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SHORT COMMUNICATION

Direct Isolation Method for Tomato-Infecting *Phytophthora infestans*

Metode Isolasi Langsung untuk *Phytophthora infestans* yang Menginfeksi Tanaman Tomat

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ABSTRACT

Infection of *Phytophthora infestans* at high altitudes in tropical regions causes symptoms of tomato late blight throughout the year. Even though it is very easy to find in the field, *P. infestans* is often very difficult to isolate aseptically in the laboratory. This study aims to evaluate direct isolation techniques that can increase the success of isolating *P. infestans*. Isolation was carried out on a non-specific medium, consisting of potato dextrose agar (PDA), corn meal agar (CMA), oatmeal agar (OMA), and water agar (WA) with three alternative types of antibiotics, i.e. chloramphenicol, amoxicillin, and rifampicin. Observations were carried out to determine the effect of the medium on sporangia induction and the age of the original colony's growth in CMA and OMA media. The results showed that the isolation of *P. infestans* using the direct method was successfully carried out on non-specific PDA, CMA, OMA and WA medium. The highest isolation success rate was obtained on CMA medium with the addition of 50 mg L^{-1} rifampicin. The fastest sporangia induction (8 days) was shown by colonies grown on OMA medium with the addition of 50 mg L^{-1} rifampicin based on the category of abundant sporangia after 20 days of incubation. Rejuvenation of *P. infestans* colonies for research purposes in the laboratory is recommended to be carried out routinely twice a month. This research provides practical guidance for understanding the bioecology of *P. infestans* infecting tomato plants, especially for further study on oomycetes fungi.

Keywords: incubation, late blight, non-specific medium, rifampicin, sporangia

ABSTRAK

Infeksi *Phytophthora infestans* pada dataran tinggi di wilayah tropis menyebabkan gejala penyakit busuk daun tomat sepanjang tahun. Meskipun sangat mudah ditemukan di lapangan, tetapi *P. infestans* seringkali sangat sulit diisolasi secara aseptik di laboratorium. Penelitian ini bertujuan untuk mengevaluasi teknik isolasi langsung yang dapat digunakan untuk meningkatkan keberhasilan isolasi *P. infestans*. Isolasi dilakukan pada medium non-spesifik, yang terdiri atas agar-agar dekstrosa kentang (PDA), agar-agar jagung (CMA), agar-agar oatmeal (OMA), dan agar-agar air (WA) dengan tiga alternatif jenis antibiotik yaitu kloramfenikol, amoksisilin, dan rifampisin. Pengamatan dilakukan untuk mengetahui pengaruh medium terhadap induksi sporangia dan pengaruh umur koloni tetua

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terhadap pertumbuhannya pada medium CMA dan OMA. Hasil pengujian menunjukkan bahwa isolasi *P. infestans* dengan metode langsung berhasil dilakukan pada medium non-spesifik PDA, CMA, OMA dan WA dengan tingkat keberhasilan isolasi tertinggi diperoleh pada medium CMA dengan penambahan 50 mg L^{-1} rifampicin. Induksi sporangia tercepat (8 hari) ditunjukkan oleh koloni yang ditumbuhkan pada medium OMA dengan penambahan 50 mg L^{-1} rifampicin, yaitu berdasarkan pada kategori jumlah sporangia yang berlimpah setelah 20 hari inkubasi. Peremajaan koloni *P. infestans* untuk keperluan penelitian di laboratorium disarankan untuk dilakukan secara rutin dua kali dalam sebulan. Penelitian ini memberikan panduan praktis untuk memahami bioekologi *P. infestans* yang menginfeksi tanaman tomat, terutama bagi para peneliti oomycetes dalam mengisolasi dan mengkaji patogen ini lebih lanjut.

Kata kunci: busuk daun, inkubasi, medium non-spesifik, rifampisin, sporangia

Phytophthora infestans (Mont.) de Bary, the most invasive pathogen causing late blight disease, has become a constraint to many horticultural crops, including potatoes and tomatoes (Nelson 2008; Bush *et al.* 2012; Nowicki *et al*. 2012; Dangi *et al*. 2020). The symptoms of *P. infestans* infection are easily found in tropical countries like Indonesia, particularly in tomato-producing regions in high land with cold temperatures. The typical symptoms manifest on the leaves and fruits, resulting in increased losses in marketable production. Nelson (2008) explains that this pathogen can spread rapidly in cool and wet conditions, causes plant death within a few days, and results in total yield loss. In contrast to studies on potatoes, there have been relatively few reports of this disease on tomatoes in Indonesia over the past five years.

The current most common method to isolate and culture *P. infestans* is based on zoospores collection. In these methods, zoospores were collected from infected leaf tissues or rotten fruits from the field (Griffith *et al*. 1995; Oyarzun *et al*. 1998; Tumwine *et al*. 2000). However, these methods have several drawbacks that lead to a poor isolation success rate. Since it takes time to travel from the field to the laboratory, it is possible that sporangia on the leaf surface were discharged into the atmosphere and mixed with other pathogens. Moreover, according to Plancarte *et al*. (2017), *Phytophthora* spp. should not be isolated from necrotic tissue due to the highly potential presence of saprophytic bacteria and fungi. In an attempt to isolate *P. infestans* in the field, Suzuki *et al*. (2019) applied a method in which the infected leaf surface was taped onto the agar medium. Despite the use of a specific agar medium (Rye-B agar), the isolation failure rate remained high due to contamination by diverse microorganisms.

It is quite challenging to culture *P. infestans* axenically due to its near-obligate biotrophic nature, making it prone to agar culture contamination (Griffith *et al*. 1995). This pathogen and bacteria coexist in plant-microbe interactions. However, the mechanism by which *P. infestans* interacts with the microbes in its environment to develop ecological adaptation remains unexplained (Berendsen *et al*. 2012; Wang *et al*. 2020). Kong and Hong (2016) have demonstrated that bacteria are essential sources of virulence signals that help *Phytophthora* sp. infect plants. We therefore hypothesize that one of the main challenges facing scientists studying this oomycete is its isolation process. Another area for improvement with culture is obtaining the expensive and hard-to-find components for the specific medium (e.g. V8 agar or rye agar). To isolate *P. infestans* from the field, we evaluated some non-specific medium in addition to a simple isolation method. It is essential to add antibiotics to the agar medium during the isolation process to suppress the growth of various microorganisms. Oomycetes researchers have extensively studied the use of different antibiotics on agar medium in their quest for optimal isolation methods. A notable study by Masago *et al*. (1976) demonstrated the successful suppression of bacteria, non-pythiaceous fungi, and twelve species of *Pythium* spp. when isolating

Phytophthora spp. from soil and infected plant tissues. Their findings clearly show the effectiveness of nystatin, rifampicin, and ampicillin in inhibiting various *Phytophthora* spp. without affecting mycelial growth or spore germination.

This research was conducted at the Plant Mycology Laboratory, Department of Plant Protection, IPB University. We sampled and isolated *P. infestans* directly in the tomato fields from IPB Experimental Station at Pasir Sarongge, Pacet District, Cianjur Regency, West Java. This field location was ideal for direct isolation because it had a high prevalence of *P. infestans* infection for the last three years.

The field visit was conducted in the early morning, right after rainfall the previous day. This made it easier to identify symptoms in the form of relatively new lesions on the leaves of infected tomato plants (Figure 1a and 1b). Direct sporangia sampling was carried out on various medium including potato dextrose agar (PDA, HiMedia Laboratories Pvt. Ltd), corn meal agar (CMA, HiMedia Laboratories Pvt. Ltd), oat meal agar (OMA: rolled oats and Bacto agar), and water agar (Bacto agar, Difco Laboratories, USA) which were supplemented with three types of antibiotics, i.e. chloramphenicol (500 mg L^{-1}), rifampicin (50 mg L^{-1}), and amoxicillin (100 mg L^{-1}). Antibiotics and medium were used to stimulate the growth of sporangia. In each agar plate, sporangia were obtained by scraping the sterile inoculation loop from the freshly created lesion and sealed (Figure 1c). The samples were transported to the laboratory and incubated for 48 hours at 16 °C. The isolated fungal colonies were identified by morphological observation, as described in Drenth and Sendall (2001). Additionally, the growth of the colonies was assessed every 12 to 72 hours.

Compared to the other three agar medium, CMA supplemented with 50 mg L^{-1} rifampicin yielded the best results (Table 1). However, on the same media, the fungi failed to grow with the addition of 500 mg L^{-1} chloramphenicol and only 1 plate of the fungi grew with the addition of 100 mg L^{-1} amoxicillin. Isolation using OMA also provides quite effective results when 50 mg L^{-1} rifampicin or 100 mg L^{-1} amoxicillin is added. Meanwhile, the use of PDA and water agar as a medium only gives a low rate of success.

Figure 2 shows the pure colonies of *P. infestans* on PDA, CMA, and OMA supplemented with 50 mg L^{-1} rifampicin on each medium. These results prove that rifampicin is the most effective antibiotic for isolating *P. infestans*. Rifampicin inhibits bacterial DNA-dependent RNA synthesis and prevents the production of host bacterial proteins, targeting mycobacteria and gram-negative bacteria (Sarker *et al*. 2020).

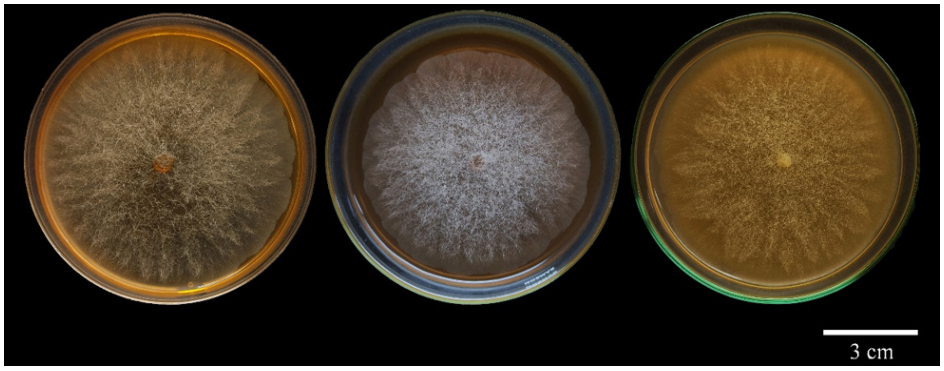
a b c

Figure 1 Direct isolation of *Phytophthora infestans* from lesion of tomato late blight in the field. a-b, infected leaf with sporangia mass; and c, inoculating the sporangia onto corn meal agar (CMA) medium supplemented with 50 mg L^{-1} rifampicin after picking them up with sterile inoculation loop.

Table 1 Comparison of four agar medium and three antibiotics for direct isolation of *Phytophthora infestans* from infected leaves in the field

Agar medium	Antibiotic		Number of petri dishes (.... of 5)		Contamination on failure dishes
	Name	Dose	Success	Failure	
PDA	Chlorampenicol	500 mg L^{-1}	1	4	Mostly fungi
	Rifampicin	50 mg L^{-1}	1	4	Mostly bacteria
	Amoxicillin	100 mg L^{-1}	0	5	Mostly fungi
CMA	Chlorampenicol	500 mg L^{-1}	0	5	Mostly fungi
	Rifampicin	50 mg L^{-1}	5	0	-
	Amoxicillin	100 mg L^{-1}	1	4	Mostly bacteria
OMA	Chlorampenicol	500 mg L^{-1}	0	5	Mostly fungi
	Rifampicin	50 mg L^{-1}	3	2	Mostly fungi
	Amoxicillin	100 mg L^{-1}	1	4	Mostly bacteria
Water agar	Chlorampenicol	500 mg L^{-1}	0	5	Mostly fungi
	Rifampicin	50 mg L^{-1}	2	3	Mostly fungi
	Amoxicillin	100 mg L^{-1}	0	5	Mostly fungi

Note: PDA, potato dextrose agar.; CMA, corn meal agar; OMA, oatmeal agar



a b c

Figure 2 Two-day-old colony of *Phytophthora infestans* on agar medium supplemented with 50 mg L^{-1} rifampicin. a, corn meal agar (CMA); b, potato dextrose agar (PDA); and c, oatmeal agar (OMA).

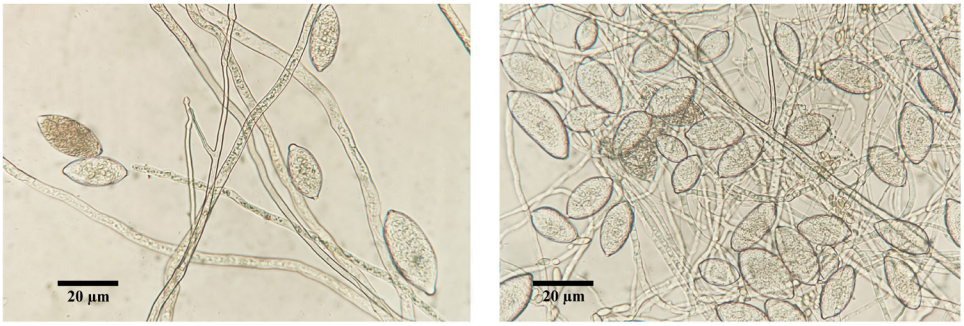
As a nutrient-poor medium, CMA is very suitable for isolating *Phytophthora* from infected tissue (Drenth and Sendall 2011). Several researchers have successfully isolated oomycetes using CMA supplemented with rifampicin. Bakker *et al*. (2017) effectively isolated *Pythium* spp. on CMA supplemented with rifampicin and nystatin. Similarly, Nam and Choi (2019) used CMA supplemented with rifampicin to investigate the cultural characteristics of *Phytopythium* and *Pythium* species.

In our study, the isolates were successfully sub cultured on PDA, CMA, and OMA. Aseptate hyphae were observed, but no sporangia were visible. To induce *P. infestans* sporangia production on agar medium and the rate of its induction, CMA or OMA combined with rifampicin or amoxicillin was examined as the recommended options during the isolation stage. The optimal medium for inducing sporangia production at 16 °C in the dark was OMA supplemented with 50 mg L^{-1} rifampicin. The incubation period was the shortest (eight days) (Table 2). Furthermore, after 20 days of incubation, a high density of sporangia was formed on OMA supplemented with 50 mg L^{-1} rifampicin (Figure 3b). In contrast, sporangia density was observed to be scarce on CMA supplemented with 50 mg L^{-1} rifampicin (Figure 3a).

Table 2 Induction of sporangia formation on corn meal agar (CMA) and oatmeal agar (OMA) media incubated at 16 °C

Agar medium	Antibiotic		Incubation period for first sporangia formed[a] (days)	Category of sporangia density after 20 days[b]
	Name	Dose		
CMA	Rifampicin	50 mg L^{-1}	10.5 ± 0.16 c	Scarce
	Amoxicillin	100 mg L^{-1}	14.0 ± 0.37 a	Scarce
OMA	Rifampicin	50 mg L^{-1}	8.0 ± 0.00 d	Abundant
	Amoxicillin	100 mg L^{-1}	12.7 ± 0.28 b	Scarce

Note: CMA, corn meal agar; OMA, oatmeal agar
[a]Data followed by the different letter show significant difference by Tukey test (α 5%). Data are given as mean ± SE; [b]Based on 10× observations from 5 mm diameter of discs cut from random site of colony plate.



a b

Figure 3 Microscopical observation of *Phytophthora infestans* sporangia 20 days after incubation on agar medium (40 × 10 optical zoom). a, corn meal agar (CMA) supplemented with 50 mg L^{-1} rifampicin; and b, oat meal agar (OMA) supplemented with 50 mg L^{-1} rifampicin.

Our further examination involved subculturing the various ages of the original *P. infestans* colonies (8, 13, 18, 23, and 28-day-old colonies) to ascertain how frequently these oomycetes should be sub-cultured to a fresh medium and which age of the original colony is still ideal for sub-culture. We sub-cultured each colony of different ages onto a new agar medium, with ten replicates on CMA and OMA. We observed the colonies' diameter while incubating for up to 72 hours at room temperature (22 °C). *P. infestans* colonies in both CMA and OMA expanded quickly upon subculturing them from 8 and 13 days of original colonies (Figure 4 and Figure 5). New colonies that are 18, 23, or 28 days old either exhibit abnormal development or fail to revive. Subculturing *P. infestans* isolates from colonies that are 23 or 28 days old (both CMA and OMA) carries an increased risk of bacterial contamination during the incubation period. This observation demands that biweekly subculturing steps be carried out if the cultures are to be utilized for routine tests before being stored for an extended period.

According to this study, direct isolation with CMA supplemented with 50 mg L^{-1} rifampicin was strongly recommended. Sporangia sampling should be conducted in the early morning since it provides maximum relative humidity and it is easier to discover fresh lesion symptoms. Furthermore, colonies that successfully expanded in the first one to two days should be sub-cultured immediately to the fresh medium to avoid bacterial contamination. This approach saves time and helps the oomycetes researcher face challenges with the shortcomings of a specific growing medium.

In conclusion, this study has successfully presented new insights that have practical implications for understanding the bioecology of *P. infestans* infecting tomato plants. These insights enable researchers to isolate *P. infestans* more efficiently and handle the isolates in the laboratory for continuous

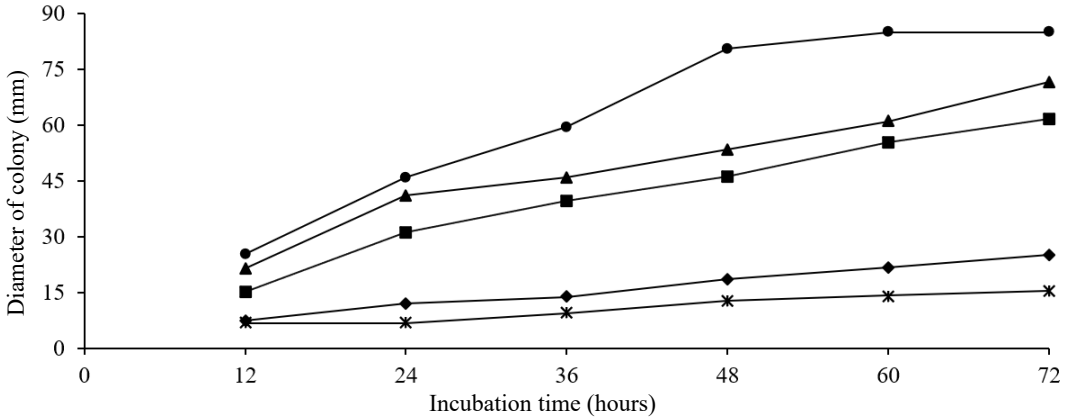


Figure 4 Growth of *Phytophthora infestans* from different ages of original colony on corn meal agar (CMA) medium supplemented with 50 mg L^{-1} rifampicin. —●—, 8 day-old; —▲—, 13 day-old; —■—, 18 day-old; —◆—, 23 day-old; and —✱—, 28 day-old.

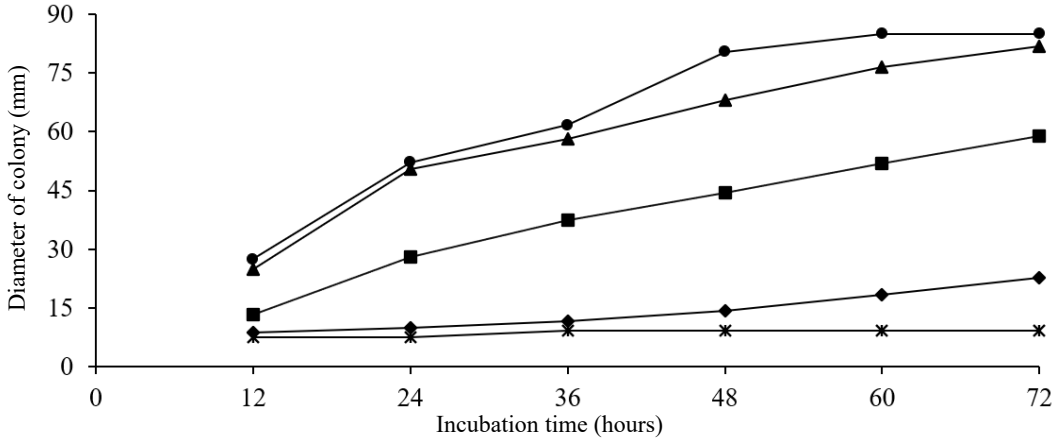


Figure 5 Growth of *Phytophthora infestans* from different age of original colony on oatmeal agar (OMA) supplemented with 50 mg L^{-1} rifampicin. —●—, 8 day-old; —▲—, 13 day-old; —■—, 18 day-old; —◆—, 23 day-old; and —✱—, 28 day-old.

research purposes. Notably, the results of this study provide practical guidance for researchers and lay the foundation for the development of effective control strategies for this pathogen in the field, potentially making a significant impact in the future.

REFERENCES

Bakker MG, Moorman TB, Kaspar TC, Manter DK. 2017. Isolation of cultivation-resistant oomycetes, first detected as amplicon sequences, from roots of herbicide-terminated winter rye. Phytobiomes. 1(1):24–35. DOI: https://doi.org/10.1094/PBIOMES-10-16-0011-R.

Berendsen RL, Pieterse CMJ, Bakker PAHM. 2012. The rhizosphere microbiome and plant health. Trends in Plant Science. 17(8):478–486. DOI: https://doi.org/10.1016/j.tplants.2012.04.001.

Bush EA, Rideout SL, Waldenmaier CM. 2012. Late blight of tomato and potato. Virginia cooperative extension. 1–8. https://vtechworks.lib.vt.edu/items/e3e599ce-d706-40d0-a6a2-60f495f78336

Dangi S, Wharton P, Ambarwati AD, Santoso TJ, Kusmana, Sulastrini I, Medendorp J, Hokanson K, Douches D. 2020. Genotypic and phenotypic characterization of *Phytophthora infestans* populations on Java, Indonesia. Plant Pathology. 70(1):61–73. DOI: https://doi.org/10.1111/ppa.13269.

Drenth A, Sendall B. 2001. Practical Guide to Detection and Identification of

Phytophthora. Brisbane (AU): CRC for Tropical Plant Protection.

Griffith GW, Snell R, Shaw DS. 1995. Late blight (*Phytophthora infestans*) on tomato in the tropics. Mycologist. 9(2):87–89. DOI: https://doi.org/10.1016/S0269-915X(09)80222-X.

Kong P, Hong C. 2016. Soil bacteria as sources of virulence signal providers promoting plant infection by *Phytophthora* pathogens. Scientific Reports. 6:33239. DOI: https://doi.org/10.1038/srep33239.

Masago H, Yoshikawa M, Fukada M, Nakanishi N. 1976. Selective inhibition of *Pythium* spp. on a medium for direct isolation of *Phytophthora* spp. from soils and plants. Phytopathology. 67:425–428. DOI: https://doi.org/10.1094/Phyto-67-425.

Nam B, Choi YJ. 2019. *Phytopythium* and *Pythium* species (oomycota) isolated from fresh water environments of Korea. Mycobiology. 47(3):261–272. DOI: https://doi.org/10.1080/12298093.2019.1625174.

Nelson SC. 2008. Late blight of tomato (*Phytophthora infestans*). College of Tropical Agriculture and Human Resources (CTAHR, University of Hawai'i Plant Disease Issue. 45:1–10.

Nowicki M, Foolad MR, Nowakowska M, Kozik EU. 2012. Potato and tomato late blight caused by *Phytophthora infestans*: an overview of pathology and resistance breeding. Plant Disease. 96(1):4–17. DOI: https://doi.org/10.1094/PDIS-05-11-0458.

Oyarzun PJ, Pozo A, Ordoñez ME, Doucett K, Forbes GA. 1998. Host specificity of *Phytophthora infestans* on tomato and potato in Ecuador. Phytopathology. 88(3):265–271. DOI: https://doi.org/10.1094/PHYTO.1998.88.3.265.

Plancarte AS, Alvarado GR, Pavía YLF, Santos MEP, Pérez LL, Celaya MD, Pavía SPF. 2017. Isolation and diagnosis protocols of *Phytophthora* spp. applied research approach. Revista Mexicana de Ciencias Agrícolas. 8(8):1867–1880. DOI: https://doi.org/10.29312/remexca.v8i8.708.

Sarker SR, McComb J, Burgess TI, Hardy GESJ. 2020. Antimicrobials in *Phytophthora* isolation media and the growth of *Phytophthora* species. Plant Pathology. 69:1426–1436. DOI: https://doi.org/10.1111/ppa.1322.

Suzuki N, Osawa H, Akino S, Uedo A, Miyachi M, Kondo N. 2019. An easy direct zoosporangia sampling method for collecting *Phytophthora infestans* isolates. Journal of the Research Faculty of Agriculture, Hokkaido University. 74:1–5.

Tumwine J, Frinking HD, Jeger MJ. 2000. Isolation techniques and cultural media for *Phytophthora infestans* from tomatoes. Mycologist. 14(3):137–139. DOI: https://doi.org/10.1016/S0269-915X(00)80096-8.

Wang J, Shen D, Ge C,Du Y, Lin L, Liu J, Bai T, Jing M, Qian G, Dou D. 2020. Filamentous *Phytophthora* pathogens deploy effectors to interfere with bacterial growth and motility. Frontiers in Microbiology. 11:581511. DOI: https://doi.org/10.3389/fmicb.2020.581511.