Thesis of doctoral dissertation

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Gödöllő
2024

HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE SCIENCES

INVESTIGATION OF RESISTANCE AGAINST *PYRENOPHORA TERES* F. *TERES* DRECHS. INFECTION IN DIFFERENT BARLEY GENOTYPES

THESIS OF DOCTORAL (PHD) DISSERTATION

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GÖDÖLLŐ

2024

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1. Background and objectives

Barley (*Hordeum vulgare* L.), in terms of yield and cultivated area, is one of the most significant crops globally and the cereal with the widest range of applications. According to FAOSTAT data in 2021, approximately 49 million hectares were cultivated worldwide, producing a total of 145 million tonnes of barley, of which about one-third, 52 million tonnes, was harvested in Europe. Regarding its role in European agriculture, it can be said that although the total harvested area decreased from 11.3 million hectares to 10.2 million hectares between 2010 and 2021, the average yield per hectare increased by 21% (from 4.2 tonnes to 5.1 tonnes). Recently, about two-thirds of barley production was used for feed, one-third for malt industry applications, and about 2% was directly used for human food production.

In Hungary, based on the area cultivated and the quantity of the harvest, it is one of our most important field crops, which in 2021 was harvested from a total area of 268 thousand hectares with a production volume of 1.7 million tonnes, yielding an average of 6.3 t/ha, surpassing the European average, according to the data from the Hungarian Central Statistical Office (KSH, 2021). The cultivated area significantly increased in 2023, with a harvest of 2.2 million tonnes from nearly 400 thousand hectares (KSH, 2023). The data highlights that barley plays a significant role in both Hungary's and the world's food supply.

Considering the effects of climate change, changing dietary needs, sustainable production methods, and challenges related to nutrient management, the economical and successful cultivation of barley faces increasing challenges. The changing environmental conditions have altered the quality and quantity of stressors affecting the plants. Therefore, one of the primary goals of plant breeding programs is to increase abiotic and biotic stress tolerance, which is a fundamental requirement for successful cultivation and sustainable agriculture. In this regard, breeding and cultivating disease-resistant, resistant, or tolerant varieties is particularly important.

Among the pathogens of cereals, including barley, various fungi play a significant role, in infecting the leaf surfaces of the plants. The net blotch disease caused by *Pyrenophora teres* f. *teres* (Drechsler) (PTT) is of paramount importance among the leaf disease-causing pathogens, which can lead to yield losses of 30-40% in addition to deteriorating quality parameters.

In the case of plant pathogen infection, oxidative stress is induced in the plant, which can disrupt normal cell functioning and even lead to cell death. To defend against oxidative stress, cells have developed numerous antioxidant defence mechanisms, including enzymatic and non-enzymatic antioxidants. Various antioxidant enzymes such as superoxide dismutase (SOD), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX), as well as non-enzymatic

antioxidants like vitamins C and E, play an important role in the plant's defence system (Ivanov & Khorobrykh, 2003).

There is increasing research on the background of resistance to pathogens, but the literature mainly reports results from studies conducted on spring barley. It is not yet clear how *Pyrenophora teres* f. *teres* infection influences the activity of various antioxidant enzymes (SOD: superoxide dismutase, APX: ascorbate peroxidase, GPX: guaiacol peroxidase) in different genotypes of autumn barley, and whether these differences are correlated with the sensitivity of barley varieties to the pathogen. Expanding knowledge through studying different resistance mechanisms can contribute to enhancing the resistance of both autumn and spring barley, allowing for environmentally friendly cultivation, reducing production costs, and leading to greater yield security. Thus, investigating the resistance of barley to *Pyrenophora teres* f. *teres* is important not only from a scientific but also from an economic perspective.

The objectives of the thesis during the research were as follows:

- Investigating the resistance of barley genotypes to *Pyrenophora teres* f. *teres* Drechs. in juvenility under greenhouse conditions.
- Investigating the resistance of barley genotypes to *Pyrenophora teres* f. *teres* Drechs. in adulthood under greenhouse conditions.
- Investigating the resistance of barley genotypes to *Pyrenophora teres* f. *teres* Drechs. in the field.
- Examining the role of the antioxidant system in defence reactions following *Pyrenophora teres* f. *teres* Drechs. infection, with particular focus on changes in the activity of SOD, APX, and GPX.

2. Material and method

In our experiment, we examined 206 different barley varieties from the barley collection of Martonvásár, thus obtaining a comprehensive picture of the resistance of genotypes from various barley-growing regions of the world. The varieties examined in the experiment included both two-row and six-row spike types, autumn and spring life forms, and they also differed from each other in their resistance to net blotch disease based on the results of preliminary research.

2.1. Determination of resistance of barley genotypes to net form net blotch disease

The field experiments were conducted in Martonvásár, at the field of the Agricultural Research Centre of the Hungarian Research Network, Institute of Agriculture (HUN-REN ATK MGI), between 2017 and 2021, over five field seasons. From each of the 206 barley genotypes, 10 untreated seeds were sown in three replicates in every season studied, using the same experimental layout. As a cover, barley straw, on which the net blotch disease pathogen overwinters provided the source of infection for the following year, was spread over the plants at the tillering stage. The examined genotypes were assessed four times per growing season at two-week intervals, where we evaluated the extent of infection and the type of lesions formed.

During the greenhouse resistance studies, the resistance of 206 barley genotypes was examined against four different monosporic *Pyrenophora* sp. isolates at two developmental stages of the plants, young (Z12 two-leaf stage) and adult (Z39-41 after the flag leaf development), to better understand the susceptibility of the examined barley varieties.

To study the juvenile resistance of barley varieties, 5 untreated seeds from each of the 206 genotypes were sown in three replicates, using the same experimental layout as in the field sowing.

The resistance of the 206 barley varieties was also examined in adulthood under greenhouse conditions, for which 10 barley seeds from each genotype were germinated, followed by a 50-day vernalization period. The plants were transplanted into plastic growing containers in three replicates (2 plants/genotype/container). The adult plant infection was carried out after the appearance of the flag leaf.

In the greenhouse experiments, monosporic PTT isolates (H-618, H-774, H-949) and PTM isolate (H-502) collected from barley leaves from various domestic locations were used. The isolates were prepared according to the description by Kunos et al. (2022). Once the appropriate development stage was reached, the plants were infected with the selected isolate's spore suspension (10,000 conidia/ml).

In the field, to record lesion types, the Tekauz scale (1-10) was used (Tekauz, 1985), based on which the developed symptoms were evaluated. The size of the infected leaf surfaces was assessed on the Saari-Prescott scale (0-9) (Saari & Prescott, 1975). In the greenhouse, the Tekauz scale was also used to determine lesion types. An exponential transformation was performed on the evaluation data (Hinfner & Békési, 1973).

$$y = A + (B - A)\left(\frac{(x - C)}{(D - C)}\right)^{k}$$

To be able to infer the severity of the symptoms caused and the progression of the disease, the area under the disease progression stairs was determined based on the values, using the Area Under Disease Progress Stairs (AUDPS) method:

Can be calculated (1):

$$AUDPS = \left[\sum_{i=1}^{n-1} \frac{y_i + y_{i+1}}{2} \times (t_{i+1} - t_i)\right] + \left[\frac{y_1 + y_n}{2} \times \frac{D}{n-1}\right] \qquad (1)$$

where, y_i is the percentage of the evaluation value of the infection; t_i is the time elapsed from the inoculation; D is the time elapsed between the first and last evaluation ($D = t_n - t_1$), n is the number of evaluations.

For comparability of multi-observational assessments of disease progression from associated trials, the relative form of AUDPS values (R-AUDPS) was used (Simko & Piepho, 2012):

$$rAUDPS = \frac{AUDPS \times (n-1)}{D \times n \times y_{max}} \qquad (2)$$

where, y_{max} is the maximal potential AUDPS value; D is the time elapsed between the first and last evaluation, and n is the number of evaluations.

2.2. Study of the activity of the antioxidant enzymes

Based on resistance data from preliminary greenhouse and field trials, the activity of antioxidant enzymes (SOD, APX, GPX) was investigated in five barley genotypes: Canela, Harrington, Manas, Mv Initium and Antonella. The varieties were selected based on their different susceptibility to PTT and grown in a greenhouse up to the age of two leaves (Z12 developmental stage) in the same way as described in juvenile resistance tests. The plants were infected with monospore *Pyrenophora* isolates (PTM: H-502, PTT: H-612, H-774, H-949) using the method described in greenhouse tests. Leaf samples were collected at 0 hours (control) before infection, 24 (day 1), 48 (day 2) and 72 hours (day 3) after infection. When treated with H-949 isolate, samples were taken from infected plants on days 7 and 15 after inoculation. Sampling during the longer incubation period was based on the earlier work of Pál et al. (2011, 2013) to examine changes in enzyme activity in the later stages of infection.

The enzyme activity of SOD was measured at 550 nm based on the work of Bergmeyer et al. (1974), and then the changes in the measured absorbency

values were converted into enzyme activity (Zhang et al., 2016). Absorbance changes in APX activity were tracked at 290 nm. GPX activity measurements were made at 470 nm by tracking the oxidation of guaiacol.

During the statistical analysis of the experiments, a hierarchical cluster analysis was performed to classify genotypes based on infection. To determine the optimal number of clusters the WSS (Within-Cluster Sum of Squares) method was used. The Kruskal-Wallis ANOVA (non-parametric) test was used to compare the clusters, followed by the Dunn post-hoc test to determine significant differences between the clusters. The difference in infection between the five selected genotypes was analyzed using a single-factor analysis of variance (ANOVA) and the genotypes were compared using a Tukey HSD post-hoc test. We created a heatmap for analysis of all experiments and evaluation of the results together. The interaction of genotypes and their environment was analyzed using an AMMI model (Additive Effects and Multiplicative Interaction). We also analyzed the relationships between all variables using a Pearson correlation matrix and examined the regression between AUDPS values and evaluation results to understand the relationship between the two variables.

We used the Microsoft O365® software for data sorting, while IBM SPSS® V27 (IBM® Corp, 2020) and RStudio software (R Core Team, 2021) were used for statistical analysis. In RStudio, the primary packages were: agricolae, emmeans, dplyr, multcomp, ggstatplot, pheatmap, ggcorrplot, and ggplot2.

3. Results and discussion

To gain insight into the resistance of the 206 genotypes to *Pyrenophora* sp., we summarized the field infection data from the 5 years under study (2017, 2018, 2019, 2020, 2021) and the data on adult resistance against two PTT isolates (H-774, H-949) and juvenile resistance against four isolates (PTM: H-502, PTT: H-618, H-774, H-949). We analyzed the average relative R-AUDPS values obtained from various experiments using a heatmap (Figure 1), which shows the aggregated results of the hierarchical cluster analysis not only by genotype but also grouped by experiment.

Based on the heatmap diagram, we determined that based on the aggregated average results of the experiments, 32% of the examined barley varieties belonged to Cluster 1, with a median R-AUDPS value of 0.23, and the varieties classified into this cluster were moderately resistant to infection. The susceptible genotypes were placed in Cluster 2, with a median R-AUDPS value of 0.33, comprising a total of 44 genotypes, which was 21.3% of the entire set of varieties examined. Furthermore, in this cluster, we recorded the highest average infection value (R-AUDPS=0.424). Out of the examined genotypes, 65, or 31.5% of the collection, were categorized into Cluster 3, which had the smallest median (0.19); this cluster included the genotypes resistant to the disease. The last, Cluster 4, had a median R-AUDS value of 0.25, and into this group fell 15% of the genotypes, totalling 31 moderately susceptible varieties. The Harrington (0.21±0.13) and Mv Initium (0.21±0.08) varieties also fell into the second cluster. The greater variance in the average relative R-AUDPS values of both varieties was significantly influenced by the results of field experiments (FSZF-2017, FSZF-2018, FSZSF-2019), as well as two greenhouse juvenile infection experiments (FUHS-H618 and H-774). The Antonella (0.07±0.06) and Canela (0.1±0.07) barley varieties, which were placed in the third cluster, had a smaller variance in their aggregated results, indicating greater resistance demonstrated during greenhouse experiments to net form net blotch. The Manas genotype (0.11±0.07), found in the fourth cluster, received the third smallest average R-AUDPS value among the selected varieties but showed less resistance in field experiments compared to greenhouse experiments.

Based on the cluster analysis of the aggregated results of the 206 genotypes examined across a total of 11 different experiments, it can be determined that there was a significant difference among the clusters overall [$H(3) = 115.343$; $p = 0.00$]. The overall experimental average R-AUDPS value was 0.239±0.69, with a minimum value of 0.104 and a maximum value of 0.424. Upon evaluating the clusters, it was found that Cluster 1 (0.218±0.043) significantly differed from all the other clusters, Cluster 2 (0.333±0.049) differed from Cluster 3 (0.189±0.040) and Cluster 4 (0.256±0.046).

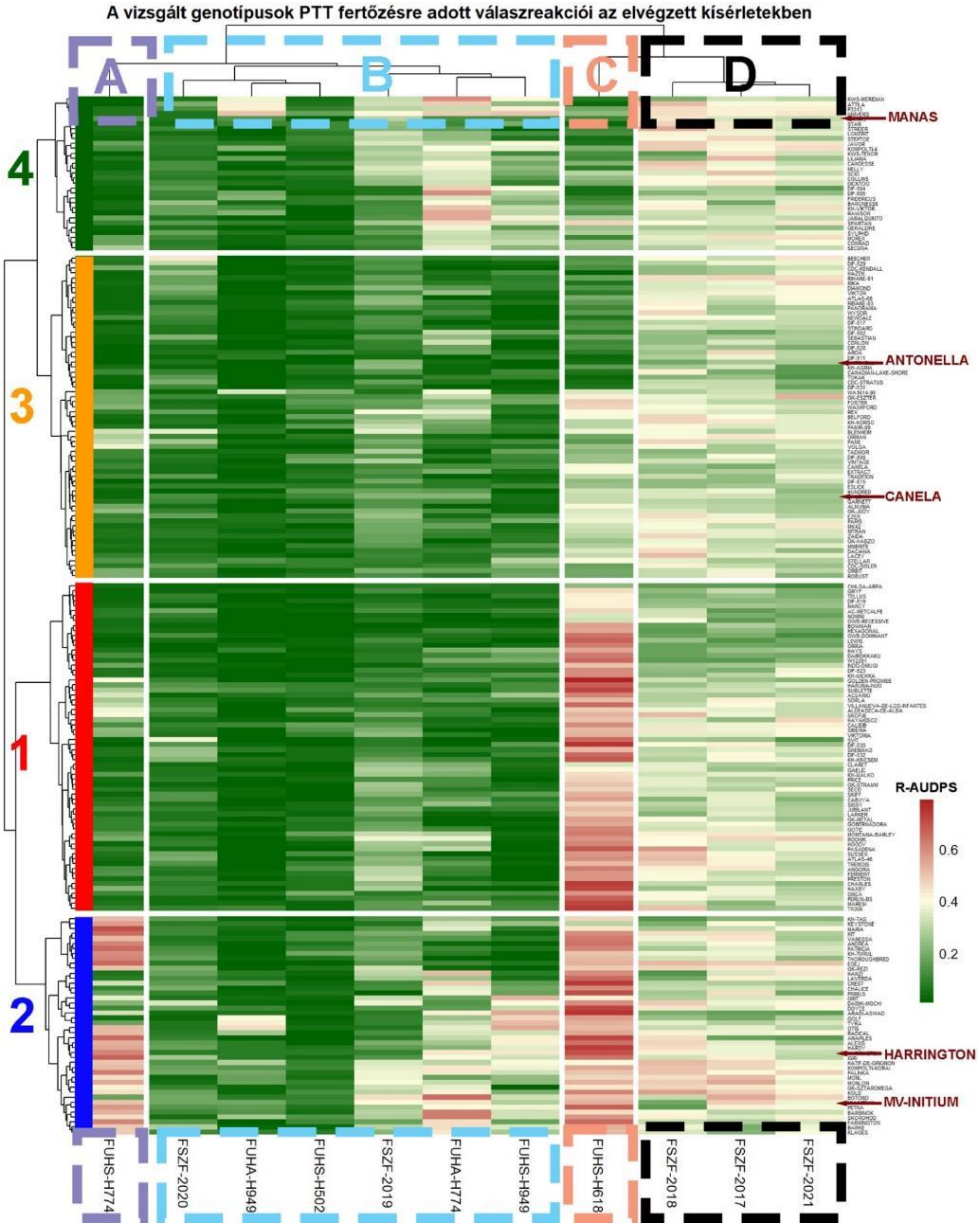

Figure 1: The 206 barley genotypes are *Pyrenophora sp*. infection response (R-AUDPS) analysis in all experiments. Evaluation of trials set in 5 field years (2017, 2018, 2019,2020,2021), greenhouse adulthood (H-774, H-949) and juvenile age (H-502, H-618, H-774, H-949). The letters A, B, C, and D denote the clustering of experiments.

When creating the heatmap, the various experiments were also divided into four different clusters (A, B, C, D). Two greenhouse juvenile infection experiments (FUHS-H774 and FUHS-H618) were completely separate, forming clusters "A" and "C" on their own, respectively. Furthermore, it was determined that among all the isolates and experimental seasons we examined, the PTT isolate used in the FUHS-H618 experiment had the highest

infectivity. The three years of field experiments (FSZF-2017, FSZF-2018, FSZSF-2019) were placed into cluster "D". The 206 barley varieties examined showed mixed results in terms of resistance to net form net blotch during the field experiments, which could be due to the negative or positive interactions of other factors occurring in nature. Cluster "B" contained a mix of field (FSZF-2020, FSZF-2021), juvenile greenhouse (FUHS-H502, FUHS-H949), and adult (FUHA-H774, FUHA-H949) experiments. The field experiments classified into this category likely diverged from the other field experiments in cluster "D" due to the effects of the year, as spring 2019 was wetter than the multi-year average, while 2020 saw less precipitation throughout the year, affecting the 2021 growing season as well. Both years experienced cooler springs than the multi-year average by approximately 0.5-1 °C, so the spring temperature and the extremely low or high amounts of precipitation could have influenced the results of the experiments. The examined genotypes showed greater resistance in adulthood against the H949 isolate, which could be due to the fact of adult infection (following the appearance of the flag leaf) and the infectivity of the isolate alike. The experiments that caused the most variance in cluster "B" were the FUHA-H774 and the FSZF-2019 experiments.

Further analysis of the data was performed using the AMMI (Additive Main Effects and Multiplicative Interaction) model. We found that the variance of environmental factors (ENV) contributed the most to the deviation of the data, explaining 62.7% of the total deviation, and in this case, it was the different isolates and the year in the case of the field experiment. The environmental repetitions (REP(ENV)) factor accounted for only 0.8% of the total data variability, indicating that the repetitions of experimental treatments did not cause significant dispersion in the data. The genetic factor (GEN) explained 23.1% of the total variance, while the interaction between environmental and genetic factors (ENV x GEN) accounted for 13.4% of the total variance.

As part of the AMMI model, principal component analysis (PCA) was also performed. The first major component (PC1) explains 42.1% of the total variance, while the second major components (PC2) and third major components (PC3) account for 19.8% and 14.3%, respectively. The remaining major components (PC4 to PC10) together account for less than 7% of total data variability. On the AMMI model (Figure 2), the PC1 and PC2 axes show the relative position of the main components containing genotypes and environment variables.

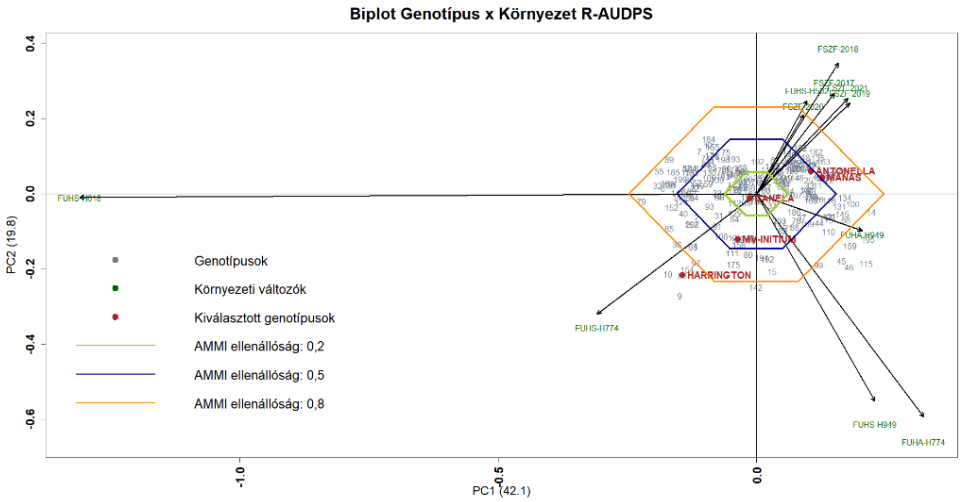

Figure 2: AMMI (Additive Main Effects and Multiplicative Interaction) model. The PC1 and PC2 axes show the relationship between genotype x environmental conditions, green shows the location of the experiments, grey shows the location of the 206 barley genotypes tested and red shows the location of the five genotypes selected for antioxidant tests from the origin based on their R-AUDPS values.

Genotypes that are closer to the origin of the biplot show more resistance to PTT infection in various experiments, while varieties that are farther from the origin are more susceptible to *Pyrenophora* sp. infection. Moreover, genotypes that are located on the horizontal axis (abscissa) and do not deflect on the vertical axis (ordinate) are considered more resistant. Genotypes that differ significantly from the abscissa in a negative or positive direction, on the other hand, can be considered susceptible to the pathogen.

Based on these, it can be stated that the four zones created by the contour lines group the varieties susceptible to PTT infection and those resistant to it. The least susceptible genotypes were in the AMMI resistance group 0.2, where the model showed that the maximum distance of genotypes from the origin was 0.068, and this group included 30 genotypes. The second resistance group of 0.5, according to the model, consisted of genotypes at a maximum distance of 0.15 from the origin, for a total of 159 barley varieties. The third group consisted of genotypes located at a maximum distance of 0.24 from the origin, there were 199 pieces. The fourth, at 0.8 distance, we were able to identify a total of seven genotypes farther from the origin with the help of the model. It is important to mention that the 4th group, identified as the most susceptible to PTT infection, also included the Harrington variety previously selected for antioxidant testing, which is confirmed by Harrington's average R-AUDPS value (0.22±0.14) during the experiments. Of the four other genotypes selected, the third group was 0.5 to 0.8 for Manas with an average R-AUDPS value of 0.11±0.09, and for Antonella (R-AUDPS: 0.072±0.071) and Mv Initium (R-AUDPS: 0.21±0.09) for the 0.5-0.2 group. The group

considered to be the least susceptible closer than 0.2 to the origin was the Canela genotype with an average relative R-AUDPS of 0.1±076.

Our results correlate with the research of Murphy et al. (2009), which showed that environmental indicators with shorter vectors, i.e. closer to the origin of the biplot, cannot be considered factors with significant impact. In contrast, the greatest variance in our studies was caused by the experiment infected with H-618 isolate (FUHSH-618) in the case of greenhouse juvenile resistance tests, which is due to the virulence of the examined isolate, which is confirmed by the fact that this experimental environment was furthest from the origin.

To investigate the relationship between the PTT resistance of selected genotypes and changes in the activity of antioxidant enzymes, a Pearson correlation coefficient matrix was prepared to observe the relationships of the investigated parameters. We found that the credit scores taken at different time points (BON1-4) show a strongly positive, significant correlation with R-AUDPS values (R=0.78-0.97). This relationship can be inferred from the method of calculating R-AUDPS, i.e. the higher the evaluation value, the higher the R-AUDPS value will be (the genotype is more sensitive to the pathogen). Also positively significant but moderate to weak correlations with evaluation values were mean SOD enzyme activity when infected with H-618 isolate (R=0.38-0.53) and mean GPX activity for H-949 isolate (R=0.33-0.46). Furthermore, we found that there was a weak, but significant and positive relationship between the overall change in APX values (DC) and recorded evaluation values for H-618 (R=0.11-0.41), H-774 (R=0.24-0.42) and H-949 (R=0.16-0.51) isolates.

Comparing the results of antioxidant results by isolate, it was found that the mean SOD value for H-618 isolate showed a positive, moderately strong correlation with GPX values (R=0.62) of plants infected with H-949 isolate, but a negative, moderate correlation for GPX activity when analyzing the results of treatment with H-774 isolate (R=-0.73). A mean, significant and positive relationship between mean APX and GPX values was found following infection with H-774 isolate (R=0.42).

It can be observed that the result of cluster analysis was strongly influenced by the evaluation results (R=(-0.31 and -0.59) and R-AUDPS values (R=-0.55) since there is a negative and significant correlation between them. This is due to the interdependence of the methodologies of evaluation, R-AUDPS calculation and cluster analysis. In addition, cluster analysis results show a significant and positive relationship with mean APX values as a result of infection with H-502 isolate (R=0.9), H-774 isolate (R=0.74), H-618 isolate (R=0.54) and H-949 isolate (R=0.78), and cluster analysis results were positively correlated with mean GPX activity values for H-774 isolate (R=0.48). Furthermore, we found that APX values generally show a

downward trend as infection progresses, in contrast to changes in SOD enzyme activity, which shows an increasing trend.

During our studies, we observed a significant increase in SOD activity in the case of PTT infection, but this increase was genotype- and isolate-dependent. This observation is also supported by previous literature (Able, 2003; Shetty et al., 2007; Urbanek et al., 1996; Vanacker et al., 1998). It is noteworthy that previous research on the measurement of antioxidant enzyme activities was generally limited to just one resistant and one sensitive genotype. Furthermore, the examination of activity changes due to abiotic stress is a more frequently researched area, for example, the effect of salt stress on the increase of SOD activity was observed (De Azevedo Neto et al., 2006; Meloni et al., 2003). In the case of five barley genotypes selected based on preliminary data for antioxidant studies, we found that the Antonella variety showed greater resistance during infection with two PTT isolates (H-618, H-774), which can be partially explained by the genotype's increasing trend of SOD enzyme activity during infection. This result supports the finding of Able (2003) that resistance of barley to net form net blotch correlated with an increase in antioxidant enzyme activities. The studies also highlight that the SOD enzyme activity, elevated after PTT infection, continues to increase, especially after 48 hours following the infection (Urbanek et al., 1996). The rapid production of ROS during the response to pathogens helps activate various defensive barriers against pathogens (Torres et al., 2006). However, we did not find a clear correlation between the juvenile resistance of the genotypes and SOD enzyme activity; therefore, resistance cannot be estimated merely based on changes in SOD enzyme activity after infection. Further research is needed to clarify the role of antioxidant enzymes in different types of biotic stress, such as during pathogen infections. These studies will allow a better understanding of the relationship between the accumulation of ROS and early pathogen infection (Jasso-Robles et al., 2020; Pandey et al., 2021).

Previous studies have addressed changes in APX enzyme activity under stress, but very few have examined the relationship between APX and net form net blotch. Sang et al. (2005) observed that salt stress increased APX activity in the root system of barley, but there was a decreasing trend in the shoots. During studies on abiotic stress, Chen and colleagues (2014) found that APX activity increased in Arabidopsis thaliana under stress conditions such as osmotic, salt, or heat stress. Zhang et al. (2013) also determined that APX plays a significant role in the defense of young plants against abiotic stress in rice by scavenging ROS, leading to an increase in APX activity. Furthermore, Moloi (2016) noted that monitoring APX activity could be an important biochemical marker for examining drought tolerance in soybeans. The results also demonstrate that APX plays a crucial role in complex stress response mechanisms. As a biotic stressor, previous studies mainly focused on powdery mildew (*Blumeria graminis* Bgh.), a biotrophic pathogen, but did not study

the consequences of PTT infection (Harb et al., 2015; Vanacker et al., 1998; Zlatev et al., 2006). These experiments found that *Blumeria graminis* Bgh. infection reduced APX activity in barley by more than 60%. Pál et al. (2011) described an opposite trend in wheat, where APX activity significantly increased following powdery mildew infection. In contrast, based on our current results, we cannot unequivocally determine an increase in APX activity due to PTT infection. Although the level of APX activity largely depends on the genotype and environmental variables, the infectivity of the pathogen isolates primarily has a significant effect. Based on our current findings, we cannot definitively ascertain an increase in APX activity due to PTT infection as a biotic stressor. While the level of APX activity depended greatly on the specific genotype and environmental factors, the infectivity of the pathogen isolate was primarily significant.

Regarding GPX activity, we obtained similar results to those of Milone et al. (2003), who reported that abiotic stress leads to a significant increasing trend in GPX activity. Investigating the relationship between frost tolerance and antioxidant capacity in cereals, including barley, it was found that the highest correlation between enzyme activity and frost tolerance was observed for GPX and APX (Janda et al., 2003). In cases of abiotic stress, it was established that cadmium and mercury contamination led to an increase in GPX activity in barley root tips, which correlated with the inhibition of root growth (Halušková et al., 2009). Conversely, Radotic et al. (2000) recorded a decrease in GPX activity in the later stages of cadmium contamination, as demonstrated in pine needles. The effect of salt stress, as an abiotic stressor, was examined in maize genotypes, and an increased GPX, SOD, and APX activity was observed in the salt-sensitive genotype, while in the salt-tolerant variety, SOD activity decreased, and GPX and APX activities remained nearly the same as the control (De Azevedo Neto et al., 2006). When investigating maize stress responses, the activity of GPX and plasma membrane-bound peroxidase enzymes significantly varied under stress effects, especially due to different pathogens (*Fusarium graminearum, F. culmorum*), highlighting the importance of peroxidases in defence against pathogens (Mika et al., 2010). Among the selected genotypes, the Antonella variety showed the most significant change in GPX activity due to PTT infection in juvenile experiments infected with the H-774 isolate, supporting the statement by Tayefi-Nasrabadi et al., (2011) that increased GPX activity may provide at least partial protection against oxidative damage induced by stress. This fact could not be confirmed in the case of the H-949 isolate, likely due to its presumably lower pathogenicity. Publications on the changes in various peroxidases due to biotic stress are scarce, with the roles of APX and GPX mainly investigated in abiotic stress research. Our results contribute to a deeper understanding of stress responses to pathogens. However, based on our current research results, we did not always demonstrate a clear relationship

between the juvenile resistance of the genotypes and GPX activity. This is supported by the findings of Moloi (2016), who indicated that GPX selectively participates in soybean drought resistance. Furthermore, it was determined that the effect of biotic stress on GPX activity is complex and depends on other environmental stress factors and the type of plant tissue stressed. Methyl jasmonate and copper treatments effectively reduced the activity of SOD, APX, and GPX in maize leaves under insect attack as a biotic stressor, and increased enzyme activity in the roots (Hanaka et al., 2018).

Among the genotypes we examined, Antonella showed the lowest SOD activity among the selected genotypes (Kunos et al., 2022). The APX enzyme activity did not change significantly due to PTT infection, however, there was a 50% increase in GPX activity compared to the initial value measured at 0 hour. Thus, it can be said that Antonella showed greater resistance to PTT infection, which was partly due to the lower level of SOD and increased GPX enzyme activity. This result contradicts previous research by Able (2003), which found that the resistance of barley to net form net blotch correlated with an increase in SOD activity. The increased GPX activity in Antonella also partially confirms the statement (Tayefi-Nasrabadi et al., 2011) that elevated GPX activity can provide at least partial protection against oxidative damage induced by stress.

Based on the results, it can be determined that the R-AUDPS values calculated from the data of the recordings can also help in selecting varieties resistant to the disease.

Among the genotypes we studied, Antonella showed the lowest SOD activity among the selected genotypes (Kunos et al., 2022). The APX enzyme activity did not change significantly due to PTT infection, however, there was a 50% increase in GPX activity compared to the initial value measured at 0 hours. Thus, it can be said that Antonella demonstrated greater resistance to PTT infection, which was partly due to the lower SOD and increased GPX enzyme activity. This result contradicts earlier research by Able (2003), which found that barley's resistance to net form net blotch correlated with an increase in SOD activity. The increased GPX activity in Antonella partially confirms the finding (Tayefi-Nasrabadi et al., 2011) that increased GPX activity can at least partly protect against oxidative damage induced by stress. The results indicate that R-AUDPS values calculated from the recordings can also help in selecting disease-resistant varieties.

Based on the experiments conducted, we compiled a ranking of the 206 barley genotypes and categorized them into different resistance groups based on their susceptibility to the pathogen. The ranking was based on relative R-AUDPS values, as this methodology was most suitable for comparing results across different experiments. The formation of resistance groups was carried out using the results of cluster analysis. Considering the aggregated results,

the genotypes that proved to be resistant (R - resistant) were placed into Cluster 3 (65 genotypes), which is 31% of the variety series. The barley varieties considered resistant are likely to carry a resistance gene that mitigates the extent of PTT infection and the appearance of disease symptoms. Therefore, the use of resistant genotypes in commercial cultivation and breeding programs is recommended. The moderately resistant varieties (MR – moderately resistant) are the genotypes located in Cluster 1, making up 32% of the variety collection we analyzed. Like the resistant varieties, the moderately resistant varieties are considered valuable breeding material; however, it is not clear what other factors (e.g., drought stress, precipitation stress, combined stress) play a role in their resistance to PTT infection. This trend was confirmed in our field experiments, where the year effect significantly influenced the extent of genotype infection. The moderately susceptible (MS – moderately susceptible) varieties can be categorized into Cluster 4, totalling 31 genotypes. For the moderately susceptible varieties, it can be said that their resistance to PTT infection did not always reach the desired level in every case and experiment. Thus, in commercial cultivation, more emphasis is placed on fungicide treatment, and they are not necessarily recommended as combination partners to increase resistance in resistance breeding programs. Finally, the PTT infection-sensitive or susceptible varieties (S – susceptible) belonged to Cluster 2, accounting for 21% of the 206 barley varieties we examined. The resistance of susceptible genotypes is not satisfactory, and during cultivation in a potentially epidemic year, stable yield and proper quality cannot be guaranteed.

The top 25 most resistant genotypes under local conditions predominantly belong to the R (resistant) and MR (moderately resistant) resistance groups. The Chilga barley from China ranked first, which, although classified as moderately resistant in some experiments, showed the lowest average infection value based on R-AUDPS values during our experiments. It is important to mention that among the five genotypes previously selected for enzyme activity studies, Antonella ranks third in the overall ranking and can be categorized into Cluster 3 and the resistant (R) resistance group. Its average R-AUDPS value was 0.069.

Among the top 25 ranked genotypes, it's worth mentioning the Canadian-Lake-Shore barley variety, which several studies have found to be resistant to PTT infection (Afanasenko et al., 2009; Dinglasan et al., 2019). Based on the results of previous studies and our experiments, it can be classified into the resistant (R) group. Canela ranked 39th and belonged to the resistant (R) group, while Manas was ranked 76th and became a member of the moderately resistant (MR) group.

Examining the last 25 elements of the 206 genotype ranking, it can be established that the most susceptible varieties had average R-AUDPS values between 0.204 and 0.251 and fell into Clusters 2 and 4. These genotypes were

placed in the susceptible (S) group, except for two genotypes (UNIVERS and P3313), which were placed in the moderately susceptible (MS) group. The PALINKA genotype ranked last in the ranking.

It's important to note that two of the five genotypes we selected, Mv Initium and Harrington, were placed into the susceptible group and among the last 25 varieties. After PTT infection, the SOD enzyme activity of Mv Initium showed a decreasing trend compared to other varieties, then increased at a greater rate (Kunos et al., 2022). In contrast, the infection had no significant effect on APX enzyme activity, but GPX activity increased by approximately 40% following infection.

The Harrington genotype follows Mv Initium in the ranking with an average AUDPS value of 0.216. Its SOD enzyme activity significantly increased after PTT infection, however, both APX and GPX activities significantly decreased as the infection progressed, by 8% and 20% respectively. Our results correlate with previous research (Afanasenko et al., 2009; Dinglasan et al., 2019), as these studies characterized Harrington as a susceptible variety to *Pyrenophora teres* infection. However, our findings are contrary to those of Jalli (2011), who in his study called Harrington potentially resistant and recommended its use in barley breeding programs in Finland.

In summary, the classification of the 206 barley genotypes from our Martonvásár collection according to their resistance to PTT can facilitate the work of future breeding programs. The importance of such research is highlighted by several studies, for example, the results of Lammari and colleagues' (2020) experiment on barley indicate that integrated pest management is essential in areas most infected with fungal diseases. Furthermore, it recommends that barley breeding programs should always evaluate disease resistance for the selection of resistant (R) and moderately resistant (MR) types. Net form net blotch has caused significant yield losses in barley growing areas, with infections resulting in up to 60% yield loss, especially in cases of highly infectious pathotypes (Aliyi Mohammed et al., 2021). This highlights that increasing resistance is crucial for the sustainable cultivation of barley. Volkova & Yakhnik (2022) point out that the cultivation of barley varieties resistant (R) and moderately resistant (MR) to PTT could promote the emergence of more virulent and diverse pathogen populations. This suggests that the cultivation of disease-resistant barley varieties leads to a more virulent and diverse pathogen population, complicating agricultural pest management and breeding efforts, and posing a continuous challenge for plant breeders.

4. Conclusions and recommendations

During our experimental work, we examined 206 barley varieties across a total of 11 different experiments, aiming to investigate the infectivity of *Pyrenophora teres* f. *teres* Drechs. under both field and greenhouse conditions. To gain a more comprehensive understanding of the resistance of barley varieties and the virulence of PTT isolates, we examined the activity of three important enzymes of the plant's antioxidant system (SOD, APX, GPX) in five selected genotypes from the variety series. In various field and greenhouse experiments, PTT infection was evaluated using Saari-Prescott and Tekauz scales, from which R-AUDPS values were calculated based on the evaluation results. The application of this method is more optimal in cases where disease evaluation occurs at different times and places greater emphasis on the first and last assessments, thereby improving the estimation of disease progression. To categorize the genotypes, we performed hierarchical cluster analysis based on the R-AUDPS values, resulting in the barley varieties being sorted into four clusters per experiment.

As a result of the cluster analysis, we observed significant differences between the clusters. Moreover, the aggregated average R-AUDPS value was 0.239±0.69, with minimum and maximum values ranging between 0.104 and 0.424. The 11 different experiments were also categorized into four different clusters (A, B, C, D), with the two greenhouse juvenile infections (FUHS-H774 and FUHS-H618) being completely separate, forming clusters "A" and "C" on their own. The likely reason is that the experiments could be set up under controlled conditions, thus eliminating confounding factors and allowing the selected isolates to provoke a stronger reaction. It was also determined that the PTT isolate used in the FUHS-H618 experiment had the highest infectivity. The majority of field experiments (FSZF-2017, FSZF-2018, FSZF-2019) were placed into cluster "D". The resistance of barley varieties during field experiments did not depend on the year, despite the negative or positive interactions of other natural factors. This means that the resistance of genotypes to PTT can be reliably estimated under field conditions. Cluster "B" included a mix of field (FSZF-2020, FSZF-2021), juvenile greenhouse (FUHS-H502, FUHS-H949), and adult greenhouse (FUHA-H774, FUHA-H949) experiments. The field experiments included here likely differed from the other field experiments in cluster "D" because the effect of the year was more pronounced in these years. Notably, the spring of 2019 was wetter than the multi-year average, while the spring of 2020 experienced drier weather. In both years, the spring was cooler than the multi-year average by approximately -0.5 to -1 °C, so the spring temperature and the extremely low or high amounts of precipitation could have influenced the results of the experiments. Despite the differing weather conditions, the AMMI model analysis showed a close correlation between the infection levels

of the varieties in all years. The examined genotypes showed greater resistance in the FUHA-H949 experiment, which could have been due to the fact of adult infection (Z41 developmental stage) and the lesser infectivity of the isolate alike. The experiments causing the most variance in cluster "B" were the FUHA-H774 and FSZF-2019 experiments.

Based on the results of the AMMI model, we determined that the variance of environmental factors (ENV) contributed most significantly to the deviation in the data, explaining 62.7% of the total variability. This primarily originated from the variance of experiments infected with greenhouse monosporic isolates. The environmental repetitions (REP(ENV)) factor accounted for only 0.8% of the total data variability. The genetic factor (GEN) explained 23.1% of the total variance, while the interaction between environmental and genetic factors (ENV x GEN) accounted for 13.4% of the total variance. Based on these, it can be said that the four zones created by contour lines on the biplot group the barley varieties susceptible to PTT infection and those resistant to it. The fourth group, identified as the most sensitive to PTT infection, included the Harrington variety previously selected for antioxidant studies as well, which was confirmed by the genotype's average AUDPS value (0.22±0.14) during the experiments. Our results indicate that the AMMI model can be applied to determine the resistance of different genotypes.

In our studies, we selected five genotypes (Manas, Harrington, Canela, Mv Initium, Antonella) to examine the effect of PTT infections on barley's SOD, APX, and GPX activity. We found that infection led to significant changes in SOD activity in barley genotypes, although the extent of these changes depended on the specific genotype and isolate. The observed increase in SOD activity following PTT infection supports literature data suggesting that the presence of reactive oxygen species indicates a plant's defensive mechanism. However, despite previous research indicating that higher SOD activity correlates with resistance to PTT infection, our results could not confirm this for all selected genotypes. In our experiments, we found that varieties fundamentally susceptible showed a greater increase in SOD activity. Our results highlight the importance of considering multiple factors when analyzing the relationship between SOD activity and plant defence mechanisms. The resistance of barley varieties cannot be estimated based solely on their SOD activity, as other antioxidant enzymes also contribute to the fine-tuning of the plant's defensive response.

The examination of APX activity did not provide a clear correlation between PTT infection and changes in enzyme activity, as there was significant variance in the data across the genotypes examined. This suggests that APX activity is significantly influenced by environmental factors and the natural PTT resistance of barley varieties.

However, more definitive results were obtained regarding GPX activity. It was evident that GPX activity significantly increased in the case of PTT infection. Moreover, the results of SOD and GPX showed a positive, albeit moderate, correlation with each other. In contrast, a negative correlation trend was observed for APX, although the result was not significant.

We also conducted a ranking and categorization into resistance groups for all 206 barley genotypes, based on relative AUDPS values. The resistant (R) genotypes were found in Cluster 3, representing 31% of the collection, while the moderately resistant (MR) varieties represented 32% of the collection in Cluster 1. The moderately susceptible varieties were in Cluster 4, and the varieties most susceptible to PTT infection were placed in Cluster 2. Based on the ranking of genotypes, the Chilga-Barley, which showed the lowest infection value, and Antonella, selected for antioxidant studies and ranked third, were among the top 25 most resistant barley varieties. Antonella demonstrated significant resistance to PTT infection, thanks to its high SOD and GPX enzyme activities. Among the selected barley varieties, Canela ranked 39th and was categorized into the resistant (R) group, while Manas was ranked 76th and became a member of the moderately resistant (MR) group. The last 25 genotypes had average relative AUDPS values between 0.204 and 0.251, with most belonging to the susceptible (S) group. The Palinka genotype was ranked last. The Mv Initium and Harrington genotypes were placed in the susceptible (S) group, and previous research also characterized the Harrington genotype as sensitive to PTT infection. The Klages variety, one of the parental lines of the Harrington variety, was also included, suggesting that its susceptibility to net form net blotch could have been inherited from this genotype.

Based on our results and the literature, it can be said that resistant and moderately resistant barley varieties are recommended for breeding programs and economical cultivation (due to the reduced need for fungicide application) alike, while greater attention must be paid to plant protection for moderately susceptible and susceptible varieties. Genotypes identified as sensitive are not recommended for use in breeding programs aimed at resistance to PTT.

In future research, a greater role could be given to the investigation of other antioxidant enzymes to better understand their role in biotic stress, such as infections by pathogens. The increase in SOD enzyme activity following infection appears to be an important aspect of plant defence, but its relationship to resistance is not yet clear. Understanding the interaction between antioxidant enzymes and defence mechanisms is a key process in developing strategies to improve the resistance of barley varieties and other crops to various pathogens. Moreover, extending studies related to antioxidant enzymes to more genotypes and isolates could provide deeper insights into the relationships between the accumulation of reactive oxygen species and early pathogen infection.

4.1. Literature

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5. New scientific results

1. For the first time in determining the resistance of barley genotypes to net blotch disease (*Pyrenophora teres* f. *teres* Drechs., PTT), we used the AUDPS (Area Under Disease Stairs) method published in 2012 instead of the previously used AUDPC (Area Under the Disease Progress Curve). Based on our results, AUDPS is demonstrably suitable for assessing the extent of infection, especially when it is important to consider the difference between the initial and final recording times since the values of the Tekauz scale and Saari-Prescott scale used for evaluating disease symptoms showed a strong, positive correlation with AUDPS values.
2. Based on studies of five consecutive field seasons using 206 genotypes, we established that the resistance of barley to *P. teres* can be determined based on the R-AUDPS value, which is based on our evaluation methods.
3. We examined the infectivity of four monosporic PTT isolates on 206 barley varieties under greenhouse conditions in two developmental stages (Z12, Z41), showing a significant, moderate strength correlation ($R2=0.349$ $p<0.001$), and established that the different isolates were not equally infective on the tested varieties. The severity of disease development greatly depended on the pathogen isolate.
4. Based on their average infection values in different experiments, barley genotypes were categorized into four resistance categories. We ranked the varieties studied under local conditions based on their susceptibility to net blotch disease, which results could contribute to the identification of resistance sources against the pathogen.
5. By examining enzyme activity changes in five barley varieties, we confirmed that superoxide dismutase (SOD) enzyme activity shows an increasing trend in susceptible varieties following *P. teres* infection.
6. During the investigation of ascorbate peroxidase (APX) enzyme activity, we determined that the enzyme activity change induced by *P. teres* significantly depended on the susceptibility of the genotypes to infection and the infectivity of the isolate.
7. We verified the increase in guaiacol peroxidase (GPX) enzyme activity during PTT infection, however, the changes in APX and GPX activity after infection showed a decreasing trend, which was influenced by the pathogenicity of the isolate used for infection.

6. The author's publication activity

6.1. Publications in international scientific journals with IF

Kunos, V., Cséplő, M., Seress, D., Eser, A., Kende, Z., Uhrin, A., Bányai, J., Bakonyi, J., Pál, M., Mészáros, K. (2022): The Stimulation of Superoxide Dismutase Enzyme Activity and It's Relation with the *Pyrenophora teres* f. *teres* Infection in Different Barley Genotypes. Sustainability, 14(5), 2597. **IF: 3,9**

Éva, C., Moncsek, B., Szőke-Pázsi, K., **Kunos, V.,** Mészáros, K., Makai, S., Sági, L. & Juhász, A. (2023): bZIP transcription factors repress the expression of wheat (*Triticum aestivum* L.) high molecular weight glutenin subunit genes in vegetative tissues. Acta Physiologiae Plantarum, 45(2). **IF: 2,6**

Schwarczinger, I., Kolozsváriné Nagy, J., Király, L., Mészáros, K., Bányai, J., **Kunos, V.**, Fodor, J., Künstler, A. (2021): Heat Stress Pre-Exposure May Differentially Modulate Plant Defense to Powdery Mildew in a Resistant and Susceptible Barley Genotype. GENES, 12(5) 776. **IF: 4,1**

Eser, A., Kassai, K. M., Kató, H., **Kunos, V.**, Tarnava, Á., Jolánkai, M. (2020): Impact of Nitrogen Topdressing on the Quality Parameters of Winter Wheat (*Triticum aestivum* L.) Yield. Acta Alimentaria: An International Journal of Food Science 49(3), 244–253. **IF: 0,57**

6.2. Proceedings

Kunos, V., Cséplő, M., Buza, Z., Bányai, J., Seress, D., Csorba, I., Pál. M., Bakonyi, J., Mészáros, K. (2019): *Pyrenophora teres* f. *teres* fertőzés hatása az árpa szalicilsav/jázmonsav és antioxidáns enzimrendszerére. In Karsai, I. (szerk.): *Növénynemesítés a 21. század elején: kihívások és válaszok: XXV. Növénynemesítési Tudományos Nap,* Budapest, Magyarország, MTA Agrártudományok Osztálya, Növénynemesítési Tudományos Bizottság (2009), 502 p., pp. 139–143, 5p.

Cséplő, M., Bakonyi, J., **Kunos, V.**, Seress, D., Csorba, I., Vida, G., Mészáros, K. (2019): Leválasztott levéltechnika alkalmazása árpa genotípusok *Pyrenophora teres* f. *teres*-szel szembeni fiatalkori ellenállóságának vizsgálatában. In Karsai, I. (szerk.): *Növénynemesítés a 21. század elején: kihívások és válaszok: XXV. Növénynemesítési Tudományos Nap,* Budapest, Magyarország, MTA Agrártudományok Osztálya, Növénynemesítési Tudományos Bizottság (2009), 502 p., pp. 252–256, 5p.

Kunos, V., Cséplő, M., Pál, M., Bakonyi, J., Mészáros, K. (2019): Investigation of the phytohormonal and antioxidant enzymatic changes caused by *Pyrenophora teres* f. *teres* infection in barley. In In: Kende, Z., Bálint, Cs., Kunos, V. (szerk.) 18th Alps-Adria Scientific Workshop: Alimentation and Agri-environment: Abstract book. Gödöllő, Magyarország: Szent István Egyetemi Kiadó (2019) 186 p. pp. 100–101.

6.3. Scientific lectures, posters

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