ARTIGOS

Development of a single uredinium inoculation method for *Puccinia kuehnii,* the causal agent of sugarcane orange rust

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ABSTRACT

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The use of resistant varieties is the ideal method to control rusts. Nevertheless, knowing the pathogen's diversity is fundamental to the success of this measure. Diversity can be analyzed phenotypically and/or genotypically. For phenotypic diversity, the reaction of genotypes is assessed by means of inoculations of the pathogen generally obtained from several uredinia. One handicap of this technique is its impossibility to detect diversity among these uredinia, assuming that they are all homogenous. Therefore, the aim of the present study was to develop a single uredinium technique for *Puccinia kuehnii* to be used in studies of rust diversity in sugarcane. The comparison between the two inoculation methods was done by employing urediniospores from SP89-1115 on the varieties SP89-1115 (susceptible) and RB975201 (resistant). The adopted design was completely randomized with five and seven replicates, respectively, examining incubation, latency, disease score, and injured area at 14 and 21 days. The two inoculation techniques were significantly equal for the susceptible variety, considering all evaluated parameters. For the resistant genotype, a significant difference was identified in the injured area and such difference did not interfere in the classification of the reaction of the material since values were below 1%. The single uredinium technique developed in this study showed to be reliable since the genotypic profile of the inoculated fungus was similar to that of pathogens from the produced lesions.

Keywords: *Saccharum spp*, infection, disease, technology

RESUMO

Porto, L.N.R.; Urashima, A.S. Desenvolvimento do método de inoculação monopústula de *Puccinia kuehnii*, agente causal da ferrugem alaranjada da cana-de-açúcar. ***Summa Phytopathologica,*** v.44, n.4, p.311-316, 2018.

O uso de variedades resistentes é o método ideal de controle das ferrugens. Entretanto, o conhecimento da diversidade do patógeno é fundamental para o sucesso dessa medida. Essa diversidade pode ser analisada fenotipica e/ou genotipicamente. Na diversidade fenotípica, a reação dos genótipos é avaliada em inoculações do patógeno advindo geralmente de várias pústulas. Uma falha nesse esquema reside no fato de não se detectar diversidade entre essas pústulas, assumindo que todas são homogêneas. Assim, o objetivo do presente trabalho foi desenvolver a técnica de inoculação monopústula de *Puccinia kuehnii* a ser utilizada para os estudos de diversidade de ferrugens em cana-de-açúcar. A comparação entre os dois métodos de inoculação foi feita a partir dos urediniósporos da SP89-1115 nas variedades SP89-1115 (suscetível) e RB975201 (resistente). O delineamento utilizado foi o inteiramente casualizado com cinco e sete repetições, respectivamente, examinando-se incubação, latência, escala de notas e área lesionada, aos 14 e 21 dias. As duas técnicas de inoculação mostraram-se significativamente iguais na variedade suscetível em todos parâmetros avaliados. Com relação ao genótipo resistente, diferença significativa foi identificada na área lesionada, diferença essa que não interferiu na classificação da reação do material, já que os valores foram abaixo de 1%. O método monopústula desenvolvido deste trabalho mostrou-se confiável, pois o perfil genotípico do fungo inoculado foi similar aos dos patógenos das pústulas produzidas.

Palavras-chave: *Saccharum spp*, infecção, doença, tecnologia

Sugarcane is the raw material for the production of ethanol, sugar, molasse, bagasse and other products subsequently employed as sugarcane-based bioenergy, as well as fertilizer (24). Brazil is the major sugarcane producer in the world, yielding 684.8 million tons of sugarcane, with predicted production of 76.3 ton/ha for the 2016/2017 crop season (11).

To reach this yield, abiotic/biotic stresses such as diseases, insect pests and droughts must be controlled during all stages of sugarcane development. Orange rust has been one of the most important threats to sugarcane production in Brazil since 2009 (4), after considerable damage to cultivar Q124 in Australia (19). Yield losses caused by this disease in Brazil have ranged from 20 to 40% tons of cane per hectare and 15 to 20% sucrose content (13). Furthermore, a survey carried out in the states of São Paulo and Mato Grosso do Sul identified that 22.6% most cultivated cultivars were susceptible or had intermediate resistant to orange rust and that the disease will remain important in the future years since 20.4% cultivars in newly established fields were susceptible (8, 9).

Orange rust, caused by *Puccinia kuehnii*, is characterized by its extraordinary capacity of dissemination by the wind, producing several disease secondary cycles in a short period, even when initiated from a tiny initial source (5). As sugarcane is cultivated in a large area in Brazil,

overlaps with conditions favorable to the germination of urediniospores of *P. kuehnii* are common: 17 to 24ºC and high humidity of 97 to 99% (20); thus, measures to keep orange rust under control are necessary to prevent epidemics.

The most important method for orange rust control is the development of resistant genotypes (14). Nevertheless, its efficacy depends on the knowledge of the pathogen's diversity since a change in the pathogen's population can determine the life span of a resistant genotype (28), a phenomenon already observed in Australia for cultivar Q124 in 2000 (19).

The phenotypic diversity of *Puccinias* in sugarcane has been examined through the inoculation of pathogen originated from multiple uredinia. Nevertheless, this technique is based on the premise that no uredinium presents diversity, which has not been documented (15). Therefore, single uredinium inoculation is essential for population diversity analyses, as already employed for *P. graminis* f. sp. *tritici* (16, 23). This technique has not been established for *Puccinias* from sugarcane, which has morphological traits different from those of the pathogen from wheat.

Thus, the present study aimed to develop a technique of single uredinium inoculation for *P. kuehnii* to be employed in diversity analyses of rust pathogens in sugarcane.

MATERIAL AND METHODS

Host

Individual buds of sugarcane cultivars SP89-1115 (susceptible control) and RB975201 (resistant control) were planted in 250-mL plastic cups containing substrate and kept at the Molecular Genetics Laboratory (LAGEM), located in the Center of Agrarian Sciences (CCA), Federal University of São Carlos (UFSCar), Araras Campus, São Paulo State, under natural photoperiod and protected from the rain, to avoid secondary infection, for 30 days.

Pathogen

Leaves of the cultivar SP89-1115 exhibiting visual symptoms of orange rust were collected at CCA (22º18' S and 47º23' W, average altitude of 700 m) and taken to the laboratory for analyses. Confirmation of orange rust was obtained under an optical microscope considering the morphological traits of urediniospores: orange, ellipsoidal, surrounded by echinulate ornamentation and presenting thickened apical wall (12).

Inoculation

Inoculations were carried out by using two different methods on the same day the disease was confirmed. The employed methods were multiple and single uredinium inoculation.

Multiple uredinia method

The inoculum suspension was prepared with urediniospores collected with a stiff-bristle brush from several uredinia on the leaf surface. The plants were individually placed in a black plastic bag, representing one replicate, and were inoculated by manually spraying a 14-mL volume, at a concentration of $1.8.10^5$ spores.mL added of Tween20 at 0.01%; then, they were kept for 48h at 25ºC, under water-saturated atmosphere. Subsequently, the plants were taken out of the bag and transferred to a greenhouse with natural photoperiod and humidity, but protected from rain to prevent secondary infection.

Single uredinium method

One uredinium classified as lesion type 6 according to the disease rank by Tai et al. (32) was selected. An area of $5cm^2$ of the abaxial part of a leaf was previously selected and a 20-µL volume of autoclaved milliQ water, added of Tween20 at 0.01%, was deposited. Then, urediniospores from the selected lesion were scattered over the water surface by using a scalpel. For the negative control, the same inoculation procedure was carried out in another area of the same leaf, employing leaf pieces of the same size but from a healthy area. Afterwards, inoculated plants were individually transferred to a plastic bag under the same conditions as those for the multiple uredinia inoculation. The concentration of the spore suspension for this method was determined by selecting lesion type 6, retrieving urediniospores deposited in a 20-µL volume with a scalpel and checking by using a hemocytometer.

Evaluation

The parameters employed to compare single and multiple uredinia inoculations were: incubation and latency periods, disease score, and percent diseased area. Incubation and latency periods were measured by taking plants to the laboratory and checking their leaves for the presence of rust symptoms under a stereomicroscope (Olympus Sz40); the check was performed daily since the end of the infection process, i.e., soon after the plants were removed from the plastic bags. The incubation period was defined as the time elapsed between the host infection by the pathogen and the first symptom, and the latency period was considered the time between the infection and the onset of reproductive structures of the pathogen (29).

Plants subjected to multiple uredinia inoculation had the entire surface of their leaves checked, whereas those that underwent single uredinium inoculation had only the inoculated area of their leaves examined, either for the positive or the negative control.

The disease rank employed by Amorim et al. (1) for brown rust of sugarcane was used for the disease score in this study, where rating 1= 0%; 2 = 0.5%; 3 = 1%; 4 = 5%; 5 = 10%; 6 = 25%; 7 = 35%; 8 = 50%; 9 = > 50%. This scale was applied to evaluate an area of $5cm^2$ leaf surface, either in the single uredinium or in the multiple uredinia inoculation, at 14 and 21 days post-inoculation. The percent injured area was assessed based on the image of the lesion area, using Assess 2.0 software (APS Press, American Phytopathological Society, St. Paul, MN, USA), for the same area employed in the disease rank.

The genotypic profiles of urediniospores of four uredinia used in the single uredinium inoculation and eight uredinia retrieved from symptoms resulted of this inoculation were compared to eliminate possible spurious contamination during the inoculation procedure. Thus, total DNA was extracted from each uredinium, according to Murray & Thompson (27). Briefly, each uredinium was placed in a 1.5-mL microtube containing extraction buffer (0.7M NaCl, 1% CTAB, 50mM Tris-HCl (pH 8.0), 10mM EDTA) and 1% 2-mercaptoetanol, where they were kept for 2h at 65°C. Subsequently, microtubes were centrifuged at 5939g for 5 min at room temperature. A 100-µL volume of the supernatant was collected, transferred to another microtube and an equal volume of chloroform:ethanol (24:1) was added, followed by homogenization for 2 min. Then, samples were centrifuged at 15,203g for 5 min at room temperature, 100 µL supernatant were transferred to another 1.5mL microtube, and 70µL isopropanol were added, homogenized and kept at -20°C for 1h. Subsequently, the solution was centrifuged again at 15,203g for 20 min, at 4°C, the whole volume was discarded and 1 mL alcohol 70% was added and centrifuged at 15,203g for 10 min, at 4°C. After DNA washing, it was dried for 3 min in the

concentrator (Eppendorf, 5301), dissolved in 20 µL autoclaved milliQ water, and kept in the dark for two days before storage at - 20°C.

PCR reaction was performed in a 10-µL volume, with 10 mM Tris-HCL, pH 8.8, 50 mM KCL, 2.0 mM $MgCl_2$, 1µM each primer, 200µM dNTPs, 0.5 unit Taq DNA polymerase and 2 µL DNA. The employed molecular markers were the microsatellites RST_135_B and RST_382_sptnk_a, developed for *P. kuehnii* by Arias et al. (2). The thermocycler BIO-RAD (C1000 Touch Thermal Cycler) was employed for DNA amplification under the following conditions: 95 ºC for 1 min, 60 ºC for 1 min (2 cycles), 95 ºC for 30 s, 60 ºC for 30 s, 68 ºC for 30 s (27 cycles) and final extension of 68 ºC for 4 min. Following amplification, PCR products were visualized in agarose gel (3%), after ethidium bromate staining, and photos were taken (Loccus Biotecnologia, L-Pix).

Statistical design

A completely randomized design was adopted for the entire experiment, with five replicates for the challenge of cultivar SP89-1115, and seven for RB975201. Data were subjected to normality test and converted to $\sqrt{x + 0.5}$. Tukey's test at 5% probability was applied to examine differences among treatments, using Software Assistat 4.1.

RESULTS

Incubation period of 7.5 days was observed for the susceptible cultivar (SP89-1115) in the single uredinium inoculation, varying from a minimum of 7 and a maximum of 8 days, whereas in the multiple uredinia inoculation the average was 9 days, varying from 8 to 10 days. Latency period was 9 days for the single uredinium inoculation and 10 days for the multiple uredinia. When these parameters were analyzed for the resistant cultivar (RB975201), the incubation period lasted an average of 9.5 days, varying from 9 to 10 days for single uredinium and 10.5 for multiple uredinia, a variation of 9 to 10 days. The latency period could not be observed in both inoculation methods for this cultivar since uredinium sporulation did not occur (Table 1).

Urediniospore concentration in the single uredinium inoculation was 1.10^5 spores.mL^{-1}, demonstrating that this inoculation method had the same concentration as that of multiple urenidia inoculation when this area was employed. The single uredinium inoculation in the susceptible genotype (SP89-1115) caused a disease score of 3.67 at 14 days after inoculation, whereas the multiple uredinia technique caused a lesion of 3.83. When the same parameter was examined at 21 days post-inoculation, single uredinium led to a disease score of 4.8 and multiple uredinia to a score of 5.0. There was no significant difference between both methods, regardless of the evaluation date since the disease increase, reflected in the disease score, was similar in both methods. As to diseased area, the single uredinium inoculation resulted in an area of 3.68% at 14 days after inoculation, whereas the multiple uredinia led to an area of 3.58%; at day 21, the diseased area was 10.43 and 14.88% for single uredinium and multiple uredia, respectively. There was no significant difference in the affected area between both methods in these evaluation periods because the variation in the diseased area was proportional between dates (Table 2).

The single uredinium inoculation led to a disease score of 1.14 for the resistant cultivar (RB975201) evaluated at 14 days post-inoculation, which did not differ significantly from multiple uredinia inoculation which also caused a disease score of 1.14. The disease score at 21 days was 2.0 for single uredinium and 1.42 for multiple uredinia. The percent diseased area at day 14 was 0.10% for the single uredinium inoculation technique and 0.03% for the multiple uredinia inoculation technique, which were significantly different. The same trend occurred at day 21, with 0.46% for the single uredinium inoculation and 0.14% for the multiple uredinia inoculation (Table 3).

The percent area affected by orange rust in two sugarcane cultivars according to the inoculation method assessed at 21 days after inoculation is shown in Figure 1. The single uredinium technique led to a diseased area of 6.28% in the susceptible cultivar (SP89-1115), whereas the multiple uredinia inoculation resulted in an area of 7.48%. When the resistant genotype (RB975201) was examined, the single

Table 1. Reaction of sugarcane cultivars to *Puccinia kuehnii* in terms of incubation and latency (days), according to the inoculation method.

	SP89-1115		RB975201	
	Incubation	**Latency**	**Incubation**	**Latency**
Single uredinium	7.5	9	9.5	Not observed
Multiple uredinia	9	10	10.5	Not observed

Table 2: Reaction of sugarcane cultivar SP89-1115 to *Puccinia kuehnii* in terms of disease score and percent diseased area, evaluated at 14 and 21 days post-inoculation, according to the inoculation method.

	days 14		days 21	
	Disease score[1]	**(%) Area**	**Disease score**	**(%) Area**
Single uredinium	3.67a	3.68a	4.8a	10.43a
Multiple uredinia	3.83a	3.58a	5.0a	14.88a
(%) CV	17.21	28.08	18.82	38.94

[1]Amorim et al. (1987): score 1 = 0%; 2 = 0.5%; 3 = 1%; 4 = 5%; 5 = 10%.

Table 3. Reaction of sugarcane cultivar RB975201 to *Puccinia kuehnii* in terms of disease score and percent diseased area, evaluated at 14 and 21 days post-inoculation, according to the inoculation method.

	days 14		days 21	
	Disease score[1]	**(%) Area**	**Disease score**	**(%) Area**
Single uredinium	1.14a	0.10a	2.0a	0.46a
Multiple uredinia	1.14a	0.03b	1.42a	0.14b
(%) CV	10.56	5.44	12.81	12.58

[1]Amorim et al. (1987): score 1 = 0%; 2 = 0.5%; 3 = 1%; 4 = 5%; 5 = 10%.

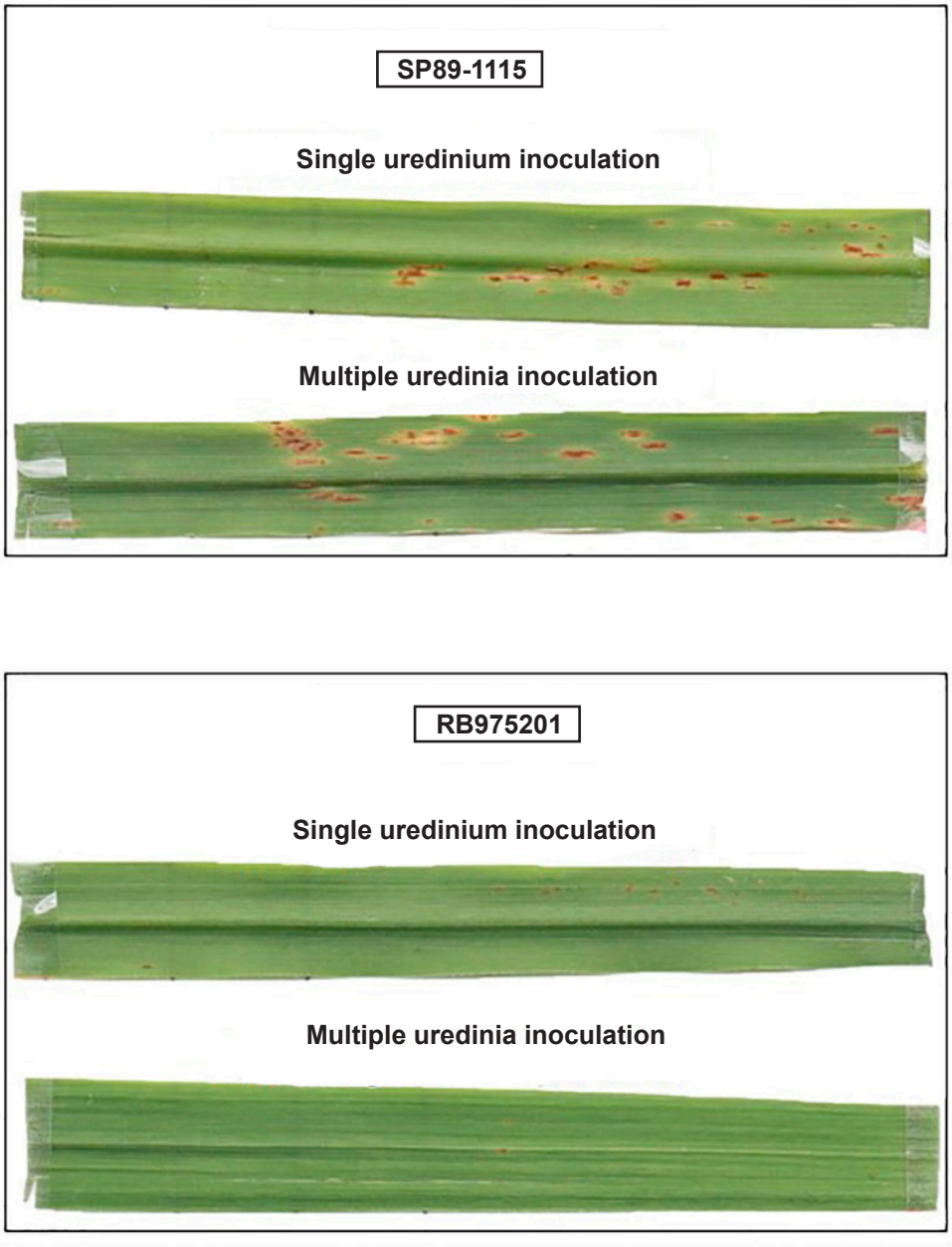

Figure 1. Reaction of sugarcane cultivars SP89-1115 and RB975201 to *Puccinia kuehnii* in terms of percent diseased area, evaluated at 21 days post-inoculation, according to the inoculation method.

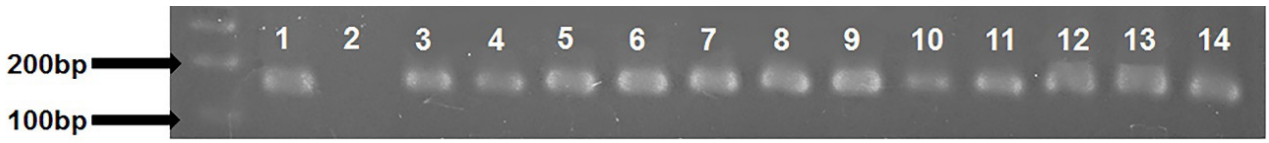

Figure 2. PCR products of *Puccinia kuehnii* amplified by primers RST_135_B and RST_382_sptnk_a (Arias et al., 2011). Lane 1: Positive control; 2: Negative control (water); DNA from fungus employed in the inoculation: lanes 3, 6, 10, 13; DNA retrieved from fungus isolated from symptoms: lanes 4, 5, 7, 8, 9, 11, 12, 14.

uredinium inoculation caused 0.41% diseased area, while the multiple uredinia, 0.07%. For both inoculation methods, the distribution of uredinia occurred evenly along the inoculated surface, with no visual distinction of symptoms between both inoculation methods.

One important component to validate the single uredinium technique is the absence of contamination by unwanted uredioniospores. Therefore, a genotypic profile was employed to show that urediniospores retrieved from orange rust symptoms were similar to those used in the inoculation and caused uredinia of orange rust. As shown in Figure 2, the urediniospores used in the inoculation (wells 3, 6, 10 and 13) had the same genotypic profile as those obtained from uredinia of symptoms caused after inoculation (wells 4, 5, 7, 8, 9, 11, 12 and 14).

DISCUSSION

The obtained data showed that the single uredinium inoculation technique developed in this study can replace the multiple uredinia inoculation to examine either the diversity of *P. kuehnii* or the reaction of sugarcane genotypes because both inoculation methods had significantly similar results for all tested parameters (incubation and latency periods, disease score, and percent diseased area). The parameters analyzed in the comparison between methods were selected because they were used in correlated studies (3, 7, 22, 26). Nevertheless, applying the single uredinium technique developed in this study requires caution: the age of the sugarcane should be around 45 days, the area of the abaxial leaf to be inoculated should be 5cm^2, and the host reaction should be evaluated between 14 and 21 days post-inoculation, a period also employed in previous studies (7, 15, 25).

The single uredinium inoculation technique is recommended for laboratory studies and has already been employed for *P. graminis* f. sp. *trittici* and *P. striiformis* f. sp. *hordei* (17, 21). As to *P. kuehnii,* the methodology developed in this study was validated since the single uredinium inoculation exhibited the same reaction as that of multiple uredinia inoculation in two sugarcane cultivars (compatible/incompatible) which, in turn, yielded a result similar to that of field evaluation for these two sugarcane cultivars. In the latter, host reaction to rust pathogen is visually examined based on the disease score, allowing evaluation of a great number of materials (7). According to this disease rank, the score varies from 1 to 9, from the least to the most affected materials (1), and genotypes are classified as resistant when scores range between 1 and 3, while scores from 4 to 9 indicate that the genotypes are susceptible (10). Sugarcane cultivars SP89-1115 and RB975201 were classified as susceptible and resistant, respectively, for both inoculation methods evaluated at 14 and 21 days (Tables 2 and 3). The significant difference in the percent diseased area for the resistant cultivar (RB975201) did not interfere in its classification as a resistant genotype since this value still fits into the range of a resistant material, which is from 0 to 1% (1, 10). It is worth mentioning that genotypes classified as resistant encompass materials with varying diseased area (up to 1%), which is an important step in the breeding process because it allows the incorporation of minor genes in a sugarcane cultivar (18). On the other hand, selection of genotypes based solely on score zero (symptomless) considers only qualitative resistance, which could eventually lead to the vertifolia effect (6), which is the erosion of horizontal resistance during the breeding process of a crop, increasing the probability of resistance breakdown for a resistant cultivar.

Single uredinium inoculation shows to be a useful tool for pathogen diversity studies, either phenotypic or genotypic studies, especially for fungi causing rust, since the efficient dissemination of urediniospores can result in multiple and varied inoculum sources even in a single leaf symptom, which could not be identified in a multiple inoculation. The single uredinium inoculation evidenced that a great number of distinct individuals initiated an outbreak of oat stem rust (*P. graminis* f. sp. *avenae*) in fields of Sweden, indicating that a large number of individuals (inoculum source) should have initiated the disease in each field and not within each field (5). This useful information could only be obtained when each pathogen was individualized by uredinium, demonstrating that the entire diversity can only be evaluated when single uredinium inoculation is employed (17).

The possibility that orange rust symptoms caused by external urediniospores mixed with those caused by the single uredinium inoculation is minimal despite efficient aerial dissemination of these spores, since low humidity was maintained throughout the host development. Moreover, the genotypic profiles of inoculated urediniospores and spores retrieved from lesions were similar, indicating absence of unwanted spores during the infection process (Figure 2).

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