From lichens to crops: Pathogenic potential of *Pseudomonas syringae* from *Peltigera* lichens is similar to world-wide epidemic strains

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Ethics and integrity policies

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Abstract

The presence of bacteria belonging to the *Pseudomonas syringae* complex (*P. syringae*) in the natural vegetation of several Icelandic habitat types has been recently reported, raising questions about the risk to Icelandic crops, particularly given the expected increase in agricultural activity due to climate warming. This study takes advantage of Iceland's unique characteristics and the discovery of *P. syringae* in *Peltigera* lichens to gain a better understanding of the potential risk posed by this newly discovered ecological niche. The main objective is to evaluate the pathogenic potential and fitness in crops of *P. syringae* strains isolated from *Peltigera* lichen sampled in Iceland, focusing on strains that belong to phylogroups 1 and 2, which commonly contain epidemic strains. The results indicate that *P. syringae* isolated from Icelandic *Peltigera* lichen demonstrate comparable fitness to well-known epidemic strains in eight out of the ten plant species tested, and certain strains exhibit similar pathogenicity in the three plant species analyzed. These findings provide valuable

insights into the potential risks posed by *P. syringae* from Icelandic natural habitats and illustrate how strains from these habitats have a wide pathogenic potential to crops without having encountered these crops in the last several thousand years of their presence in Iceland.

Introduction

Bacterial strains within the *P. syringae* complex are present in connection with diverse biological and non-living substrates worldwide. The ability of *P. syringae* to adapt to a wide range of habitats linked to the water cycle is thought to be a driver of its broad host range (Morris et al. 2013). This idea is reinforced by the observation that numerous strains from this group of bacteria isolated from non-agricultural environments are phylogenetically closely related to plant associated strains and have also been shown to be pathogenic on plants such as kiwifruit and tomato (Bjelland et al. 2011; Garg et al. 2016; Spribille et al. 2016). For this reason, recent research has continued to explore the ecology and pathogenicity of *P. syringae* outside the context of crops.

According to Ellis et al. (2010), agricultural land is becoming increasingly prevalent compared to other vegetated areas on Earth. However, Iceland stands out as an atypical region with a limited amount of cultivated land, thereby providing a unique opportunity to study the adaptation of *P. syringae* without a predominant influence of local agriculture. Iceland's flora is characterized by a relatively small number of native species of vascular plants, comprising around 530 species (Wąsowicz 2020). Additionally, the country is home to a diverse range of lichens (755 species) (Kristinsson and Heiðmarsson, 2009) and mosses (around 600 species) (Jóhannsson 2003). However, the vegetation of Iceland evolved in the absence of large herbivores and subsequently is vulnerable to grazing and human activities (Runólfsson 1987). Furthermore, Iceland’s position on the border between Arctic and Atlantic waters and air masses, known as the polar front, makes for an interface

(Jónsdóttir et al. 2005) which creates favourable climate conditions for *P. syringae* with average temperatures between 2 to 14°C throughout the year (Ogilvie and Jónsson, 2001).

Previous research has shown that *P. syringae* is prevalent in Iceland on wild vascular plants and moss (Morris et al. 2022), which, coupled with observations of *P. syringae* genes in the lichen metagenome of *Peltigera membranaceae* (Sigurbjörnsdóttir et al. 2015; Sigurbjörnsdóttir, 2016). This prompted researchers to investigate its ubiquity across several types of plants and lichens in Iceland, hypothesizing that lichens may serve as non-host reservoirs for *P. syringae* (Vilhelmsson et al. 2016). Morris and colleagues (Morris et al. 2022) unveiled how the genetic lines of *P. syringae* in the Icelandic region are monophyletic, indicating that they may have evolved separately from the *P. syringae* populations elsewhere in the world during the relatively short geological history of Iceland. However, these monophyletic haplotypes represent different phylogroups (PGs) (Morris et al. 2022). This illustrates the extraordinary adaptive properties throughout the *P. syringae* complex.

P. syringae were found in lichens in Iceland, specifically in species of the genus *Peltigera* (Ramírez et al. 2023). To delve deeper into this discovery, a phylogenetic analysis of *P. syringae* strains collected from various sources, including lichens, tracheophytes, and moss was conducted. The analyses revealed significant differences in strains between geographical locations, showing greater similarity of *P. syringae* within a site across all vegetation types compared to within vegetation types across sites. However, *Peltigera* thalli harbored a consistent population density of *P. syringae* although it was lower than that on moss and tracheophyte samples (Ramírez et al. 2023). This finding underscores the adaptability of *P. syringae* to inhabit a diverse range of vegetation beyond higher plants, offering novel insights into its evolutionary dynamics.

Assigning *P. syringae* strains to phylogroups based on their citrate synthase gene sequences (Berge et al. 2014) is a useful tool for understanding their phenotypic variations. Many of the strains isolated from *Peltigera* lichens can be assigned to phylogroups PG01 and PG02. These phylogroups contain a wide range of epidemic strains, as documented by Berge and colleagues (Berge et al. 2014). Roughly fifty percent of the lichen thalli were found to host PG01 and/or PG02 strains. Among the *P. syringae* isolates, PG02 constituted approximately 14%, while PG01 accounted for a mere 4% of the overall *P. syringae* population derived from *Peltigera* (Ramírez et al. 2023).

In light of the ubiquity of bacteria in the *P. syringae* complex on vegetation in Iceland and, in particular the presence of phylogroups with high frequencies of strains with functional T3SS such as PG01 and PG02 (Berge et al. 2014), our goal was to assess the pathogenic potential and fitness – mainly on crops - of strains from PG01 and PG02 from Icelandic *Peltigera* compared to strains in the same phylogroup isolated from epidemics on crops elsewhere in the world.

Materials and methods

Bacterial strains

P. syringae strains belonging to PG01 and PG02 were selected from a collection of strains isolated from *Peltigera* lichen to represent the range of diversity of these phylogroups associated with this group of lichens (Supplementary 1). As positive controls, strains belonging to PG01 and PG02 from epidemic occurrences were chosen due to their well-established pathogenic potential and consistent behavior, as revealed by previous work (Morris et al. 2019). For the evaluation of pathogenicity, CC0125, CC0094, and CFBP1906 were employed as reference controls, aligning with the findings outlined previously (Morris et al. 2000). In terms of fitness assessment, the reference strains comprised CC0094 and DC3000.

Inoculum preparation

Bacterial inoculum was prepared from 24-72 hr growth on King's B (KB) medium (King et al. 1954). A loopful of growth was resuspended in phosphate buffer (TP1: 8,75g of K_2HPO_4 and 6,75g of KH_2PO_4 diluted in 1L of distilled water) and adjusted with a spectrophotometer to 10^8 CFU mL^{-1} ($OD_{600\ nm}$ = 0.1). The inoculum was further diluted in phosphate buffer, resulting in a concentration of 10^7 CFU mL^{-1} for the pathogenicity tests and 10^6 CFU mL^{-1} for the in planta in the fitness tests. Inoculum concentration was verified by dilution plating.

Plant material

Pathogenic potential was evaluated for plant species cultivated in Iceland using cultivars available in France: kale (*Brassica oleracea*), cucumber (*Cucumis sativus*) and barley (*Hordeum vulgare*). For fitness tests, plant species belonging to 10 families used were: rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), thale cress (*Arabidopsis thaliana*), annual mugwort (*Artemisia annua*), spinach (*Spinacia oleracea*), garlic chives (*Allium tuberosum*) and tobacco (*Nicotiana tabacum*) in addition to the three plant species used for the pathogenicity studies. Cultivar details, growing conditions, and cultivation dates can be found in Supplementary 2. The duration of cultivation in the greenhouse was tailored to the specific plant species. In the greenhouse environment of Montfavet, France, the plants were cultivated during a period spanning from September 2022 to January 2023. Adequate watering was provided in accordance with the plants' needs. Plants were grown in TS3 substrate mixture from Klasmann-Deilmann (Geeste, Germany).

Evaluation of fitness *in planta*

Fitness of strains in plants was determined in terms of population growth after inoculation. Strains were inoculated into plants by placing a 5µL-drop of inoculum (10^6 CFU mL^{-1}) on wounded leaf tissue and letting the inoculum be absorbed by the plant. An emery board/emery paper was used gently to create a fresh wound on the surface of the leaf. Each plant received a single inoculation, and there were 10 replicate plants per strain.

Three to five plants were inoculated with phosphate buffer as negative control for each plant species. For a given plant species, all strains were inoculated simultaneously to facilitate between-strain comparisons. Via a randomized complete block design, the plants were set up in a growth chamber and incubated at 24 °C (14 h light, 10 h dark) and 75% humidity.

At 1 and 8 days post inoculation (dpi), five leaves were collected from each plant species for each strain, except in the case of *A. thaliana*, where six leaves were collected. Prior to maceration, leaves bearing soil residues were gently cleaned using dry paper. Each leaf was stomached for 2–3 min in sterile phosphate buffer (10µL of 0.1 M buffer per mg of fresh plant weight amounting to between 1-30 ml depending on the leaf size) in sterile stomacher bags. Aliquots of serial dilutions of the macerate were plated on KB medium. The plates were placed in a dark environment and incubated at room temperature for a period of 48-72 hours. *P. syringae* colonies were identified based on their shape, size, color, texture, elevation, and margin (Morris et al. 2022). The abundance of *P. syringae* in the inoculated leaves was expressed per leaf.

Inoculation, incubation, and disease assessment for pathogenicity assays

One leaf per plant was infiltrated with a needleless syringe with 5-10 µL of inoculum (10^7 CFU mL^{-1}). Six to eight replicate plants were utilized per plant species per strain. The negative control consisted of phosphate buffer. Plants were arranged in a growth chamber in a randomized complete block design and incubated at 24 °C (14 h light, 10 h dark) and 75% humidity for 14 days.

Symptoms were scored at 2, 5, 9 and 14 dpi in terms of the maximum length of the necrosis that developed at the inoculated site except for cucumber that was observed only until 9 dpi. Symptoms were also photographed and described at each scoring date. Strains were classified as pathogenic if the necrotic length was significantly different than the negative control.

To verify that symptoms were caused by *P. syringae*, isolations were made from at least three infected tissue replicates per strain. Sections of tissue (at the interface of necrotic and the green surrounding tissue) were aseptically collected and placed on KB plates and incubated at 24°C in darkness. After 2-3 days, colony morphology, colour and fluorescence under 366 nm were assessed.

Statistical analyses

Utilizing Microsoft Excel and R Studio, statistical analyses were conducted. This included t-tests, graph plotting with the ggplot2 package, and one-way ANOVA. The t-test was applied to determine the differences between strains inoculated into the same plant species at various time points. Moreover, one-way ANOVA was employed, followed by post hoc Tukey-Kramer analysis to unravel strain variations.

Results

P. syringae from *Peltigera* and from crop epidemics have similar pathogenic potential

To account for non-specific reactions of the plants due to wounding, the size of the necroses on inoculated plants was compared to those observed on control plants. Nine out of 17 strains of *P. syringae* produced necrotic symptoms that were significantly longer than any of the necroses observed on the negative controls in barley and cucumber. These same nine strains and four additional strains also caused lesions that were significantly longer on kale when compared to necroses on the control plants (Fig. 1). Symptoms were most severe on barley and cucumber compared to kale, and symptoms caused by PG02 strains tended to be greater and more variable than those caused by PG01 strains. The intensity of the symptoms caused by some of the strains from *Peltigera* lichens were within the same range as those caused by the strains from crop epidemics (Fig. 1).

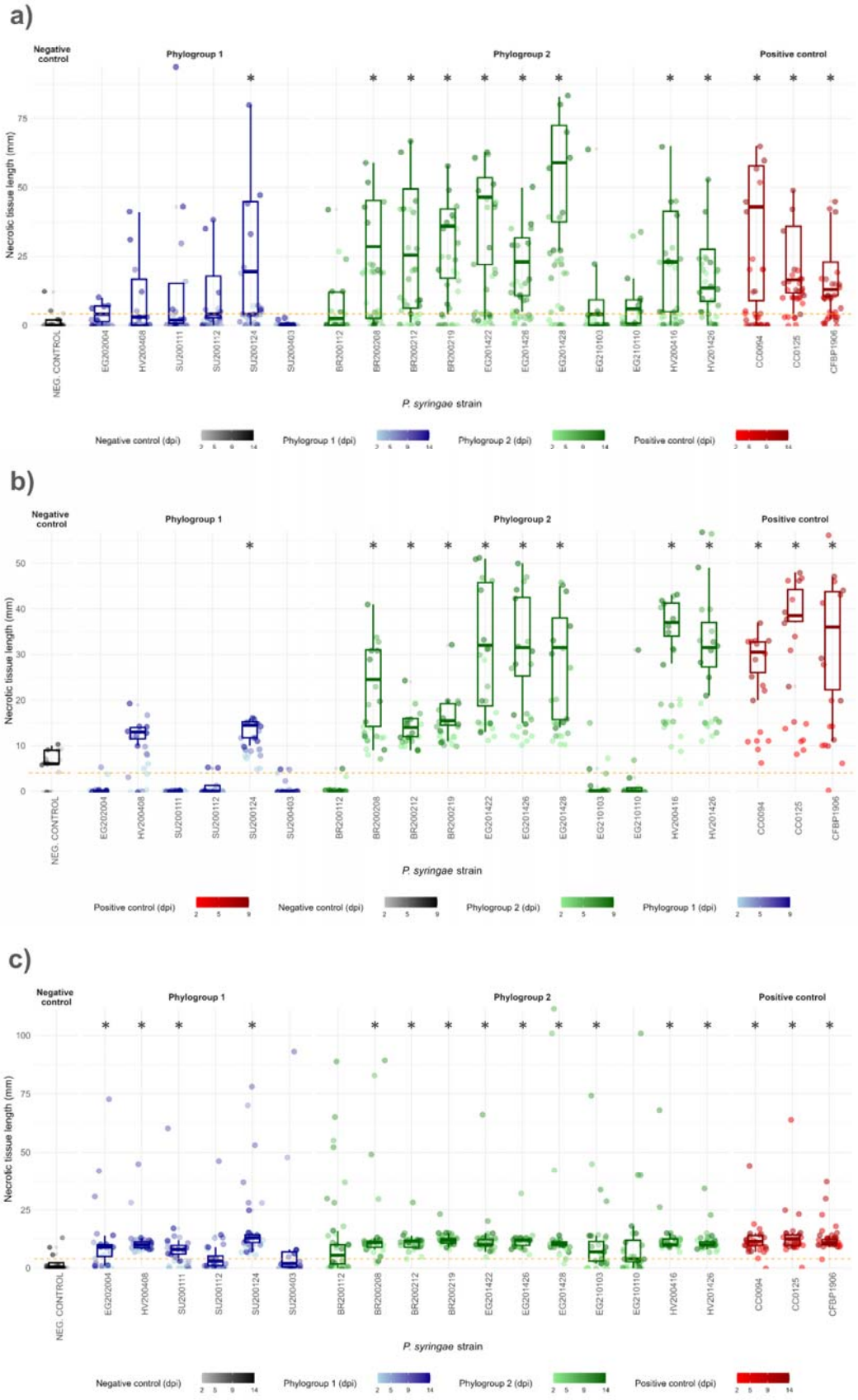
a)
Negative control
Phylogroup 1
Phylogroup 2
Positive control
Necrotic tissue length (mm)
NEG. CONTROL
EG202004
HV200408
SU200111
SU200112
SU200124
SU200403
BR200112
BR200208
BR200212
BR200219
EG201422
EG201426
EG201428
EG210103
EG210110
HV200416
HV201426
CC0094
CC0125
CFBP1906
P. syringae strain
Negative control (dpi)
Phylogroup 1 (dpi)
Phylogroup 2 (dpi)
Positive control (dpi)
b)
Negative control
Phylogroup 1
Phylogroup 2
Positive control
Necrotic tissue length (mm)
P. syringae strain
Positive control (dpi)
Negative control (dpi)
Phylogroup 2 (dpi)
Phylogroup 1 (dpi)
c)
Negative control
Phylogroup 1
Phylogroup 2
Positive control
Necrotic tissue length (mm)
P. syringae strain
Negative control (dpi)
Phylogroup 1 (dpi)
Phylogroup 2 (dpi)
Positive control (dpi)

Fig.1 Necrotic tissue length in mm on leaves infiltrated with *P. syringae*. Dots represent all measurements at different time points distinguished by the darkness of the color while the boxplots summarize the necrotic length on: a) Barley at 14 dpi; b) Cucumber on 9 dpi and; c) Kale on 14 dpi. All plants were incubated at 24 °C (14 h light, 10 h dark) and 75% humidity for up to 14 days. The *P. syringae* strains that exhibit statistical significance from the negative control (p value < 0.05) are marked with an asterisk on this graph

In addition to necrosis, symptoms such as chlorosis and water-soaked areas sometimes surrounded by a yellow halo were observed after infiltration of the *Peltigera* strains in kale.

In barley, in addition to necrosis, other symptoms such as chlorosis, wilting, or a slightly pale appearance were also recorded (Fig. 2). The observed symptoms in cucumber included necrosis, chlorosis, leaf collapse, leaf curling, and lesions with a yellow halo (images not available).

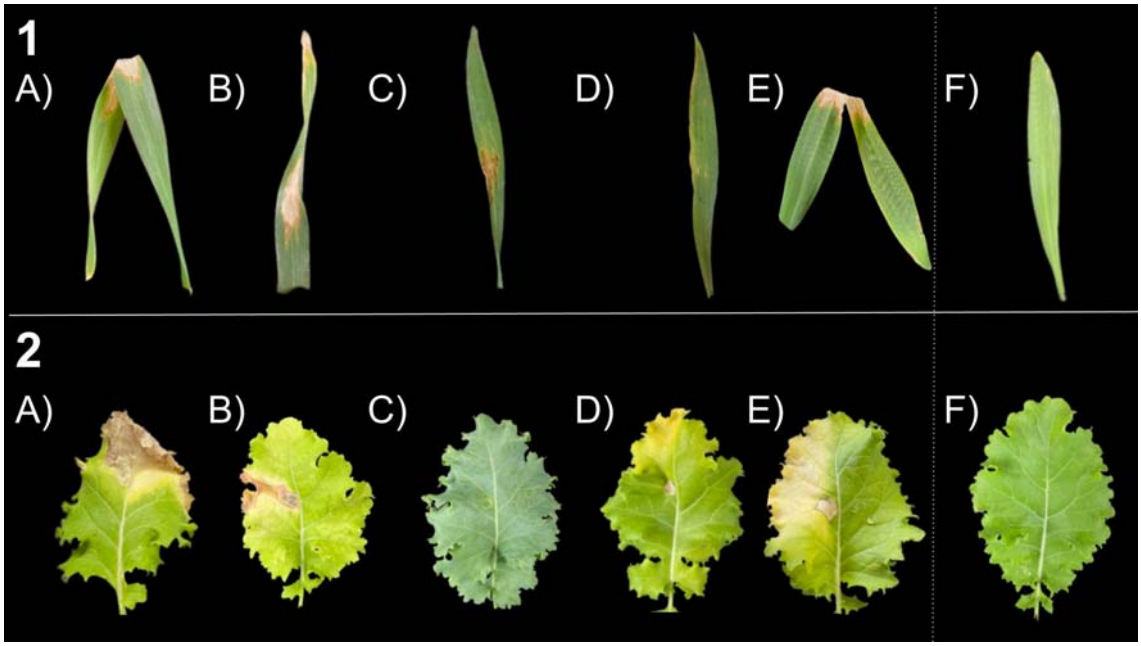

Fig.2 Leaf symptoms at 14 dpi. The leaves represent some of the most common symptoms observed on each plant species.1. Barley infected with strains a) HV201426; b) EG201422; c) EG201428; d) HV201426 e) Positive control CFBP1906; and f) Negative control. 2. Kale infected with strains a) HV201426; b) EG210110; c) SU200403; d) EG201426; e) Positive control CFBP1906; and f) Negative control

P. syringae isolated from Icelandic *Peltigera* lichens and epidemic strains have similar fitness in plants

The fitness analysis revealed that *P. syringae* strains isolated from Icelandic *Peltigera* lichen exhibited population growth levels similar to the epidemic strains CC0094 and DC3000 at both 1 and 8 dpi under the specified culture conditions across the ten plant species tested, except for cucumber. Notably, differences emerged in tomato, with the population size of *Peltigera* lichen *P. syringae* strains being higher at 1 dpi (Supplementary 3) and in barley, where the epidemic strains showed a higher population at 8 dpi (Fig. 3). However, the total population at both dpi was statistically higher for lichen than for epidemic strains (p value < 0.05).

Fig. 3 *P. syringae* population in inoculated leaves of ten plant species on the 8th day post-infection. Violin and boxplot graphs resume the values of *P. syringae* per leaf for 5 or 6 replicates per strain inoculated in each

plant. The Icelandic strains isolated from lichens are represented by a solid color while the epidemic strains used as control are plotted as transparent. Grey dots indicate the minimum detection level. Asterisk denotes a plant species where statistically significant differences in *P. syringae* population sizes between epidemic and *Peltigera* strains were observed

When examining individual strains, a discrepancy appeared at the 1st dpi in tobacco, where CC0094 differed from all other strains used in the fitness analyses, except HV200426 (Fig. 4). Conversely, at 8 dpi, distinctions were observed only in cucumber, with DC3000 having greater population sizes than all lichen strains, and in barley, where CC0094 had a significantly higher population size than SU200124 (p value <0.05) (Fig. 3).

Discussion

Here we have revealed that *P. syringae* isolated from *Peltigera* lichen in Iceland has fitness and pathogenicity levels comparable to strains isolated from epidemic crops worldwide. Numerous studies have demonstrated that *P. syringae* retains its pathogenic capability even in the absence of significant agricultural pressures (Morris et al. 2008; Morris et al. 2013; Morris and Moury, 2019), especially in those strains that have a wider range of habitat. However, the unique context of the Icelandic *P. syringae* strains that have evolved isolated for thousands of years makes them an interesting case study (Morris et al. 2022) as it illustrates the ancestral nature of the traits that confer pathogenicity and their maintenance in populations in natural habitats (Xin et al. 2018).

While our results do suggest the potential threat posed by *P. syringae* in Iceland, it is crucial to keep in mind that *P. syringae* is ubiquitous in the environment. In this light, it is notable that there have been no reported instances *of P. syringae* causing diseases in local crops in Iceland nor to the native vegetation. This highlights the role that environmental factors such as cold temperatures that reduced the cultivation season or average temperatures under the optimal for *P. syringae* to develop aggressive traits (Bender et al. 1999) likely play in

constraining bacterial proliferation and safeguarding plant health. The interplay of environmental conditions significantly contributes to the assessment of disease potential (Morris et al. 2023).

P. syringae in Iceland has evolved over the past 10000 years, at least, separate from contact with plants that are common to agricultural landscapes such as rice and tomato (Morris et al. 2022). Nevertheless, strains from Iceland have some level of fitness in these plants which could be considered as novel hosts for these stains. This raises intriguing questions about the establishment of host specificity. However, the aggressiveness detected on Icelandic *P. syringae* might be related to the adaptation to environmental habitats which have been linked to the evolution of their pathogenicity (Morris et al. 2010).

The finding of *P. syringae* raised concerns about a possible threat to Icelandic crops in case the strains isolated showed pathogenic properties. Icelandic cool climate can play an important role in pathogenicity of *P. syringae*, due to tissue damage that intensified by frost episodes and humid environments, as it has been shown that the presence of a layer of free water is essential for infection (Lamichhane et al. 2015). Furthermore, certain studies have indicated that lower temperatures enhance the functionality of T3SS (Type III Secretion System) and T6SS (Type VI Secretion System), which are typically associated with increased aggressiveness (Tribelli and López, 2022). Despite the limited agricultural land in Iceland (Denk et al. 2011), certain crops are cultivated on an industrial scale in greenhouses, with yields exceeding 1000 tonnes annually. These include tomatoes and cucumbers, while outdoor cultivation involves crops like barley and potatoes. Nevertheless, the anticipation is that the range of plant species grown outdoors and their yields in Iceland will rise over the coming decades due to the predicted rise in temperatures (Parry 1991). The expansion of cultivable land, coupled with the anticipated milder, yet still relatively cold temperatures in Iceland, may pose a potential threat to Icelandic crops.

The results of this study are one more example of *P. syringae* strains isolated from environmental habitats, where they are not causing any obvious damage, or where they might even be beneficial (Morris et al. 2013). However, these strains can affect some plant species under specific conditions – conditions that might be increasingly probable with the changing climate and land use in Iceland.

Disclosure statement

The authors affirm that the research was conducted without any commercial or financial affiliations that could be construed as potential conflicts of interest.

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