

## ARTIGOS

# Quantification of incubation, latent and infection periods of *Phakopsora pachyrhizi* in soybean, according to chronological time and degree-days

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### ABSTRACT

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In experiments conducted in a growth chamber, the chronological time and the accumulated degree-days were determined for the duration of incubation, latent and infectious periods of *Phakopsora pachyrhizi* cultivars BRSGO 7560 and BRS 246 RR. Detached soybean leaflets were placed in gerbox-type acrylic boxes and inoculated with  $20 \times 10^3$  uredospores/mL. The study was conducted at 12-h photoperiod and temperatures of 10°C, 15°C, 22°C, 25°C and 30°C for 30 days. Lesions and uredia/cm<sup>2</sup> were evaluated and the number of uredia per lesion was quantified after the beginning of

sporulation. The sporulation potential was also quantified for cultivars BRSGO 7560 and BRS 246 RR. The steps of the infection process can be quantified based on both the chronological time and the accumulated heat. The cultivar BRSGO 7560 produced 4,012.8 spores/cm<sup>2</sup> and BRS 246 RR, 7,348.4 uredospores/cm<sup>2</sup>. The largest number of uredia was produced at 25°C in both cultivars; however, BRS 246 RR presented 372.7 uredia/cm<sup>2</sup> and BRSGO 7560, 231.6 uredia/cm<sup>2</sup>. At 10°C and 30°C, leaf infection did not occur in both cultivars.

**Keywords:** *Glycine max*, temperature, Asian soybean rust

### RESUMO

Danelli, A.L.D.; Reis, E.M. Quantificação dos períodos de incubação, latente e infeccioso de *Phakopsora pachyrhizi*, em soja, por tempo cronológico e por graus-dia. *Summa Phytopathologica*, v.42, n.1, p.11-17, 2016.

Em experimentos conduzidos em câmara de crescimento determinou-se o tempo cronológico e os graus-dias acumulados para a duração dos períodos de incubação, latente e infeccioso de *Phakopsora pachyrhizi* nas cultivares BRSGO 7560 e BRS 246 RR. Folíolos de soja destacados foram acondicionados em caixas de acrílico tipo gerbox e inoculados com  $20 \times 10^3$  uredosporos/mL. O trabalho foi conduzido com fotoperíodo de 12 horas e nas temperaturas de 10°C, 15°C, 22°C, 25°C e 30°C por 30 dias. Foram avaliadas lesões e urédias/cm<sup>2</sup> e o número de urédias por lesão quantificados após o início da esporulação.

Também foi quantificado o potencial de esporulação nos cultivares BRSGO 7560 e BRS 246 RR. As fases do processo de infecção podem ser quantificadas tanto pelo tempo cronológico como pelo calor acumulado. A cultivar BRSGO 7560 produziu 4.012,8 esporos/cm<sup>2</sup> e a BRS 246 RR 7.348,4 uredosporos/cm<sup>2</sup>. O maior número de urédias foi produzido na temperatura de 25°C em ambas as cultivares, entretanto, a BRS 246 RR apresentou 372,7 urédias/cm<sup>2</sup> e a BRSGO 7560, 231,6 urédias/cm<sup>2</sup>. Nas temperaturas de 10°C e 30°C não ocorreu infecção foliar em ambas as cultivares.

**Palavras-chave:** *Glycine max*, temperatura, ferrugem asiática

Asian soybean rust (ASR) is caused by *Phakopsora pachyrhizi* Syd. & P. Syd. This disease has led to yield losses in many grain-producing regions; in the Planalto Region of Rio Grande do Sul, in Southern Brazil, losses can be as high as 48% (24).

The first report of ASR in South America was in 2001, causing damage to soybean crop in Paraguay and Brazil (33).

The main control strategy for ASR is fungicide application on the leaves. In resistant cultivars, the pathogen cannot fully develop, resulting in decreased number of lesions, larger uredinium number/lesion, decreased uredospore production and increased latent and infectious periods. These mechanisms result in reduced pathogen population by decreasing the amount of inoculum and consequently

the disease intensity (3, 10).

Living organisms respond to stimuli that are environmental signals, such as water, temperature, and light (34). The growth and development of plant pathogens and their hosts generally respond to the temperature of their environment (35). The thermal time can be useful in modeling and evaluating the development of many pathogens that are important for agriculture (13).

The temperature acts as a catalyst, accelerating or slowing the development of the biological life cycle of the fungus, and interfering with uredospore development and germination (11), aplerium formation, uredinium development and sporulation (19), and teliospore formation and germination (25).

For *P. pachyrhizi*, the infectious process is halted below 9°C and above 29°C (19). Alves et al. (2) reported the thermal limits of 9.6°C and 30°C. Caldwell et al. (6) also studied the effect of temperature on the infectious process and did not find disease development at 15°C and 30°C. Bonde et al. (4) studied the effect of temperature on uredospore germination, germ tube development and lesion density/cm<sup>2</sup>, and reported thermal limits ranging from 10.4°C to 29.6°C.

A large number of microorganisms have the ability to maintain their body temperature constant and are classified as endothermic; others do not demonstrate this ability and are known as exothermic (31). So far, this principle has been little explored in plant pathology. Fungi have their body temperature regulated by the environmental heat, which determines the duration of their life cycle and phase shift (14, 27).

One of the first studies using degree-days to measure the latent period was reported in 1983 for apple (*Malus* spp. (Borkh.)) and *Venturia inaequalis* Cooke. pathosystem by Tomerlin & Jones (30). Scherm & Van Bruggen (26) studied the effect of temperature, measured by degree-days, on the latent period of *Bremia lactucae* Rengel. Sun & Yang (29) studied the influence of different light intensities quantified by degree-days on apothecium production of *Sclerotinia sclerotiorum* (Lib.) de Bary. Alves & Fernandes (1) studied the influence of temperature and relative humidity on the sporulation of *Magnaporthe grisea* (Hebert) Barr. in wheat, showing that the conidium formation rate was related to the accumulative degree-days. Zearfoss et al. (35) developed a model based on degree-days to measure the latent period of *Stagonospora nodorum* (Berk) in winter wheat.

A warning model for ASR based on accumulated heat was developed by Reis et al. (23). The accumulated growing degree-days were calculated from the day of rust occurrence (1% leaflet incidence) in the current season up to the new occurrence in the next growing season. A thermal constant of 890°C was determined in a five-year experiment.

The hypothesis is that the subphases of the infection process can be measured based on accumulated degree-days.

This study aimed to determine incubation, latent and infectious periods for cultivars showing different reactions to ASR, using chronological time, expressed as days, and heat units, expressed as accumulated degree-days (ADD).

## MATERIAL AND METHODS

The experiment was conducted in the Laboratory of Plant Pathology, University of Passo Fundo-UPF, Passo Fundo/RS. The ASR inoculum came from infected soybean plants collected in the UPF experimental field and kept, by frequent inoculation, in potted-soybean plants grown in a greenhouse at 25°C and 12-h photoperiod.

Soybean cultivars BRSGO 7560, resistant to rust, 7.5 maturation group, and BRS 246 RR, susceptible, 7.2 maturation group, were used in this study.

Throughout this experiment, detached soybean leaflets were used (9). Healthy soybean trifoliolate leaves were placed in acrylic boxes (11 x 11 x 3.5 cm high) containing, at the bottom, nylon foam, 0.5cm thick, and covered with an aluminum foil. Using a pair of tweezers, a hole was made in the aluminum foil and nylon foam to introduce the leaf petiole. Each box was supplemented with 20 ml nutrient solution containing macro and micro nutrients and vitamin (20). The containers were kept in a growth chamber at 25°C and 12-h photoperiod until complete petiole rooting.

For inoculation, leaves with abundant *P. pachyrhizi* sporulation were introduced in a plastic bottle containing 200 mL water and a

drop of Tween 20/L water, and the suspension was stirred to remove the spores. Then, the spores were counted in a scanning microscope slide containing one drop of known volume. The spore suspension concentration was adjusted to a minimum of 20 x 10<sup>3</sup> uredospores/mL (3) and the leaves were inoculated with a plastic manual sprayer.

After inoculation, the boxes were placed in a BOD-type incubator (biological oxygen demand), remaining in the dark for 8 hours for spore germination and leaf penetration. As the time elapsed, the boxes were transferred to a climatic chamber, 12-h photoperiod (supplied by eight white 40-Watts fluorescent light bulbs at 30 cm above the boxes), and supplemented with the nutrient solution at every three days.

Five constant temperatures, 10°C, 15°C, 22°C, 25°C and 30°C, were tested. The evaluations were daily performed under a stereomicroscope (Zeiss 40 x), observing the development of domes (closed uredia) and the beginning of sporulation (open uredia with visible spores). Evaluations were made at 5, 10, 15, 20, 25 and 30 days after sporulation had begun. For each replicate, 1.0cm-diameter circles were marked with a stopper punch on the two leaf halves, one on the right side and the other one on the left side, totaling six assessed sites per leaf, where the lesions and uredinium number/cm<sup>2</sup> were counted. Data were expressed as lesion number/cm<sup>2</sup> and uredinium number/lesion.

Additional leaf discs, 1.0cm diameter, at seven-day intervals, were transferred to test tubes with 10 mL distilled water and one drop of Tween 20/L water. A 0.01-mL aliquot was taken with a micropipette and three drops were deposited on a microscope slide. Spore counting was performed by scanning the drops under a microscope. Data were expressed as uredospores/cm<sup>2</sup>.

Degree-days (DD) were calculated according to the method of Villa Nova et al. (32), in which: DD = (MT - LTT), where: MT = mean daily temperature and LTT = lower threshold temperature. Similarly, ADD (accumulated degree-days) =  $\sum$  DD (degree-days) along 30 days. The LTT was taken from the study of Carlini (7), where basal, optimum and maximum temperatures for spore germination and germ tube growth of *P. pachyrhizi* uredospore were determined. In the experiments of Carlini, a temperature range at degree intervals from 5°C to 36°C and exposure times of 3, 6, 12 and 24 h were tested. The considered basal temperature was 6°C, optimal temperature 21°C, and upper threshold 35°C.

The latent period was considered the period from spore deposition on the leaf surface to the appearance of the first uredospores in the open uredia. The infectious period was considered the period from the end of the latent period to the final 30 days of the experiment, pre-determined to avoid leaf senescence.

The experiments were repeated twice.

The study was conducted in a completely randomized block design with five replicates for each temperature, in a 2 x 5 factorial arrangement (soybean cultivars and temperatures). Data were subjected to analysis of variance (ANOVA) and means compared according to Tukey's test. The equations expressed in each graph were used for regression, determining the Pearson's correlation coefficient among spores, lesions, uredia, and uredinium number/lesion.

## RESULTS

Analysis of variance showed that the factor cultivar was significant only for uredia ( $p = 0.005$ ), while the factor temperature showed significance for lesions ( $p < 0.001$ ); however, for all tested variables, spores ( $p = 0.04$ ), lesions ( $p = 0.0138$ ), uredia ( $p = 0.005$ ), and uredinium number/lesion ( $p = 0.004$ ), there was a significant interaction between temperature and cultivar (Table 1).

**Table 1.** Mean squares (MS) and their significance (*p*) according to F test for the studied sources of variation in the quantification of spores (no./cm<sup>2</sup>), lesions (no./cm<sup>2</sup>), uredia (no./cm<sup>2</sup>) and uredia (no./lesion) of Asian soybean rust. UPF, Passo Fundo, RS, 2015

Factor	Spore (no./cm <sup>2</sup> )			Lesion (no./cm <sup>2</sup> )			Uredia (no./cm <sup>2</sup> )			Uredia (no./lesion)		
	MS	F	<i>p</i>	MS	F	<i>p</i>	MS	F	<i>p</i>	MS	F	<i>p</i>
Cultivar	1737001.3	2.4	0.06 <sup>ns</sup>	6.75362	0.0486	>0.050 <sup>ns</sup>	3430.4	4.4	0.005**	16.2	2.2	0.08 <sup>ns</sup>
Temperature	475275.5	0.6	>0.05 <sup>ns</sup>	1660.86283	11.9474	<0.001**	205.7	0.2	>0.05 <sup>ns</sup>	6.8	0.9	>0.05 <sup>ns</sup>
Interaction	1925930.9	2.7	0.04*	8.30594	0.0597	0.0138*	3412.4	4.4	0.005**	32.7	4.4	0.004**

\*\* : Significant at 1% probability \* : Significant at 5% probability: <sup>ns</sup> not significant

**Table 2.** Mean squares (MS) and significance (*p*) according to F test for the studied and quantified causes of variation related to incubation, latent and infectious periods based on accumulated degree-days (ADD) and chronological time (CT) of Asian soybean rust. UPF, Passo Fundo, RS, 2015

Factor	Incubation period ADD			Latent period ADD			Infectious period ADD		
	MS	F	<i>p</i>	MS	F	<i>p</i>	MS	F	<i>p</i>
Cultivar	36205.1	296.8	<0.001**	49928.5	313.9	<0.001**	187695.7	1206.6	<0.001**
Temperature	238.3	1.9	0.16 <sup>ns</sup>	519.5	3.3	0.07 <sup>ns</sup>	539.1	3.5	0.069 <sup>ns</sup>
Interaction	23459.5	192.9	<0.001**	38057.9	239.3	<0.001**	133932.3	860.9	<0.001**
Factor	Incubation period CT			Latent period CT			Infectious period CT		
	MS	F	<i>p</i>	MS	F	<i>p</i>	MS	F	<i>p</i>
Cultivar	200.6	166.3	<0.001**	281.6	208.9	<0.001**	609.6	454.1	<0.001**
Temperature	5.8	4.8	0.03*	4.1	2.9	0.0912 <sup>ns</sup>	4.1	2.3	0.09 <sup>ns</sup>
Interaction	200.7	166.4	<0.001**	298.6	221.6	<0.001**	409.8	305.3	<0.001**

\*\* : Significant at 1% probability \* : Significant at 5% probability: <sup>ns</sup> not significant.

Considering the analysis of variance for incubation, latency and infectious periods quantified according to ADD and chronological time, the factor cultivar was significant for all periods. The temperature was only significant for the incubation period quantified according to the chronological time (Table 2). The interaction between cultivars and temperature was significant (*p* < 0.001) for all periods, when quantified according to either ADD or chronological time (Table 2).

At 22°C, the number of produced spores differed between cultivars: BRSGO 7560 showed the smallest number, 2,500.2/cm<sup>2</sup>, and BRS 246 RR, the largest number, 8,456.3/cm<sup>2</sup>. BRSGO 7560 produced the smallest spore number/cm<sup>2</sup>, 4,012.8, and BRS 246 RR, the largest number, 7,348.4/cm<sup>2</sup>. The largest number of spores was produced at 22°C for BRS 246 RR, and at 25°C for BRSGO 7560 (Figure 1A).

Regarding lesion/cm<sup>2</sup>, the means did not differ among cultivars: on average, 23.8 lesions/cm<sup>2</sup> were counted for BRSGO 7560 and 24.5 lesions/cm<sup>2</sup> for BRS 246 RR. At 15°C, the lowest means were recorded, 2.8 lesions/cm<sup>2</sup>, and at 22°C and 25°C, the highest means were obtained, 24.9 and 25.7 lesions/cm<sup>2</sup> (Figure 1B).

BRSGO 7560 produced the smallest number of uredia and BRS 246 RR, the largest number. At 15°C, 22°C and 25°C, BRSGO 7560 produced 81.5, 168.8 and 231.6 uredia/cm<sup>2</sup>, respectively, while BRS 246 RR produced 141.5, 291.5 and 372.7 uredia/cm<sup>2</sup>, respectively (Figure 1C).

The cultivar BRSGO 7560 showed the smallest uredinium number/lesion. At 15°C, BRSGO 7560 showed 3.6 uredia/lesion, while BRS 246 RR presented 6.7 uredia/lesion. At 22°C, BRSGO 7560 produced 6.8 uredia/lesion and BRS 246 RR showed 11.5 uredia/lesion. At 25°C, BRSGO 7560 had 9.5 uredia/lesion and BRS 246 RR formed 13.7 uredia/lesion. The largest uredinium number/lesion was found at 25°C (Figure 1D).

The effect of temperature on the incubation period, assessed based

on DD, for both cultivars, indicated that the lowest ADD was found at 15°C and the highest one at 25°C (Figure 2A). The effect of temperature on the incubation period, measured according to the chronological time, indicated that the smallest number of days was found at 25°C, and the largest number at 15°C for both cultivars (Figure 2B).

Considering the duration of the latent period assessed based on the ADD, the highest ADD values were found at 25°C, and the lowest values at 15°C (Figure 2C). When analyzed according to the chronological time, the largest number of days was observed at 15°C and the smallest one at 25°C (Figure 2D).

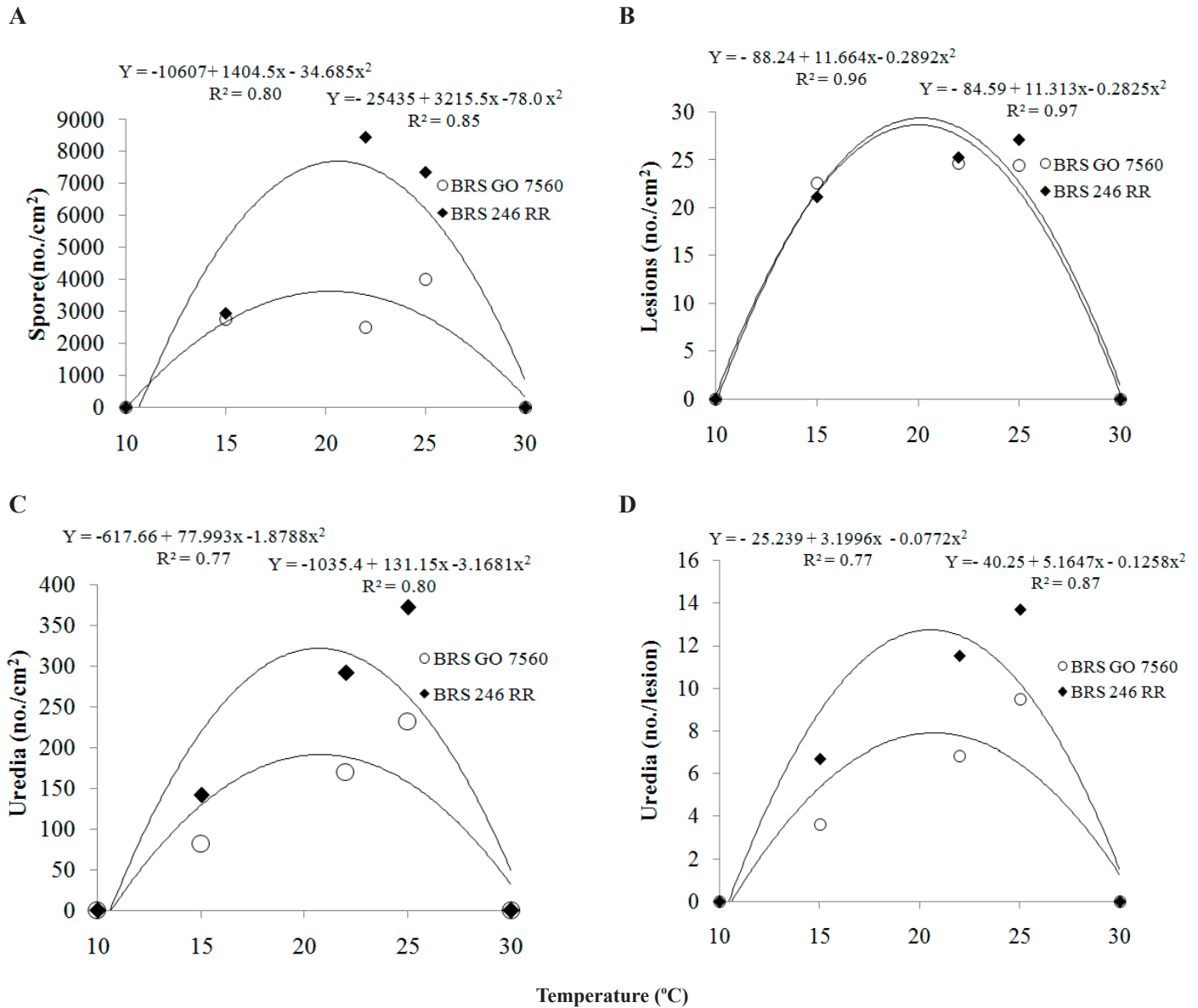
Regarding the infectious period according to ADD, the highest values were found at 25°C for both cultivars and the lowest values were found at 15°C (Figure 2E). In the chronological analysis for the infectious period, the largest number of days was observed at 25°C, and the smallest one at 15°C (Figure 2F).

In the evaluation of incubation, latent and infectious periods, as temperature was reduced (15°C), ADD also decreased, thus taking longer for the first symptoms to appear. At 22°C and 25°C, the ADD was higher, reducing the number of days for the onset of spores.

The latent period at the temperatures of 15°C, 22°C and 25°C was 17, 10.2 and 8 days, respectively, for BRSGO 7560, and 19, 9.3 and 8 days, respectively, for BRS 246 RR. The longest latent periods were found at the temperature of 15°C and the shortest ones at 22°C and 25°C (Figure 2D).

The infectious period at 15°C, 22°C and 25°C was 13, 19.7 and 22 days, respectively, for BRSGO 7560, and 11, 20.7 and 22 days, respectively, for BRS 246 RR. The longest latent periods were found at 22°C and 25°C and the shortest ones at 15°C (Figure 2F).

Correlation analysis showed a high degree of positive linearity between spores (no./cm<sup>2</sup>) and lesions (no./cm<sup>2</sup>) (*r* = 0.91; *p* = 0.0034), spores (no./cm<sup>2</sup>) and uredia (no./cm<sup>2</sup>) (*r* = 0.94; *p* < 0.0001), lesions (no./



**Figure 1.** Spores (no./cm<sup>2</sup>) (A), lesions (no./cm<sup>2</sup>) (B), uredia (no./cm<sup>2</sup>) (C) and uredia (no./lesion) (D), produced by *Phakopsora pachyrhizi* inoculated in BRS GO 7560 and BRS 246 RR soybean cultivars at different temperatures. UPF, Passo Fundo, RS, 2015.

cm<sup>2</sup>) and uredia (no./cm<sup>2</sup>) ( $r = 0.90$ ;  $p < 0.0001$ ), and uredia (no./cm<sup>2</sup>) and uredia (no./lesion) ( $r = 0.82$ ;  $p < 0.0001$ ). The lowest correlation coefficients were found for the relationship between lesions (no./cm<sup>2</sup>) and uredia (no./lesion) ( $r = 0.59$ ;  $p < 0.0001$ ), and spores (no./cm<sup>2</sup>) and uredia (no./lesion) ( $r = 0.34$ ;  $p < 0.0145$ ) (Table 3). There was no foliar infection at 10°C and at 30°C.

## DISCUSSION

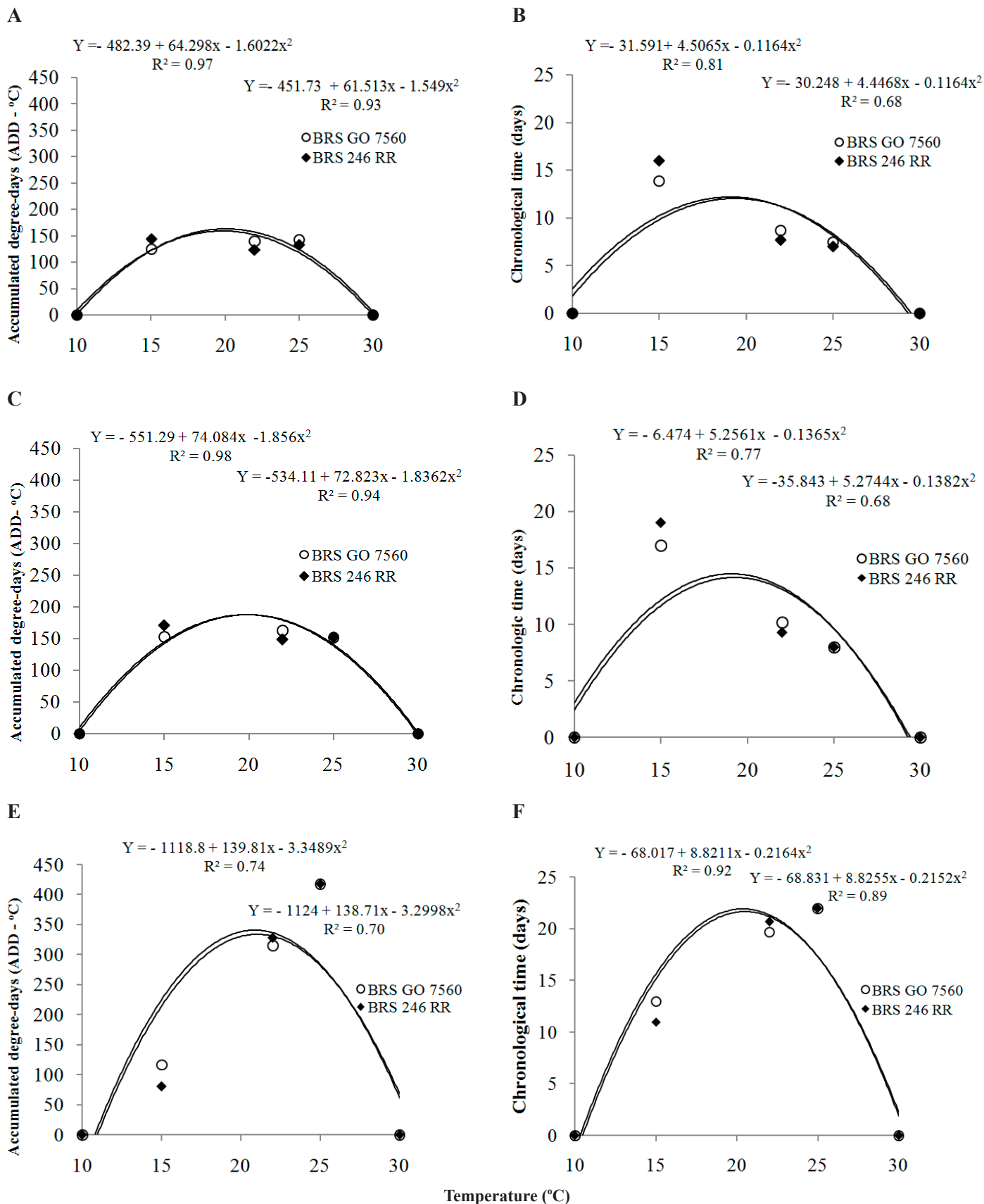
According to Souza et al. (28), the cultivar BRS GO 7560 carries a major gene that confers vertical resistance to soybean rust. At a high inoculum density, this cultivar can show susceptible lesions of tan color, which can be indicative of the lowest number of lesions found in this study. Another important aspect is that a resistance break can occur along the growing seasons due to the development of new races.

Comparing the cultivars BRS 246 RR and BRS GO 7560, regarding

spore number/cm<sup>2</sup>, uredinium number/cm<sup>2</sup>, and uredinium number/lesion, the statistical analysis showed differences between cultivars: BRS 246 RR showed the highest values, while BRS GO 7560 showed the lowest values.

Soybean lines with partial resistance have been identified and characterized based on the uredinium number/lesion (5). Our data for uredia/lesion are similar to those reported by Reis et al. (22), who found 14 uredia/lesion. In a study conducted by Azevedo et al. (3), the mean uredinium number/cm<sup>2</sup> ranged from 4.5 to 7.8. Regarding the total number of produced spores, Melching et al. (17) reported sporulation values of different isolates similar to those found in our study.

Our results showed that the optimal temperature ranged between 22°C and 25°C, with the largest number of spores, lesions, uredia, and uredia/lesion. At 10°C and 30°C, there was no leaf infection; this is probably due to constant temperature, which does not occur in the field. The environmental conditions required for soybean rust infection have been well defined in two temperature/leaf wetness combination studies



**Figure 2.** Effect of temperature on incubation period (A), latent period (C) and infectious period (E) of *Phakopsora pachyrhizi*, rated based on accumulated degree-days (ADD - °C), and effect of temperature on incubation period (B), latent period (D) and infectious period (F) (days) of *Phakopsora pachyrhizi*, rated based on chronological time (days) and inoculated in BRS GO 7560 and BRS 246 RR soybean cultivars.

**Table 3.** Level of significance (*p*) and correlation coefficients (*r*<sup>2</sup>) of spores (no./cm<sup>2</sup>), lesions (no./cm<sup>2</sup>), uredia (no./cm<sup>2</sup>) and uredia (no./lesion) of Asian soybean rust. UPF, Passo Fundo, RS, 2015

	Spores (no./cm <sup>2</sup> )	Lesions (no./cm <sup>2</sup> )	Uredia (no./cm <sup>2</sup> )	Uredia (no./lesion)
Spores (no./cm <sup>2</sup> )	1.00000	0.912702 <i>p</i> =0.0034**	0.938397 <i>p</i> <0.0001**	0.344724 <i>p</i> =0.0145 *
Lesions (no./cm <sup>2</sup> )		1.00000	0.901299 <i>p</i> <0.0001**	0.588767 <i>p</i> <0.0001***
Uredia (no./cm <sup>2</sup> )			1.00000	0.817991 <i>p</i> <0.0001**
Uredia (no./lesion)				1.00000

\*\* : Significant at 1% probability \* : Significant at 5% probability.

(15, 18). The optimal temperature for infection was 20°C-25°C and at least 6-8 hours of continuous leaf wetness.

The high degree of positive linearity found in the correlation analysis for spores (no./cm<sup>2</sup>) and lesions (no./cm<sup>2</sup>), spores (no./cm<sup>2</sup>) and uredia (no./cm<sup>2</sup>), uredia (no./cm<sup>2</sup>) and uredia (no./ lesion) was expected, because when the number of lesions increase, the number of spores and uredia also increase.

*Phakopsora pachyrhizi* has a relatively short latent period, compared to other pathogens. Nevertheless, it varies with temperature, i.e., 9 to 14 days (11), 8 to 10 days (18) 9 days (3), 11 to 16 days (16), 8 to 9 days (8) 14 to 28 days (7), 9 to 12 days (12).

According to Trudgill (31), the duration of the life cycle and the change in phenological stages for ectothermics can be modeled by using degree-days or accumulated heat, i.e., it can be inferred that the annual cycle duration of the fungus is governed by the accumulated heat of the environment. The most relevant results of our study regarding the thermal time to describe the stages of the disease cycle were that, regardless of temperature, the ADD are the same for both latent and incubation periods.

Chronological variation was expected since it is influenced by temperature. Considering the infectious period alone, as there was an increase in temperature, the infectious period also increased. These results agree with those reported by Carlini (7), Lange et al. (12) and Reis et al. (23) when the phases and sub-phases of *P. pachyrhizi* in soybean were quantified by the use of degree-days or accumulated heat.

The study of weather effects, such as temperature, on the pathogen development phases (incubation period and latent infection) are important to model the epidemics and thus assist in the management of diseases (21, 23, 29, 35).

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