POLYMERASE CHAIN REACTION (PCR) - BASED DIAGNOSIS OF HUANGLONGBING (CITRUS GREENING) DISEASE IN SALYAN DISTRICT OF NEPAL

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ABSTRACT

Greening, Huanglongbing is a cosmopolitan and financially burdening disease of Citrus. The disease has been reported from major citrus pockets of Nepal. Salyan is one of the major citrus producing districts of Nepal. The present study was carried out to understand the spread of disease in selected citrus orchards of different Village Development Committees of the district and raise its awareness among farmers. Of the 79 suspected samples collected on the basis of their phenotype, positive infection was observed by Polymerase Chain Reaction in 17samples. Among 17 samples, 16 trees were seedling origin while one was graft in origin. During field survey, vector Diaphorina citri could be observed in orchards of VDCs, Kotmala and Marke. Due to terminal nature of this disease, it has become imperative for its timely and efficient diagnosis so as to avoid further spread of the disease and protect the farmers from needless financial burden.

Keywords: Candidatus liberibacter asiaticus, citrus greening disease, *Diaphorina citri*, Nepal

INTRODUCTION

Citrus decline is becoming a major problem of Nepalese citrus industry. This decline can be attributed various underlying factors such as soil status, use of poor plantlets, pest damage, poor management practices among farmers as well as bacterial and viral pathogenesis of the plant (Chaudhary et al., 1994). Among these, Citrus Greening Disease (CGD) or Huanglongbing Disease (HLB) has been considered as one of the most devastating factor for deterioration of citrus fruit production around the world (Batool et al., 2007). Reported from Nepal in 1968 for first time, after the initial discovery in China at the end of 19th century as "yellow shoot disease", the disease has now been reported from

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more than 40 countries around the world and is considered as a major contributing factor for citrus decline in Nepal (Thrower, 1968; Bové, 2006; Paudyal, 2015). The disease has been known to be caused by the infection of unculturable, gram negative bacterium, belonging to genus *Liberibacter* (Garnier and Bové, 1977). This phloem restricted bacteria has been given the prefix *Candidatus* owing to its unculturable nature (MacLean and Hogenhout 2012). The bacterium is transmissible between the trees either via graft or with the help of a psyllid insect vector. Altogether, three species of bacterium has been reported around the world causing HLB, *Candidatus Liberibacter asiaticus* from Asia, *Candidatus Liberibacter africanus* from Africa and *Candidatus Liberibacter americanus* from Brazil (Jagoueix *et al.*, 1994; Teixeira *et al.*, 2005). Among these *Candidatus Liberibacter asiaticus* has been known to be prevalent in Asian countries, including Nepal and the psyllid, *Diaphorinacitri* has been identified as the responsible vector (Batool *et al.*, 2007). Since its discovery in the late 1960s, HLB has been reported from numerous citrus producing districts of Nepal, which may not be exhaustive and remains incomplete, as there is still lack of awareness about the disease among farmers as well as an extensive survey is yet to be carried out by concerned government authorities about the incidence and prevalence of the disease (Thrower, 1968; Bové, 2006; Regmi and Yadav, 2007; Regmi *et al.*, 2010).

Salyan district is one of the important districts both in area coverage and production of citrus in mid- western development region. Citrus farming is done in 860 hectares of the district. The annual citrus production was approximately 8170 metric ton in the fiscal year 2013/14 (Nepal Agriculture Atlas, 2018).

OBJECTIVES

- Field survey of various orchards of Salyan district to know the prevalence of Citrus greening disease
- Molecular confirmation for *Candidatus Liberibacter asiaticus* in suspected citrus samples.

MATERIALS AND METHODS

FIELD SURVEY

The citrus production in the chosen field of study, Salyan, shown in Figure 1, has been observing decline over the years. Hence, field survey was conducted in commercial citrus orchards of Kotmala, Khalanga and Marke Village Development Committees (VDCs), Salyan.

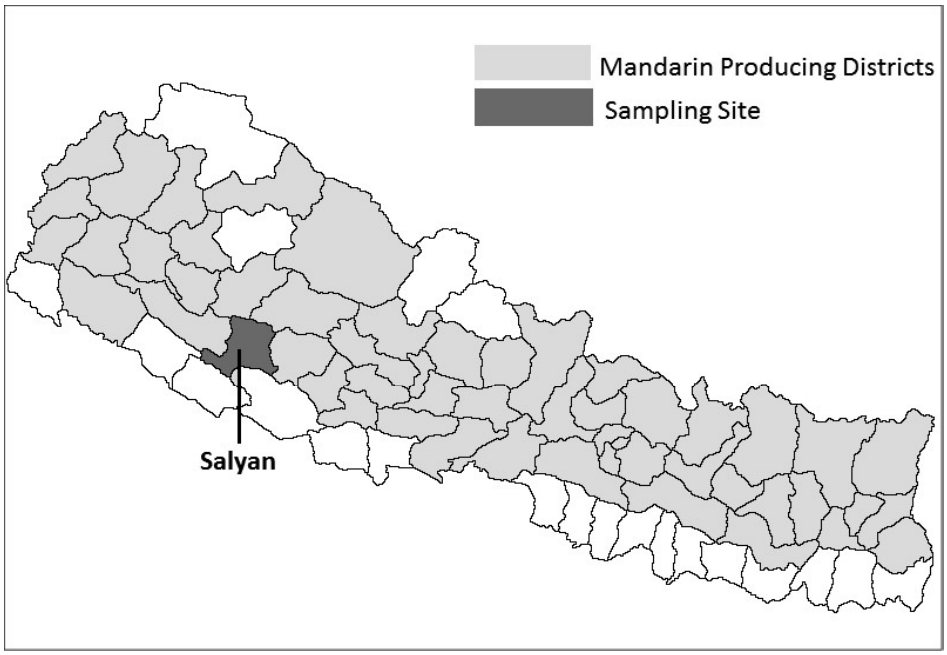

Figure 1. Map of Nepal indicating the citrus growing districts as well as sample collection site (Salyan: Also one of the prime mandar in producing districts) (Nepal Agriculture Atlas, 2018)

Sample collection from field visit

Sample collection was done in the month of April, 2013. A total of 79 samples were collected under suspicion for HLB infection. The inclusion criteria for sampling conferred were, for the number of trees to be more than 50; only those orchards growing mandarin (*Citrus reticulate* Blanco) were chosen; individual trees were selected based on their phenotypic appearance as well as fruiting status of the tree, the information for which was obtained upon interview with the local farmers. Only those trees symptomatic for HLB were chosen. The suspected samples were then placed in sterile zip lock polythene bags with proper labeling and kept in icebox. Samples were brought to Molecular Biotechnology Unit, Nepal Academy of Science and Technology, where it was stored at 4^{o}C until further analysis (Table 1).

Table 1: List of collection sites and number of samples collected and positive results obtained from each of the Village Development Committees (VDCs)

S.N.	VDCs	No. of orchards	No. of samples tested	No. of HLB positive samples	Damaged during Transport	Percentage of HLB Positive Samples
1.	Kotmala	Gauragaun	11	5	0	45.4
		Kotgaum	5	0	0	0
		Chaitegade	3	0	0	0
		Malekafal	22	5	0	22.7
2.	Marke	Jamunepani	7	1	0	14.3
		Tallotole	8	0	1	0
3.	Khalanga	Sijwaltakura	5	1	0	20
		Ratamata	13	2	3	20
		Siruwar	5	3	1	75
Total			**79**	**17**	**5**	**22.9**

DNA EXTRACTION

DNA extraction was carried out from the midribs of suspected leaf using Qiagen DNeasy Plant Mini Kit (cat. nos. 69104 and 69106 Qiagen) as per manufacturer's instructions. The extracted DNA was then stored at -20°C until further analysis.

AMPLIFICATION BY POLYMERASE CHAIN REACTION (PCR)

PCR was used for detection of HLB organism as per the protocol described by Jagoueixet *et al.,* in 1996. The principle is based on amplification of 1160bp long fragment of 16S rDNA of HLB organism using primer OI1, OAI and OI2C (Jagoueixet *et al.,* 1996). PCR reaction was performed using the primers OI1 (5' -GCGCGTATGCAATACGAGCGGCA-3'),OA1 (5'-GCGCGTATTTTATACGAGCGG-CA-3'), and OI2C (5'-ACAAAAGCACGAACAA -3'). The optimized PCR condition is as follows: 94°C for 2 minutes followed by 35 cycles of 92°C for 45 seconds and 72°C for 90 seconds, final extension at 72°C for 10 minutes and hold at 4°C for 2 minutes. The amplicons were then observed in gel documentation system after agarose gel electrophoresis.

RESULT AND DISCUSSION

SURVEY

Orchards with more than fifty trees were surveyed and these predominantly were made up from seedling origin. Of the seventy-nine samples collected (details provided in supporting information file), seventy-two were of seedling origin while seven were derived from graft. Five seedling origin samples were damaged during transport, hence could not be processed further (Table 1). Farmers in these orchards preferred seedling than grafted plant due to its easy availability and cheaper price. They are primarily dependent upon traditional collection of locally collected planting materials as a starter planting material for initiation of new orchards. During field survey, the vector, *Diaphorina citri* could be observed in the orchards of VDCs, Kotmala and Marke.

PCR

A band length corresponding to approx. 1160 bp confirmed the presence of bacterium responsible for causing Citrus Greening Disease (CGD) (Figure 2 A and B). Seventeen out of seventy-four (~ 23%) tested samples were found to be positive as these gave band length similar to that of the positive control, previously confirmed in the laboratory (Table 1). Among these 17 samples, there were 23.88 % (16 out of 67) seedling originated plants and 14.28 % (1 of 7) grafted plants.

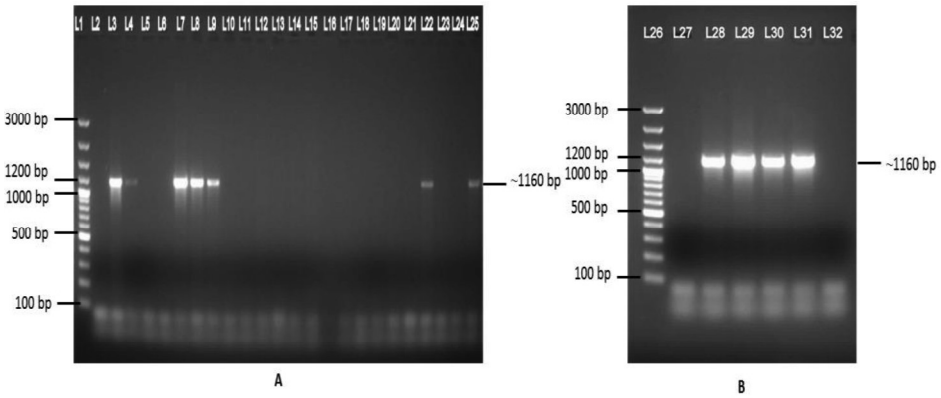

Figure 2. Representative Image of Agarose Gel Electrophoresis for PCR amplicons showing band length (~ 1160 bp) corresponding to that obtained from the positive control. (A) L1: GeneRuler™ 100 bp Plus DNA Ladder; (A) L2- L25: Samples 1 to 24; (B) L26: GeneRuler™ 100 bp Plus DNA Ladder; (B) L27 – L30: Samples 77 to 79; (B) L31: Positive Control; (B) L32: Negative Control.

Supplementing previously published studies, this study reports on a Polymerase Chain Reaction based diagnosis performed for detection of the bacterium *Candidatus Liberibacter asiaticus* from the citrus sample collected from Salyan district of Nepal. It is third highest mandarin producing district out of the total 54 districts from which mandarin production has been reported (Nepal Agriculture Atlas, 2018). In this study, we found that 17 of the collected 79 suspected citrus plant samples were infected by the bacterium, with infection detected from all 3 of the VDCs. To our knowledge, this study presents first report on Citrus Greening Diseases from Salyan District of Nepal. Although the responsible vector had been previously reported from the district, existence of HLB was not confirmed till now (Lama et al., 1988). As majority of confirmed samples were from plants of seedling origin, it is also possible that farmers have been unintentionally spreading the pathogen from one orchard to the other, apart from the obvious transmission by the psyllid vector (Batool et al., 2007).

In the context of Nepal, most of the citrus plants are supplied by private nurseries which produces seedling as well as grafted nursery trees. However, in Salyan, it was observed that people still heavily relied upon traditional method of locally grown seed collection and starting new orchards from these. Although this helps farmers in recuperating from economic loss of having to buy new plantlets from nurseries, it also inadvertently increases the chances for spread of asymptomatic, dormant lying bacteria to new orchards.

Huanglongbing (HLB) disease is considered to be a devastating disease due to its terminal nature and rapid progression causing death of the plant within few years after infection. The symptomatic fruits also have flavor problems which could be due to increase in bitter compounds (Baldwin et al., 2009). Despite development of several visual as well as molecular techniques for detection of disease as well as implementation of several management strategies, fruition is yet to be realized and destruction of entire infected trees is the only viable option that in turn can create a huge economic burden for farmers (Bassanezi and Bassanezi, 2008; Paudyal, 2015). This failure in control may partly be blamed to unculturable nature of the infecting pathogen which renders researchers with hindrance in understanding the pathogenicity of the microbe (Garnier and Bove, 1977).

The combination of primer sets OI1-OI2C and OA1-OI2C amplified 16S DNA regions from the bacterium *Candidatus Liberibacter asiaticus* and *Candidatus Liberibacter africanus* respectively, however, due to funding deficiency, sequencing of the PCR products could not be carried out (Jagoueix *et al.*, 1996). Although the actual pathogen could only be surmised as either of the

aforementioned bacteria, this study showed the presence of the causative agent in the tested samples.

Apart from the role played by vector and pathogen, rapid spread could also be partly explained as due to lack of proper orchard management system, lack of supply for healthy and certified bud wood and effective preventive measures and surveillance strategy. Hence, the disease itself may be accidentally being transported to the previously healthy orchards by the anthropogenic movements of infected seedlings as well, apart from the direct role played by the vector as well as grafts (DOA, 2011).

Despite the declining citrus production, here the disease alone could not be blamed for infection by the pathogens, as many other physical as well as biological etiologic agents could be responsible for citrus decline including but not limited to zinc deficiency, which also shows symptoms similar to *Liberibacter* infection as well as infestation by *Phytophthora* fungi and other abiotic and biotic factors. Due to this complex interplay between plant, pathogens and the environment, the determination of the disease becomes highly doubtful based only on morphology of plant. Furthermore, the co-relation between the presence of disease and PCR results cannot be determined as identical plant phenotype tend to have variable PCR results. Hence, the exploration for all the possible courses was beyond the scope of this research.

Similarly, the newly infected plants would also be missed as they remain asymptomatic during initial years. Furthermore, as the sampling criteria included only those orchards with more than fifty trees, this would subsequently leave out the small plantations that could act as a reservoir for the pathogens. The presence of infecting pathogen in vector as well as vector distribution was beyond the scope of this study. Apart from these limitations, the complexity of disease brought on due to uncultivable nature of the bacteria outside of plant phloem as well as low level bacterial DNA extraction resulting in reduced sensitivity of PCR could lead to false negative results from plant samples. However, the problem of sensitivity could be solved using novel primer sets developed by Fujikawa and Iwanami that has higher sensitivity when compared to the conventional primer sets (Fujikawa and Iwanami, 2012).

For the time being, in the resource limited settings, Polymerase Chain Reaction presents a reliable method of detection for identification as well as confirmation of HLB in the suspected citrus plant samples (Jagoueix *et al.*, 1996; Shrestha *et al.*, 2003). Hence, incorporation of such modern diagnostic techniques into the surveillance policy could both help in correct identification of the disease as well as avoid unnecessary destruction of trees and orchards

under disease suspicion. The technique has been routinely applied for laboratory diagnosis at Molecular Biotechnology Unit, NAST, which is currently the only place providing such facility in Nepal. However, with increasing interest in citrus cultivation among farmers as well as the incentives being supplemented by the national bodies, it has become a necessity to introduce a strong stratagem if the current citrus production is to be increased. Lack of attention in the current citrus decline will, slowly but certainly, not only wipe out the prospect of high citrus production and economic progress of local farmers but also at the extreme end, might make it completely un-inhabitable for cultivation of citrus fruits due to terminal nature of the infecting pathogen.

CONCLUSION

Due to rapid progression of disease as well as its heavy economic burden upon the farmers, it has become imperative for researchers around the world to carry out extensive research on the pathogen as well as possible mechanisms for control and eradication. Failure to fulfill this could result in significant loss of economy as well as possible extinction of the viable citrus species. Hence, under the current predisposition, although cure is far from realization, timely diagnosis of Citrus Greening Disease (CGD) could at least impart some relief to the farmers, the world over, from the ensuing economic losses.

ACKNOWLEDGEMENT

The authors would like to thank Dr. Chiranjibi Regmi, Ms. Nisha Rana, Ms. Jaishree Sijapati and Dr. Sangita Shrestha for their invaluable support and guidance during sample collection and laboratory work. The authors would also like to thank Nepal Academy of Science and Technology for providing the necessary technical and financial support.

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