SHORT COMMUNICATION

DEVELOPMENT OF GLOMERELLA LEAF SPOT IS ENHANCED IN VIRUS-INFECTED MAXI GALA APPLES

D.S. Guerra[1], O. Nickel[2], E.M. Del Ponte[1], R.M.V. Sanhueza[2], T.V.M. Fajardo[2] and G.A.B. Marodin[1]

[1] *Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, 91540-000, Porto Alegre, RS, Brasil*
[2] *Embrapa Uva e Vinho, 95700-000 Bento Gonçalves, RS, Brasil*

SUMMARY

Apples are commercially grown in Brazil in a subtropical environment that favors the development of fungal diseases such as Glomerella leaf spot (GLS) caused mainly by *Glomerella cingulata* (anamorph *Colletotrichum gloeosporioides*). The main objective of this work was to evaluate the effect of mixed infections by *Apple stem grooving virus* (ASGV) and *Apple stem pitting virus* (ASPV) on the infection and the colonization processes of *C. gloeosporiodes* in cv. Maxi Gala plants. Leaves of 16-month-old potted plants were spray-inoculated and both the disease incidence and lesion count were monitored over time and leaf severity was assessed in the final evaluation using an image analysis tool. Results showed that initial infection estimated from a monomolecular model fitted to progress of lesion count was higher and the incubation period (time to reach 50% incidence) was on average 10 h shorter in virus-infected plants compared to non-infected plants. It is hypothesized that initial events such as conidial germination and fungal penetration into plant cells were facilitated by the presence of viral infection. Also, final GLS severity was significantly higher in the virus-infected plants. Mixed infections by ASGV/ASPV seemed to make apple leaves more susceptible to the initial infection and colonization by *C. gloeosporioides*.

Key words: *Malus domestica*, susceptibility, fungal disease, virus infection, pathogen interactions.

Apples (*Malus domestica*) are commercially grown in Brazil on approximately 40,000 ha, genotypes of the cv. Gala group representing around 60% of the germplasm in use (Mello, 2006). Most of the major Brazilian apple-growing regions are in humid environments that favour the development of fruit rots and fungal foliar diseases such as apple scab and Glomerella leaf spot (GLS) (Crusius *et al.*, 2002; Santos *et al.*, 2005). In Brazil, GLS is associated with at least three species: *Colletotrichum gloeosporioides* (75% of the isolates), *C. acutatum* (8%), and *Colletotrichum* sp. (17%) and the primary inoculum for the epidemics overwinters in dormant twigs and buds (Crusius *et al.*, 2002). As a typical polycyclic disease, when successive cycles develop during the season, severe losses are induced in susceptible cultivars such as Royal Gala, Golden Delicious, Belgolden, Granny Smith and Pink Lady (Araujo *et al.*, 2010).

Apple propagative material from Brazilian nurseries and commercial orchards are usually infected with one or more latent viruses (Nickel *et al.*, 1999, 2001) such as *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV), *Apple chlorotic leaf spot virus* (ACLSV) [family *Betaflexiviridae*; (Martelli *et al.*, 2007)], which are transmitted by grafting and have no known vector (Yoshikawa *et al.*, 1992; Magome *et al.*, 1999; Jelkmann, 1997). Virus-infected plants undergo ultrastructural, physiological and/or biochemical changes during the course of infection (Bertamini *et al.*, 2004; Fajardo *et al.*, 2004; Liu *et al.*, 2006; Radwan *et al.*, 2007) that may increase their susceptibility to other diseases (Meyer and Pataky, 2010; Soto-Arias and Munkvold, 2011). To our knowledge, there is no information on the impact of the presence of latent viruses on GLS epidemic components. Therefore, the objective of this study was to quantify and compare components related to the monocyclic processes of the GLS disease cycle between virus-infected (mixed ASGV/ASPV infections) and non-infected young apple plants under a controlled environment.

'Maxi Gala' plants free of latent viruses (ASGV, ACLSV, ASPV) and ApMV were selected for the experiments after indexing and RT-PCR analysis. In March 2005, bud sticks of cv. Maxi Gala were grafted onto uniform cv. Gala seedlings (5-8 mm in diameter) grown in 3-litre plastic bags. Virus inoculation was initially made in July 2005 using two buds removed from ASGV- and ASPV-positive cv. Fuji Suprema plants, as determined by indexing, and were repeated approximately one year later. The presence of virus infection was also ascertained by RT-PCR at three times, i.e. December 2005, October 2006 and May 2007. Total nucleic acids were extracted from twig bark (Boom *et al.*, 1990), and subjected to

Corresponding author: E.M. Del Ponte
Fax: +55.5133086908
E-mail: emerson.delponte@ufrgs.br

PCR using primers and thermocycling conditions described elsewhere (Nickel *et al.*, 1999, 2001; Radaelli *et al.*, 2006). The molecular assays confirmed the presence of ASGV and ASPV but not of ACLSV and ApMV in bud-inoculated plants. Uninoculated and RT-PCR-negative plants were used as non-infected controls.

A virulent isolate of *C. gloeosporioides* (CGF1) from Embrapa Uva e Vinho was used in the experiments. Three-day-old cultures growing on PDA at 22°C and a 16/8 h light/dark cycle were used as a source of inoculum. A conidial suspension was obtained by filtering fungal mycelium through muslin and adjusting its final concentration to 10^6 conidia/ml.

Forty (twenty in each treatment) sixteen-month-old plants were spray-inoculated, in each experiment, following the procedure of Crusius *et al.* (2002) with a minor modification that included a 24 h conditioning treatment by moving plants (0.80-1.0 m in height) into a humid chamber (*ca.* 99% relative humidity) at 22°C. The number of leaves in each inoculated plant varied from 10 to 20. Following inoculation, plants were maintained under the same conditions for an extra three-day period, then moved into a chamber at 22°C and 60% relative humidity under continuous light during the disease development period.

Several disease-related variables were used to describe GLS intensity such as disease incidence (proportion of symptomatic leaves), lesion count (number of lesions per leaf), and disease severity (proportion of leaf area covered by symptoms) as estimated digitally from scanned images. The experiments, repeated twice, were named experiment I and II. GLS incidence and lesion count were recorded seven times from the first to the eighth day (192 h) after inoculation in experiment I, and seven times from the first to the sixth day (144 h) after inoculation in experiment II. For the temporal data (mean incidence and lesion count), monomolecular and linear models, that usually describe development of monocyclic processes, were fitted to each progress curve using linear regression for the model-transformed incidence values against time. Choice of the model was based on graphs of the residuals and statistical parameters (Madden *et al.*, 2007). Three model parameters, initial inoculum (y_0), apparent infection rate (r) and maximum incidence or lesion count (y_{max}) for each treatment were compared in each experiment. The area under the disease progress curve (AUDPC) for the cumulative incidence and lesion count data over time was estimated using the trapezoidal integration method. Based on the model that fitted disease progress best, the time to achieve 50% disease (t_{50}) incidence, which was an indicative of the incubation period, was calculated (Madden *et al.*, 2007). Finally, GLS severity (%) was estimated on the final assessment time (144 h) in the experiment II only. Diseased leaves were scanned and Image J (Abramoff *et al.*, 2004) was used to determine the severity based on the proportion of pixels of the diseased area relative to the total leaf area.

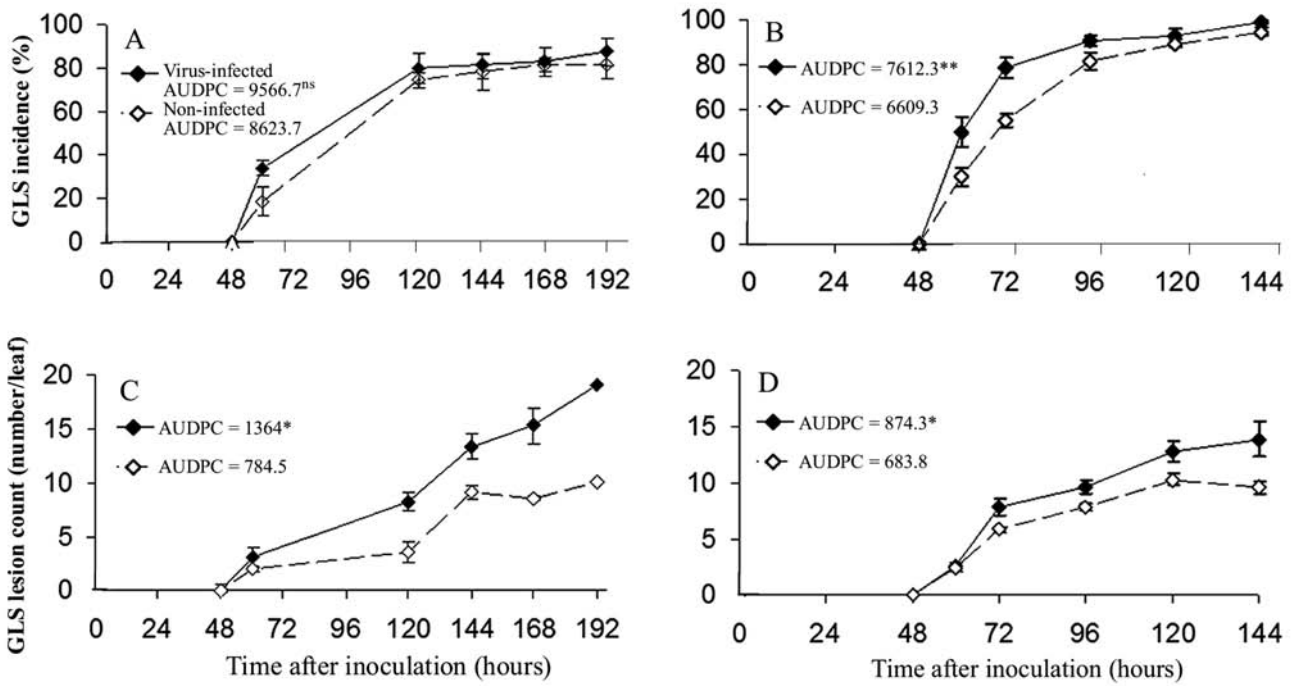

Fig. 1. Disease progress curves and respective AUDPC values of GLS incidence (A and B) and lesion count (C and D) on virus-infected (ASGV-ASPV) and non-infected apple plants of cv. Maxi Gala at different times following inoculation with *C. gloeosporioides* in a controlled environment in experiment I (A and C) and experiment II (B and D). * and ** indicate significant differences between treatments at the 0.05 and 0.01 level as determined by analysis of variance.

Table 1. Parameters of a monomolecular and a linear model fitted to mean GLS incidence and mean lesion count (number of lesions per leaf) progress over time, respectively for both variables measured in apple cv. Maxi Gala infected with ASGV/ASPV and inoculated with *C. gloeosporioides* in two experiments.

	GLS incidence data			
	Experiment I		Experiment II	
Model parameter[1]	Virus-infected	Non-infected	Virus-infected	Non-infected
y_0	-0.73	-0.51	-2.32*	-2.95
r_M	0.01	0.01	0.05	0.06
y_{max}	87.3	81.3	99.07	94.92
t_{50}	81.0*	92.2	58.0*	69.1
	GLS lesion count data			
	Experiment I		Experiment II	
Model parameter	Virus-infected	Non-infected	Virus-infected	Non-infected
y_0	-0.29	-0.28	-0.35	-0.31
r_L	0.01	0.01	0.01	0.01
y_{max}	19.13*	10.01	13.95*	9.75

[1] y_0 = model intercept (initial inoculum); r_M and r_L= disease progress rate for the monomolecular and linear model, respectively; t_{50} = time to reach 50% incidence (incubation period); *are means significantly different (t test, $p<0.05$) between treatments (same row) and the same experiment.

In each experiment, groups of four plants composed an experimental unit and three replications were used. A t-test was used to compare values of the three disease model parameters from each treatment. Correlation analysis (Pearson coefficient) was used to determine the relationship among all variables (disease incidence, severity and lesion count). For the AUDPC data, fixed effects included the two treatments that were considered significantly different at the 0.05 and 0.01 level according to a linear model (SAS Institute, USA).

Typical GLS symptoms developed in both experiments with incidence levels higher than 80%. The first small necrotic lesions were not visible to the naked eye earlier than *ca.* 50 h after inoculation. A continuous increase in the proportion of diseased leaves occurred up to *ca*. 100 h after inoculation, when infection reached the maximum value and remained constant until the last evaluation. On the other hand, a continuous increase in the number of lesions per leaf was recorded until the end of both experiments. While maximum incidence was similar among the treatments in both experiments, virus-infected plants consistently showed lesion count values at all assessment times higher than the non-infected plants (Fig. 1).

The monomolecular model adequately described disease incidence progress (R^2= 0.84 to 0.94), whereas a linear model described the lesion count progress data best (R^2=0.84 to 0.98) (Table 1). A significant difference was observed for the incubation period, which was determined based on t_{50}, taking all inoculated leaves of the plants into account. In both experiments, the estimated incubation period was approximately 10 h shorter in virus-infected plants than in the control. AUDPC for the foliar incidence progress data differed between treatments in Experiment II, but not in experiment I (Fig. 1A). There was no significant difference for the other model parameters, with the exception of the estimated initial inoculum, which was higher for the virus-infected than the non-infected plants.

As to the lesion count data, significant differences were observed for the mean maximum estimated lesion number per leaf in both experiments, which was 2 and 1.5 times higher in virus-infected than the non-infected plants in experiment I and II, respectively. Also AUDPC was around 2 and 1.25 times higher in the virus-infected plants than in the non-infected plants, in experiment I and II, respectively (Fig. 1B, 1D). The other lesion count variables based on linear model parameters did not differ between the treatments (Table 1). The digitally estimated disease severity was significantly higher in the virus-infected plants than the non-infected ones (Fig. 2).

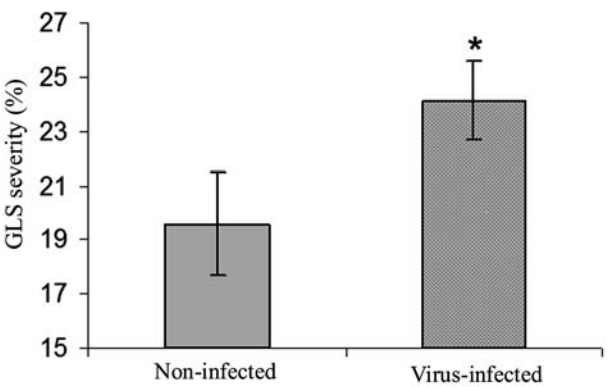

Fig. 2. GLS severity on virus-infected (ASGV/ASPV) and non-infected apple cv. Maxi Gala 144 h after *C. gloeosporioides* inoculation in a controlled environment. * represents means significantly different at 0.05 level, as determined by a t-test.

A positive and significant association was also observed between GLS incidence and lesion count in both treatments. However, a lower correlation coefficient (R=0.87; P <0.001) was determined for the relationship between incidence and lesion count in virus-infected plants compared to the same relationship in the non-infected plants (R=0.92; P<0.001).

In our study, a clear difference in susceptibility between ASGV/ASPV-infected and non-infected plants was inferred from measurements of GLS monocyclic components. Incubation period, which comprises the average time interval between infection and the appearance of disease symptoms for all developing lesions (Bergamin Filho and Amorim, 2002), was shorter in virus-infected plants. Under conditions favorable for effective sporulation leading to secondary inoculum and further infections, GLS disease developed faster in virus-infected plants during field epidemics.

The apparent infection rate is usually an useful parameter to differentiate susceptibility among cultivars (Spósito *et al.*, 2004). However, it did not differ between virus-infected and non-infected plants, suggesting that colonization rate was affected less than other components of the disease cycle in virus-infected plants. Conversely, the intercept of the model describing incidence progress, an indicator of the initial infection, was higher in virus-infected plants in one of the experiments, suggesting that conidial germination and/or fungal penetration into plant cells was facilitated in virus-infected plants. Previously, Liu *et al.* (2006) had reported that in the *Fusarium graminearum*-wheat pathosystem the presence of *Barley yellow dwarf virus* (BYDV) facilitated fungal penetration but not the earliest events, such as germination of macroconidia and germtube formation.

Higher lesion count in virus-infected plants may have contributed to a higher disease severity in cv. Maxi Gala plants infected with ASGV/ASPV. Although a positive association was found between lesion count and severity in virus-infected plants (R= 0.46; P =0.022), the relatively low correlation coefficient between these two variables suggests that lesion size (not measured in our study) may have contributed to higher disease severity in the virus-infected plants.

The lower correlation in the relationship between disease incidence and lesion count in the virus-infected plants may be due to the irregular distribution or the localized occurrence of viruses within the woody hosts. Contrary to the uniform distribution of virus particles in herbaceous plants, individual buds on an apple twig may be virus-free, particularly towards the tip of the shoot (Fridlund, 1982). As a consequence, twigs of virus-infected plants may bear some virus-free leaves, affecting the uniformity of infection. This is especially relevant because ASGV is usually more concentrated in the stems than leaves (Knapp *et al.*, 1995), as confirmed by the higher concentrations of ASGV RNA in the shoots detected by Wang *et al.* (2010). Thus, the reaction of individual leaves could be attributed to an infection by ASPV alone. However, plants in this study had a vigorous and uniform leaf growth that made them physiologically more active, and more susceptible to *C. gloeosporioides*. Increased severity of southern corn leaf blight (*Bipolaris maydis*), usually a minor disease of this crop, has been observed in *Maize dwarf mosaic virus* (MDMV)- and *Sugarcane mosaic virus* (SMV)-infected maize (Meyer and Pataky, 2010). Similarly, the presence of *Bean pod mottle virus* (BPMV) infection increased seed infection by *Phomopsis longicolla* and induced delayed maturation of soybeans (Soto-Arias and Munkvold, 2011).

A likely consequence of the observed increase in susceptibility of apples to GLS is the larger size of the necrotic leaf areas that could lead to reduced photosynthesis and increased respiration rate (Fajardo *et al.*, 2004; Meyer and Pataky, 2010). This can negatively affect fruit quality and yield and extends the maturation period, thus favoring additional GLS attacks of cv. Maxi Gala.

In conclusion, although the mechanism is still unclear, our results suggest that apple plants infected by latent viruses such as ASGV and ASPV undergo physiological, biochemical and structural changes that make them more susceptible to the initial infection by the GLS pathogen. This increased susceptibility may result from the suppressed expression of defense genes, a condition that deserves further investigations aimed at elucidating the mechanisms involved.

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