Inoculum production and evaluation of temperature and leaf wetness for *Passalora sojina* inoculation in soybean

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ABSTRACT

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The objective of this study was to develop a methodology for inoculum production and inoculation of *Passalora sojina* in soybean to assess the reaction to frogeye leaf spot. Thus, sporulation of fungal races was quantified in five substrates under two light regimes. The temperature and the leaf wetness period that resulted in greater infection efficiency were also determined. Petri dishes containing the culture media Oat Flour Agar (OFA), Tomato Juice (FTJ), V8 agar juice (V8), Infant Food (IF) and Potato Sucrose Agar (PSA) plus isolates of *P. sojina* races 23, 24 and 25 were subjected to 12-h photoperiod and 24-h continuous dark. Inoculated plants were incubated at temperatures of 15, 20, 25, 30 and 35 ºC and leaf wetness periods of 12, 24, 36, 48 and 72 hours. Experimental design was completely randomized with five replicates. There was interaction among isolate, culture medium and photoperiod. The highest sporulation (conidia/cm^2) was obtained in the culture media FTJ (race 23) and V8 (races 24 and 25) under 12-h photoperiod. The optimum temperature for the disease development was 27ºC under 72 hours of continuous leaf wetness.

Keywords: sporulation, frogeye leaf spot, leaf wetness, infection critical period.

RESUMO

Camera, J.N.; Deuner, C.C.; Ghissi, V.C.; Reis, E.M.; Koefender, J. Produção de inóculo e avaliação de temperatura e molhamento para inoculação de *Passalora sojina* em soja. ***Summa Phytopathologica,*** v.44, n.2, p.122-126, 2018.

O objetivo desse trabalho foi desenvolver uma metodologia para produção de inóculo e inoculação de *Passalora sojina* em soja visando avaliar a reação à mancha foliar "olho-de-rã". Para isso foi quantificada a esporulação de raças do fungo em cinco substratos, sob dois regimes luminosos. Também foi determinada a temperatura e duração do período de molhamento foliar que resultasse na maior eficiência da infecção. Placas de Petri com os meios de cultura Farinha de Aveia Ágar (FAA), Suco de Tomate Temperado (STT), Suco V8 ágar (V8), Alimento Infantil (AI) e Batata Sacarose Ágar (BSA) contendo isolados das raças de *P. sojina* 23, 24 e 25 foram submetidas aos fotoperíodos de 12 h e 24 h de escuro contínuo. Plantas inoculadas foram incubadas a temperaturas de 15, 20, 25, 30 e 35 ºC e períodos de molhamento foliar de 12, 24, 36, 48 e 72 h. O delineamento experimental foi inteiramente casualizado com cinco repetições. Houve interação entre isolado, meio de cultura e fotoperíodo. A maior esporulação (conídio/cm^2) foi obtida no meio de cultura STT (raça 23) e no meio V8 (raças 24 e 25), sob fotoperíodo de 12 h. A temperatura ótima para o desenvolvimento da doença foi de 27 ºC com 72 horas de molhamento foliar contínuo.

Palavras-chave: esporulação, mancha foliar "olho-de-rã", molhamento foliar, período crítico à infecção.

Frogeye leaf spot caused by *Passalora sojina* (Hara) H. D. Shin & U. Braun (syn. *Cercospora sojina* Hara) is among the important fungal diseases that reduce the yield of soybean [*Glycine max* (L.) Merrill] (22). The use of resistant cultivars is the most effective and profitable control method (13). Therefore, an easy and reproducible methodology needs to be developed in order to assess the reaction of soybean genotypes to *P. sojina*.

The objectives of this study were to evaluate a methodology for inoculum production and to detect the optimum temperature and leaf wetness period for plant inoculation with *P. sojina*. Therefore, the most suitable medium and light exposure regime for the fungus sporulation were also determined, as well as the optimal temperature and the leaf wetness duration that result in the highest disease intensity.

MATERIAL AND METHODS

Sporulation of *Passalora sojina* in different culture media and light exposure

The races 23, 24 and 25 of *P. sojina* used in this study were provided by Embrapa Soybean. Race 23 was isolated from soybean cultivar Doko (Niquelândia-GO), race 24 from soybean cultivar BRS 28 (Santa Filomena-PI) and race 25 from soybean cultivar Cariri (Balsas-MA).

The following culture media were assessed for fungal sporulation: 1) oat flour agar (OFA) (8); 2) flavored tomato juice (FTJ) - 200 mL tomato juice Super Bom®; 16 g agar; 3.2 g $CaCO_3$, 0.2 g streptomycin and 800 mL distilled water (21); 3) V8 juice agar (V8) (21); 4) infant food (IF) (2); and 5) potato sucrose agar (PSA) (1). After preparation, the media were autoclaved at 121 ºC for 20 minutes and poured into 9.0cm-diameter plastic Petri dishes. Then, a 4.68mm-diameter mycelial disk containing the fungus from the monosporic isolation was placed onto the media and grown for 25 days under 12-h photoperiod and 24-h continuous dark. After this period, sporulation was quantified by using a hemocytometer-type Neubauer chamber (1), where the number of conidia cm^{-2} was calculated.

The experiment was carried out in a completely randomized design, triple factorial arrangement 3x5x2 (*P. sojina* races x culture media x light regimes), and five replicates. Data were subjected to analysis of variance and means were compared according to Tukey's test at 5%.

Interaction between temperature and leaf wetness period on frogeye leaf spot intensity

Interactions between the temperatures 15, 20, 25, 30 and 35 ºC and the leaf wetness periods 12, 24, 36, 48, 60 and 72 hours were assessed. Each temperature consisted in an experiment and the different leaf wetness periods consisted in the treatments.

Monosporic culture of *P. sojina* race 25 was multiplied in V8 agar (21) at 25 ºC and 12-h photoperiod. By using a hemocytometer-type Neubauer chamber (1), a conidial suspension $4x10^4 mL^{-1}$ was inoculated. The susceptible cultivar Don Mario **7**.0i was grown in pots in a growth chamber at 25 ºC and 12-h photoperiod. The plants were inoculated in V3 – when the second trifoliolate leaf was fully developed (7) by spraying the conidial suspension. Plants in five pots were sprayed with water alone and kept under the same environment as a control treatment. At the end of each wetness period, the plants were dried by using an electric fan and kept at 25 ºC and 12-h photoperiod

Fifteen days after inoculation, the disease intensity was assessed, considering: I) leaf area severity: the percentage of necrosed leaf area in relation to the total leaf area, assigning scores from 0 to 100% (6); II) lesion number per leaflet: in the leaflets, where the affected leaf area was quantified and the number of lesions was counted; and III) lesion diameter per leaflet: the diameter of four lesions per leaflet was quantified with a digital caliper.

The mean values of leaf area severity, the diameter and the number of lesions per leaflet, as well as temperature data, underwent regression analysis. Temperature data and leaf wetness followed the quadratic polynomial equation $Y = X^2 B1 - B2 + B3 X$, where Y = dependent variable (leaf area severity, diameter and number of injuries per leaflet), X = independent variable (temperature), B1 = estimated variable, B2 = parameter related to the initial inoculum, and B3 = disease progression rate. The means of all leaf wetness periods were calculated for the graphs of temperature variables and the average of all temperatures was calculated for the graphs of leaf wetness period.

The experiment was conducted in randomized design with five replicates, in which each temperature consisted in an experiment. The obtained data were subjected to analysis of variance and regression (19).

RESULTS AND DISCUSSION

Sporulation of *Passalora sojina* in different culture media and light regimes

Regarding the number of conidia cm^{-2}, there was significant interaction among the three factors. For the interaction between races and culture media, race 23 showed the largest number of conidia cm^{-2} in the FTJ substratum and the smallest number of conidia in OFA. As to race 24, the highest value was obtained in V8, while the lowest means were found in PSA and OFA, which were statistically similar. Considering race 25, the largest number of conidia was produced in V8 and was statistically superior to the others, whereas the smallest number of conidia was determined in PSA. Regardless of the culture medium, race 25 was statistically superior to the other races for the produced number of conidia cm^{-2}, and there was no significant difference between races 23 and 25 in PSA. The lowest fungal sporulation was found in IF and OFA for races 23 and 24, in V8 for race 23, and in PSA and FTJ for race 24 (Table 1).

The largest number of conidia cm^{-2} was produced under 12-h photoperiod. Race 25 showed the highest value, regardless of the light regime, and was statistically superior to the other races. The 12-h photoperiod was statistically superior to continuous darkness for the number of conidia cm^{-2}, which was statistically superior in V8 than in the other media, followed by FTJ (Table 1).

Table 1. Sporulation (conidia cm^{-2}) of races 23, 24 and 25 of *Passalora sojina* under different light regimes in different culture media. UPF, Passo Fundo/ RS. 2011

Races	Continuous dark	12-h Photoperiod [2]
23	B 137.8 c[1]	A 6462.2 b
24	B 540.0 b	A 6734.8 b
25	B 1061.5 a	A 15805.8 a
CV %	9.90	

Culture Media	Continuous dark	12-h Photoperiod [2]
AI	B 206.14 c[1]	A 10894.96 c
STT	B 720.46 b	A 14653.44 b
BSA	B 7.218 c	A 2158.15 d
V8	B 1196.63 a	A 18271.60 a
FAA	B 768.63 ab	A 2360.10 d
CV %	8.76	

Races	IF[4]	FTJ	PSA	V8	OFA
23	B 4,208.8 b[3]	A 8,402.8 b	D 1,026.3 a	C 2,418.1 c	E 444.1 b
24	B 4,227.8 b	C 3,414.8 c	D 677.7 b	A 9,255.0 b	D 411.7 b
25	C 8,214.9 a	B 11,243.1 a	E 1,344.0 a	A 17,529.1 a	D 3837.2 a
CV %			10.81		

[1]Means followed by the same lowercase letters in the column and capital letters on the line did not differ according to Tukey's test at 5% error probability. [2] 12-h Photoperiod: 12 h light and 12 h dark. [3]Means followed by the same lowercase letters in the column and capital letters on the line did not differ according to Tukey's test at 5% error probability. [4]Oat flour agar (OFA); Flavored Tomato Juice (FTJ); V8 agar juice (V8); Infant Food (IF); Potato sucrose agar (PSA).

As regards substrata, Hanada et al. (9) showed that the greatest sporulation of *Paracercospora fijiensis* (Morelet) Deighton was verified in V8 juice agar under 12-h photoperiod and sequential light exposure. These authors reported that there was no fungal sporulation in the dark for the majority of the tested culture media. Similarly, Brunelli et al. (3) found that V8 and FTJ media and 12-h photoperiod showed higher induction of sporulation, resulting in the production of $22.4x10^4$ and $28.62x10^4$ conidia.mL^{-1}, respectively. The results obtained in the present study indicate that the media V8 and STT (which is a modification of V8) increased *P. sojina* sporulation. OFA substratum did not show satisfactory sporulation and was the second best substrate for the fungus growth. V8 and tomato extract (variation of V8) media have the highest nutritional value and the greatest concentration of complex carbohydrates. V8 medium has been reported as an inductor of sporulation for several *Cercospora* spp. (9). Tomato extract agar was indicated by Queiroz & Menezes (16) as capable of inducing *Cercospora nicotianae* Ellis & Everh sporulation. Comparing V8 and FTJ, the latter seemed to be more useful from the practical point of view, since it is easily obtained in the market.

One characteristics of *Cercospora* species is the slow growth and the scarcity of sporulation in artificial media (3). For other *Cercospora* spp., there are reports indicating the positive effect of light exposure on sporulation in regimes alternated with dark periods. This was reported for *Cercospora arachidicola* Hori isolates by Moraes & Salgado

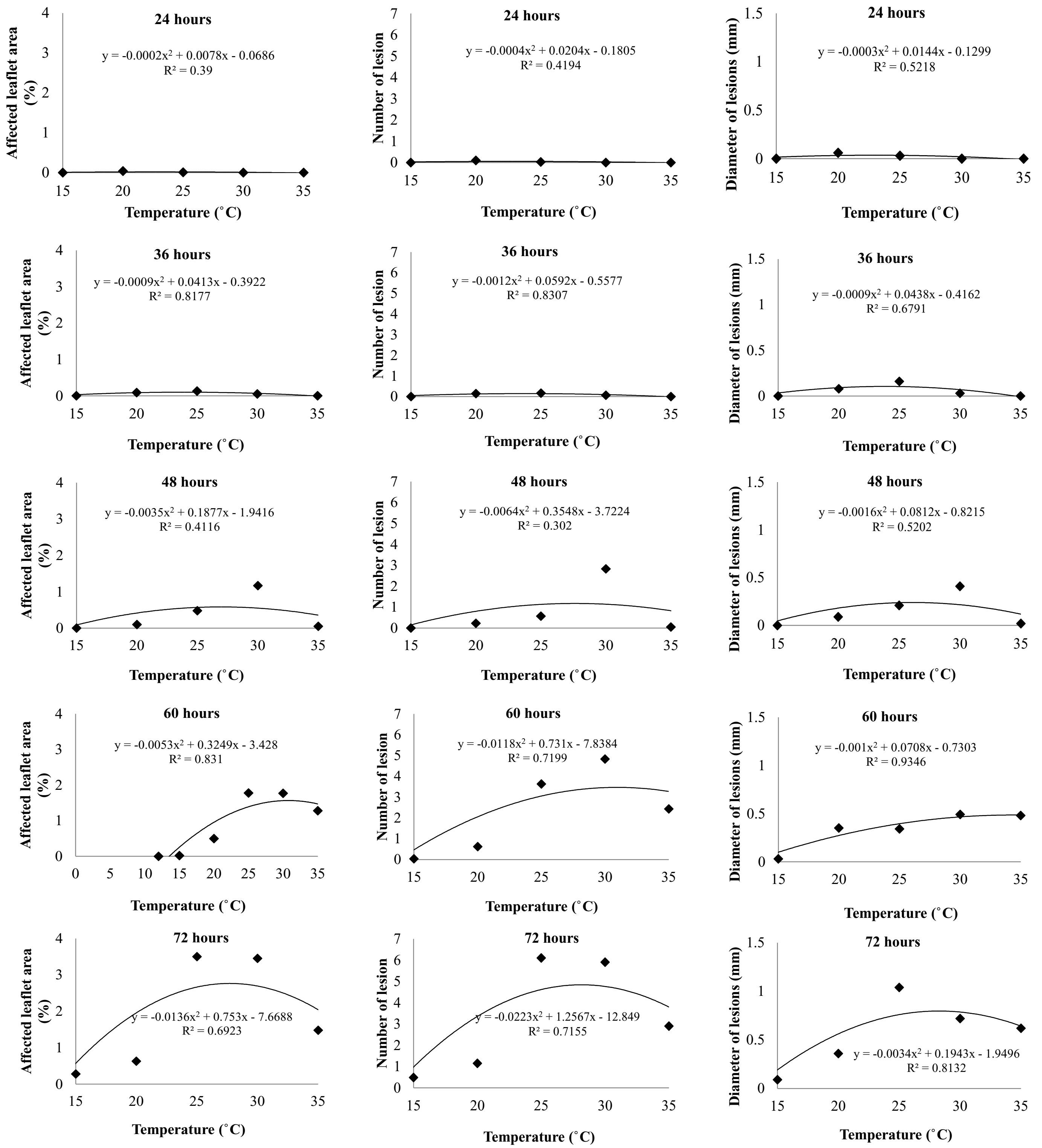

Figure 1. Quadratic polynomial model of frogeye leaf spot variables versus temperature for each leaf wetness level. Affected leaflet area (%), number of lesions per leaflet and diameter of lesions per leaflet (mm) for frogeye leaf spot in soybean cultivar Mario 7.0i.

(14) and for *Cercospora kikuchii* (MATSUMOTO & TOMOYASU) Gardner] by Dela-Cueva et al. (5). Partial inhibition of sporulation under continuous dark conditions was observed by Kilpatrick & Johnson (10) for *C. nicotianae* Ellis & Everh, by Stavely & Nimmo (20) for *Cercospora arachidicola* Hori, and by Hanada et al. (9) for *P. fijiensis*. According to Brunelli et al. (3), the best condition for incubation, aiming at conidial production for *Cercospora zeae-maydis* Tehon & Daniels, was 12-h photoperiod, as shown in our study for soybean x *P. sojina.*

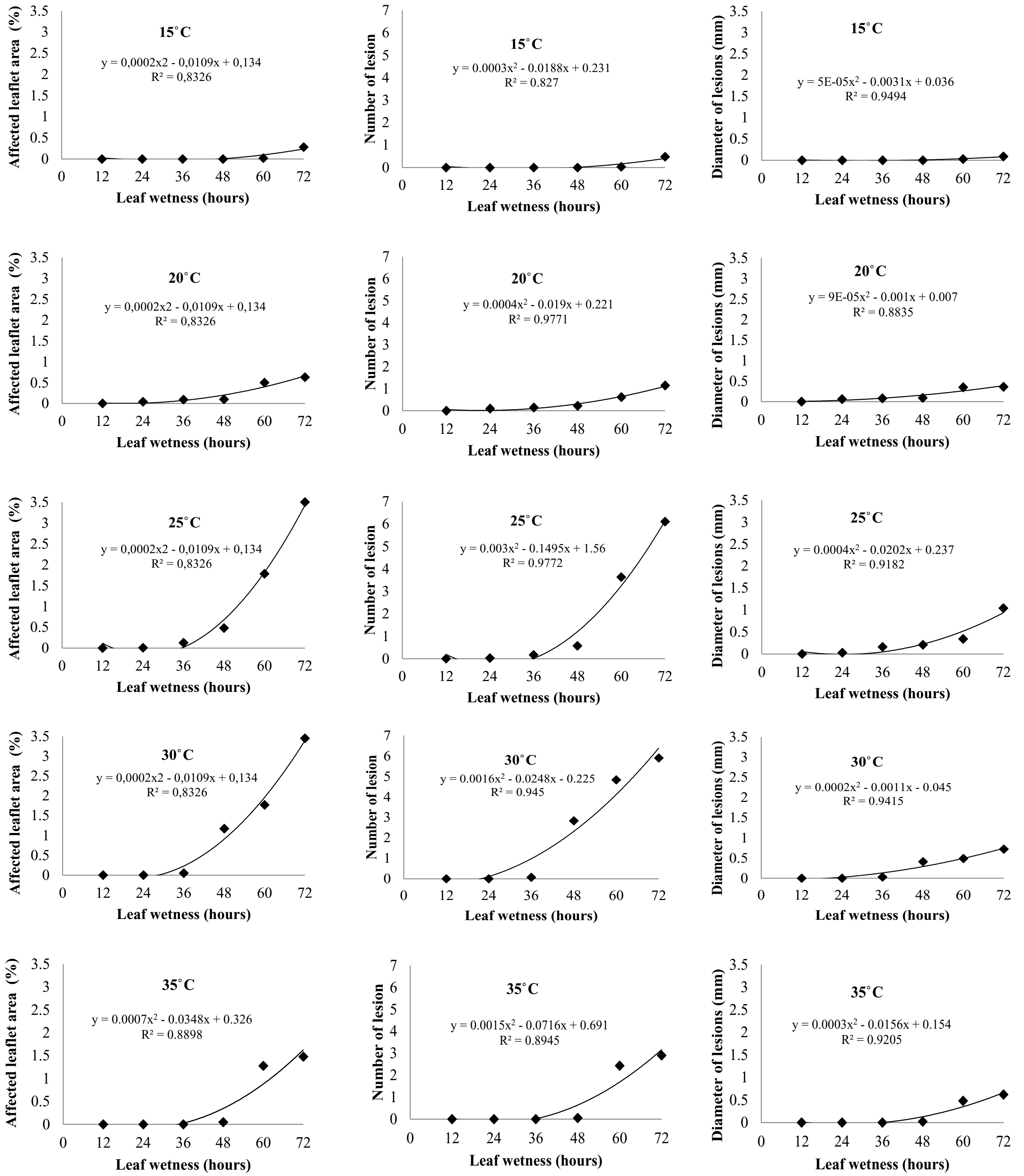

Figure 2. Quadratic polynomial model of frogeye leaf spot variables versus leaf wetness for each temperature level. Affected leaflet area (%), number of lesions per leaflet and diameter of lesions per leaflet (mm) for frogeye leaf spot in soybean cultivar Mario 7.0i.

Interaction between temperature and leaf wetness period on frogeye leaf spot intensity in soybean

The disease did not occur with 12 hours of leaf wetness, regardless of the temperature. The optimum temperature was determined for affected leaf area, number of lesions and diameter, considering 27 ºC, 28 ºC and 27 ºC, respectively (Figure 1). Both temperature and leaf wetness duration influenced the intensity of frogeye leaf spot. As regards temperature, when soybean plants were subjected to 15 ºC, the disease was observed only with 60 hours leaf wetness, at 20 and 25 ºC with 24 hours, at 30 ºC with 36 hours and at 35 ºC with 48 hours (Figure 2). Considering leaf wetness period, the disease intensity increased with the increase in the leaf wetness period; however, during 12 h the disease was not detected and the highest intensity was detected with 72h (Figure 2).

The relevance of studying the interaction between temperature and leaf wetness is explained by Zadoks & Schein (23), who support the idea that different intensities of a determined disease can be obtained by using different temperatures and leaf wetness periods, and such interaction is important for the disease occurrence and intensity. The critical period is related to the hours during which the environmental conditions are favorable to infection, in order to allow germination, penetration and establishment of the parasite in the host. Each pathosystem has its own critical infection period (17).

In *in vitro* studies, the optimum temperature for *P. sojina* spore germination was 25ºC (4). The infection and the development of *in vivo* disease symptoms were favorable at temperatures between 25 and 30°C, and relative humidity higher than 90%, once under such conditions sporulation may occur within 48 hours after the first onset of symptoms. Similarly, according to this same author, conidia can germinate on the leaf surface one hour after inoculation, in the presence of water and at 25 and 30°C, developing lesions from 8 to 12 days after inoculation. In this study, both the temperature and the leaf wetness period affected frogeye leaf spot intensity at 27.3ºC. In studies with the same pathosystem, Phillips (15) verified that the optimal temperature for mycelial development of *P. sojina* was 25ºC, and the pathogen development was limited at > 32 ºC.

Considering leaf wetness period, Kudo (11), working with this same fungus, proved that the disease intensity was found with 72 h leaf wetness, but Veiga (22) reported that 48 h leaf wetness resulted in *P. sojina* infection in soybean. Scandiani et al. (18) found data similar to that of Kudo (11) for leaf wetness at 26 °C, aiming at the selection of soybean genotypes; under such conditions, they observed symptoms on the eighth day after inoculation.

These data can be useful in selection studies of soybean genotypes for genetic breeding programs.

There is an interaction among *Cercospora sojina* race, culture medium and photoperiod.

The highest sporulation of *P. sojina* is verified in flavored tomato juice agar and V8 juice agar. The best light regime for sporulation is 12-h photoperiod.

The optimum temperature for the occurrence of frogeye leaf spot is 27 ºC, at which the disease intensity increases linearly as the wetness leaf period increases, and the highest intensity of the disease is verified in the period of 72 hours.

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