Evidence of apple blotch resistance in wild apple germplasm (*Malus* spp.) accessions

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Accepted: 10 November 2020 / Published online: 27 November 2020
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Abstract *Diplocarpon coronariae* causes apple blotch, which results in significant economic losses. Resistance to apple blotch in commercial apple cultivars has not been reported in recent literature. The *Malus* germplasm collection from the Fruit Genebank of the Julius Kühn-Institut that is maintained in Dresden, Germany consists of 516 accessions of about 49 *Malus* species. This apple gene pool was evaluated for resistance to *Diplocarpon coronariae*. A four-year field survey was subsequently followed by artificial inoculation of field-selected accessions. Fifteen accessions that represent nine different species and hybrids were confirmed to be highly resistant following a repeated inoculation assay in the laboratory. The results from this investigation is of high interest to breeders for future apple breeding programmes and investigations of resistance to *Diplocarpon coronariae*.

Keywords *Diplocarpon coronariae* · *Malus* · Wild species · Germplasm · Resistance · *Marssonina coronaria*

The genus *Malus* Mill belonging to the *Rosaceae* family of plants, is subdivided into six sections including

between 25 to 47 species, depending on the taxonomic classification (Robinson et al. 2001, Forsline et al. 2003, GRIN, <https://npgsweb.ars-grin.gov>). The domesticated apple (*Malus × domestica* Borkh.), a prominent member of this genus, is one of the most important fruit crops cultivated in the temperate zone (Hanke et al. 2020). The largest apple producers in the world are China, USA and Poland. Although, apple production for 2018 was 86.1 MMT (FAOSTAT 2020), several diseases threaten its production. One of such diseases is apple blotch, caused by the fungus *Diplocarpon coronariae* (Ellis and Davis) Wöhner and Rossmann) previously designated as *Marssonina coronaria* (Crous et al. 2020).

The disease has increasingly caused economic losses in apple cultivation in China and in organic cultivation orchards in Europe (Wöhner and Emeriewen 2019). Apple blotches appear on leaves between June and August followed by premature defoliation of the tree crown before fruit harvest. Fruit blotches (Sutton et al. 2014) also cause several losses but are less frequent. Different factors and stages of the fungus lead to typical apple blotch symptoms and occurrence worldwide (Reviewed in Wöhner and Emeriewen 2019). Disease control by fungicide application has been shown to protect commercial apple orchards against apple blotch (Dang et al. 2017; Sharma et al. 2018).

Breeding for disease resistance would be another possibility to manage the disease (Aldwinckle et al. 1997), but unfortunately, most apple cultivars are susceptible to the disease (Wöhner et al. 2019). Nevertheless, Yin et al. (2013a) described sources for resistance in wild apple accessions. However, there are no studies

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describing apple blotch resistance/susceptibility in a diverse *Malus* germplasm over several years.

In the present study, we assessed the field occurrence of *D. coronariae* by the assessing leaf blotch symptoms in the *Malus* germplasm collection of the JKI Fruit Genebank between 2014 and 2018. The collection of the Julius Kühn-Institut, Institute for Breeding Research on Fruit Crops consists of 516 accessions representing 49 species and several *Malus* hybrids of the genus *Malus* (Höfer et al. 2014). The ages of the assessed trees ranged from 9 to 15 years old. The collection was originally grafted on the rootstock ‘Bittenfelder Sämling’ in 1998 and planted in 1999. However, several trees were lost due to disease outbreaks, hence were replanted and therefore the trees consist of different ages. The trees were not sprayed with any disease protective agents during the evaluation period.

Apple blotch, cause by *D. coronariae*, was first confirmed in the experimental field of the JKI in 2014 (Wöhner 2015). The assessment of typical symptoms (Fig. 1a) was conducted in September each year (except 2017). Crown defoliation (Fig. 1b) was scored between 2015 and 2018 for trees of each accession. Up to four trees per accession were assessed for crown defoliation, although only one and two trees were assessed for several accession (Fig. S1). The disease incidence was assessed for each accession that showed symptoms of *D. coronariae* as follows: no symptom in all four years = no incidence; symptoms in only one year = low incidence; symptoms in two years = intermediate incidence; symptoms in three years = high incidence; and symptoms in all four years = very high incidence. The percentage incidence of apple blotch was calculated by dividing the number of incidence from four years of evaluation by the number of times evaluations were carried out in the four-year period, and multiplied by 100. To confirm the results of the field observations, we inoculated up to six leaves of the same age of 136 accessions from the *Malus* wild species collection that showed no disease symptoms in the field, in two separate experiments. The *M. ×domestica* cv. ‘Golden Delicious’, which is susceptible to the disease, was used as positive control in the laboratory assay with spores of *D. coronariae*. Briefly, symptomatic leaves collected from the field were used to prepare conidia suspension by squeezing the leaves in a 5 l beaker containing tap water and subsequently filtering through a thin metal filter to

separate the obtained solution from leaf debris. The concentration of the conidia was determined with a haemocytometer (Thoma, Germany). Finally Tween 20 (0.01%) was added to the final inoculum suspension. Conidia concentration of 6×10^5 per millilitre and 79–90% conidial germination was obtained on water agar after 24 h, and used for inoculation. Detached leaves of the 136 accessions were sprayed three times (Wöhner et al. 2019) with inoculum on the abaxial and adaxial side and kept humid over the infection period at a temperature of 24 °C, 12 h diffuse light and 12 h of darkness. After inoculation, petri dishes were sealed with parafilm. Symptom assessment and diseased leaf area measurement using the leaf doctor software (Pethybridge and Nelson 2015) was carried out 14 days post inoculation. The percentage of mean diseased leaf area was calculated. Statistical analyses included Shapiro-Wilk test on normal distribution of the data. The Mann Whitney U test procedure was performed to analyse the data for significant differences between highly resistant accessions (mean disease leaf area < 10%) and the percentage of mean diseased leaf area of susceptible control *M. ×domestica* cv. ‘Golden Delicious’.

In the field observations (Fig. 2a), 183 accessions showed no disease symptom. The disease incidence of 143 accessions was classed as low, 101 accessions were classified as intermediate, and in 65 accessions disease incidence was classified as high. Twenty-four accessions showed symptoms in all four years of field observation and hence their disease incidence was classified as very high (Fig. 2a). The frequency of the disease incidence for each year and the average incidence are outlined in Fig. 2b. The mean number of accessions with typical symptoms of *D. coronariae* was 159 (SD $\pm$ 84.4) whilst the mean number of accessions without symptoms was 285 (SD $\pm$ 110).

Table 1 shows the percentage incidence of apple blotch symptoms per *Malus* species. Twenty-three of the 49 species and *Malus* hybrids evaluated were represented by a number of ≥ 5 accessions and therefore selected for calculation, whereas species with $n < 5$ accessions were excluded from the calculation. None of the 6 accessions of *M. sikkimensis* and 18 accessions of *M. sieboldii* expressed symptoms of *D. coronariae* between 2014 and 2018. Between 59.6% and 71.3% of the accessions from the species *M. prunifolia*, *M. floribunda*, *M. ×domestica*, *M. asiatica* and *M. sieversii*

Fig. 1 (a) *D. coronariae* symptoms on accessions of *Malus* wild species in the gene bank of the Julius Kühn – Institut in Dresden. To avoid mistakes, a scab symptomatic leaf of *M. prunifolia*

(MAL0709) was added to the figure and clearly shows differences between the two leaf diseases. (b) Rating scale for estimating the defoliation of tree crowns in field assessments

developed apple blotch symptoms for the evaluated period.

For the crown defoliation assessment, an overall mean defoliation score of 2.16 (SD $\pm$ 0.81) was obtained for all accessions in the field plot. The lowest mean defoliation score was observed for *M. ×heterophylla* (accession MAL0390) and the highest for three accessions of *M. pumila*. Figure 3 shows parts of the results of crown defoliation assessment for *Malus* wildspecies with accessions $n > 5$.

Results of the two detached leaf experiments showed that several of the 136 accessions, which developed no symptoms in the field observation, developed disease. Only 15 accessions had less than 10% mean diseased leaf (Table 2). Of these, 10 accessions developed no symptoms of *D. coronariae* in both experiments, whereas the other five accessions developed typical symptoms in one or both performed experiments. Figure 4 shows

M. prunifolia accession MAL0175 (without symptoms) and *M. domestica* cultivar ‘Golden Delicious’ (with symptoms) following artificial inoculation. A correlation analyses performed to compare the mean percentage diseased leaf area from all accessions of both experiments showed a correlation coefficient of $\rho = 0.71$ (Fig. 5). The susceptible control ‘Golden Delicious’ developed 90.3% diseased leaf area, and was significantly different ($p < 0.01$) from other accessions.

Diplocarpon coronariae, a major disease pathogen of apple in China, is increasing in significance in European organic production (Wöhner and Emeriewen 2019). A major impact of apple blotch disease is premature defoliation of the tree crown before fruit harvest. Massive defoliation over several years weakens the physiological balance of the host, leading to a reduced vigour and decreased yields (Yin et al. 2013a). Screening for the disease and the subsequent identification of

Fig. 2 (a) Number of *Malus* accessions with different frequencies of *D. coronariae* during a time interval from 2014 to 2018 (without 2017). (b) averaged number of *Malus* accessions with present and absent symptoms of *D. coronariae* for each evaluation year

resistance donors will be crucial for future apple blotch resistance breeding programs. Assessment of large germplasm collections can be performed through field surveys, laboratory assays or with the use of molecular markers, if available (Li et al. 2019). In this study, we performed a four-year field screening for resistance/susceptibility to apple blotch in more diverse *Malus* germplasm than previously reported (Yin et al. 2013a; Li et al. 2012). Following field observations, we identified and selected 136 accessions for further testing. The field observations indicated that disease spreads within the plot and that disease incidence increased from west to the east in the field plot (Fig. S2). This is not surprising given the main wind direction in Dresden-Pillnitz; Germany is from west to east (Surke et al. 2013). Precise determination of resistance/susceptibility to apple blotch is essential for the identification of resistance donors and hence, a detached leaf bioassay is recommended to confirm results from the field screening. Detached leaf inoculation with spores of the pathogen allowed for the identification of 15 highly resistant accessions, a sharp reduction of the number of accessions thought to be resistant from field observation. The discrepancy between field and laboratory observations could be due

to optimal conditions during pathogenesis in the laboratory assay and low infection pressure or unsuitable conditions in the field (Wöhner and Emeriewen 2019). Moreover, artificial inoculations of plants with causative pathogens usually overestimate susceptibility, nevertheless, is the usual practice in determining resistance/susceptibility to plant pathogens (Peil et al. 2020; Kellerhals et al. 2012). However, it is important for future work in apple blotch resistance/susceptibility screening, to develop an automated field phenotyping system such as reported for other apple pathogens and plant species (Wang et al. 2017; Stewart and McDonald 2014; Jarolmasjed et al. 2019).

A variability of susceptible and resistant reactions to *D. coronariae* was observed in this study. Plant susceptible reactions are responses in which infection and spread of the disease occur, producing varying amounts of damage (Dayton et al. 1983). Typical susceptible reactions were observed both in field and laboratory experiments. The multiplication of the pathogen due to the development of acervuli and the spread of the pathogen over the whole leaf induces chlorosis and diseased leaf area. Thus, the measurement of diseased leaf area as performed in this study could prove suitable for

Table 1 Percentage of incidence of apple blotch per evaluated *Malus* species from 2014 to 2018 (without 2017), $n > 5$

<i>Malus</i> species	No. of accessions	No. of observations within 4 years ^a	Incidence of apple blotch ^b	Percentage incidence of apple blotch from evaluations ^c
<i>Malus sikkimensis</i>	6	24	0	0.0
<i>Malus sieboldii</i>	18	72	0	0.0
<i>Malus hupehensis</i>	7	28	1	3.6
<i>Malus coronaria</i>	9	36	2	5.6
<i>Malus sargentii</i>	10	32	2	6.3
<i>Malus sylvestris</i>	38	126	8	6.3
<i>Malus ×zumi</i>	8	32	3	9.4
<i>Malus ioensis</i>	5	20	2	10.0
<i>Malus fusca</i>	5	17	2	11.8
<i>Malus baccata</i>	50	200	32	16.0
<i>Malus toringoides</i>	5	20	4	20.0
<i>Malus komarovii</i>	6	24	6	25.0
<i>Malus spectabilis</i>	8	32	10	31.3
<i>Malus ×robusta</i>	6	24	8	33.3
<i>Malus</i> hybr.	48	182	61	33.5
<i>Malus ×moerlandsii</i>	5	20	7	35.0
<i>Malus ×purpurea</i>	7	26	12	46.2
<i>Malus orientalis</i>	11	43	20	46.5
<i>Malus prunifolia</i>	49	188	112	59.6
<i>Malus floribunda</i>	7	28	17	60.7
<i>Malus ×domestica</i>	34	127	84	66.1
<i>Malus asiatica</i>	5	15	10	66.7
<i>Malus sieversii</i>	109	237	169	71.3

^a Number of observation for each accession per species. Discrepancies in the number of accessions and observations is a result of loss of genotypes due to other pathogens in the field

^b Number of apple blotch incidence per observation

^c The percentage incidence of apple blotch was calculated by dividing the number of incidence from four years of evaluation by the number of times evaluations were carried out in the four-year period, and multiplied by 100

identification of resistance trait to *D. coronariae*. However, both characters should be strongly correlated..

Disease resistance or plant defense mechanisms against fungal diseases can be classified as constitutive (passive) or inducible (active) (Prell and Day 2001). Both mechanisms aim to reduce the extent of pathogen invasion and include structural and chemical factors (Prell and Day 2001). Therefore, future investigation of the 15 highly resistant accessions should include microscopic investigation after inoculation to determine whether spores of *D. coronariae* will develop germ tubes and if structural factors of the host inhibit further spread of the pathogen in the host tissues. Induced resistance can only be observed after the initiation of infection by the fungal pathogen (post-infectious or

active defense). Active responses are cell or tissue responses that limit the severity of infection. Those responses are often seen in single cells (appositions or papillae) or on the tissue level (zone of barrier cells that separates infected cells from healthy tissue). Both interfere with fungal growth by: 1) limiting the translocation of water and nutrition out and toxins or enzymes into the plant cells, 2) producing plant cell walls that are more resistant to fungal penetration, and 3) impending fungal development allowing phytoalexins to reach effective levels. However, these defense mechanisms underlie genetic factors. In addition, major resistance genes against fungal diseases prevent the formation of sporulating lesions, whereas minor gene resistance conditions induce more subtle changes

Fig. 3 Mean crown defoliation of evaluated *Malus* species from 2014 to 2018 (without 2017). At least $n \geq 5$ per species were evaluated for four years. Accessions without defoliation (0%) were scored - 1, less defoliation (up to 20%) – 2, intermediate (up to

40%) – 3, high (up to 60%), very high (up to 100%). The mean of evaluated accessions was calculated per species. The error bars indicate the standard deviation for each species

such as the extent of colonization, growth and sporulation by a pathogen.

D. coronariae – apple host interactions appear to be complex and involve several genes and pathways. Zhou et al. (2012) reported differential expressions of genes of catalase (CAT), hypersensitive-induced response protein (HIR) and calmodulin (CaM), and the involvement of reactive oxygen species (ROS) in a first defense reaction against *D. coronariae* for an apple cultivar, ‘Qinquan’. Yin et al. (2013b) demonstrated a similar defense reaction on

M. sieversii, the primary progenitor of the cultivated apple, when studying the expressions of marker genes for salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) triggered defense pathways. Due to the hemibiotrophic lifestyle of *D. coronariae*, observed incompatible interactions of early defense responses were more associated with SA-signaling and ROS accumulation (biotrophic stage) followed by JA-mediated defense at later infection stages; typical for necrotrophic stage (Yin et al. 2013b). Similarly, resistance related proteins have recently been identified

Table 2 *Malus* wild species accession of the Julius Kühn-Institut collection resistant to apple blotch

Acc no.	<i>Malus</i> species	% mean disease leaf area	SD	Apple blotch leaf symptoms		Mann Whitney U significance (p) compared to ‘Golden Delicious’
				Exp. I	Exp. II	
MAL0419	<i>baccata</i>	0.0	0.0	–	–	<0.01
MAL0778	<i>baccata</i>	0.1	0.3			<0.01
MAL0364	<i>×zumi</i>	0.7	1.3			<0.01
MAL0302	<i>×platycarpa</i>	2.1	3.5			<0.01
MAL1144	‘Evereste’	2.3	3.0			<0.01
MAL0969	<i>sargentii</i>	2.5	3.6			<0.01
MAL0175	<i>prunifolia</i>	3.1	3.9			<0.01
MAL0722	<i>sieboldii</i>	5.4	10.3			<0.01
MAL0278	<i>×sublobata</i>	8.0	6.4			<0.01
MAL0547	<i>×sublobata</i>	8.8	16.4			<0.01
MAL1020	<i>baccata</i>	7.9	8.4	–	+	<0.01
MAL0710	<i>sargentii</i>	8.9	6.9			<0.01
MAL1018	<i>baccata</i>	6.2	6.6	+	–	<0.01
MAL1005	<i>transitoria</i> <i>× floribunda</i>	4.7	3.2	+	+	<0.01
APF0239	<i>× domestica</i> cv. ‘Golden Delicious’	90.3	21.9	+		–

Mann Whitney U significance was calculated according to <https://www.socscistatistics.com/tests/mannwhitney/default2.aspx>

Fig. 4 Leaves of (a) *M. prunifolia* accession MAL0175 and (b) *M. x domestica* ‘Golden Delicious’ 14 days post inoculation with *D. coronariae* spores

using transcriptomic approaches (Feng et al. 2019; Li et al. 2014; Xu et al. 2015). Although, no monogenic resistance

Fig. 5 Correlation of mean percentage of diseased leaf area between inoculation experiment 1 & 2. The correlation coefficient obtained was $\rho = 0.71$

against *D. coronariae* has been identified yet, recent study of Feng et al. (2019) indicate the existence of quantitative resistance. Feng et al. (2019) described the resistance of *Malus baccata* to *D. coronariae*. Interestingly, *M. baccata* is one of the accessions found to be highly resistant in the current study. Although, the resistant accessions found in our study would require further investigations, they present clear evidence of resistance to apple blotch and pave the way for future genetic mapping studies.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10658-020-02156-w>.

Funding Open Access funding enabled and organized by Projekt DEAL.

Data availability The data supporting the findings of this study are deposited into the Open Agrar repository (Wöhner et al. 2020, <https://doi.org/10.5073/20201002-102537>).

Compliance with ethical standards

Ethics declarations The authors declare that ethical standards have been followed and that no human participants or animals were involved in this research.

Competing interests The authors declare that they have no competing interests.

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