Breeding of multipurpose willows on the basis of *Salix daphnoides* Vill., *Salix purpurea* L. and *Salix viminalis* L.

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Abstract

Willows are planted in short rotation coppices (SRC) to produce biomass. Another potential use of willows is the extraction of natural salicylates as alternatives to chemically synthesized acetyl salicylic acid (medical use). Available clones in Europe based on *Salix viminalis* L. do not contain salicylates and have high water requirements.

In order to breed willows that can serve both as bioenergy and salicylate source under dry soil conditions, a collection of several hundred wild type genotypes of *Salix daphnoides* Vill. and *Salix purpurea* L. was used for an extensive crossing program with selected *S. viminalis* biomass clones. Altogether 578 inter- and intraspecific crossings resulted in 203 progenies and 622 genotypes. After selection in nursery trials 62 genotypes have been cloned and planted into two field trials with different soil moisture.

Crossings of salicylate free *S. viminalis* clones with salicylate containing *S. daphnoides* and *S. purpurea* clones resulted in progenies that contained both salicylate and triandrin (from *S. viminalis*). The highest salicylate contents were found in *S. daphnoides x daphnoides* crosses followed by *S. viminalis* x *daphnoides* and *S. purpurea* x *viminalis*. Rust infection was low for almost all genotypes. While at the wet site the highest biomass was recorded for *S. viminalis* clones, the highest biomass at the dry soil site was recorded for *S. daphnoides* x *daphnoides* clones. This shows that it is possible to produce clones with high biomass and high salicylate content by intraspecific crossings of *S. daphnoides*. Because of the different cutting regimes at the two sites (first year cut at the dry site, no first year cut at the wet site), further investigations are necessary to confirm the superiority of *S. daphnoides* clones over *S. viminalis* clones at dry sites.

Keywords: *Short rotation coppice, salicortin, phenolic glycosides, interspecific crossing, Melampsora resistance*

Zusammenfassung

Züchtung von Weiden zur Mehrfachnutzung auf der Basis von *Salix daphnoides* Vill., *Salix purpurea* L. und *Salix viminalis* L.

Weiden werden in Kurzumtriebsplantagen (KUP) angebaut, um Biomasse für die Energiegewinnung zu produzieren. Eine weitere Nutzungsmöglichkeit ist die Herstellung von Salicylat-haltigen Rindenpräparaten als Ersatz für synthetische Schmerzmittel auf der Basis von Acetylsalicylsäure. Da die bisher in Europa verwendeten Biomasse-Klone auf *Salix viminalis* L. basieren, die kaum Salicylate enthalten, fallen diese für eine mögliche Mehrfachnutzung aus.

Auf der Basis eines Klonarchivs der Salicylat-haltigen Arten *Salix daphnoides* Vill. und *Salix purpurea* L. wurden 578 Kreuzungen mit Klonen von *S. viminalis* durchgeführt, welche in 203 Nachkommenschaften mit insgesamt 622 Genotypen resultierten. Nach Vorprüfungen wurden 62 Genotypen in zwei Feldversuchen mit unterschiedlichen Bodenfeuchten gepflanzt.

Die höchsten Salicylat-Gehalte bei gleichzeitig hoher Frischmasse hatten intraspezifische Kreuzungen *S. daphnoides* x *daphnoides*, gefolgt von *S. viminalis* x *daphnoides* und *S. daphnoides* x *purpurea*. Während am feuchten Standort die höchste Biomasse für *S. viminalis*-Klone gemessen wurden, war sie am trockenen Standort am höchsten für *S. daphnoides* x *daphnoides*-Klone. Aufgrund des unterschiedlichen Schnittmanagements (Erstjahresschnitt am feuchten Standort, kein Erstjahresschnitt am trockenen Standort) sind weitere Untersuchungen notwendig, um die Überlegenheit von *S. daphnoides* an trockenen Standorten zu bestätigen.

Schlüsselwörter: *Kurzumtriebsplantage, Salicortin, Phenolglykoside, interspezifische Kreuzung, Melampsora-Resistenz*

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1 Introduction

Species of the genus *Salix* can be grown in short rotation coppices (SRC). This form of cultivation in agricultural fields uses the ability of willows to sprout from stools of cut plants to produce high biomass yields within 2 to 3 years after cutting, so that the rotation time is shorter than in conventional forest plantations (Lindegaard et al., 2016; Makeschin, 1999). The main use is as fuel in biomass heating and power plants (Djomo et al., 2011). Short rotation coppices are widely planted in Sweden and Great Britain (Aylott et al., 2008; Mola-Yudego and Gonzalez-Olabarria, 2010). In these and other countries breeding programs are finished or underway to provide high biomass genotypes by crossing and selection (Gebhardt, 2012; Larsson, 1998; Lindegaard and Barker, 1996; Pohjonen, 1991).

The species *Salix viminalis* L. is widely used in short rotation coppices (Begley et al., 2009; Lindroth and Bath, 1999). According to the classification of Skvortsov (1999), *S. viminalis* belongs to the section *Vimen* and is widely distributed across Northern Eurasia. Among other *Salix* species used for selection or as breeding partners are *Salix dasyclados* Wimm/ *burjatica* Nasarov or *Salix schwerinii* E. Wolf (Berlin et al., 2011; McCracken and Dawson, 1992; McCracken et al., 2011). They also belong to the Section *Vimen*. Thus, considering that Skvortsov (1999) recognizes 26 sections with 135 species in Eurasia, the genetic basis for biomass willows is relatively small.

Apart from use as biomass for bioenergy *Salix* species can also be planted for the use of substances within the bark. The most prominent among them are phenol glycosides such as salicylates (e.g. salicin and salicortin (Boeckler et al., 2011). The concentration ranges from 0.1 % to 10 % in dry bark depending on species (Shao, 1991). Salicylates from willow bark are used as substitute for synthetic analgesics based on acetyl salicylic acid (Meier, 2001). Other phenolic substances in the bark of willows are condensed tannins like catechin with a concentration of 0.2 % to 1 % and flavonoids in the form of glycosides of naringenin, eriodictyol and isoquercitrin (0,4 % to 3,8 %, Shao, 1991). A potential further use of bark extracts is as replacement of chromium in leather tanning, as it is already proposed for other tree barks (Seabra et al., 2018).

SRC willows are especially suitable for the extraction of substances from the bark since the large numbers of shoots emerging after cutting allows for a high percentage of young bark in relation to the total volume. Thus, willows could be used both for bioenergy generation and the production of phenolic substances. One problem is that the biomass producing species *S. viminalis* contains only relative low amounts of salicylates (under 1 %, Minakhmetov et al., 2002; Shao, 1991) while other species such as *Salix purpurea* L. and *Salix daphnoides* Vill. contain more than 1 % salicylates in dry weight of bark (Julkunen-Tiitto, 1989; Shao, 1991). If a dual use of biomass and salicylates is required *S. viminalis* is not a suitable species. One possible solu-tion is the breeding of willow genotypes that produce both biomass and salicylates. This goal can be reached by interspecific crossing of *S. viminalis* with salicylate containing species or by improving the biomass yield of salicylate containing species through intraspecific crossings and clone selection.

Not only biomass and phenol glycoside content are goals for new genotypes of biomass willows but also resistance to biotic and abiotic stresses. Among biotic stresses the colonization of leaves by rust fungi of the genus *Melampsora* can lead to substantial losses of biomass (Pei et al., 2004). Therefore, one breeding goal is the provision of rust resistant willow genotypes (Pei et al., 2008). Because of the high specificity of some races of *Melampsora spp.*, the yield of willow plantations can be improved by mixed planting of several genotypes of willow (McCracken and Dawson, 1998).

Another motivation for breeding new willow genotypes is the requirement for drought tolerance. The established *S. viminalis* clones are selected for and planted in areas with relatively high precipitation. In the lowlands of Eastern Germany precipitation is around 600 mm or even below (Hänsel et al., 2007). When breeding for dryer re-gions those species should be considered as crossing partners that are known to have lesser requirements for water supply. For instance, *S. daphnoides* Vill. var. *pomeranica* from the Baltic Sea coast is growing on sand dunes and known to resist drought to some degree (Chmelar and Meusel, 1979).

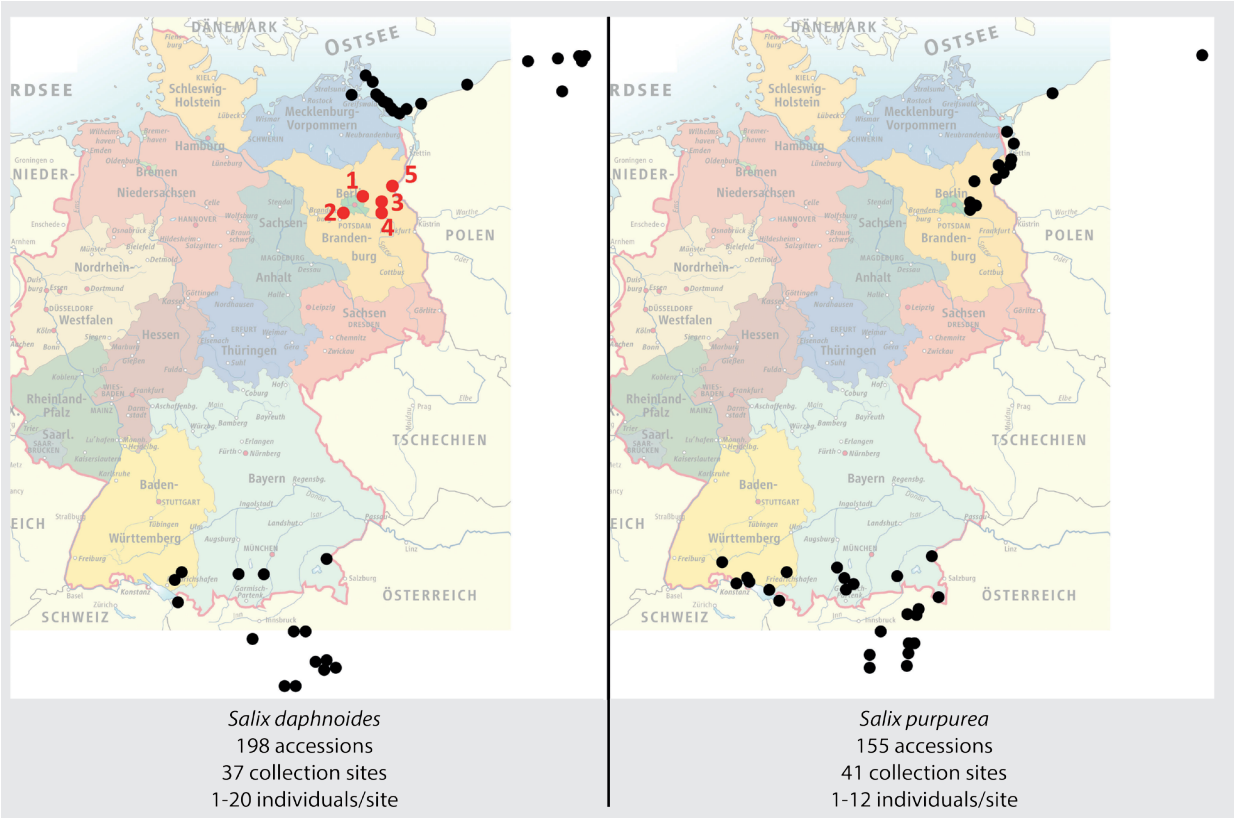
In the following we present results of a German breeding program that was supported by the German Fachagentur für Nachwachsende Rohstoffe. In contrast to other breeding programs, important crossing partners were *S. daphnoides* and *S. purpurea*. Both intraspecific crossings and interspecific crossings with established *S. viminalis* genotypes were performed. The breeding goals were as follows:

- High biomass yield
- High content in phenol glycosides especially salicylates
- Resistance against willow rusts
- Drought tolerance.

2 Material and Methods

2.1 Genotype collection

A genotype collection of *Salix daphnoides* and *S. purpurea* was the basis for breeding (Figure 1). The genotypes have been collected in two areas: the coast of the Baltic Sea in northeastern Germany and Poland and the Alps in Germany, Austria and Italy. They have been planted in the stock collections Zepernick and Berlin-Dahlem at Humboldt-Universität zu Berlin (Zander et al., 2010). A second source for crossing partners was a stock collection in Waldsieversdorf at the Thünen Institute. It comprised established genotypes of the species *S. viminalis* that are widely used for short rotation coppices in Sweden but also genotypes of other *Salix* species such as *Salix humboldtiana* Willd. *S. humboldtiana* has been chosen for testing the principal possibilities to cross foreign species into native species



Salix daphnoides
198 accessions
37 collection sites
1-20 individuals/site

Salix purpurea
155 accessions
41 collection sites
1-12 individuals/site

In the left panel the location of the three stock collections and the two field trials is indicated:
1 stock collection Zepernick
2 stock collection Berlin-Dahlem
3 stock collection Waldsieversdorf
4 field trial Wei15
5 field trial Wriezen 2

Figure 1
Provenance of the breeding material

2.2 Controlled crossings

Directed crossings were performed in spring 2011, 2012, 2013 and 2014. Branches of male *Salix* genotypes were cut in early spring. Pollen was collected after flowering in the green house (Figure 2b) and stored at 4 °C for max 4 weeks. Two methods have been applied for pollination:

A. Direct method: Catkins of female genotypes in the genotype collection Zepernick were bagged before flowering and directly pollinated on the plant with a sterile brush. The capsules ripened within a few weeks in the bag and the catkins were collected when the seeds were released. This method was mainly used for intraspecific crossings of *S. daphnoides*.
B. Greenhouse method: Catkins on branches of female genotypes (Figure 2a) were pollinated in crossing chambers in the greenhouse with a sterile brush. The capsules ripened on the branch in the crossing chamber (Figure 2c). This method was mainly used for interspecific crossings of *S. purpurea*, *S. daphnoides* and *S. viminalis*.

Seeds were cleaned from hairs with a sieve (Figure 2d and e) and sown on vermiculite that was moistened with ½ concentration of liquid Schenk/Hildebrand Medium (Duchefa, Haarlem, Netherlands). Until germination and the first two weeks after germination cultivation took place under 100 % air moisture (Figure 2f). Seedlings were transferred to soil and cultivated in pots in the greenhouse for a few weeks (Figure 2g) until they were transferred outside the greenhouse.

2.3 Terminology for seedlings and clones

Each seedling was given a name consisting of an abbreviation of the crossing partners and a running number; the first genotype is the female and the second genotype the male parent. At the end a letter follows for the nursery trial as place of selection (D = Berlin-Dahlem, W = Waldsieversdorf, Z = Zepernick). An example is 79036 x DA57_1_W. This is a crossing of the female *S. viminalis* genotype 79036 and the male *S daphnoides* genotype DA57. It is the seedling number 1 in the nursery trial Waldsieversdorf. Note that 79036 x DA57_1_Z was selected in the nursery trial Zepernick and is another seedling with a different genotype.

In this paper the following terminology will be used for differentiating clones and ramets: The original seedling is the genet. The propagation by cuttings (i.e. cloning) results in ramets. The ramet together with the genet forms the clone. Thus, the name 79036xDA57_1_W can denote both the orig-inal seedling and the clone. Usually the name will be

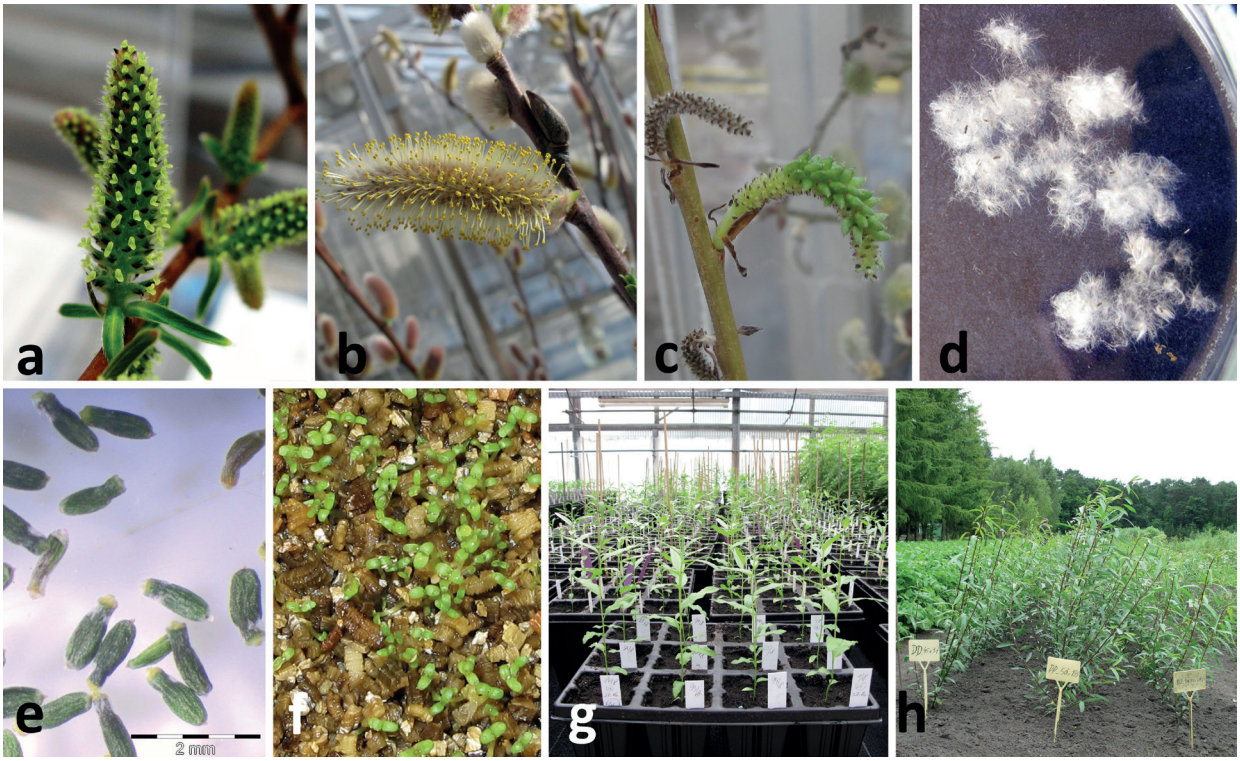


Figure 2
Stages of the crossing process: a) catkin of female *S. humboldtiana* genotype SH2; b) catkin of male *S. daphnoides* genotype DA65; c) developing capsules of the intraspecific crossing PU59 x PU108 on branches in crossing chambers; d) "wool" with seeds; e) seeds of crossing *S. viminalis x S. daphnoides* Tordis x DA135; f) seedlings after germination; g) seedlings in quick-pots in the greenhouse; h) plants in July of the second year after germination, they are not yet propagated by cuttings.

used as clone name. Where a distinction between clone and the original seedling is necessary it will be explicitly stated.

2.4 Cultivation and propagation of clones

The selection and propagation process is exemplified for the crossing season 2011 (Table 1).

In the year of crossing seedlings were cultivated in pots. When a crossing yielded numerous seedlings, excess seedlings were planted into nursery trials in the fall of the crossing year. Routinely, seedlings were planted in spring of the second year into nursery trials (Figure 2h). Fast growing seedlings were selected for propagation by micro cuttings. Micro cuttings were prepared from one year old seedlings by cutting the shoot in 7 to 10 sections with at least two buds in March. They were grown in 0.5 l pots with seedlings substrate with perlite (Klasmann-Deilmann, Geeste Germany) under plastic cover at 2 to 20 °C. Acclimatization started in May by removing part of the foil and installing shading. Beginning from May, the ramets were planted into nursery trials together with the rootstock of the original seedling.

A second method for propagation was the use of conventional cuttings (25 cm length, diameter 1 to 2 cm). When cuttings were prepared in the spring of the third year after crossing (either from original seedlings or from ramets) they were rooted in the greenhouse in early spring and planted from pots into the field in May. Cuttings from clones in the fourth year or older (mostly ramets) were also directly planted into field trials in May. In the first year, irrigation was provided.

2.5 Nursery trials and field trials

Nursery trials have been established next to the stock collections in Berlin-Dahlem (Humboldt-Universität zu Berlin), Zepernick (Humboldt-Universität zu Berlin) and Waldsieversdorf (Thünen Institute). They served to survey the growth rate, number of shoots and rust colonization in the second and third year of cultivation after crossing. All seedlings of a crossing progeny (usually 7 to 9) or all ramets of one clone were planted into a consecutive row. Nursery trials were surveyed from the years 2012 to 2015.

Field trial Wriezen2 has been established in spring 2013 with rooted cuttings. It is situated in the lowlands of the river Oder (Oderbruch: 52.741591° N, 14.121651° E), where the groundwater level is high. The clones are planted in a randomized block design with three blocks. Each block consisted of 36 plots with 15 plants per plot. 34 clones from crossings 2011 and two standard clones of the section Vimen (Tordis and Zieverich 3N) were planted. Tordis is a hybrid of S. schwerinii and S. viminalis. Zieverich is originally a S. viminalis clone. In the Salix clone collection of Hann. Münden it is

Table 1
Time course of cultivation at the example of the crossing progeny of 2011

Consecutive timing	Time	Event
1st year	Spring 2011	crossing and sowing, cultivation in pots
	Summer 2011	cultivation in pots
2nd year	Spring 2012	selection of 145 seedlings by shoot length, diameter and rust resistance; preparation of 7-10 micro cuttings for each seedling
	May 2012	planting of ramets together with the original rootstocks into nursery trials
	Fall 2012	selection by shoot lengths, shoot number, rust resistance: 67 genotypes remain in nursery
3rd year	February 2013	propagation by cuttings and rooting in the greenhouse
	May 2013	planting of rooted ramets of 34 genotypes in the field trial Wriezen2
	Winter 2013/14	first cut
4th year	Winter 2014/15	Second cut with measurements of fresh weight and phenol glycoside content

female and diploid by flow cytometry (Fehrenz, 2015). The clone in the clone collection Waldsieversdorf is triploid and female. Therefore the used standard is called Zieverich 3N to make clear that it is distinct from Zieverich. According to morphological traits Zieverich 3N is at least relative of S. viminalis.

Field trial Wei15 has been established in spring 2015 with rooted cuttings in 30 km distance to Wriezen2 near the town Müncheberg (52.522007° N, 14.135644° E). In comparison to the field trial Wriezen2 the soil conditions are dry with a low ground water level. The randomized block design consisted of 4 blocks with 50 plots and 21 plants in each block. Altogether 48 clones have been tested: 20 clones already planted in Wriezen2 and 28 clones from the crossing years 2012 to 2014. Standard clones are Tordis and Zieverich 3N.

The following three sections describe the different measurements. Rust infection and biomass/growth measurements served as criteria to select clones for planting in the field trials. Rust infection, biomass/growth, and phenolic glycosides served to evaluate the selected clones in the field trials.

2.6 Scoring rust infection

Rust infection was surveyed by a rating scheme with 7 grades (Figure 3). Scores are the estimated average leave surface covered by rust pustules on all leaves of the plant. Score 1 means that no rust pustules are observed on the plant, while 7 means that more than 50 % of the leave surface of the whole plant is covered with rust pustules. Surveys in the nursery trials were conducted in the first two weeks of September. Within the progeny of a crossing the rust score was determined for each individual seedling. Seedling with a score higher than three were excluded from further cultivation except for the case that they showed exceptional growth rates (putative cases of tolerance). In the case of clones generated by micro cuttings the values for the ramets were averaged. The threshold for further cultivation was also three.

Screening for rust resistance in the field trial Wriezen2 took place on 16th September 2014. The rating scheme has been adjusted in a way that the score was averaged visually for all 15 plants of a plot resulting in one rust score per plot. Preliminary test had shown that the variation between the ramets of one plot is very low, allowing to apply a summarizing score. Comparison of rust infection between genotypes was based on the average score of three blocks.

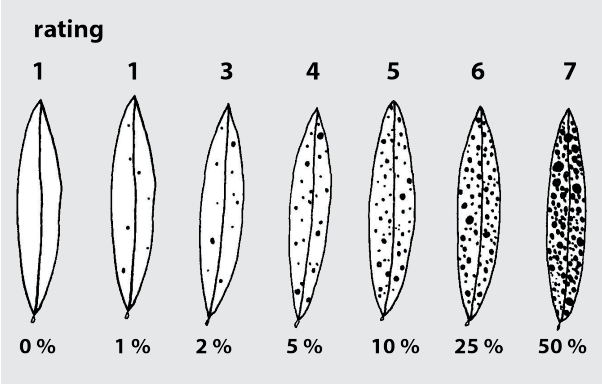


Figure 3
Rating for surveys of rust infestation in nursery trials and field trials, adapted from McCracken and Dawson (1992)

2.7 Measurements for growth and biomass

In the nursery trials following parameters have been measured: height of the highest shoot, diameter of the strongest shoot and the number of shoots. It was based on 6 to 9 ramets from the propagation by micro cuttings. Seedlings or clones were selected after two years for further testing in field trials. In the field trial Wriezen2 biomass was measured as fresh weight directly from the cut shoots in winter 2014/2015 after cutting in winter 2013/2014. That is stools grew two growing periods and the shoots one growing period.

An indirect approach was applied to estimate biomass in the field trial Wei15. The diameters at breast height (1.3 m) of all shoots per plant were measured and the basal area was calculated in autumn 2017 after three growing periods

without cutting. The biomass function for dry weight has been determined in a previous field trial in Müncheberg (Wei9) with following parameters:

Oven dry weight [g] = 118.22 x basal area +112.13.

2.8 Measurements of phenolic glycosides

Sampling: Sampling took place in winter to early spring 2014. Bark was removed in stripes from shoots of three plants of each genotype at a height of 30 to 60 cm above ground. The bark was shock frozen in liquid nitrogen and stored at -20 °C until further processing.

Extraction: Phenolic glycosides were extracted as described in Förster et al. (2008). Briefly, samples were lyophilized and homogenized in a ball mill. Fifty mg of each sample were mixed with 750 µl 80 % methanol and 100 µl internal standard resorcin (50 mM), incubated in an ultrasonic bath on ice, and centrifuged to collect the supernatant. The pellet was re-extracted with 80 % methanol two more times. The extract was concentrated in a vacuum concentrator and refilled to 1 ml with ultrapure water. It was filtrated (SpinX filters, 0.22 µm) and stored at -20 °C until HPLC analysis.

HPLC analysis: The HPLC system consisted of a DIONEX P680 pump, an ASI-100 auto sampler, a TCC-100 thermostated column department, and an UltiMate 3000 Photodiode Array Detector. Reversed phase chromatography was carried out on an Acclaim PolarAdvantage C16 column (3 µm, 120 Å, 2.1 x 150 mm, Thermo-Fisher) protected by a pre-column (5 µm, 120 Å, 2 x 10 mm, Thermo-Fisher). Eluents used for HPLC analysis were (A) 2 % tetrahydrofuran, 0.5 % phosphoric acid in ultrapure water and (B) 100 % methanol. The applied gradient program (flow rate 0.35 ml/min) was: 0 % B (min 0-5), 0-15 % B (min 5-10), 15-25 % B (min 10-20), 25-35 % B (min 20-30), 35-50 % B (min 30-40), 100 % B (min 40-42), 100-0 % B (min 42-44), 0 % (min 44-49). Injection volume was 10 µl and peak detection was carried out at 270 nm. Qualitative analysis of phenolic glycosides was based on their retention times, specific UV-spectra (Shao, 1991), and mass spectrometry. Peak evaluation of chromatograms was performed with the program Chromeleon 6.8.

Quantification: Quantification of phenolic glycosides was based on the peak area relative to the internal standard. The content of phenolic glycosides is given in mg/g dry weight as sum of all phenolic glycosides including salicylates (salicin and salicortin) and separately only for salicylates.

2.9 Statistics

Fresh weight per plant (15 for Wriezen2, 21 for Wei15) was averaged for each plot. These plot means were used as input for testing for ANOVA. Significance of difference of clone means with the mean of Tordis (standard clone) was tested by Dunnett´s test. The GLM procedure of SAS 9.3 was used. The statistics for oven dry ton (10^6 g) at Wei15 started from dry mass per plot by using the sum of all basal areas as input for the biomass function. With the plot size of 21 m^2 the value was converted into t ha^{-1}.

Correlation of biomass and salicylate content was calculated as Spearman´s rank correlation coefficient and tested on significance by using SPSS 23.

3 Results

3.1 Success of crossings

Altogether 578 crossings with 64 mother and 48 father genotypes have been performed. They resulted in 203 progenies with altogether 622 seedlings (Table 2). In the years 2011, 2012, and 2013 around 50 % of crossings resulted in seeds. The low number of successful crossings in 2012 has two causes. First, the catkins have been damaged by biotic (larvae of different insects) and abiotic (frost) stresses. Second, in 2012 mostly interspecific crossings have been performed that resulted in lower number of successful fertilizations as compared to intraspecific crossings. The original aim was to clone 10 seedlings per progeny by cuttings or micro cuttings. This was only possible in 2011 for a larger number of crossings whereas in the following years only two to three seedlings per progeny could be cloned. This was especially true for interspecific crossings that often resulted in only one clone. The female clone of the foreign species *S. humboldtiana*, SH2, proved to be a good mother clone. Several interspecific crossings with *S. viminalis*, *S. purpurea* and *S. daphnoides* yielded viable seedlings.

Table 2
Success of crossing over four crossing seasons

Consecutive timing	2011	2012	2013	2014
Mother genotypes	24	35	35	32
Father genotypes	16	27	30	29
Crossings	79	185	199	115
Crossings yielding seeds	40 (50 %)	36 (19 %)	99 (50 %)	73 (63 %)
Progenies	36 (46 %)	35 (19 %)	74 (37 %)	58 (50 %)
Seedlings (= new clones) in nursery trial	268	82	161	111
Average number of clones per progeny	7.4	2.3	2.2	1.9

Percentages in brackets refer to the number of performed crossings.

Table 3
Clones selected for field trials

crossings	Selection for Wriezen2 (2013)	Selection for Wei15 (2015)
S. daphnoides x daphnoides (in brackets DA135 as father)	21 (21)	11 (8)
S. humboldtiana x viminalis	0	1
S. humboldtiana x purpurea	0	1
S. humboldtiana x daphnoides		1
S. purpurea x viminalis	2	4
S. viminalis x daphnoides	8	7
S. viminalis x viminalis	3	3
Sum	34	28

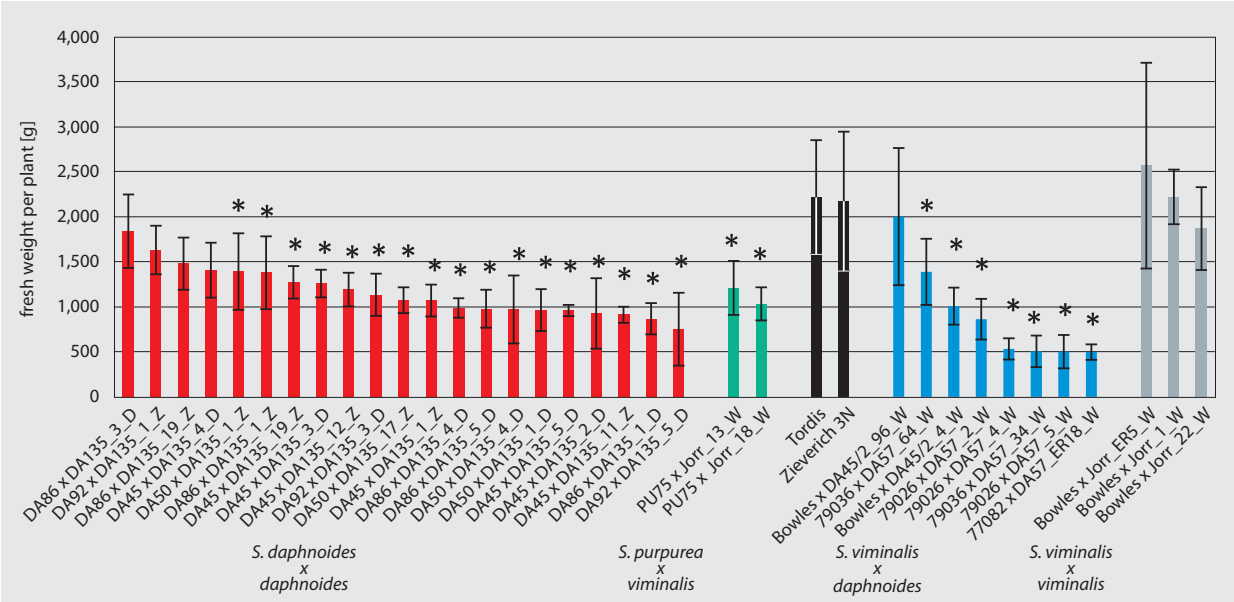
3.2 Selected clones for planting in field trials

Cloned for the field trials were selected according to the growth, number of shoots and rust infestation in the nursery trials. After at least two years of observation in the nursery trials altogether 62 clones from originally 622 clones were selected for plantings in field trials Wriezen2 and Wei15 (Table 3 and clone names in Figure 4 to 6). The number of selected clones for Wriezen2 was 34 and comprised clones from the crossing year 2011. Another 28 clones from the crossing years 2012 to 2013 were selected for field trial Wei15. They were planted together with 20 clones that showed good growth in Wriezen2, so that the total number of clones for Wei15 is 48.

Among 32 intraspecific clones of *S. daphnoides*, 29 of them were offspring of genotype DA135 as father (Table 3). This is especially noteworthy considering the relative weak growth of DA135 in the stock collections as compared to other fathers (data not shown). A larger number of intraspecific clones of *S. viminalis* could also be selected. Crossings of *S. purpurea* x *purpurea* resulted in seeds (Figure 2c) and clones in the nursery trials but none could be selected for field trials. Among the interspecific crossings the largest number of clones could be selected for the crossings *S. viminalis* x *daphnoides*. When used as crossing partner with other species *S. purpurea* was involved in six selectable crossings. Although crossing with *S. humboldtiana* is principally possible, the resulting seedlings were relatively weak. Nevertheless, three seedlings with a father of *S. viminalis*, *S. purpurea*, *and S. daphnoides* respectively, could be selected for the field trial Wei15.

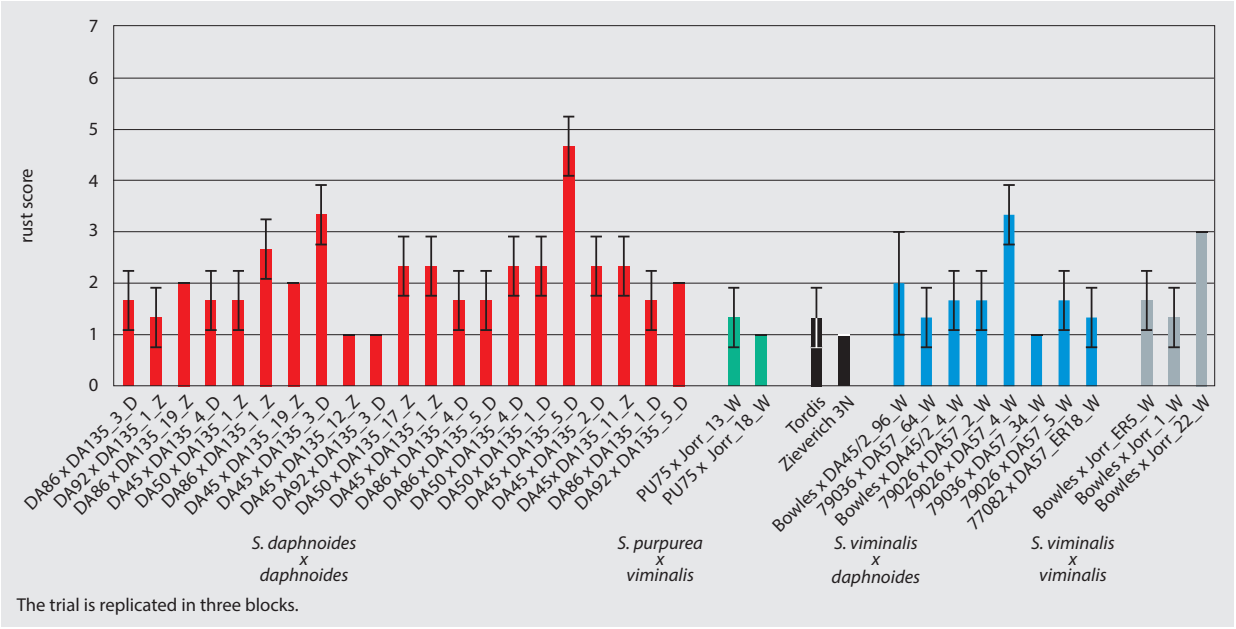
3.3 Biomass and rust at the moist field trial Wriezen2

Biomass: Under moist soil conditions the highest biomass is recorded for an intraspecific crossing *S. viminalis* x *viminalis* (Bowles x Jorr_ER5_W) with 2.6 kg per plant and a high standard deviation of 1,1 kg. However no clone has a significant higher



The trial is replicated in three blocks. Asterisks mark significant differences compared to the standard Tordis. (Dunnett´s test, p = 0.05)

Figure 4
Moist site Wriezen2, 2014. Fresh weight of the second cut (two vegetation periods after planting).



The trial is replicated in three blocks.

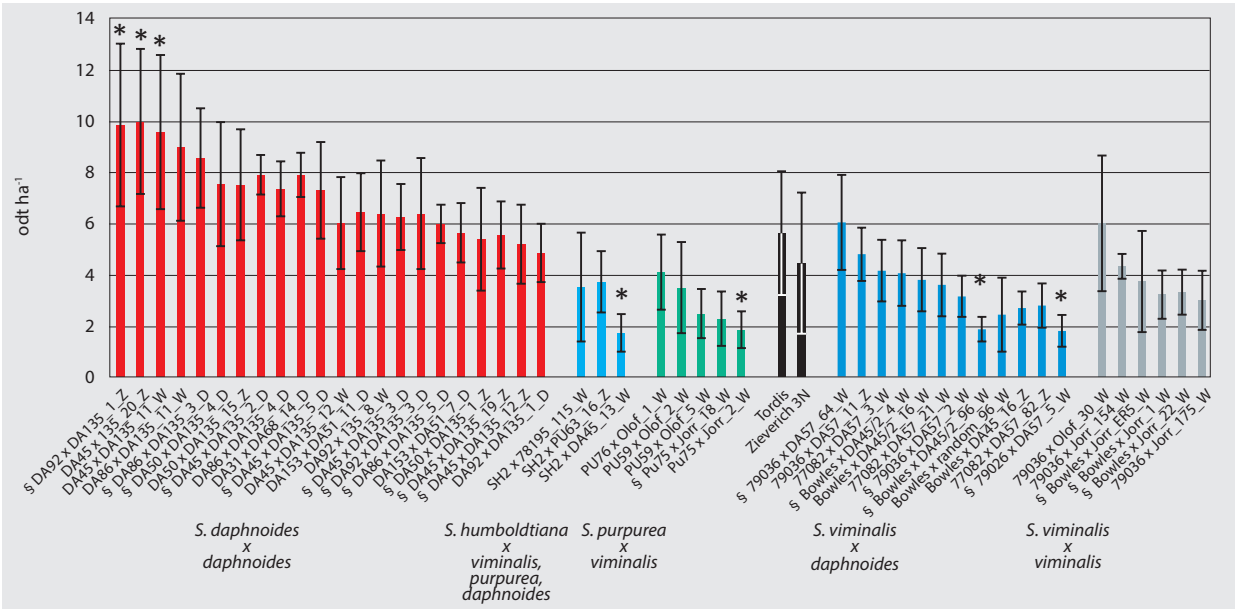
Figure 5
Moist site Wriezen2, rust survey on 16th September 2014 (two vegetation periods after planting).

biomass than the standard clone Tordis (Figure 4). A significant lower biomass is determined for 26 clones. However, there are 8 clones that have no significant lower biomass than the standard. Among them are the second standard Zieverich 3N, four intraspecific crossings *S. daphnoides* x *daphnoides* with DA135 as father, three intraspecific crossings *S. viminalis* x *viminalis*, and one interspecific crossing S. *viminalis* x *daphnoides*.

Rust infestation: Rust score above 5 was not observed and the score 5 was only observed once in one plot of the clone DA45 x DA135_5_D. Five clones had a score of 1 (i.e. no rust at all) over all three plots while the majority of clones showed a score around 2. Overall rust infestation was low (Figure 5).

3.4 Biomass at the dry field trial Wei15

Under the dry soil conditions of Wei15 nineteen *S. daphnoides* clones had higher dry weight than the standard *S. viminalis* Tordis. For three of them, DA92 x DA135_1_Z, DA45 x



The trial is replicated in three blocks. Asterisks mark significant differences (Dunnett´s test p = 0.05) compared to the standard Tordis. Clones marked with § have also been planted in field trial Wriezen 2.

Figure 6
Dry site Wei15. Estimated odt ha-1 three vegetation periods after planting (calculated from stem diameter).

Table 4
Contents of phenolic glycosides in the field trial Wriezen2

Crossing family/parent	Salicylate in mg g^{-1}		Further phenolic glycosides in mg g^{-1}				Total phenolic glycosides in mg g^{-1}
	Salicin	Salicortin	Syrengin	Triandrin	Naringenin-5-glucoside II	Naringenin-7-glucoside	
S. daphnoides x daphnoides							
DA45 x DA135 (n=8)	1.74 ±0.59	31.95 ±3.52	4.36 ±1.15	0	12.55 ±4.79	14.13 ±3.53	79.10 ±9.78
DA50 x DA135 (n=4)	2.02 ±1.09	31.16 ±5.93	4.66 ±0.88	0	11.43 ±2.97	13.46 ±1.56	77.40 ±11.34
DA86 x DA135 (n=6)	1.48 ±0.69	33.10 ±2.37	5.37 ±1.60	0	9.11 ±2.88	15.92 ±3.14	78.59 ±9.23
DA92 x DA135 (n=3)	1.39 ±0.20	32.55 ±5.98	4.90 ±2.18	0	12.59 ±2.11	12.63 ±7.72	77.62 ±17.84
S. purpurea x viminalis							
Pu75 x Jorr (n=2)	0.35 ±0.13	9.13 ±2.07	1.35 ±0.57	55.37 ±2.50	2.70 ±1.61	0.12 ±0.04	72.74 ±7.16
S. viminalis x daphnoides							
Bowles x DA45/2 (n=2)	n.a.	n.a.	n.a.	n.a,	n.a.	n.a.	n.a.
77082 x DA57 (n=1)	0.38	10.69	2.26	86.4	2.14	0.88	105.27
79026 x DA57 (n=3)	0.88 ±0.13	15.81 ±5.96	2.91 ±0.20	43.19 ±11.96	2.67 ±0.38	2.19 ±2.21	71.40 ±17.01
79036 x DA57 (n=2)	0.42 ±0.12	15.88 ±1.84	2.76 ±1.19	58.44 ±2.21	3.04 ±1.45	2.10 ±0.52	86.66 ±1.94
S. viminalis x viminalis							
Bowles x Jorr (n=3)	0	0	0.73	101.09 ±9.20	0	0	111.20 ±4.93
parents (for all: n=1)							
DA45[1]	1.99	46.88	2.38	0	14.68	13.63	95,23
DA45/2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
DA50[1]	1.93	35.31	1.68	0	13.26	12.11	79.54
DA86[1]	2.43	44.87	2.59	0	10.42	21.98	101.72
DA92[1]	2.16	44.65	2.15	0	9.93	24.55	100.74
DA135[3]	1.49	21.42	8.02	0	9.37	9.51	60.02
PU75[2]	0.98	25.62	2.71	0	8.53	1.31	51.71
Bowles[1]	0	0	1.45	101.52	0	0	108.76
Jorr[1]	0	0	0.68	109.18	0	0	114.25
77082[2]	0	0	2.7	133.73	0	0	170.97
79026[1]	0	0	0.54	90.08	0	0	95.04
79036[2]	0	0	2.6	140.1	0	0	147.99

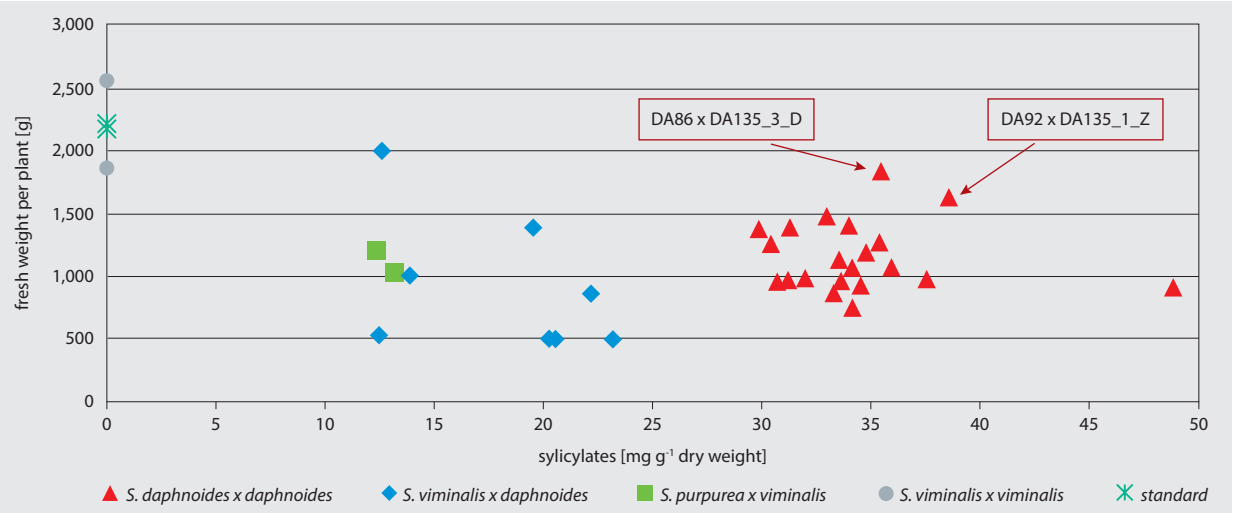
The measurements are from block 3 and are given in mg g^{-1} dry weight. For each crossing family the average and the standard deviations are given. Measurements for the parents are from the field trial Wriezen1 ([1]) or the stock collections in Dahlem ([2]) and Zepernick ([3]). Apart from the listed phenol glycosides following phenolic compounds have been measured (included in the column "total phenol glycosides"): different catechins, vimalin, ampelopsin, eriodictyol-7-glucoside, naringenin-5-glucoside I, salidrosid, purpurein, unknown phenolic glycoside (data adapted from Köhler 2016).

DA135_20_Z, DA45 x DA135_11_W, the difference was significant (Figure 6). Only three interspecific crosses had a significantly lower dry weight than the standard *S. viminalis* Tordis. The three intraspecific *S. viminalis* clones that showed no significant difference to Tordis in Wriezen2 had also no significant differences at the field trial Wei15. The estimated dry masses for the three best clones are 9.84 odt (oven dry ton) ha^{-1} (DA92 x DA135_1_Z), 9.98 odt ha^{-1} (DA45 x DA135_20_Z) and 9.56 odt ha^{-1} (DA45 x DA135_11_W, Figure 7).

Preliminary rust screenings in 2016 and 2017 showed no rust infestation at Wei15. Therefore, that it was not scored.

3.5 Measurement of phenolic glycosides at Wriezen2

The phenol glycosides have been measured once for each clone and are summarized according to their crossing



Each data point represents one clone. Salicin and salicortin are summarized as salicylates and are given as average of three replicates (one measurement per block, data taken from Köhler 2016). The fresh weight is the mean of three replicates as in Figure 4.

Figure 7
Joint plot of fresh weight and salicylate content at the field trial Wriezen2

families. Data are taken and adapted from Köhler (2016). Different substance classes are separately listed in Table 4.

Qualitative differences for intraspecific crossings and parents: The main phenolic glycoside in S. *daphnoides and S. purpurea* (PU75) is the salicylate salicortin. Both species contain low amounts of salicin. Other common phenolic glycosides in these two species are syrengin, (+/-)-naringenin -5-glucoside (naringenin-5-glucoside I and II) and naringenin -7-glucoside. Both species do not contain triandrin. In contrast, triandrin is the main phenolic glycoside in *S. viminalis*. The only substance detected in all of these three species is syrengin.

Qualitative differences of interspecific crossings and parents: The phenolic glycoside spectrum of the offspring of interspecific crossings is a combination of the parents. The crossings *S. viminalis* x *S. daphnoides* and *S. purpurea* x *viminalis* contain both salicylates and triandrin and all minor phenolic glycosides listed in Table 4. There are several substances that are measured in minor amounts (for instance catechins) that are not listed in Table 4. For them is also true that they can be crossed into the offspring, that is they are present in the hybrid when they were present in one of parent species (data not shown).

Quantitative differences: The highest contents of total phenol glycosides are found in the barks of *S. viminalis* parents and *S. viminalis* crossings (both intraspecific and interspecific) but the phenolic glycosides are dominated by one substance, triandrin. When considering the salicylates the highest content is recorded for the parent *S. daphnoides* DA45 with 1.99 mg g^{-1} dry weight (dw) salicin and 46.88 mg g^{-1} dw salicortin. Intraspecific *S. daphnoides* crossings have an average salicortin content of 31.96 to 33.1 mg g^{-1} dw. Interspecific crossings of *S. daphnoides* x *S. viminalis* have a lower salicortin content with the lowest content in the interspecific crossing *S. purpurea* x *S. viminalis* (9.13 mg g^{-1} dw).

Intraspecific crossings are intermediary between their parents in the amount of specific substances. For instance, progeny of the crossing DA45 x DA135 has a salicortin mean of 31.95 mg g^{-1} dw, which is intermediary between the parents DA45 (46.88 mg g^{-1} dw) and DA135 (21.42 mg g^{-1} dw).

3.6 *Joint assessment of salicylates and biomass at Wriezen2*

There is no significant correlation between salicylate content and biomass (Spearman correlation 0.183, Figure 7). However, the plot of Figure 7 is useful for distinguishing clones for dual use as salicylate source and biomass producer. Promising clones are DA86 x DA135_3_D and DA92 x DA135_1_Z because they have both high biomass and high salicylate content. There are also some clones of *S. daphnoides* x *viminalis* with a biomass comparable to the best *S. daphnoides* crossings, but their salicylate content is much lower. The two S. *purpurea* x *viminalis* crosses show a similar combination of salicylate content and biomass. The best biomass producers in Wriezen2 are *Salix viminalis* clones, but they do not contain salicylates.

4 Discussion

The aim of this breeding program was to combine the high biomass yield of *S. viminalis* genotypes with the high content of salicylates in other *Salix* species.

Salicylate content: We could confirm previous reports that *S. viminalis* contains no or only traces of salicin and other salicylates (Minakhmetov et al., 2002; Poblocka-Olech et al., 2007). *S. daphnoides* and *S. purpurea* contain salicin and salicortin, with salicortin being the main salicylate. The measured salicortin concentrations in *S. daphnoides* (30 to 45 mg

g^{-1} dw) and in *S. purpurea* (25 mg g^{-1} dw) are less than previously reported for these species (87 mg g^{-1} dw and 72 mg g^{-1} dw respectively (Julkunen-Tiitto, 1989)). The measured concentrations are still sufficient for use as phytopharmaceutical since the required salicin concentration in the willow bark as drug is at least 1.5 % (ESCOP, 2003). Salicin and salicortin are similar in their physiological effect because they are both hydrolyzed to saligenin (Julkunen-Tiitto and Meier, 1992), which is metabolized in the human body to salicylic acid, the physiological active substance (Meier, 2001).Thus, also the bark of the *S. viminalis* x *daphnoides* crossing families 79026 x DA57 and 79026 x DA57 with an average of 15 mg g^{-1} dw (= 1.5 %) salicortin is suitable as phytopharmaceutical.

The salicortin content in the two tested *S. purpurea* x *viminalis* clones is on average below 1.5 %. This is a result of intermediate inheritance because a clone with 2.5 % salicylates (PU75) is crossed with a clone without salicylate (Jorr). When both parents would have salicylate contents above the threshold value of 1.5 % also the progeny had a salicylate content above 1.5 %. In the same way it could be argued that *S. daphnoides* x *daphnoides* crosses could be further improved. All tested intraspecific *S. daphnoides* crosses had DA135 as father, because those crosses have been selected in the nursery trials for their growth and rust resistance. However, DA135 had the lowest content of salicylates among the used parents. Pollination with a salicylate rich father could lead to a further improvement of the salicylate content in intraspecific crossings.

With the interspecific crossings we could show that it is possible to cross the ability to produce salicylates into salicylate free *Salix* species. Despite the lack of salicylates, *S. viminalis* clones could be interesting as producers of phenolic substances because they contain more than 10 % phenolic glycosides (parent 77082: 17 %), dominantly as triandrin. In comparison, Thieme (1965) reports 6.7 % triandrin for *Salix viminalis* bark. *Salix* phenolic glycosides other than salicylate can become an item of substantial interest for the "chemoceutical" industry that uses plant substances for a bio based economy (Brereton et al., 2017). Therefore, future scaled up trials for phenolic glycoside yield should also include *S. viminalis* clones. Moreover, recent studies showed not only salicylates to be effective as bioactive substances in willow bark, other compounds such as flavonoids and procyanidins are also known for their bioactivity potential (Agnolet et al., 2012; Fiebich and Chrubasik, 2004, Ishikado et al., 2013; Nahrstedt et al., 2007).

Drought tolerance: At the site with a high ground water level (Wriezen2) a new *S. viminalis x viminalis* clone (Bowles x Jorr_ER5_W) had the highest biomass. None of the other two species (neither an interspecific nor an intraspecific crossing) had a significantly higher biomass than the standard *S. schwerinii* x *viminalis* Tordis. The high biomass of the crossing Bowles x Jorr_ER5_W demonstrates that *S. viminalis* and relatives have the potential to be further improved by intraspecific crossings.

At the site with the dry soil conditions the picture is different. Because of differences in the setup of the field trials we cannot compare the biomass yields of both sites directly. They have been planted in different years, the genotypes overlap only partially, and the cutting regime differed (second rotation, second year for Wriezen2; first rotation, third year for Wei15). Furthermore, the biomass at Wei15 has not been measured, but calculated as oven dry ton, not as fresh weight, for better comparison with published literature. Nevertheless, we can compare the relation of the different species and crossing families to each other and to the standard *S. schwerinii* x *viminalis* Tordis. The significantly best biomass producers are three intraspecific clones of *S. daphnoides* and further 16 *S. daphnoides* x *daphnoides* clones show a higher biomass (though not significantly) than the standard. At the first glance, this seems to indicate that *S. daphnoides* performs superior to *S. viminalis* and related species at the dry site. This could be due to the expected drought tolerance of S *daphnoides*. Almost all *S. daphnoides* crossings have at least one crossing partner originating from the Baltic Sea coast. There, *S. daphnoides,* normally a small tree grows, more bushy and on sand dunes (Chmelar and Meusel, 1979) indicating an adaption to sites with dry soils.

The interpretation of draught tolerance is problematic since the plants at site Wriezen2 were cut after the first growing season, while they were grown for three seasons without cutting at Wei15. *S. viminalis* is a shrub willow and sprouting from stool with many shoots and even needs cutting to develop full biomass capacity (Karp et al., 2011). *S. daphnoides* from the Alps is small tree (Chmelar and Meusel, 1979) regenerating from stools with a smaller number of shoots and is not promoted as strongly by cutting as *S. viminalis*. This also applies to most of the tested crosses of *S. daphnoides*, although they have been selected in the nursery trials for a high number of basal shoots. Therefore it is possible that *S. viminalis* did not show its optimal growth at the dry site due to the uncut condition and not due to drought stress. The three years growth on the hand may have been favorable for *S. daphnoides*. Further field trials are necessary to finally conclude whether *S. daphnoides* is superior at dry sites in comparison to *S. viminalis* and relatives.

Biomass production: The estimated dry weight of the three best S. *daphnoides* x *daphnoides* clones is with 9 odt (oven dry ton) ha^{-1} below the values reported for *S. viminalis*/ *S. dasyclados* with 10 to 20 odt $ha^{-1}a^{-1}$ and exceptionally 30 odt ha^{-1} a^{-1} in experimental plots (Mola-Yudego and Aronsson, 2008). This difference is even higher considering that literature values are annual yield estimates, whereas Wei15 was measured three years after planting. On the other hand, the yield of the first rotation will be increased after cutting (Tahvanainen and Rytkonen, 1999). Thus, a higher yield could be expected over a longer operation time of Wei15. If this is also true for *S. daphnoides*, which is normally sprouting from stools with less shoots than *S. viminalis* has to be shown in future field trials.

It should also be considered that in poplar experimental field plots yields are overestimated 4 to 7 times as compared to plantations (Hansen, 1991). In commercial willow plantations of Southern and Central Sweden average yield was 4.47 odt ha^{-1} a^{-1} with an average rotation length of 4.1 years (Mola-Yudego and Aronsson, 2008). The biomass value of

S. daphnoides at Wei15 should also be seen under the aspect that the best clone *S. daphnoides* x *daphnoides* DA92 x DA135_1_Z is also a clone that is valuable as source for salicylates (Figure 7).

While the interspecific crosses of S. *viminalis x daphnoides* show both weak clones and clones that are not significantly different to *S. viminalis*, crossings with *S. purpurea* as mother are not under the promising clones. This is surprising since *S. purpurea* is routinely used as starting material both for biomass and salicylate improvements (Serapiglia et al., 2013; Smart et al., 2005; Sulima et al., 2017) and also in interspecific crosses (Fabio et al., 2017). Since *S. purpurea* produces its biomass from many thin shoots as compared to *S. daphnoides* with few but strong shoots it is possible that *S. purpurea* crossings need repeated cuts before they develop higher biomass yields from the stools.

The three hybrids of *S. humboldtiana* show that crossings of *S. humboldtiana* with several native S*alix* species are possible. The hybrids could be easily propagated by cuttings so that they could be planted into the field trial. The relatively low yield at Wei15 could have been the same reason as for S. purpurea. The question of the number of shoots from stools can only be solved in further field trials with repeated cuts.

***Melampsora* resistance:** *Melampsora* infestation was low at Wriezen2. At the field trial Wei15 rust infestation was so low that it was not measured separately. The reason could be that the choice of the parent genotype was already guided by rust resistance (e.g. DA135 is rust resistant) and that the offspring with a high rust susceptibility have been eradicated in the nursery trials. If this is true, then the selection at an early stage of the breeding process is an easy approach to reduce rust susceptibility.

Another reason for the low rust incidence could be the absence of the alternate host from the field trial Wriezen2. In a previous study in the stock collections and nursey trials of Zepernick, Berlin-Dahlem and Waldsieversdorf it could be shown that the *Salix* species *S. viminalis*, *S. daphnoides* and *S. purpurea* are mainly infested by two formae speciales of the rust species *Melampsora larici-epitea* Kleb. (Bubner et al., 2014). The alternate host of this *Melampsora* species is *Larix decidua* Mill (Gäumann, 1959; Klenke and Scholler, 2015). This tree species is not present in the vicinity of Wriezen2 which is surrounded by meadows and agricultural fields. For future scaled up field trials their location should be chosen for the presence of the potential alternate host. This approach should ensure that the rust surveys give a realistic view of the rust resistance.

Processing of multipurpose willows: When growing a dual or multipurpose plant it should be possible to use the same feedstock for different purpose e.g. as bioenergy and for chemoceuticals (Joyce and Stewart, 2012; Lyko et al., 2009). In the case of salicylate containing willows the bark will be peeled off and the remaining wood can be processed to wood chips. Although commercial willow bark peeling machines for basketry are available, machines for the industrial use in short rotation coppice have still to be developed. Another problem is that the rotation time in willow plantation is four years (Mola-Yudego and Aronsson, 2008) but bark for phenolic glycosides extraction is usually harvested from one or two year old shoots (Sulima et al., 2017; Sulima et al., 2006). In a future field trial it should be tested whether four year old bark contains as much salicin as two year old shoots.

One approach to circumvent the peeling problem is the use of alternate rotation times. One rotation could comprise four years to produce biomass and a second rotation is one year to provide shoot for extraction of phenol glycosides. The phenols could be extracted directly from the shoot, although the industrial application of such an approach has still to be developed. In future project the phenolic glycoside yield from shoots and from bark should be compared. This includes measuring the percentage of bark dry weight and the total dry weight separately.

5 Conclusion

We could show that we are able to cross *S. viminalis* with *S. daphnoides* and *S. purpurea* to yield both salicylates and improved biomass. Crossings with *S. daphnoides* produced better results than crosses with *S. purpurea*. Besides interspecific crossing the crossing of two *S. daphnoides* genotypes is an equivalent approach to combine high salicylate content and high biomass. This approach should be further pursued. A next step is the planting of scaled up field trials to verify the results for agricultural application. They should also include sites with wet and dry soil conditions to further investigate the possible superiority of *S. daphnoides* over *S. viminalis* on dry sites. The necessary cuttings could be provided through the field trial Wei15.

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