SZENT ISTVÁN UNIVERSITY

Identification and application of resistance sources against black rot (*Guignardia bidwellii* (Ellis) Viala *e*t Ravaz) in grape resistance breeding

Thesis of Dissertation (PhD)

Dóra Roznik

Budapest

2019

PhD School
Name: Doctoral School of Horticultural Sciences

Field: Crop Sciences and Horticulture

Head of
Ph.D. School: Prof. Dr. Éva Németh Zámboriné
Doctor of the Hungarian Academy of Sciences
Head of Department of Medicinal and Aromatic Plants
Szent István University,Faculty of Horticultural Sciences

Supervisor: Dr. Pál Kozma
Head of department, PhD senior researcher
PTE RIVE, Pécs

Co-Supervisor: Dr. Róbert Oláh
PhD senior researcher
NARIC RIVO, Experimental Station of Kecskemét

The applicant met the requirement of the PhD regulations of the Szent István University and the thesis is accepted for the defence process.

....	....
Prof. Dr. Éva Zámboriné Németh	Dr. Pál Kozma
Head of Ph.D. School	Supervisor

1. Background and objectives

Guignardia bidwellii (Ellis) Viala *et* Ravaz, [anamorph *Phyllosticta ampelicida* (Engleman)] is the causal agent of black rot of grapevine, originates from North America. It has been introduced to Europe/ France in 1885, but has not caused severe epidemics in larger areas of Europe till 2000. The first epidemics in Hungary appeared in 2010 demonstrating that climatic conditions (long rainy periods in May and June) can favour this pathogen. Since 2010 epidemics have spread all over in grape growing regions of Europe, including Hungary, causing severe crop losses. Application of fungicides is the primary strategy used to control black rot, but vineyards of mildew-resistant cultivars are not protected chemically against fungal diseases suffer significant economic losses. However, extended periods of high temperature and humidity are favour for the infection by this pathogen. Uncultivated, abandoned vineyards served as reservoirs of inoculum for black rot.
The fungus is able to infect all green parts of the plant (leaf, shoot, tendril, cluster). In epidemic years it can cause even 80-100 % crop loss. The ideal solution from economic environmental and human health aspects would be production of grape free of chemicals on the base of cultivars with multiple resistances against black rot and mildews.

At the Research Institute Viticulture and Enology of the University of Pécs (PTE RIVE) breeding program was started in 2000 with the aim to create innovative varieties with high resistance against powdery and downy mildews. Newly developed innovative grapes, cultivated without any chemical plant protection, are susceptible to black rot, so in order to avoid considerable economic losses plant protection is necessary. So, it is definitely necessary to incorporate black rot resistance into the newly developed fungal resistant grapes.

The main goal of my PhD research work was the identification of different effective resistance sources for the breeding program, and observation of the inheritance of the putative resistance genes. This study wants to improve our knowledge about the biology of *G. bidwellii*.

To accomplish our goals, we've set the following tasks:

- selection of germplasm materials, producing woody cuttings for 3-4 replicates to screen their black rot resistance, and to identify source materials for breeding program,

- inoculation large-scale experimental plant materials with spores of *in vitro* propagated *Guignardia. bidwellii* and phenotyping of plants for black rot resistance,

- developing effective method for *in vitro* propagation of *G. bidwellii,* and for the infection and incubation process,

- developing effective infection and scoring method to determine the accurate level of black rot resistance on grape berry and cluster,

- determining the correlation between the leaf and berry resistance to black rot,

- creating and artificially infecting different hybrid families to study the inheritance of black rot resistance.

2. Materials and methods

2.1. Methods for *in vitro* propagation and identification of *Guignardia bidwellii*

Guignardia bidwellii isolates were collected in vineyards of the RIVE of Pécs and Eger between 2011-2014. Cultures of *G. bidwellii* were obtained by using a method described by Molitor (2009). Isolates were maintained *in vitro* as described by Jailloux (1992) on ½ PDA and oatmeal agar under continuous fluorescent lights. *G. bidwellii* can be readily identified by morphological characteristics of cultures and conidia.

Identity of isolates used in artificial inoculations were verified two times with molecular techniques, including ITS regions and ribosomal RNA genes sequencing by co-worker of KRF RIVE, Eger. Spores were washed off with purified water from 14-16 days old cultures. The conidium suspension was diluted to 4-6 x 10^4-10^5 spore/ml.

2.2. Experiments for *in vitro* cultivation of *G. bidwellii* and methods to increase the sporulation

The aim of experiments with different media was to increase spore production and proper timing of sporulation. In this experiment, the growth intensity of *G. bidwellii* was tested on three different media (1/2 potato dextrose, oatmeal and malt extract) and those were completed with 5 or 10 V/V% grape must, in 5 replicates. Mycelial growth, colony diameter, colour and form of *Guignardia* colonies were evaluated on the 7th, 14th and 20th days after inoculation. Data processing and statistical analysis were performed with Microsoft Office Excel 2010. Inoculum production was examined on the three basic media and on their modified varieties completed with 10 V/V% grape must. The intensity of sporulation was categorized into 4-scale/group based on the percentage of the pycnidia formed and sporulated at the same time.

2.3. Plant material

Artificial infection for black rot resistance were carried out on grape plants grown in pots. Woody cuttings for 4 replicates were produced from altogether 168 accessions: **12** accessions of French-American hybrids, **61** accessions of Seyve-Villard hybrid's progenies created in European breeding programs, **26** accessions of *Vitis amurensis* x *Vitis vinifera* F_2, BC_1 and BC_2 hybrids created in Hungarian breeding programs, **7** accessions of *Muscadinia rotundifolia* x *V. vinifera* x *V. amurensis* x French-American complex hybrids created in Pécs, **57** accessions of *V. vinifera* cultivars indigenous in subtropical areas of Georgia and **2** accessions of *Vitis vinifera* cultivars indigenous in central Asia. The rootstock variety 'Börner' (*Vitis riparia* x *Vitis cinerea*) and 3 seedlings of *M. rotundifolia* were used as resistant controls, and a *V. vinifera* cultivar ('Furmint') was used as a sensitive control.

Hybrid families created for study of inheritance of black rot resistance can be seen in Table 1. BR5, BR10 and BR16 resistant sources were chosen from the genbank collections of Cornell University USA, Geneva. Based on our experiences a mapping population was created with the black rot resistant variety 'Csillám' in 2015.

Table 1. Hybrid populations created for study of black rot resistance (Resistance source: underlined)

Family	Origin	Pieces
12-14	BR5 (***V. cinerea*** '6524-219' x *V. vinifera* 'Tannat') self-pollination	550
12-15	BR10 (***V. berlandieri*** x *V. vinifera* cv 'Fresno' 58-22) self-pollination	56
11-19	NY06.0516.04 open pollinated	50
11-20	BR16 (***V. rupestris*** 65-43 x Galibert 114-11) x 'Chardonnay'	580
15-4	'SK 00-1/7' (*M. rotundifolia* x *V. vinifera* BC4) x 'Panonija') x 'Csillám' (**Seibel 4643** x Blaufrankish)	126

2.4. Infection method

Leaf, berry and cluster infection with black rot was accomplished in climate chamber and in the field between 2013-2018. For accurate description of the phenological development stages of grapes the BBCH scale was used. Infection was made on three replications per genotypes. Young clusters were infected artificially in case of accessions which showed tolerance or resistance based on the leaf tests. In a 2-weeks-post-bloom stage, clusters of the potted plants were inoculated the same way and incubated under the same conditions as for the leaf tests.

In climate chamber leaves and berries were infected in period of intensive growing, 4-8 leaf stage as well as growing berries using manual sprayer. The infection was carried out under controlled conditions: temperature of 24-27 ^{o}C, diurnal cycle of 16/8h day/night. The relative humidity was 92-96% during the first day after inoculation, then it was reduced to 65-80%. In field the artificial infection of berries of variety 'Csillám' was made at the PTE RIVE in Pécs, on summer of 2017, in parcel 45.

For evaluation of natural infection a special garden (without any chemical protection) was established for testing of resistance to black rot.

2.5. Evaluation of experiments of infection

Disease severity on leaves was evaluated 21 days post-inoculation (dpi). Scoring was based on the number and size of lesions on leaves and shoot, and the presence of pycnidia, using the 5 classification scale described by VIVC: (http://www.vivc.de/resistances/Guignardia-bidwellii-Blatt.pdf). Disease severity on berries was evaluated 21 days post-inoculation for the first time, thereafter resistant phenotypes were observed until full ripening. Scoring system of berry resistance was developed based on our experience of the tests, four categories were distinguished for evaluation (Roznik et al. 2017).

2.6. Statistical analyses of artificial infections

Correlation calculation of Pearson was used to determine the correlation between leaf and berry resistance, by IBM SPSS Statistical Program. χ^2 assay was completed to investigate inheritance of black rot resistance using the critical value table χ^2, as described by Sváb (1973).

3. Results

3.1. Results of experiments with different media and propagation of *G. bidwellii in vitro*

Isolates collected from infected leaves in different times and vineyards developed uniformly on each medium, as it was described in former publications. Our colonies were homologous with the reference control culture got from DSMZ genbank, morphologically, too. Molecular assays were also carried out two times during a four-year experiment.

Results from molecular analysis of the *G. bidwellii* isolates used for artificial infections showed identity with the *G. bidwellii* (accession CBS 237.48) sequence in genbank.

The basic media served as controls, and compared to these there were screened the effect of 5 and 10 V/V% grape must on the mycelial growth of *G. bidwellii*. Media completed with 5 V/V% must (PDAIII, ZABIII, MEAIII) caused a small enlargement of mycelial growth compared to control basic media (PDAI, ZABI, PDAI), it was found as non-significant difference ($p>0,05$). Considering the intensity of mycelial growth, the fastest growth was observed on media containing 10 V/V% grape must (Figure 1).

Sporulation of pycnidia began on oatmeal media contained 10% must (ZABII) on the 7th day after inoculation, and it was followed by other types of medium from the 10th day. There was observed absence or low sporulation on the 14th day on malt extract agar medium (MEAI) and on malt extract completed with 10 V/V% must (MEAII). The most intensive outflow of spores on the entire colony was observed on the ZABII medium. On the 14th and 21st days, the rate of sporulation in each repetition was intense or good.

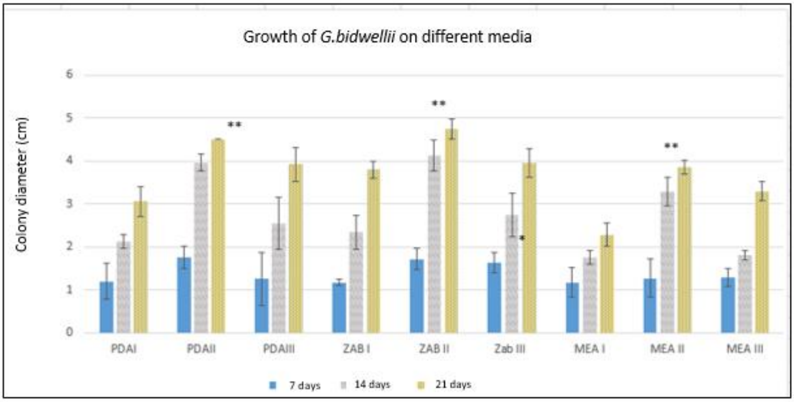

Figure 1. Means of colony diameter of *G. bidwellii* on different types of media for days (** p<0,001).

3.2. Results of searching of resistance sources

Evaluation of the artificial infections of accessions was grouped according to their origin. From the French-American group of varieties, 'Csillám' and 'Chancellor' ('Seibel 7053') showed symptomless (class 9) leaf resistance at each test. 'Seyval blanc' ('Seyve-Villard 5276') also had outstanding resistance, it showed resistance of high level in leaf tests (classes 7-9). In this group, varieties 'Merzling', 'Felicia', 'Villard blanc', 'Teréz', 'GM318-57' and 'Villard noir' were classified as moderately resistant varieties (classes 5-8). All the other tested French-American accessions were classified as susceptible or highly susceptible (classes 1-3). Among *V. amurensis* x *V. vinifera* F_2 hybrids two accessions ('5-11-2', '5-10-6') showed high level of resistance, a third accession ('5-11-6') had medium level of resistance, while all others were classified as susceptible or highly susceptible genotypes. *M. rotundifolia* x *V. vinifera* x *V. amurensis* x French-American complex hybrids, which are the newest candidates for state approval in the breeding program, were equally highly susceptible to black rot disease. The 57 *V. vinifera* cultivars originating from Georgia were in majority susceptible or highly susceptible to black rot. However, two cultivars ('Muradouli' and 'Odjaleshi') showed resistance of medium level (class 4-6) to the disease. The well-known resistant rootstock variety 'Börner' and *M. rotundifolia* seedlings were also symptomless resistant (class 9). The *V. vinifera* variety ‘Furmint’, used as susceptible control, showed very serious symptoms covering the entire leaf.

22 varieties of medium or high leaf resistance were selected for artificial infection of clusters in 2016 to state the correlation between the leaf and berry resistance for black rot. In 2017, cluster infections were repeated four times from bloom until veraison to determine the accurate score of black rot resistance. In case of ‘Csillám’ leaf and berry resistance was coherently symptomless, they were in each phenological stages highly resistant. Variety ‘Seyval blanc’ showed high resistance in each time of evaluation after cluster inoculation. Lesions appeared on the surface of berries similar symptoms were observed only on muscadine grapes. Small, black, superficial, scabby lesions appeared on the berry skin which did not cause the decay of the entire berry as occurs on sensitive cultivars. Few pycnidia developed in the spots. As the berry grew in size the scabs detached from the berry in some cases, leaving intact skin tissue behind. These superficial lesions were observed in case of another four genotypes: ‘Merzling’, ‘5-11-2’, ‘5-10-6’ and ‘5-11-6’ in two successive years. However, in case of the last four hybrids it was observed one short susceptible period after bloom, when berries turned into mummies, infected rate of 5-15% in 2017.

Clusters of varieties with moderate leaf resistance were susceptible or highly susceptible to infection, it depends on variety and their developmental stages. Low correlation was discovered in case of ‘Chancellor’. It showed symptomless leaf resistance at each test, however, two-thirds of the berries developed black rot when clusters were inoculated, and 50-90% of the berries turned into mummies. The control varieties (‘Furmint’ and ‘Bianca’) had almost complete yield loss.

Disease did not develop on berries of ‘Csillám’ when artificial infection was carried out in field at different developmental stages, even though they were left on the shoot until full ripening and the pressure of infection was high. However, there were two phenological stages (BBCH71 and BBCH73) when superficial, scabby lesions were developed on skin of berries. These lesions peeled off traceless under ripening progress, therefore fruits could be harvested. These results confirmed the high level of black rot resistance of ‘Csillám’. Complementary field observations in the special test garden in 2017 under high natural black rot infection pressure were in accordance with results from climate chamber. Appearance and severity of symptoms were the same in both locations.

3.3. Results of black rot resistance infections in hybrid population

The **12-14** and **12-15** seedling populations were evaluated for inheritance of black rot resistance. Mainly susceptible seedlings were found in **12-14** population during 2015-2017 under artificial infections in climate chamber. A high level of black rot resistance (class 6-9) was found in 15 progenies and a moderate level of resistance (class 4-6) at 29 seedlings, while other seedlings showed susceptibility (class 1-3) to black rot. After that, parents being resistant according to database were infected artificially in 2017. The hybrid **BR5** as the parent of **12-14** population was susceptible, black rot symptoms were observed at each test. Our results contradicted the symptomless black-rot resistance of **BR5** hybrid, however, the results of the **12-14** family in climate chamber were confirmed. By summarizing our results, we explored that the family **12-14** is not appropriate for track the black rot resistance gene, similarly to the hybrid population **12-15**. 56 progenies of population **12-15** showed serious black rot symptoms in climate chamber in two successive years (2015, 2016). Hybrid **BR10**, the parent of family was susceptible for rot like all of its seedlings.

Testing the **11-20** hybrid family, our aim was to find black rot resistant sources for our breeding program. Symptomless resistant seedlings were identified in large rate in climate chamber in three successive years. 23 individuals were highly resistant for black rot (class 7-9) consequently regarding both leaf and cluster infection under artificial and field conditions, too. Additional positive characters were observed on selected valuable seedlings derived from ‘Chardonnay’ (recurrent parent of the backcrossing programme), as *V. vinifera* habitus and quality. Only 6 individuals of population **11-19** were resistant, others were susceptible, tested in climate chamber during 2015-2016. Although the individuals of the family had strong growth potential, their degree of resistance was low to pull out them as a breeding material.

Hybrid population **15-4** derived from a black rot resistant 'Csillám' was produced for tracking the inheritance of black rot resistance gene. 126 seedlings were infected artificially. 49 progenies were symptomless resistant, 11 seedlings were moderate resistant and symptoms of black rot developed on 66 individuals. Based on one-year phenotypic evaluation the result of khi square (χ^2) test was 0,28, it confirmed, that the ratio of resistant and susceptible seedlings is 1: 1 at 0,05 significance level. Based on this result it can be supposed that the inheritance of the black rot resistance of variety 'Csillám' is probably dominant - recessive.

3.4. New scientific achievements

1. A fast, reliable, advanced method was developed for the *in vitro* propagation of *Guignardia bidwellii*. The most suitable mycelial growth and sporulation of *G. bidwellii* was obtained on oatmeal agar completed with 10V/V% grape must compared to control media.

2. We described at first the appearance of superficial, scabby black rot lesions on non-muscadine grapes. The scabs detached from the berries in some cases, leaving intact skin tissue behind during the ripening process. Genotypes with this symptom were called resistant, because the internal tissues of the berries were not damaged, with the clusters remaining intact.

3. No close correlation was found in black rot resistance of the leaves and berries of different grape varieties. Consequently, cluster infection must also be carried out to determine the accurate black rot resistance level of genotypes.

4. Variety 'Csillám' was identified as a new and relevant resistance source for breeding work, concerning their outstanding leaf and berry resistance against black rot. Variety 'Csillám' is able to transmit/inherit the high level of black rot resistance to progenies, confirmed by the numerous resistant seedlings in population **15-4**.

5. In hybrid family **11-20**, we found a large number of black rot resistant seedlings, similarly to their symptomless resistant parent **BR16,** which suggests that this crossing combination may also be appropriate to create basic breeding material and map the resistance gene.

4. Conclusions and proposal

Guignardia bidwellii in vitro experiments demonstrated the successful isolation of the pathogen from black rot symptomatic leaves. Isolates were identified as *G. bidwellii* using morphological and molecular process.
The results of *in vitro* assay showed that 5 V/V% must caused low enlargement of mycelial growth, but did not influence consequently the pycnidia formation and inoculum production. Colony diameter enlarged significantly on media completed with 10 V/V% must. However, the effect of must on sporulation activity were found non-significant. The most intensive sporulation was observed on oatmeal agar completed with 10% must. In conclusion, the most suitable mycelial growth and sporulation of *G. bidwellii* was obtained on oatmeal agar completed with 10 V/V% grape must. In order to make adequate inoculum for artificial infection, we used this media in Pécs.

Parallel tests for leaf and berry resistance showed that symptoms found on leaves are not always in accordance with symptoms found on berries. Results of the correlation calculation proved, that there was no close correlation between the results of leaf and cluster infections. Consequently, cluster infection must be carried out to select appropriate resistance sources, and to determine the accurate black rot resistance level of genotypes. Only in the case of 'Csillám' was the leaf and berry resistance coherently symptomless.
High level resistance to black rot of 'Seyval blanc' and 'Merzling' varieties had been reported earlier, but systematic phenotypic analysis both on leaf and cluster was described firstly here. The above mentioned three varieties, derived from North-American *V. rupestris* species, live in coevolution with *G. bidwellii*. Interestingly, among *V. amurensis* x *V. vinifera* F_2 hybrids two genotypes ('5-11-2', '5-10-6') showed high level of resistance both on leaves and berries.
A new type of black rot symptoms was noticed under process of cluster infection, when superficial, scabby lesions appeared on the berry skin, which did not cause the decay of the berry. It was not described earlier in case of the *Vitis* genus before, only on muscadine grapes. Cultivars possessing this kind of berry resistance are of high economic importance, there is no loss of yield.

Vitis vinifera cultivars, including varieties from Georgia, were susceptible to black rot, we did not find any resistance source among them.

Data of the field evaluation and the climate chamber trials gave similar results, but artificial infection must be carried out to determine reliable level of black rot resistance of genotypes.

Variety 'Csillám' could be a potential genetic source for black rot resistance. Owing to its symptomless resistance to black rot and valuable wine quality we suggest 'Csillám', as an appropriate breeding material.

We found families **12-14** and **12-15** as not appropriate for study the black rot resistance gene, because their seedlings were not consequently resistant to black rot. Parents (**BR5, BR10**) being resistant to black rot were selected by results of experiments made in the USA, were susceptible after artificial infection using inoculum collected in Pécs. We did not find any breeding basic material from the population of hybrid family **11-19.** By the study of hybrid family **11-20** (**BR16** x 'Chardonnay') we found a large number of black rot resistant seedlings, similarly to their symptomless resistant parent **BR16**, it is an excellent resistance source and may also be suitable for monitoring the resistance gene. Based on phenotypic data, viticultural and organoleptic examinations, several valuable seedlings were selected for further breeding work.

Evaluating the breeding family **15-4** ['SK 00-1/7' ((*M. rotundifolia* x *V. vinifera* BC_4) x 'Panonija') x 'Csillám'], half of the seedlings (60 pc) showed resistance to black rot. These results can be regarded as 1:1 segregation, it was confirmed by the khi square (χ^2) test calculation.

From this segregation rate we suppose that one dominant gene is responsible for black rot resistance that originates from 'Csillám', so it is a promising resistance source.

5. References

1. Jailloux, F. (1992): *In vitro* production of the teleomorph of *Guignardia bidwellii*, causal agent of black rot of grapevine. *Canadian Journal of Botany,* 70:254-257. p.

2. Molitor, D. (2009): Biologie und Bekämpfung der Schwarzfäule (*Guignardia bidwellii*) an Weinreben. Geisenheimer Berichte Bd. 65. Gesellschaft zur Förderung der Forschungsanstalt Geisenheim, Germany

3. Roznik D., Hoffmann S., Kozma P. (2017): Identification of grapevine accessions highly resistant to *Guignardia bidwellii*, the causal agent of black rot. *Mitteilungen Klosterneuburg,* 67:149-157. p.

4. Sváb J. (1973): Biometria módszerek a kutatásban. Mezőgazdasági Kiadó, 517. p.

6. Publications connected to the dissertation

Papers in impact factored journals:

Roznik D., Hoffmann S., Kozma P. (2017): Screening a large set of grape accessions for resistance against black rot (*Guignardia bidwellii* (Ell.)) *Mitteilungen Klosterneuburg,* 67:149-157. p.

Kiss E., Tóth-Lencsés K., Szőke A., Roznik D., Kerekes A., Veres A., Kozma P. (2017): Origin of cv. 'Csillám' a promising source for black rot resistance. *Vitis,* 56:53-54. p.

Other papers:

Roznik D., Hoffmann S., Csikász-Krizsics A., Kozma P. (2015): Szőlőnemesítési alapanyagok feketerothadás ellenállósága. *Borászati Füzetek*, Külön kiadvány, 150-152. p.

Kozma P., Hoffmann S., Roznik D., Horváth B., Dula Bencéné, Csikász-Krizsics A., Szabó A. (2015): Magas fokú, tartós rezisztenciával és versenyképes minőséggel rendelkező borszőlő fajták nemesítése Pécsett. *Borászati Füzetek*, Külön kiadvány 6-9. p.

Roznik D., Hoffmann S., Kozma P. (2017): Feketerothadás, az új kihívás a szőlő rezisztencia nemesítésében. *Jelenkori Társadalmi és Gazdasági Folyamatok,* 12:(3) 113-117. p.

Roznik D., Hoffmann S., Csikász-Krizsics A., Váczy K., Kozma P. (2014): A feketerothadás (*Guignardia bidwellii*) elleni rezisztencia beépítése az új innovatív szőlőfajtákba. Növénynemesítés a megújuló mezőgazdaságban. Magyar Tudományos Akadémia, Budapest, 384-389. p.

Conference papers (abstract):

Roznik D., Hoffmann S., Kozma P. (2018): A *Vitis rupestris* feketerothadás ellenállóságának kiaknázása a borszőlő rezisztencia nemesítésben. XXIV. Növénynemesítési Tudományos Nap, 2018, Budapest, Összefoglalók, 120. p.

Roznik D., Hoffmann S., Kozma P. (2017): Feketerothadás (*Guignardia bidwellii*) ellenálló források azonosítása szőlőben. XXIII. Növénynemesítési Tudományos Nap, 2017, Budapest, Összefoglalók, 62. p.

Roznik D., Hoffmann S., Kozma P. (2016): Szőlő Feketerothadás ellenálló génforrások felkutatása a rezisztencianemesítés számára. XXII. Növénynemesítési Tudományos Nap, 2016, Budapest, Összefoglalók, 110. p.

Roznik D., Hoffmann S., Csikász-Krizsics A., Kozma P. (2015): Feketerothadás ellenálló szőlő rezisztenciaforrások keresése a nemesítés számára. 61. Növényvédelmi Tudományos Napok, 2015, Budapest, Összefoglalók, 95. p.