Scientific article

Genetic inheritance analysis of the resistance to a virulent isolate of *Colletotrichum acutatum* in strawberry based on an octoploid model

Análisis de la herencia genética de la resistencia a un aislado virulento de *Colletotrichum acutatum* en frutilla basado en un modelo octoploide

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Abstract

In the present work we propose a hypothesis to explain the mechanism of the genetic control of resistance to anthracnose and we show an experimental model that may support our hypothesis. The inheritance of the strawberry resistance to a virulent strain of *Colletotrichum acutatum* isolated in Northwestern Argentina was assessed with a progeny obtained from the cross between the susceptible cultivar 'Pájaro' as female and the resistant cultivar 'Sweet Charlie' as male. Infections were carried out under controlled conditions by spraying a conidial suspension on leaves of young plants and lesions were evaluated from days 10-50 after infection. The disease severity ratings (DSR) values exhibited a continuous distribution. We propose a model based on a gene-for-gene interaction in an octoploid genomic background to explain the results. The model assumes that the genetic control of the resistance is determined by different allelic variants of an R-gene. According to this model, results suggest that susceptibility to this isolate is partially dominant over resistance and that the defensive response would be modulated by the allele dosage, although other gene interactions may also be involved.

Keywords: Anthracnose; *Colletotrichum acutatum*; *Fragaria ananassa*; Heritability; Resistance inheritance.

Resumen

En el presente trabajo proponemos una hipótesis para explicar el mecanismo del control genético de la resistencia a la antracnosis y mostramos un modelo experimental que puede apoyar nuestra hipótesis. La herencia de la resistencia en plantas de frutilla a una cepa virulenta de *Colletotrichum acutatum* aislada en el noroeste de Argentina se evaluó con una progenie obtenida del cruce entre el cultivar susceptible 'Pájaro' como hembra y el cultivar resistente 'Sweet Charlie' como macho. Las infecciones se llevaron a cabo en condiciones controladas pulverizando una suspensión conidial en hojas de plantas jóvenes y las lesiones se evaluaron entre los 10 y 50 días posteriores a la infección. Los valores obtenidos en las evaluaciones de severidad de la enfermedad (DSR) exhibieron una distribución continua. Proponemos un modelo basado en una interacción gen-por-gen en un fondo genómico octoploide para explicar los resultados. El modelo supone que el control genético de la resistencia está determinado por diferentes variantes alélicas de un gen R. De acuerdo con este modelo, los resultados sugieren que la susceptibilidad a este aislamiento es parcialmente dominante sobre la resistencia y que la respuesta defensiva sería modulada por la dosificación del alelo, aunque también pueden estar involucradas otras interacciones génicas.

Palabras claves: Antracnosis; *Colletotrichum acutatum*; *Fragaria ananassa*; Heredabilidad; Herencia de la resistencia.

Introduction

Anthracnose is an important disease of strawberry caused by several species of the fungus *Colletotrichum*. The genetics of the resistance to anthracnose in strawberry has been studied by several authors. Gupton and Smith (1991) suggested that the action of a major gene may be involved

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in this resistance. Other authors proposed that resistance to the isolate 1267b of *Colletotrichum acutatum* was monogenically controlled, being the resistance dominant over susceptibility, whereas resistance to other isolates of *C. acutatum* seemed to be polygenic (Denoyes and Baudry, 1995; Denoyes-Rothan, 1997). In contrast, Giménez and Ballington (2002), determined that resistance to runner infection appeared to be quantitative, with important non-additive and additive effects on the inheritance of resistance. The screening studies performed by Ballington *et al.* (2002) supported the quantitative gene action model for resistance to anthracnose. Also, Mori (2002) working with F1 progenies of different strawberry cultivars inoculated with *Colletotrichum fragariae*, reported that the frequency distribution of resistance indexes varied continuously. These apparently contradictory results could be explained because in the genetic analysis the progenitors and isolates used were different, suggesting that there would not be a single mechanism valid for all the pathogen strains to explain the genetic control of anthracnose resistance. Consequently, results should be analyzed considering the specific interaction pathogen genotype x plant genotype.

In the published results no reference to the octoploid nature of *Fragaria ananassa* has been made to explain the genetic control of resistance to anthracnose. However, it is known that the genome redundancy created by polyploidy makes difficult the genetic analysis for a given trait.

In this work we propose a hypothesis to explain the mechanism of the genetic control of resistance to anthracnose that includes polymorphism of the resistance (R) gene and allele-dosage effects, in the strawberry octoploid genetic background, and we show an experimental model that may support our hypothesis. We examined the inheritance of the resistance to an isolate of *C. acutatum* in a progeny resulting from the cross between a susceptible and a resistant cultivar of *F. ananassa.* This study was performed by using a local strain of *C. acutatum*, which showed to be one of the most virulent isolates found in a pathogenic survey carried out in Northwest Argentina when tested against the cultivar 'Pájaro' (Ramallo *et al.,* 2000). Since we detected that the cultivar 'Sweet Charlie' was highly resistant to this isolate, we considered that the progeny obtained from the cross between this cultivar and the susceptible cultivar 'Pájaro', would be a proper model to analyze the genetic control of resistance. We also report the time evolution of the disease symptoms on leaves of young plants (before flowering) and discuss possible mechanisms involved in the control of resistance.

Material and methods

Plant materials

A progeny of 48 hybrids derived from the cross of cvs. 'Pájaro' x 'Sweet Charlie' was used. In a previous study, cv. 'Pájaro' was highly susceptible to a *C. acutatum* isolate called M11 (Racedo *et al*., 2013), whereas cv. 'Sweet Charlie' was very resistant (Ramallo *et al*., 1997). Seeds obtained from the cross were germinated on pasteurized substrate (humus:perlome, 2:1), then planted in 8cm pots and runner-propagated to obtain 10-12 seedlings from each genotype. Seedlings were grown for 14-16 weeks in growth cabinets at 28 °C, 70% RH and 16 h photoperiod.

Fungal cultures

The isolate M11 used in this experiment was obtained from the crown of strawberries plants (cv. 'Pájaro') cultivated in Tucumán (Argentina) that showed the characteristic symptoms of the anthracnose disease. Culture was carried out in PDA medium (potato-dextrose-agar) supplemented with streptomycin (300 mg/ml) at 28 ºC for 12 days under continuous fluorescent light (Smith and Black, 1990). The M11 isolate was identified as *Colletotrichum acutatum* according to classical microbiological (cultural characteristics and conidial morphology) and molecular criteria (Code IMI 386395; CABI BioScience International Mycology Institute, UK Center, Egham, England).

Inoculum

Fungal isolates were grown on PDA in Petri dishes for 10 days under continuous fluorescent light at 28 °C to induce conidia formation. Conidia were obtained from the surface of the colony, filtered through gauze to remove mycelial debris under axenic conditions and suspended in distilled water. Concentration of the inoculum was evaluated with a hematocytometer and adjusted to 1.5 x 10^6 conidia/ml.

Experimental design

The experimental design was randomized with 8 plants per genotype and per experimental unit, 4 corresponded to the challenged plants and 4 to the control plants. Each genotype was evaluated three times in successive independent experiments. Two replicates of the parental genotypes were included in each experiment. Prior to inoculation, all the leaves were removed from the plants with the exception of the youngest three leaves. All plant parts were sprayed to runoff with the conidial suspension. Controls were sprayed with sterile distilled water containing two drops of Tween 20 per liter. Immediately after inoculation, plants were placed in a dew chamber with 100% RH at 28-30 °C (infection chamber) for 48 h in the dark (Smith and Black, 1990). Then, plants were transferred to a glasshouse held at 30 ºC ± 2 ºC, and a 16 h-photoperiod, where they were maintained during the 50-day-period of evaluation.

Disease severity rating (DSR)

Plants were evaluated for disease severity from 10 days after inoculation up to 50 days, every ten days. A 1 to 5 severity scale based on petiole symptoms (lesion length and extent) was used. Disease severity was rated as follows: 1 = healthy petiole (no lesion); 2 = lesions less than 3 mm long; 3 = lesions 3-10 mm long; 4 = lesions 10-20 mm long, girdling petiole and 5 = entirely necrotic petiole and plant dead (Delp and Milholland, 1981). An average disease score for each hybrid was calculated based on the disease scores of the four plants representing each genotype, in each of the three replicate experiments.

Data analysis

Data based on three replicate experiments were subjected to ANOVA. Where the F-test showed significant differences, means were compared by means of a LSD-test (Dudley and Moll, 1969; Snedecor and Cochran, 1971; Freeman, 1973). Broad-sense heritability was estimated as follows: $H^2 = \sigma^2_G / \sigma^2_E + \sigma^2_G$, assuming that variation within genotypes (among replicates) could be attributed only to environmental effects (σ^2_E), while the one occurring among genotypes to both, environmental and genetic (σ^2_G) effects (Falconer, 1981).

Results and discussion

A continuous distribution of DSR (with values ranging between 1 and 5) was obtained when the progeny derived from the cross between the susceptible cultivar 'Pájaro' and the resistant cultivar 'Sweet Charlie' was evaluated for resistance to the M11 isolate of *C. acutatum*.

The assessment of DSR was performed during a period of 50 days post inoculation because in preliminary studies we observed that certain genotypes began to recuperate 30 days after the infection. For that reason, in order to determine the best period for evaluation of resistance/susceptibility of individuals, the broad-sense heritability (H^2) was estimated at each time of assessment of DSR (Figure 1). Results showed that values tend to increase from days 10 to 30 and reached to a plateau after 30 days. Accordingly, we assumed that the level of resistance or susceptibility of each hybrid corresponds to the average value of the DSR obtained at days 30, 40 and 50.

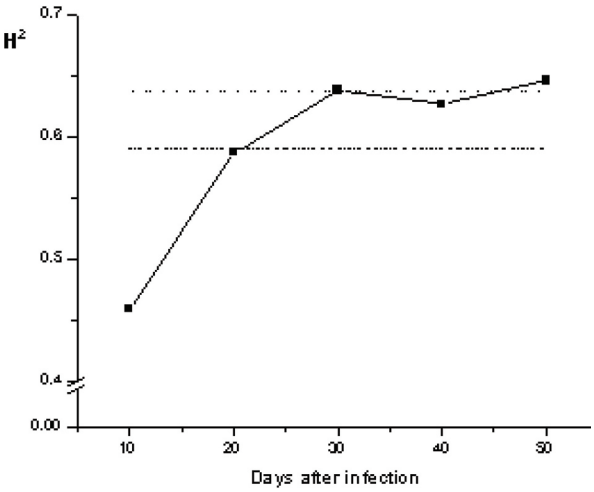

Figure 1. Broad-sense heritability (H^2) at each time of evaluation (___). H^2 mean value from 10 to 50 day = 0.591 ± 0.035 (_ _ _ _). H^2 mean value from 30 to 50 day = 0.637 ± 0.005 (- - -) (Mean ± standard error).

Based on these values and taking into account the severity of damage caused by the pathogen after 30 days, we determined four phenotypic classes : very resistant (DSR ≤ 1.5); tolerant (DSR = 1.6 to 3); susceptible (DSR = 3.1 to 4.5) and very susceptible (DSR > 4.5) (Figure 2). Plants were included in each class according to their most frequent values of DSR and if those values were maintained after 30 days. Only four genotypes did not fulfill the latter condition (see Figure 3c). Even though, they were included in the class that corresponded to the average value of DSR.

Table 1. Genetic structure of the genotypes involved in this study assuming an octoploid model. The corresponding DSR (Disease Severity Ratings), phenotypes, observed and expected segregation ratios are indicated (χ^2 = 3.496; $p < 0.01$). A 1 to 5 severity scale is used for resistance to the M11 isolate of *Colletotrichum acutatum*.

	Genotype	Phenotype	DSR	Segregation ratios	
				Observed	Expected
´Pájaro´	aa A´a´ Bb B´B´	very susceptible	5		
´S. Charlie´	aa a´a´ bb B´b´	very resistant	1.4		
Offspring	aa A´a´ Bb B´B´ aa A´a´ Bb B´b´	very susceptible	> 4.5	17	12
	aa a´a´ Bb B´B´ aa A´a´ bb B´B´	susceptible	3.1-4.5	11	12
	aa a´a´ Bb B´b´ aa A´a´ bb B´b´	tolerant	1.6-3	8	12
	aa a´a´ bb B´B´ aa a´a´ bb B´b´	very resistant	≤ 1.5	12	12

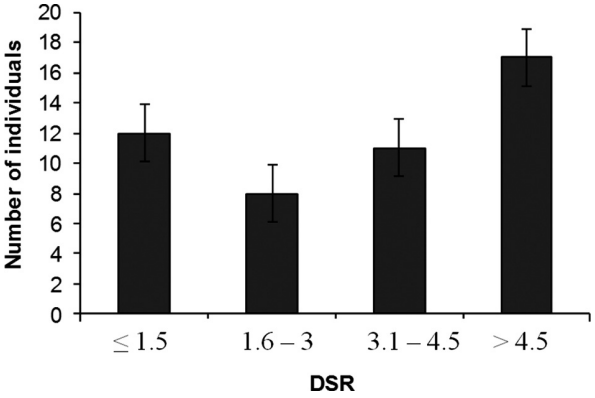

Figure 2. Distribution of mean DSR (Disease Severity Ratings) of the progeny resulting from the 'Pájaro' x 'Sweet Charlie' cross. A 1 to 5 severity scale is used for resistance to the M11 isolate of *Colletotrichum acutatum*.

Although the criterion of grouping in four classes may look artificial, we considered that it correlates better with an agronomical point of view because in the field the yield of plants with a DSR of 2 or 3 will be different than the yield of plants with DSR of 4 or 5. We assume that the criterion of other authors (e.g. Denoyes-Rothan, 1997) that consider only two phenotypic classes (resistant and susceptible) tend to oversimplify the complex biological phenomenon that we have observed, and would not take into account an agronomic point of view.

The continuous range of DSR observed, from highly susceptible to highly resistant, suggests that a complex genetic mechanism is involved in the defense response toward the isolate M11. This data may support the hypothesis that resistance to this isolate of *C. acutatum* is quantitative, as reported by Giménez and Ballington (2002) and Ballington *et al*. (2002). However, if we consider the four classes mentioned above (Figure 2), the frequency distribution of lesions appears bimodal and this agrees with data obtained by Gupton and Smith (1991) and Denoyes-Rothan (1997) that suggested the action of a major gene in resistance to anthracnose.

In order to explain our results, we propose a genetic model based on the gene-for-gene system, taking into account the octoploid structure of *Fragaria ananassa* and assuming a four-locus genetic model (Arulsekar *et al*., 1981), in which the resistance (R) gene locus at each of the four genomes presents different alleles that interact with distinct but related determinants of the same Avr protein of the isolate.

Since cytological and genetic evidence suggest that the genomic structure of the modern *F. ananassa* is 2A 2A' 2B 2B' with a diploid behavior (Bringhurst, 1990), as confirmed by molecular studies (Arulsekar *et al*., 1981; Haymes *et al*., 1997), we may speculate that various allelic variants of the R gene, present in the four genomes, determine the response of the plant toward the tested isolate of *C. acutatum*. Thus, assuming that susceptibility is determined by partially dominant alleles (A, A', B and B'), and resistance by partially recessive alleles (a, a', b, and b'), we can propose for the susceptible cultivar 'Pájaro' the genotype: aa A'a' Bb B'B', and for the resistant cultivar 'Sweet Charlie' the genotype: aa a'a' bb B'b'. In Table 1 we present the possible genetic structure of genotypes derived from the cross between a very susceptible (aa A'a' Bb B'B') and a very resistant (aa a'a' bb B'b') parental genotypes. In this model it is assumed that: i) the degree of incompatibility conferred by the homozygous set and the heterozygous one is not equivalent. The latter would allow us to discriminate levels of susceptibility among different phenotypes according to the occurrence of

the sets homozygous or heterozygous in each individual; ii) the alleles a, a-, b and b' do not exert identical effects; iii) likewise, the alleles A, A', B and B'; iv) alleles A, A', B and B' that determine susceptibility are partially dominant over a, a-, b and b'. According to this model, these genotypes can be clustered in four groups, each one containing a different combination of homozygous or heterozygous sets of alleles, that would determine the four phenotypic classes (e.g. very susceptible, susceptible, tolerant and very resistant). Thus, the segregation ratios are 17:11:8:12 for the observed and 12:12:12.12 for the expected ones (Table 1).

We observed that some genotypes displayed a particular phytopathological behavior. The evolution of disease from the days 10 to 50 showed three different patterns (Figure 3).

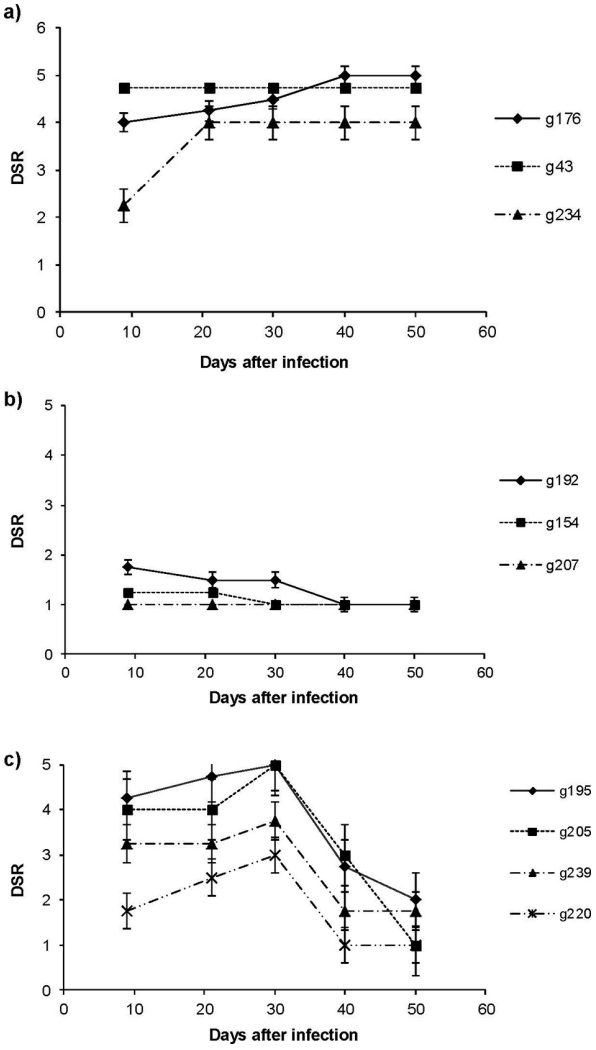

Figure 3. Evolution of the DSR (Disease Severity Ratings) values of selected hybrids coming from the cross 'Pájaro' x 'Sweet Charlie' evaluated during the 50 days experiment for resistance to the M11 isolate of *Colletotrichum acutatum*. a) hybrids that show constant and high DSR values (very susceptible) 30 days after infection; b) hybrids that show constant but low DSR values (very resistant) from the day 30 after infection and c) hybrids showing a typical recovery profile 30 days after infection. Symbols identifying each genotype are indicated.

The behavior of the majority of plants (about 92 %) is similar to those genotypes shown in Figure 3a and 3b, whose symptoms observed on the day 30 were maintained until the end of the evaluation. However, there are four genotypes (e.g. 195, 205, 239, 220), that exhibit high DSR values on the day 30 and then, they overcame the symptoms decreasing their DSR values toward the day 50 (Figure 3c). The most striking recuperation was observed in the hybrid 205 that became a completely "healthy" plant on the day 50 (its DSR changed from 5 to 1, Figure 3c). These data might indicate that an intricate mechanism is involved in the control of resistance to the studied isolate of *C. acutatum* (see discussion).

The elucidation of the genetic control of interesting traits in the cultivated strawberry was difficult due to its octoploidy. For some traits, however, the genetics has been elucidated, and it was demonstrated that they are ruled by a single locus. In host–pathogen interactions studies, resistance to *Phytophtora fragariae* var *fragariae* was demonstrated to be ruled by a single gene (Van de Weg, 1997). For the phosphoglucoismerase (PGI) isozymes, on the other hand, Arulsekar *et al*. (1981) demonstrated that it is monogenically inherited, but in this case they proposed a four-locus genetic model for the octoploid strawberry at the PGI locus, in which the four "loci" represent the gene site at each of the four homologous genomes of the *F. ananassa.* According with this model, we suggest that the response of the studied hybrids to the *C. acutatum* isolate is determined by different allelic variants of the R gene at the four genomes of the strawberry. It is known that plant resistance (R) genes contain loci that segregate for a large number of alleles. This allelic diversity can represent transient polymorphism arising during the adaptive spread of novel resistance alleles, or evolutionary stable polymorphism (Bergelson *et al*., 2001). It is possible that variants of the R gene possess distinct but partially overlapping specificities, as occur in the three members of the RPP1 complex locus in *Arabidopsis* (Botella *et al.,* 1998). In addition, it is possible that allele dosage can also influence the degree of incompatibility. Reported examples of

resistance resulting from homozygous recessive alleles at a locus can be explained by allele-dosage effects in which the heterozygous host genotype is classed phenotypically as susceptible, although pathogen growth is more restricted than it would be in the homozygous susceptible genotype (Crute and Pink, 2001). In diploids, allele-dosage effects have been observed for many genes, including key regulatory genes of developmental processes. Such effects are observed in heterozygous genotypes as intermediate gene expression levels and phenotypic effects, as compared with null/low expressing alleles or high-expressing alleles in homozygous genotypes. Thus, for genes having allele-dosage effects, polyploidy increases the potential variation in expression levels (Osborn *et al.*, 2003). Now, if we hypothesize that the response to the pathogen varies with the allele dosage and also with the distinct interactions between the pathogen and each allele present in each genome, we could explain the continuous variation in resistance levels observed in hybrids derived from the cross of the mentioned genotypes.

Besides the effects of gene dosage and polymorphism of the R-gene, the wide variability of DSR observed in response to the M11 isolate of *C. acutatum* could also be due to the influence of other factors, such as R and Avr protein expression levels, timing of Avr gene expression during pathogen development, efficiency of delivery of the Avr products or compatibility signals to the plant (Botella *et al*., 1998). It has been demonstrated that the phenotypic expression of R genes can be determined by the expression of other complementary genes like those identified in bean, barley, and tomato. It is likely that these genes "required for resistance", encode components of the signal transduction pathway that leads to the activation of defense responses (Crute and Pink, 2001). We can speculate that the behavior of those genotypes that became "healthy" on the day 50, in spite of their initial "susceptibility", is due to a late activation of complementary genes "required for resistance".

According with our hypothesis based on polymorphism of the resistance (R) gene and allele-dosage effects, the results obtained from our experimental model suggest that susceptibility is partially dominant over resistance, in contrast with data obtained by Denoyes-Rothan (1997). This author shows, by analyzing the response to isolate 1267b of *C. acutatum* of different progenies, that resistance is monogenically controlled, being resistance dominant over susceptibility. However, their two class of disease rating scheme may bias their results. Alleles mediating resistance have often been described as dominant over susceptibility alleles, but there are also examples of resistance resulting from recessive alleles, such as a recessive gene conferring anthracnose resistance in sorghum (Boora *et al.,* 1998), or the *mlo* gene, that confers resistance against all barley powdery mildew fungus *Erysiphe graminis* (Jorgensen, 1994).

Since variation in dosage-regulated gene expression has not been extensively studied in polyploids, and as yet there are no concrete examples (Osborn *et al.,* 2003), we propose a model of genetic control of resistance to *C. acutatum* in the octoploid strawberry that can explain the results obtained in our system. In this model, we assume a diploid meiotic behavior of the R locus involved in resistance toward the M11 isolate of *C acutatum*. Although it has been recently suggested that the genome of the *F. ananassa* presents a di-polysomic behavior (Lerceteau-Köhler *et al.,* 2003), a disomic segregation was demonstrated for certain regions of the genome, like the PGI locus (Arulsekar, 1981) and for the resistance gene *Rpf1* (Haymes *et al*., 1997). According to the proposed model, the resistance to the tested isolate would be determined by partially recessive alleles of an R-gene present at the four loci of the homologous genomes of the octoploid *F. ananassa*. The response to the pathogen would be modulated by different factors that determine a wide variability in phenotypic expression of the R-gene, being the dosage effect the more important. Also, the analysis of the broad-sense heritability obtained from 30 day suggests that the selection for resistance in the progenies is feasible, and moderately effective, particularly after the day 30 post-inoculation.

Conclusion

Although a larger experimental design would be desirable to confirm our model, we think that in the studied progeny the widest range of variability expected for the resistance to anthracnose in a population is fairly well represented.

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