Note

Vegetative and generative maintenance of self-incompatibility in six accessions of German chamomile

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Self-incompatible (SI) plants are able to form ideal mother lines for hybrid crossing in hermaphroditic plants, assuring fertilization from the desired father line. To find out suitable ways to maintain SI was the aim of this study. Among 220 plants of German chamomile (*Matricaria recutita* (L.) Rauschert) within six accessions SI-genotypes were selected. SI was determined as staying seedless in three flower heads per plant. Initial SI-plants formed the basic paternal generation (P1) of i) maintaining the same genotypes over six months and repeating seed set analysis (P2) and of ii) conducting crossings in three versions (SI × SI, SI × NSI (not SI evaluated plants) and NSI × SI), thereby producing the F_1 population. F_1 exhibited 78% SI and P2 62% SI, indicating a higher environmental than genetic influence on SI. But heritability, calculated from the results of SI × SI crossings, showed high values ($h^2 = 0.71$). Within generative propagation, the influence of generation/crossing version was highly significant ($p = 0.001$) and the cultivar 'Degumille' explored the highest value of SI (86%) after SI × NSI crossings. Therefore, the intra-cultivar combination of 'Degumille' SI mother plants crossed with NSI father plants can be recommended as the most promising version to maintain SI in chamomile.

Key Words: chamomile, self-incompatibility, *Matricaria recutita*, breeding tool, hybrid crossing, mother lines, heritability.

Introduction

German chamomile (*Matricaria chamomilla* L. (syn. *M. recutita* (L.) Rauschert)) is a valuable medicinal plant, delivering essential oil and dried flower heads. Traditionally German chamomile is used as herbal tea, primarily effective against gastric and intestinal complaints (Ravindran and Pillai 2012). To develop high quality cultivars with desired agronomical traits breeding efforts have been undertaken for more than 60 years (Franke 2009). Pank and Bluethner (2009) expressed the aims of breeding as an increase of medicinally valuable components and suitability for mechanical harvesting.

Specific crossings are the method of choice to combine desired maternal and paternal traits and/or to use heterosis effects in outcrossing crops (Wricke and Weber 1986). In hermaphroditic *Asteracae* like chamomile, possible self-fertilization is to be avoided to ensure fertilization with the chosen crossing partners (Becker 1993, Richards 1997, Stoskopf *et al.* 1993). A—vegetatively propagated—mother line of self-incompatible (SI) plants could form an ideal base for crossings, ensuring the provided partner plants to be the father and the seeds deriving from mother plants to be progenies of desired parents. Without using SI, mother plants would have to be castrated manually or chemically, which would cause enormous stress and decrease reproductivity (Becker 1993).

The challenge in this scope is to develop suitable strategies to maintain self-incompatibility (SI), either vegetatively by recurring cuttings or generatively by using a suitable maintainer line as partner. Other papers already stated the search for genetic constitution of maintenance of SI (e.g. Sijacic *et al.* 2004, Vieira and Charlesworth 2002). In consequence, the present research focused on exploring the effects of vegetative or various generative maintaining methods on SI occurrence in a population of chamomile plants. Furthermore, partners with differing SI constitutions were individually crossed to get an idea of maternal or paternal inheritance and to work towards best breeding results in the next generation. As already done for other intrinsic values of German chamomile (Franz 1993), investigating the heritability of the SI trait would give an answer to the question of suitability of individual selection procedures in breeding. Some previous work on self-incompatibility in German chamomile showed that there is a significant effect of cultivar on

this trait and that diploid cultivars tend to stronger self-incompatibility (higher outcrossing rate) than tetraploid ones (Faehnrich *et al.* 2013a). Therefore focus was laid on diploid (2*x*) cultivars in this study. Four diploid accessions ('Degumille' (DEG), 'Germania' (GER), Promyk' (PRO) and 'USA' (USA)) and two tetraploid ones ('Hungarian 1' (HUN 1), 'Lutea' (LUT)) were comprised in the experiments.

Normally genetics of plants should not change during life time; solely a certain genetic variability and consecutively an occasional loss of chromosomes in auto-tetraploid genotypes (like in the colchicine induced 4*x*-accessions of chamomile) could happen (Cassells and Morrish 1987, Faehnrich *et al.* 2013b, Gottschalk 1976, Mar'yakhina and Butenko 1974, Otto 2007). According to earlier trials with German chamomile, exploring ploidy level and formation of aneuploidy at the beginning and at the end of a five month lasting period, no directed change or loss of chromosomes should be expected (Faehnrich 2013, personal communication). Genetic stability therefore could be assumed and phenotypic alteration should be due to environmental changes. Nevertheless, differing gene expression as a response to environmental changes/stress and/or elapsed time/senescence concerning labile phenotypic traits is an often observed effect in commercial plants (Havaux 2014, Li *et al.* 2012, Wen *et al.* 2015, Westneat *et al.* 2015). This effect could also play a role in recent maintenance experiments, and could be a reason for the high environmental influence on SI in spite of the high heritability values. However, cultivar specific reactions suggest possible certain SI maintaining strategies.

Materials and Methods

General

To start the recent trials a population of 220 German chamomile (*Matricaria recutita* (L.) Rauschert) plants among six varieties ('Degumille' (DEG), 'Germania' (GER), 'Hungarian 1' (HUN 1), 'Lutea' (LUT), 'Promyk' (PRO) and 'USA' (USA)) was grown in the green house of

the University of Veterinary Medicines, Vienna (**Table 1**). 'Degumille', 'Germania', 'Lutea', and 'Promyk' are described varieties since 1977, 1962, 1995 and 1992, respectively (Heine *et al.* 2009). 'Hungarian 1' is an accession provided by the Faculty of Horticulture of the University of Budapest and was proven to be 81% tetraploid (Faehnrich *et al.* 2013a). 'USA' is a trade accession from the referring country. 'Degumille', 'Germania', 'Promyk' and 'USA' are diploid; 'Lutea' is a tetraploid variety (Plescher and Sonnenschein 2013, Schilcher 1987).

SI-examination

Cuttings were produced from each plant of the starting population before evaluation of self-incompatibility. To prove SI, single plants were isolated with micro-perforated Crispac-bags (Baumann, Waldenburg, Germany) and hand pollinated not fewer than three times. As soon as flower heads ripened (ray flowers turned downwards, disk flowers turn from yellow to brown), three flower heads were collected, dried in an oven (30°C, not shorter than one week) and seed set was examined under light microscope at 100-fold magnification. Ripe and normal shaped achenes (with a length of 1–2 mm and a width of app. 0.3 mm, striped, slightly curved) were assumed to bear fertile seeds and were counted. Plants displaying a negative seed set in all three examined flower heads were determined as self-incompatible.

Vegetative maintenance

Plants raised from cuttings from initially selected SI-plants were kept as the first parental population (P1). In Oct. 2014 this P1 population included 61 individuals among five chamomile varieties (**Table 1**). The first part of the trial maintained the individuals (partly by generating cuttings) and therefore the genetic material of P1 for six months, until new SI determination in March 2015. Except of some perished plants, this population survived and was called P2. Thereby the SI maintenance over varying environments (elapsed time, changing growing conditions) at stable genetic conditions could be assessed (**Table 1**).

Table 1. Total number of plants in the starting population and number of evaluated plants in each other determined population of the trial (N). Mean values of SI-development (SI = 1.00, non SI = 0.00 per plant), standard deviation (SD) of all mean values in all determined populations, separated by variety. Missing values indicate that there are no evaluable plants available in the respective group

	Starting population			P1			P2			F_1 after SI × SI			F_1 after SI × NSI			F_1 after NSI × SI			F_1 total		
Variety	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
LUT	40	0.20	0.41	7	1.00	0.00	0			3	0.33	0.58	0			2	0.50	0.71	5	0.40	0.55
HUN 1	49	0.36	0.50	14	1.00	0.00	6	0.50	0.55	0			0			0			0		
USA	41	0.00	0.00	0			0			0			0			0			0		
GER	40	0.38	0.52	1	1.00		2	0.00	0.00	0			1	1.00		3	0.33	0.58	4	0.50	0.58
DEG	40	0.34	0.48	38	1.00[i]	0.00	16	0.75	0.45	68	0.82[hi]	0.38	35	0.86[hi]	0.36	31	0.71[h]	0.46	134	0.81	0.40
PRO	10	0.60	0.55	1	1.00		2	0.50	0.71	0			0			0			0		
Total	220	0.31	0.46	61	1.00[aef]	0.00	26	0.62[b]	0.50	71	0.80[cd]	0.40	36	0.86[de]	0.35	36	0.67[c]	0.48	143	0.78[g]	0.41

[a,b] Different letters indicate significant differences at probability error level α = 0.05 between P1 and P2.

[c–e] Different letters indicate significant differences at probability error level α = 0.05 between P1 and crossing versions of F_1.

[f,g] Different letters indicate significant differences at probability error level α = 0.05 between P1 and F_1 total.

[h,i] Different letters indicate significant differences at probability error level α = 0.05 between P1 and F_1 crossing versions in 'Degumille' (DEG).

Table 2. P-values after two way univariat analyses of variance for the influence of generation/crossing/environment, cultivar and interactions, respectively, on the occurrence of self-incompatibility (SI) in the relevant populations (databases)

Factor of influence	P1 + P2	P1 + F_1 crossing versions	P1 + F_1 total	P2 + F_1 total	F_1 crossing versions
Generation/crossing/environment	<0.001*	0.001*	0.004*	0.143	0.257
Cultivar	0.064	0.606	0.282	0.014*	0.345
Interactions	0.057	0.207	0.126	0.241	0.416

* Significant at a level of error probability of $\alpha = 0.05$.

Generative maintenance

The second part of the trial used cuttings of the P1 population for intra-cultivar crossings. Defined SI plants of P1 as mother were crossed with defined SI plants as father (SI × SI) and with 'normal', not SI evaluated plants (so called NSI plants) as father (SI × NSI). The third version (NSI × SI) incorporated crossings of NSI plants as mother and SI plants as father. To avoid misleading fertilization all plants were isolated as a whole with Crispac-bags and hand pollinated. Self-fertilization of NSI mother plants therefore could happen and was not eliminated to avoid additional stress through emasculation. Total F_1 generation comprised 215 progenies within all three crossing versions, among which 143 could be SI evaluated (**Table 1**).

Statistics

Statistical analyses were performed with SPSS 20 program (IBM, Armonk, USA). Experimental unit was the single plant individual. Influence of cultivar and environment, generation and/or crossing version on SI development in defined groups of plants was assessed by two way univariat analyses of variance. If applicable, post hoc tests were performed according to Student-Newman-Keuls. All statistical analyses were conducted at a level of probability of 5%. Determined SI (3 × 0 seeds) was given the value 1.00 (equal to 100% SI), plants with positively evaluated seed set were given the value 0.00 (equal to non SI). The mean values displayed in tables vary between 0.00 and 1.00, indicating SI percentages of the respective group between 0% and 100%. In case fewer than three flower heads ripened to seed harvesting stage, data of this plant were not included in the calculations. Cross tables and chi-square tests (Pearson) evaluated the concordance of SI distribution with expected values. Realized heritability (h^2) of SI was calculated via equation $h^2 = R/S$ (Becker 1993). S indicates the difference of mean of the starting population and the mean of the SI selected population P1 and R indicates the success of selection, i.e. the difference between the mean of the starting population and the mean of the F_1 after SI × SI.

Results

Influence of environment—comparison of P1 and P2

To evaluate the influence of changing environment at stable genetics, SI appearance of P1 in Oct. 2014 compared with P2 in Mar. 2015 was tested over all cultivars/varieties by analysis of variance. The environment showed highly significant influence with a p-value of <0.001. Cultivar turned out not being significant (p = 0.064). Still, this p-value indicates a certain tendency, even underlined by the p-value for the interaction cultivar*environment of 0.057 (**Table 2**). Despite of non-significant results, post hoc tests for the influence of cultivars on SI maintenance displayed two subgroups (**Table 3**). The high mean value of 'Lutea' (1.00) over both populations (P1 and P2) can be neglected, as 'Lutea' only occurs in P1 (**Table 1**). Nevertheless, remarkable is the high value of 'Degumille' of 0.93, as this can be seen as a sign of a high SI stability over changing environments for this cultivar (**Table 3**). Also the strong tendency of the cultivar*environment interaction as described above underlines this approach. Therefore, a special focus on heritability calculations of 'Degumille' will be given. Chi-square tests checking the distribution of self-incompatible plants (16 in P2) and non-self-incompatible plants (10 in P2) against the expectations of the original P1 population (61 SI plants, 0 non-SI plants) showed highly significant values for the asymptotic p-value of 0.000 and $\chi^2 = 2346141.54$ (**Table 4**). Also the studentized residuals of −10 for SI plants in P2 and of 10 for non SI plants in P2

Table 3. Student-Newman-Keuls post hoc tests for SI development of cultivars over P1 and P2. Mean values of homogenous groups in subgroups are displayed, $\alpha = 0.05$. Value 1.00 indicates 100% SI of the respective group, value 0.00 indicates 0.00% SI

Cultivar	N	Subgroups	
		1	2
GER	3	0.33	
PRO	3		0.67
HUN 1	20		0.85
DEG	54		0.93
LUT	7		1.00

Table 4. Results of chi-square-tests (Pearson), displaying asymptotic significance (p-value) and chi-square-value (χ^2-value) to reveal differences of observed and expected SI distribution in respective populations

Observed distribution of SI in	Against the expectation of			
	100% SI (P1)		Mean values of F_1 total (78% SI, 22% non SI)	
	P-value	χ^2-value	P-value	χ^2-value
F_1 after SI × SI	<0.001*	168371.89		
F_1 after SI × NSI	<0.001*	42352.50	0.115	4.327
F_1 after NSI × SI	<0.001*	243984.00		
P2	<0.001*	2346141.54	0.066	3.373

* Significant at a level of error probability of $\alpha = 0.05$.

show a highly significant decrease of SI occurrence and a highly significant increase of non SI plants in the course of time. These results indicate a strong influence of environment on SI development, maybe stronger than genetic influence. Yet, some cultivars exhibit more stable SI maintenance ('Degumille', mean 0.93) than others ('Germania', mean 0.33, or 'Promyk', mean 0.67).

Influence of generation—comparison of P1 and F_1

In this research approach the situation is more complex because three crossing versions (SI × SI, SI × NSI, NSI × SI) act besides cultivars and generation as additional influencing factors. Distribution of SI development and number of plants in all cultivars and test populations can be checked in **Table 1**. Noticeable is the general high number of 'Degumille' plants in all variants, revealing a strong vigor and robustness of this cultivar. Per univariat two-way analysis of variance the SI development was checked taking into account the influencing factors 'generation/crossing' with the mean values of P1, F_1 after SI × SI, F_1 after SI × NSI and F_1 after NSI × SI and the 'cultivar'. Cultivar does not show any effect, but effects of generation/crossing is highly significant (p = 0.001, **Fig. 1**). No influence of interactions between cultivar and generation/crossing on SI development is identifiable (p = 0.207, **Table 2**). Exploring post hoc tests of the generation/crossing-versions, the F_1 generation after SI × NSI with the mean value of 0.86

exhibits the closest value to the 100% SI of the P1 generation and can be found in the same subgroup (**Table 1**). By looking at the results over all cultivars and generation/crossing versions again 'Degumille' with the F_1 version of SI × NSI attracts attention by developing the highest value (0.86, **Table 1**) of SI. Therefore analysis of variance for all generation/crossing versions was conducted for 'Degumille' only and showed significant results (p = 0.007, **Table 1**). Apparently, 'Degumille', with self-incompatible mother plants crossed with non-self-incompatible father plants provides the best option to keep SI over generations.

Considering the distribution of SI and non SI plants in the different crossing combinations and comparing by chi-square-test with the expected distribution of the parental generation (P1; 61 SI and 0 non SI plants), a highly significant asymptotic deviation (p < 0.001) occurs for all crossing variations. χ^2 is 168371.89 for SI × SI, 42352.50 for SI × NSI and 243984.00 for NSI × SI (**Table 4**). Studentized residues show –/+ 14, –/+ 5 and –/+ 12 for SI and non SI plants in F_1 after SI × SI, after SI × NSI and after NSI × SI, respectively, and therefore indicate a highly significant deviation of the 100% SI—population P1.

Statistically comparing the mean values of P1 and F_1 total a highly significant effect of generation is displayed (p = 0.004, **Table 2**), whereas the effect of cultivar and interactions, respectively, show no influence (p = 0.282 and p = 0.126, resp.). However, it seems clear that in general there will be a lessening of SI abundance from the P1 to the F_1 generation. The question is rather, if and how there is a difference of SI distribution between the different crossing variations of the F_1, because—in case of genetic determination of the trait—paths of heredity and in consequence preferable ways of breeding could be revealed. Due to the high number of SI × SI progenies and consecutively high number of SI plants in this population a significant distinction of SI × SI against the other F_1 populations seems probable (**Fig. 1**). But chi-square-tests (Pearson) after generated cross tables of the SI/non SI distribution in F_1 populations and against the expectation of the mean values of these populations show an asymptotic p-value of 0.115 and $\chi^2 = 4.327$. Therefore no oriented distinctness can be stated (**Table 4**). Even the two way analysis of variance shows no influence of cultivar or crossing version on SI occurrence (**Table 2**).

Influence of maintaining method—comparison of P2 and F_1

The phenotypic appearance of a trait depends on genetics, on the environment and on interactions between both (Falconer 1984). To weigh the impact of environment and genetic heredity on SI development, starting from a general 100% SI test group (P1), the comparison of P2 and of F_1 can give valuable information. As the percentages of SI in P2 displayed 62%, and in F_1 total 78%, the assumption of higher impact of environment than of genetic determination on loss of SI is self-evident (**Table 1**). But one-way analysis of variance, exploring the effect of environment vs. generation, revealed these differences in SI occurrence not to be
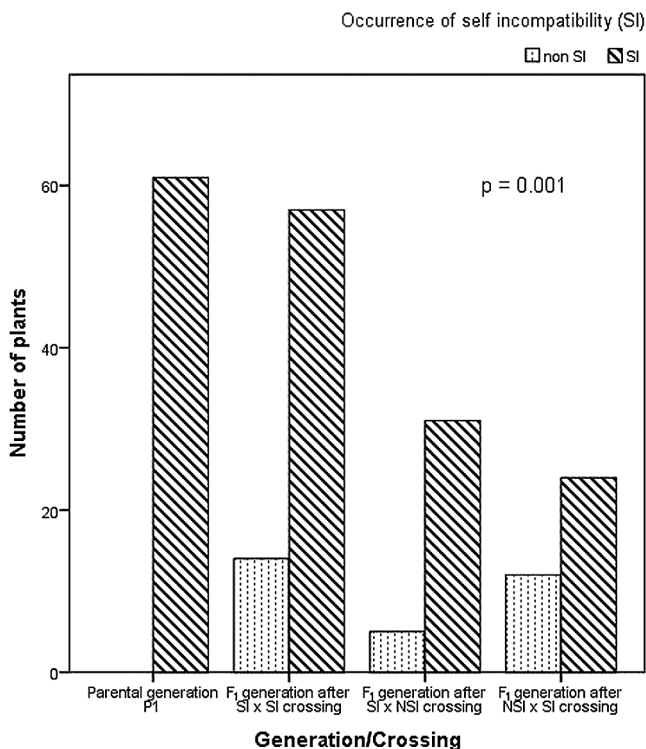

Fig. 1. Number of self-incompatible (SI) plants and non SI plants in the respective generation/crossing version. Included p-value refers to results of two-way analysis of variance for the influence of generation/crossing version on SI development.

significant. Calculated over all cultivars p-value exhibited 0.067, calculated for 'Degumille' only p-value was 0.600. However, the cultivar 'Degumille', displayed with 75% SI in P2 and 81% SI in F_1 total the highest values of all cultivars (**Table 1**) 'Degumille' indicates thereby a high genetic stability and is worth a closer look.

Influence of genetics—calculations of heritability

This naturally leads to calculations of heritability of SI over generations. Broad sense heritability estimates how far variability of a phenotypic trait in a population depends on genetic differences (Becker 1993, Falconer 1984, Wricke and Weber 1986). As we specifically selected SI plants from a basic starting population and then crossed within this selected fraction (SI × SI), heritability of SI occurrence can be estimated. Realized heritability (h^2) in general is described as the response to selection (R) relative to the strength of selection (S) (Becker 1993, Falconer 1984, Wricke and Weber 1986). S is the phenotypic difference between the mean of the selected fraction (in our case P1) and the mean of the starting population. R is the difference between the mean of the progeny of the selected fraction (in our case F_1 after SI × SI) and the starting population. According to the equation $h^2 = R/S$ and the values displayed in **Table 1**, h^2 of SI development displays the value 0.71 over all cultivars and 0.66 for 'Degumille'. These values exhibit a genetic determination of 71% and 66%, respectively, of the phenotypic trait of SI in German chamomile.

Discussion

For breeding purposes self-incompatibility in hermaphroditic plants is a desired trait. To maintain once assessed self-incompatibility, two ways—vegetative and generative maintenance—seem possible. As a general outcome of this study an intra-cultivar, generative maintenance of self-incompatibility with the cultivar 'Degumille', using defined SI plants as mother and normal, not SI evaluated plants as father, can be recommended.

Reasons for this recommendation can be found on several levels of breeding efforts, e.g. in the practical fact that it will be easier to maintain a specific breeding line of annual and hapaxanth plants like German chamomile by generating successions of generations instead of recurrently producing vegetative cuttings of the same genetic material (Arbury *et al.* 1997, Bareja 2010, Guse and Larsen 2001). Moreover, the influence of environment on SI development can be high (p < 0.001). To exactly reveal the mechanisms of environmental influence of SI in German chamomile further investigations, including molecular biology and gene expression would be necessary, like Sijacic *et al.* (2004) and Thomann *et al.* (2015) showed for other plants.

Moreover, the high general heritability of SI in German chamomile ($h^2 = 0.71$) is notable. Commonly h^2-values over 0.6 are stated as a high heritability (Dorfman 2001). In previous investigations of intrinsic chamomile traits (essential

oil compounds) equally high values of heritability (0.72–0.95) were discovered (Franz 1993). High co-heritability, as a description of genetic association within pairs of quantitative traits and a sign for low genetic variability and high inheritance (Janssens 1979), was discovered for a number of economic traits in German chamomile, like for fresh flower yield and for spread area (Lal *et al.* 2000). For breeding purposes, in particular, the heritability of reproductive traits in plants is an important and is therefore often investigated value. Moghe and Shiu (2014), for example, discovered significant genetic influence for the inheritance of unreduced gamete formation in crops. Whereas Austen *et al.* (2015) balanced heritability and environmental influence on sex allocation in hermaphroditic plants. The present work seems to be the first one investigating heritability values of reproductive traits in German chamomile.

Another remarkable finding is the co-occurrence of the SI × NSI crossing version and the P1 in the same subgroup after post hoc tests of analysis of variance for the influence of generation/crossing versions on SI development (p = 0.001), which shows the high affinity of this special crossing version to develop towards 100% SI in the P1 population. No statistical significance between crossing versions to guarantee the best maternally or paternally dominated crossing version can be provided (p = 0.257), but the above mentioned fact stresses the proximity of the crossing version SI (mother) × NSI (father) to the pure SI starting population and therefore serves as trend indicator for the best crossing version.

The outstanding features of the cultivar 'Degumille', exhibiting high SI stability over varying environments (mean value 0.93 over P1 and P2) and vigorous plants throughout the experiment, support the set up recommendation. Additional investigations to test interspecific but diploid crossing lines with 'Degumille' SI mother plants to find out ideal SI maintainer lines are a valuable future approach. Also to extend SI investigations to the tetraploid cultivar 'Manzana', an autopolyploid enhancement of 'Degumille', could be a reasonable approach, in particular to combine specific, desired reproductive traits like SI with economic interests of high drug yield and other useful characteristics (Franke *et al.* 2005).

Acknowledgements

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