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Do Morphological Variations in *Sclerotinia sclerotiorum* Strains Indicate Differences in Aggressiveness?

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Abstract: White mold (*Sclerotinia sclerotiorum* de Bary) is one of the most important fungal diseases of winter oilseed rape (OSR). Since the pathogen can persist in the soil for a long time with its sclerotia, prevention and non-chemical methods (specifically biological agents) are important pillars in the integrated plant protection strategy against this pathogen. Mapping the intraspecific variability of the pathogen is an important step in the development of resistance to *S. sclerotiorum*. *S. sclerotiorum* isolates were collected from different OSR growing locations in Hungary during the 2020/21 and 2021/22 growing seasons. The morphological characteristics of sclerotia obtained from infected OSR stems were studied in the laboratory, and seedlings of four OSR hybrids were infected in vitro with isolates. The strains from four locations have different morphological characteristics. Significant differences in the level of aggressivity were also observed between strains; a correlation was also found between mycelial growth after 24 h, weight of sclerotia, and aggressivity. Among the four tested hybrids, OSR PT271 proved to be the most susceptible to most *S. sclerotinia* strains.

Keywords: winter oilseed rape; stem rot; *Sclerotinia sclerotiorum*; morphological characteristics; level of aggressivity

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1. Introduction

Winter oilseed rape (*Brassica napus* L.) (hereinafter referred to as OSR) is an important oilseed crop around the world, including in Hungary. Globally, OSR is cultivated on nearly 34 million hectares of land, of which 200 thousand hectares of sowing areas are present in Hungary. The average harvested yield of oilseed rape has not shown an increasing trend over the last 10 years, with the lowest average yield of 15.38 million tons observed in 2019, while the highest yield of 21.82 million tons was observed in 2014 for all EU Member States. This may be due to several abiotic and biotic factors that, depending on weather conditions, can cause significant yield and quality losses [1,2].

A major limiting factor for OSR production is the fungal pathogen *Sclerotinia sclerotiorum*, which is the causal agent of white mold disease [3–5]. According to some studies, yield losses for OSR due to *S. sclerotiorum* can be as high as 50%; however, yield losses of up to 80% have also been reported due to severe infection [5]. The extent of yield loss is determined by the severity of the disease and the phenological stage at which the plant is infected. In addition to yield loss, the pathogen also causes quality damage, which is associated with a reduction in oil content and deterioration in oil quality. In varieties without erucic acid, the disease causes qualitative and quantitative changes in the fatty acid composition of the crop. In addition, the oil content of highly infected plants may also increase the content of erucic acid and glucosinolate [6]. Previous studies have shown that the yield of siliquae per plant is reduced to almost half compared to healthy plants, and that the length of the siliqua is also negatively affected by the onset of the disease. Infected seeds had a thousand-kernel weight ranging from 0.47 to 0.81 g and were also characterized by lower oil content (on average, 9.1% lower oil content was observed in infected seeds) [3].

S. sclerotiorum has a wide host range in arable cropping systems (e.g., sunflower, OSR, and soybean), with more than 400 host plants in the aforementioned system (including important weeds and wild plants) [7,8]. Sclerotia of variable sizes survive in the soil, which makes them persistent and aids the spread of the pathogen. Sclerotia are found in the siliquae that can contaminate the harvester and in the stem during the stubble–hilling operation, and in some cases, they are carried to the soil surface or deeper into the soil by the seed [9,10]. Their persistent form may survive in the soil for several years, but its viability is significantly affected by the depth, the soil temperature, the soil organic matter content, and the biological activity of the soil [11]. The production of sclerotia also depends on the number of infected plants and the type of host plant; however, little information is available on the latter. In their study, Taylor et al. [12] inoculated different *S. sclerotiorum* host plants (bean, carrot, lettuce, and OSR) with three *S. sclerotiorum* isolates, and the number and weight of sclerotia produced on them were determined. Their results concluded that the number of sclerotia depended on both the type of crop and the isolate, but most sclerotia were produced on OSR crops and lettuce. The sclerotia serve as an inoculum for subsequent infections and thus are able to persist in the soil for a longer period [11]. Depending on environmental factors and the sclerotia, they germinate in two different ways, i.e., carpogenic and mycelial [8,13,14]. The size of the sclerotia affects their germination, with larger sclerotia producing more apothecia, and a higher germination rate for these sclerotia persists for longer time periods in the soil [13,14].

The main mode of infection of the pathogen in OSR is by carpogenic germination, during which apothecia develop, followed by wind-borne ascospore dispersion and infection of above-ground parts of OSR plants [8,15,16]. The optimal conditions for carpogenic germination are 15 °C and −0.03 or −0.007 MPa [17]. Fruiting bodies (apothecia) develop close to the soil surface, on which ascites are formed, and they produce 8-8 ascospores [15]. Heran et al. [18] found that the efficiency of infection was highly dependent on the age of the petals, as lesions on the so-called "old" petals were less variable and larger than those on "young" petals [16].

In mycelial germination, the pathogen directly infects plants by developing a mycelium and causing a "base infection" on the plants [5,19]. In OSR, the mycelial infection mode is less common and can be influenced by several factors (e.g., sclerotial maturity, bark melanization, temperature, and moisture) [16]. Immature sclerotia primarily infect plants with direct mycelium [20], whereas in mature sclerotia, the outer dark crust inhibits mycelial development [16,21]. The mycelial germination mode is promoted by low temperatures, and Huang [22] has shown that the mycelial growth of sclerotia stored at −20 °C is more vigorous than that of sclerotia stored at −10 °C. Matheron and Porchas [23] found that sclerotia in irrigated soil at 5 cm depth had a lower germination rate than in dry soils, and that irrigated soil at 32 °C had significantly reduced germination rates compared to sclerotia in irrigated soil at 26 °C.

S. sclerotiorum in OSR may already cause seedling blight or complete death during the germination and emergence growth stages [24,25]. The most typical symptoms of OSR are often found on its stem and branches and infrequently on its leaves and siliquae [8,9]. Symptoms on the stem appear as bleached light-brown fast-spreading lesions, which are just above the soil level but at any height of OSR. Symptoms on the stems are caused by the mycelial germination of sclerotia, whereas the infection of the mid-stem and siliqua is the result of the arm germination mode [16]. Following the lesions on the stem (irrespective of their location), infected plants turn yellow and finally die, as the concerted action of several extracellular lytic enzymes degrade the plant tissues [26–28]. In an advanced infection, sclerotia develop inside and on the surface of the bleached parts of the OSR stem. In rare cases, siliquae also may become infected, becoming creamy white and usually containing white moldy seeds and sclerotia [9,29].

Prevention and containment are important pillars in integrated plant protection against the white mold pathogen with several practices present to reduce the risk of infection [30]. Prevention includes avoiding low-lying, waterlogged areas, following a minimum of 4 years of replanting [11], avoiding late sowing [31], an appropriate seeding rate of high phytosanitary level and optimum nutrient supply [32], and by preferring tolerant hybrids [33]. The excessive use of nitrogenous fertilizers increases the susceptibility of the plants [34], while boron is associated with a suppressive effect on diseases caused by this pathogen [35]. Among biological control agents, the *Paraphaeosphaeria minitans* (W.A. Campb.) (syn. *Coniothyrium minitans* Campbell) [36] antagonist can be successfully used against *S. sclerotiorum*, thereby reducing the number of sclerotia in the soil [37–39]. In addition to the above control methods, fungicides are usually required for this pathogen in all OSR areas, but these fungicides must be applied before the plant becomes infected [30,38].

Knowledge of the intraspecific variability of *S. sclerotiorum* is necessary for the success of breeding programs for resistant hybrids because pathogens with a wide host range, e.g., *S. sclerotiorum*, are characterized by many different virulence genes that are less host-specific [40]. The pathogen's interspecific variation is demonstrated by the rate of mycelial growth of isolates, sclerotial production, oxalic acid production, and levels of aggressiveness [41]. The geographical regions play an important role as the environment of these isolates because certain genotypes may have an advantage under different environmental conditions [42,43]. This study aimed to compare the mycelial growth, sclerotial production, and level of aggressiveness of *S. sclerotiorum* isolates collected from different locations in Hungary over two growing seasons.

2. Results

2.1. Mycelial Growth

After 24 h of simultaneous testing under controlled conditions, a noticeable variation in mycelial growth among eight strains of *S. sclerotiorum* (Figure 1) was observed. The V20 strain exhibited the most rapid mycelial growth, displaying a significantly larger colony diameter compared to that of strains from the 2021/22 growing season (V21 $p = 0.00$, O21 $p = 0.02$, RSZ21 $p = 0.00$, and JA21 $p = 0.02$). Conversely, the RSZ21 strain exhibited the slowest mycelial growth, significantly slower than strains from the 2020/21 growing season (V20 $p = 0.00$, O20 $p = 0.01$, RSZ20 $p = 0.01$, and JA20 $p = 0.00$). A significant difference in mycelial growth was observed between the V21 strain and the O20 and JA20 strains, with the V21 strain displaying significantly slower growth ($p < 0.04$). Furthermore, disparities in mycelial growth were noted between strains collected in the 2020/21 and 2021/22 growing seasons, with isolates from the former displaying higher growth rates after 24 h. Specifically, in the 2020/21 growing season, the V20 strain showed the highest mycelial growth, whereas in the 2021/22 growing season, the RSZ21 and V20 strains showed an average mycelial growth of 4.16 cm or lower. After 48 h, the O20, RSZ20, V21, and RSZ21 strains had smaller colony diameters, although no significant differences were observed in the other strains. After 72 h, none of the tested strains exhibited significant differences in mycelial growth, as most strains had achieved full mycelial growth on the 9 cm diameter PDA plates (except for the RSZ21 strain) (Figure 2).

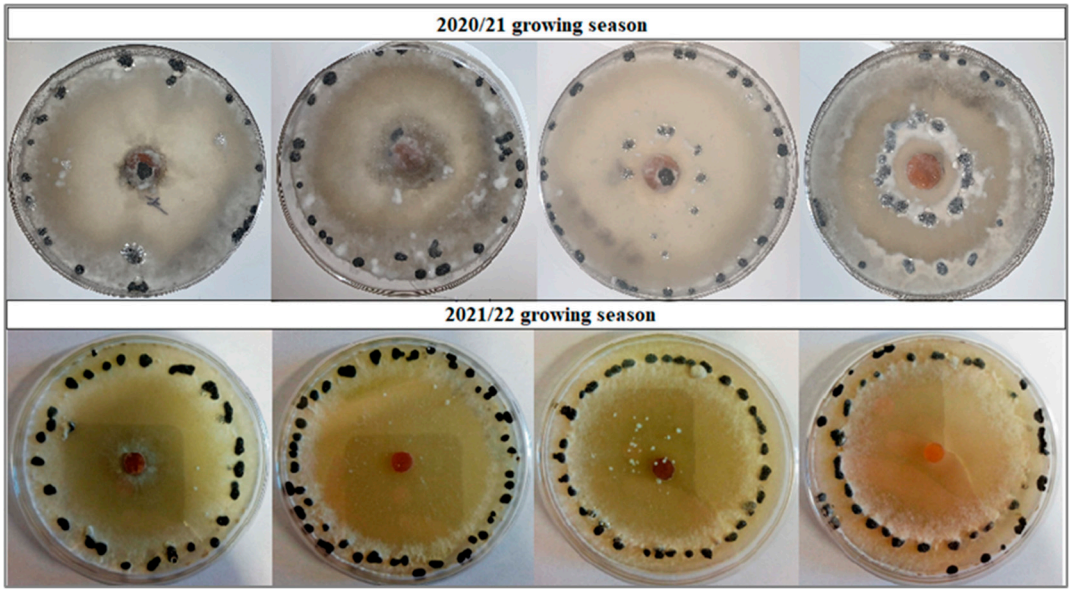

Figure 1. Isolates from the two growing seasons (2020/21 and 2021/22).

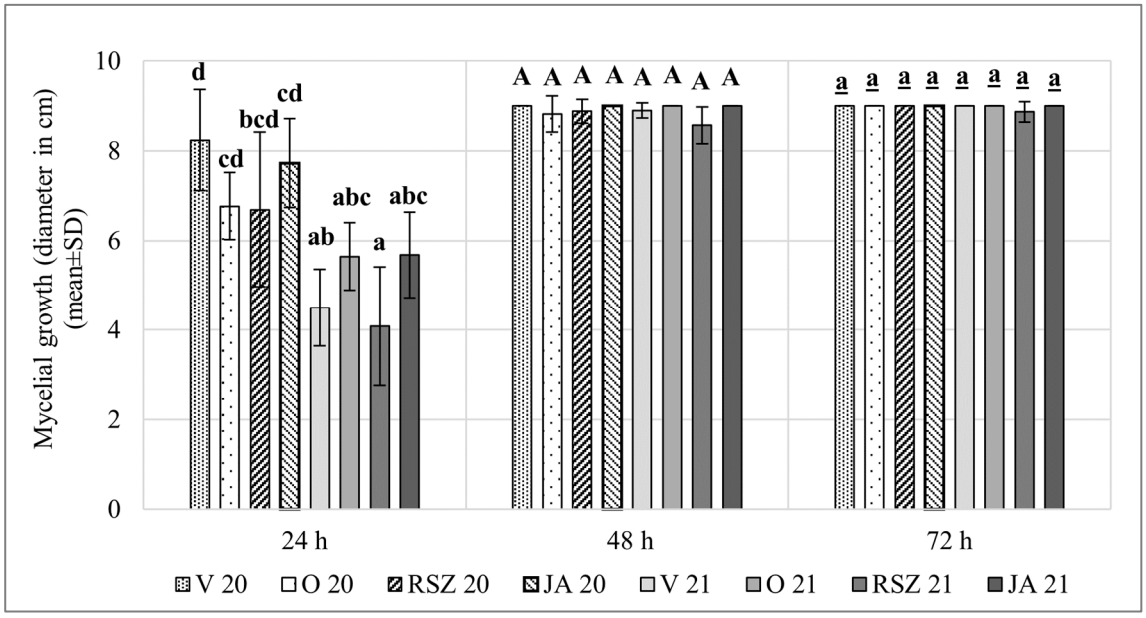

Figure 2. Mycelial growth of the *Sclerotinia sclerotiorum* strains after 24, 48, and 72 h. Bars with different letters are significantly different.

2.2. Sclerotial Production

IThe RSZ21 strain exhibited the highest sclerotial production, averaging 45.33 sclerotia per PDA plate, with a significant difference compared to that of the V20 and O21 strains ($p < 0.04$). Conversely, the V20 strain displayed the lowest average number of sclerotia per PDA plate (averaging 24.2 sclerotia per PDA plate) followed by the O21 strain (averaging 18.33 sclerotia per PDA plate). The other strains studied produced between 24.6 and 31.67 sclerotia per PDA plate (Figure 3).

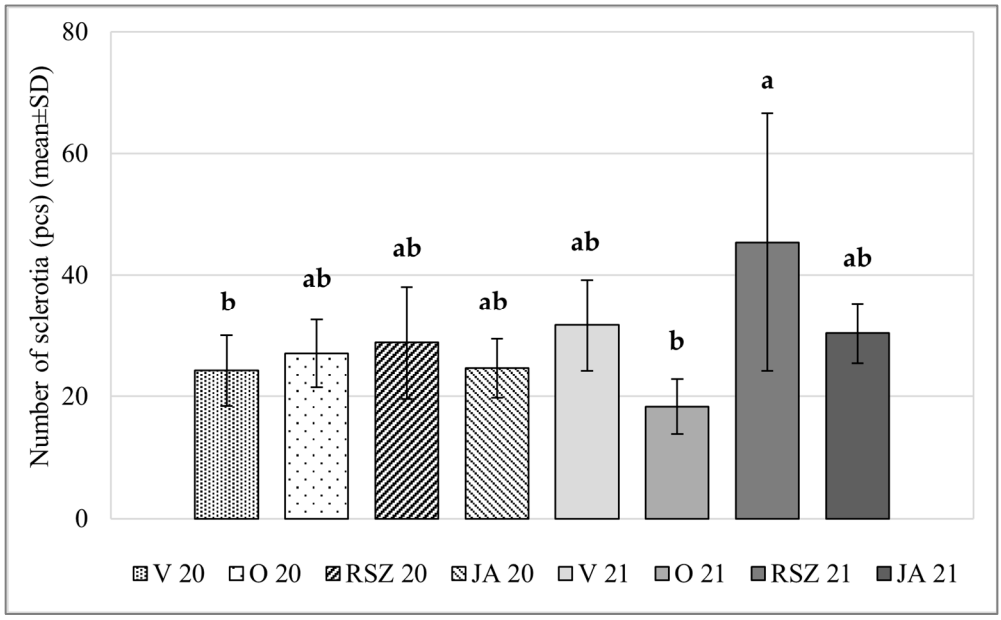

Figure 3. Sclerotial production per PDA plate of *Sclerotinia sclerotiorum* strains at 240 h after inoculation. Bars with different letters are significantly different.

Significant differences were observed in the total sclerotial weight, with strains from the 2021/22 growing season exhibiting a higher sclerotial mass per PDA plate compared to that of strains from the 2020/21 growing season. The JA21 strain showed the highest sclerotial weight, averaging 1.04 g per PDA plate, which was significantly higher than that of strains from the 2020/21 growing season (V20 $p = 0.04$, O20 $p = 0.00$, RSZ20 $p = 0.00$, and JA20 $p = 0.00$). Conversely, the RSZ20 strain exhibited the lowest sclerotial weight, averaging 0.46 g per PDA plate, which was significantly lower than that of strains from the 2021/22 growing season (V21 $p = 0.00$, O21 $p = 0.00$, RSZ21 $p = 0.01$, and JA21 $p = 0.00$). Furthermore, significant differences were noted between the JA20 and V21 strains ($p = 0.01$), as well as between the V20 and RSZ20 strains ($p = 0.01$) (Figure 4).

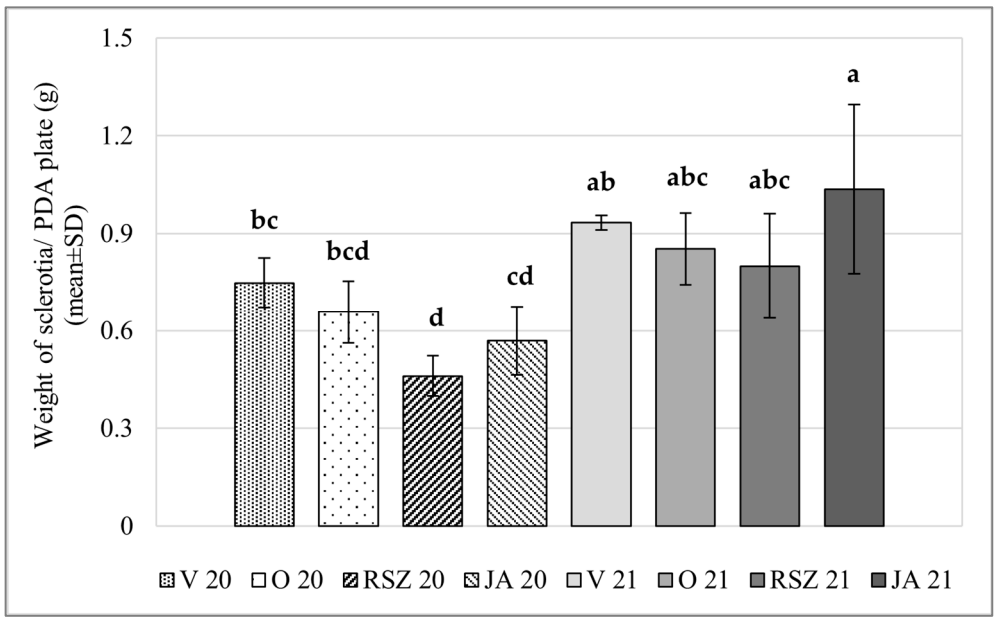

Figure 4. Total sclerotial weight per PDA plate of *Sclerotinia sclerotiorum* strains at 240 h after inoculation. Bars with different letters are significantly different.

2.3. Aggressivity on OSR

Variations in aggressivity levels were observed among the eight strains. In the case of the Architect and Umberto hybrids, no significant differences in aggressiveness were observed among strains from the 2020/21 growing season; however, they exhibited significantly higher aggressiveness compared to that of strains from the 2021/22 growing season ($p < 0.02$). Notably, significant differences in aggressiveness were noted within both the Architect and Umberto hybrids between the O21 and RSZ21 strains ($p < 0.02$). For the Architect hybrid, the highest level of aggressivity was shown by the O20 strain (average: 2.94), whereas the RSZ21 strain exhibited the lowest aggressivity (average: 1.42). Conversely, within the Umberto hybrid, the RSZ20 strain displayed the highest aggressiveness level (average: 2.89), while the RSZ21 strain (similar to the Architect hybrid) showed the lowest aggressiveness (average: 1.25).

For the Bluestar hybrid, significantly higher aggressivity was observed in the V20, O20, RSZ20, JA20, and O21 strains compared to the other strains ($p < 0.02$). A significant difference was observed between the RSZ21 and JA21 strains ($p = 0.02$). Within this hybrid, the highest aggressivity was displayed by the V20 strain (average: 2.92), while the lowest aggressivity was exhibited by the RSZ21 strain (average: 1.33).

In the case of the PT271 hybrid, where all strains exhibited high aggressivity compared to the other tested hybrids, the O20 strain showed the highest aggressivity (average: 2.92), while the RSZ21 strain showed the lowest aggressivity (average: 2.33). A significant difference was noted between the O20 and RSZ21 strains, with the O20 strain displaying significantly higher aggressivity ($p = 0.03$) (Figure 5).

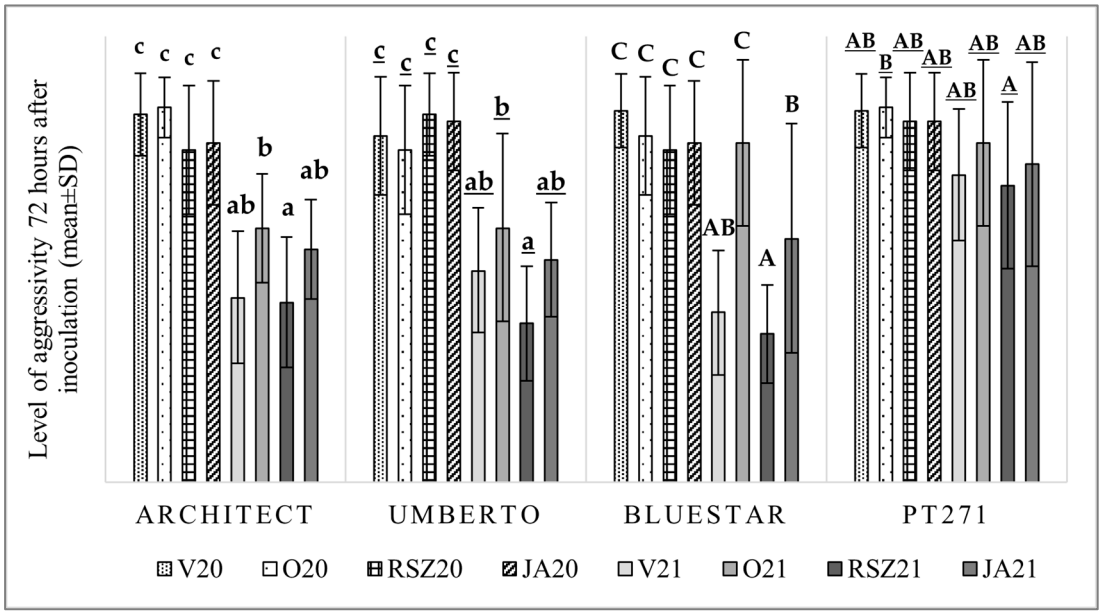

Figure 5. Level of aggressivity of *Sclerotinia sclerotiorum* strains on the four hybrids. Bars with different letters are significantly different.

2.4. Regression Analysis

There was a strong correlation between the level of aggressivity and mycelial growth after 24 h as well as between the level of aggressivity and sclerotial weight in the case of all four tested hybrids. As the level of aggressivity increased, larger colony sizes were observed, while the weight of sclerotia per PDA plate decreased with higher aggressivity levels. However, no correlation was found between the level of aggressivity and mycelial growth after 48 h or between the level of aggressivity and the number of sclerotia in any of the hybrids (Figure 6).

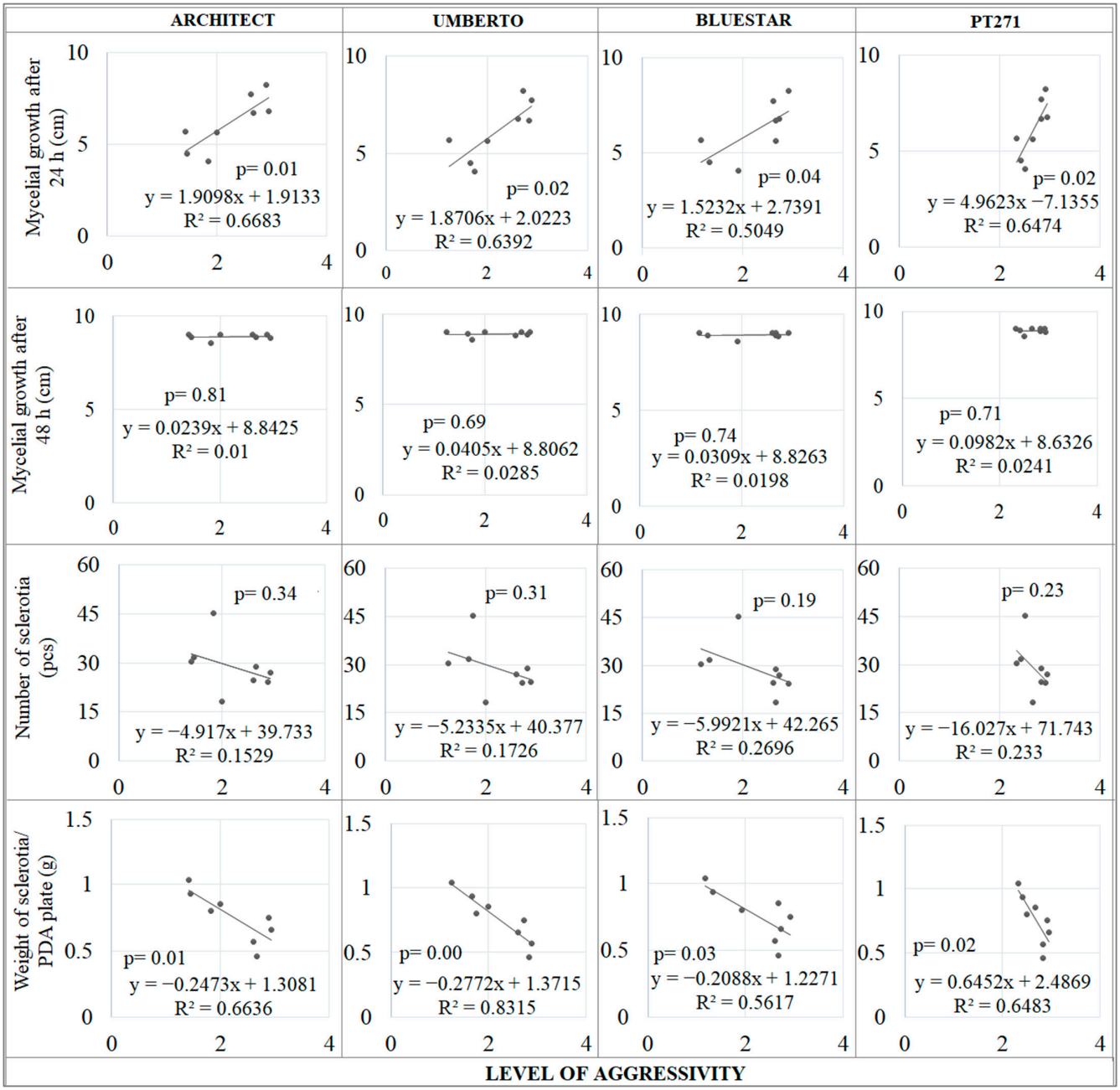

Figure 6. The correlations between the mycelial growth after 24 h and 48 h, number of sclerotia, weight of sclerotia, and aggressivity of the eight strains on the four hybrids.

A strong correlation was observed for the aggressivity levels of all four hybrids between the 2020/21 and 2021/22 growing seasons ($p < 0.03$). For all four examined hybrids, the aggressiveness levels observed during the 2020/21 growing season were higher (Figure 7).

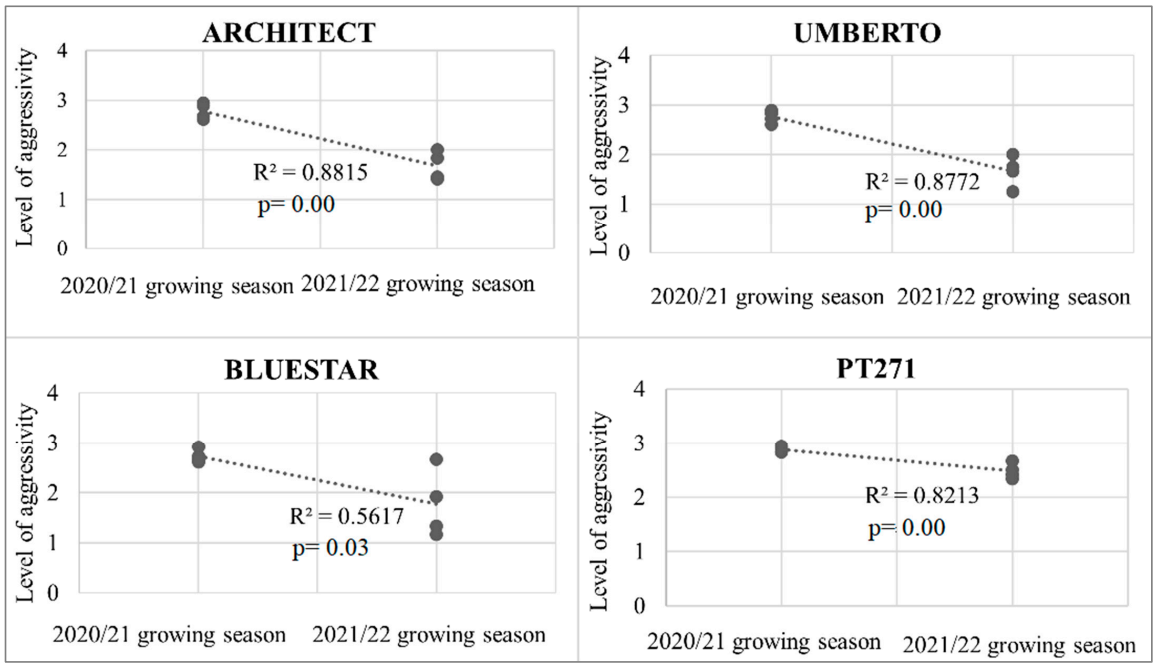

Figure 7. The level of aggressivity is correlated between the two growing seasons for the four examined hybrids.

3. Materials and Methods

3.1. Sample Collection

S. sclerotiorum isolates were collected from OSR fields in four different locations in Hungary in the 2020/21 and 2021/22 growing seasons. The sampling locations were as follows: Répceszentgyörgy (47.3512406°; 16.846689°), Olaszfalu (47.2416704°; 17.9115042°), Jászapáti (47.512244°; 20.142285°), and Vaskút (46.1080968°; 18.9861524°) (Figure 8). In each location, based on visual symptoms, 10 to 30 white mold-infected OSR stems were randomly collected across the OSR fields. The sclerotia were air-dried, placed in paper bags, provided with a unique identifier, and stored at −4 °C until further processing.

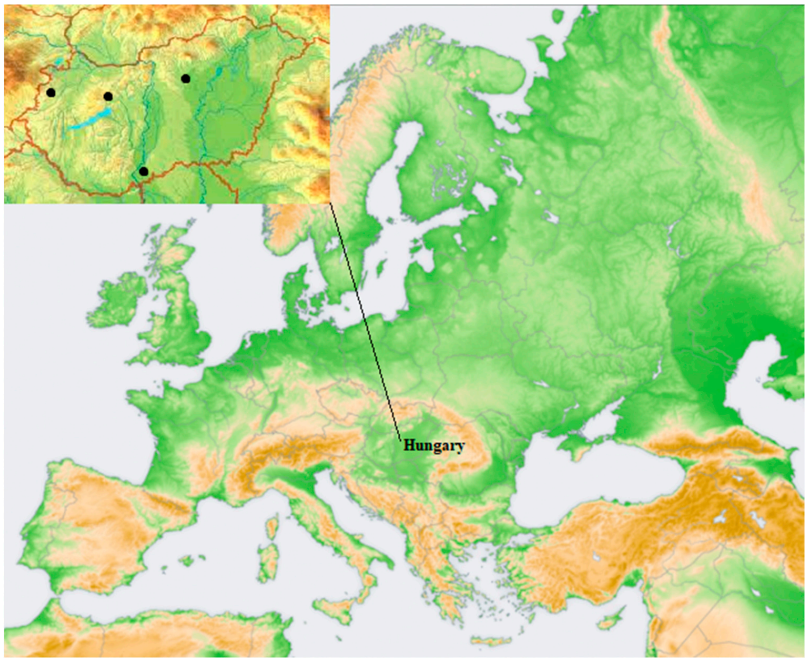

Figure 8. The four different sampling locations for *Sclerotinia sclerotiorum* isolates in Hungary [44].

3.2. Subcultures of Isolate

This research was conducted according to the methods of Dhingra and Sinclair [45], and the *S. sclerotiorum* isolates were purified using the hyphal tip technique. The cultures of each tested isolate were produced from sclerotia that were surface-sterilized in bleach (2% Sodium Hypochlorite (NaOCl)) for 3 min and then washed in sterile water for 4 min. Sclerotia were placed on 9 cm Petri dishes containing potato dextrose agar (PDA) supplemented with 50 µg/mL chloramphenicol. Petri dishes were incubated at room temperature (~20–25 °C) until actively growing mycelium was observed (3–5 days). These tested isolates were subcultured twice onto PDA plates by extracting an agar plug from the actively growing margins of mycelial growth. Based on mycelial growth characteristics, one isolate from each location and growing season was selected for the further experiment: RSZ20, RSZ21, O20, O21, JSZ20, JSZ21, V20, and V21 strains from the locations of Répceszentgyörgy, Olaszfalu, Jászapáti, and Vaskút, respectively. From 5-day-old cultures of the isolates, mycelium plugs with 0.5 cm diameters were cut and placed on 9 cm potato dextrose agar (PDA, Difco Laboratories, Detroit) plates for all tested isolates, and three replicates were performed. Then, these plates were incubated in the dark at 25 °C. The mycelial growth (in cm) was measured after 24, 48, and 72 h of inoculation [46,47]. The number of sclerotia produced from each inoculum per PDA plate (pcs) and the weight of individual sclerotium (g) were measured 240 h after inoculation.

3.3. In Vitro Experiment

The aggressivity of these strains was tested in vitro on four OSR hybrids (Architect, Umberto, Bluestar, and PT271). For each strain and each hybrid, 6 plants were tested in 3 replicates. The rape seeds were disinfected in 2% Sodium Hypochlorite (NaOCl) on-site for 10 min, then washed with sterile water, and then dried on filter paper. Then, the seeds were placed on aqueous agar and germinated with a 16 h photoperiod at 23 °C and 40 to 60% humidity for 48 h. A 5 mm mycelial plug from the freshly growing fungal cultures was placed on the hypocotyl part of the rootlet. This was performed using a similar procedure to that described in the study by Kull et al. [48], but the inoculum was placed on the hypocotyl part of the plant instead of the cotyledon. The inoculated plants were incubated with a 16 h photoperiod at 23 °C and 40 to 60% humidity in ambient illumination, and they were assessed 72 h after inoculation. The level of aggressivity was assessed on a banded scale from 0 to 4 as follows: 0—no symptoms; 1—browning is visible on young plants at the contact of the mycelium plug; 2—the mycelium of the pathogen covers a maximum of half of the rootlet; 3—the mycelium of the pathogen completely covers the rootlet; and 4—sclerotia appear on young plants (Figure 9).

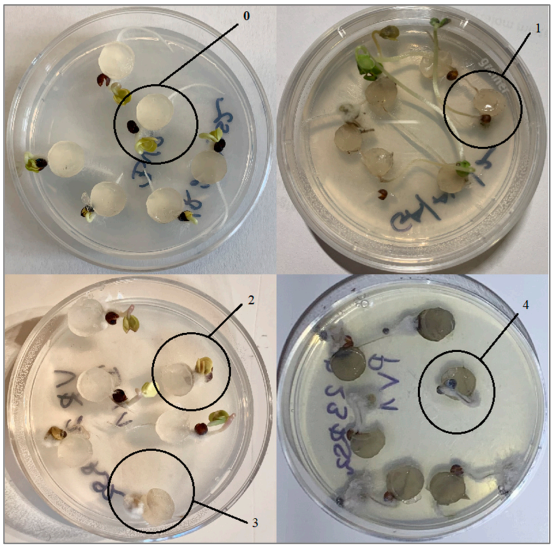

Figure 9. A scale from 0 to 4 used to determine the level of aggressivity.

3.4. Statistical and Regression Analyses

All statistical analyses were performed using the PAST program. The data were analyzed using the ANOVA and Tukey's pairwise comparisons, and *p*-values of 5% or less were considered to be a statistically significant difference. Regression analyses were performed for all tested hybrids between the level of aggressivity and the mycelial growth after 24 h, the mycelial growth after 48 h, the number of sclerotia, and the weight of sclerotia.

4. Discussion

Strains of the pathogen *S. sclerotiorum* from four different OSR-growing locations in Hungary were tested. The mycelial growth of the strains showed significant differences in colony growth among the strains after 24 and 48 h, as pointed out by Garg et al. [49]. Rathi et al. [47] classified the strains into fast and slow mycelial growth groups. After 48 h, average colony diameters between 1.23 and 2.5 cm were considered as slow mycelial growth, while rapid growth after 48 h was considered as an average colony diameter of 6.2–6.5 cm and after 72 h as an average colony diameter of 7.9–9.0 cm. Strains tested in this research also showed rapid mycelial growth after 48 and 72 h, similar to the results obtained by Ahmadi et al. [50]. This can be inferred from the fact that, in some rape-growing areas of Hungary, there are strains of *S. sclerotiorum* with a higher colonization capacity, which could even displace the strains that lagged in the "struggle for living space".

The number of sclerotia produced differed between the strains, consistent with several studies [29,51,52], which explain that these differences are indicative of the morphological variability of the pathogen [53]. Taylor et al. [12] assessed the number and mass of sclerotia produced from several host plants on PDA plates and observed differences in both studies for different isolates. The three isolates that they studied showed a consistent pattern; however, isolate L6 produced several small sclerotia, while L44 produced a few large sclerotia, and for L17, the value ranged between the values of the other two studied isolates. In this study, the RSZ21 isolate developed the most sclerotia, averaging 45.33 sclerotia per PDA plate.

The total weight of sclerotia produced by the strains ranged from 0.46 to 1.04 g/PDA plate, while the results of Kyryk et al. [54] did not show such a wide range (0.15–0.46 g). Several reports (similar to our result) indicate that different degrees of virulence are associated with different strains of *S. sclerotiorum* [49,55,56].

Pratt and Rowe [57] infected alfalfa cultivars and Alavi and Dalili [58] infected OSR cultivars with different *S. sclerotiorum* strains, and similar to our result, they concluded that both cultivars and strains affect the sensitivity.

Research by Taylor et al. [59] on Brassica plants showed no significant correlation between the number of sclerotia, the weight of sclerotia, the weight per sclerotia on a PDA plate, and aggressivity. Similar results were reported by Li et al. [29], who compared *S. sclerotiorum* strains from sunflowers. The results of this study show that a positive correlation was observed between mycelium growth after 24 h and aggressivity, while a negative correlation was observed between the weight of sclerotia and aggressivity. The latter results agree with those of Vleugles et al. [41], where a negative correlation was observed between the number of sclerotia produced by *S. trifoliorum* strains and the aggressivity of red clover. These results contradict the results obtained in the research by Garg et al. [49], who found no association between mycelial growth and aggressivity. The aggressivity levels of all four considered hybrids varied across individual years due to the vintage effect. This statement contradicts the findings of Otto-Hanson et al. [60], who discovered that the isolates they investigated exhibited no significant difference between the collection years.

5. Conclusions

This study examined *S. sclerotiorum* strains from four different oilseed rape (OSR) growing locations in Hungary, revealing significant differences in mycelial growth and

sclerotial formation. Results from two growing seasons did not indicate the strain or region with the highest colonization capacity, possibly due to different strain dominance across seasons in the same OSR growing regions. Regarding strain aggressivity, notable differences were observed in aggressivity levels and morphological characteristics. Hybrids (Architect, Bluestar, Umberto, and PT271) showed diverse responses to the strains, with PT271 showing the highest aggressivity across all strains. Strong correlations were noted between mycelial growth after 24 h and aggressivity, as well as between sclerotial weight and aggressivity. Strains exhibiting higher mycelial growth after 24 h displayed greater aggressivity, while the level of aggressivity decreased for strains producing higher sclerotial weight per PDA plate. The investigated parameters offer a potential avenue for assessing strain colonization ability and survival in a given region.

Understanding pathogen characteristics enables us to prioritize integrated pest management strategies, particularly prevention. Given that specific morphological traits of *S. sclerotiorum* strains correlated with aggressivity levels, it is useful to know the morphological characteristics of strains occurring in OSR fields. This knowledge can aid in selecting tolerant or less-susceptible hybrids/varieties of OSR, thereby minimizing yield and quality losses caused by *S. sclerotiorum*.

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