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Antimicrobial treatment of citrus budwood to control *Candidatus* Liberibacter asiaticus: An effort to develop low-cost technology for farmers

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Abstract

Huanglongbing (HLB), also known as citrus greening, is one of the most devastating diseases of citrus worldwide menacing the survival of Pakistan and the world's citrus industry. HLB is caused by different species of *Candidatus* Liberibacter bacterium: *Candidatus* Liberibacter asiaticus, *Candidatus* Liberibacter americanus and *Candidatus* Liberibacter africanus. The bacterium is transmitted from one plant to another by psyllid vector and through budding/grafting of infected plant material. As potential control strategies for citrus HLB, the effectiveness of different doses of penicillin including 25ppm, 50ppm, 75ppm and 100ppm in solution form were evaluated on HLB infected scions grafted on a rough lemon. Infected grafts were immersed in penicillin solutions for 2, 4, 6 and 8 hours. Biochemical analysis for the elimination of bacterial pathogens from treated sprouts was performed using iodo starch test. Survival of disease-free scion after the treatment with the antimicrobial agent is a landmark for HLB management. Highly significant differences were obtained for *Candidatus* liberibacter asiaticus detection in penicillin treated grafts sprouts at 8 hours immersion periods (F= 42.12, P= 0.0001). Our results revealed that scions immersed for 8 hours in 75 ppm solution of penicillin provided the highest efficiency in graft survival, sprouting and suppressing the HLB. This may provide a useful tool for the management of citrus HLB for citrus growers.

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Introduction

Fruit plants have to deal with many biotic and abiotic stresses which can negatively affect crop production by curtailing the yield, disturbing the biomass and ultimately shortened the productive life of plants. Among various biotic stresses, citrus greening, or huanglongbing (HLB) has struck as a prime problem to the citrus industry worldwide (Gottwald, 2010). In 1995 the disease got the universal name "Huanglongbing" in the 13th Conference of International Organization of Citrus Virologists. HLB is one of the most fatal diseases that affect citrus plants. In Africa, the disease exists only in cooler areas while in Asia HLB symptoms occur even when temperatures are well above 30°C (Jagoueix *et al*., 1994). Greening affected trees show diverse symptoms on leaves and fruits. HLB affected plants have scattered yellow foliage, generally slow in growth and exhibit twig dieback (Khan, 1989). Symptoms are only seen on one part of the canopy at the early stages of infection (Su and Chen, 1990) and most typical symptoms are mottling and chlorosis of leaves. The fruits can have a bitter or sour taste and are usually small and lopsided and color development on affected fruits starts at peduncular ends rather than the stylar end as in the case of normal fruit (Jepson, 2009).

Huanglongbing is caused by a phloem-restricted, Gram-negative, unculturable, 2µm long and 0.2µm in diameter bacterium (Weinert *et al.,* 2004; Garnier *et al.* 1984; Bove, 2006). There are three isolate types of the bacterium; *Candidatus* Liberibacter asiaticus (Las), *Candidatus* Liberibacter africanus (Laf) (Da Graca, 1991) and *Candidatus* Liberibacter americanus (Lam) (Teixeira *et al.,* 2005). There are two species of insect vector; the Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama (Hemiptera: Sternorryncha: Lividae) and *Trioza erytreae* Del Guercio (Hemiptera: Sternorrynca: Triozidae) reported in case of citrus greening (Aubert, 1987). The ACP can transmit both Las and Lam. The most abundant bacterium species among huanglongbing infected trees is Las (Jagoueix *et al.,* 1994; Bove, 2006) that can also be transmitted by grafting (Lin, 1956). The severity of the disease may vary with citrus genotypes and all commercial cultivars are affected by this pathogen (Folimonova, 2009). The flow of nutrients is disturbed by this pathogen and as a result, causes a rapid decline of the tree. HLB exhausts root system, causes fruit abscission and eventually rate of tree mortality increases. New cells of phloem are produced in vegetative growth up to 6 months until phloem plugging. For HLB management, a few weeks before and after spring and summer bloom are the best (Brodersen *et al.*, 2014).

In Asia and African countries during the early 1990s, more than 60 million trees had been ruined by HLB (Aubert, 1993; Timmer *et al.*, 2003). In Asia, about 100 million citrus trees have been destroyed by greening and about 1 million trees were removed in Brazil. In Florida since 2006, HLB is responsible for $3.63 billion in lost revenues and 6,611 lost jobs in the orange juice production industry (Duan *et al.*, 2009; Hodges and Spreen, 2012; Stansly *et al.*, 2014). HLB diagnosis, only based on symptoms is difficult as the HLB symptoms are confused with nutrient deficiency symptoms. The most reliable diagnostic test is DNA based PCR test for HLB (Li *et al.*, 2006). PCR test is costly, laborious and difficult for a huge number of samples in limited time. A biochemical test based on starch reaction has been successfully practiced for HLB diagnosis. It is very simple, easy and economical. The level of starch increases up to six times in HLB affected leaves than healthy ones. The results of this iodo starch test have found to be 90 to 95% correct when compared with PCR results (Taba *et al.*, 2006; Etxeberria *et al.*, 2007). A combination of photochemical and non-photochemical quenching and chlorophyll fluorescence parameters proved to be efficient for advanced diagnosis of HLB in asymptomatic trees (Sagaram and Burns, 2009).

In Pakistan and Asian countries, sweet orange (*Citrus sinensis*), mandarins (*C. reticulata*) and grapefruits (Citrus paradiseae) are favourite but susceptible to HLB. For the treatment of bacterial diseases the introduction of antibiotics revolutionized the field of medicine. For more than fifty years different antibiotics were used to control HLB but at present

no proper cure is available for infected trees. Due to a lack of useful integrated management and lack of resistant citrus species, control of HLB is urgently needed. An effective short-term strategy for control of HLB is chemotherapy. According to the previous reports antibiotics have been applied by injecting into trees in several regions of the world to control citrus HLB (Nariani *et al.,* 1971; Nariani *et al.,* 1975; McManus *et al*., 2002). Beta-lactam antibiotics such as Penicillin (Pen), and Ampicillin (Amp), can hamper the growth of sensitive bacteria by immobilizing enzymes located in the bacterial cell membrane, known as penicillin-binding proteins, which are involved in the synthesis of the cell wall (Spratt and Cromie, 1988). In previous studies, it has been reported that Amp or Pen when applied via root drench, foliar spray, or trunk injection can eradicate Las bacteria in HLB-affected periwinkle and citrus (Yang *et al*., 2015). Penicillin exhibits the greatest antimicrobial activity against Las showing no phytotoxicity on citrus in graft-based assays (Yaqub *et al*., 2019). Citrus can easily take Penicillin. Additionally, as a response to Amp and Pen treatments, the Las role on HLB progression and structure within the citrus leaf was modified (Zhang *et al*., 2014).

The main aim of the study was to diagnose HLB using biochemical tests and evaluate the effectiveness of this test and different doses of penicillin on HLB infected scion buds grafted on rough lemon for *Candidatus* Liberibacter asiaticus elimination and graft survival for the citrus growers.

Materials and methods

Experimental Layout and Conditions

The current experiment was conducted on Citrus at the Department of Horticultural Sciences, University College of Agriculture and Environmental Sciences, The Islamia University of Bahawalpur. The experimental area was situated in the sub-tropical climatic zone, characterized by seasonal rainfall, high temperature and relatively long days during the summer season and mild to cool winters. The rainfall here averages 143 mm. The plants of rough lemon were already grown in the controlled conditions of the greenhouse for grafting. The experiment was designed with three replications of each treatment. Budwood was taken from PCR tested HLB positive sweet orange source tree. For the collection of budwood, those branches of source trees were selected that had leaves with HLB symptoms like vein yellowing and blotchy mottling (Fig 1A&B).

Selection, preparation and treatment of budwood

Mature, round and white striped scion wood with at least three buds were cut with the help of pruning scissor and were given slanting cuts with the help of budding knife. Leaves were removed with the help of scissor but petiole remained intact. To assess the effect of penicillin on HLB infected budwood six treatments including control were made. Citrus stems containing 3-5 buds per stick prepared for antibiotic treatment (Fig. 2A). Budwoods were submerged in beakers containing different doses of antibiotic solution for 2, 4, 6 and 8 hours (Fig. 2B-2F; Table 1). Budsticks treated with different doses of penicillin were grafted on rough lemon rootstock immediately after treatment. Three plants for each dose of antibiotic-treated budstick were grafted. Two budsticks were grafted on each replicate plant. For controls, HLB positive budwood without antibiotic treatment and positive budwood immersed only in water were also grafted on a rough lemon. After 3 weeks of grafting, data collection started and polythene sheets were removed from grafts to check the condition either they are alive, dead, or sprouted.

Biochemical test for huanglongbing diagnosis

Leaf samples for the confirmation of elimination or suppression of *Candidatus* Liberibacter asiaticus from the penicillin treated sprouted grafts were collected after twenty-eight weeks or 210 days and subjected to a biochemical test. The iodo starch test was performed according to Etxeberria *et al.* (2007). For this test, a commercially available tincture of iodine was purchased that contained iodine and sodium iodide dissolved in alcohol and water. For iodo starch test, one part of iodine solution i.e. 1 mL iodine mixed with 9 mL of distilled water. It formed

10 times of a dilute solution to prevent the faulty result due to the intense reaction of pure iodine solution. The solution was kept in a dark-tinted bottle for downstream processes. For sterilization of scissor we took 20ml bleach and dissolved it in 180ml distill water to make ten times dilute solution. Selected leaves were cut in the form of a strip with the help of scissors sterilized with the bleach solution and rinse with distilled water. Leaves strips were immersed in the prepared iodine solution for 2 minutes. Leaves strips were rinsed with distilled water to remove the excess dye and observed with the help of a hand lens. The leaf strip with dark brown edges confirmed the HLB (Fig. 3).

Statistical analysis

Differences among antibiotic treatment levels were assessed by two way ANOVA using StatPlus software for the level of significance at $P \leq 0.05$. Commulative data of grafts after treatment with different doses of antibiotics for graft immersion for different periods were analyzed in percentages.

Results

Antimicrobial activity of the penicillin against Candidatus Liberibacter asiaticus elimination and graft survival

Four concentrations of penicillin antimicrobial drug (25 ppm, 50 ppm, 75 ppm and 100 ppm) were assessed for the elimination of *Candidatus* Liberibacter asiaticus from HLB positive budsticks.

Grafts were immersed in the solutions of given concentrations for 2, 4, 6 and 8hours to find the significant treatment for graft survival and bacterial elimination. For two hour immersion period, out of 36 grafts, only 5 were sprouted, 4 remained alive and 21 died (Fig. 4A). Similarly, in four hours immersion 20 grafts died, 7 sprouted and 4 remained live (Fig. 4B). In six and eight-hour immersion timings 18 died, 8 sprouted, 5 lived and 16 died, 8 sprouted and 6 remained alive respectively (Fig. 4 C&D).

Table 1. Penicillin treatment doses and durations for the elimination of *Candidatus* Liberibacter asiaticus from HLB positive bud wood.

Treatment	Penicillin doses	Immersion time (hours)	Immersion time (hours	Immersion time (hours)	Immersion time (hours)	Plant replicates grafted	No. of grafts
T1/ HLB positive control	Without antibiotic or water treatment	0	0	0	0	3	2
T2/ Water control	Grafts dipped in distilled water	2	4	6	8	3	2
T3	25 ppm	2	4	6	8	3	2
T4	50 ppm	2	4	6	8	3	2
T5	75 ppm	2	4	6	8	3	2
T6	100 ppm	2	4	6	8	3	2

Biochemical studies for the diagnosis of HLB in chemotherapied sprouted leaves were performed using iodo starch test. Very clear dark brown to purple red margins generated in the leaf strips of HLB positive leaves in a biochemical reaction between the starch present in the leaf strips and iodine solution. Margins with purple, brown or dark brown colour generation also developed in leaf strips of water immersed; 25 ppm for 2, 4, 6 and 8 hours treatment; 50 ppm for 2, 4, 6 and 8 hours treatment; 75 ppm for 2 and 4 hours treatment and 100 ppm for 2 and 4 hours treatment. No colored margins were observed in healthy leaf strips and strips immersed in iodine solutions from 75 ppm penicillin treated leaf for 6 and 8 hours as well as 100 ppm for 6 and 8 hours (Fig. 3). Highly significant differences were obtained for Candidatus liberibacter asiaticus detection in penicillin treated grafts sprouts at various immersion periods ($F= 42.12$, $P= 0.0001$). Penicillin doses at 50 ppm for 8 hours immersion of grafts and 75 ppm for 6 and 8 hours graft immersion proved best for graft survival and sprouting, whereas, 75 ppm for 8 hours graft immersion proved best for graft survival and HLB bacteria elimination as well.

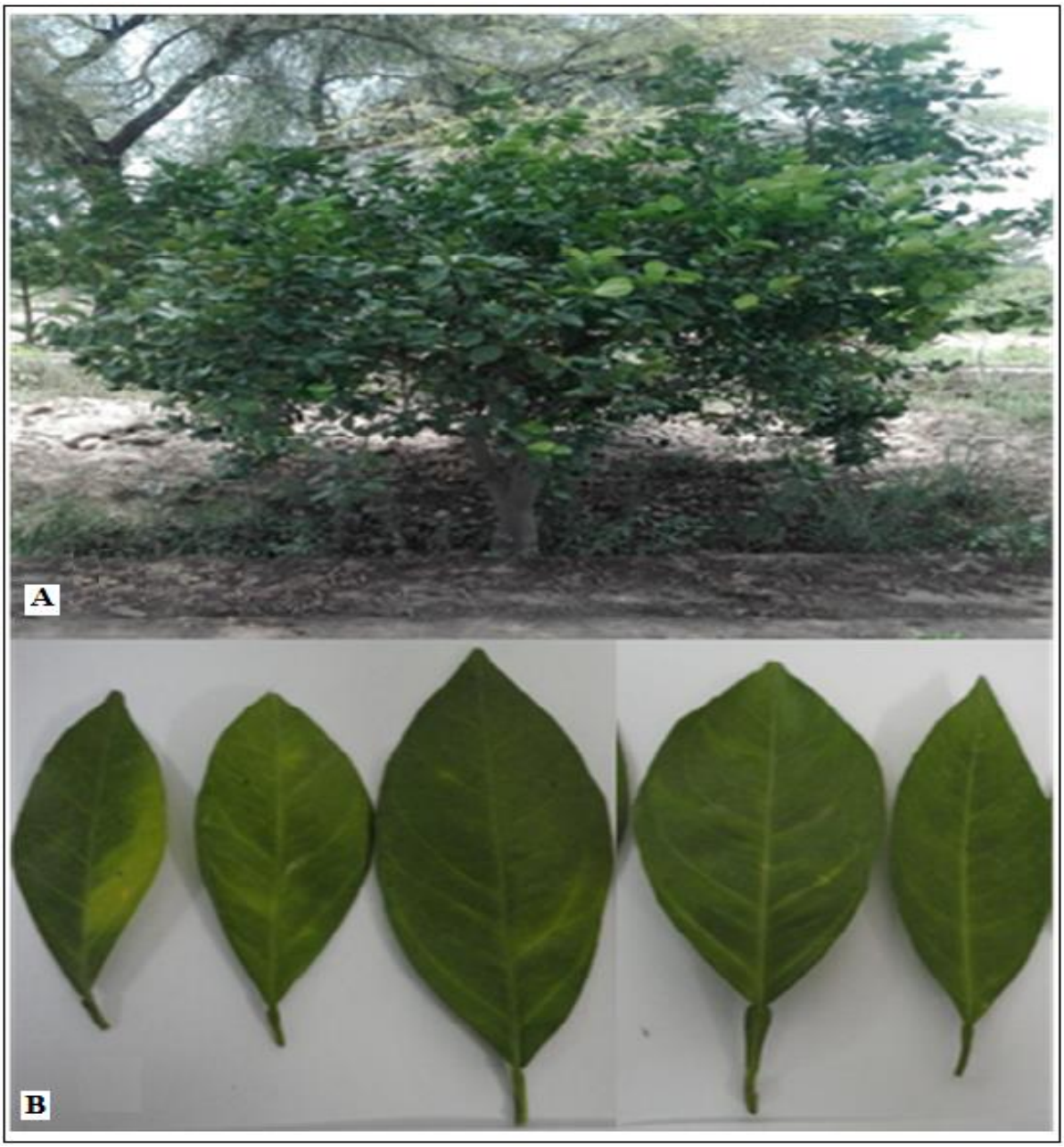

Fig. 1. PCR tested HLB positive graft source from citrus field: A, Sweet orange HLB positive graft source tree; B, HLB positive sweet orange leaves with blotchy mottle and yellow veins symptom.

Discussion

Effect of penicillin therapy on graft survival

In the case of positive control 83.33% and water control treatments 75% of the grafts died. It may presumably due to the accumulation of starch in HLB affected phloem sieve tubes resulting in the obstruction of sap movement and ultimately the graft's death. During the present study, graft survival and sprouting rate were found quite better as compared to previous studies. Previously, most of the grafts died during penicillin and tetracycline treatment with grafts immersion period of 4 hours. No bud was sprouted in any therapied grafts except 75 ppm that was also died after twenty-nine weeks (Yaqub *et al.*, 2019).

Huanglongbing infected citrus plants can be identified by the appearance of symptoms but they do not appear immediately after infection. Older leaves express specific blotchy mottled HLB symptoms and the yellow shoots express zinc deficiency like symptoms in the canopy of the tree.

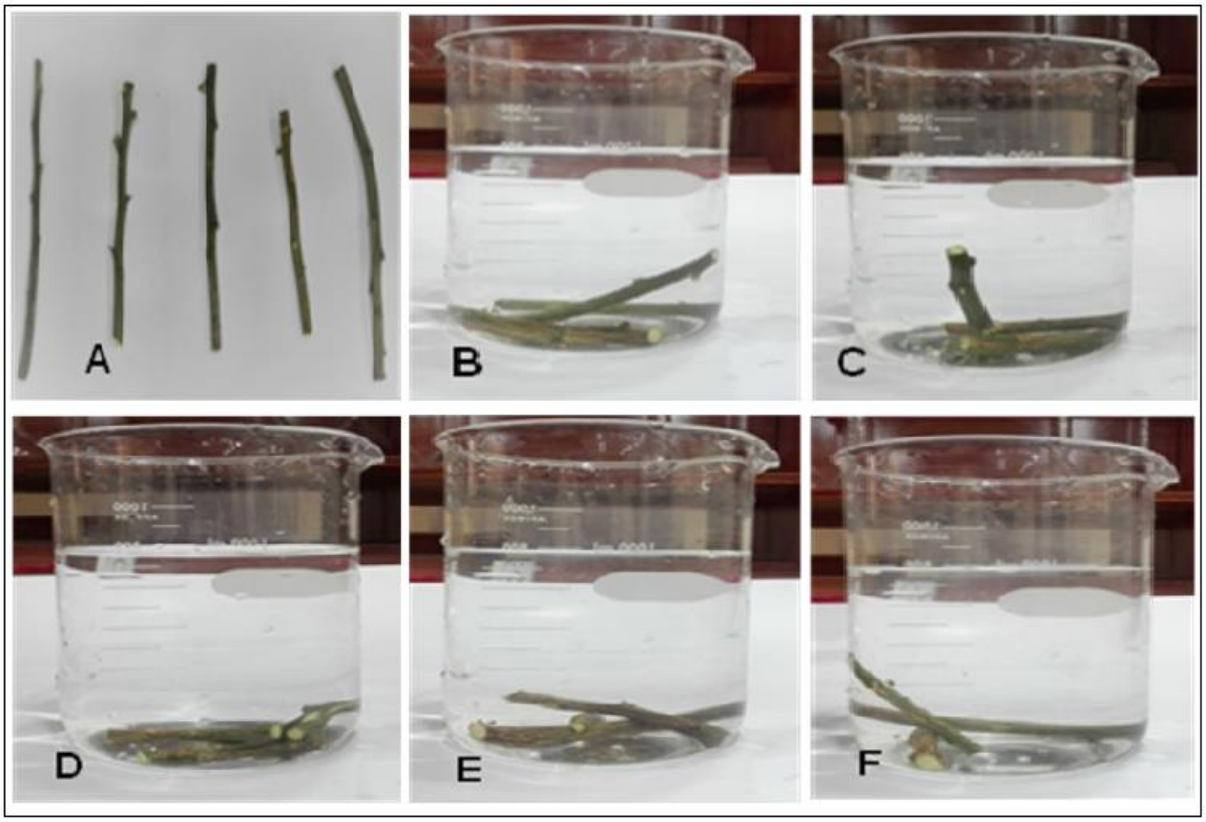

Fig. 2. Antibiotic treatment of HLB positive budsticks: A, HLB positive grafts having 3-5 buds; B, distill water immersed grafts; C, penicillin treatment with 25 ppm solution; D, penicillin treatment with 50ppm solution; E, penicillin treatment with 75ppm solution; F, penicillin treatment with 100ppm solution.

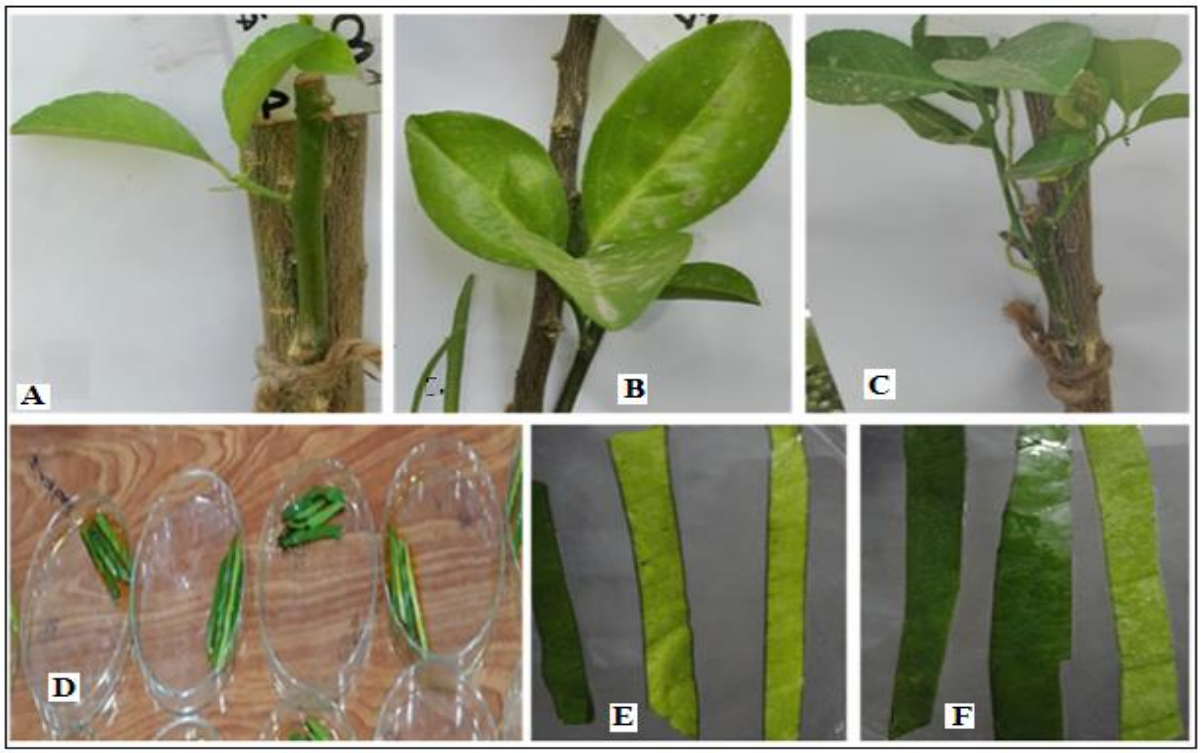

Fig. 3. Graft sprouting after penicillin treatment and Iodostarch test for huanglongbing diagnosis: A - C, Pencillin treated sproured grafts; D, Sample preparation for iodo starch test; E, HLB positive samples having dark grey margins of leaf strips; F, Iodostarch tested healthy control leaf strips.

Some citrus and its relatives do not express symptoms even after infection e.g. trifoliate orange (*Poncirus trifoliata*) (McClean and Schwarz, 1970; Miyakawa, 1980). From the results of the present study, it is suggested to the farmers and growers of citrus that they should treat the grafts with penicillin at 75 ppm dose for 6 or 8 hours before grafting to save their money, energy and citrus orchards; because the grafts they have taken from non-symptomatic trees don't need to be healthy.

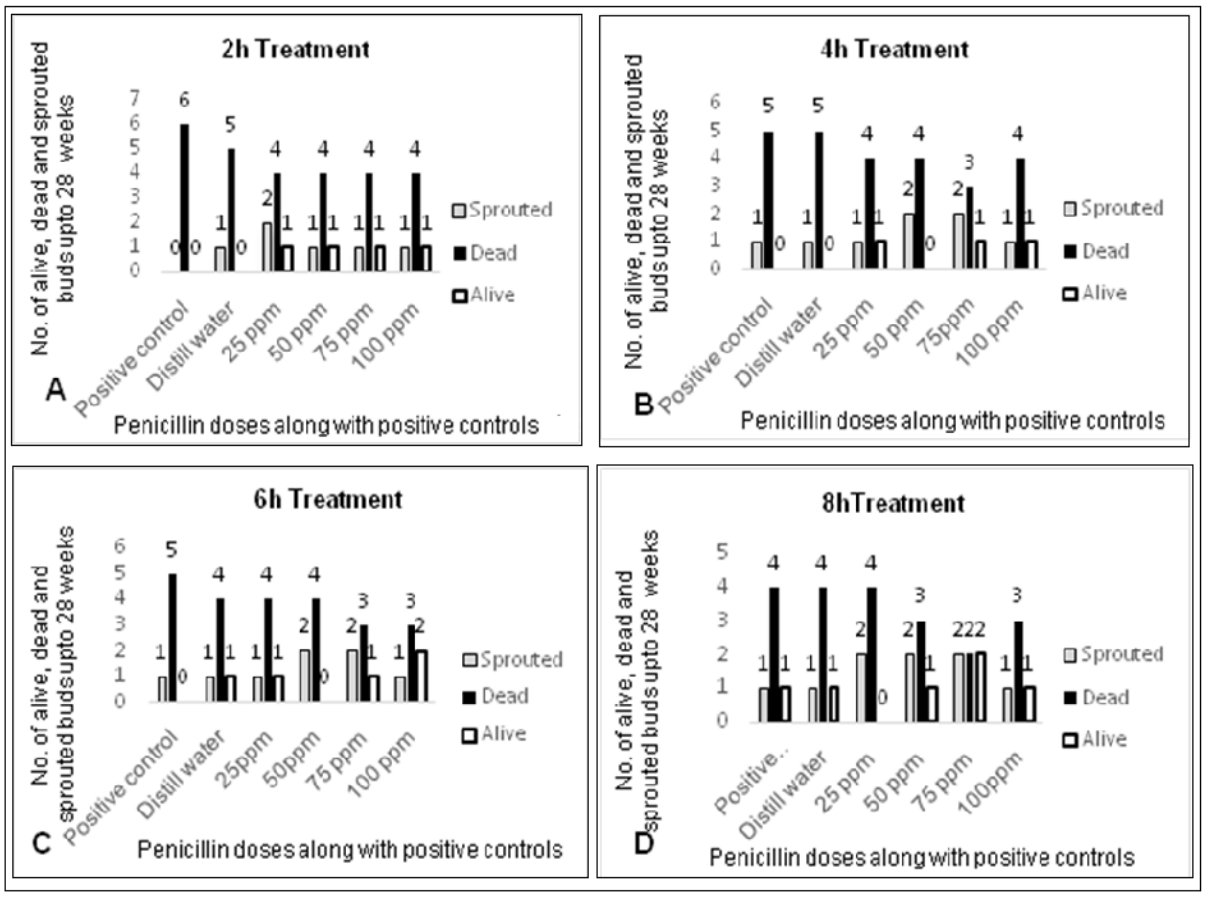

Fig. 4. Effect of penicillin treatment on HLB positive grafts survival: A, Grafts survival upon penicillin treatment for 2 hour immersion; B, Grafts survival upon penicillin treatment for 4 hour immersion; C, Grafts survival upon penicillin treatment for 6 hour immersion; D, Grafts survival upon penicillin treatment for 8 hour immersion.

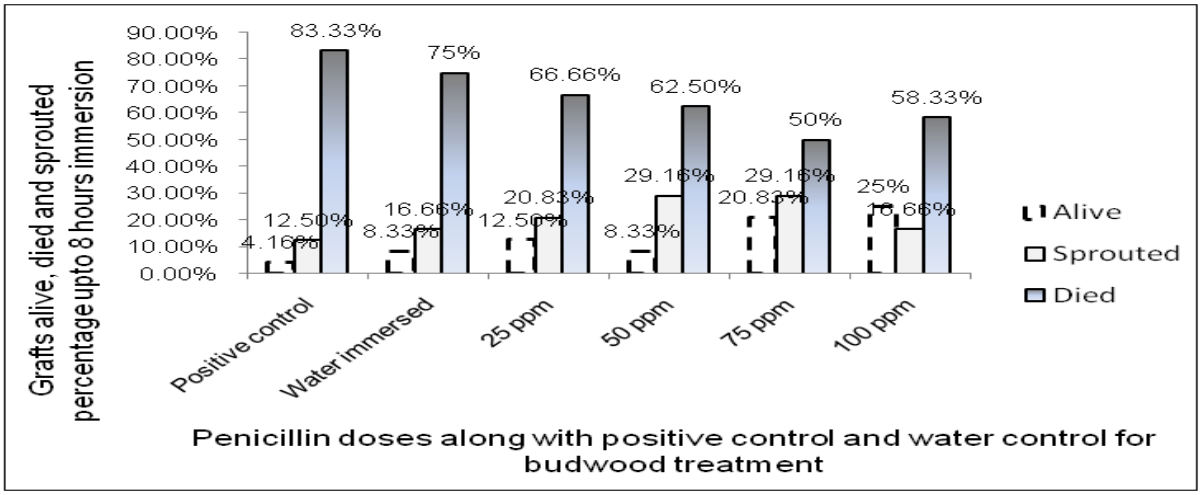

Fig. 5. Percentage of HLB positive grafts survived, sprouted and died upto 8 hours immersion period in penicillin soltions for elimination of *Candidatus* Liberibacter asiaticus.

The uprooting of the HLB infected trees is not the only solution to escape from this disease. There is some germplasm of citrus that is very rare and important to save, so currently the best solution is the treatment of grafts with antimicrobial drugs instead of their spray as most of the antibiotics are heat sensitive and lose their effect in hot weather.

Iodo starch test for HLB diagnosis

The level of starch increases up to six times in HLB affected leaves than healthy ones (Etxeberria *et al.*, 2009). Starch readily reacts with iodine resulting in a very dark grey to black stain.

The iodo starch test was performed according to Etxeberria *et al.* (2007). As this test is very simple and economical, farmers can easily do this test in the fields.

The significance of this test is that it is about 95% accurate and there are only 5% chances of false-positive results for HLB diagnosis as described by Taba *et al.* (2006). It means that a farmer is collecting 95% HLB free buds if he performs this test for the collected grafts and graft source tree.

Conclusion

It is concluded from the results of the current study that penicillin doses at 50 ppm for 8 hours immersion of grafts and 75 ppm for 6 and 8 hours graft immersion proved best for graft survival and sprouting, whereas, 75 ppm for 8 hours graft immersion proved best for graft survival and HLB bacteria elimination as well. The farmers and growers of citrus can use to treat the grafts with penicillin and diagnose HLB easily with iodo starch test.

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