Content Index

Table index xv

Figure index xvii

Chapter 1. General Introduction 3

Loquat cultivation 3

Loquat scab caused by *Fusicladium eriobotryae* 6

Chapter 2. Objectives 15

Chapter 3. Effect of environmental factors on mycelial growth and conidial germination of *Fusicladium eriobotryae*, and the infection of loquat leaves 19

Chapter 4. Dispersal of conidia of *Fusicladium eriobotryae* and spatial patterns of scab in loquat orchards in Spain 41

Chapter 5. Development and validation of a standard area diagram set to aid assessment of severity of loquat scab on fruit 63

Chapter 6. Development and validation of a weather-based model for predicting infection of loquat fruit by *Fusicladium eriobotryae* 79

Chapter 7. A nested-polymerase chain reaction protocol for in planta detection of *Fusicladium eriobotryae*, causal agent of loquat scab 105

Chapter 8. Evaluation of fungicides to control loquat scab caused by *Fusicladium eriobotryae* 115

Chapter 9. Identification of resistance to difenoconazole and thiophanate-methyl in field populations of *Fusicladium eriobotryae*, and molecular characterization of thiophanate-methyl resistant isolates 125

Chapter 10. General discussion 139

Chapter 11. Conclusions 147

References 151

Table Index

Table 1.1. Loquat world production 4

Table 1.2. Diseases affecting loquat 7

Table 1.3. Fungicide schedule recommended for the management of loquat scab in Alicante province (Spain) (GVA, 2013) 12

Table 3.1. Equations used to fit data on mycelial growth (1), conidial germination (2), effect of dry period on conidial germination (3), disease incidence (4) and severity (5) of *Fusicladium eriobotryae* as a function of environmental variables 28

Table 3.2. Parameter estimates and summary statistics of nonlinear regression analysis relating the relative growth of *Fusicladium eriobotryae* at different temperatures 29

Table 4.1. Collection of conidia of *Fusicladium eriobotryae* on vertical and horizontal microscope slides in relation to rainfall events during the sampling periods in two loquat orchards (A and B) in Alicante, Spain, in 2011 and 2012. 48

Table 4.2. Evaluation of rainfall to predict collection of *Fusicladium eriobotryae* on slidesa placed under loquat trees in orchards A and B in Alicante, Spain, in 2011 and 2012 52

Table 5.1. Effect of use of SADs as an assessment aid on the bias, precision and overall agreement of estimates of scab severity on loquat fruit made by raters with or without experience in disease severity assessment 71

Table 5.2. Effect of rater experience on the bias, precision and overall agreement of estimates of scab severity on loquat fruit made by ten raters either unaided or aided by a standard area diagram (SAD) 72

Table 5.3. Inter-rater reliability (reproducibility) of visual estimates of scab severity on 50 loquat fruit by 20 raters either unaided (one-time assessment) or aided by SADs measured by the intra-class correlation coefficient (ICC). Two assessments were made using the SADs with a two-week interval 74

Table 5.4. Intra-rater reliability (repeatability), measured by Lin's concordance correlation coefficient (ρc), for estimates of severity on 50 diseased loquat fruit by two groups of ten raters either with or without experience but using SADs during two consecutive SAD-aided assessments 74

Table 6.1. List of variables used in the model 85

Table 6.2. Statistics and indices used for evaluating the goodness-of-fit of loquat scab infection predicted by the model versus disease observed in field 95

Table 7.1. List of *Fusicladium eriobotryae* isolates and other fungi used to determine specificity of the species-specific primer 107

Table 7.2. Detection of *Fusicladium eriobotryae* in samples from loquat trees using either single or nested PCR assays 111

Table 8.1. Fungicides selected for in vitro sensitivity testing 117

Table 8.2. EC50 values for inhibiting in vitro mycelial growth and conidial germination of *Fusicladium eriobotryae* by fungicides representing different chemical classes 120

Table 9.1. Characteristics of *Fusicladium eriobotryae* isolates selected for molecular characterization of thiophanate-methyl resistance 129

Table 9.2. Primers used in the molecular characterization of *Fusicladium eriobotryae* to thiophanate-methyl 132

Figure Index

Figure 1.1. A, young loquat twig affected by scab; B, first symptoms of scab (circular chlorotic spots) in a loquat leaf; C, conidia of *Fusicladium eriobotryae* arising from the cracked cuticle of a loquat leaf; D, loquat fruit showing initial scab lessions (olive colored and velvety); E, stroma producing conidia in a loquat leaf; F, fruit showing several lesions that coalescence; G, loquat leaf severely affected by scab; H, fruit severely affected by scab with old (brown, corky) and young (olivaceous) lesions; I, loquat tree severely affected by scab9

Figure 3.1. Relationship between temperature and relative growth of *Fusicladium eriobotyae* mycelium as predicted by Equation 1 of Table 3.1. Symbols represent observed data and whiskers are the standard errors calculated over different experiments and fungal isolates. Relative growth is calculated by dividing growth (mm/day) in each observation by the maximum growth at optimum temperature 26

Figure 3.2. Relationship between temperature, wetness duration and relative germination of conidia of *Fusicladium eriobotryae* as predicted by Equation 2 of Table 3.1. Relative conidial germination is calculated by dividing percentage of germinated conidia in each observation by the maximum germination found in each experiment 27

Figure 3.3. Predicted versus observed data on relative germination of *Fusicladium eriobotryae* conidia. Data are predicted by Equation 2 of Table 3.1. Whiskers are the standard errors calculated over different experiments and fungal isolates..... 27

Figure 3.4. Relationship between length of dry periods and relative germination of *Fusicladium eriobotryae* conidia as predicted by Equation 3 of Table 3.1. Symbols represent observed data and whiskers are the standard errors calculated over different experiments and fugal isolates. Relative conidial germination is calculated by dividing percentage of germinated conidia in each observation by the maximum germination found in each experiment..... 30

Figure 3.5. Effect of temperature and wetness duration on scab incidence (A) and severity (B) in loquat leaves inoculated with *Fusicladium eriobotryae*. Each column represents the mean disease incidence of two experiments with five replicate plants each. Incidence was assessed as the percentage of leaves with scab lesions per plant and severity as the percentage of leaf area with scab lesions. Whiskers represent the standard erros..... 33

Figure 3.6. Relationship between temperature, wetness duration and relative scab incidence of *Fusicladium eriobotryae* on loquat leaves as predicted by Equation 4 of Table 3.1. Relative disease incidence is calculated by dividing incidence for each plant by the maximum incidence found in the experiment 34

Figure 3.7. Relationship between temperature, wetness duration and relative scab severity of *Fusicladium eriobotryae* on loquat leaves as predicted by Equation 5 of Table 3.1. Relative disease severity is calculated by dividing percentage of severity for each plant by the maximum severity found in the experiment 35

Figure 3.8. Predicted versus observed data on relative incidence (A) and severity (B) of *Fusicladium eriobotryae* infection on loquat leaves. Data are predicted by Equation 4 and 5 of Table 3.1, respectively 36

Figure 4.1. Weekly number of conidia of *Fusicladium eriobotryae* collected on horizontal slides, and scab incidence in 2011 in loquat orchard A (A) and loquat orchard B (B) relative to daily temperature and rainfall (C). In A and B, horizontal black lines (▬) indicate weeks in which at least one *Fusicladium eriobotryae* conidium was detected in the rain collectors. Full triangles (▼) indicate weeks in which <100 conidia were captured on horizontal slides; empty triangles (▽) indicated weeks in which <1 mm of rain was recovered. Whiskers in A indicate standard errors for percentage of fruits with disease ..50

Figure 4.2. Weekly number of conidia of *Fusicladium eriobotryae* collected on horizontalslides, and scab incidence in 2012 in loquat orchard A (A) and loquat orchard B (B) relative to daily temperature and rainfall (C). In A and B, horizontal black lines (▬) indicate weeks in which at least one *Fusicladium eriobotryae* conidium was detected in the rain collectors. Full triangles (▼) indicate weeks in which <100 conidia were captured on horizontal slides; empty triangles (▽) indicated weeks in which <1 mm of rain was recovered. Whiskers in A indicate standard errors for percentage of fruits with disease ..51

Figure 4.3. Receiver operating characteristic (ROC) curve for the weekly accumulated rainfall (mm) in relation to the probability of collecting conidia of *Fusicladium eriobotryae* (in orchard A and B during 2011 and 2012). The dotted line represents no discrimination. Also indicated are values for area under the receiver operating characteristic curve (AUROC), AUROC standard error (SE), and the probability the AUROC is different to 0.5 (*P*). 53

Figure 4.4. Values of the area under the disease progress curve (AUDPC) for incidence of loquat scab in orchard A in southeastern Spain in 2011 (A) and 2012 (B). Each point corresponds to the AUDPC value of one of the 46 trees in the orchard (the orchard was surveyed 14 times in 2011 and 16 times in 2012)..... 54

Figure 4.5. Frequency distribution of the dispersion index (D_{tree}) of fruit with symptoms of loquat scab (caused by *Fusicladium eriobotryae*) in 46 trees of loquat in orchard A in southeastern Spain during seven sampling periods in 2011 (A: 17 January, B: 28 February, C: 14 March, D: 28 March, E: 04 April, F: 18 April and G: 02 May) and in 2012 (H: 05 January, I: 26 January, J: 16 February, K: 01 March, L: 15 March, M: 28 March and N: 12 April). The following are indicated for each sampling period: the dispersion index for the orchard ($D_{orchard}$), the probability (P) that $D_{orchard}$ is significantly >1, and the average disease incidence (inc). Values of D_{tree}>1 (dotted line) indicate aggregation in the spatial pattern of disease 55

Figure 4.6. Relationship between the logarithm of the binomial variance and the logarithm of the variance of the incidence of loquat scab in orchard A in Alicante, Spain. The grey circles and black circles represent the observations from 2011 and 2012, respectively. The solid line represents the linear regression, and the dotted line represents the binomial line (i.e., when the observed variance equals the binomial variance). The regression equation between ln(*Vbin*) and ln(*Vobs*) and the adjusted R^2 are indicated; the probability that the intercept and slope of the regression line are > 1 is <0.001 in both cases 56

Figure 5.1. Fruit of loquat with typical symptoms of scab..... 64

Figure 5.2. The standard area diagram (SAD) set developed as an aid for assessment of scab on fruit of loquat. Each value represents the percentage of loquat scab severity on that image..... 67

Figure 5.3. Relationship between the estimates and true severity (A, B) and the absolute error (estimate minus true severity) (C, D) of assessments of a set of 50 images of scab-diseased fruit of loquat by 20 raters without (A, C) and with (B, D) the use of a standard area diagrams (SADs). Dashed line (A, B) represents the regression line 70

Figure 5.4. The relationship between gain (difference between the estimate with and without use of a standard area diagram, SAD) for measures of accuracy, precision and agreement of visual severity estimates made by 10 inexperienced (circles) and 10 experienced (triangles) raters for a set of 50 images of scab-diseased fruit of loquat (A, agreement, measured by Lin's concordance correlation coefficient; B, accuracy measured by the bias correction factor; and C, precision, measured by the correlation coefficient)..73

Figure 6.1. Disease cycle of loquat scab caused by *Fusicladium eriobotryae*82

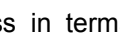
Figure 6.2. Relational diagram showing how the model simulates infection by *Fusicladium eriobotryae*. Legend: (\ state variable), (\flux and direction of states), (\ flux and direction of information), (\parameter), (\intermediate variable), (\switch) (\outgoing variable), ( \valve in a flux (rate)). See Table 6.1 for acronym explanation 84

Figure 6.3. Schematic representation of the infection process in terms of lesion units (LU). Each circle represents an LU that can have different states: healthy (LUH, in white), occupied by ungerminated conidia (LUUC, in green), occupied by germinated conidia (LUGC, in blue), with latent infection (LULI, in orange), or with visible scab (LUVI, in brown). Deposition, death of conidia, germination, infection, and latency progress are the phenomena that define the change of LUs from one state to another. In the example presented and in relative units, LUHC=1 at time zero and LUUC=1 at the first time step (deposition of splashing conidia). At the second time step: LUUC+LUGC+LUH=0.16+0.40+0.44=1. At the third time step: LUUC+LUGC+LULI+LUH=0.12+0.04+0.20+0.64=1. At the fourth time step: LUVI+LUH=0.20+0.80=1. Therefore, at the end of the infection process, 20% of the lesion units became infected in this example 87

Figure 6.4. Dynamics of lesion units (LUs) during an infection period of *Fusicladium eriobotryae*. The graph shows the relative frequency of LUs occupied by ungerminated conidia (LUUC, in green), germinated conidia (LUGC, in red), and latent infections (LULI, in blue). Blue bars at the top indicate hours with free water on the fruit surface. An infection period starts when a rain event splashes conidia on LUs and ends when no viable conidia are present on any LUs, i.e., when LUUC≤0.01 88

Figure 6.5. Weather data and model output in 2011. A: daily weather data; B: predicted frequency (%) of lesion units (LUs) with ungerminated conidia; C: predicted increase of LUs with latent infections (LULIs) for each infection period (arrows represent clusters of infection periods, clustering is based on an interval of at least 5 days between the beginning of two consecutive clusters); D: predicted seasonal dynamics of the cumulative values of LULI (ΣLULI)……90

Figure 6.6. Weather data and model output in 2012. A: daily weather data; B: predicted frequency (%) of lesion units (LUs) with ungerminated conidia; C: predicted increase of LUs with latent infections (LULIs) for each infection period (arrows represent clusters of infection periods, clustering is based on an interval of at least 5 days between the beginning of two consecutive clusters); D: predicted seasonal dynamics of the cumulative values of LULI (ΣLULI)……91

Figure 6.7. Comparison between model output and scab observed on loquat fruit in southeastern Spain. (A) data from 2011 and (B) data from 2012. Blue lines represent the rescaled infection predicted by the model as the seasonal summation of the lesion units with latent infections (ΣLULI). Points represent rescaled incidence of loquat fruit with scab observed in the orchards; rescaled incidence is shifted back by 21 days (red points) or 420 DD (base 0°C, green points) to account for the latency period, i.e., the time elapsed between infection and visible symptoms in the form of sporulating scab lesions..... 96

Figure 6.8. Comparison between model output and scab on loquat fruit in single-exposure experiments. Experiments were carried out in a loquat orchard in southeastern Spain in 2013. Observed data (X axis) are expressed as the rescaled disease severity in 11 groups of fruits that were exposed (for 7-day-long moving periods) to splashing rain in a severely affected orchard; model output (Y axis) is expressed as the summation of the lesion units with latent infections (ΣLULI) in the exposure period 97

Figure 6.9. Comparison between model output and expert assessment in southeastern Spain from 2006 to 2013. Loquat severity was estimated as low, medium, or high by an expert advisor; bars show the average number (+ SE) of scab outbreaks predicted by the model in each severity category. A disease outbreak was defined as ΣLULI>0.1 in 1 day, when no outbreaks were predicted on the previous 5 days 98

Figure 8.1. Relative severity on loquat plants inoculated with *Fusicladium eriobotryae* and treated with five different fungicides at different times of application. Relative disease severity was calculated as the percentage of severity relative to the median value of severity on control plants. For each combination of fungicide and time of application eight plants were inoculated. Bars represent the standard error. 121

Figure 9.1. Schematic representation of the β-tubulin gene showing the binding sites of the primers used in this study and the point mutations associated to thiophanate-methyl resistance. Black boxes denote protein-coding sequences (exons), and white boxes denote introns. 128

Figure 9.2. Distributions of relative growth (RG) values of *Fusicladium eriobotryae* isolates (n=249) on A, difenoconazole and B, thiophanate-methyl. Bars represent number of isolates in each RG category and dotted vertical lines indicate the average RG value for each fungicide. 130

Figure 9.3. Map of Spanish locations from which isolates of *Fusicladium eriobotryae* resistant to difenoconazole (black circle), thiophanate-methyl (black diamond) or both (black triangle) were found. White circles indicate locations at which no resistant isolates were found. Provinces: ALM, Almería; ALC, Alicante; CA, Castellón; GR, Granada; and VA, Valencia. 131