Breeding of resistant strawberry cultivars for organic fruit production – Diallel crossing strategies and resistance tests for *Botrytis cinerea* and *Xanthomonas fragariae*.

M. Bestfleisch[1], M. Höfer[1], K. Richter[2], M.-V. Hanke[1], E. Schulte[3], A. Peil[1] and H. Flachowsky[1]

Abstract

Organic strawberry production suffers from high yield losses caused by numerous fungal and bacterial diseases. Two of the most important diseases are the grey mould disease caused by Botrytis cinerea *Pers. (teleomorph* Botryotinia fuckeliana*), and the bacterial angular leaf spot disease caused by* Xanthomonas fragariae *(Kennedy & King). Beside cultivation methods and organic plant protection measures, the development of resistant cultivars seems to be the most promising strategy in order to improve the productivity in organic strawberry cultivation. Therefore, we established resistance tests to determine resistant and susceptible strawberry cultivars and breeding selections. In a first run, 40 different cultivars and selections were tested for their susceptibility towards* B. cinerea *by artificial inoculation of fruits and leaves and evaluation of the disease symptoms. Plants of 40 cultivars were tested for susceptibility to* X. fragariae *by artificial inoculation in the greenhouse. In a diallel crossing approach, 12 commonly cultivated strawberry cultivars have been crossed reciprocally and propagated in a field trial. Important characteristics of the progeny such as ripening time, yield, morphological traits and occurrence of diseases have been evaluated for a period of two consecutive years and lead to the determination of general (GCA) and specific (SCA) combining abilities. Together with the results of the resistance tests we identified a set of genotypes that show resistant characteristics towards* B. cinerea *and might be suitable for use in organic cultivation systems. Furthermore, they can be used for targeted breeding experiments in the future.*

Keywords: *Fragaria × ananassa*, grey mould, angular leaf spot, combining ability

Introduction

The necrotrophic fungus *B. cinerea* causes severe damage in a broad spectrum of host plants (Elad *et al.,* 2007; Williamson *et al.,* 2007) and the control requires high efforts especially in organic farming (Boff *et al.,* 2001) due to the fact that botryticides are not permitted. Warm temperatures and high humidity lead to a high rate of sporulation (Sosaalvarez *et al*., 1995). Hence, the released conidia are spread widely by wind and cause infections of flowers, leaves and fruits. Once the conidia germinated in strawberry flowers, the mycelial growth is temporarily suppressed by high levels of the Flavan-3-ols Catechin, Epicatechin and Proanthocyanidin (Puhl & Treutter, 2008). The content of those substances decreases during ripening of the fruits and the fungus continues its life cycle. Due to the fact that there are numerous factors influencing the disease progress and the pathogen reacts in a rather unspecific way, there are no incidences for a monogenic

[1] Markus Bestfleisch, Monika Höfer, Magda-Viola Hanke, Andreas Peil, Henryk Flachowsky, Julius Kühn-Institut (JKI), Institute for Breeding Research on Horticultural and Fruit Crops, 01326 Dresden (Germany), markus@bestfleisch.de

[2] Klaus Richter, Julius Kühn-Institut (JKI), Institute for Resistance Research and Stress Tolerance 06484 Quedlinburg (Germany)

[3] Erik Schulte, Bundessortenamt - Prüfstelle Wurzen, 04808 Wurzen (Germany)

resistance against *B. cinerea* (Chandler *et al.,* 2006). Therefore breeding of resistant cultivars is challenging and as a first step we tried to find sources of resistance by testing a high number of genotypes.

X. fragariae was first reported by Kennedy and King (1962) in Minnesota, USA. From there it spread worldwide, dispersal occurred mainly through trade and propagation with infected strawberry runner plants. The gram negative bacterium is highly host specific for strawberries. For Europe the disease was first reported in Italy in 1973, later in Greece, Portugal, Spain, Romania and since the 1990's also in Germany. It is listed as a quarantine pest at the European and Mediterranean Plant Protection Organization (EPPO) with the status A2 (OEPP/EPPO, 1986). Yield loss is reported from 5-8 % (Roberts *et al.*, 1997), up to 75 % (Epstein, 1966). The main yield reducing effect is caused by brown spotted calyx which reduces the marketability of the fruit. Until now, there are neither suitable plant protection products nor resistant strawberry cultivars available. Resistant breeding selections US4808 and US4809 have been found (Maas *et al.*, 2000; Maas *et al.*, 2002) but there are no targeted resistance breeding activities. Therefore, our approach is to test a large number of cultivars, selections and Fragaria species to generate beneficial data about the susceptibility towards *X. fragariae* and potential usability in breeding experiments.

In a diallel crossing approach, different genotypes are crossed in a reciprocal way. Each genotype is used both as mother and as father. This approach gives us insight into the inheritance of different morphological traits by calculating the general combining abilities (GCA) of the parents and the specific combining abilities (SCA) of the progeny (Aalders & Craig, 1974; Vieira *et al.*, 2009).

Material and Methods

The field trial area is located near Dresden, Germany at an altitude of 113-118 m. The soil belongs to the soil class Luvisol and the soil type can be characterized as a sandy loam with a pH of 6.0 (2010) and an average soil value of 65. The previous crops were a grass mixture followed by *Tagetes*. The experimental plots were fertilized by 30 t/ha manure in spring 2010 and supplemented with 60 kg/ha P (TSP) and 40 kg/ha N (CAN) in spring 2011. The plant protection strategy follows the guidelines of integrated pest management. In 2011, fungicides were avoided in order to evaluate fungal diseases in the experimental plots.

Diallel crosses of the cultivars 'Antea', 'Arosa', 'Clery', 'Daroyal', 'Darselect', 'Elsanta', 'Florence', 'Galia', 'Madeleine', 'Marmolada', 'Polka', 'Sonata' and 'Yamaska' were carried out in march 2009 in the greenhouse. Each cultivar was used as mother and father except 'Yamaska' which has only female flowers. The seedlings were raised in the greenhouse and planted in the field in august 2009. The total number of crosses made was n=144, with 13 mother and 12 father genotypes. For each cross 15 seedlings were planted in a randomized block design with two replications. Yield, fruit firmness, flowering time, leaf health, position of inflorescences, and number and color of fruits have been measured and evaluated in 2010 and 2011.

The general (GCA) and specific (SCA) combining abilities of the parents and the progeny in the diallel crossing approach was calculated according to Falconer (1984). For the main effects, the GCA corresponds to the difference between the line mean of the cultivar and the total mean of all crosses. Conferring to the interaction between the parents, the SCA can be calculated as the difference between the mean of each crossing and the expected value. Therefore, the expected value consists of the paternal GCA_P, the maternal GCA_M, and the total mean.

For the resistance test against *B. cinerea* we inoculated 15 fully ripened fruits of 40 strawberry genotypes with 5µl droplets of a 10^5 CFU/ml conidial suspension after surface sterilization. We tested 32 cultivars and 8 selections from the Dresden-Pillnitz strawberry breeding program (Figure 1). Inoculated fruits were placed upon wet filter paper in aluminum boxes and incubated at 20°C (14h light, 10h dark) in a climate cabinet for nine days. The boxes were randomized and their positions changed within the cabinet. The degree of fruit rot by *B. cinerea* was evaluated on a scale from 0 (no symptoms), 1 (symptoms <10%), 2 (symptoms 11-25%), 3 (fruit rot 26-50%) to 4 (full fruit rot >50%).

Resistance to *X. fragariae* was evaluated for 40 strawberry cultivars (Figure 2) by targeted infection of the plants in the greenhouse. Frigo plants of the cultivars were brushed with a 10^9 CFU/ml inoculum of the bacteria on the abaxial side of the leaves. For each cultivar, eight to nine plants were tested in three replicates using a randomized block design. After the inoculation guttation was forced by increasing humidity and temperature at the afternoon and cooling down the cabin over night, so that the bacteria could enter the plants through open hydathodes. The infections were evaluated 15, 21, 35 and 62 days after inoculation using a scale from 1 (no symptoms) to 9 (necrotic leaf).

Statistical analysis was carried out with SAS 9.2 using the procedure NPAR1WAY with the Wilcoxon operation (Kruskal-Wallis-Test, α=0.05) and ANOVA (α=0.05). To fulfill the requirements for the analysis of variance, the data from the *B. cinerea*-test were transformed according to Fisher and Yates.

Results

In the *Botrytis cinerea* resistance test the artificially inoculated fruits showed first symptoms of fruit rot after one to three days post-inoculation, depending on the cultivar. The symptoms of rotting increased during the incubation period and after six days the differences between the cultivars were clearly visible. As expected, we could not find totally resistant cultivars.

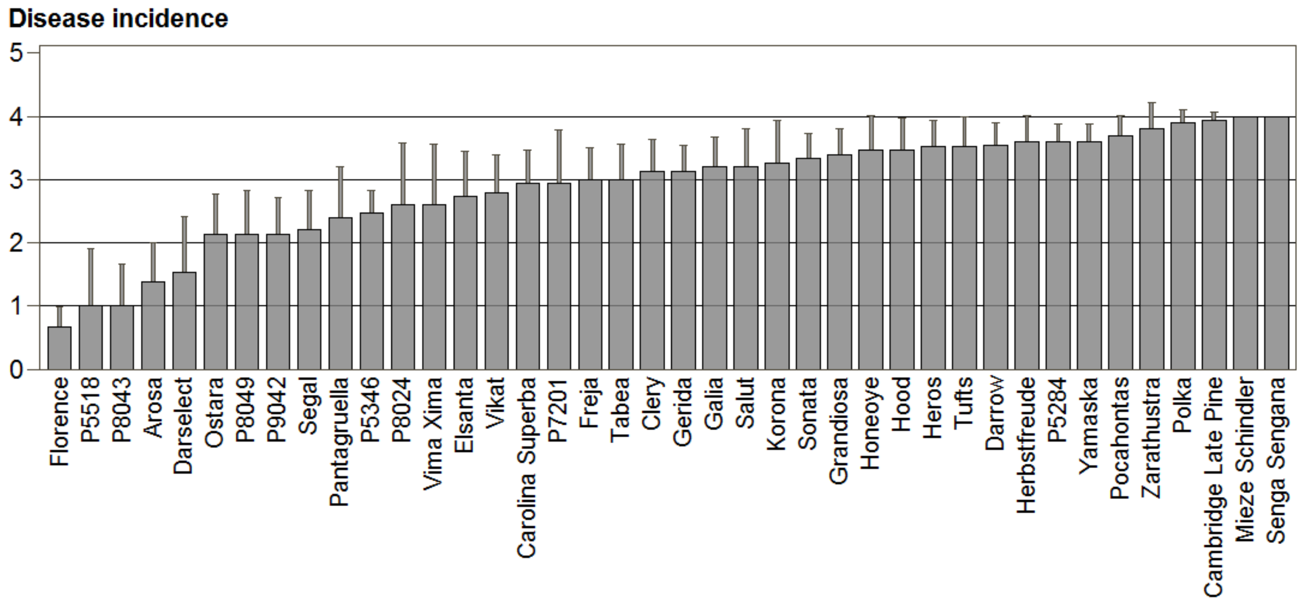

Figure 4: Susceptibility of 40 different strawberry genotypes towards grey mold caused by *B. cinerea*, 6dpi. Scale from 0 (no symptoms) to 4 (full fruit rot), error bars show positive standard error of the means.

The lowest fruit rot symptoms showed the cultivar ‘Florence’ with a mean score of 0.7 which refers to <10 % disease incidence. For P5580, P8043, ‘Arosa’ and ‘Darselect’ we found mean scores for fruit rot between 1 and 2. They are still significantly less susceptible than the cultivars ‘Mieze Schindler’ and ‘Senga Sengana’ with a fruit rot score of 4 (data not shown). The fruits of these cultivars were completely rotten after six days of incubation (Figure 1).

Resistance to *X. fragariae* was evaluated 15, 21, 35 and 62 dpi. The typical symptoms of the angular leaf spot disease became visible after 15 dpi and spread slowly on the infected leaves. The largest differences in susceptibility between the tested cultivars were found at the end of the experiment after 62 dpi. The highest level of disease incidence was recorded for the cultivar ‘Malwina’, followed by ‘Darselect’ and ‘Korona’. With a mean disease score of 3.9 the early flowering ‘Clery’ is the least susceptible cultivar in the test. A similar level of disease incidence showed the cultivars ‘Diana’, ‘Donna’ and ‘Florin’ (Figure 2). We found no resistant cultivar towards *X. fragariae*.

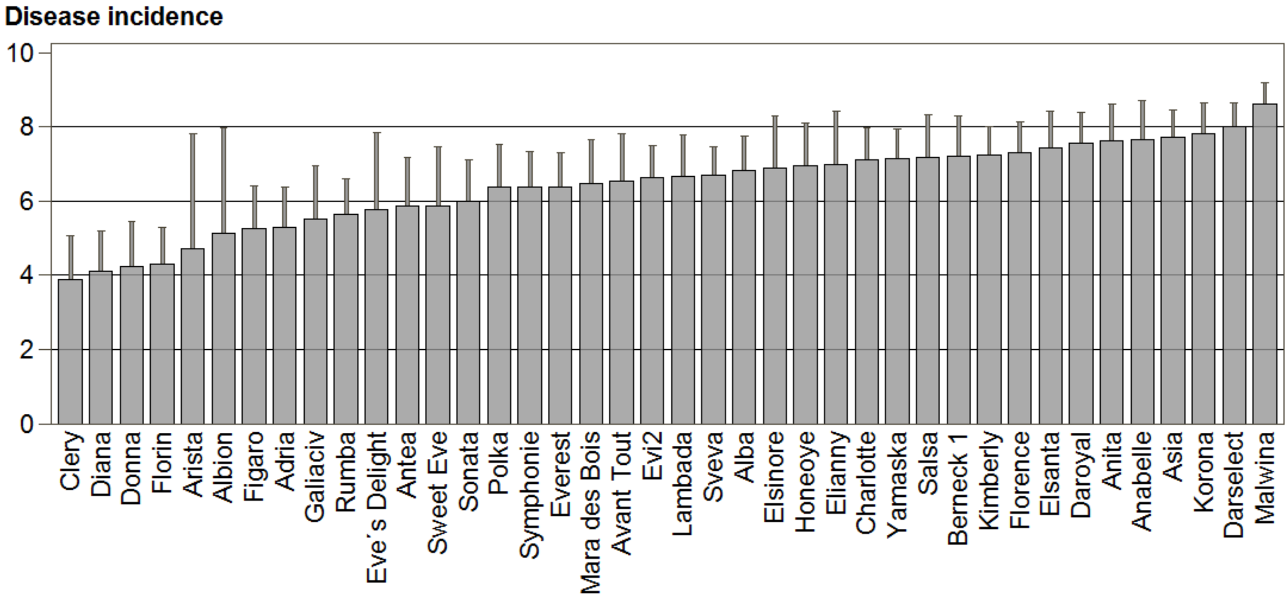

Figure 5: Susceptibility of 40 different strawberry genotypes towards bacterial angular leaf spot disease caused by *X. fragariae*, 62dpi. Scores from 1 (no symptoms) to 9 (necrotic), error bars show positive standard error of the means.

For the ten commonly grown cultivars ‘Clery’, ‘Darselect’, ‘Elsanta’, ‘Florence’, ‘Galia’, ‘Honeoye’, ‘Korona’, ‘Polka’, ‘Sonata’ and ‘Yamaska’ results from the resistance tests to both pathogens, *B. cinerea* and *X. fragariae* are shown in detail in Table 1.

The data show that there is no cultivar with low susceptibility against both pathogens. The cultivars ‘Darselect’ and ‘Florence’ show significantly the slightest fruit rot symptoms, whereas ‘Darselect’ is highly susceptible against the angular leaf spot disease. For ‘Clery’, which is less susceptible to *X. fragaria*, we have to face a *B. cinerea*-score of 3.1 that is not significantly different from the highly susceptible ‘Polka’. ‘Elsanta’ is significantly more susceptible to the grey mold disease than ‘Darselect’ and ‘Florence’ but not different compared to ‘Clery’, ‘Galia’, ‘Honeoye’, ‘Korona’, ‘Sonata’ and ‘Yamaska’ and less susceptible than ‘Polka’. ‘Clery’ and ‘Galia’ have a significantly lower disease score for *X. fragariae* than ‘Darselect’ whereas the differences between ‘Elsanta’, ‘Florence’, ‘Honeoye’, ‘Korona’, ‘Polka’, ‘Sonata’ and ‘Yamaska’ are not significantly different.

Table 4: Susceptibility of selected strawberry cultivars towards *X. fragariae* (*Xf*) and *B. cinerea* (*Bc*) after artificial inoculation.

Cultivar	**Score *Xf* 62 dpi (1-9)**	*	**Score *Bc* 6 dpi (0-4)**	*
Clery	3,9	a	3,1	bc
Darselect	8,0	c	1,5	a
Elsanta	7,4	bc	2,7	b
Florence	7,3	bc	0,7	a
Galia	5,5	ab	3,2	bc
Honeoye	6,9	bc	3,5	bc
Korona	7,8	bc	3,3	bc
Polka	6,3	bc	3,9	c
Sonata	6,0	abc	3,3	bc
Yamaska	7,1	bc	3,6	bc

* Means indicated with different letters are significantly different (Tukey-HSD, α=0.05).

The results of the diallel crossing approach shown in Table 2 are summarized for the paternal and maternal GCA values of the characteristics yield, fruit firmness and leaf health. The general combining ability can be interpreted as a prediction for the mean crossing performance of a cross between two cultivars.

Table 2: General combining abilities (GCA) of 13 strawberry cultivars in a diallel crossing system, GCA is given for each cultivar as mother (maternal) or father (paternal) in the reciprocal cross.

Traits:	**Yield**		**Fruit firmness**		**Leaf health**	
Cultivar	**GCA** paternal	**GCA** maternal	**GCA** paternal	**GCA** maternal	**GCA** paternal	**GCA** maternal
Antea	-2	-19	-0,16	0,27	-0,40	-0,24
Arosa	-4	**77**	0,22	**0,74**	-0,04	-0,11
Clery	-34	-48	-0,12	-0,33	-0,25	-0,17
Daroyal	0	-38	-0,08	-0,34	0,21	0,25
Darselect	1	21	**0,44**	0,23	-0,25	-0,52
Elsanta	22	19	0,42	-0,35	-0,16	-0,42
Florence	-30	-10	-0,05	-0,13	0,05	0,02
Galia	-58	-34	-0,44	-0,06	**0,45**	**0,43**
Madeleine	-5	-52	0,23	0,21	-0,27	-0,04
Marmolada	6	-43	-0,26	-0,26	0,01	0,03
Polka	**85**	**81**	-0,22	-0,37	**0,48**	0,23
Sonata	21	44	0,02	0,15	0,18	0,20
Yamaska		2		0,24		0,31

For the different GCA-effects we find that crosses using ‘Polka’ as a father resulted in yield of 85 g/plant more than the mean of all crosses, which was 245 g/plant. With ‘Arosa’ as mother, fruit firmness was 0.74 scores higher than the mean of all crosses. Leaf health in progenies from crosses with ‘Polka’ and ‘Galia’ as father is 0.48 scores / 0.45 scores higher than the allover mean. In context with the GCA's we calculated the specific combining abilities (SCA's) for each of the 144 crosses (data not shown). Small SCA effects of a cross mean that the prediction of a trait can be estimated by GCA-effects of the parents in a reliable way. For 'Polka' x 'Galia' regarding trait 'yield' we find a very small SCA of 2 and a yield of 269 g/plant; in this case the performance of the cross is realized based on GCA-effects. For the combination 'Darselect' x 'Polka' with a SCA of 1 and a yield of 362 g/plant, the performance of the combination is mainly influenced by the parental GCA effects. In contrast, the progeny of the cross 'Sonata' x 'Madeleine' reach the highest yield in the test field with 410 g/plant and a very high SCA of 117; in this case the performance of the cross could not be estimated truly by GCA-effects of the parents. For fruit firmness, 'Arosa' x 'Elsanta' with a SCA of -0,03 and a firmness index of 6,28 also indicates, that in this case the trait is mainly influenced by the parental GCA effects.

Discussion

As a conclusion it can be expected that the cultivation of less susceptible cultivars like 'Florence', 'Arosa' and 'Darselect' might be a chance to overcome high yield losses due to *B. cinerea* in organic farming, when botryticides are not permitted. In comparison to this approach with artificially inoculated fruits the results may differ under natural conditions. Disease incidence of *B. cinerea* also depends on environmental factors and on the morphology of the plants. High leaf density and the position of inflorescences under the leaves and close to the soil increase the amount of infestation. Furthermore, the results revealed storage characteristics of the fruits of different cultivars at room temperature. Fruits of the mentioned cultivars can be stored longer, in case of 'Florence' six days at 20°C with very little symptoms of fruit rot. To improve the statistical power of the data, the resistance tests have to be repeated over several years. Additionally we investigate the resistance of leaves and flowers to *B. cinerea*.

For *X. fragariae* we found no sources of resistance among the 40 cultivars in the test. Therefore, we will continue of the test using *Fragaria* species from strawberry genetic resources of the Fruit genebank at JKI in Dresden-Pillnitz. In contrast to descriptions in literature, a systemic distribution of bacteria in the plants could not be detected. Subsequently, molecular analysis will be carried out using PCR methods according to Zimmermann *et al.* (2004).

Further crossing experiments based on the best combinations from the diallel crossing approach will be performed and other traits will be evaluated, such as early flowering and susceptibility to the widespread pathogens *Mycosphaerella fragariae* and *Diplocarpon earliana* that cause the common leaf spot disease in strawberry plants. Although the conditions on the experimental field do not fit the guidelines for organic farming, the results improve a targeted resistance breeding process for organic farming.

Acknowledgements

Parts of the presented research activities are funded by grants from the 'Bundesanstalt für Landwirtschaft und Ernährung' (BLE) in the frame of the BÖLN-programme, project no. 2809OE084.

References

Aalders, L. E. and D. L. Craig (1974). Analysis of Fruit Yield and Related Factors in a Diallel of 7 Inbred Strawberry Clones. *Canadian Journal of Genetics and Cytology* **16**(2): 381-387.

Boff, P., P. Kastelein, et al. (2001). Epidemiology of grey mould in annual waiting-bed production of strawberry. European Journal of Plant Pathology **107**(6): 615-624.

Chandler, C. K., J. C. Mertely, et al. (2006). Resistance of Selected Strawberry Cultivars to Anthracnose Fruit Rot and Botrytis Fruit Rot. *Acta Horticulturae* **708**: 123-126.

Elad, Y., B. Williamson, et al. (2007). *Botrytis: Biology, Pathology and Control*. Dordrecht, The Netherlands, Springer.

Epstein, A.H. (1966). Angular leaf spot of strawberry. *Plant Disease* **50**: 167

Kennedy, B. W. and T. H. King (1962). Angular Leaf Spot of Strawberry Caused by Xanthomonas-Fragariae Sp Nov. *Phytopathology* **52**(9): 873f.

Maas, J. L., C. Gouin-Behe, et al. (2000). Sources of resistance for two differentially pathogenic strains of Xanthomonas fragariae in Fragaria genotypes. *Hortscience* **35**(1): 128-131.

Maas, J. L., C. C. Gouin, et al. (2002). Strawberry parent clones US 4808 and US 4809 resistant to bacterial angular leafspot disease caused by Xanthomonas fragariae. *Hortscience* **37**(4): 716-717.

OEPP/EPPO (1986) Data sheets on quarantine organisms No. 135, *Xanthomonas fragariae*. *Bulletin OEPP/EPPO Bulletin* **16**, 17-20.

Puhl, I. and D. Treutter (2008). Ontogenetic variation of catechin biosynthesis as basis for infection and quiescence of Botrytis cinerea in developing strawberry fruits. *Journal of Plant Diseases and Protection* **115**(6): 247-251.

Roberts, P. D., R. D. Berger, et al. (1997). Disease progress, yield loss, and control of Xanthomonas fragariae on strawberry plants. *Plant Disease* **81**(8): 917-921.

Sosaalvarez, M., L. V. Madden, et al. (1995). Effects of Temperature and Wetness Duration on Sporulation of Botrytis-Cinerea on Strawberry Leaf Residues. *Plant Disease* **79**(6): 609-615.

Vieira, R. A., C. A. Scapim, et al. (2009). Diallel analysis of leaf disease resistance in inbred Brazilian popcorn cultivars. *Genetics and Molecular Research* **8**(4): 1427-1436.

Williamson, B., B. Tudzynsk, et al. (2007). Botrytis cinerea: the cause of grey mould disease. *Molecular Plant Pathology*. **8:** 561-580.

Zimmermann, C., J. Hinrichs-Berger, et al. (2004). Nested PCR (polymerase chain reaction) for detection of Xanthomonas fragariae in symptomless strawberry plants. *Journal of Plant Diseases and Protection* **111**(1): 39-51.