Evaluation of *Actinidia* spp cultivars' resistance to Ceratocystis wilt

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CONTENT

1. Introduction

At the beginning of it's domestication, the kiwifruit (*Actinidia* spp.) was considered a disease-free crop. However, the rapid growth of world production and the expansion of its cultivation have led to the emergence of diseases (Huang, 2016). Among the diseases that have arisen, the Ceratocystis wilt, caused by the fungus *Ceratocystis fimbriata* (Ellis & Halsted), has drawn attention because of the damages caused to kiwifruit in Brazil (Piveta et al., 2016). Although *C. fimbriata* has already been recognized as a pathogen in Brazil for more than 75 years (Ferreira et al., 2010), Ceratocystis wilt was first reported on kiwifruits in 2010, in the state of Rio Grande do Sul, Brazil (Sonego et al., 2010). Currently, it is also found in the state of Santa Catarina. (A. C. Alfenas - personal communication, 2015). The disease leads to the loss of turgescency and brightness of the leaves, which coils up, followed by dryness and defoliation, which culminates in the death of the plant. The affected plants are mainly observed in patches. In internal tissues, radial lesions of reddish-brown colour are observed. The disease causes a reduction on fruit production and when fruits are produced they are usually smaller and rejected for commercialisation (Piveta et al., 2016).

Ceratocystis wilt is currently one of the most important diseases of arboreal plants, due to the wide range of hosts it can affect (Baker et al., 2003; Harrington et al., 2005; Ferreira et al., 2010; Firmino el al., 2012; Valdetaro et al., 2015), because of the high genetic and physiological variability of the pathogen (Ferriera el al., 2010; Oliveira et al., 2016), the vascular nature of infections and also because of the great damages caused to crops (Alfenas et al., 2009). After the first report, the plant mortality rate of some orchards of the state of Rio Grande do Sul were between 25-30% (Ferreira et al., 2017), mainly from the use of grafts and rootstocks highly susceptible to Ceratocytis wilt. Considering a production of 30,000 kg / ha of the fruit, at a price of US $ 1.00 / kg, and a mortality rate that can reach 30% per year, the loss can reach US $ 9,000 / ha / year (Piveta et al., 2016).

Pathogenicity tests in some kiwifruit cultivars have shown that they are all susceptible to Ceratocystis wilt (Piveta et al, 2016). The report of this disease in kiwifruit, associated to the fact that all the cultivars evaluated to date, are susceptible to it, emphasizes the importance of identifying sources of resistant to Ceratocystis wilt. Thus, the objective of this work was to evaluate the resistance of a broad range of kiwifruit cultivars and progenies, in order to select resistant graft and / or rootstocks.

2. Materials and methods

2.1 Obtaining, multiplying and developing cultivars

Plants of grafted cultivars Alisson, *Actinidia arguta*, *Actinidia arguta* Ken's Red, Bruno, Chieftain, Hayward, Monty and Tomury cultivars (Table 1) were made available by the Rio Grande do Sul state Agricultural Research Foundation (Fepagro) to ***Clonar Resistance to Forest Diseases*** (located in the municipality of Cajuri, state of Minas Gerais), with the purpose of developing and multiplying them. The plants were rooted in plastic tubes (55 cm^3), containing a substrate (Carolina) ®, supplied with 0.5 g Osmocote ® Plus (15% N, 9% P2O5, 12% K2O, 1% Mg, 2.3% S, 0.05% Cu, 0.45% Fe, 0.06% Mn, 0.02% Mo) and 1g of single super phosphate. In order to increase rooting efficiency, the cuttings were immersed in a solution containing indolebutyric acid (IBA), at a concentration of 6,000 ppm (Ferri et al., 1996). The cuttings were kept in a rooting house (28 – 35º·C.) until it was possible to visualise healthy roots growing from the base of the tubes. This done, the plants were kept for 20 days at an acclimatation house, followed by 30 days in a rustification house. After the rustification step, the cuttings were transferred into 15x20cm polyethylene bags containing the same substrate (Carolina®) used for rooting and kept in a greenhouse.

2.2 Screening of isolates

The isolates of *Ceratocystis fimbriata*, used in this study, were obtained from infected kiwifruit plants (*Actinidia chinense* var. *deliciosa*) collected during 2011, 2014 and 2015. From the available pool of 119 isolates, 14 isolates from different geographical and genotypically distinct regions were selected (Table 2), based on 14 microsatellite molecular markers. The 14 selected isolates were inoculated on "Monty". The selected isolates, stored in 15% glycerol at -80°C, were transferred separately to Petri dishes (9cm), containing Malt-Yeast-Agar-Extract (EMLA) medium (2% malt, 0.2% yeast extract and 2% agar) and incubated at 28°C with a 12-h photoperiod for the duration of 15 days. Given the incubation period, 10 mL of sterile distilled water were added to the plates and the surface of the colonies were scraped using a brush. The spore suspensions were filtered on a double layer of gauze and the inoculum concentration was adjusted to $1x10^7$ spores per mL^{-1} utilising a haemocytometer. The isolates were individually inoculated into five three-months-old

cuttings of Monty cultivar. A wound was made at the base of the plant stem, and then 200 μL of inoculum suspension was deposited in the wound. Five plants were inoculated with each isolate. The same numbers of plants were inoculated with 500 μL of distilled water to serve as control and the treatments were arranged in a completely random design. The aggressiveness was evaluated 45 days after inoculation, by calculating the percentage of diseased tissue (dark wood) in relation to the total height of the plant. The data was submitted to a variance analysis (ANOVA) and the treatments were compared by utilising the Scott & Knott grouping test ($p < 0.01$) (Scott & Knott, 1974) and using the ASSISTAT software version 7.7 (Silva & Azevedo, 2016).

2.3 Evaluation of cultivar resistance

To minimise the clone/isolate interaction, we tested if the mixture of inoculum of the five most aggressive isolates (PG01, FI05, CE10, FI22 and A15) could be used to screen for resistance of kiwifruit cultivars. Results of these inoculations on the cultivar Monty, showed no differences between the mixture of inoculum compared to the most aggressive isolate (PG01). Therefore, based on these results, the resistance of the cultivars was determined by using a mixture with the five most aggressive isolates (PG01, FI05, CE10, FI22 and A15). For the production of the inoculum, the isolates were grown on Petri dishes (9cm), containing EMLA at 25ºC and 12h photoperiod, for 15 days. Thereafter, 10 mL of sterile distilled water were added to the plates and the surface of the colonies was scraped with a brush. Then, the spore suspensions were filtered on a double layer of gauze and the inoculum concentration was adjusted to $1x10^7$ spores per mL^{-1}. The inoculum suspension was obtained by mixing aliquots of the same volume from each suspension at the same concentration. On each plant, 200 μL of the mixture of inoculum were deposited in a wound (2 cm long), made at the base of the stem, of five plants of each cultivar. At 45 days after inoculation, the lesion length was measured (Fig. 1). The experiment was arranged in a completely random design and repeated twice. Data were submitted to a variance analysis (ANOVA) and the treatments were grouped by utilising the Scott & Knott test ($p < 0.01$) using ASSISTAT software version 7.7 (Silva & Azevedo, 2016).

3. Results

3.1 Screening of isolates

A significant difference in aggressiveness was found amongst the isolates tested ($F = 16.07$, $p < 0.0001$). They were separated into four distinct groups, with a severity range of approximately 10% to 95% (Fig. 2). Un-inoculated control plants remained asymptomatic, presenting only wound reaction. The isolate A16 was the least aggressive one and the only isolate that did not differ from the control (Fig. 2). The isolates PG01, FI05, CE10, FI22 and A15 were the most aggressive ones and were therefore chosen to compose the mixture of isolates used as an inoculum for evaluation of the cultivar's resistance.

3.2 Evaluation of the *Actinidia spp* cultivar's resistance.

There were no significant differences between the results of the two inoculation experiments carried out independently in different times for both lesion size ($F = 1.9711$, $p = 0, 0624$) and disease severity ($F = 0,9120$, $p = 0,5116$).

All evaluated cultivars were susceptible to Ceratocystis wilt and disease severity ranged from 41% to 100% (Table 3). Four distinct groups related to disease severity amongst the cultivars tested (Table 3) were found. Actinidia Arguta Ken's Red, Monty, Tomury and Hayward presented the highest disease severity values (ranging from 92% to 100%) . "Actinidia Arguta Ken's Red" was the most susceptible. As early as 10 and 15 days after inoculation, dead plants were observed, and at the end of the experiment all inoculated plants died. Control plants showed only wound reaction. "Bruno" and "Chieftain" were the most resistant cultivars (Table 3). The fact that cultivars that presented severity levels statistically distinct grouped together when tests were applied to the wound can be explained by differences in plant height. .

4. Discussion/Outcome/Recommendations

As *C. fimbriata* is a soil borne pathogen and has a high genetic variability (Ferreira et al., 2010; Ferreira et al., 2013; Oliveira et al., 2016) the best option for managing this disease is to utilise resistant materials (Piveta et al., 2016), selected based on inoculation of highly aggressive isolates. To reduce the isolate/cultivar interaction, for resistance screening inoculum composed of a mixture of isolates should be used. The choice of inoculating the mixture of isolates rather than a single highly aggressive isolate is due to the fact that there is cultivar-isolate interaction, as demonstrated by Piveta et al. (2016). Therefore, an isolate highly aggressive to one cultivar may be non-aggressive to another. If this isolate was used only to evaluate the resistance of the cultivars, then there is a risk that we could generate false positive results for disease resistance. This can, however, be avoided by utilising a selection of the isolates in the mixture. Rarely a susceptible cultivar will be classified as resistant, since it is very likely that at least one of the isolates will be highly aggressive to it.

Although all cultivars evaluated were susceptible, it was possible to observe different degrees of resistance to Ceratocystis wilt. The cultivars Actinidia Arguta Ken's Red, Hayward, Monty and Tomury were highly susceptible, with an average severity level greater than 90%, whereas the Bruno and Chieftain cultivars had an average severity level of 42% and 48%, respectively, making these, the most resistant cultivars (Table 3). In separate inoculation experiments of plants originated from Bruno seedlings, some genotypes were resistant to the disease and these could be used as rootstock in the near future. Variation in the degree of resistance between the evaluated cultivars demonstrates that the resistance to Ceratocystis wilt in kiwifruit as well as in *Eucalyptus* spp, *Mangifera indica* and *Acacia mangium*, is possibly a quantitative trait. In other words, it is controlled by a large number of genes (Rosado et al., 2010, Brawner et al., 2015, Arriel et al., 2016). However, a more detailed study on the inheritance of Ceratocystis wilt resistance in kiwifruit is needed. As most commercial cultivars belong to the same species (*Actinidia chinensis*), the introduction of wild genetic material should be considered in breeding programs, in order to expand the genetic basis of the crop and favour the development of commercial cultivars resistant to Ceratocystis wilt.

As demonstrated by Baker et al. (2003) and Thorpe (2004), isolates of the fungus from other plant species in Brazil did not have a complete specialisation per host and this is likely to occur in isolates from other countries. Therefore, the establishment of kiwifruit plantations, near the regions containing the pathogen should be avoided in countries such as China and New Zealand; two of the largest kiwi producers in the world, where the fungus has been reported to cause disease in other crops (Slade et al., 1960; Huang et al., 2008, Li et al., 2014, Li et al., 2016). In the case of China, where *C. fimbriata* has already been reported in Yunnan province, the region with the highest biodiversity of the genus *Actinidia* and one of the main kiwi producing provinces of China (Huang, 2016), the best alternative to avoid future losses is by planting sturdy materials. For these reasons, the identification of materials resistant to Ceratocystis wilt, additional to being important to save the kiwifruit plantations of Brazil, is of great value for the kiwi production hubs as a measure of prevention against future damages and loss to the crop caused by the introduction of the pathogen.

5. References

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Table 1 Cultivars utilised in the evaluation for resistance to *Ceratocystis wilt*.

SPECIES	APPLICATION	CULTIVAR
Actinidia chinensis var. *deliciosa*	Producer of fruits (Pistilada)	Allison
		Bruno
		Hayward
		Monty
	Pollinizers (Estaminadas)	Chieftain
		Tomury
Actinidia arguta	Producer of fruits (Pistilada)	Actinidia arguta
		Actinidia arguta Ken's Red

Table 2 Origin and genotypes of *C. fimbriata* kiwi isolates that underwent screening in Monty cultivars.

ISOLATE	GENOTYPE	CITY AND STATE	GEOGRAPHIC COORDINATES
A8	DEDBFEABAAADCA	Farroupilha-RS	29°07’40’’S 51°24’00’’W
A11	CDDBDEABBCADBC		
A15	DCDBCBACBACLBA		
A16	EDCBDAABACADCC		
A32	BCDBCBACBACLBA		
PG01	ECDBCBACBACMBA		
CE07	DCDBCBACBACJBA	São Joaquim-SC	28°17’38’’S 49°55’54’’W
CE10	DCEBCCACBACMBA		
FI05	DCEBBBACBACMBA	Fraiburgo-SC	27°01’34’’S 50°55’17’’W
FI11	DCDBBDADBACQBA		
FI13	DCEBBBACBACRBA		
FI22	BCABDBACBACHBA		
JO03	CAEDEDABAECBAA	Campo Belo do Sul-SC	27°53’57’’S 50°45’39’’W
JO04	DCDBCBACBACMBA		

KEY: RS = Rio Grande do Sul | SC = Santa Catarina

Table 3 Number of dead plants (NPM), wound length (CL) and severity in eight kiwi cultivars inoculated with the mixture of isolates of *Ceratocystis fimbriata*.

CULTIVAR	NPM	CL(cm)	SEVERITY (%)
Actinidia arguta Ken's Red	5/5	13,0 a[a]	100 a[b]
Monty	4/5	16,4 a	98,9 a
Hayward	4/5	10,5 a	92,3 a
Tomury	2/5	12,6 a	92,2 a
Alisson	1/5	13,7 a	82,6 b
Actinidia arguta	2/5	13,8 a	82,0 b
Chieftain	0	10,2 a	48,4 c
Bruno	0	6,0 b	41,9 c
Controle	0	2,0 c	9,0 d

[a]Averages, followed by the same letter, did not differ statistically from one another by the Scott-Knott test ($p < 0.01$).

[b] Averages, followed by the same letter, did not differ statistically from one another by the Scott-Knott test ($p < 0.01$).

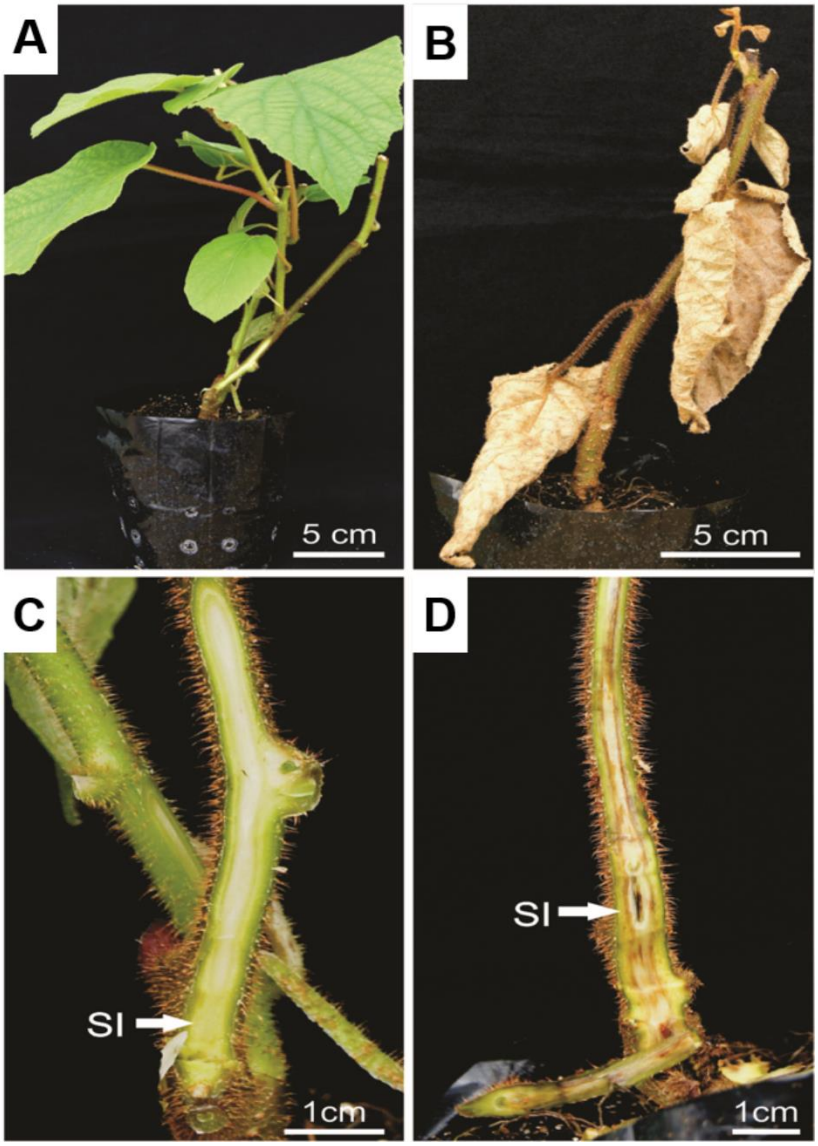

Fig. 1 Control plant and susceptible plant (*Actinidia spp.).* **A** and **C**, control plant, without wilt and with no darkening of internal tissues; **B** and **D,** susceptible plant, showing wilt and darkening of internal tissues; **SI**, inoculation site

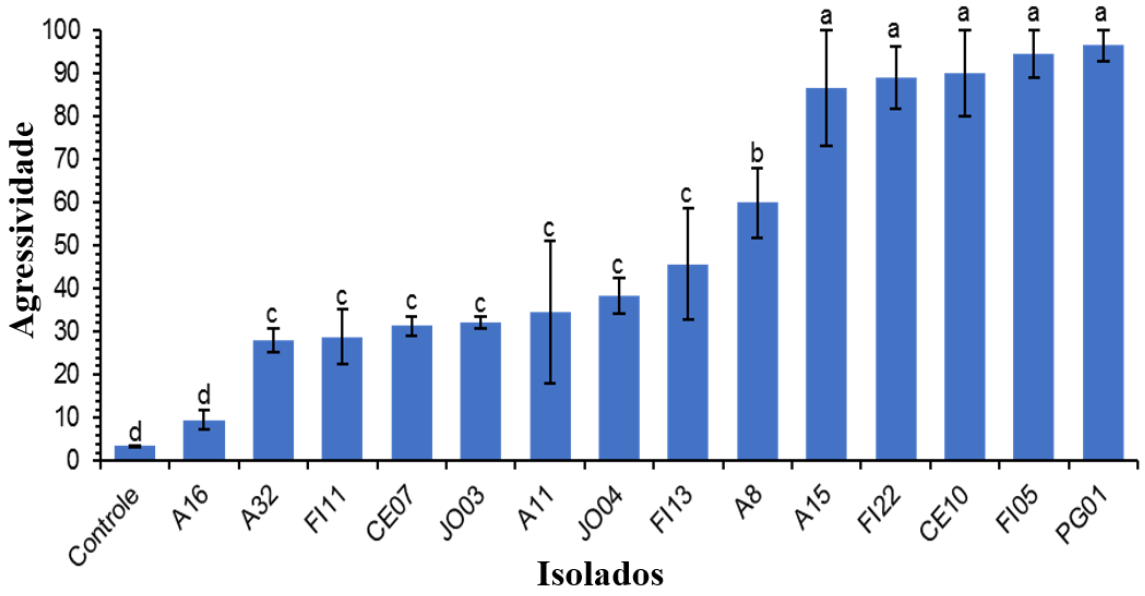

Fig. 2 Aggressiveness of 14 isolates of kiwifruit *Ceratocystis fimbriata* in Monty *(Actinidia chinensis var. Deliciosa).* Means, followed by the same letter, did not differ statistically from one another (Scott-Knott test, $p < 0.01$).