such as STG.
- Citrus mother plants foundation blocks should be established throughout citrus growing districts of Nepal.
- PCR diagnosis of HLB should be made mandatory for the certification of citrus planting materials.

- Health certified high quality citrus saplings such as Mankamana mandarin and Sindhuli junar should be exported abroad as well as its cultivation and production within country should also be promoted.
- Vector control by chemical and Biological means should also be practiced.
- Due to climatic changes the vector might have migrated to high altitudes also, so detail study on the vector distribution need to be carried out for the successful management of HLB in Nepal.
- In order to maintain the healthy citrus orchards in Nepal, Government must practice the sanitary and phyto-sanitary measures during import and export of citrus planting materials.

INTERNET SURVEY

Internet visit 1:	http://anrcatalog.ucdavis.edu
Internet visit 2:	http://lifesciences.asu.edu
Internet visit 3:	www.nlm.nih.gov
Internet visit 4:	http://en.wikipedia.org/wiki/Real-time_Polymerase_chain_Reaction
Internet visit 5:	http://www.dorak.info/genetics/realtime.html
Internet visit 6:	http://en.wikipidia.org
Internet visit 7:	www.agnet.org
Internet visit 8:	http://www.answers.com/topic/immunology

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PHOTOPLATE I

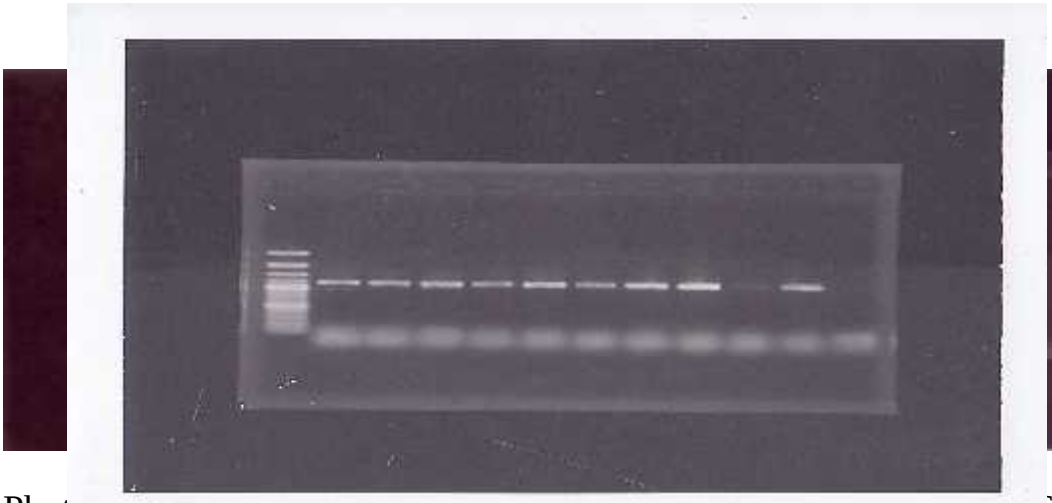

Photo 1: Gel photograph of 16S-PCR assay using OAI, OI1 & OI2C primers. Lanes 4, 5, 6, 7, 8 & 10 are HLB positive samples. Lane marked M is 100bp ladder molecular weight marker.

Photo 2: Gel photograph of 16S-PCR assay using OAI, OI1 & OI2C primers. Lanes 1, 2, 3, 4, 5, 6, 7, 8 & 10 are HLB positive samples. Lane marked M is 100bp ladder molecular weight marker.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

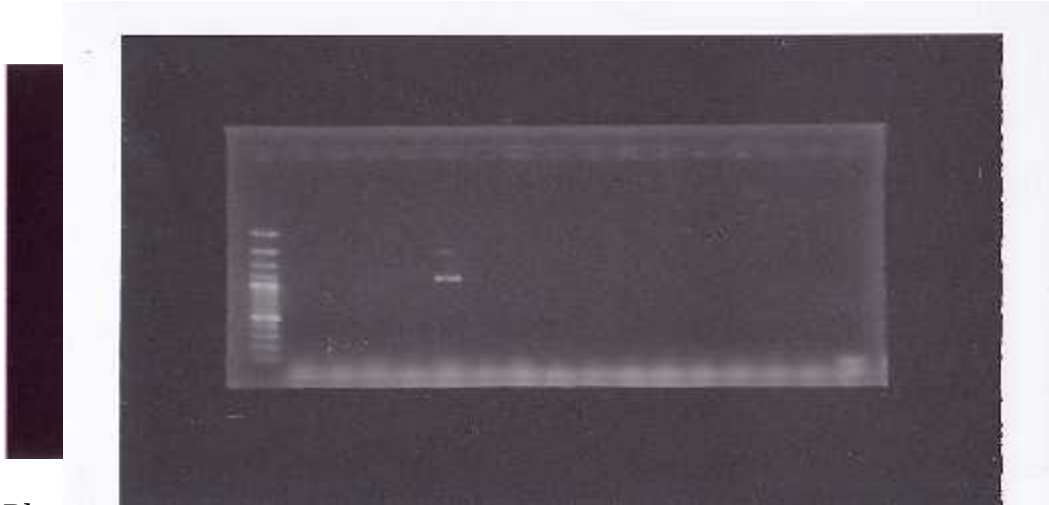

Photo 3: Gel photograph of rpl-PCR assay. Using primers A2 & J5 M is 100bp ladder, molecular weight marker.

Photo 4: Gel photograph of 16S-PCR assay using primers OAI, OI1 & OI2C primers Lanes 1, 7 & 8 are HLB positive HLB samples. Lane marked M is 100bp ladder molecular weight marker.

M 1 2 3 4 5 6 7 8 9 10 11

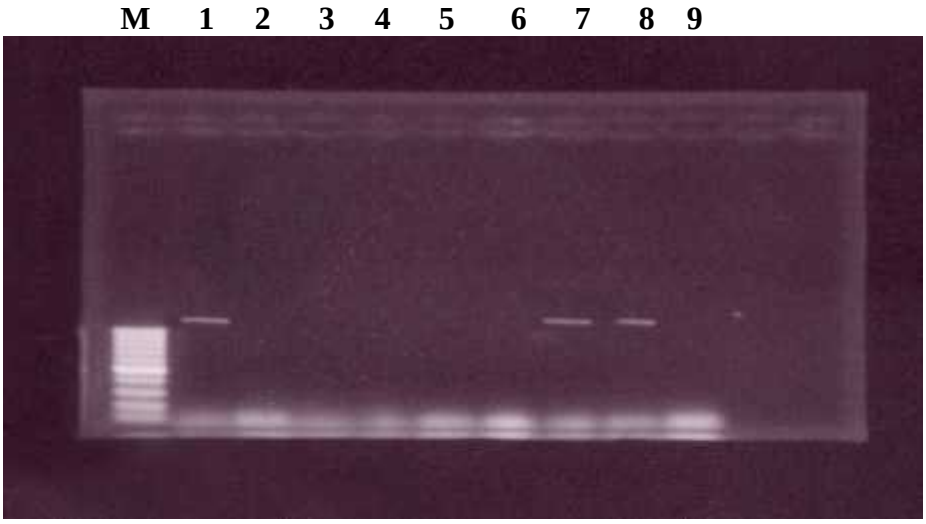

Photo 4: Gel photograph of 16S-PCR assay using primers OAI, OI1 & OI2C primers. Lanes 1, 7 & 8 are HLB positive HLB samples. Lane marked M is 100bp ladder molecular weight marker.

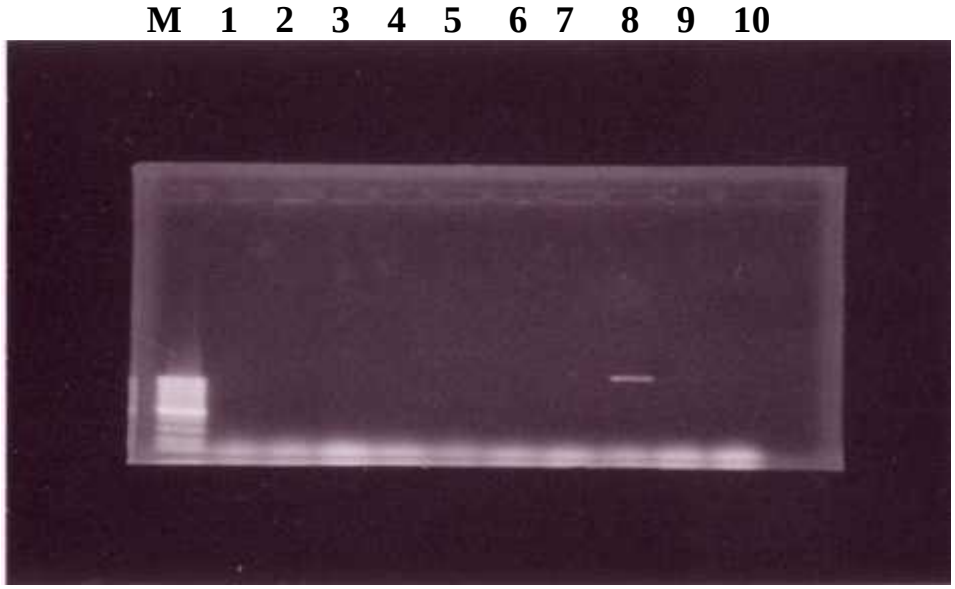

Photo 3: Gel photograph of rpl-PCR assay. Using primers A2 & J5 primers. Lane 9 is HLB positive sample. Lane marked M is 100bp ladder molecular weight marker.

ANNEXES

Annex- I Area, Production and Yield of Fruits in Nepal

Year	Total area (ha)	Increasing productive area (ha)	Productive area(ha)	Production (mt)	Yield (mt/ha)
1997/1998	17023.18	1103	10034	100352	10.00
1998/1999	18007.20	981.02	10592	107250	10.13
1999/2000	19017.95	1020.75	11277	115067	10.20
2000/2001	20672.80	1654.85	11891.6	121665.3	10.23
2001/2002	22423.37	1750.57	12615.5	130927.74	10.38
2002/2003	23662.97	1239.60	13311.86	139109.55	10.45
2003/2004	24798.89	1135.92	13930.86	148010.22	10.62
2004/2005	25909.54	111065	14605.95	156955.90	10.75
2005/2006	27021.74	1112.20	15206	163877.07	10.78
2006/2007	27979.8	158.06	15831.9	171874.5	10.86
2007/2008	30790.3	2810.5	19979.73	227070.62	11.36

Source: Horticulture Development Programme (2007/08), Nepal Agriculture and Cooperative Ministry

Annex- II. QUESTIONNAIRE FOR THE CITRUS GROWING FARMERS

1. Name:
2. Address (VDC/District):
3. Questionnaire date:
4. Affiliated to the farmers group:
5. From where the citrus plants have been brought?

Source	**No. of plants**		**Quality of plants**		**Self byed**	**Dist. Agri. Div.**
	Grafted	**Seedling**	**height**	**affected /non-affected with disease/insects**		**recommendation**
Own nursery						
Private nursery						
Horticulture farm						

6. How were the plants provided?
   i from own nursery
   ii purchase on their own
   iii provided by District Agriculture Division Office
   iv provided by the NGOs
7. Particulars/information on different species of citrus fruits cultivated in the orchards:

Name of citrus species	**No. of citrus plants**				
	under age of five	**beyond age of five**		**total**	**Total productivity**
		good yielding	**non productive affected by diseases/insects**		
Citrus reticulata					
C. sinessis					
C. limon					
others					

8. No. of trees infected with disease/insects in the orchard

Disease / insect	**No. of Diseased/insect affected tree**				**Remarks**
	Citrus reticulata	***C. sinessis***	***C. limon***	**Others**	
Phytophthora					
Powdery mildew					
Canker					
Foot rot					
Sooty mould					
Leaf minner					
Huanglongbing					
Zn deficiency					
others					

9 Pesticides and manures used in recent year for controlling disease/ insect pest

Name of manure/pesticides used	used month	amounts	effectiveness		
			excellent	satisfactory	ineffective

10 On whose suggestion the above mentioned manures and pesticides have been used?

i self known

ii known from training

iii referred from district agriculture office

iv known from District Agricultural Section

11 Are you more interested in increasing citrus farming orchards?

if yes, which species of citrus?

…….

if no, why?

.....

12 what should be done to develop healthy citrus farming? Suggestions

Annex –III. Record of the samples received from different districts of Nepal

Samples received from Salyan District

E.N.	S.N.	Owner/ locality	Species	Origin	Symptoms	Received date
Cr 35	1	Buddhi Raj Bista Khalanga-8 (Tree 1)	*Citrus reticulata*	Seedling	Mosaic like Zinc deficiency	13/12/2007
Cr 36	2	Buddhi Raj Bista Khalanga-8 (Tree.2)	*C. reticulata*	Seedling	Mosaic like Zinc deficiency	13/12/2007
Cr 37	3	Ganga Ram Bista Khalanga-8 (Tree 1)	*C. reticulata*	Seedling	Mosaic like Zinc deficiency	13/12/2007
Cr 38	4	Ganga Ram Bista Khalanga-8 (Tree 2)	*C. reticulata*	Seedling	Mosaic like Zinc deficiency	13/12/2007
Cr 39	5	Kasi Ram Lamichane Khalanga-8 (Tree 1)	*C. reticulata*	Seedling	Zinc deficiency	13/12/2007
Cr 40	6	Kasi Ram Lamichane Khalanga-8 (Tree 2)	*C. reticulata*	Seedling	Zinc deficiency	13/12/2007
Cr 41	7	Fatteh Bahadur Khatri Khalanga-8	*C. reticulata*	Seedling	Zinc deficiency	13/12/2007
Cr 42	8	Indra Bahadur Khatri Khalanga-8 (Tree 1)	*C. reticulata*	Seedling	Mosaic like Zinc deficiency	13/12/2007
Cr 43	9	Indra Bahadur Khatri Khalanga-8 (Tree 2)	*C. reticulata*	Seedling	Mosaic like Zinc deficiency	13/12/2007
Cr 44	10	Dhakavir Sejwal Khalanga-8	*C. reticulata*	Seedling	Mosaic like Zinc deficiency	13/12/2007
Cr 45	11	Amar Bahadur K. C. Khalanga-8 (Tree 1)	*C. reticulata*	Seedling	Zinc deficiency	13/12/2007
Cs 46	12	Amar Bahadur K. C. Khalanga-8 (Tree 2)	*C. reticulata*	Seedling	Zinc deficiency	13/12/2007
Cs.47	13	Nar Bahadur Sejwal Khalanga-8 (Tree 1)	*C. reticulata*	Seedling	Zinc deficiency	13/12/2007
Cr 48	14	Om Bahadur Sejwal Khalanga-8 (Tree 1)	*C. reticulata*	Seedling	Mosaic like Zinc	13/12/2007

					deficiency	
Cr 49	15	Om Bahadur Sejwal Khalanga-8 (Tree 2)	*C. reticulata*	Seedling	Mosaic like Zinc deficiency	13/12/2007
Cr 50	16	Bhoj Raj Paudel Padane- 1, Simlae	*C. reticulata*	Seedling	Mosaic like Zinc deficiency	13/12/2007
Cs 81	17	Bishnu Prasad Basnet Khalanga-6	*C. sinensis*	Seedling	Mosaic like Zinc deficiency	22/02/2008
Cr 82	18	Man Bahadur Sejwal Khalanga-6	*C. reticulata*	Seedling	Yellow green patches	22/02/2008
Cr 83	19	Buddhi Raj Bista Khalanga- 5	*C. reticulata*	Seedling	Zinc deficiency	22/02/2008
Cr 84	20	Ramesh Basnet Khalanga- 6	*C. reticulata*	Grafted	Mosaic like Zinc deficiency	22/02/2008

Samples received from Dhading District

E.No.	**S.N.**	**Owner/ locality**	**Species**	**Origin**	**Symptoms**	**Received date**
Cr 53	21	Rana Bahadur Thapa Magar, Grasebas- 9	*Citrus reticulata*	Seedling	Damaged Sample	22/02/2008
Cr 54	22	Kedar Nath Silwal Dhusha- 9, Laksmibas	*C. reticulata* 5 yrs old	Seedling	Damaged Sample	22/02/2008
Cs 55	23	Laksmi Silwal Dhusha- 7, Gyaja	*C. sinensis* (17 yrs old)	Seedling	Yellowing of leaves	22/02/2008
Cr 56	24	Tanka Prasad Silwal Dhusha- 8, Bhanjyang	*C. reticulata* (26 yrs old)	Seedling	Yellowing of leaves	22/02/2008
Cr 57	25	Bishnu Prasad Silwal Dhusha- 8, Bhanjyang	*C. reticulata* (35 yrs old)	Seedling	Yellowing of leaves	22/02/2008
Cr 58	26	Hem Prasad Silwal Dhusha- 8, Bhanjyang	*C. reticulata* (22 yrs old)	Seedling	Damaged Sample	22/02/2008
Cr 59	27	Birendra Prasad Pandey Dhusha- 8, Bhanjyang	*C. reticulata* (21 yrs old)	Seedling	Yellowing of leaves	22/02/2008
Cr 60	28	Shiva Silwal Dhusha- 8, Bhanjyang	*C. reticulata* (17 yrs old)	Seedling	Mosaic like Zinc deficiency	22/02/2008

Cr 61	29	Maan Bahadur Thapa Naulothur-8	*C. reticulata* (17 yrs old)	Seedling	Damaged Sample	22/02/2008
Cr 62	30	Hem Prasad Silwal Dhusha- 8, Bhanjyang	*C. reticulata* (4 yrs old)	Seedling	Damaged Sample	22/02/2008
Cr 63	31	Krishna Man Shrestha Nalang- 1, Patale	*C. reticulata* (5 yrs old)	Grafted	Damaged Sample	22/02/2008
Cr 64	32	Kamal Gurung Nalang- 1, Patale	*C. reticulata* (9 yrs old)	Seedling	Damaged Sample	22/02/2008
Cr 65	33	Govinda Shrestha Nalang- 1, Patale	*C. reticulata* (11 yrs old)	Seedling	Damaged Sample	22/02/2008
Cs 66	34	Dhum Narayan Shrestha Nalang- 1, Patale	*C. reticulata* (11 yrs old)	Seedling	Mosaic like Zinc deficiency	22/02/2008
Cr 67	35	Mrs. Man Kumari Shrestha Nalang- 1, Patale	*C. reticulata* (11 yrs old)	Grafted	Damaged Sample	22/02/2008
Cr 68	36	Bijay Silwal Nalang- 5, Patale	*C. reticulata* (11 yrs old)	Seedling	Mosaic like Zinc deficiency	22/02/2008
Cr 69	37	Ek Bahadur Baram Nalang- 5, Jayabhadre	*C. reticulata* (10 yrs old)	Seedling	Mosaic like Zinc deficiency	22/02/2008
Cr 70	38	Mrs. Hira Maya Shrestha Nalang- 5, Jayabhadre	*C. reticulata* (3 yrs old)	Seedling	Damaged sample	10/02/2008

Samples received from Dhankuta District

E N.	**S N.**	**Owner/ locality**	**Species**	**Origin**	**Symptoms**	**Received date**
Cr 95	39	Chandra Kala Sahu Anargadhi M. 5	*Citrus reticulata*	Grafted	Mosaic like Zinc deficiency	22/02/2008
Cr 96	40	Siddha Raj Panta Anangadi N. Pa – 5	*C. reticulata*	Seedling	Decayed sample	22/02/2008
Cr 97	41	Kamala Devi Sahu Anargadhi M. 5	*C. reticulata*	Seedling	Mottling of leaves	22/02/2008
Cr 98	42	Bisnu Devi Sahu Anargadhi M. 5	*C. reticulata*	Seedling	Decayed sample	22/02/2008
Cr.177	43	Gyanu Devi Sahu Anargadhi M. 5	*C. reticulata*	Seedling	Zinc deficiency	22/02/2008

Cs.178	44	Bharat Raj Panta Anargadhi M. 5	*C. sinensis*	Seedling	Zn-deficiency	22/02/2008
Cr. 179	45	Laxmi Devi Sahu Anargadhi M. 5	*C. reticulata*	Seedling	Mottling of leaves	22/02/2008
Cr. 180	46	Narayan Panta Anargadhi M. 5	*C. reticulata*	Seedling	Zinc deficiency	22/02/2008
Cr. 181	47	Prabha Devi Sahu Anargadhi M. 5	*C. reticulata*	Seedling	Mottling of leaves	22/02/2008
Cr. 182	48	Bhim Raj Panta Anargadhi M. 5	*C. reticulata*	Seedling	Mottling of leaves	22/02/2008

Samples received from Kailali District

E N.	**S N.**	**Owner/ locality**	**Species**	**Origin**	**Symptoms**	**Received date**
Cr 74	49	Indra Bahadur Rajbar, Nigale- 5	*Citrus reticulata*	Seedling	Mottling	22/02/2008
Cr 99	50	Ganesh Bahadur Singh, tree no.1, Nigale- 3,	*C. reticulata*	Seedling	Mosaic like Zinc deficiency	22/02/2008
Cr 100	51	Ganesh Bahadur Singh tree no.2, Nigale- 3,	*C. reticulata*	Seedling	Dried sample	22/02/2008
Cr 101	51	Chatra Bahadur Saudth Nigale-5,	*C. reticulata*	Seedling	Zinc deficiency	22/02/2008
Cr 102	52	Padam Bahadur Saudth Nigale- 5,	*C. reticulata*	Seedling	Zinc deficiency	22/02/2008
Cr.183	53	Shiva Bdr Saudht Nigale-5,	*C. reticulata*	Seedling	Damaged sample	22/02/2008
Cs.184	54	Bijay Bdr. Saudth Nigale-5	*C. sinensis*	Seedling	Zinc deficiency	22/02/2008
Cr.185	55	Ganesh Saudth Nigale-5	*C. reticulata*	Seedling	Zinc deficiency	22/02/2008
Cr.186	56	Manju Devi Saudth Nigale-5	*C. reticulata*	Seedling	Mosaic like Zinc deficiency	22/02/2008
Cr.187	57	Tara Bdr Saudth Nigale-5	*C. reticulata*	Seedling	Mosaic like Zinc deficiency	22/02/2008

Samples received from Dailekh District

	S N.	Owner/ locality	Species	Origin	Symptoms	Received date
Cr 72	58	Maha Singh Malla Tichanda-5	*Citrus reticulata*	Seedling	Mosaic like Zinc deficiency	22/02/2008
Cr 73	59	Nar Bahadur Malla Tichanda- 5	*C. reticulata*	Seedling	Mosaic like Zinc deficiency	22/02/2008
Cs 77	60	Laxmi Shah Dullu-5	*C. sinensis*	Seedling	Dried sample	22/02/2008
Cr 78	61	Padam Nath (10/6) Barkurali- 2	*C. reticulata*	Seedling	Dried sample	22/02/2008
Cr 79	62	Ghana Nath Barkurali- 2	*C. reticulata*	Seedling	Yellow green patches	22/02/2008
Cs 80	63	Padam Singh Karki Dashara M-2	*C. sinensis*	Seedling	Mosaic like Zinc deficiency	22/02/2008
Cr 87	64	Narayan Bahadur Malla, Tichada- 5	*C reticulata*	Seedling	Damaged sample	22/02/2008
Cr 88	65	Nirmala Nath Barkurali- 2	*C. reticulata*	Grafted	Mosaic like Zinc deficiency	22/02/2008
Cs 89	66	Indra Kumari Shah Dullu- 5, Dailekh	*C. sinensis*	Seedling	Mosaic like Zinc deficiency	22/02/2008
Cr 90	67	Chandra Bdr Shah Dullu- 5, Dailekh	*C. reticulata*	Seedling	Zinc deficiency	22/02/2008

Samples received from Baitadi District

E N.	S N.	Owner/ locality	Species	Origin	Symptoms	Received date
Cs 85	68	Dipak Prasad Bhatta Naganjung- 5	*C. sinensis*	Seedling	Mosaic like Zinc deficiency	22/02/2008
Cr 86	69	Padam Singh Karki Naganjung- 5	*C. reticulata*	Seedling	deficiency	22/02/2008
Cr 75	70	Laxman Chanda Naganjung- 5	*C. reticulata*	Grafted	Zinc deficiency	22/02/2008

Cr 76	71	Jay Singh Bhandari Naganjung- 5	*C. reticulata*	Seedling	Mottling of leaves	22/02/2008
Cs.188	72	Babu Ram Bhatta Naganjung- 5	*C. sinensis*	Seedling	Zn-deficiency	22/02/2008
Cr.189	73	Bimal Bdr. Karki Naganjung- 5	*C. reticulata*	Seedling	Mottling of leaves	22/02/2008
Cr.190	74	Hem Raj Bhandari Naganjung- 5	*C. reticulata*	Seedling	Mottling of leaves	22/02/2008
Cr.191	75	Khem Bhandari Naganjung- 5	*C. reticulata*	Seedling	Zn-deficiency	22/02/2008
Cs.192	76	Dayananda Karki Naganjung- 5	*C. sinensis*	Seedling	Mottling of leaves	22/02/2008
Cr.193	77	Jeevan Bhandari Naganjung- 5	*C. reticulata*	Seedling	Mottling of leaves	22/02/2008

Samples received from Sindhupalchawk District

E N.	**S N.**	**Owner/ locality**	**Species**	**Origin**	**Symptoms**	**Received date**
Cr 103	78	Ganga Ghimire Chautara- 5	*Citrus reticulata*	Seedling	Yellow green patches	13/01/2008
Cr 104	79	Jwala Kasju Chautara- 9	*C. reticulata*	Grafted	Mottling like Zinc deficiency	13/01/2008
Cr 105	80	Ratna Bahadur Shrestha Chautara- 7	*C. reticulata*	Grafted	Mottling like Zinc deficiency	13/01/2008
Cs 106	81	Indra Mani Shrestha Chautara- 7	*C. sinensis*	Seedling	Yellow green patches	13/01/2008
Cr 107	82	Hariman Kasaju Chautara- 9, Gaurigaun	*C. reticulata*	Grafted	Mottling like Zinc deficiency	13/01/2008
Cr 108	83	Ramesh Kaji Shrestha Chautara- 5	*C. reticulata*	Seedling	Dried sample	13/01/2008
Cr 194	84	Jyoti Ghimire Chautara- 5	*C. reticulata*	Seedling	Blotchy leaves	13/01/2008
Cr 195	85	Mahendra Kasaju Chautara- 5	*C. reticulata*	Seedling	Zn-deficiency	13/01/2008

Cr 196	86	Dependra Shrestha Chautara- 5	*C. reticulata*	Seedling	Mottling of leaves	13/01/2008
Cr. 97	87	Dharma kaji Shrestha, Chautara- 5	*C. reticulata*	Seedling	Mottling like Zinc deficiency	13/01/2008

Samples received from Okhaldhunga District

E N.	**S N.**	**Owner/ locality**	**Species**	**Origin**	**Symptom**	**Received date**
Cr 109	88	Maya Gurung Rumjatar- 7	*Citrus reticulata* (Tree no. 1)	Seedling	Mottling like Zinc deficiency	25/12/2007
Cs 110	89	Obadhan Rai Kalikasthan- 3	*C. sinensis* (Tree no. 1)	Seedling	Mottling like Zinc deficiency	25/12/2007
Cr 111	90	Mukti Nath Dhamala Rumjatar- 8	*C.reticulata* (Tree no. 1)	Seedling	Mottling like Zinc deficiency	25/12/2007
Cr 112	91	Sabitri Gurung Rumjatar- 8	*C reticulata* (Tree no. 1)	Seedling	Yellow green patches	25/12/2007
Cs 113	92	Sabitri Gurung Rumjatar- 8	*C. sinensis* (Tree no. 2)	Seedling	Yellow green patches	25/12/2007
Cr 114	93	Mukti Nath Dhamala Rumjatar- 8	*Citrus reticulata*	Seedling	Mottling like Zinc deficiency	25/12/2007
Cr 115	94	Krishna Gopal Gurung Rumjatar- 5,	*C reticulata* (Tree no. 1)	Seedling	Dried sample	25/12/2007
Cs 198	95	Gayatri Gurung Rumjatar-5	*C. sinensis*	Seedling	Mottling like Zinc deficiency	25/12/2007
Cr 199	96	Kamal Dhamala Rumjatar-5	*C reticulata*	Seedling	Yellow green patches	25/12/2007
Cr 200	97	Poornima Gurung Rumjatar-5	*C reticulata*	Seedling	Mottling like Zinc deficiency	25/12/2007

Samples received from Doti District

E N.	S,N.	Owner/ locality	Species	Origin	Symptoms	Received date
Cr 116	98	Gangan Singh Rawat Saraswatinagar-7, Jorapal	*Citrus reticulata*	Seedling	Mottling like Zn Deficiency	11/03/2008
Cr 117	99	Jit Bahadur Bhandari Saraswatinagar- 9	*C reticulata*	Seedling	Mottling like Zn Deficiency	11/03/2008
Cr 118	100	Prem Prakash Mede Laxminagar-2 Lilingbag	*C reticulata*	Seedling	Yellowing of leaves, vein clearing	11/03/2008
Cs 119	101	Gagan Singh Rawat Saraswatinagar- 7	*C. sinensis*	Seedling	Mottling	11/03/2008
Cs 120	102	Dharma Devi Saud Laxminagar-1, Faledi	*C. sinensis*	Seedling	Yellowing of Midrib , Mottling	11/03/2008
Cs 121	103	Dipa Devi Sarki Laxminagar-1, Faledi	*C. sinensis*	Seedling	Mottling	11/03/2008
Cr 122	105	Dipa Devi Sarki Laxminagar-1, Faledi	*Cs reticulata*	Seedling	Mottling	11/03/2008
Cs 123	106	Chakra Bdr. Kapadyal Laxminagar-1	*C. sinensis*	Seedling	Mottling like Zn deficiency	11/03/2008
Cr 124	107	Chakra Bdr. Kapadyal Laxminagar-1, Talal	*Cs reticulata*	Seedling	Mottling like Zn deficiency	11/03/2008
Cr 125	108	Khem Bdr. Saud Laxminagar-1	*C reticulata*	Seedling	Mottling, Midrib Yellowing	11/03/2008
Cs 126	109	Khem Bdr. Saud Laxminagar-1	*C. sinensis*	Seedling	Mottling, Midrib Yellowing	11/03/2008
Cs 127	110	Sher Bdr. Kapadyal Laxminagar-1, Kuchhekhel	*C. sinensis*	Seedling	Mottling, Midrib Yellowing	11/03/2008
Cr 128	111	Sher Bdr. Kapadyal Laxminagar-1	*C reticulata*	Seedling	Mottling like Zn deficiency	11/03/2008

Cr 129	112	Dhama Devi Saud Laxminagar-1, Faledi	*C reticulata*	Seedling	Mottling like Zn deficiency	11/03/2008
Cr 130	113	Shreedhar Bohara Laxminagar-1, Faledi	*C reticulata*	Grafted	Slight mottling, Mid rib yellowing	11/03/2008
Cs 131	114	Prem Prakash Mede Laxminagar-2, Silingbag	*C. sinensis*	Seedling	Mottling, Mid rib yellowing	11/03/2008
Cr 132	115	Thaguli Saud Laxminagar-1, Kuchhekhola	*C reticulata*	Grafted	Mottling	11/03/2008

Samples received from Dadeldhura District

E N.	SN.	Owner/ locality	Species	Origin	Symptoms	Received date
Cs 91	116	Chandra Datta Joshi, Bagarkot- 5	*C. sinensis*	Seedling	Mottling	22/02/2008
Cr 92	117	Hari Lahar Bagarkot- 5	*C reticulata*	Grafted	Mottling	22/02/2008
Cr 93	118	Ganesh Datta Joshi Bagarkot- 5	*C reticulata*	Seedling	Mosaic like Zinc deficiency	22/02/2008
Cr 94	119	Bishnu Bahadur Maheta Serat- 5, Bagarkot	*C reticulata*	Seedling	Decayed sample	22/02/2008
Cr 133	120	Rajendra Prasad Panta, Jhurkali-5	*C reticulata*	Seedling	Mottling	22/02/2008
Cr 134	121	Khagendra Prasad Panta, Jhurkali-5	*C reticulata*	Seedling	Mottling like Zn deficiency	22/02/2008
Cr 135	122	Parbati Devi Panta Jhurkali-5	*C reticulata*	Seedling	Slight Mottling	22/02/2008
Cr. 201	123	Shankar Prasad Panta, Jhurkali-5	*C reticulata*	Grafted	Mottling of leaves	22/02/2008
Cr. 202	124	Prakash Panta Jhurkali-5	*C reticulata*	Seedling	Slight Mottling	22/02/2008
Cr. 203	125	Bhaskar Panta Jhurkali-5	*C reticulata*	Seedling	Slight Mottling	22/02/2008

Samples received from Lamjung District

E N.	S N.	Owner/ locality	Species	Origin	Symptom	Received date
Cr 136	126	Plant No. 1	*C reticulata*	Seedling	Mottling on leaf	27/08/2008
Cr 137	127	Plant No.2	*C reticulata*	Seedling	Mottling on leaf	27/08/2008
Cr 138	128	Plant No.3	*C reticulata*	Seedling	Mottling on leaf	27/08/2008
Cr 139	129	Plant No.4	*C reticulata*	Seedling	Mottling on leaf	27/08/2008
Cr 140	130	Plant No.5	*C reticulata*	Grafted	Yellowing of veinlets	27/08/2008
Cr 141	131	Plant No.6	*C reticulata*	Seedling	Yellowing of veinlets	27/08/2008
Cr 142	132	Plant No.7	*C reticulata*	Seedling	Yellowing of veinlets	27/08/2008
Cr 143	133	Plant No.8	*C reticulata*	Seedling	Yellowing of veinlets	27/08/2008
Cr 144	134	Plant No.9, Fidafa Nursery, Kupling, Bajhakhet-6	*C reticulata*	Grafted	Mottling on leaf	27/08/2008
Cr 145	135	Plant No.10, Fidafa Nursery, Kupling, Bajhakhet-6	*C reticulata*	Grafted	Mottling on leaf	27/08/2008

Samples received from Rukum District

E N.	S N.	Owner/ locality	Species	Origin	Symptom	Received date
Cr 146	136	Plant No. 1	*C reticulata*	Seedling	Yellowing of veinlets	20/06/2008
Cr 147	137	Plant No. 2	*C reticulata*	Seedling	Mottling of leaves	20/06/2008
Cr 148	138	Plant No. 3	*C reticulata*	Seedling	Yellowing of veinlets	20/06/2008
Cs 149	139	Plant No. 4	*C. sinensis*	Seedling	Yellowing of veinlets	20/06/2008
Cr 150	140	Plant No. 5	*C reticulata*	Seedling	Yellowing of veinlets	20/06/2008

Cs 151	141	Plant No. 6	*C. sinensis*	Seedling	Yellowing of veinlets	20/06/2008
Cr 152	142	Plant No. 7	*C reticulata*	Seedling	Yellowing of veinlets	20/06/2008
Cr 153	143	Plant No. 8	*C reticulata*	Seedling	Mottling	20/06/2008
Cr 154	144	Plant No. 9	*C reticulata*	Seedling	Mottling	20/06/2008
Cr 155	145	Plant No. 10	*C reticulata*	Seedling	Mottling	20/06/2008

Annex–IV. Record of the samples collected from Syangja, Kaski and Kathmandu districts of Nepal

Samples collected from Syangja District

E N.	S N.	Owner/locality	Species	Origin	Symptom	Date of collection
Cr.204	146	Prem N.Adhikari, Putalibazar-1, Rangkhola,	*C reticulata*	Seedling	Yellowing of veinlets	07/07/2008
Cr 205	147	Krishna Acharya Putalibazar-1, Rangkhola,	*C reticulata*	Seedling	Mottling	07/07/2008
Cs 206	148	Kiran Pd. Aryal Putalibazar-1, Rangkhola,	*C. sinensis*	Seedling	Mottling	07/07/2008
Cr 207	149	Hom Adhikari Putalibazar-1, Rangkhola,	*C reticulata*	Seedling	Yellowing of midrib	07/07/2008
Cr 208	150	Pashupati Adhikari Putalibazar-1, Rangkhola,	*C reticulata*	Seedling	Mottling	07/07/2008
Cr 209	151	Rukmagat Adhikari Putalibazar-1, Rangkhola,	*C reticulata*	Seedling	Yellowing of veinlets	07/07/2008
Cr 210	152	Ram Bdr. Gurung Putalibazar-1, Rangkhola,	*C reticulata*	Seedling	Mottling of leaves	07/07/2008
Cr 211	153	Jhabindra Adhikari Putalibazar-1, Rangkhola,	*C reticulata*	Seedling	Yellowing of leaves	07/07/2008
Cr 212	154	Khagendra Adhikari, Putalibazar-1, Rangkhola,	*C reticulata*	Seedling	Yellowing of leaves	07/07/2008
Cr 213	155	Manju. Aryal Putalibazar-1, Rangkhola	*C reticulata*	Seedling	Zn-deficiency	07/07/2008

Samples collected from Kaski District

E N.	S N.	Owner/locality	Species	Origin	Symptom	Date of collection
Cr 214	156	Malepatan Horticulture, Tree no. 1, Malepatan,	*Citrus reticulata*	Grafted	Mottling like Zn-deficiency	10/07/2008
Cr 215	157	Malepatan Horticulture, Tree no. 2, Malepatan,	*C reticulata*	Grafted	Mottling like Zn-deficiency	10/07/2008
Cs 216	158	Malepatan Horticulture, Tree no. 3, Malepatan,	*C reticulata*	Grafted	Mottling like Zn-deficiency	10/07/2008
Cr 217	159	Malepatan Tree no. 4, Horticulture, Malepatan,	*C reticulata*	Grafted	Zn-deficiency	10/07/2008
Cr 217	160	Malepatan Tree no. 5, Horticulture, Malepatan,	*C reticulata*	Seedling	Yellowing of leaves	10/07/2008
Cr 217	161	Malepatan Tree no. 6, Horticulture, Malepatan,	*C reticulata*	Seedling	Zn-deficiency	10/07/2008
Cr 217	162	Tara Aryal Hansapur-5	*C reticulata*	Grafted	Mottling like Zn-deficiency	10/07/2008
Cr 217	163	Sita Ram Adhikari Hansapur-5	*C reticulata*	Grafted	Mottling of leaves	10/07/2008
Cr 217	164	Biraj Bdr Kunwar Hansapur-5	*C reticulate* Tree no. 1	Grafted	Yellowing of leaves	10/07/2008
Cr 217	165	Biraj Bdr Kunwar Hansapur-5	*C reticulate* Tree no. 2	Grafted	Mottling like Zn-deficiency	10/07/2008

Samples collected from Kathmandu District

E N.	S. N.	Owner/locality	Species	Origin	Symptom	Date of collection
Cs 158	128	Block 'A' No.48	*Citrus sinensis*	Mother plant	Mottling of leaves	08/07/2008
Cs 159	129	Block 'A' No.37	*C sinensis*	grafted	Mottling	08/07/2008
Cr 160	130	Block 'A' No.34	*C reticulata*	Grafted	Yellowing of leaves	08/07/2008
Cp 161	131	Block 'A' No.2	Grapefruit	Grafted	Blotched leaves	08/07/2008
Cg 162	132	Block 'B' tree no. 4	*C. grandis*	Seedling	Yellowing of leaves	08/07/2008
Cr 163	133	Collection Block 'B' tree no. 6	*C reticulata*	seedling	Blotching of leaves	08/07/2008
Cs 164	134	Collection Block 'B' tree no. 14	*C. sinensis*	Grafted	Blotching of leaves	08/07/2008
Cs 165	135	Block 'C2' tree no. 3	*C. sinensis*	Grafted	Yellowing of leaves	08/07/2008
Cs 166	136	Block 'C2' tree no. 52	*C sinensis*	Grafted	Zn-like deficiency	08/07/2008
Cr 167	137	Block 'E'	*C. reticulata*	Grafted	Yellowing of veinlets	08/07/2008
Cs 168	138	Not Blocked	*C. sinensis*	Grafted	Mottling	08/07/2008
Cr 169	139	Glass house tree no.1	*C. reticulata*	Grafted	Yellowing of veinlets	08/07/2008
Cr 170	140	Glass house nursery	*C. reticulata*	Grafted	Yellowing of leaves	08/07/2008
Fj 171	141	Glass house	*Fortunela japonica*	Mother plant	Yellowing of leaves	08/07/2008
Cr 172	142	Glass house nursery	*C. reticulata*	Mother plant	Yellowing of leaves	08/07/2008
Cr 173	143	Durga Sakya, Tau daha	*C. reticulata*	Seedling	Yellowing of shoot	08/07/2008
Cs 174	144	Rajan Pd. Maharjan, Tree no. 1, Tau daha	*C. sinensis*	Seedling	Yellowing of leaves	08/07/2008
Cs 175	145	Rajan Pd. Maharjan, Tree no. 2, Tau daha	*C sinensis*	Seedling	Yellowing of leaves	08/07/2008

Annex V Preparation of Stock Solutions and reagents

1 Tris Hcl (1M, p_H 8.0)

Tris Hcl (1M, p_H 8.0) (Promega co-operation, Maidson, Spain) stock solution was prepared by adding Tris base (60.55g) to double distilled water (400ml). The p_H was adjusted to 8.0 by the addition of concentrated HCl. The final volume was made up to 500ml, autoclaved and stored at room temperature until needed for preparation of extraction buffer.

2 EDTA (0.5M, p_H 8.0)

Disodium ethylene-diaminetetra-acetate.$2H_2O$ (EDTA; 93.05g) was added to a Schott bottle containing double distilled water (400ml), mixed on a magnetic stirrer and the p_H was adjusted to 8.0 by adding NaOH pellets (approximately 10g). The volume was adjusted up to 500ml with double distilled water, autoclaved and stored at room temperature until needed.

3 DNA Extraction buffer

1ml (0.01M, p_H 8.0) Tris and 80ml of (0.4M, p_H 8.0) EDTA was added to a sterilized Schott bottle. 1gm of SDS (1%) was added to the solution followed by the addition of 25mg of Protinase K (i.e. 0.025mg/µL) and final volume was made up to 100ml. DNA Extraction buffer (DEB) thus prepared was stored at 4˚C for future use.

4. Primer, dNTPs and DNA Dilution

Primers were initially diluted to a stock of 100 µM, using sterile distilled water, then ultimately diluted to the 10 µM (working solution), using the standard formula of volumetric analysis (Mitra, 1998).

$$V_1XS_1 = V_2XS_2$$

Where V_1 is the volume of stock solution of primer to be taken to make desired concentration and volume of diluted primer, S_1 is the concentration of the stock solution of the primer, V_2 is the final volume of the diluted primer to be prepared and S_2 is the concentration of the diluted primer to be prepared. Required DNA dilution was also carried out using the same formula.

5. 10(x) TAE stock buffer (Tris, glacial acetic acid and EDTA)

Tris base (121g), glacial acetic acid (28.6ml) and EDTA (50ml, 0.5M, p^H 8.0) were placed into a Schott bottle (1L), was dissolved in double distilled water (50ml). The

final volume made up to 500ml. This tris, glacial acetic acid and EDTA, TAE stock (50x) was diluted to (1x) with further double distilled water prior to being used for gel running.

6. Gel Loading Buffer (GLB)

Sucrose (2.5g) was dissolved in double distilled water (7ml) in which bromophenol blue (20gm) was added and the final volume made up to 10ml. This gel loading buffer (GLB) was added to the sample in the proportion as 1(GLB) to 4 (PCR product) by volume.

7. Agarose Gel (1%)

Agarose (1%, 1g) (Promega co-operation,Maidson, Spain) was dissolved in 100ml, 1 X TAE/TBE buffer in a glass bottle in a microwave oven. It was then cooled to approximately 55˚C and poured onto the gel casting tray using a comb with an appropriate number of dents (14 to 20 toothed). When the gel was solidified, the comb was gently removed and the gel with assembly was transferred to the electrophoresis tray (EMBI TEC Santiago, CA) filled with 1 X TAE/TBE buffer.