Advances in Biological Sciences Research, volume 19
Proceedings of the 2nd International Conference on Smart and Innovative Agriculture (ICoSIA 2021)

Accumulation of Experimental Data for the Introduction of Smart Plant Protection Systems

Cases of Transboundary and High-risk Diseases

Takashi Fujikawa[1,*]

[1] *Institute for Plant Protection, National Agriculture and Food Research Organization (NARO), Japan*
[*] *Corresponding author. Email: ftakashi@affrc.go.jp*

ABSTRACT
Smart agriculture will be essential for sustainable food production. Above all, the implementation of smart plant protection is highly expected as a way to protect crops from pests. However, for this purpose, it is necessary to collect a lot of experimental data, link it with various information, and analyze them. This topic introduces three transboundary and high-risk diseases and shows how each experimental data relates to smart plant protection.

Keywords: *citrus greening, huanglongbing (HLB), Candidatus Liberibacter asiaticus (Las), Candidatus Liberibacter solanacearum (Lso), Pseudomonas syringae pv. actinidiae (Psa), PCR detection, genome, seed-transmissibility.*

1. INTRODUCTION

Securing and streamlining the workforce, passing on farmers' skills, increasing productivity and improving economic outcomes are important challenges in the agriculture around the world. Furthermore, as a global effort to achieve sustainable development goals (SDGs), farmers and agronomists need to be serious about increasing yields and securing food, paying attention to overuse of chemical pesticides and fertilizers. And it is expected that "smart agriculture" will solve these problems [1, 2]. The essence of "smart agriculture" is outlined as follows; the sensing data of the physical spaces of agriculture (which may be crop fields or inspection sites) is analyzed in cyberspace, and the optimal real-time responses/actions in the physical spaces are introduced. Therefore, it is essential to develop the internet and intranet as the communicating means of information, but more importantly, we need to clarify what kind of sensing data is collected, how to analyze the data, and what countermeasures can be taken. Works such as data collection, data analysis, and proposing countermeasures are dependent on big data mining, and the use of artificial intelligence (AI) technology is indispensable. As you know, AI technology has made great progress recently due to the improvement in machine learning accuracy and the dramatic development of computing machines. However, it is often misunderstood by the general publics that "because AI, not humans, analyzes the data, AI derives the results which humans don't think of, and the countermeasures proposed by AI must be very good." The cause of this misunderstanding seems to be that people have the following unreasonable hopes for AI as; "AI knows facts that humans cannot understand because they can perform calculations that humans cannot do", "AI has information that people cannot collect", and "even data values are somewhat sloppy, AI derives statistically meaningful conclusions", and "AI replaces missing data values with reasonable numbers". However, whether it is AI or human beings, data analysis containing unexplained missing values or unknown events must be inaccurate. There is no choice but to upgrade the accuracy the data values by properly cycling PDCA (plan-do-check-act) for data mining. Especially, it will be essential to link and analyze "wet" experimental data with various environmental information (geographical, meteorological, botanical, pedological, etc.).

Although the above-mentioned issues remain in smart agriculture, I think that the lack of information is remarkable in the field of plant protection especially. At first glance, "smart plant protection" seems to be a

technology that can be implemented at an early stage because methods for calculating the outbreak prediction of pests based on meteorological information have been known for a long time. It seems also possible to predict the QUALITATIVE prevalence of disease depending on between the race types of pathogens and the presence/absence of resistance of the host variety. However, it is difficult to predict at present how the local effects of micrometeorology affect the occurrence of various diseases, and the QUANTITATIVE prevalence of disease depending on between the race types of pathogens and the presence/absence of resistance of the host variety. In the first place, it is not possible to calculate a model of damage spread without clarifying how widespread the diseased plants are in the field, and it is not possible to determine what to plant in the field without knowing about the host range of the pathogens. When actually implementing the AI-driven smart plant protection systems, it seems to be an obstacle for data mining that no one (also AI) knows enough the disease information (the pandemic/endemic epidemiology, biological characteristics of pathogens, environmental effects to pathogens and plants, and the pathogen/host relationships, and so on). Therefore, in order to promote the smart plant protection systems, it is necessary to collect information by conducting sufficient on-site disease surveys, "wet" experiments and observations. In this topic, I will introduce some examples of conducting on-site investigations and "wet" experiments while being aware of how they are involved in smart plant protection. In particular, among our previous studies, I will focus on transboundary and high-risk diseases that are not yet fully understood and are required to be dealt with worldwide.

2. CASE OF CITRUS GREENING DISEASE

2.1. Citrus greening and Candidatus Liberibacter asiaticus

Citrus greening (huanglongbing; HLB) disease is a devastating disease of citrus trees with high economical costs to the worldwide citrus industry. Symptoms include blotchy chlorosis and/or mottling of leaves; yellowish shoots; vein corking; stunted growth; poor root growth; small, green, and malformed fruits; and finally, death [3]. This disease is caused by three species of phloem-limited fastidious bacteria, "Candidatus Liberibacter asiaticus" (Las), "Ca. L. africanus, " and "Ca. L. americanus" [3]-[7]. And these pathogens are transmitted by grafting and by the sap-sucking psyllids Diaphorina citri and Trioza erytreae [3]. And Las is in the most widespread (e.g., Asia, Brazil, and North America) [3], [8], [9]. All major commercial citrus cultivars are susceptible to this species, and no effective control in practical use is known other than the removal of infected trees. Therefore, in areas in which greening has not become established, rapid identification and culling of infected trees and budwoods in quarantine are the most important control measures. Various DNA amplification methods, including polymerase chain reaction (PCR) have been used to test greening-infected plants [10], but obtaining stable results is often difficult at an early stage of infection when the bacterial density in trees is low. If the test results differ depending on the PCR method, it will be difficult to confirm the infected trees. Namely, the data on the spread status in the survey area may change depending on the detection accuracy of PCR. In order to prevent this, it is important to use the PCR method with as high detection accuracy as possible. Therefore, our group developed a highly sensitive PCR method [11].

2.2. Highly sensitive and accurate endpoint PCR of Las

In a re-examination of the specificity of primers used in PCR tests for greening-infected plants, we found a Las-specific sequence region after aligning and comparing the various bacterial 16S rDNA sequences and were able to design new primers with high sensitivity and accuracy [12]. The endpoint PCR (conventional PCR) using the new primer set (Las606/Lss) was more sensitive than with any other known primer sets, including those often used at inspection sites, and we confirmed the high specificity for Las. We have also developed a direct PCR method that does not require DNA extraction, enabling easy and rapid genetic testing [13]. This has made it possible to increase the number of trees to be inspected.

2.3. Use of detection method for greening disease in smart plant protection

In Japan, greening disease occurs in some islands in Kagoshima prefecture and in Okinawa prefecture [14, 15]. By introducing our methods and the related technologies, inspections are becoming more efficient. In the area where greening disease occurs, the number of tests can be increased to clarify the presence or absence of infection. As shown here, depending on accuracy and number of tests, PCR detection influences the determination of the number of infected trees and the area where greening disease occurs. The improvement of the PCR method is a "wet" experiment, but which makes it possible to collect accurate disease test result data. By using these data, it is expected that it will be possible to understand the spread situation, predict the onset of the disease, and set the inspection area. It is thought to lead to outbreak prediction in smart plant protection.

3. CASE OF BACTERIAL CANKER OF KIWIFRUIT

3.1. Bacterial canker disease of kiwifruit

Bacterial canker is a serious disease that causes severe damage to Actinidia plants including kiwifruit, and production countries around the world have been taking precautions to prevent its invasion [16]. Nevertheless, the area where this disease is present has expanded, and its occurrence has been currently confirmed in 18 countries, including Japan. Therefore, this disease is now thought to be in a pandemic state [17]. Actinidia plants are considered to be native to East Asia, and more than 50 kinds of wild species grow naturally in the mountains of the East Asia region, centering on China. The genus Actinidia is also distributed widely in Japan. And the bacterial canker disease of kiwifruit was first recognized around 1980 in Japan [16]. The causative agent of this disease is *Pseudomonas syringae* pv. *actinidiae* (Psa), which causes systemic symptoms, such as spots on leaves, dying of flower buds, shoots and branches, cracking or canker in branches and trunks, white or reddish-brown exudates leaking from pruning cuts, scars, lenticels and lesions. In particular, infection with Psa biovar 3 (the variety-level taxon that includes strains causing the pandemic), which is considered highly virulent, can lead to the death of heavily diseased trees. Interestingly, various groups have been found in Japan, not just the first reported group in the world (currently classified as biovar 1) and the group causing the pandemic (biovar 3). So far, Psa biovar 1, 3, 5, 6, and *Pseudomonas syringae* pv. *actinidifoliorum* (this pathogen has been classified to another pathotype from biovar 4, currently) have been found in various parts of Japan [16]. In Japan, nationwide surveys of "bacterial canker of kiwifruit disease" have succeeded in finding such various Psa biovars. Then, to clarify the genetic relationship of these biovars, genome analysis of each biovar was required.

3.2. Genomes of various biovars of Pseudomonas syringae pv. actinidiae

So far, we have been sequencing the genomes of domestic Psa biovars [18-21]. We compared these genomes and clarified the characteristics among biovars. The biggest difference among Psa biovars is that they have different types of phytotoxins. Many biovar 1 have the synthetic gene cluster of phaseolotoxin [20], and biovar 6 has the synthetic gene clusters of phaseolotoxin and coronatine, respectively [19]. However, no known phytotoxin synthetic gene cluster has been identified for biovar 3 [21] and biovar 5 [18]. In addition, we found that there are common type III effectors of Psa and biovar-specific type III effectors. The type III effectors are known as virulence factors involved in pathogen-plant interactions (mainly in the field of plant immunity) [22]. By pursuing the functions of phytotoxins and the combination of type III effectors among biovars, it will be possible to understand the essence of pathogenicity to kiwifruit and to estimate the host range.

3.3. Use of genome information for bacterial canker of kiwifruit in smart plant protection

From genome analysis, we found that Psa is a highly diverse pathogen with respect to both phenotypes and genotypes. It is possible that unknown biovars are still hidden in the source population distributed on the phyllosphere of wild *Actinidia* plants throughout East Asia [16]. In recent years, ecological research including analysis of various environmental DNAs using next-generation sequencers (NGS) has been progressing. The number of gene sequences registered in DDBJ/EMBL/GenBank is increasing day by day, but there are many gene sequences that no one knows yet in the world. In smart plant protection, it is important to accumulate and utilize a large amount of pathogen genomic information in order to put NGS-based comprehensive pathogen detection into practical use. By analyzing big data consisting of a large amount of genomic information, it may be possible to find the key to infer the existence of a pathogen even with a small gene sequence.

4. CASE OF LSO CONTAMINATED CARROT SEEDS

4.1. "Candidatus Liberibacter solanacearum" in carrot seeds

"*Candidatus* Liberibacter solanacearum" (Lso) is an unculturable gram-negative bacterium and the causal agent of potato zebra chip disease and carrot Lso disease [23, 24]. Symptoms of Lso carrot disease include leaf curling, leaf discoloration, stunted shoots and roots, secondary root proliferation, and reduced edible root volume [23, 25]. Although Lso is known to be normally transmitted by insect vectors, grafting, and vegetative propagation, Lso was also reported to be seed transmitted [26]. If seedlings (plants developed from seeds) derived from Lso-infected plants are naturally infected with Lso, the spread of this disease in carrot-producing regions is unavoidable. Since the major vegetable seeds are distributed internationally every day and sown in the production areas worldwide, understandably, carrot seed producers, carrot farmers, seed companies, and plant quarantine agencies in various countries are concerned about potential economic damage. On the other hand, recent studies have also reported that Lso seed transmission is unlikely to occur in some circumstances [27-29]. Since it was unclear whether seed transmission occurs or not, our group also investigated the possibility of seed transmission of this disease.

4.2. Seed-transmissibility of Lso in carrot seeds

First, we updated the Lso-specific detection primers for PCR. Following our Las-specific primer set design for citrus greening disease [12], we have developed primers (and a TaqMan probe) that can be used in both endpoint and real-time PCR [30]. Next, we obtained carrot seeds that were actually contaminated with Lso, and we confirmed that the Lso on seeds was alive [31]. Since Lso is difficult to culture and its survival cannot be confirmed in the culture test, we could confirm the survival of Lso by detecting Lso RNA. Then, we sowed the seeds that are contaminated with live Lso, and we investigated whether Lso was detected from the seedlings grown out and symptoms appeared. Namely, we evaluated the possibility of carrot seed transmission of Lso using grow-out tests and probability analyses [32]. Based on the contamination rate of contaminated seeds and the number of diseased seedlings (actual number was 0), the upper limit of the proportion of transmission was calculated by a statistical method. Consequently, we found that the proportion of transmission was extremely small, resulted in that seed transmission of Lso is unlikely in practice.

4.3. Use of seed transmissibility information in smart plant protection

A big problem with AI-driven information collection and analysis in smart plant protection is thought to be how to assess the accuracy of the collected information. Can we scoop up old (and essential) information that has sunk into the deep sea of information? Can we monitor high impact but unrealistic information? There are still many challenges in the informatics of a smart society. Here, seed transmissibility is a plant pathological theme that has long been a concern. The fact that pathogens are transmitted via seeds is high impact and easy to be publicly known than the fact that pathogens are not transmitted via seeds. Information on the pathogenicity and transmissibility of plant pathogens is often biased. AI may be able to correct these biases in the future, but at least for now, scientists need to be very careful. Research on seed transmissibility of pathogens may be a typical example.

5. CONCLUSION

The most actively researched in smart agriculture are high-spec sensor devices, automated machines and vehicles, remote sensing by artificial satellites, image analysis and data mining of legacy agricultural data [1, 2]. These research outcomes have been increasing rapidly in recent years. In our group as well, AI-driven smart plant protection technology including image analysis, drone work, utilization of satellite photographs, chemical composition analysis and genetic information analysis, are studied and developed for various diseases including citrus greening [33]. On the other hand, biochemical research on pests, which is a conventional agricultural study, may be regarded as a sober and muddy work. However, as shown in this topic, the results of long-standing research will eventually be used as analytical data in smart agriculture. Accumulation of experimental data is increasingly needed and contributes to smart and innovative agriculture.

ACKNOWLEDGMENTS

We also thank the members of the transboundary and high-risk pest control group, division of core technology for pest control research, Institute for plant protection (IPP), NARO, for their helpful discussions.

REFERENCES

[1] MAFF (2021) Promotion of Smart Agriculture. [https://www.maff.go.jp/e/policies/tech_res/smaagri/robot.html]. Accessed 12 October 2021

[2] FAO (2021) Climate-Smart Agriculture. [http://www.fao.org/climate-smart-agriculture/en/]. Accessed 12 October 2021

[3] Bové JM (2006) Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. J Plant Pathol 88: 7–37.

[4] Jagoueix S, Bové JM, Garnier M (1994) The phloem-limited bacterium of greening disease of citrus is a member of the α subdivision of the proteobacteria. Int J Sys Bacteriol 44: 379–386.

[5] Garnier M, Bové JM (1996) Distribution of the huanglongbing (greening) liberobacter species in fifteen Africans and Asian countries. In: Proceedings of the 13th Conference of the IOCV, Riverside, CA. IOCV. pp. 388–391.

[6] Garnier M, Jagoueix-Eveillard S, Cronje PR, Le Roux HF, Bové JM (2000) Genomic characterization of a liberibacter present in an ornamental rutaceous tree, Calodendrum capense, in the Western Cape province of South Africa. Proposal of 'Candidatus Liberibacter africanus subsp. capensis'. Int J Sys Evol Microbiol 50: 2119–2125.

[7] Teixeira DC, Saillard C, Eveillard S, Danet JL, de Costa PI, et al. (2005) 'Candidatus Liberibacter americanus', associated with citrus huanglongbing (greening disease) in São Paulo state, Brazil. Int J Syst Evol Microbiol 55: 1857–1862.

[8] Halbert SE (2005) The discovery of huanglongbing in Florida. In: Proceedings of the 2nd International Citrus Canker and Huanglongbing Research

Workshop, Florida Citrus Mutual, Orlando, FL. p. H-3.

[9] Teixeira DC, Danet JL, Eveillard S, Martins EC, De Jesus WC Jr, et al. (2005) Citrus huanglongbing in São Paulo state, Brazil: PCR detection of the 'Candidatus' Liberibacter species associated with the disease. Mol Cell Probes 19: 173–179.

[10] National Research Council (2010) Strategic planning for the Florida citrus industry: addressing citrus greening disease. The National Academies Press, Washington DC. http://www.nap.edu/catalog/12880.html

[11] Fujikawa, T (2014) Studies on early and highly sensitive detection of citrus greening pathogenic bacterium. J Gen Plant Pathol 80, 523–524.

[12] Fujikawa T, Iwanami T (2012) Sensitive and robust detection of citrus greening (huanglongbing) bacterium "Candidatus Liberibacter asiaticus" by DNA amplification with new 16S rDNA-specific primers. Mol Cell Probes 26: 194–197

[13] Fujikawa T, Miyata S, Iwanami T (2013) Convenient detection of the citrus greening (huanglongbing) bacterium 'Candidatus Liberibacter asiaticus' by direct PCR from the midrib extract. PLoS One 8: e57011

[14] Miyaji, K (2012) A successful eradication program in Amami islands, Kagoshima prefecture, Japan. FFTC-NTU International Symposium on Epidemiology and Disease Management of Citrus Huanglongbing Disease for Sustainable Citrus Production in the Asian-Pacific Region. S17. Food and Fertilizer Technology Center.

[15] Uesato, T (2012) Protection of citrus production areas by regional control in Okinawa Island. FFTC-NTU International Symposium on Epidemiology and Disease Management of Citrus Huanglongbing Disease for Sustainable Citrus Production in the Asian-Pacific Region. S16. Food and Fertilizer Technology Center.

[16] Sawada H, Fujikawa T. 2019. Genetic diversity of Pseudomonas syringae pv. actinidiae, pathogen of kiwifruit bacterial canker. Plant Pathol 68: 1235-1248

[17] CABI (2021) Pseudomonas syringae pv. actinidiae (bacterial canker of kiwifruit). [https://www.cabi.org/isc/datasheet/45002]. Accessed 12 October 2021

[18] Fujikawa T, Sawada H. 2016. Genome analysis of the kiwifruit canker pathogen Pseudomonas syringae pv. actinidiae biovar 5. Sci Rep 6: 21399

[19] Fujikawa T, Sawada H. 2019. Genome analysis of Pseudomonas syringae pv. actinidiae biovar 6, which produces the phytotoxins, phaseolotoxin and coronatine. Sci Rep 9: 3836

[20] Fujikawa T, Hatomi H, Sawada H. 2020. Draft genome sequences of 10 strains of Pseudomonas syringae pv. actinidiae biovar 1, a major kiwifruit bacterial canker pathogen in Japan. Microbiol Resour Announc 9: e00759-20.

[21] Fujikawa T, Sawada H. 2020. Draft genome sequences of nine Japanese strains of the kiwifruit bacterial canker pathogen Pseudomonas syringae pv. actinidiae biovar 3. Microbiol Resour Announc 9: e01007-20.

[22] Coombes BK. 2009. Type III secretion systems in symbiotic adaptation of pathogenic and non-pathogenic bacteria. Trends in Microbiol 17(3): 89–94

[23] EPPO (2013) Candidatus Liberibacter solanacearum. Bull OEPP/EPPO Bull 43: 197–201

[24] EPPO (2017) PM 9/25 (1) Bactericera cockerelli and 'Candidatus Liberibacter solanacearum'. Bull OEPP/EPPO Bull 47: 513–523

[25] Munyaneza JE, Fisher TW, Sengoda VG, Garczynski SF, Nissinen A, Lemmetty A (2010) First report of "Candidatus Liberibacter solanacearum" associated with psyllid-affected carrots in Europe. Plant Dis 94:639

[26] Bertolini E, Teresani GR, Loiseau M, Tanaka FAO, Barbe S, Martinez C, Gentit P, Lopez MM, Cambra M (2015) Transmission of 'Candidatus Liberibacter solanacearum' in carrot seeds. Plant Pathol 64:276–285

[27] Loiseau M, Renaudin I, Cousseau-Suhard P, Poliakoff F, Gentit P (2017) Transmission tests of 'Candidatus Liberibacter solanacearum' by carrot seeds. Acta Hortic 1153: 41–46

[28] Loiseau M, Renaudin I, Cousseau-Suhard P, Lucas PM, Forveille A, Gentit P (2017) Lack of evidence of vertical transmission of 'Candidatus Liberibacter solanacearum' by carrot seeds suggests that seed is not a major transmission pathway. Plant Dis 101: 2104–2109

[29] Oishi M, Hoshino S, Fujiwara Y, Ushiku S, Kobayashi Y, Namba I (2017) A comparison of protocols to detect Candidatus Liberibacter solanacearum from carrot seeds, research on the effectiveness of propidium monoazide treatment and evaluation of seed transmission in carrot seeds. Res Bull Plant Prot Japan 53:111–117 (in Japanese with English summary)

[30] Fujiwara K, Fujikawa T (2016) Primers for both conventional and realtime PCR in diagnosis of ‘Candidatus Liberibacter solanacearum. J Plant Pathol 98: 63–68

[31] Fujikawa T, Taguchi M, Kido K, Kusano S, Enya J, Mihara T, Kaku H, Sato M (2019) Evaluation of an RNA-based PCR assay to detect viable Candidatus Liberibacter solanacearum (Lso) in Lso contaminated carrot seed using different disinfection methods. J Plant Pathol 102: 205–211

[32] Fujikawa T, Yamamura K, Osaki K, Onozuka N, Taguchi M, Sasaki A, Sato M (2020) Seed transmission of ‘Candidatus Liberibacter solanacearum’ is unlikely in carrot. J Gen Plant Pathol 86: 266–273

[33] Iwanami T (2021) Occurrence and control of citrus greening (huanglongbing, HLB) in Japan. JARQ (under publication)