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Inheritance of *Sclerotinia* Midstalk Rot Resistance in Elite Sunflower Breeding Germplasm

Abstract: *Sclerotinia sclerotiorum* (Lib.) de Bary is a yield-limiting factor and the major disease of sunflower (*Helianthus annuus* L.) in the temperate regions of the world. In this study, we characterized resistance to *S. sclerotiorum* midstalk rot and morphological traits in a population derived from a cross between two *Sclerotinia* resistant lines. Phenotypic data for 114 $F_{3:4}$ lines and the two parents, $NDBLOS_{sel}$ and K04, were obtained under artificial infection in field experiments which yielded moderate to high heritabilities. Our results suggest that *S. sclerotiorum* resistance is highly quantitative and that different genomic regions may mediate the resistance in different tissues of the plant. We found transgressive segregation for all three resistance traits suggesting that both resistant parents carried complementary QTL. In addition, we investigated the segregation of two known QTL for midstalk resistance and found that one of them also acts as a major QTL in this cross between two resistant lines. Collectively, our results suggest that a QTL stacking approach is a promising way to increase resistance to *S. sclerotiorum* in elite sunflower germplasm.

Keywords: sunflower, Sclerotinia, midstalk resistance, QTL stacking

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Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a major pathogen of sunflower that can cause devastating yield losses of up to 100% (Gulya *et al.*, 1997; Sackston, 1992).

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The fungus is an omnivorous and nonspecific plant pathogen that causes three distinct types of disease on sunflower: wilt, midstalk rot, and head rot. Midstalk rot is of particular importance in Germany and originates from a leaf infection caused by airborne ascospores which enter leaves through wounded tissue and subsequently colonize in the leaves. The infection then progresses down the petioles of the leaves and results in stem lesions. The stalks usually break at the site of infection which in turn results in the loss of the tissues above, including the head.

Genetic and quantitative trait loci (QTL) mapping studies indicated a polygenic inheritance of resistance to *S. sclerotiorum* (Tourvieille *et al.*, 1996; Degener *et al.*, 1998; Micic *et al.*, 2004, 2005a, 2005b; Fusari *et al.*, 2012; Talukder *et al.*, 2014a). Consequently, most of the identified QTL have small effects and explain only a small proportion of the genotypic variance. Previous studies have, however, also identified major QTL affecting stem lesion, for example on linkage group (LG) 8 and 10 (Micic *et al.*, 2004, 2005a, 2005b). In two independent studies the QTL on LG8 explained 36.7 and 25.5% of the genotypic variance, respectively (Micic *et al.*, 2004, 2005a), while the QTL on LG10 explained 24.0% of the genotypic variance (Micic *et al.*, 2005b). Additionally, significant associations between candidate genes and resistance to *S. sclerotiorum* were found (Fusari *et al.*, 2012; Talukder *et al.*, 2014a) indicating that there is a chance to stack different genes for increasing resistance to *S. sclerotiorum*.

No sources of complete resistance to *S. sclerotiorum* are available in elite sunflower germplasm (Talukder *et al.*, 2014b) and thus the development of resistant lines is a major aim in sunflower breeding programs. This may be facilitated by simultaneously introgressing resistance genes from different sources. The line $NDBLOS_{sel}$ has been identified as promising source of resistance against *S. sclerotiorum* and has also been used in the QTL mapping studies by Micic *et al.* (2004, 2005a). The source of resistance in this $NDBLOS_{sel}$ line is unknown because the germplasm pool NDBLOS was derived by bulking 49 B lines selected for high oil (Roath *et al.*, 1987). In our sunflower breeding program, we have identified a second line, K04, which in field trials showed a comparably high resistance to *S. sclerotiorum*. The source of this resistance is also unknown and consequently, both resistant lines may achieve their resistance by the same or different QTL.

We crossed the two resistant lines, $NDBLOS_{sel}$ and K04, to generate a segregating $F_{3:4}$ population with 114 progenies. The objectives of our study were to (1) assess the genotypic variation in this population in order to examine whether both lines carry the same or different *S. sclerotiorum* resistance QTL, (2) test if progenies can be identified in this cross that are superior to their parents

with regard to *S. sclerotiorum* resistance, and (3) investigate if the two major QTL on LG8 and LG10 segregate in this population and assess their contribution to the genotypic variance for the resistance.

Materials and methods

Plant materials, field experiments, and molecular markers: This study was based on a segregating population of 114 diploid $F_{3:4}$ sunflower (*Helianthus annuus* L.) lines derived from a cross between the two parents $NDBLOS_{sel}$ (Degener *et al.*, 1998) and K04 (proprietary line of KWS SAAT AG, Einbeck, Germany). All lines, including the two parents, were evaluated in routine plant breeding trials with three replicates at two locations in 2010. *S. sclerotiorum* culture and plant infection of five plants per replication were done following Micic *et al.* (2004). In addition to flowering time, two morphological and four resistance traits were analyzed:

1. Flowering time measured as days after sowing.
2. Petiole length (PL) measured in centimeters.
3. Petiole length plus leaf lamina (total leaf length, TLL) measured in centimeters.
4. Leaf lesion length (LLL) measured in centimeters around the explant as the length of the brown rotted zone along the leaf vein, one week after infection.
5. Time to stem infection (TSI) measured in days as the time from leaf inoculation until the fungus reached the stem.
6. Speed of fungal growth (SFG) measured in centimeters per day and calculated from the length of the leaf including petiole and the time to stem infection.
7. Stem lesion length (SLL) measured in centimeters as the length of the rotted zone on the stem one month after inoculation.

All genotypes were fingerprinted following standard protocols with the two SSR markers, ORS70 and ORS537. Based on the map of Yu *et al.* (2003) ORS70 is located on LG8 at 58.1 cM and 4.1 cM proximal to ORS243 which in the studies of Micic *et al.* (2004, 2005a) has been mapped 5.3 cM and 1.5 cM distal to the QTL, respectively. ORS537 is located on LG10 at 64.3 cM and 0.5 cM distal to ORS1129, the marker identified by Micic *et al.* (2005b) to be closest to the QTL. Both ORS70 and ORS537 can therefore be expected to be located in close proximity to the two major QTL identified on LG8 and LG10.

Phenotypic data analyses: Lattice analysis of variance was performed using plot means calculated from individual plant measurements for each trait. Components of variance were estimated by the restricted maximum likelihood

(REML) method considering all components in the statistical model as random. Significance for variance component estimates was tested by model comparison with likelihood ratio tests where the halved P values were used as an approximation (Stram and Lee, 1994). Heritability (h^2) on an entry-mean basis was estimated as the ratio of genotypic to phenotypic variance according to Melchinger *et al.* (1998). Furthermore, genotype was regarded as fixed effect and best linear unbiased estimates (BLUEs) were determined for all genotypes and traits. All mixed model calculations were performed using the software ASReml 2.0 (Gilmour *et al.*, 2006).

QTL detection: Significance of the two markers was tested in linear models based on the BLUEs for all seven traits. Markers were declared QTL at $P < 0.01$. The α-effect was obtained from the linear model. The total proportion of genotypic variance (p_G) explained by the significant QTL was calculated by fitting the QTL in a linear model to obtain R^2_{adj}. The ratio $p_G = R^2_{adj}/h^2$ yielded the proportion of genotypic variance (Utz *et al.*, 2000).

Results

First symptoms of *S. sclerotiorum* infection were visible on the leaves of the artificially infected plants after 3 days. The time until the fungus had reached the base of the petiole and infected the stem was shorter for the parent $NDBLOS_{sel}$ than for K04 (Figure 1). However, $NDBLOS_{sel}$ also had shorter petiole and total leaf lengths and consequently the speed of fungal growth was comparable in both parental lines. With regard to leaf lesion and stem lesion K04 was more resistant than $NDBLOS_{sel}$ as smaller lesions were formed on this line. We observed significant genotypic variances for all traits and also significant genotype-by-location interaction variances, except for leaf lesion (Table 1). For all traits the genotype-by-location interaction variance was smaller than the genotypic variance. The heritabilities were intermediate to high and ranged from 0.41 to 0.78. Leaf lesion showed a heritability of 0.41 and stem lesion of 0.59.

The values for leaf lesion ranged from 2.36 to 4.75 cm, those for stem lesion varied from 2.28 to 33.05 cm, and the speed of fungal growth ranged from 0.58 to 1.01 cm per day (Table 1). Histograms of the best linear unbiased estimates (BLUEs) of the 114 $F_{3:4}$ lines are shown in Figure 1. BLUEs for all traits followed a normal distribution. For leaf lesion the BLUE of $NDBLOS_{sel}$ was 3.70 cm and for K04 3.29 cm, and for stem lesion the BLUE of $NDBLOS_{sel}$ was 16.75 cm and for K04 11.21 cm. For all resistance traits $F_{3:4}$ lines transgressed the means of the parents. For leaf lesion and stem lesion, the $F_{3:4}$ lines were on average more resistant than the mean of the parents.

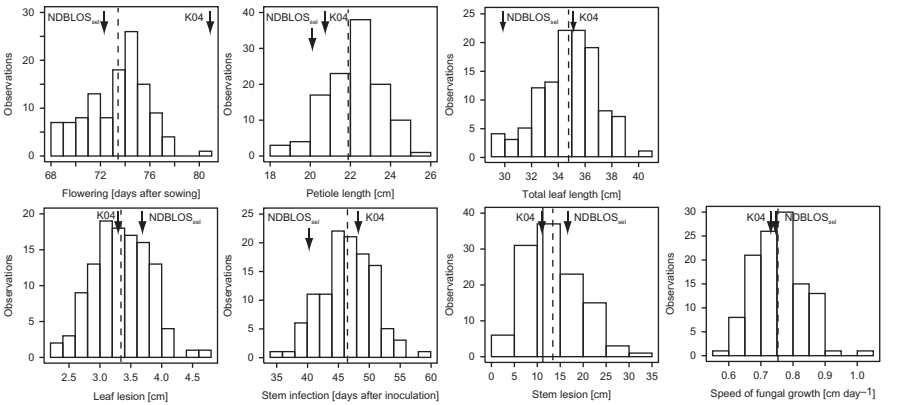

Figure 1: Histograms of the best linear unbiased estimates (BLUEs) of the seven traits analyzed in this study. The performance of the two parents of the population, $NDBLOS_{sel}$ and K04, is indicated by arrows and the population mean as dashed line

Table 1: Mean and range of the 114 sunflower genotypes for the seven traits, variances for genotypes (σ^2_G), genotype-by-location interaction effect ($\sigma^2_{G\times L}$), residual (σ^2_e), and heritabilities (h^2) for flowering time (Flowering [days after sowing]), petiole length (PL [cm]), total leaf length (TLL [cm]), leaf lesion length (LLL [cm]), time to stem infection (TSI [days after inoculation]), stem lesion length (SLL [cm]), and speed of fungal growth (SFG [cm day^{-1}])

Parameter	Flowering	PL	TLL	LLL	TSI	SLL	SFG
Min	68.43	18.79	29.37	2.36	35.75	2.28	0.58
Mean	73.36	22.22	34.76	3.34	46.48	13.45	0.75
Max	80.93	25.00	40.32	4.75	59.73	33.05	1.01
σ^2_G	4.51**	1.14**	3.01**	7.7e-2**	11.25**	20.69**	0.004**
$\sigma^2_{G\times L}$	1.57**	0.60**	1.58**	3.0e-8	5.52**	13.33**	0.001*
σ^2_e	3.50	1.75	5.03	6.8e-1	26.01	46.31	0.009
h^2	0.78	0.66	0.65	0.41	0.61	0.59	0.65

Note: *,**Indicates significance at $P < 0.05$ and $P < 0.01$, respectively.

The two morphological traits were not correlated with leaf lesion, showed a weak correlation with stem lesion, and a medium correlation with the speed of fungal growth (Figure 2). Leaf lesion and stem lesion were not significantly correlated with each other. Whereas leaf lesion showed only a weak correlation with the speed of fungal growth, the correlation between stem lesion and the speed of fungal growth was high.

ORS70, the marker located on LG8, was strongly associated with stem lesion and to a lower extent also with the speed of fungal growth (Table 2).

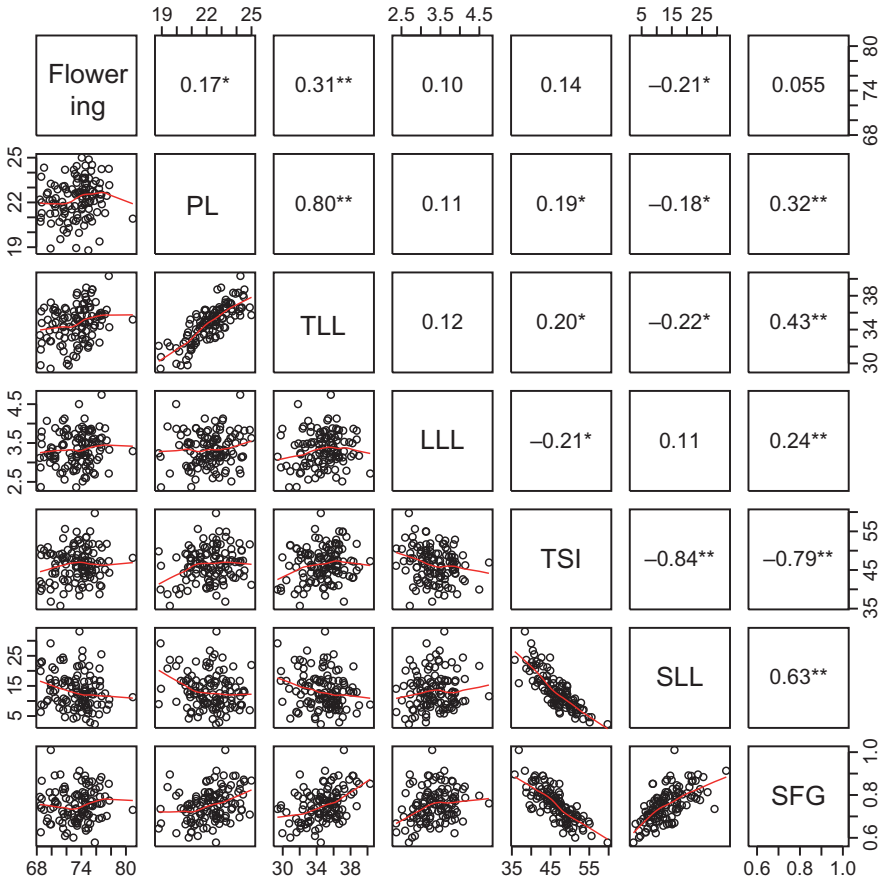

Figure 2: Correlations between phenotypic values (BLUEs) of the 114 sunflower genotypes evaluated for seven traits. The lower part shows the bivariate scatterplots with a fitted line. Flowering time (Flowering [days after sowing]), petiole length (PL [cm]), total leaf length (TLL [cm]), leaf lesion length (LLL [cm]), time to stem infection (TSI [days after inoculation]), stem lesion length (SLL [cm]), and speed of fungal growth (SFG [cm day^{-1}]). (*, ** significantly different from zero with $P < 0.05$ or $P < 0.01$, respectively)

The marker explained 28.0% of the genotypic variance for stem lesion and 8.5% of the genotypic variance for the speed of fungal growth. For stem lesion the allele from the parent $NDBLOS_{sel}$ had an α-effect of −3.86 and for the speed of fungal growth of −0.03. In contrast to its effect on stem lesion, ORS70 exerted no effect on leaf lesion as shown in Figure 3. The marker ORS537 was not associated with neither leaf lesion, stem lesion nor with the speed of

Table 2: *P* values of both tested SSR markers, ORS70 (LG8) and ORS573 (LG10), for all seven traits. Flowering time (Flowering [days after sowing]), petiole length (PL [cm]), total leaf length (TLL [cm]), leaf lesion length (LLL [cm]), time to stem infection (TSI [days after inoculation]), stem lesion length (SLL [cm]), and speed of fungal growth (SFG [cm day^{-1}]). Proportion of explained genotypic variance (p_G in %) and α-effect for the allele coming from parent NDBLOS$_{sel}$ for the QTL (markers significant at $P < 0.01$)

Trait	ORS70			ORS537		
	***P* value**	**p_G**	**α-effect**	***P* value**	**p_G**	**α-effect**
Flowering	0.32			0.57		
PL	0.40			0.008	8.0	0.46
TLL	0.60			0.0002	16.8	1.03
LLL	0.46			0.61		
TSI	0.002	12.0	1.93	0.002	12.1	1.72
SLL	3.9e-6	28.0	−3.86	0.15		
SFG	0.006	8.5	−0.03	0.59		

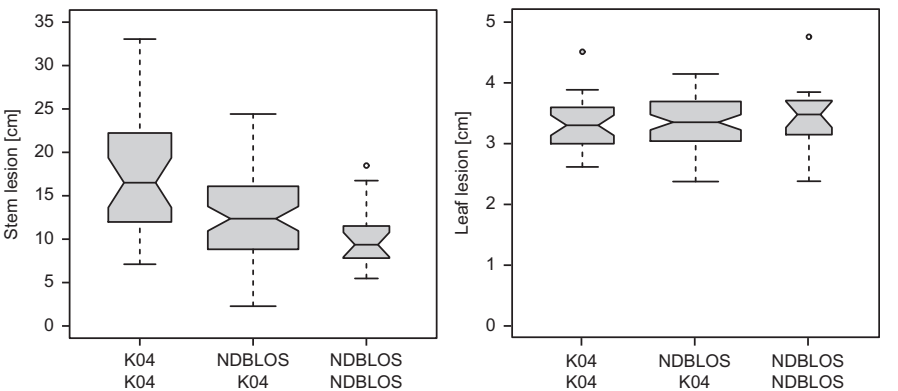

Figure 3: Boxplot for the three genotypic classes at marker ORS70 on LG8 for stem lesion and for leaf lesion. Circles indicate outliers which are outside the extreme of the upper whisker. Non-overlapping notches between plots indicate that the medians of the genotypic classes differ. The variable widths indicate the different numbers of plants in each genotypic group

fungal growth. ORS537 did show significant associations with the two morphological traits and with the time the fungus reached the stem. The proportion of genotypic variance explained by the marker was 16.8% for the total leaf length and 8.0% for petiole length. The allele coming from NDBLOS$_{sel}$ had a positive effect on both morphological traits.

Discussion

Inheritance of midstalk rot resistance

The inheritance of resistance against *S. sclerotiorum* has been reported to be polygenic with medium heritability (Mestries *et al.*, 1998). The frequency distributions for the resistance traits and the observed heritabilities confirmed these findings. In this study, we have evaluated the progeny from a cross between two highly resistant lines, $NDBLOS_{sel}$ and K04. $NDBLOS_{sel}$ had been employed in two previous QTL mapping experiments (Micic *et al.*, 2004, 2005a) where it was crossed with a susceptible line. We found that the other parental line, K04, showed an even higher resistance than $NDBLOS_{sel}$ for both leaf lesion and stem lesion (Figure 1). The quantitative nature of the resistance suggests that many genes are involved, most of them with small effects and only few with medium or large effects. The source of resistance of both parental lines is unknown and may therefore be based on the same or a different set of QTL. The observed transgressive segregation for all four resistance traits suggests that both parents carry in part complementary QTL. In the progeny different favorable QTL alleles from the parents are combined resulting in an even higher degree of resistance against *S. sclerotiorum* than in either parent (see Figure 1). This result illustrates that both resistant parents achieve their resistance by different QTL and consequently a QTL stacking approach appears a promising avenue to increase resistance to midstalk rot in elite sunflower material. The highly resistant plants identified in this cross will serve as a valuable source to further increase the resistance for example by crossing them to other elite lines which likely carry additional favorable QTL alleles. Thus, pyramiding of QTL for *S. sclerotiorum* resistance does not necessarily require crosses with exotic, agronomically less adapted germplasm, but can be achieved by crosses within elite germplasm.

Correlation between traits

We observed weak but significant negative correlations between the two morphological traits, petiole length and total leaf length, with the resistance trait stem lesion (see Figure 2). The time until the fungus reached the stem was also different between the two parental lines. However, while the petiole length between them was comparable, $NDBLOS_{sel}$ had a shorter total leaf length compared to K04 (Figure 1). The difference in time to stem infection can therefore be attributed to the morphological difference between the two lines. This

becomes obvious as the speed of fungal growth was comparable in both parents. The reason for this effect of leaf and petiole length on *S. sclerotiorum* resistance is that the shorter the distance from the site of infection on the leaf to the stem, the faster the fungus can reach the stem, and the more severe the infection. Long leaves are, however, not desired in sunflower breeding and the low correlation suggests that progress in selection can also be achieved based on true resistance genes. Nevertheless, the mechanisms of the true resistance genes are still open. In addition, we observed a low negative correlation between flowering time and stem lesion (Figure 2).

Previous QTL mapping studies (Mestries *et al.*, 1998; Bert *et al.*, 2002) identified different genomic regions responsible for resistance against stem and head rot. In our experiment the correlation between leaf lesion and stem lesion was close to zero and not significant (Figure 2). This indicates that different QTL may be involved in the expression of resistance to mycelial extension in leaves and in the stem as described for example for the resistance of sunflower to *Phomopsis* (Langar *et al.*, 2002). Consequently, to obtain lines with a maximum resistance against *S. sclerotiorum* in stem, head, and root, QTL for resistance in the different tissues may have to be combined.

Assessment of known QTL for *S. sclerotiorum* resistance

In two previous QTL mapping experiments, the resistant line $NDBLOS_{sel}$ was crossed with a susceptible line (Micic *et al.*, 2004, 2005a). These experiments identified a major QTL on LG8 explaining 36.7% and 25.5% of the genotypic variance. The same genomic region also exerted an effect on leaf lesion and the speed of fungal growth and harbored a closely linked QTL for leaf with petiole length. In addition, this QTL was also identified in a QTL mapping experiment based on USDA germplasm (Yue *et al.*, 2008). We found that the marker ORS70, which is located in the QTL region on LG8, showed a strong association with stem lesion and to a lower extent also with the speed of fungal growth (Table 2) but not for leaf lesion (Figure 3). For stem lesion, this marker explained a high amount of the genotypic variance comparable to that in the crosses of $NDBLOS_{sel}$ with a susceptible line. This suggests that K04 did not carry this QTL, despite its high resistance to midstalk rot. We observed no effect of ORS70 on the morphological traits suggesting that both parents may carry identical alleles at this linked locus and that this QTL is, therefore, not segregating in this population.

In another QTL mapping experiment, with a line carrying resistance genes originating from *Helianthus tuberosus*, a major QTL for stem lesion was

identified on LG10 (Micic *et al.*, 2005b). The same genomic region also harbored major QTL for the morphological traits, leaf and petiole length. We did not observe an effect of this QTL on the resistance traits in the population under study (Table 2). There was an effect on leaf length and total leaf length, and as a consequence of that also on the time the fungus needed to reach the stem.

Despite the high proportion of genotypic variance explained by the QTL on LG8, the major part of the genotypic variance must be attributed to as yet unknown QTL. The transgressive segregation observed in the progeny from this cross suggests that also K04 may carry major QTL alleles not present in $NDBLOS_{sel}$. The identification of these QTL will have to await a genome-wide QTL mapping in this population.

Conclusions

We have investigated the inheritance of midstalk rot in a cross between two highly resistant elite sunflower lines. The observed transgressive segregation for all three resistance traits underlines the quantitative nature of this resistance and indicates that an enhanced resistance against *S. sclerotiorum* can be achieved by stacking QTL from different resistance sources. Our results suggest that the resistance against this devastating disease can be further increased through crosses with additional resistance sources.

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Conflict of interest: The authors declare no conflict of interest.

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