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A Decade of Variety Testing for Resistance of Red Clover to Southern Anthracnose (*Colletotrichum trifolii* Bain et Essary) at the Bavarian State Research Center for Agriculture (LfL)

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Abstract: Southern anthracnose is caused by the fungal pathogen *Colletotrichum trifolii* Bain et Essary and affects red clover (*Trifolium pratense*) cultivation, causing severe losses in plant stands. Artificial inoculation with the pathogen in the greenhouse has been proven to effectively differentiate varieties for their resistance based on the survival rates of plants. Additionally, this method was successfully used to improve red clover populations via recurrent selection. However, not much is yet known on its association with resistance behavior in the field. In this study, results from 10 years of artificial inoculation trials at the Bavarian State Research Center for Agriculture were analyzed and compared to official German variety descriptions that are based on field data. A good congruency between survival rates from the greenhouse and official susceptibility ratings were observed. Thus, data from greenhouse tests have great potential to complement official variety lists where gaps exist. It was shown that within only three generations of recurrent selection using the greenhouse test, an existing variety could be significantly improved in terms of its resistance to Southern anthracnose without changing its DUS characteristics. A continuously increasing resistance level in the varieties registered in Germany since 2005 indicates that breeders can successfully respond to the threat imposed by this relatively new disease.

Keywords: greenhouse test; recurrent selection; red clover; resistance; Southern anthracnose; variety differentiation

1. Introduction

Red clover (*Trifolium pratense* L.) is an important crop for diverse crop rotations. As a fodder legume, it fixes atmospheric nitrogen and delivers high protein forage for the feeding of ruminants, making it an essential crop, especially for organic farming. The fungal pathogen *Colletotrichum trifolii* Bain et Essary causes the disease Southern anthracnose in red clover and was first reported by Bain and Essary in 1905 [1] (cited in [2]). The final species designation for the pathogen was then given in 1906 [2]. Southern anthracnose can lead to severe losses of red clover plants in pure stands, as well as in clover-grass mixtures, as observed in the USA and in Switzerland [3–5]. The first report on the occurrence of the pathogen in Germany was published in 2004 [6]. Subsequently, it exerted an increasing impact on red clover cultivation and, in 2009, the characteristic "susceptibility to Southern anthracnose", as determined from natural infections in the field, was for the first time included in the descriptive red clover variety list for Germany [7]. Data available from field trials on resistance to Southern anthracnose in alfalfa (*Medicago sativa* L.) are so far still too limited to include official rankings for this species in the variety list [8].

From 2009 to 2012, an existing testing protocol for variety differentiation based on artificial inoculation with *Colletotrichum trifolii* Bain et Essary in the greenhouse [4] was implemented at the Bavarian State Research Center for Agriculture (LfL) within the project "Securing and improving the availability of organically produced red clover seed by developing selection procedures against seed- and soil-borne fungal diseases for breeding sustainably resistant varieties" (Funding code 28060E161; funding body: Federal Office of Agriculture and Food (BLE)). Since red clover cultivars are heterogeneous populations with different frequencies of alleles conferring resistance to the plant, the level of resistance in a cultivar can be determined by the rate of surviving plants after artificial inoculation. The first results from the experiments showed that resistance levels of various cultivars could be differentiated based on this method and were published in 2010 [9–11]. After its establishment, the testing protocol was adapted to local conditions. Adaptations included the addition of special jets for inoculation on the jet carrier of the automatic irrigation system (irrigation itself was blocked) for spraying, allowing a large number of trays to be inoculated very uniformly and replicable. The protocol was then employed in routine tasks. For example, all varieties of red clover and alfalfa that were newly registered in Germany were tested for their resistance against Southern Anthracnose. By testing each variety at least three times, the aim was to obtain a solid basis of data for the provision of advisory material for Bavarian farmers.

In the above-mentioned project, it was also shown that varieties could be improved in their level of resistance to Southern anthracnose by applying the artificial inoculation protocol in a recurrent selection program [10]. The initial application of this method resulted in an improved level of resistance against Southern anthracnose in the variety Titus. A comparison of DUS (distinctiveness, uniformity, stability) traits in the original and recurrently-selected plant material of variety Titus was performed by the Federal Plant Variety Office (BSA). It confirmed that a given variety could be improved for its resistance against Southern anthracnose without changing its other DUS characteristics.

The resistance of different red clover varieties to Southern anthracnose is also determined by the BSA and this information is published in the official descriptive variety list. However, these classifications are based on spontaneous occurrences in field trials, although it is not known how they are related to resistance determined using artificial inoculation in the greenhouse. In this study, results from the long-term greenhouse trials conducted at LfL were analyzed and compared with the official classification. Thereby, our aims were (i) to assess the level of resistance in red clover varieties registered in different time periods, (ii) to test the relation between levels of resistance determined using artificial inoculation with values reported in the official variety list, and (iii) to test the effect of recurrent selection using artificial inoculation in the greenhouse to improve resistance against Southern anthracnose at the field level with the example of the variety Titus.

2. Materials and Methods

This study is based on 46 red clover and 17 alfalfa varieties that were registered in Germany within the period from 2010 to 2021. These varieties were evaluated for their resistance to Southern anthracnose according to a test based on a method by Schubiger et al. [4]. This method was adapted to the technology available at LfL and optimized for high throughput [9].

From 2011 to 2021, one experiment with a subset of the complete set of varieties was performed per year. Among the experiments, the set of varieties tested was partly overlapping (Details Table S1). In each experiment, single plants of the tested varieties were planted in Quickpots™ trays (single plant pots of 2.8 × 2.5 × 5.5 cm) consisting of 15 rows and 20 columns, resulting in a total of 300 single pots per tray. Each tray was filled with one replicate of two varieties (9 columns × 15 rows = 135 plants each), separated by two columns (30 plants) of a control variety. The four replicates per experiment were arranged in a randomized complete block design. Because the different experiments were conducted over several years, the seeds of the control variety in each experiment were derived

from the same seed lot, which was stored at −20 °C in multiple single portions. Thereby, partial amounts could be thawed for every experiment, ensuring the standardization of the control variety with regard to both genetic composition and quality.

Plants were grown at 22 °C and cut 50 days after emergence. Before inoculation, which occurred 10 days after this cut, the number of plants alive was recorded. The inoculum of *Colletotrichum trifolii* Bain et Essary used in this study was originally isolated from naturally infected plants at the Agroscope field station at Ellighausen, Switzerland (47.6099° N, 9.1403° E). For the production of conidia spores, the pathogen was incubated on potato dextrose agar plates and was grown for two weeks at 18 °C under ultraviolet light. Conidia were then washed from the plates with water and spore density in the solution was set to 3×10^6 conidia per ml. One drop of Tween was added per liter of the conidia suspension. The inoculation was carried out via spray infection with a jet carrier of the automatic irrigation system, amended with additional carriers for the inoculation technique and a compressed air bottle (2 bar). This allowed a uniform speed (6 km/h) to be set and thus a uniform amount of suspension to be applied over all replicates of the experiment. To provide a humid climate for optimal fungal growth, plants were covered with PVC foil. Three days after inoculation, the foil was removed for half an hour and covered again. Five days after inoculation the foil was removed again. Fourteen and 40 days after inoculation, plants were cut. The number of surviving plants was determined 48 days after inoculation.

This test was also the basis for the recurrent selection cycles that led to the improved resistance in the variety Titus (RKL 105), whereby the trays were only filled with seeds of the variety Titus. The selection process was again adapted to the conditions in the greenhouses of the breeding company "Saatzucht Steinach".

The surviving plants were then allowed to pollinate together as a population for the production of seeds. The next test cycle was started with seeds harvested on these plants. To avoid genetic drift in DUS or other traits, a high number of plants per cycle was tested (approx. 15,000 per cycle; the survival rate for the first cycle was approximately 33%, for the second cycle it was approximately 66%, and for the third cycle it was approximately 75%).

The statistical analysis was performed with the SAS program version 9.4 (SAS Institute Inc.). Before statistical analysis, survival rates per replicate of 135 plants, given in %, were angle-transformed. Data were then analyzed using the GLIMMIX procedure [12], which fits statistical models to data with correlations or nonconstant variability and where the response is not necessarily normally distributed.

The following model was used to analyze data from all experiments together

$$y_{ijk} = \mu + \alpha_i + \beta_j + b_{jk} + \varepsilon_{ijk}$$

where y_{ijk} represents the observation for survival rate on a "per replicate" basis, μ denotes the overall mean, α_i is the main effect of *i*-th variety, β_j is the main effect of *j*-th experiment (i.e., testing year), b_{jk} is the random block effect that is nested within the experiment and ε_{ijk} is the residual error. The distributional assumptions are:

$$b_{jk} \sim N(0, \sigma_b^2)$$

$$\varepsilon_{ijk} \sim N(0, \sigma^2)$$

Least square means per variety, experiment, or ploidy group were then calculated using the lsmeans statement function. The letter display for pairwise LS-mean differences was created using the lines option.

The REG procedure from SAS [13] was used to calculate Pearson's correlation coefficients between survival rates and the official susceptibility classification ("susceptibility to Southern anthracnose") by the BSA.

3. Results

The least square means of survival rates per experiment (i.e., testing year) showed considerable variation, ranging from 42.26% in the 2017 experiment to 88.49% in the 2011 experiment (Table 1). This indicates that, despite all efforts to maintain constant technical test conditions, that year effects were clearly present. Least square means of survival rates per variety showed a similar variation, ranging from 44.7% in the tetraploid variety Amos to 79.9% in the tetraploid variety Osmia (Table 2). Significant differences were observed among the 45 red clover varieties, indicating that significant genotypic variation for resistance against Southern anthracnose is present in red clover.

Survival rates per variety, determined based on the inoculation trial in the greenhouse, corresponded well with the official "Susceptibility to Southern anthracnose" classification by the BSA, the latter being based on results from field trials. The coefficient of determination (R^2) from the linear regression of survival rates on official classification ratings was 0.70, with lower susceptibility ratings associated with higher survival rates (Figure 1). Varieties with the highest official susceptibility rating of six showed survival rates from 48.9% (cv. Maro) to 54.8% (cv. Magellan). A somewhat larger range of survival rates was observed for varieties with the lowest official susceptibility rating of two, with survival rates varying from 65.7% (cv. Megalic) to 79.9% (cv. Osmia). This indicates that the accordance between official ratings and survival rates was lower for resistant materials compared to clearly susceptible materials.

The variety Titus could be significantly improved in its resistance against Southern anthracnose via recurrent selection using artificial inoculation in the greenhouse: the survival rate increased from 50.7% to 65.7% and the official susceptibility rating was lowered from six to four (Table 2 and Figure 1, based on preliminary information from the BSA [8] for the reclassification of "Titus" as "Titus recurrent"). Recurrent selection was, therefore, effective to improve resistance to Southern anthracnose also under field conditions.

Table 1. Least square means of survival rates from artificial inoculation trials in the greenhouse per test year (experiment). Least square means with the same letter are not significantly different ($\alpha = 0.05$).

Test Year	Survival Rates (%)		
2011	88.49	A	
2012	87.52	A	
2021	68.70	B	
2010	66.74	B	C
2013	64.25		C
2015	60.84	D	
2014	60.12	D	
2019	53.00	E	
2016	52.54	E	
2018	47.21	F	
2020	44.70	G	
2017	42.26	H	
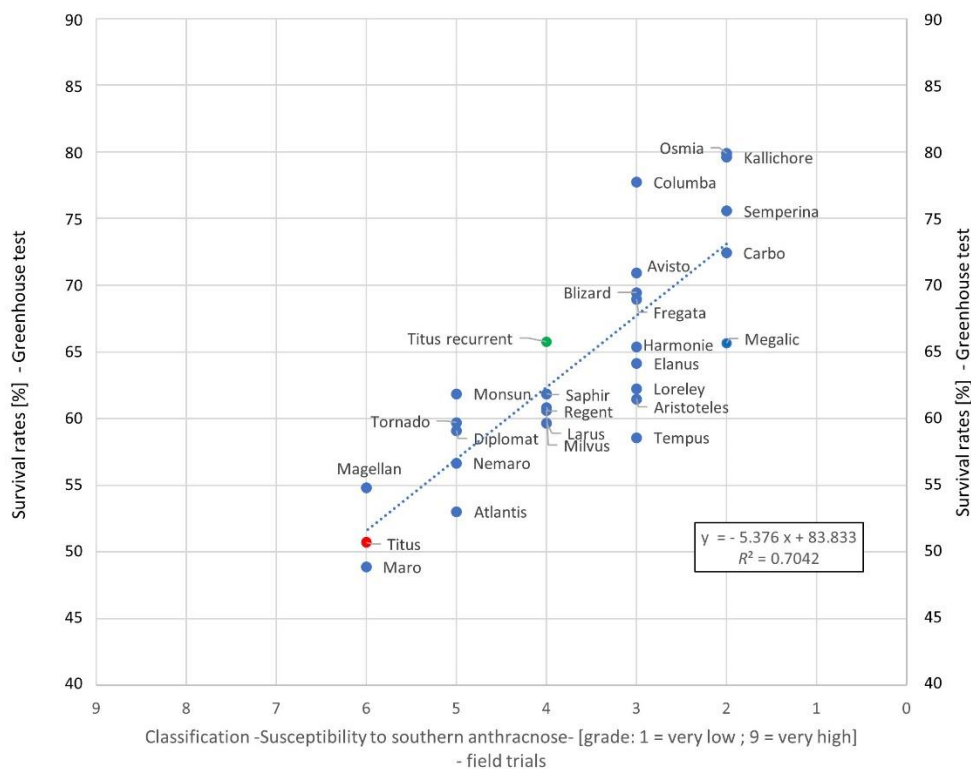
Figure 1. Regression of survival rates from artificial inoculations in the greenhouse on the official susceptibility classification (- Susceptibility to Southern anthracnose -) by the BSA on the basis of data from field trials (Descriptive Variety List 2020). Regression equation and coefficient of determination (R^2) are shown in the box.

Table 2. Ploidy (T = tetraploid, D = diploid), least square means of survival rates from artificial inoculation in the greenhouse and year of registration for red clover varieties. Least square means with the same letter are not significantly different (conservative T grouping, $\alpha = 0.05$).

Variety	Ploidy	Survival Rate [%]	*SE*					Registration Granted	Cancelled
Osmia	T	79.9	1.98			A		2021	
Kallichore	D	79.6	1.41			A		2019	
Columba	D	77.7	1.07	B		A		2017	
Semperina	D	75.6	0.94	B				2015	
Carbo	T	72.4	1.26			C		2019	
Avisto	D	70.9	1.07	D		C		2017	
Avanti	T	69.5	1.40	D		C		2010	2016
Blizard	T	69.5	1.07	D		C		2016	
Pavo	D	69.1	0.65	D				2002	2010
Fregata	T	69.0	1.07	D	E			2015	
Titus (recurrent)	T	65.7	1.26	F	E			(2016) [(1)]	
Megalic	D	65.7	1.41	F	E	G		2019	
Harmonie	D	65.4	0.68	F	E	G		2007	
Lemmon	D	65.3	1.62	F	E	G		2003	
Merula	D	65.1	1.00	F		G		2003	
Elanus	T	64.2	0.90	F		G		2005	
Loreley	D	62.3	1.00		H	G		2015	
Monsun	T	61.9	0.85	I	H	G		2014	

Variety	Ploidy	Survival Rate [%]	*SE*							Registration Granted	Cancelled
Astur	T	61.9	1.26		I	J	H	G		2004	2014
Saphir	D	61.9	1.00		I	J	H	G		2015	
Aristoteles	D	61.5	1.26	K	I	J	H	G		2020	
Montana	D	61.4	1.62	K	I	J	H	G		2001	
Regent	D	60.8	1.06	K	I	J	H	G		2008	
Milvus	D	60.6	0.85	K	I	J	H	G		1997	
Global	D	60.6	1.15	K	I	J	H	G		2002	
Rotra	T	60.6	2.29	K	I	J	H	G	L	1974	
Tornado	T	59.7	1.00	K	I	J	H		L	2010	
Larus	T	59.7	1.15	K	I	J	H		L	2000	
Kontiki	D	59.3	1.26	K	I	J	H		L	2010	2020
Diplomat	D	59.1	1.26	K	I	J			L	2001	
Tempus	T	58.6	1.26	K		J	M		L	1995	
Odenwälder Rotklee	D	57.6	0.70	K			M		L	1955	
Pirat	D	57.5	1.63	K	N		M		L	1995	2013
Taifun	T	57.0	1.07		N		M		L	2004	
Nemaro	D	56.7	1.00		N		M		L	1986	
Magellan	T	54.8	0.94		N		M			2009	
Heges Hohenheimer	D	54.4	1.99		N		M	O		2017	
Lucrum	D	53.2	0.89		N			O		1968	
Atlantis	T	53.1	0.70		N			O		2005	
Merviot	D	51.9	2.81		N		P	O		1983	2003
Titus	T	50.7	0.70				P	O		1994	
Mars	T	49.7	1.26				P			1997	2013
Kvarta	T	49.3	0.66				P			1983	2018
Maro	T	48.9	1.26				P			1990	
Amos	T	44.7	2.81				P			1999	2009

[1] BSA proved that DUS-register characteristics are still indistinguishable from the original variety.

If the least square means of survival rate per variety are compared with the year of registration, a trend towards more resistant varieties is visible (Figure 2). The correlation between survival rate and year of registration was positive but, with an R^2 value of 0.05, non-significant for the period before 2005. However, this correlation became significant and stronger for the period after 2005, with an R^2 value of 0.44, indicating selection progress for resistance against Southern anthracnose.
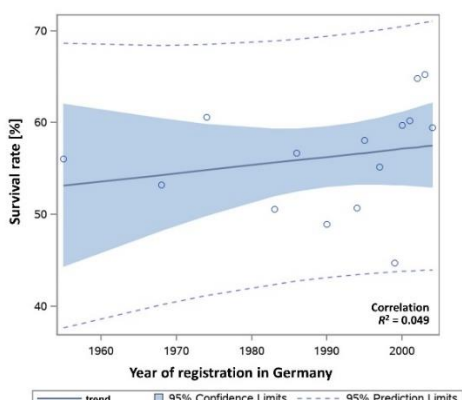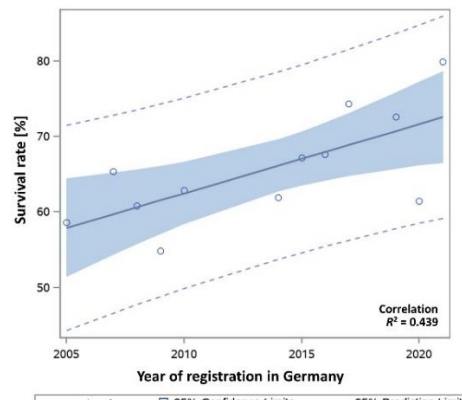
(a)　　　　　　　　　　　　　　　　　　　　　(b)

Figure 2. Mean resistance to Southern anthracnose of red clover varieties newly registered in Germany and the linear correlation of survival rate and year of registration in Germany, including 95% confidence limits (blue area borders) and prediction limits (stitched lines) (**a**) from 1955 to 2004 and (**b**) from 2005 to 2021. Graphs produced by PROC REG in SAS.

The comparison of all tested varieties grouped by their ploidy showed a significant difference in favor of the diploid varieties (Table 3). The analysis of survival rates from the experiments in which alfalfa varieties were also included (test years 2017 to 2021; Table S2) showed significantly higher resistance levels in red clover compared to alfalfa: the mean survival rate of red clover varieties (52.5%) was nearly six percentage points higher than the mean survival rate of alfalfa varieties (46.8%) (Table 3). The comparison among individual varieties itself also showed that the best performing alfalfa variety "Dakota" performed significantly worse than the best red clover varieties, Kallichore, Osmia, and Columba (statistics not shown).

Table 3. Comparison of mean survival rate between diploid and tetraploid red clover varieties, as well as between red clover and alfalfa varieties. Least squares means per ploidy level and species are given. Least squares means with the same letter are not significantly different ($\alpha = 0.05$).

Ploidiy/ Species	Survival Rate (%)	Standard Error	DF	*t*-Value	Pr > [t]	
diploid vs. tetraploid red clover						
diploid	63.09	0.3245	1246	194.32	<0.0001	A
tetraploid	60.03	0.3485	1246	172.28	<0.0001	B
red clover vs. alfalfa						
red clover	52.47	0.4585	627	114.42	<0.0001	A
alfalfa	46.84	0.5730	627	81.74	<0.0001	B

4. Discussion

In this study, we summarized results obtained over a decade of artificial inoculation trials conducted at LfL for testing the resistance of red clover against Southern anthracnose. This represents an update to the results published earlier by Jacob et al. [7] and closes the gaps in the description of varieties registered in Germany with regard to their reaction to inoculation with *Colletotrichum trifolii* Bain et Essary in greenhouse tests. The employed protocol proved to be appropriate for the comparison of open pollinating populations, which are standard in red clover breeding, and significant differences could be found among the different varieties tested. The large differences observed among experiments (i.e., test years) confirmed the need to include a relatively large proportion of plants from a control variety (10% in our case) to control for such experiment-specific effects and additional nuisance variations. In addition, the inclusion of each variety in at least three experiments helped to improve contrasts among them.

4.1. Description of Resistance against Southern Anthracnose in the Greenhouse and in the Field

The comparison of resistance against Southern anthracnose as determined by artificial inoculation in the greenhouse showed a good correlation with the official classification of the BSA, which was based on field data and therefore on natural infections. Hence, our data confirm the earlier observations about the good correlation of greenhouse and field data [14]. The large variation in survival rates for varieties with low official susceptibility ratings (i.e., scores of three or two, Figure 1) were discussed with the BSA. Therefore, the small number of field observations with only lower infestations with Southern anthracnose might be a reason for this weaker association. It is also assumed that, especially with low infestation in the field, mix-ups with damage caused by other biotic/abiotic factors occurs more frequently. In consequence, a scoring of low Southern anthracnose incidence in field trials will be less precise than a scoring of medium-to-high infestation

or a complete lack of disease incidence. Survival rates based on artificial inoculation trials in the greenhouse are, therefore, a valuable source to close the gaps in descriptive lists regarding the classification for susceptibility against Southern anthracnose.

Continuing the comparison of greenhouse and field data (as they have been generated anyway) would be recommended in order to discover any significant changes in the correlation between them. A breakdown in the positive correlation between field and greenhouse data could potentially occur when new races of *Coletotrichum trifolii* Bain et Essary emerge. If these new races overcome the plants' resistance, which has been selected based on the standard races, e.g., those used in this study, a breakdown of the resistance in the field would be observed. Several authors [14–16] have so far reported the occurrence of different races in *Coletorichum trifolii* Bain et Essary. However, the correlation between greenhouse test results and German field results was stable over the comparatively long observation period presented in this study. Thus, at least no races appear to have emerged during this period that could significantly break the existing resistances. We propose monitoring the pathogen over a large proportion of the relevant cultivation areas in Europe in order to gain an overview on its evolution. Some previous studies have already made progress in this respect [14,17]. As described in other studies [14,18], other *Coletorichum* species should not be ignored either.

4.2. Selection for Improved Resistance against Southern Anthracnose

The results for the variety "Titus" are a proof of the concept proposed by Jacob et al. [10] and demonstrate the successful implementation of a scientific project result in practical breeding. Within only three generations of recurrent selection for survival after artificial inoculation in the greenhouse, the official susceptibility rating could be improved by two points. A reclassification of Titus will be implemented in the forthcoming descriptive list of varieties [8]. Our results further show that resistance against Southern anthracnose in red clover is obviously not coupled with DUS traits that allow the determination of the distinctiveness, uniformity, and stability of the variety. Apparently, no co-selection effects on these traits were affected when selecting for the increased frequency of alleles conferring resistance against Southern anthracnose during recurrent selection. At present, it is not yet clear whether the resistance is controlled by only one recessive gene, as already proposed in 1958 by Athow and Davis [19] or whether resistance is controlled by dominant genes [19,20] or by an even more complex process of quantitative inheritance.

The rapid transfer of breeding progress, evident in the improvement of the mean varietal resistance of the subsequent new registrations after the first report in 2004 on the occurrence of the pathogen in Germany, is a positive example of the rapid response of the breeding industry to farmers' needs. The comparison of all the tested varieties grouped by their ploidy shows a significant positive difference in favor of the diploid varieties (Table 3). The selection progress per cycle is generally greater in diploid material as there are fewer masking effects of dominant alleles compared to tetraploid material. An additional reason might be that tetraploid varieties are often created by treating advanced diploid material with colchicine to convert it to the tetraploid state. Therefore, more time is needed for the breeding progress made in terms of resistance against Southern anthracnose to be transferred to the breeding of tetraploid varieties. This is possibly the main reason for the difference, because the group of diploid varieties has on average an older age of acceptance than the tetraploid varieties.

The comparison of the species red clover and alfalfa now shows significant advantages of red clover. This advantage was only made possible through the rapid breeding progress in red clover, as the list of alfalfa varieties registered in Germany changed only slightly in the same period. For a real comparison of the two species in the European area, a larger number of European alfalfa varieties should therefore be included in future trials. Since only a small amount of material has been registered for testing their value for cultivation and use (VCU) in Germany over a long period of time, official trials are only conducted every 4th year at only few sites by the BSA, in addition to simultaneous variety

trials established by the federal states of Germany. Even fewer field data are available for alfalfa compared to red clover (trials are started every 2nd year and at most sites). In order to ensure yield results for alfalfa, these trials also tend to be located in favorable locations that often have lower pathogen pressure. To fill this data gap, it would be helpful to have access to Pan-European field results for alfalfa resistance against Southern anthracnose. With this broader database, researchers should then examine whether the gaps in the official variety lists of alfalfa for susceptibility against Southern anthracnose could also be closed using greenhouse tests. In the field of breeding, [21] shows possible gene sources in annual *Medicago* sp. which may be used for improvements in *Medicago sativa*.

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