

The Aggressiveness of *Diaporthe longicolla* and *Diaporthe pseudolongicolla* Isolates Tested by the Soybean Seed's Artificial Inoculation

Agresivnost izolata *Diaporthe longicolla* i *Diaporthe pseudolongicolla* testirana umjetnom infekcijom sjemena soje

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THE AGGRESSIVENESS OF *Diaporthe longicolla* AND *Diaporthe pseudolongicolla* ISOLATES TESTED BY THE SOYBEAN SEED'S ARTIFICIAL INOCULATION

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SUMMARY

This study's objective was to determine if there was a difference in aggressiveness between the *Diaporthe longicolla* and the *Diaporthe pseudolongicolla* isolates infecting the soybean seeds. An artificial inoculation was performed under laboratory conditions and in a greenhouse on a soybean cultivar (Tisa) created in the breeding program of the Agricultural Institute Osijek and represented in a large-scale production in the Republic of Croatia. An attempt was made to discover whether the isolates of the recently described *D. pseudolongicolla* were more aggressive to the soybean seeds than the *D. longicolla* isolates, which have manifested a high aggressiveness to the soybean seeds throughout a series of earlier experiments. The *D. pseudolongicolla* isolates used in the experiment were isolated from the infected seeds pertaining to a wide production in the vicinity of Osijek. The experiments have demonstrated that there were statistically significant differences ($P < 0.05$) in the aggressiveness between the isolates tested, with the *D. longicolla* isolate manifesting the highest aggressiveness in both trials.

Keywords: soybean, seed, *D. longicolla*, *D. pseudolongicolla*, artificial inoculation, pathogenicity

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is listed as the one of the most significant oil and protein crops in the world (FAO, 2016). It has been estimated that the wild soybean (*Glycine soja*) was domesticated as a cultivated soybean approximately 7,000–9,000 years ago in Asia (Lee et al., 2011). Soybean's significance principally originates from its seed quality (chemical composition), whose value and usage versatility are continually being confirmed in a scientific and technological research (Matoša Kočar et al., 2017). An important barrier to an increased soybean production and seed quality is the large number of biotic factors that affect the soybean production (Maldonado dos Santos et al., 2019). More than 200 phytopathogenic microorganisms have been described on the soybean (Hartman, 2015), while about 30 species can inflict a significant economic damage (Roy et al., 2000). The most harmful are the fungi, then

the bacteria, viruses, and phytoplasmas (Vidić et al., 2013). Most of them attack the soybean seed, decrease germination, and cause a poor seed quality. One of the major causes of a poor seed quality in most soybean-growing regions worldwide is the *Diaporthe* seed decay (Li, 2011; Vidić et al., 2013; Petrović et al., 2018). This disease is caused primarily by the fungus *Diaporthe longicolla*, along with *D. sojae*, *D. caulivora*, *D. aspalathi* and the *D. eres* species complex, *D. foeniculina*, and *D. radis* (Li, 2011; Petrović et al., 2015; Petrović et al., 2016; Hosseini et al., 2020). The members of the *Diaporthe* genus and its anamorph *Phomopsis* have long been recognized as the pathogens responsible for sever-

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al deleterious diseases of an enormous economic importance on a wide spectrum of host plants worldwide (Hosseini et al., 2020). The infected soybean seeds are usually small, shrunken, flattened and elongated, with a cracked seed coat and a chalky-white mycelium and may introduce the pathogen in the new areas (Gleason and Ferriss, 1985). The affected seeds do not germinate or germinate slowly. Seed inoculation can also result in a pre- or post-emergence damping-off, and under severe conditions the stands can be affected to the point of yield reduction (Li et al., 2015). According to Hepperly and Sinclair (1978) and Sinclair (1993), some of these pathogens are the most important organisms causing an off-color of the soybean seeds, which is one of the primary quality-rating factors that negatively influences a soybean market grade. Nevertheless, a latently infected seed can have a normal appearance, without disease symptoms, but the germination, vitality, and quality will be reduced as a result of a latent infection (Kmetz et al., 1978). The isolates of *D. longicolla* were equally present on the soybean seeds and stems, while the *D. pseudolongicolla* was earlier isolated only from seeds (Petrović et al., 2018). A difference between these two species was that the isolates of *D. pseudolongicolla* become rapidly sterile when grown in a culture and lost their ability to form the reproductive organs. In many *Diaporthe* species, both anamorph (asexual) and teleomorph (sexual) stages have been described, as well as their ability to form pycnidia and perithecia (Petrović et al., 2018). The *D. longicolla* has long been described as a fungus without a teleomorph stage, whose pycnidia has the very long necks and releases the alpha conidia only. In the past few years, the new studies have revealed some

unknown details on this fungus, such as the atypical symptoms on the soybean stems, the pycnidia with the beta conidia, and an ability to form a teleomorph (Santos et al., 2011; Vidić et al., 2013; Olson et al., 2015).

Due to a fact that a fungus from the *Diaporthe* species could significantly reduce a soybean grain yield and quality, this paper's objective was to evaluate the aggressiveness of the *D. pseudolongicolla* isolates recently extracted from a soybean seed in our region, in comparison with a *D. longicolla* isolate proved most aggressive in the previously conducted investigations.

MATERIAL AND METHODS

A number of isolates used in this investigation was small because only a small quantity of isolates in the *Diaporthe* species were identified as *D. pseudolongicolla* (Petrović et al., 2018). In this research, the isolates of *Diaporthe longicolla* (PI 027) and *Diaporthe pseudolongicolla* (CBS127269, CBS127270, and CBS127271 – Fig. 1) were used. In an earlier research (Duvnjak, 2004), the isolate PI 027 has manifested the highest aggressiveness and pathogenicity among the tested *D. longicolla* isolates. The isolates CBS127269, CBS127270, and CBS127271 were identified as the *D. novem* (Santos et al., 2011). All three isolates can be found in the CBS-KNAW Database (CBS-KNAW Westerdijk Fungal Biodiversity Institute). Petrović (2012) and Petrović et al. (2018) considered the *Diaporthe pseudolongicolla* to be an appropriate name for the unidentified group of *Phomopsis* sp. 9 due to a strong similarity in terms of a morphological characteristic and pathogenicity among the *D. longicolla* and *Phomopsis* sp. 9 (syn. *D. novem*) isolates.

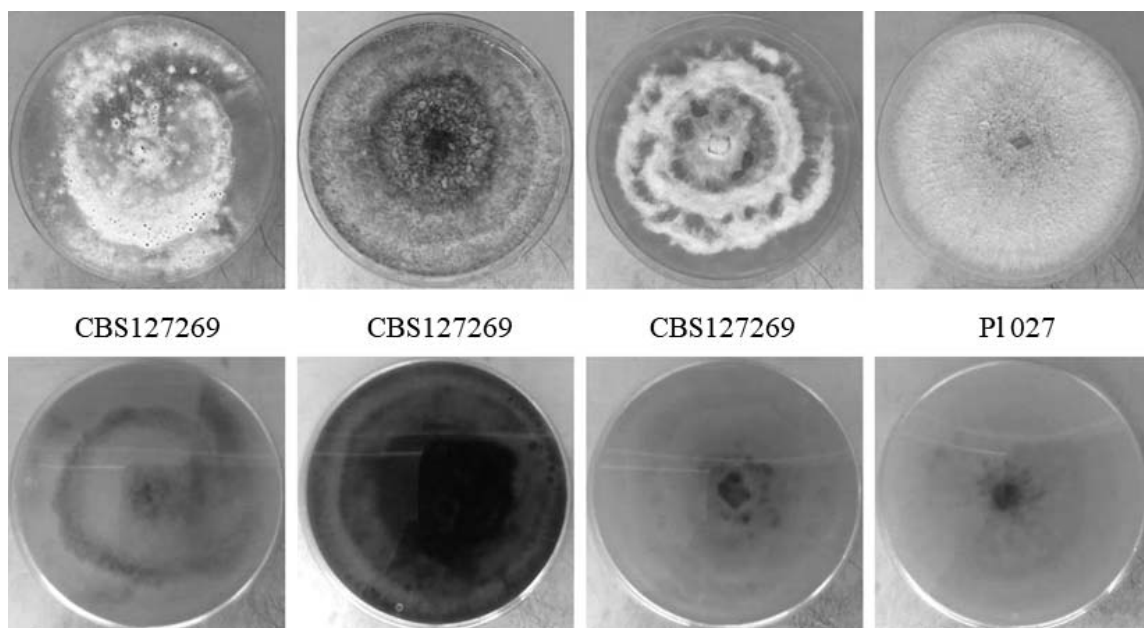


Figure 1. A colony of the *D. longicolla* (PI 027) and *D. pseudolongicolla* (CBS127269, CBS127270, and CBS127271) isolates

Slika 1. Kolonije izolata *D. longicolla* (PI 027) i *D. pseudolongicolla* (CBS127269, CBS127270 i CBS127271)

A taxonomy of the *Diaporthe longicolla* (Hobbs), J. M. Santos, K. Vrandečić & A. J. L. Phillips, *Persoonia* 27:13.2011.

Basionym. *Phomopsis longicolla* Hobbs, *Mycologia* 77:542.1985. *Cultural characteristic:* On the potato dextrose agar (PDA), it had a white, compact aerial mycelium with a typical yellowish-green ring around a colony center. Subsequent to five to seven days, the colony formed the massive, black stromatic structures, irregular in shape, which completely covered the bottom of the Petri dishes. The *Pycnidial conidiomata* on an autoclaved soybean stem with the PDA in the culture were globose, black, aggregated and rarely solitary. They had the long necks, ranging 250-700 μm in length. The *Pycnidia* formed within black stroma had white to yellowish mucous droplets observed on ostiole. The *Alpha conidia* were ellipsoidal, 2-guttulate, with the dimensions of 4.9-7.5 \times 2.2-3.0 μm . The *Beta conidia* were detected in the two-month-old cultures. They were unicellular, comma-shaped, with a size of 22.3-29.2 \times 1.0-1.3 μm . The *Perithecia* were not formed.

A taxonomy of the *Diaporthe pseudolongicolla*, K. Petrović, L. Riccioni & M. Vidić, *nom. nov.* – MycoBank MB564245.

Etymology: The prefix *pseudo-* is used to mark a fungus very similar to the *D. longicolla* in terms of the morphological and pathogenic features. = *D. novem*, J. M. Santos, K. Vrandečić & A. J. L. Phillips, *Persoonia* 27:14.2011. *Basionym.* *Phomopsis* sp. 9 (van Rensburg et al., 2006). *Cultural characteristic:* The fresh strains were identical with *D. longicolla* in terms of white, compact aerial mycelia on the PDA. A main difference between these two species was that the isolates of *D. pseudolongicolla* quickly become sterile and lost an ability to generate the reproductive organs. The *Pycnidial conidiomata* on the autoclaved soybean stems with the PDA in the culture were spherical, up to 550 μm in diameter. They were black, aggregated with a stroma, without necks or with the long necks, up to 450 μm long. A yellowish drop with the alpha and beta conidia exuded from the ostiole. The *Alpha conidia* were unicellular, bi- to multiguttulate, ellipsoidal, with the dimensions of 6.2-9.7 \times 2.3-3.1 μm . The *Beta conidia* were unicellular, filiform, curved at one end, sized 19.5-29.1 \times 0.9-1.2 μm and rarely formed. The *Perithecia* were not formed.

The differences in aggressiveness among these isolates were tested in the greenhouse and laboratory trials by artificial inoculation.

Laboratory-Based Artificial Inoculation

The experiment was set up in the Agricultural Institute Osijek. In a laboratory, a soybean seed was artificially infected with a conidia and mycelium suspension of the tested isolates. A suspension concentration amounted to 4.5 $\times 10^6$ spores ml^{-1} (Duvnjak et al., 2007). Subsequent to two weeks on the PDA, previously acidified

to a pH of 4.5, a mycelium with the pycnidia was mixed with 100 ml of sterile water. The soybean seeds of the *Tisa* cultivar (cv.) were sterilized in a 1-percent sodium hypochlorite (NaOCl) for one minute and submerged in the suspension, in which they were kept for 24 hours. The trial was set in four repetitions (four Petri dishes with 25 seeds each, all together 100 seeds were inoculated with each isolate). In the control, the seeds were kept in sterile water for 24 hours and were subsequently transferred to the sterile Petri dishes. The Petri dishes with the inoculated seeds were put into a climatic chamber (*Binder KWBF 240*) at a temperature of 25°C and a 12/12-hour light régime, as well as the control. The Petri dishes were occasionally moisturized by sterile water to improve the inoculation development. Subsequent to seven days, a number of rotten and healthy seeds, a total number of nongerminated seeds (i.e., the rotten + healthy but nongerminated seeds) were counted, and a germ length, as well as the length of germ necrosis, were measured by a ruler.

Greenhouse-Based Artificial Inoculation

In the procedure described earlier, the conidia and mycelium suspension of the each tested isolate was prepared. The soybean seed (cv. *Tisa*), previously sterilized in a 1-percent sodium hypochlorite (NaOCl) for one minute, was soaked in each of these suspensions for five minutes. Subsequently, the inoculated seeds were planted in the sterile soil pots having an 8-centimeter diameter. For each of the tested isolates, 40 inoculated seeds per replication were planted in the pots, in three replications (120 seeds in total per a tested isolate). In the control, the seeds were previously soaked for five minutes in sterile water. During the emergence, the soil temperature in the pots was amounted to 20-24°C. The number of emerged plants was counted 10 days subsequent to the planting. In the next 10 days, a plant decay dynamic was monitored every two days and the symptoms were recorded. At the end (30 days subsequent to the planting, third examination), a number of the emerged and a number of the decayed plants were counted. Thereafter, an isolation was obtained on the PDA to confirm a causal agent of the decayed plants.

A significance of differences between the data obtained by the laboratory- and greenhouse-based artificial inoculations was tested by an Analysis of Variance (ANOVA) applying the LSD test. The ANOVA was conducted using a GLM procedure in the *SAS Software 9.3* (2011).

RESULTS AND DISCUSSION

Laboratory-Based Artificial Inoculation

The results of pathogenicity among the laboratory-tested isolates (on a filter paper) are presented in Table 1.

Table 1. Aggressiveness test of the *D. pseudolongicolla* and *D. longicolla* isolates on the artificially inoculated soybean seeds on a filter paperTablica 1. Test agresivnosti izolata *D. pseudolongicolla* i *D. longicolla* na umjetno zaraženome sjemenu soje na filter papiru

| Isolate Izolot | Species Vrsta | Number of germinated seeds Broj prokljalih sjemenaka (%) | Number of rotten seeds Broj trulih sjemenaka (%) | Germ length Dužina klice (cm) | Germ necrosis Nekroza klice (cm) | Number of nongerminated seeds Broj nekljalih sjemenaka (%) |
|---------------------|------------------|--|--|-------------------------------------|--|--|
| Control/Kontrola | | 98 ^a | 0 ^b | 10.05 ^a | 0.00 ^b | 2 ^c |
| CBS127269 | DP | 83 ^{bc} | 11 ^a | 5.26 ^b | 0.95 ^{ab} | 17 ^{ab} |
| CBS127270 | DP | 84 ^{bc} | 15 ^a | 6.73 ^b | 1.35 ^a | 16 ^{ab} |
| CBS127271 | DP | 89 ^{ab} | 10 ^{ab} | 5.41 ^b | 0.76 ^{ab} | 11 ^{bc} |
| PI 027 | DL | 75 ^c | 20 ^a | 6.95 ^b | 0.88 ^{ab} | 25 ^a |
| Average/Prosjeak | | 86 | 11 | 6.88 | 0.79 | 14 |
| LSD _{0.05} | | 12 | 10 | 2.13 | 1.04 | 13 |

Note: Species – DP (*D. pseudolongicolla*), DL (*D. longicolla*); the values with the same superscript letter are not significantly different

An effect of control and different isolates on a germ length, necrosis, germination, seedlings rotting, and nongerminating seeds was analyzed in an experiment conducted under laboratory conditions. All tested isolates decrease a seed germination and germ length while increasing the number of rotten seeds and number of nongerminated seeds. These results correspond to the earlier obtained ones (Vidić et al., 1995). Both species' isolates have significantly reduced a germination rate. The same result was obtained by Petrović et al. (2018). An Analysis of Variance revealed statistically significant differences ($P < 0.05$) between the controls and isolates for all analyzed parameters. The germ length was highest in the control (10.05), which was significantly longer than all other isolates, but no significant difference in a germ length was detected for the isolates. As expected, the control had the highest germination (98), whereas a germination of the CBS127271 isolate was only slightly lower and in the same rank (89). A germination of the PI 027 isolate was the lowest (75). According to the number

of rotting seedlings, no statistically significant differences were detected between the isolates. The highest value was detected in the isolate PI 027 (20), followed by the CBS127270, CBS127269, and CBS127271, respectively. There were no rotten seedlings in control. Identical to the previous parameter, the isolate PI 027 had the highest value (25) of nongerminating seeds, while the control had the lowest one (2), followed by the isolate CBS127271 (11). In this investigation, the *D. pseudolongicolla* isolates manifested lower aggressiveness in comparison with the PI 027 (*D. longicolla*) isolate. A former investigation has proven that the *D. longicolla* is more aggressive than the *P. sojae* isolates (Vidić et al., 1995; Vidić et al., 1999). Furthermore, the *D. pseudolongicolla* isolates were highly aggressive in the previous investigations (Petrović et al., 2018).

Greenhouse-Based Artificial Inoculation

The results of aggressiveness among the tested isolates in a greenhouse experiment (in pots) are presented in Table 2.

Table 2. The influence of the *D. pseudolongicolla* and *D. longicolla* isolates on the emergence and damping-off of soybean seedlingsTablica 2. Utjecaj izolata *D. pseudolongicolla* i *D. longicolla* na nicanje i propadanje klijanaca soje

| Isolate Izolot | Species Vrsta | Number of healthy plants / Broj zdravih biljaka | | | Average Prosjeak |
|--------------------------------------|------------------|---|------|------|---------------------|
| | | Examination / Pregled | | | |
| | | I | II | III | |
| CBS127269 | DP | 7.75 | 7.50 | 7.25 | 7.50 ^b |
| CBS127270 | DP | 8.00 | 8.00 | 8.00 | 8.00 ^b |
| CBS127271 | DP | 9.25 | 9.00 | 9.00 | 9.08 ^a |
| PI 027 | DL | 7.00 | 6.25 | 5.75 | 6.33 ^c |
| Control / Kontrola | | 9.75 | 9.75 | 9.75 | 9.75 ^a |
| Average / Prosjeak | | 8.35 | 8.10 | 7.95 | 8.13 |
| LSD _{isolate/izolat (0.05)} | | | | | 1.01 |

Note: Species – DP (*D. pseudolongicolla*), DL (*D. longicolla*); the values with the same superscript letter are not significantly different

Statistically significant ($P < 0.05$) differences were detected between the isolates in the number of healthy plants. No statistically significant differences in the number of healthy plants were detected between the first, second, and the third examinations and the isolate

x examination interaction. As expected, the highest number of healthy plants was recorded in the control (9.75). The somewhat lower numbers of healthy plants, but in the same rank as the control, were detected for the isolate CBS127271 (9.08). The isolates CBS127270

and CBS127269 had 8.00 and 7.50 healthy plants, respectively. The isolate PI 027 had a significantly lower number of healthy plants (6.33) when compared to all other tested isolates.

CONCLUSION

Statistically significant differences in aggressiveness were detected between the tested isolates. In a laboratory experiment, statistically significant differences were detected between a control and all the tested isolates. The isolate *D. longicolla* (PI 027) manifested the highest aggressiveness according to the number of nongerminated seeds in comparison to all the tested *D. pseudolongicolla* isolates. The same results were obtained in a greenhouse experiment. Regarding the differences in aggressiveness among the tested *D. pseudolongicolla* isolates, statistically significant differences were detected only in the greenhouse experiment for the isolate CBS127271, which manifested a significantly lower aggressiveness when compared to the remaining two isolates (CBS127269 and CBS127270, respectively). The *D. pseudolongicolla* isolates manifested less aggression on soybean seeds when compared to the *D. longicolla* isolate used in the experiment. Although the PI 027 isolate manifested the highest aggressiveness of the tested *D. longicolla* isolates in the previous experiments, it should not be ruled out that other *D. pseudolongicolla* isolates could significantly impact a soybean production under favorable conditions for a pathogen development.

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AGRESIVNOST IZOLATA *Diaporthe longicolla* I *Diaporthe pseudolongicolla* TESTIRANA UMJETNOM INFEKCIJOM SJEMENA SOJE

SAŽETAK

Cilj ovoga rada bio je utvrditi postoje li razlike u agresivnosti između izolata fitopatogenih gljiva *Diaporthe longicolla* i *Diaporthe pseudolongicolla* koje zaražavaju sjeme soje. Obavljena je umjetna infekcija u laboratorijskim uvjetima i u plasteniku na kultivaru soje (Tisa) kreiranome u oplemenjivačkom programu Poljoprivrednoga instituta Osijek i zastupljenom u širokoj proizvodnji u Republici Hrvatskoj. Pokušalo se doći do odgovora jesu li izolati nedavno opisane *D. pseudolongicolla*, agresivniji za sjeme soje od izolata *D. longicolla* za koji se kroz seriju ranijih pokusa utvrdila visoka agresivnost na sjemenu soje. Pokus je postavljen na Poljoprivrednome institutu Osijek, a izolati *D. pseudolongicolla* korišteni u pokusu izolirani su sa zaraženoga sjemena iz široke proizvodnje u okolici Osijeka. Pokusi su pokazali kako postoje statistički opravdane razlike ($P < 0,05$) u agresivnosti između izolata u pokusu, pri čemu je izolat *D. longicolla* pokazao najveću agresivnost u oba postavljena pokusa.

Ključne riječi: soja, sjeme, *D. longicolla*, *D. pseudolongicolla*, umjetna infekcija, patogenost

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