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The adaptive potential of a plant pathogenic fungus, *Rhizoctonia solani* AG-3, under heat and fungicide stress

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Abstract The ability to improve fitness via adaptive evolution may be affected by environmental change. We tested this hypothesis in an in vitro experiment with the plant pathogen *Rhizoctonia solani* Anastomosis Group 3 (AG-3), assessing genetic and environmental variances under two temperatures (optimal and higher than optimal) and three fungicide concentrations (no fungicide, low and high concentration of a copper-based fungicide). We measured the mean daily growth rate, the coefficient of variation for genotypic (I_G) and environmental variance (I_E) in growth, and broad-sense heritability in growth. Both higher temperature and increased fungicide concentration caused a decline in growth, confirming their potential as stressors for the pathogen. All types of standardized variances in growth—I_G, phenotypic variance, and I_E as a trend—increased with elevated stress. However, heritability was not significantly higher under enhanced stress because the increase in I_G was counterbalanced by somewhat increased I_E. The results illustrate that predictions for adaptation under environmental stress may depend on the type of short-term evolvability measure. Because mycelial growth is linked to fitness, I_G reflects short-term evolvability better than heritability, and it indicates that the evolutionary potential of *R. solani* is positively affected by stress.

Keywords Evolvability · Fungicide resistance · Genetic variation · Heat-stress · Optimal temperature

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Introduction

Stressful environmental change not only decreases individual and possibly population mean fitness, but may also impact genetic variation and the evolvability of polygenic traits (Hoffmann and Merilä 1999). To understand whether organisms can adapt to initially novel, stressful conditions, we must study how stress affects evolutionary potential (Schlichting 2008). In this study, we estimated the effect of an increase in temperature and the application of a broad-spectrum fungicide on evolvability in growth in the plant pathogenic fungus *R. solani* Anastomosis Group 3 (AG-3).

Evolutionary biologists have proposed several hypotheses on stress and genetic variation (reviewed in Hoffmann and Merilä 1999). Some of these predict an increase of additive genetic variance under stress and a positive effect on heritability, h^2 (the ratio of additive genetic variance, V_A, to phenotypic variance, V_P). Explanations involve selection that has not yet eliminated deleterious alleles in novel stressful environments, de-canalization of otherwise unexpressed genetic variants under stress, or enhanced genotypic differences when resources are limited. Other hypotheses predict a decrease in heritability, either because stress enhances environmental variance without necessarily affecting additive genetic variance, or because stress prevents organisms from reaching their genetic potential under poor nutrition with the consequence of reducing genetic variance.

Empirical studies on genetic variance and heritability under favourable and unfavourable conditions have almost entirely focused on plants, insects and vertebrates (e.g., Hoffmann and Merilä 1999; Charmantier and Garant 2005; Laperche et al. 2006). These studies have also produced conflicting results. Laboratory work on *Drosophila* and mice reveals a trend toward increased heritability under stress (Hoffmann and Parsons 1991), whereas wild populations of vertebrate and invertebrate animals may have lower heritabilities for body size under stressful conditions (Charmantier and Garant 2005). Charmantier and Garant (2005) argued that the decline in heritability under stress that they observed may be due to a decline in V_A, apart from an increase in V_E. For cultivated grasses it was found that nutritional stress commonly leads to decreased heritability (Laperche et al. 2006). Our study of microorganisms broadens the taxonomic focus and therefore helps build an understanding of the capacity of organisms to evolve under environmental stress.

Also, the unresolved relationship between heritability and stress suggests that it may be useful to explore measures of evolvability other than heritability. Houle (1992) argued that the genetic coefficient of variation (V_A standardized by the square of the mean) is the appropriate measure of evolvability for fitness-related traits, and facilitates comparison of variances across environments. While heritability predicts the response to selection (R) relative to the selection intensity (i) and phenotypic standard deviation of the selected trait before selection, σ_P (Falconer and Mackay 1996; $R = h^2 i\sigma_P$), standardized genetic variance for a fitness-related trait (I_G) predicts the selection response relative to the trait mean before selection, X (Houle 1992; $R = I_G X$). Because both types of estimate predict the selection response, they can both be considered measures of a population's evolvability or adaptive potential (Houle 1992; Willi et al. 2006).

Rhizoctonia solani AG-3 belongs to a species complex that consists of several sub-taxa unified on the basis of molecular phylogenetic analysis, somatic hyphal interactions (anastomosis grouping), and host-specificity (Ceresini et al. 2007). The life cycle has an asexual and a sexual phase with the production of basidiospores, though the former phase is the most commonly observed. During the asexual phase, the fungus grows mycelia by hyphae and—to endure unfavorable conditions—sclerotia (hardened fungal mycelia). *Rhizoctonia solani* AG-3 is associated primarily with diseases of solanaceous host plants. Infection starts with hyphae growing on the plant surface, later forming appresoria to penetrate plant cells. Initial infection is followed by the release of enzymes that degrade cell walls, kill the cells, and promote the spread of hyphae in dead cells. Mycelial growth is therefore an important fitness component for this species because the fungus grows on and damages host plants with asexual hyphae (Lehtonen et al. 2008). The stress conditions we studied were elevated temperature and two concentrations of a copper oxychloride-based fungicide. Copper 2+ ions have a broad-spectrum effect on microorganisms, disrupting cell membrane integrity, displacing essential metals within cells, and interfering with oxidative phosphorylation and the conformational structure of nucleic acids and proteins (Borkow and Gabbay 2005). The broad-spectrum effect of copper oxychloride suggests that fungal growth rate in the presence of fungicide may have a polygenic basis. The focal question was: Does stress imposed by fungicide and increased temperature influence evolvability measures?

Materials and methods

Isolates of *R. solani* AG-3 were collected in 2008 from a potato field at Agroscope Changins Research Station, near Geneva, Switzerland, and 10 of these were randomly chosen for this study. Before the start of the experiment, isolates had been clonally propagated and transferred twice to petri dishes with potato-dextrose agar (30 g/l potato dextrose, 2 g/l agar, 50 mg/l kanamycin). From the second generation of propagation, cores of 4 mm diameter were punched from the front range of mycelium growth and used as inocula for the experimental plates. Each core was placed upside-down in the center of the dish. There were three fungicide treatments: 1 g/l Cupromaag in the agar preparation (Syngenta Agro, Switzerland; 20% of the recommended concentration for controlling potato diseases), 0.1 g/l Cupromaag, and no fungicide. Cupromaag contains copper oxychloride. Two temperature treatments were tested together with fungicide in a complete factorial design: 24 or 27°C. The lower temperature is about optimal for mycelial growth of *R. solani* AG-3 on potato-dextrose agar (Ritchie et al. 2009). In total, there were 10 isolates × 3 fungicide concentrations × 2 temperatures × 4 replicates (each of a different precursor dish)—8 losses (6 of which originated from a single precursor dish and had 87% reduced growth compared to the other replicates of the same isolate in the different environments), resulting in 232 dishes. Each replicate was set up by a different person and this was accounted for in the variance partitioning (see below). After 66 h, we measured the radial growth rate (mm/day).

We used analysis of variance to test for effects of temperature and fungicide on radial growth rate to reveal their level of stress (PROC GLM, SAS Institute 2008; see Table 1 for complete presentation of model). Fixed effects involving temperature and fungicide were tested over their interactions with isolate because isolates were considered replicates for evaluating fixed effects. All other effects

were tested over the pooled error. Subsequently, we estimated variance explained by isolate, person punching, person measuring and error variance for each environment separately (PROC MIXED, restricted maximum likelihood; SAS Institute 2008). The model included the intercept as a fixed effect, and as random effects isolate, person punching, and person measuring. Wald *Z*-tests revealed whether variances were significantly different from 0. Variance explained by isolate was interpreted as genotypic variance (V_G) while the error was considered to be environmental variance (V_E). Variances were standardized by the squared trait mean for the environment (now I_P, I_G, and I_E; Houle 1992). Broad-sense heritability was calculated as the ratio of genotypic to phenotypic variance ($V_P = V_G + V_E$). Standard errors of trait-mean adjusted variances and heritabilities were calculated by jackknifing over isolates (Knapp et al. 1989).

Table 1 General linear model testing the effects of temperature, fungicide concentration, isolate, and their interactions on in vitro radial mycelial growth per day of the plant pathogen *Rhizoctonia solani* AG-3

Source of variation	$df_{num, denom}$	MS	*F*	*P*
Temperature	1, 9	178.43	69.73	**<0.0001**
Fungicide concentration	2, 18	1939.79	791.79	**<0.0001**
Temp. × fungicide conc.	2, 18	23.98	30.45	**<0.0001**
Isolate	9, 172	35.60	43.71	**<0.0001**
Isolate × temperature	9, 172	2.56	3.14	**0.0016**
Isolate × fungicide conc.	18, 172	2.45	3.01	**0.0001**
Isolate × temp × f. conc.	18, 172	0.79	0.97	0.5000
Error	172	0.81		

Fixed effects involving temperature and fungicide were tested over their interactions with isolate. *P*-values <0.05 are indicated in bold

Results

Both temperature and fungicide concentration had significant effects on radial growth rate (Table 1), with high fungicide concentration and higher than optimal temperature causing lower growth. The interaction between temperature and fungicide reflected a higher impact of fungicide at optimal temperature than at 27°C (Fig. 1). This interaction disappeared under log transformation of growth, because the proportional effect of fungicide was similar at both temperatures. Significant effects of isolate indicated that growth differences among genotypes were maintained across all environments (Fig. 1). Nevertheless, there was genetic variation in the growth effects of fungicide and temperature, because there was significant genotype-by-environment interaction (G × E; Table 1). Such GxE interactions are a prerequisite for changes in genetic variance among environments. Differences between fast and slow growing genotypes were more pronounced under stress; in a few cases reaction norms crossed (Fig. 1). The three-way interaction was not significant because all isolates experienced the temperature-by-fungicide interaction similarly (Table 1). Table 2 lists mean growth for the six environments, along with genotypic and environmental variances. Regressions of jackknifed standardized variances on mean growth (the latter indicating the level of stress caused by the environment) were significantly negative for genotypic variance and phenotypic variance (I_G: b ± SE −0.022 ± 0.003, $P < 0.01$, Fig. 2a; I_P: −0.023 ± 0.003, $P < 0.01$), nearly significant for environmental variance (I_E: −0.0015 ± 0.0006, $P = 0.08$, Fig. 2b), but not significant for broad-sense heritability (H^2: −0.016 ± 0.012, $P > 0.2$; Fig. 2c).

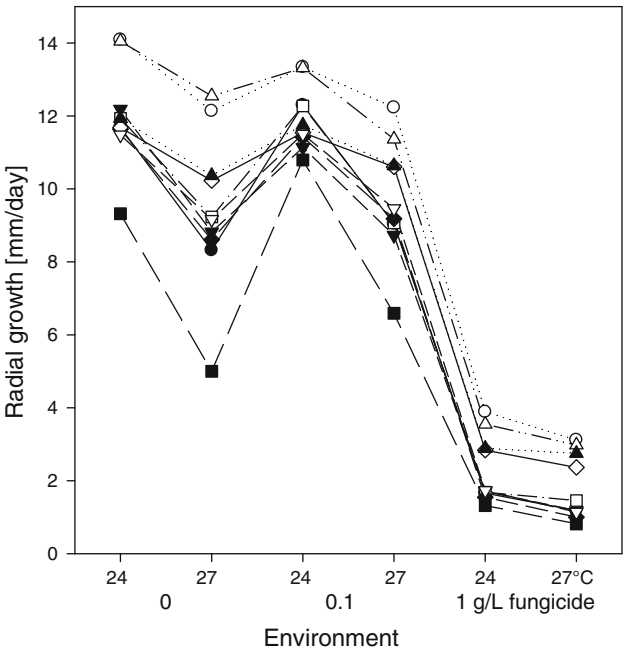

Fig. 1 Genotype means of radial growth rate across environments in *Rhizoctonia solani* AG-3. The ten genotypes are represented by *different symbols* and *line* types; environments include optimal and higher than optimal temperature (24 and 27°C), and three fungicide concentrations (0, 0.1 and 1 g/l)

Discussion

This study of a plant pathogenic fungus shows that stress due to elevated temperature and exposure to fungicide causes heightened predicted evolvability measured as genotypic variance relative to the trait mean, but no

Table 2 Means, genotypic (V_G) and environmental variances (V_E), and test statistics for mycelial growth rate of the plant pathogen *Rhizoctonia solani* AG-3 under six environmental treatments

Environment	Mean ± SE	V_G	Z	P	V_E	Z	P
24°C, no fungicide	12.00 ± 0.43	1.6794	1.98	**0.024**	0.4405	3.42	**<0.001**
24°C, low	11.94 ± 0.27	0.7306	1.91	**0.028**	0.2769	3.27	**<0.001**
24°C, high	2.28 ± 0.29	0.8500	2.07	**0.019**	0.0862	3.60	**<0.001**
27°C, no fungicide	9.44 ± 0.67	4.3713	2.03	**0.021**	0.8117	3.60	**<0.001**
27°C, low	9.69 ± 0.50	2.1212	1.73	**0.042**	1.8116	3.53	**<0.001**
27°C, high	1.80 ± 0.28	0.8034	2.06	**0.020**	0.0696	3.49	**<0.001**

P-values < 0.05 are indicated in bold. Wald's Z tests whether variances were significantly different from 0

directional change in heritability. Willi et al. (2010) observed a similar result in *Mycosphaerella*, a wheat fungal pathogen. While that study focused on the general impact of stress on evolvability estimates, here we applied treatments (fungicide and heat) that are relevant for *R. solani* AG-3 in the agricultural practice.

Our results imply that changes in heritability under stress are inherently difficult to predict because multiple components of variance can change simultaneously, but may not change relative to each other (Hoffmann and Merilä 1999). This may be one reason why meta-analyses on the relationship between heritability and stress have not revealed clear-cut patterns (Hoffmann and Merilä 1999; Charmantier and Garant 2005). We suggest that variance components determining heritability should be directly compared across environments, and this requires standardization by the squared trait mean. At the same time, the coefficient of variation may be a more meaningful measure of evolvability than heritability for some kinds of traits (Houle 1992). In our study, standardized genotypic variances increased under increasing levels of stress. The variance among isolates decreased when conditions were stressful, but the trait mean was also sharply reduced, so the evolvability of growth rate relative to the trait mean was enhanced. If evolvability is best expressed as the predicted response to selection relative to the trait mean before selection, as has been shown for fitness-related traits (Houle 1992), stress will actually increase evolvability despite a concurrent increase in environmental variance.

A more general understanding of stress and genetic variation may emerge from mechanistic information on how genetic and environmental variances change. In our experiment, the increase in genetic variance was not achieved by genotypes performing very differently relative to each other under different environments. In fact, there were few examples of crossing reaction norms. Instead, differences between fast and slow growing genotypes were simply more enhanced under stressful conditions. Of the several mechanisms outlined by Hoffmann and Merilä (1999), the most probable in our case may be increased developmental noise combined with de-canalization of otherwise non-expressed genetic variants under unfavorable conditions. Heat and other sources of stress have been linked to the increased presence of active heat-shock proteins that work as chaperones for other proteins, for example by facilitating protein folding or modulating protein activity (Craig et al. 1993; Hoffmann and Willi 2008). Heat-shock proteins operate also in the absence of stress (Kregel 2002), but when stress levels increase substantially, they may become limited in fulfilling their tasks despite their increased concentration in the cells (Rutherford and Lindquist 1998). On the one hand, this has been shown to increase developmental noise (Rutherford and Lindquist 1998), a propensity that may be underlain by genetic variation and that evolves (Pigliucci 2008). On the other hand, genetic variants may be expressed under stress that otherwise are buffered, as has been documented in both *Drosophila* and *Arabidopsis* (Rutherford and Lindquist 1998; Queitsch et al. 2002). An experiment on the reaction of yeast to copper exposure revealed that heat-shock proteins are very susceptible to oxidation, suggesting that copper stress like heat stress can increase the expression of cryptic genetic variation (Shanmuganathan et al. 2004).

Independent of stressors, we found that evolvability measures of *R. solani* AG-3 —I_G and H^2—were generally high (ranges: I_G: 0.005–0.27; H^2: 0.6–0.9). Houle (1992) reported I_A values (standardized additive genetic variance) for *Drosophila melanogaster* of 0.0006 (development time), 0.0098 (longevity), and 0.0142 (fecundity). High evolvability for quantitative traits relevant to disease control, mycelial growth, and tolerance to a copper-based fungicide likely stem from gene flow and high effective population size (N_e). Gene flow within *R. solani* AG-3 on potato is substantial, with the result of little population differentiation on local or regional scales (Ceresini et al. 2002; Ceresini et al. 2003; Ferrucho et al. 2009). With their positive effect on genetic variation in polygenic traits, high effective population sizes and migration rates will enable the pathogen to adapt rapidly to global change over

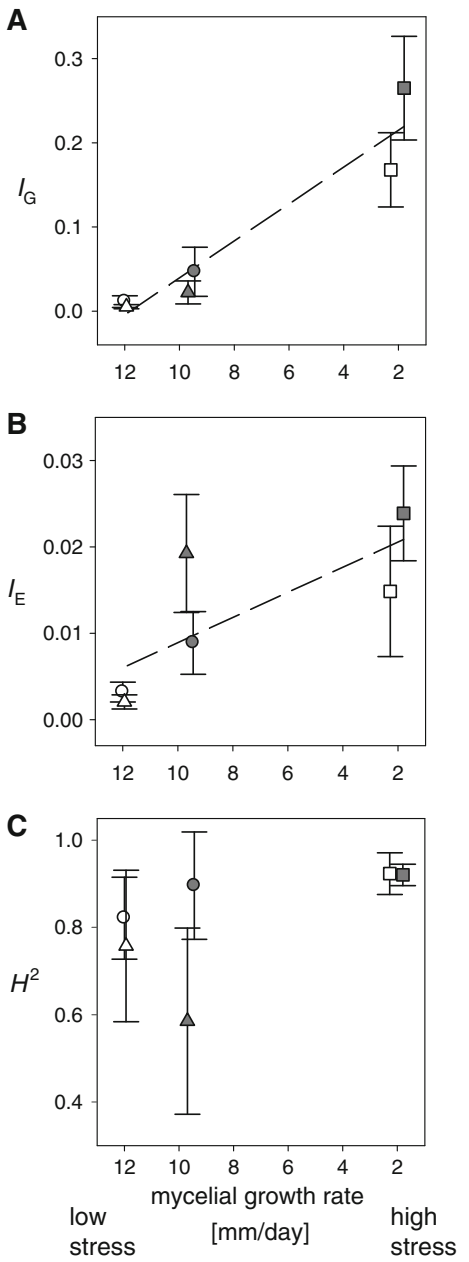

Fig. 2 Genotypic and environmental variance (I_G and I_E, respectively, standardized by squared mean growth) and broad-sense heritability (H^2) of radial growth rate depending on environmental stress (**a**–**c**) in *Rhizoctonia solani* AG-3. The level of stress in *panels* **a**–**c** is reflected in mean growth rate (higher growth and lower stress on left end of *x*-axis). *Dark grey symbols* represent jackknifed mean estimates at 27°C and *white symbols* are at 24°C; *error bars* indicate ± SE. The three symbol types indicate the three fungicide concentrations: no (*circle*), low (*triangle*) and high (*square*). While relationships between genotypic and environmental variance and level of stress were significant or nearly so, heritability showed no association with stress (see "Results" section)

its extended geographic range (McDonald and Linde 2002; Willi et al. 2006; Willi and Hoffmann 2009). High migration rates also suggest that the high evolvability parameters are likely to be found not only for the local population we analyzed but for the whole region.

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