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High altitudes limit the incidence of huanglongbing and its vector, *Diaphorina citri*, in citrus orchards

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Abstract. Citrus fruit are important crops in many regions of the world and are valuable sources of carbohydrates, fibre and health-promoting compounds. Like other crops, production of these fruits is affected by a range of pests and pathogens that can affect the sustainable development of citriculture. The most devastating of these is huanglongbing (HLB), a bacterial disease caused by species of liberibacters, pathogens that are spread by insect vectors. Currently, there is no remedy for HLB, and management relies on the use of disease-free seedlings, the rouging of diseased trees, and pesticides to suppress populations of vectors. Our studies on HLB in Indonesia and Bhutan have shown that citrus grown at higher altitudes (above 1000–1200 m ASL) can remain free from the main vector of the disease, the psyllid *Diaphorina citri*, despite climatic conditions at these altitudes being favourable for the insect. Thus, sustainable production of citrus at these altitudes without the use of polluting agrochemicals may be possible. However, this would be dependent on the production and use of HLB-free propagation material, strict control on the movement of trees into these areas and the adoption of appropriate agronomic practices for the control of other pests and pathogens.

Keywords. Altitude; Citrus; Huanglongbing; Liberibacters; Psyllids

1. Introduction

Citrus fruit are important crops in tropical and subtropical areas and have been targeted for poverty alleviation. They are valuable sources of carbohydrates, fibre and health-promoting compounds that are beneficial for human health [1], as epidemiological studies have suggested these compounds possess antioxidant, anti-inflammation, -mutagenic, -carcinogenic and -aging properties [2,3]. Like other crops, the production of citrus fruits is affected by a range of pests and pathogens, the most devastating of which is huánglóngbìng (HLB). Huánglóngbìng, known as citrus vein phloem degeneration in Indonesia, is the most serious disease facing citriculture worldwide and has become established in more than 40 countries [4]. The disease is caused by phloem-limited bacteria of the candidatus genus Liberibacter, the most devastating of which is the Asian form, '*Candidatus* Liberibacter asiaticus'

(*C*Las: α-Proteobacteria) that is predominantly vectored by *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), the Asiatic citrus psyllid (ACP). Symptoms of the disease include: leaves with asymmetric, blotchy-mottling or chlorosis resembling mineral deficiencies; small upright leaves; vein-corking or yellowing; out-of-season flushing and heavy flowering; small, irregularly-shaped, bitter-tasting fruit; branch dieback; and death of trees [5]. Affected trees may lose their fruiting capacity within 2–5 years from the start of symptoms, and their life span is reduced to 7–10 years [6,7]. In Florida, it is estimated that 90% of the production area and 80% of trees are affected by the disease [8]; losses due to the disease have been estimated to be approximately US$ 1 billion per year [9]. In 2020, HLB was first reported in the state of Bahia [10], the fourth largest citrus producing region of Brazil. Prior economic modelling estimated this incursion could cause US$ 700 million in direct losses over a 20-year period [11]. The area of citrus grown in Indonesia has ranged from 20,000 ha in 1961 to over 13,000 ha in 1996 and 1967 (FAO Statistical Databases for Agriculture: http://www.fao.org/faostat) but has since declined. Annual yields during this interval ranged from 80,000 to 1 million tonnes. Similar cyclic declines have occurred in other countries.

During the early stage of an HLB epidemic, area-wide monitoring, insecticidal vector control and the rogueing of infected trees may slow its progress [12,13], but as an epidemic worsens, the removal of infected trees becomes ineffective [14]. In the medium term, productivity can be maintained through insecticidal control of the vector [15, 16,17]. However, costs of pesticide applications may not outweigh any income from increased yields [18,19,20], and insecticide use inevitably leads to resistance in ACP populations and outbreaks of secondary pests [17]. In Florida, many growers have implemented foliar nutritional programs to help maintain productivity [16,21,22]. However, studies show that these programs may not be effective or economically viable [9,17,21], in addition, an inoculum source remains in the field. Other means of managing the disease have been suggested including the use of antimicrobials, tolerant rootstocks and scion varieties and the biological control of the vector. However, control remains problematic.

The associations among the causal bacterium, the vector and the host plant are affected by the environment. *C*Las can be distinguished from the closely related species '*Ca*. L. americanus' and '*Ca*. L. africanus' on the basis of its temperature sensitivity [23,24], and titres of *C*Las with the plant host may vary with season [25,26] and location [27]. *Diaphorina citri* can tolerate ambient temperatures as high as 50 °C [28] and low temperatures between 5.4–6.9 °C, with minimums from -5.3–7.5 °C [29]; temperature and humidity also affect flight activity [30]. Seasonal effects on the insect host have also been found. In Florida, a study [31] showed that populations of *D. citri* reached their peak in April; however, the highest incidence of *C*Las in the insect occurred in autumn suggesting seasonal effects on titres of bacteria in host plants and the vector [5]. A laboratory-based study [32] has shown that temperature affects both the acquisition of *C*Las and titres within the insect. At higher temperatures, host plants will develop their flush growth earlier and flushing will be of a shorter duration.

The principal cause of the cyclical declines in plantings and production outlined above is HLB, and the disease has prevented successful establishment and development of a sustainable industry seriously affecting the welfare of farmers and national economies. Given the need for alternative and sustainable control strategies for HLB and the effects of the environment on the HLB pathosystem, studies were made in Central Java and Bhutan on the influence of climatic and edaphic conditions and plant growth on the occurrence of the psyllid and the bacterium.

2. Studies in Indonesia

2.1. Methodology

In Indonesia, the studies commenced in 2005 at three sites in Central Java: Purworejo (7.7145 °S, 110.0096 °E, 60 m ASL), low altitude; Grabag (7.3667 °S, 110.3000 °E, 640 m ASL) medium altitude; and Ngablak (7.3963 °S, 110.3893 °E, 1288 m ASL), high altitude. Each location was planted with disease-free Siem mandarin (*Citrus reticulata* Blanco) seedlings with a 2 × 2 m^2 tree spacing. Crop phenology and *D.* citri populations were observed fortnightly on nine central trees from within nine 5 × 5 trees subplots. Automatic records were taken of total radiation, air temperature, relative humidity (RH), rainfall, wind speed and leaf temperature from weather stations at the field sites. Full details of methodologies can be found in [33].

2.1. Occurrence of D. citri *at the experimental sites in Central Java*

Average temperatures were approximately 27, 24 and 20 °C at Purworejo, Grabag and Ngablak, respectively, and at each site did not vary appreciably throughout the year (Figure 1). Rainfall ranged from ~16–470 mm over 7 months, from ~23–580 mm over 8 months, and from ~12–630 mm over 8 months at these locations, respectively, and was either low or absent during the months of June to November. Immature trees planted at each of the three locations produced spring and autumn flush growth and intermittent flush between these seasons. The occurrence of *D. citri* at the three study locations differed. Adults were first found at Purworejo in December 2005 but did not occur regularly until September 2006. At Grabag, they were first observed in March 2007, but not regularly until October 2007. In 2008, adult psyllids were found throughout the year at Purworejo but only from July to December at Grabag. No psyllids were found at Ngablak. The major impediment to tree growth at Grabag and Ngablak was *Oidium citri* (JM Yen) U. Braun with infections at Ngablak being severe.

3. Studies in Bhutan

3.1. Methodology

Studies in Bhutan were conducted in the Tsirang Dzongkhag along the Droopchhugang-Phuensoomgang mountain ridge. Two sets of plants were assessed. The first consisted of sets of twelve, two-year-old, pathogen-free mandarin seedlings planted in May 2013 within existing orchards at each of eight locations with elevations varying between 783–1473 m ASL. Seedling growth was assessed based on leaf and shoot growth. Control plants were maintained at the National Plant Protection Centre (NPPC). The second set of plants consisted of ten mature trees located next to the seedlings.

Assessments of soil chemistry (pH; total C and N; extractable cations (Ca, K, Mg, Na); percentage aluminium saturation; effective cation exchange capacity; percentage exchangeable Na, Al and acidity; and electrical conductivity) at each site were made at the start of the study.

Leaf temperatures of the seedlings were recorded from May to August 2014. Tinytag data loggers recorded ambient air temperatures and RH, and an electronic rain gauge recorded rainfall.

The incidence of *D. citri*, *Diaphorina communis* Mathur and *Cacopsylla heterogena* Li (Psyllidae) was assessed from March to August 2014, and once in April 2015 on the seedlings only. *C*Las in each of the seedlings was assayed using stem bark and fibrous roots, with samples from the plants taken in 2014–2016; mature trees were also tested for *C*Las in 2015 and 2016. Detection of *C*Las in plant samples by real-time PCR (rtPCR) used the method of [34] with analysis of the results based on [35]; conventional PCR (cPCR) used the primer pair, A2/J5 of [36] followed by Sanger sequencing. Further details of the methods can be found in [37].

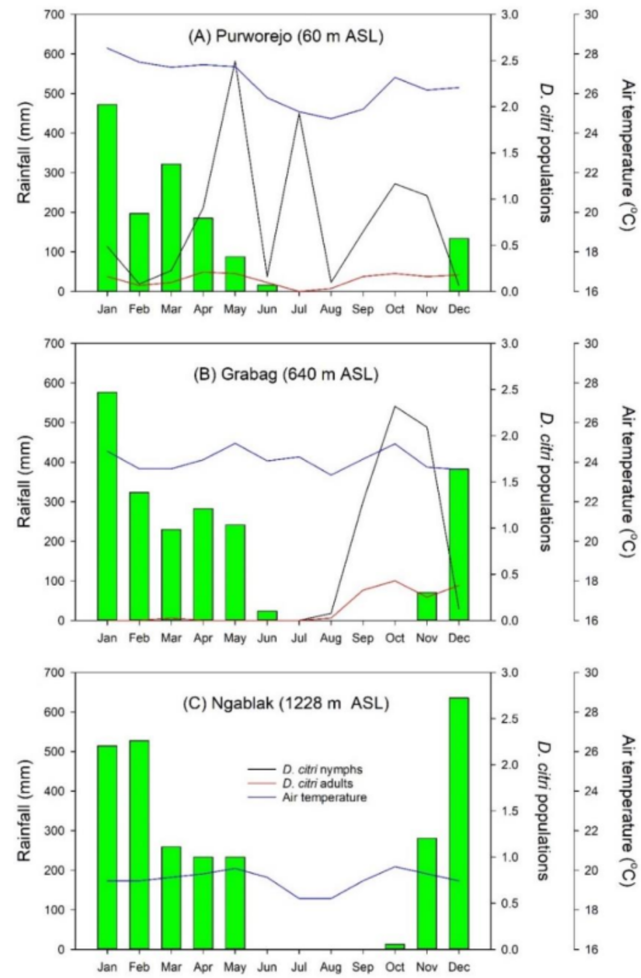

Figure 1. Environmental data and incidence of *D. citri* at the experimental sites in Central Java in 2008.

3.2. Soil chemistry and seeding growth

The soils at all sites were acidic (Figure 2), and regression analysis showed no significant relationships between most aspects of soil chemistry and elevation. The only exception was the C/N ratio; however, the range of this parameter was small.

Multilevel Poisson regression using data from 2014 (Figure 3) showed no significant relationship between leaf numbers and elevation. However, using the data collected in 2015, this technique showed a significant, negative relationship. Plants at the lowest two elevations producing an approximate five-fold higher numbers of leaves compared to those at the highest two elevations. Linear regression analyses found no significant associations of altitude with either shoot death or an absence of flush growth.

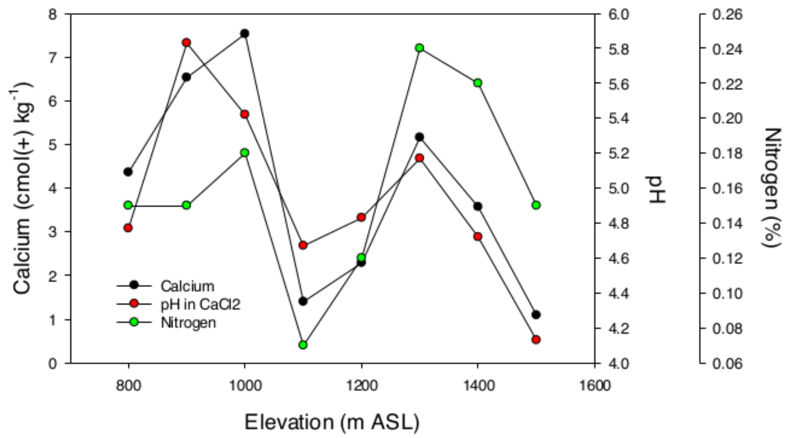

Figure 2. Chemical analyses of the soils from each of the eight experimental sites along Droopchhugang-Phuensoomgang.

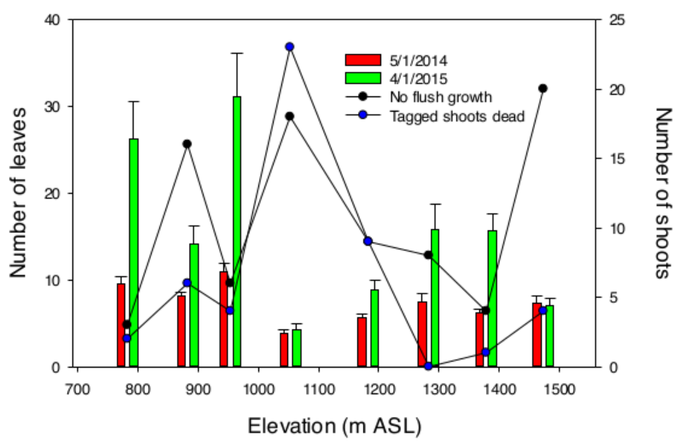

Figure 3. Mean (n = 48) numbers of leaves per terminal shoot (red and green boxes) on mandarin seedlings and numbers of shoots that did not produce flush growth or were dead. Data pertaining to leaves were collected in 2014 and 2015. Four shoots per plant were assessed at each sampling time. Data pertaining to the growth of shoots were collected in 2015.

3.3. Climatic conditions and leaf temperatures

Average temperatures declined with elevation (Figure 4). May to September were the warmest months and December and January the coolest (Figure 5). Leaf temperatures were ~1–3 °C lower than ambient air temperatures in May, and 2–7 °C lower in July−August. Relative humidity increased with elevation; mean minimum RH was higher from June to September and ranged from 66.5−73.4% at 783–1053 m ASL and from 79.3−85.2% at 1183–1473 m ASL (Figure 6).

Total monthly rainfall for 2014 at the sites along the ridge is presented in Figure 7; in 2013 and 2015, the patterns of precipitation for the months in which data were collected were similar. Rainfall during January–April was low and least in November and December. The monsoon season started in May–June and ended in September–October. Rainfall at lower altitude sites (< 1183 m ASL) tended to be less rainfall than at higher altitude sites (≥ 1183 m ASL); however, the differences were small.

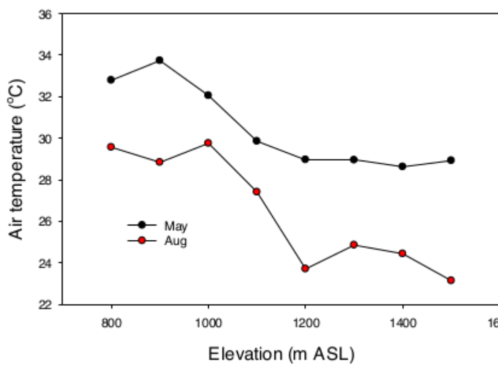

Figure 4. Average ambient temperatures at the experimental sites in 2014.

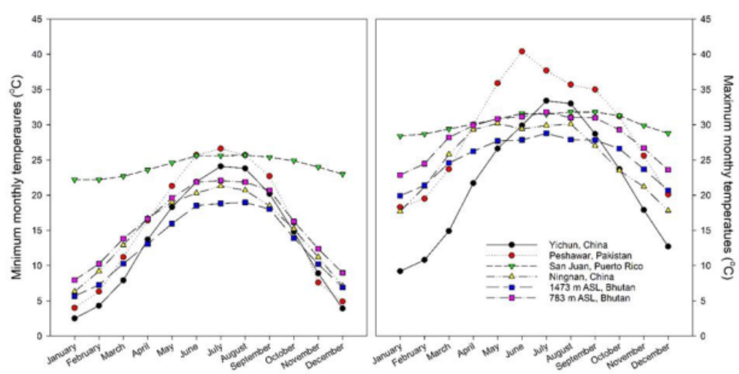

Figure 5. Monthly average minimum and maximum temperatures at locations where *D. citri* occurred and for the highest and lowest of the experiment sites along Droopchhugang-Phuensoomgang.

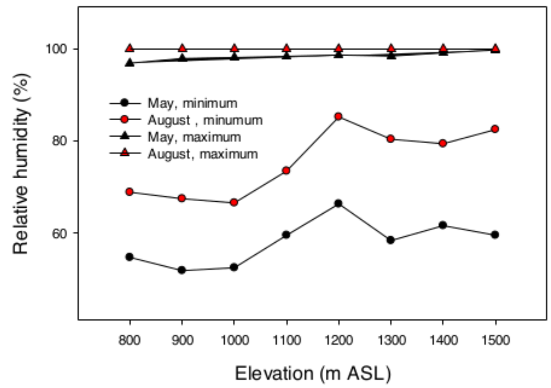

Figure 6. Comparison of average ambient relative humidities at the experimental sites along Droopchhugang-Phuensoomgang in 2014.

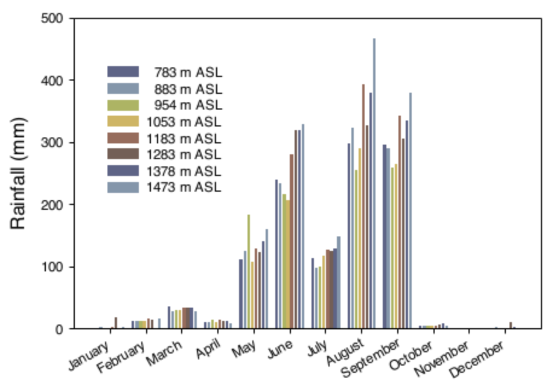

Figure 7. Monthly total rainfall recorded in 2014 at the experimental sites along Droopchugang-Phuensoomgang.

3.4. Psyllid incidence

During the assessment period, adult *D. citri* were found at sites with elevations of 783–1053 m ASL, and immature stages at 883–1053 m ASL (Table 1). Univariable negative binomial regression found a weak negative relationship between psyllid numbers and elevation and weak positive relationships with RH and temperature. There was no significant relationship with rainfall. Adults of *C. heterogena* were not observed on the seedlings at any of the experimental sites; however, immature stages of this psyllid were found within pouch galls at 1378 m ASL in April 2015. Neither immature nor adult stages of *D. communis* were found at any of the sites.

Table 1. Total numbers of *Diaphorina citri* nymphs and adults found on the mandarin seedlings between March 2014 and April 2015.

Elevation (m ASL)	Nymphs	Adults
1473	0	0
1378	0	0
1283	0	0
1183	0	0
1053	1	10
954	1	35
883	4	109
783	0	134

Table 2. Numbers of bark and root samples from young and mature mandarin trees at each elevation along the ridge.

	Young		Mature				
Elevation (m ASL)	Bark (+/T)[a]	Root (+/T)	Bark June 2014 (+/T)	Bark May 2015 (+/T)	Bark March 2016 (+/T)	Root May 2015 (+/T)	Root March 2016 (+/T)
1473	0/12	0/12	3/10	0/10	2/10	6/10	3/10
1378	0/11	0/12	3/10	0/10	1/10	0/10	2/10
1283	0/12	0/12	1/10	1/10	0/10	2/10	0/10
1183	0/11	0/12	1/10	0/10	2/10	5/10	2/10
1053	0/12	0/12	10/10	1/10	10/10	2/10	7/10
954	4/12	7/12	9/10	2/10	10/10	1/10	9/10
883	8/11	7/12	8/10	1/10	9/10	0/10	6/10
783	5/11	4/12	10/10	3/10	10/10	0/10	10/10

[a]+ = number of *C*Las-positive samples; T = total number of samples tested.

3.5. CLas detection in samples mandarin seedlings

Throughout the experiment, no symptoms of HLB occurred on the control seedlings kept at the NPPC, and rtPCR tests showed these seedlings were free of *C*Las.

The results of rtPCR tests of the samples collected from the seedlings (Table 2) showed that four or more of the bark and root samples from those planted at altitudes from 783–954 m ASL were positive for *C*Las (mean Ct values: bark, 22.1–28.3; roots, 26.1–28.6). In contrast, all samples from above 954 m ASL were negative. Sanger sequencing of positive extracts (MN563110–113 & MN563114–116) showed high identity with accessions from Japan, Iran, China and India.

3.6. Presence of CLas in mature trees

*C*Las was detected in most of the bark samples collected at 1053 m ASL or below (Table 2) but in only in a few samples from plants growing at higher elevations.

In the root samples taken in May 2015, the proportions of samples from altitudes between 1183–1473 m ASL that were *C*Las positive were higher than those from lower elevations. In contrast, the majority of the root samples collected in March 2016 from sites at 783–1053 m ASL were *C*Las positive, but the bacterium was not detected in the majority of samples collected from 1183 m ASL and above. Those that were positive had mean Ct values between 29.3–30.5.

4. Discussion

In agriculture, as in other systems, there is a global pressure for a greater use of natural resources and less reliance on synthetic inputs, while maintaining economic viability. This has led agricultural policy makers to promote sustainable agricultural systems [38] that require integrated crop production systems and the adoption of sustainable agricultural practices. These practices focus on the associations between crop plants or livestock and their environment [39] and require the redesign of cropping systems [40]. Our studies in Indonesia and Bhutan demonstrate how a focus on the environment can promote productivity and sustainability.

Results obtained in Central Java, Indonesia showed that *D. citri* was common at the low altitude site (60 m ASL), uncommon at the mid altitude site (640 m ASL) and did not occur at the high altitude site (~1300 m ASL). The trees planted at each of these locations produced seasonal and intermittent flush growth that would be suitable for the development of psyllid populations. At the high altitude site, the annual average temperature is ~17.4°C [33]. The rate of development of *D. citri* is temperature-dependent [41,42], and based on the modelling of population dynamics of *D. citri* with respect to temperature by [43], temperatures at this site should allow approximately seven generations to occur per year. Populations of *D. citri* at the low and mid altitude sites reduced substantially during the monsoon. Chavan and Shummanvar [44] linked seasonally low populations near Pune (18.5202 °N, 73.8570 °E, 558 m ASL) in Maharashtra, India, to monsoon rains. Given the above, the absence of *D. citri* at some elevation between 700 and 1300 m ASL in Central Java is likely due to the effects of seasonal rainfall and fewer generations of the psyllid each year.

In Bhutan, in our studies along Droopchhugang-Phuensoogang, *D. citri* was only found on seedlings planted below 1183 m ASL, and assays for *C*Las only detected the bacterium in the seedlings below 1053 m ASL. In the mature trees along the ridge, *D. citri* was not found at 1175 m and above; these trees retained full canopies. Away from the study site, sightings of psyllids have been made above 1200 m on two occasions, one at 1436 m ASL and the other at 1350 m ASL [45,46]. However, in general, our observations and the observations of [46] suggest that populations of the psyllid in Bhutan are generally restricted to elevations below 1200 m ASL.

In the study of the immature trees planted along the ridge, although statistical analyses showed that psyllid numbers were associated with RH and temperature, the relationships were weak, and we found no substantial evidence that either factor limited the distribution of *D. citri*. There was no relationship with rainfall. Based on [43], temperatures at the highest site could have allowed ~10 annual generations of the psyllid to occur. The average minimum and maximum temperatures at all altitudes along the ridge all lie within the range of monthly temperatures for 18 locations in mainland Asia where *D. citri* has been recorded [37], the data from the two extremes of which are shown in Fig. 5. Therefore, environmental conditions other than temperature and RH appear to restrict, either directly or indirectly, the occurrence of the psyllid and *C*Las at elevations above 1200 m ASL.

Distributions of *D. communis* and *C. heterogena* (Fig. 8) are also related to elevation. Surveys have shown that *Diaphorina communis* occurs up to 1223 m ASL in association with curry leaf and occasionally on mandarin. *Cacopsylla heterogena* was recorded on mandarin between 983 and 1500 m but rarely below 1200 m in Tsirang, up to 2444 m at Talo (27.5516 °N, 89.8238 °E) in Punakha Dzongkhag and on a putative citrus hybrid at 1929 m near Basochhu and at 2118 m near Wengkhar.

A possible explanation for the inability of *D. citri* and *D. communis* to thrive above 1200 m ASL may be ultraviolet (UV) radiation, particularly UVB. Daily totals of global, UVA and erythemal effective radiation have been shown to increase by approximately 8, 9 and 18%, respectively, per 1000 m [47], and Bhutan lies within a region where high UV irradiation occurs [48,49,50]. UV damage in plants is related to and increases with elevation [51], and the resulting changes in plant morphology, physiology and photochemistry can be detrimental to herbivores [52]. Eggs and nymphs of *D. citri* and *D. communis* are directly exposed to UV in contrast to the eggs and nymphs of *C. heterogena* that are shielded inside pouch galls (Fig. 8). Thus, the absence of *D. citri* and *D. communis* above 1200 m in Bhutan could be related to the UV impact on the psyllid through direct damage to tissues and metabolism or to indirect effects on host plants. In addition, '*Candidatus* Carsonella ruddii', the primary endosymbiont of psyllids [53,54], lacks the ability to repair damaged DNA [55]. This organism supplies essential amino acids, and psyllids cannot survive without it [56]. Thus, the inability of *D. citri* and *D. communis* to survive at the higher altitude may also be related to impacts of UV on their primary endosymbionts. Other arthropods observed during our study in Bhutan also displayed behaviours that could be associated with avoidance of UV radiation. An undescribed leaf gall midge (Diptera: Cecidomyiidae), found at elevations ranging from ~800 to 1600 m, develops within wart-like, tubular leaf galls formed by adaxial leaf surfaces rolled inwards along midribs (Fig. 8). Spider mite (Acari: Tetranychidae) colonies were found on abaxial, but not adaxial surfaces, of mandarin leaves at 650 m and *Zanthoxylum* sp leaflets at 1610 m (Fig. 8). Some spider mites are known to stay on abaxial leaf surfaces in order to avoid UV [57] and UV has been reported to reduce the oviposition by two-spotted spider mite (*Tetranychus urticae* Koch) [52].

Our studies in both Indonesia and Bhutan show that above certain altitudes *D. citri*, the main vector of HLB, cannot thrive and pass on the causal bacterium. This helps prolong the productive life of trees at these altitudes without the use of harmful pesticides to control the vector. However, at these higher altitudes, production needs to be based on suitable varieties produced under disease-free conditions. *C*Las has also been recorded in and transmitted by *Cacopsylla citrisuga* Yang & Li [58,59], a species closely related to *C. heterogena* [60]. Thus, *C. heterogena* may transmit the pathogen; however, the low incidence of the pathogen in mature orchards in Tsirang above 1200 m ASL in our study suggests negligible rates of transmission by this psyllid, if transmission does occur. Mineral oils provide an environmentally-friendly alternative to hard pesticides [61,62,63,64] and should be evaluated for suppression of populations of *C. heterogena* and other citrus psyllids. In addition, powdery mildew caused by *Oidium citri* [65] occurs on mandarin in most citrus-growing areas of Bhutan and was also found at the medium and high-altitude sites in Central Java; control of this disease is needed. Several environmentally-friendly options should be evaluated including bicarbonates [66], sulphur [67], milk [68] and mineral oils [69,70].

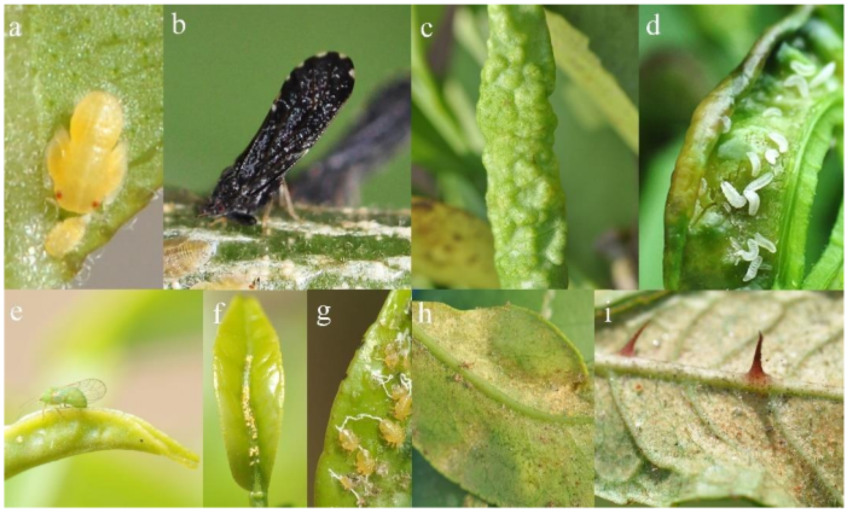

Figure 8. (a) Fifth instar *Diaphorina communis* nymph on a curry leaf (*Bergera koenigii* L.) leaflet, (b) adult *D. communis* on a *B. koenigii*, (c) wart-like leaf gall of an undescribed cecidomyid, (d), larvae of the undescribed cecidomyid, (e) adult *Cacopsylla heterogena* female ovipositing in a mandarin leaf pouch-gall, (f) eggs of *C. heterogena* lining the midrib of a mandarin leaf as the leaf begins to form a pouch gall, (g) late-stage *C. heterogena* nymphs on an adaxial surface of a prised mandarin leaf pouch-gall, (h) a spider-mite infestation on an abaxial surface of a mandarin leaf, and (i) a spider-mite infestation on an abaxial surface of a *Zanthoxylum* sp. leaflet.

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