

Article

Temperature-Mediated Plasticity Regulates the Adaptation of *Phytophthora infestans* to Azoxystrobin Fungicide

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Abstract: Fungicide is one of the main approaches used in agriculture to manage plant diseases for food production, but their effectiveness can be reduced due to the evolution of plant pathogens. Understanding the genetics and evolutionary processes responsible for the development of fungicide resistance is a key to food production and social sustainability. In this study, we used a common garden experiment to examine the source of genetic variation, natural selection, and temperature contributing to the development of azoxystrobin resistance in *Phytophthora infestans* and infer sustainable ways of plant disease management in future. We found that plasticity contributed to ~40% of phenotypic variation in azoxystrobin sensitivity while heritability accounted for 16%. Further analysis indicated that overall population differentiation in azoxystrobin sensitivity (Q_{ST}) was significantly greater than the overall population differentiation in simple sequence repeat (SSR) marker (F_{ST}), and the *P. infestans* isolates demonstrated higher level of azoxystrobin sensitivity at the higher experimental temperature. These results suggest that changes in target gene expression, enzymatic activity, or metabolic rate of *P. infestans* play a more important role in the adaptation of the pathogen to azoxystrobin resistance than that of mutations in target genes. The development of azoxystrobin resistance in *P. infestans* is likely driven by diversifying selection for local adaptation, and elevated temperature associated with global warming in the future may increase the effectiveness of using azoxystrobin to manage *P. infestans*. The sustainable approaches for increasing disease control effectiveness and minimizing the erosion of the fungicide efficacy are proposed.

Keywords: fungicide efficacy; Q_{ST}/F_{ST} comparisons; *Phytophthora infestans*; sustainable disease management; diversifying selection; global warming

1. Introduction

Sustainability is a global issue covering a range of social and ecological concerns as outlined in recent United Nations (UN) mandates [1]. Recently, many studies were carried out to formulate sustainable solutions for food production and socioeconomic development as well as health and resilient ecosystem fostering in the future [2–4]. Fungicide resistance in pathogens is a worldwide menace to an

extensive range of natural and social sustainability including food security, public and animal health, socioeconomic wellbeing, biodiversity, and environmental soundness that resemble other global issues caused by climate change [5], but can be mitigated or even prevented through the application of eco-evolutionary principles [6–8]. Hence, improved knowledge on the factors and processes leading to the evolutionary development of fungicide resistance is essential for effective and durable management of plant diseases, social stability, and development of ecological sustainability both in agricultural and natural systems [9,10]. Resistance to fungicide is an acquired genetic and epigenetic reduction in the sensitivity of a filamentous pathogen to specific chemical compounds that could occur via mutations in the nuclear or mitochondrial genes creating a heritable change (heritability) [11], or alternations of target gene expression and metabolic activity of cells, i.e., phenotypic plasticity [12], followed by the amplification and spread of the acquired traits through other evolutionary processes such as natural selection, genetic drift, recombination, and gene flow [13,14]. Empirical studies show that the relative importance of these genetic forces shaping the population genetic dynamics and evolution of biological and ecological traits varies among species [15], even though they share ecological niches.

Among the evolutionary factors, genetic variation and natural selection are most important in determining the potential and trajectory of developing fungicide resistance in pathogen populations. It was reported that species that have higher genetic variation display a better opportunity and fast rate of adaptation to changing environments such as the adaptation of plant pathogens to fungicide application [16,17]. However, the appearance of new fungicide mutants in individual pathotype by mutation alone can only have a negligible effect on the fitness of pathogen populations. To cause a major impact on fungicide efficacy, pathogens must progress to the second and also more important step of resistance development: frequency amplification of new mutants. In this second phase of evolution, the frequency of newly formed pathotypes in pathogen populations increases gradually due to their higher fitness and fungicide resistance occurs when the new pathotypes in the pathogen populations become dominant.

Determining the types and intensity of natural selection acting on quantitative traits such as the development of fungicide resistance in pathogens is a challenge but can be achieved by a comparative analysis of population differentiation in the concerned quantitative traits estimated by (Q_{ST}) and population differentiation in neutral genetic markers estimated by fixation index (F_{ST}). The theory behind this comparison correlates with Fisher's theorem of natural selection: Traits that are under selection will exhibit different levels of genetic differentiation with other parts of the genome [18,19], generating a different pattern of Q_{ST} in fungicide sensitivity and F_{ST} in simple sequence repeat (SSR) marker loci. In contrast, random genetic drift affects the entire genome equally. Consequently, neutral marker loci are expected to show a similar level of differentiation among populations, leading to the same level of Q_{ST} in fungicide sensitivity and F_{ST} in SSR marker loci. Q_{ST} can be estimated by partitioning the total phenotypic variation to within- and among-population components in a way similar to the estimate of F_{ST} in neutral genetic markers [20–22]. Many genetic and environmental factors including chemical property, pathogen biology, fungicide operational scheme, and climatic conditions can affect the types and intensity of natural selection and, therefore, influence the development of fungicide resistance in pathogens [23]. Temperature, a stronghold abiotic environmental factor that influences all biological and biochemical activities of species, could affect the evolution of fungicide resistance in pathogen populations by regulating the generation and maintenance of genetic variation in target genes, metabolic and enzymatic activities of cells, as well as types and intensity of natural selection [24–27].

Potato late blight caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary, is the most devastating disease of potato worldwide [28,29]. It causes a fast epidemic in potato and tomato and can destroy entire crops shortly once the conditions are favorable [29]. Despite the application of many control strategies, it remains a bottleneck for sustainable potato and tomato production, causing approximately 8 billion US dollars economic loss annually in potato alone globally [30]. The disease is mostly managed by frequent uses of synthetic fungicides and resistant cultivars [31,32]. Due to its

high evolutionary potential, resistance to phenyl-amide and other classes of fungicides [33,34] has gradually emerged in *P. infestans* populations. Thus, continuous application of these fungicides may not be effective in terms of disease management but could cause a risk to ecological sustainability and biodiversity conservation strategies; thereby, it is of great concern to potato growers, agro-chemical companies, and environmentalists. In this case, the choice has been shifted to new fungicides such as azoxystrobin, the efficacy of which has not yet been reportedly overcome by pathogens [35]. Azoxystrobin is a site-specific fungicide frequently used to safeguard from plant diseases of main crops globally. It is the famous synthetic fungicide within the strobilurin family [36]. The main function of this fungicide is to obstruct the mitochondrial respiration of pathogens by binding its active compound to Qo in the cytochrome bc_1 enzyme complex (Complex III), thus blocking electron transfer and freezing the production of adenosine triphosphate (ATP) [36,37].

In this study, we hypothesize that local temperature, through its impact on the spatiotemporal dynamics of *P. infestans* variation, plays a deterministic role in the adaptive evolution of its resistance to azoxystrobin (Figure 1, solid line). Our alternative hypothesis is that adaptation of *P. infestans* to azoxystrobin is mainly governed by stochastic events (Figure 1, dashed line). To verify the hypotheses, we focus on the comparison of spatial distribution in genetic variation of SSR neutral marker loci and azoxystrobin sensitivity among *P. infestans* populations originating from different thermal regions in China using a common garden approach. This approach permits us to evaluate the source of genetic variation in azoxystrobin adaptation and determine evolutionary processes shaping the possible development of azoxystrobin resistance in the pathogen populations [19,38]. Although azoxystrobin is still effective for controlling *P. infestans* in the fields at the recommended doses, this analysis is necessary for the reduction and prevention of developing azoxystrobin resistance in *P. infestans* and related pathogens for sustainable management of plant diseases to fulfill UN mandates. The specific objectives of the research are (1) understanding the sources of genetic variation contributing to azoxystrobin adaptation in *P. infestans*; (2) inferring the evolutionary processes possibly driving the development of azoxystrobin resistance in *P. infestans*; (3) evaluating the likely impact of global warming on efficacy of azoxystrobin and related fungicides on future disease management; and (4) making recommendations for sustainable management of azoxystrobin efficacy and plant diseases.

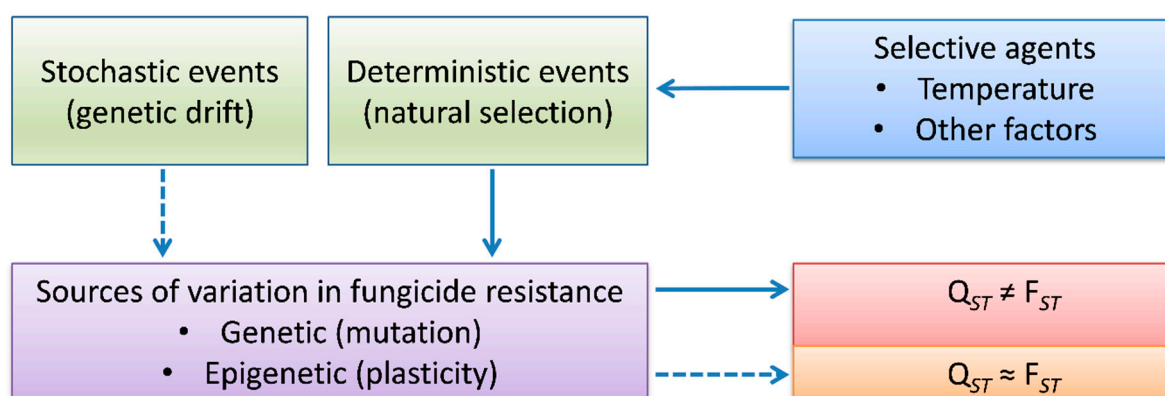


Figure 1. Schematic figure showing the hypothesis of our research. The solid lines represent our null hypothesis and the dashed lines represent the alternative hypothesis.

2. Materials and Methods

2.1. Origin of the Pathogen Populations

Samples of *P. infestans* populations were collected from nine potato fields located in Inner Mongolia, Ningxia, Gansu, Guizhou, Yunnan, Wuhan, Fujian (Xiapu and Fuzhou), and Guangxi during the 2010 and 2011 growing seasons (Figure 2), where no azoxystrobin or any member of the strobilurin family was applied. The samples were collected from infected potato leaves and taken to the laboratory for

P. infestans isolation. The collected isolates were reserved in long-term storage on culture media for upcoming use.

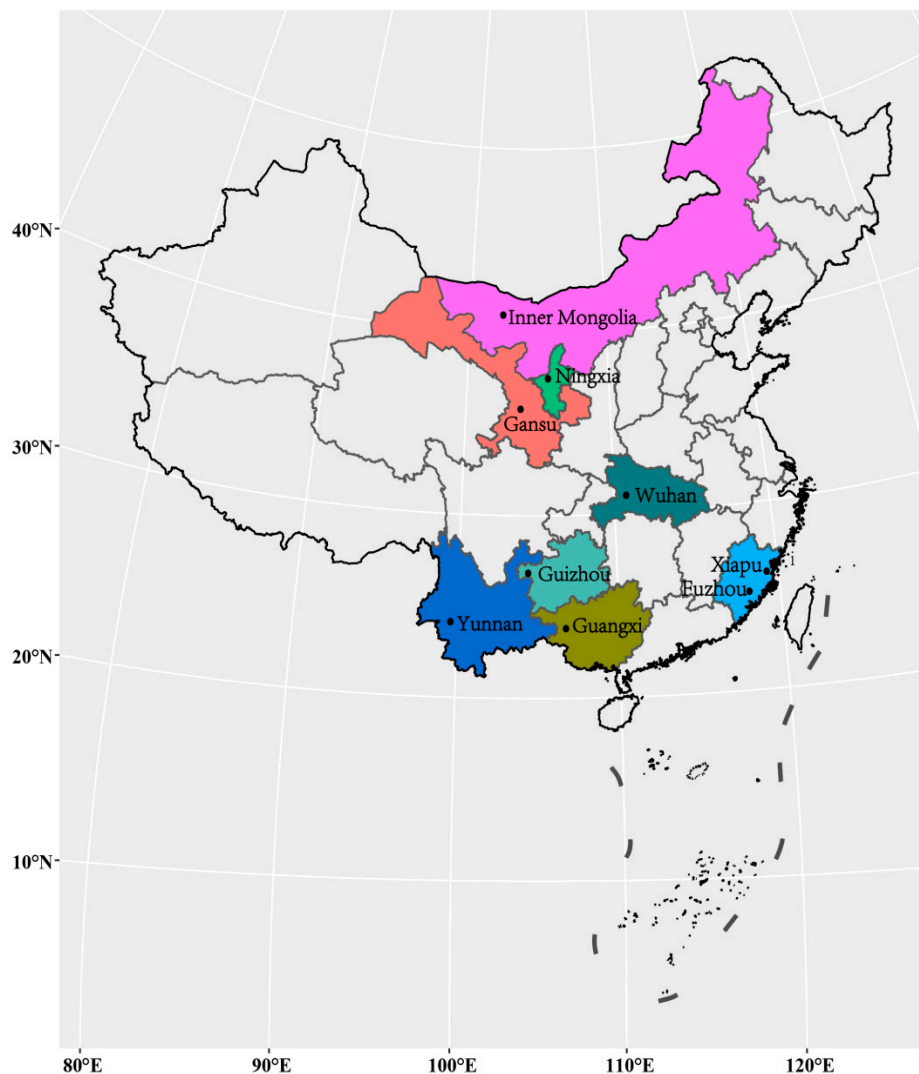


Figure 2. Map of China showing the geographical locations of the nine *Phytophthora infestans* populations sampled for this study.

2.2. SSR Genotyping

SSR data of isolates collected from seven out of the nine field *P. infestans* populations (Ningxia, Gansu, Guizhou, Yunnan, Xiapu, Fuzhou, and Guangxi) were taken from earlier publications [22,39,40]; whereas, the genotypic data of the isolates for the remaining two populations (Inner Mongolia and Wuhan) were generated following the same protocols. In summary, mycelia (~100 mg) obtained from the individual isolates of *P. infestans* grown on rye B agar at 19 °C were composed and kept in 2 mL centrifuge tubes. A vacuum freeze dryer (Alpha1–2, Christ, Germany) was used to lyophilized the mycelia, which were then crushed into a powder using a mixer mill (MM400, Retsch, Germany). Total genomic DNA was extracted using a Plant gDNA Miniprep Kit (GD 2611, Biomiga, San Diego, CA, USA) based on the company's guidelines, and the genomic DNA was suspended in 200 µL of distilled water and then kept at –20 °C for use in this study.

The simple sequence repeats (SSR) were done using eight pairs of primers (G11, Pi02, Pi04, Pi4B, Pi16, Pi33, Pi56, and Pi89) appraised by [41,42], marked by fluorescent dyes [39]. Polymerase chain reaction (PCR) volumes were 25 µL comprising 20 ng of DNA, 12.5 µL of 2 × PCR Buffer Mix (TransGen

Biotechnology Company Limited, Beijing, China), 1.0 μL in both the forward and reverse primers. Amplifications were done in a thermal cycler (Applied Biosystems, Beverly, MA, USA) set for 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 25 s at 56–58 °C (reliant on the primers) and 60 s at 72 °C, followed by a step of 5 min at 72 °C. PCR products were sent to Ruiboxingke Biotechnology Company Limited, Beijing, China, to ascertain the fragment sizes by an ABI 3730XL automated DNA sequencer (Applied Biosystems, Foster, CA, USA) whereby a DNA size ladder was involved in every sample [39]. Alleles were allocated by GeneMaker software version 3.7 using a binning technique and genotype was determined by joining alleles at each SSR locus in the same order.

2.3. Evaluating Azoxystrobin Sensitivity of *P. infestans* Using a Common Garden Experiment

A total of 180 genetically distinct isolates, 20 from each of the nine *P. infestans* populations, were recovered from long storage and restored on rye B agar medium (50 g/L rye and 15 g/L agar). The azoxystrobin sensitivity of the 180 *P. infestans* isolates was evaluated at five experimental temperature schemes (13, 16, 19, 22, and 25 °C) according to a common garden experiment [43,44]. Before setting the experiment, isolates were revived from long-term storage on culture media and grown on rye B agar at 19 °C for 8 days. Mycelia plugs of 3 mm diameter were then taken from the grown margin of the revived colonies and inoculated onto fresh rye B agar culture media supplemented with azoxystrobin (treatments) or without (control) in 9 cm Petri dishes. Three azoxystrobin concentrations (0.05, 0.10, and 0.30 $\mu\text{g}/\text{mL}$) were used in the experiment with controls. Preliminary experiments indicate these doses yielded the best resolution in differentiating azoxystrobin sensitivity among strains. Many isolates did not grow when a higher dose was used while growth rates in many isolates were not significantly changed when a lower dose was used. Inoculated plates were exposed to one of the five experimental temperatures in an incubator and were laid out in a completely randomized design (CRD) using three replications as suggested earlier [38,45]. Image analysis software ASSESS [46] was used to measure the colony sizes starting from day three to eight days after inoculation.

2.4. Data Analysis

A logistic model [47] was used to estimate the growth rate of *P. infestans* isolates under the azoxystrobin concentrations at each of the temperature schemes, using the colony sizes taken at 3–8 days after inoculation. The relative growth rate (RGR) of all isolates was assessed by dividing the growth rate of *P. infestans* in the presence with that in the absence of azoxystrobin and was used to measure the azoxystrobin sensitivity of the isolates.

Phenotypic variance for azoxystrobin sensitivity was estimated and portioned into sources attributed to “isolate” (I, random effect), “population” (P, random effect), “temperature” (T, fixed effect), and “fungicide concentrations” (C, fixed effect) using SAS GLM and VARCOMP programs (SAS 9.1.3) according to the model:

$$Y_{riptc} = M + I(P) + T + C + P + I(P) \times T + I(P) \times C + E_{riptc}$$

where Y_{riptc} refers to the fungicide sensitivity of isolate i in replicate r , population p at temperature t and concentration c ; M is the overall mean; T is the experimental temperature; C is the experimental concentration; and E_{riptc} is experimental error. The terms P , $I(P)$, $I(P) \times T$, and $I(P) \times C$ refer to genetic variance among populations, genetic variance within populations, variance due to the genotype \times temperature interaction, and different responses of populations to concentration effects, respectively [45,48].

The overall population differentiation in RGR was estimated using the following formula:

$$Q_{ST} = \frac{\delta^2_{AP} + \delta^2_{P \times E}/n}{\delta^2_{AP} + \delta^2_{P \times E}/n + \delta^2_{WP}}$$

where δ^2_{AP} , $\delta^2_{P \times E}$, δ^2_{WP} , and n represent the variance among populations, variance within population, variance in the population \times concentration interaction, and the number of environments (concentrations), respectively [44,45].

Population differentiation for SSR marker loci was estimated by the fixation index F_{ST} [49], using POPGENE [50]. Statistical comparison of the overall F_{ST} in SSR loci and overall Q_{ST} in thermal-azoxystrobin sensitivity was evaluated using the standard deviation of F_{ST} constructed from 100 bootstraps of the original data as described previously [45].

Effects of inherited genes and epigenetics on the azoxystrobin sensitivity in the *P. infestans* populations were measured by heritability and phenotypic plasticity. The heritability and plasticity were calculated separately for each population. Heritability was measured by dividing the genetic variance within a population, i.e., $I(P)$, by the total phenotypic variance, while plasticity was calculated by dividing the variance of genotype \times concentration interaction, i.e., $I(P) \times C$, by total phenotypic variance [13,51,52], as explained in the formula below;

$$\text{Heritability} = \frac{\delta^2_{WP}}{\delta^2_C + \delta^2_{IC} + \delta^2_{WP} + \delta^2_E}$$

and

$$\text{Plasticity} = \frac{\delta^2_{IC}}{\delta^2_C + \delta^2_{IC} + \delta^2_{WP} + \delta^2_E}$$

where δ^2_C , δ^2_{IC} , δ^2_{WP} , and δ^2_E are the variances due to concentration, genotype \times concentration interaction, among isolates within the population and among the replicate respectively. Simple linear correlation [53] was used to evaluate the association between all the correlated parameters.

3. Results

3.1. Frequency Distribution of *P. infestans* Natural Population to Azoxystrobin Resistance

In the current study, the distribution of fungicide sensitivity of the *P. infestans* populations to the five different experimental temperatures was investigated. The relative growth rate (RGR) of the isolates from the nine pathogen populations displayed continuous unimodal resistance distribution in all the three azoxystrobin treatments, across the thermal regimes (Figure 3 and Figure S1). Pathogen population showed high sensitivity to azoxystrobin at 22 °C in 0.05 and 0.10 $\mu\text{g/mL}$, followed by 19 °C (Figure 3a,b), respectively and were least sensitive at the lowest temperature 13 °C in 0.10 and 0.30 $\mu\text{g/mL}$ (Figure 3b,c). The pathogen populations grown at 16 °C displayed maximum sensitivity at the highest azoxystrobin concentration 0.30 $\mu\text{g/mL}$ (Figure 3c). The relative growth rate also displayed a continuous and unimodal distribution in all the three azoxystrobin concentrations when all 180 isolates grown in the five temperatures were combined (Figure 4). As fungicide concentration increased, the mean RGR of the isolates decreased (Figure 4).

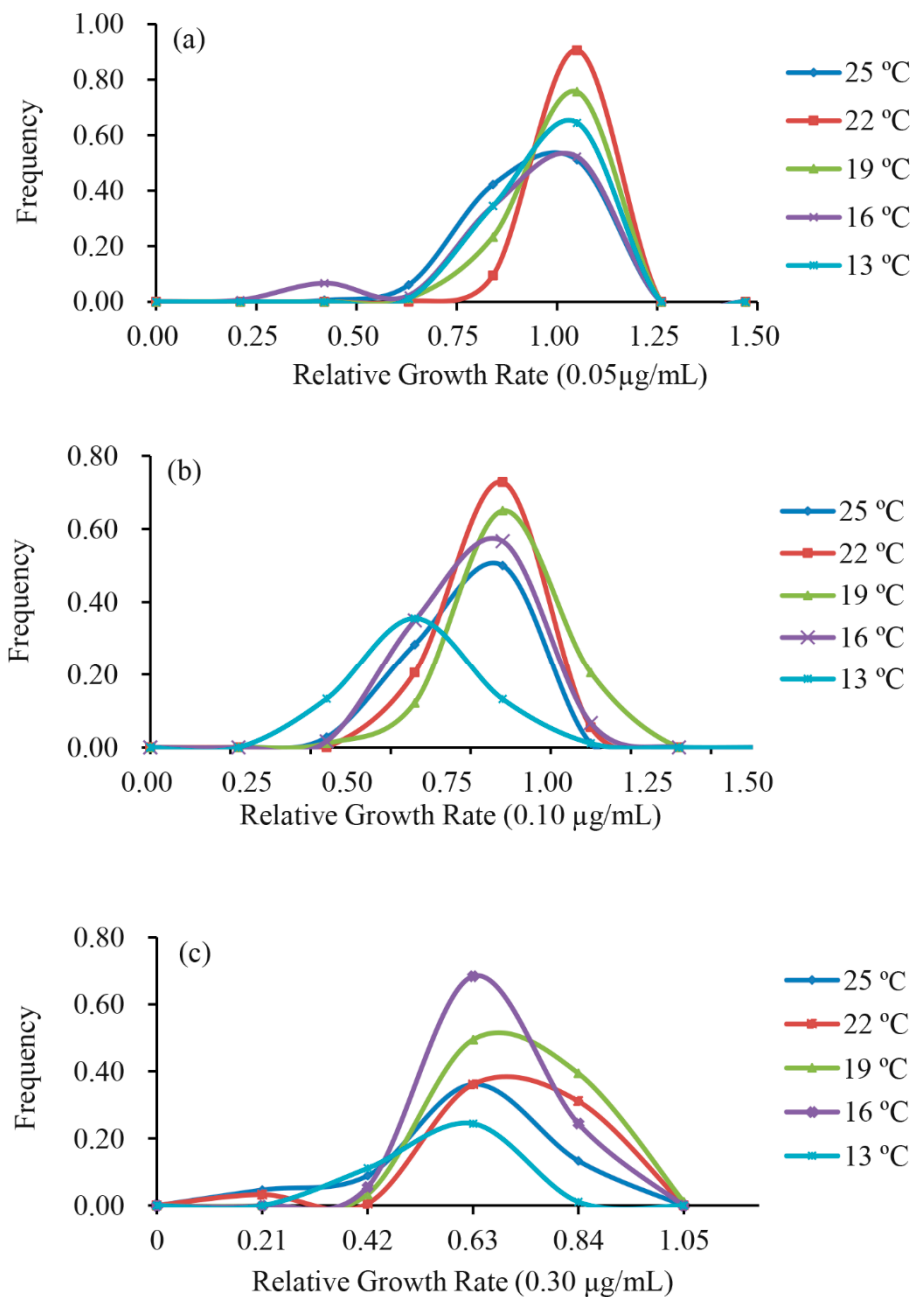


Figure 3. Frequency distribution of azoxystrobin sensitivity in the 180 isolates of *P. infestans* sampled from nine populations. The sensitivity was measured by relative growth rate (RGR) of the isolates in the presence and absence of fungicide and presented according to each of the five experimental temperatures (dark blue: 25 °C, red: 22 °C, green: 19 °C, purple: 16 °C, light blue: 13 °C): (a) 0.05 µg/mL; (b) 0.10 µg/mL; and (c) 0.30 µg/mL.

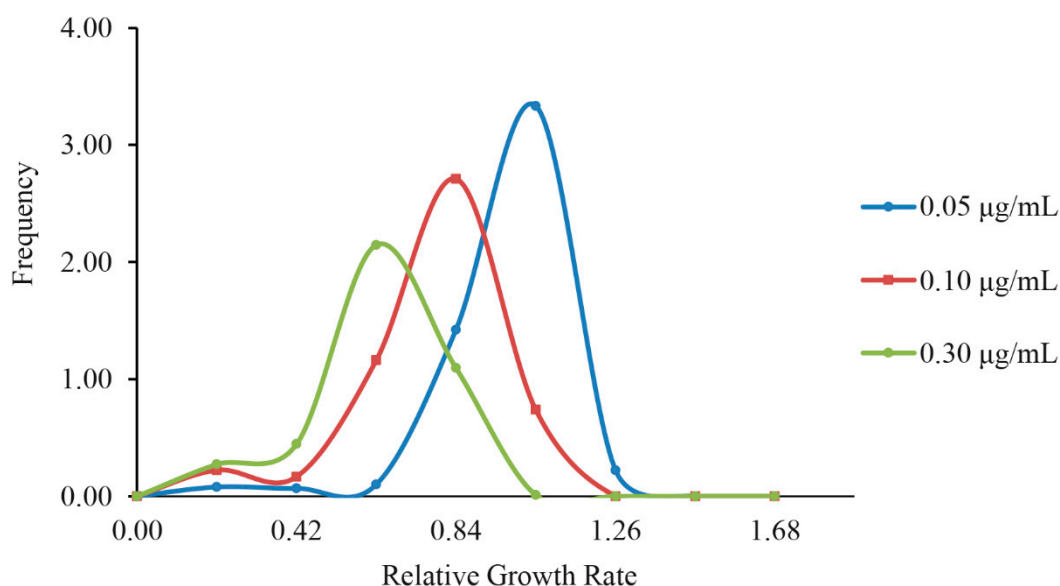


Figure 4. The frequency distribution of azoxystrobin sensitivity over five experimental temperatures in the 180 isolates of *P. infestans* sampled from nine populations. Sensitivity was measured by relative growth rate (RGR) in the presence and absence of fungicide in the five experimental temperatures.

3.2. Gene Diversity in SSR Markers, Genetic and Phenotypic Variation in Azoxystrobin Sensitivity of *P. infestans* Field Populations

The SSR diversity in the nine *P. infestans* field populations ranged from 0.40 to 0.61 with a mean average diversity of 0.47 (Table 1). The *P. infestans* population collected from Inner Mongolia exhibited the highest SSR diversity, while that sampled from Ningxia showed the lowest level of gene diversity. Estimated phenotypic plasticity in the nine *P. infestans* populations ranged from 0.21 to 0.54 with a mean of 0.40, while heritability in the populations ranged from 0.12 to 0.28 with a mean of 0.16, and ratio of plasticity to heritability ranged from 1.64 to 5.68 with an overall mean of 3.09 (Table 1).

Table 1. Gene diversity in simple sequence repeat (SSR) marker loci and variance in fungicide tolerance in the nine field populations of *P. infestans*.

Population	Isolate	SSR Diversity	Variance (RGR)		
			Plasticity	Heritability	R *
Inner Mongolia	20	0.61	0.35	0.13	2.80
Ningxia	20	0.40	0.47	0.19	2.43
Gansu	20	0.48	0.54	0.13	4.29
Guizhou	20	0.41	0.32	0.06	5.15
Yunnan	20	0.49	0.49	0.28	1.74
Wuhan	20	0.43	0.53	0.26	2.05
Xiapu	20	0.50	0.31	0.05	5.68
Fuzhou	20	0.45	0.42	0.21	1.97
Guangxi	20	0.45	0.21	0.12	1.67
Average	20	0.47	0.40	0.16	3.09

* Ratio of plasticity to heritability (calculated by dividing plasticity by heritability in each population). RGR—relative growth rate.

3.3. Associations of Variances (Phenotypic Plasticity and Heritability) in Azoxystrobin Sensitivity with Gene Diversity in SSR Marker Loci

Negative associations were observed between the gene diversity in SSR marker of the nine *P. infestans* populations with phenotypic variance as measured by plasticity ($r = -0.133$, $p = 0.733$) and genetic variance as measured by heritability ($r = -0.173$, $p = 0.656$), respectively (Figure 5a,b), but none

of them was significant, indicating an independent evolution of neutral SSR markers and azoxystrobin resistance in the pathogen.

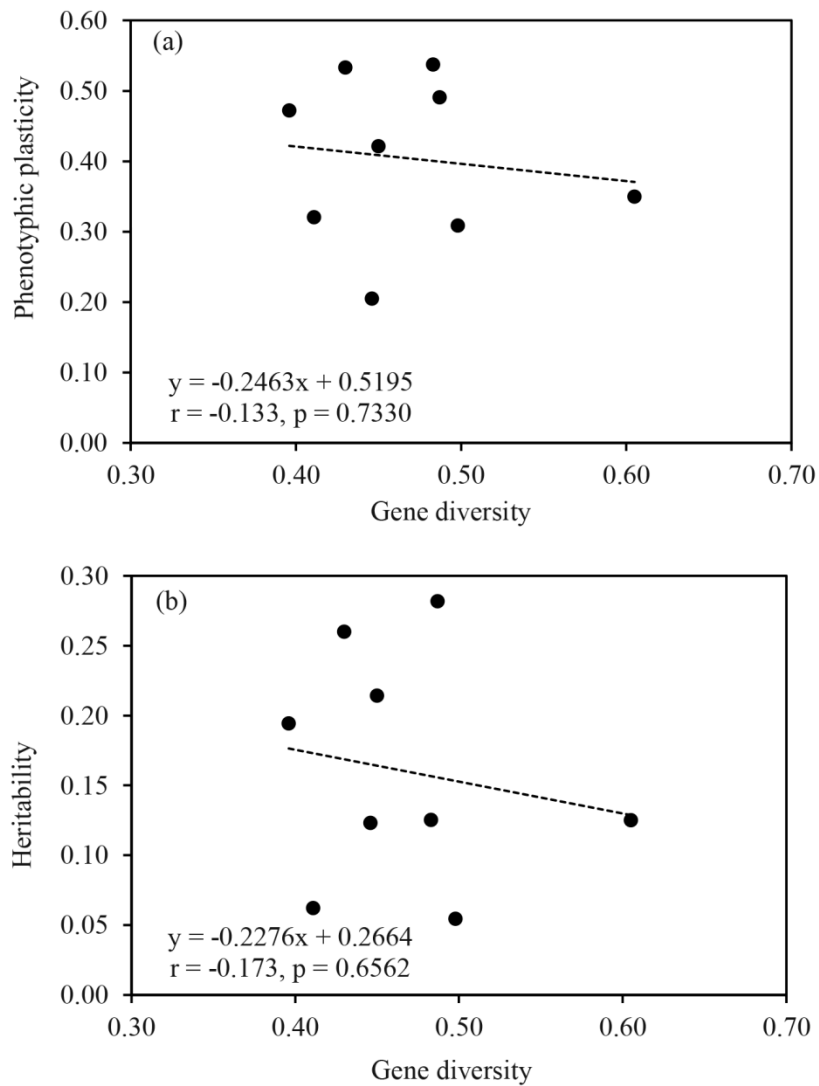


Figure 5. Correlations between gene diversity in the SSR markers loci and (a) phenotypic plasticity (variance of genotype \times concentration); (b) heritability (genetic variance).

3.4. Pairwise Q_{ST} – F_{ST} Comparisons

The overall Q_{ST} (0.24) was greater than F_{ST} (0.16). The pairwise Q_{ST} between the nine populations ranged from 0.00 to 0.72, while the pairwise F_{ST} among the populations ranged from 0.01 to 0.18 (Table 2). Negative and non-significant correlation ($r = -0.057$, $p = 0.741$) existed between the pairwise Q_{ST} and F_{ST} (Figure 6).

Table 2. Pairwise comparisons between the population differentiations for SSR marker loci (F_{ST}) and differentiations in thermal azoxystrobin tolerance measured by RGR (Q_{ST}) among the nine populations of *P. infestans*.

	Guizhou	Fuzhou	Guangxi	Gansu	Ningxia	Xiapu	Yunnan	Wuhan	Inner Mongolia
Guizhou	-	0.10	0.11	0.13	0.11	0.03	0.05	0.03	0.09
Fuzhou	0.14	-	0.13	0.13	0.05	0.07	0.06	0.08	0.04
Guangxi	0.55	0.34	-	0.08	0.15	0.05	0.09	0.10	0.03
Gansu	0.42	0.12	0.00	-	0.14	0.11	0.07	0.09	0.01
Ningxia	0.00	0.19	0.54	0.40	-	0.10	0.17	0.11	0.11
Xiapu	0.72	0.50	0.00	0.00	0.70	-	0.12	0.18	0.04
Yunnan	0.35	0.00	0.20	0.19	0.26	0.51	-	0.13	0.15
Wuhan	0.28	0.00	0.29	0.19	0.23	0.52	0.00	-	0.13
Inner Mongolia	0.44	0.00	0.00	0.01	0.41	0.22	0.04	0.09	-

Values above the diagonal are F_{ST} , and those below the diagonal are Q_{ST} . Populations with greater F_{ST} and Q_{ST} comparison are indicated by values in bold.

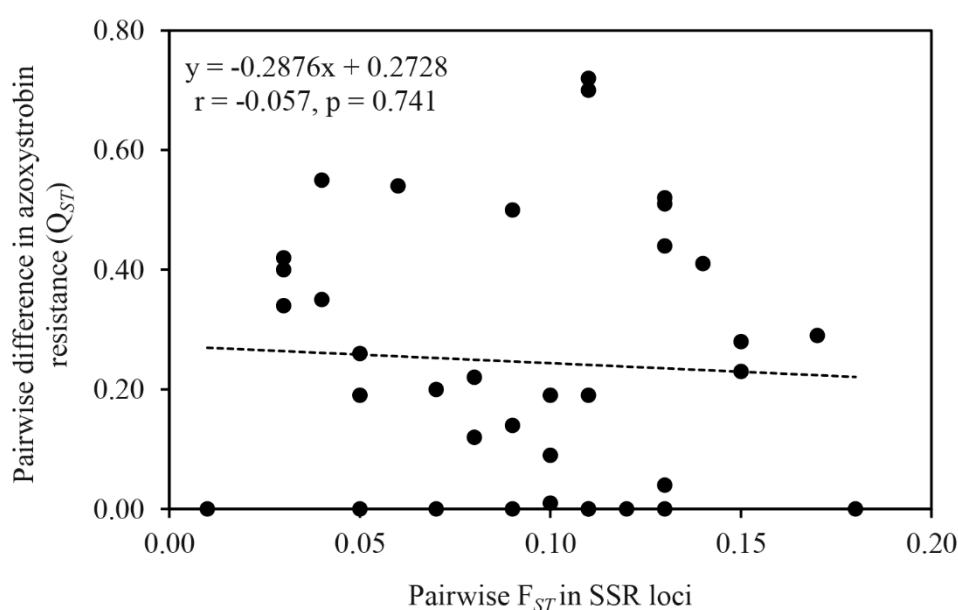


Figure 6. Correlation among the pairwise population differentiations in SSR marker loci (F_{ST}) and differentiations in azoxystrobin resistance (Q_{ST}) in the nine *P. infestans* populations. The pairwise F_{ST} and differentiations in azoxystrobin resistance Q_{ST} were measured by RGR over the three fungicide concentrations (0.05, 0.10, and 0.30 $\mu\text{g}/\text{mL}$ azoxystrobin).

4. Discussion

Evolution of fungicide resistance greatly undermines plant disease management, therefore, threatening agricultural production, food security, and socioeconomic development [9]. However, employing some management strategies guided by evolutionary principles such as rotating fungicide when it is applied more than one time in a season [54,55], mixing compounds with different modes of action [55], and/or applying when only it is necessary with right doses [56], could reduce the resistance development and ensure sustainable disease management [57]. In order to lessen the danger of efficacy loss in fungicides, knowing the genetic and evolutionary mechanisms and processes liable for the development of resistance in pathogens populations is of paramount importance.

4.1. Sources of Genetic Variation Contributing to Azoxystrobin Adaptation in *P. infestans* Populations

Our results showed that *P. infestans* populations responded and distributed differently to the azoxystrobin treatments, suggesting that the pathogens originating from different locations in China experienced different evolutionary processes. This result is supported by the comparative analyses of genetic variation and population differentiation in SSR neutral markers and azoxystrobin

sensitivity. No pairwise correlation between Q_{ST} and F_{ST} across the populations and significantly higher overall Q_{ST} than overall F_{ST} is an indication that evolution of azoxystrobin resistance was under diversifying selection driven by local adaptation. This type of selection can only be possible when some of the phenotypic traits are favored by local environments such as climatic conditions or agricultural practices [58], for example, the density of fungicide application or trade-offs associated with fungicide resistant mutants [27,59]. Previous studies demonstrated that Q_{ST} - F_{ST} comparisons are a powerful approach to infer the importance of diversifying selection in the evolution of quantitative traits [44,60], and revealed that the evolutionary mechanism serves as main driver for the development of fungicide resistance and other ecological traits in pathogen populations including virulence, pesticide resistance, and temperature tolerance in *Puccinia striiformis*, *Mycosphaerella graminicola*, *P. infestans*, and *Parastagonospora nodorum*, [38,45,61,62]. On the contrary, stabilizing selection ($Q_{ST} < F_{ST}$) was found to drive the adaptation of fungicide resistance in a barley pathogen *Rhynchosporium commune* [14]. The fields where the pathogen samples were collected were not sprayed with azoxystrobin. Therefore, the fungicide resistance observed in the study likely represents the true pattern of the local metapopulations constantly experiencing extinction and recolonization [63,64], indicating that the pathogens in different regions should be treated with different formulations of the fungicide for an improved economic and ecological benefit [65].

4.2. Evolutionary Processes Driving the Development of Azoxystrobin Resistance in *P. infestans*

The different evolutionary history among geographic populations of *P. infestans* was supported by the non-association between gene diversity in SSR markers and variances in azoxystrobin resistance (Figure 6), which is expected to be positively corrected for neutral traits [59]. Furthermore, genetic variation is the main determinant of adaptive ability of species to environments [66], and analysis of genetic variation in species can help us to understand its potential and mechanism of evolutionary adaptation [67]. Our results showed that phenotypic plasticity accounted for ~40% of the total phenotypic variation while on average the genetic variation (heritability) accounted for 16% of the variation (Table 1). Hence, this result suggests that the phenotypic variation caused by the regulation of gene expression and/or metabolic activity plays a more important role in the development of azoxystrobin resistance in *P. infestans* than the change of genetic architecture in the target genes. Supporting previous results, that resistance to site-specific fungicides in pathogens is mainly regulated by altering gene expression [12,48,68,69]. This could also contribute to the non-association of gene diversity with the plasticity, heritability in the populations (Figure 5a,b), respectively.

4.3. Impact of Elevated Temperature and Global Warming on Efficacy of Azoxystrobin Fungicide on the Management of *P. infestans*

The findings of the highest sensitivity (lowest RGR) of the pathogen to low doses of azoxystrobin (0.05 and 0.10 $\mu\text{g}/\text{mL}$) (Figure 3a,b) and the least sensitivity (highest RGR) in the lowest temperature 13 °C (Figure 3b,c) indicates that efficacy of controlling *P. infestans* with azoxystrobin fungicides tends to increase as the local air temperature increases, consistent with our previous result of negative association between azoxystrobin tolerance and mean annual temperature of *P. infestans* collection sites [48]. Global warming is a major concern of human society. It is projected that the air temperature on the earth will increase several degrees in the next decades [70], and the impact of global warming on food production and disease management is still largely unknown. Our result suggests that elevated air temperature associated with global warming in the future may increase the effectiveness of the fungicide. This result also supports the hypothesis that temperature can regulate both pathogen sensitivity to fungicides and the toxicity of chemical compounds [25,27]. The result further showed that an increase in fungicide doses could lead to a decrease in sensitivity among the pathogens (Figure 4). Hence, in agricultural practices local thermal conditions and fungicide doses need to be considered together for effective and sustainable management of plant diseases.

5. Conclusions

In conclusion, the results from this study demonstrate that variation in azoxystrobin sensitivity exists among *P. infestans* populations originating from different regions, and diversifying selection according to local environmental conditions such as temperature and the density and history of azoxystrobin application is an important evolutionary mechanism contributing to the evolutionary development of the fungicide resistance in *P. infestans* populations. Tolerance of *P. infestans* to azoxystrobin was found to be mainly physiological governed by phenotypic plasticity through the changes of target gene expression and/or metabolic activity of the species, although genetic alternation in the nucleotide sequence of target genes is also important, and warmer places tend to select for *P. infestans* populations with higher azoxystrobin sensitivity. These findings are useful toward the mitigation and prevention of developing azoxystrobin resistance in *P. infestans* populations and the formulation of sustainable late blight management strategies to boost potato production, which is playing an increasing role in meeting food demand in the growing world population. A single, cross-region protocol of azoxystrobin application such as using same doses might not work effectively and sustainably due to the spatial variation in azoxystrobin sensitivity of the pathogen. Hence, farmers may need to take local thermal conditions into account when formulating application doses and frequency of azoxystrobin to maximize the efficacy of the fungicide and minimize environmental pollution and the potential of developing azoxystrobin resistance in *P. infestans*.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2071-1050/12/3/1188/s1>, Figure S1: Examples of highly sensitive and less sensitive isolates of *P. infestans* grown on 9 cm plates at different temperatures. The left two panels show the photos of highly sensitive isolates grown at each of five temperatures under control (first column) and fungicide treatment (second column) and right two panels show the photos of less sensitive isolates grown at each of five temperatures under control (third column) and fungicide treatment (fourth column).

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