FOR A SWEET WORLD – TOWARDS SUSTAINABLE SUGAR CROPS

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FOR A SWEET WORLD – TOWARDS SUSTAINABLE SUGAR CROPS

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Haplotype Variation of Flowering Time Genes of Sugar Beet and Its Wild Relatives and the Impact on Life Cycle Regimes

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The species *Beta vulgaris* encompasses wild and cultivated members with a broad range of phenological development. The annual life cycle is commonly found in sea beets (ssp. *maritima*) from Mediterranean environments which germinate, bolt, and flower within one season under long day conditions. Biennials such as the cultivated sugar beet (*B. vulgaris* ssp. *vulgaris*) as well as sea beets from northern latitudes require prolonged exposure to cold temperature over winter to acquire floral competence. Sugar beet is mainly cultivated for sugar production in Europe and is likely to have originated from sea beet. Flowering time strongly affects seed yield and yield potential and is thus a trait of high agronomic relevance. Besides environmental cues, there are complex genetic networks known to impact life cycle switch in flowering plants. In sugar beet, *BTC1*, *BvBBX19*, *BvFT1,* and *BvFT2* are major flowering time regulators. In this study, we phenotyped plants from a diversity *Beta* panel encompassing cultivated and wild species from different geographical origin. Plants were grown under different day length regimes with and without vernalization. Haplotype analysis of *BTC1*, *BvBBX19*, *BvFT1,* and *BvFT2* was performed to identify natural diversity of these genes and their impact on flowering. We found that accessions from northern latitudes flowered significantly later than those from southern latitudes. Some plants did not flower at all, indicating a strong impact of latitude of origin on life cycle. Haplotype analysis revealed a high conservation of the CCT-, REC-, BBX-, and PEBP-domains with regard to SNP occurrence. We identified sequence variation which may impact life cycle adaptation in beet. Our data endorse the importance of *BTC1* in the domestication process of cultivated beets and contribute to the understanding of distribution and adaption of *Beta* species to different life cycle regimes in response to different environments. Moreover, our data provide a resource for haplotypes identified for the major floral regulators in beet.

Keywords: *Beta vulgaris*, ssp. *maritima*, vernalization, bolting, phenological development

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INTRODUCTION

To ensure reproductive and therewith evolutionary success, flowering plants have developed different life cycles. Sea beets (*Beta vulgaris* L. ssp. *maritima*) are wild relatives of sugar beet (*B. vulgaris* L. ssp. *vulgaris*). Annual sea beets from Mediterranean environments germinate, bolt, and flower within one season under long days, whereas most sea beets from northern latitudes

are biennial. They need prolonged exposure to cold temperatures (typically during winter) to acquire a floral competent state. Besides, there are perennial sea beets mostly from Northern Europe which exhibit an iteroparous life cycle (Hautekèete et al., 2002). While iteroparous beets revert to vegetative growth after reproduction, annual and biennial beets are semelparous and die after reproduction (Hautekèete et al., 2001). The onset of floral transition in beets is indicated by the elongation of the main shoot which is commonly referred to as "bolting." In sugar beet cultivation, early bolting (without vernalization) is a trait of high agronomic relevance because it causes severe yield loss. The genetic control of photoperiodic flowering has been elucidated in the model plant *Arabidopsis thaliana* and many of the identified genes are structurally conserved in all known plants (Capovilla et al., 2014; Pajoro et al., 2014; Blümel et al., 2015).

In beet, major components of the photoperiodic pathway have been identified. The bolting locus *BOLTING TIME CONTROL 1* (*BTC1*) determines the annual life (Pin et al., 2012). *BTC1* was identified as a pseudo-response regulator (PRR) gene, sharing sequence homology with the *PSEUDO RESPONSE REGULATOR 7* (*PRR7*) gene from *A. thaliana*. It encodes for a protein carrying a response regulator receiver (REC) and a CONSTANS, CONSTANS-Like, and TOC1 (CCT) domain. Beets carrying the dominant *BTC1* allele mainly reveal an annual growth habit such as most sea beet genotypes, while beets carrying the recessive *btc1* allele exhibit a biennial life cycle (Pin et al., 2012). Two *FLOWERING LOCUS T* (*FT*) genes, *BvFT1,* and *BvFT2* which are homologous to the Arabidopsis *FT,* were discovered acting downstream of *BTC1* (Pin et al., 2010). *BvFT1* and *BvFT2*, both belonging to the phosphatidylethanolamine-binding protein (PEBP) gene family, have evolved antagonistic functions. While *BvFT2* promotes flowering and is required for floral development, *BvFT1* acts as a floral repressor. Pin et al. (2012) proposed a model for life cycle control in beet with *BTC1* acting upstream of *BvFT1* and *BvFT2*. In annual beets, the dominant *BTC1* allele represses *BvFT1* and concurrently activates *BvFT2* to induce bolting and flowering. On the contrary, in biennial beets the expression of the recessive *btc1* allele is increasing gradually to a decreasing expression of *BvFT1* during vernalization, enabling the promotion of *BvFT2* expression to initiate flowering. The recent discovery of another bolting time regulator *BvBBX19* encoding for a DOUBLE B-BOX TYPE ZINC FINGER protein extended the model for bolting time regulation in beet (Dally et al., 2014). *BvBBX19* is diurnally regulated and acts epistatically over *BTC1* upstream of *BvFT1* and *BvFT2*. Interestingly, *BTC1* transcription was reduced in *BvBBX19* mutants suggesting a physical interaction of both proteins to jointly regulate *BvFT1* and *BvFT2* (Dally et al., 2014). In addition to those major regulators, several *CONSTANS-LIKE* (*COL*) genes have been detected, differing by their zinc-finger (B-Box) and CCT domains (Chia et al., 2008; Dally et al., 2014). To date, only *BvCOL1* has been functionally characterized by overexpression in Arabidopsis (Chia et al., 2008) but it was excluded as a functional ortholog of *CO* due to non-typical expression profile. Hébrard et al. (2013) compared gene expression and DNA methylation profiles of bolting-resistant and bolting-sensitive beet genotypes after vernalization and determined 169 differentially expressed genes and 111 differentially methylated regions as putative bolting loci. The *SBT-9*/*BR1* locus was discovered to control bolting resistance after winter (Pfeiffer et al., 2014), where a homolog of the Arabidopsis *CLEAVAGE AND POLYADENYLATION SPECIFIC FACTOR 73-I* (*CPSF73-I*) was identified as the most promising candidate gene (Tränkner et al., 2016). Recently, Tränkner et al. (2017) proposed that two QTL contribute to variation in seasonal bolting. Besides *SBT-9*/*BR1*, the *SBT-4* locus was elucidated to majorly control seasonal bolting and *BvFT2* was suggested as a candidate gene.

The adaptation to different environments is of central importance for the evolutionary success in flowering plants. In *Beta* species, adaptation to different geographical regions is processed through the evolution of different life cycles (Hautekèete et al., 2002). It was suggested that the domestication of sugar beet involved the selection of a rare partial loss-of-function allele of *BTC1*, which alters the plant's response to long day conditions (Pin et al., 2012). A *BTC1* haplotype analysis of a large number of *Beta* accessions and cultivars revealed eleven haplotypes divided into two classes, "annuals" ($BTC1_{d-k}$) and "biennials" ($btc1_{a-c}$). These two classes mainly differ by six non-synonymous single-nucleotide polymorphisms (SNPs) as well as a large insertion (∼28 kb) within the promoter of biennial *btc1* alleles (Pin et al., 2012). Intriguingly, vast majority of cultivated beets carry the recessive $btc1_a$ allele while sea beets mainly exhibited *BTC1* alleles from the "annual" class.

In contrast, information about *BvBBX19*, *BvFT1,* and *BvFT2* haplotypes and their abundance among wild and cultivated species is lacking so far. This study aims to understand the role of the four major *Beta* flowering time regulators *BTC1*, *BvFT1*, *BvFT2,* and *BvBBX19* on the adaptation to different environments. We assumed that sequence variations within the coding region of these genes have a major impact on phenological development. Consequently, a non-random distribution of haplotypes across accessions from different geographical origin was expected. Moreover, we reasoned that life cycle changes follow a latitudinal cline. For this purpose, 29 *Beta* accessions from different geographical origin were grown under standardized conditions and the onset of bolting was recorded. The coding regions of *BTC1*, *BvFT1*, *BvFT2,* and *BvBBX19* were sequenced from all accessions and found high variation within *BTC1*, whereas sequence variation among the other genes was low. A relationship between haplotype variation and life cycle regime could be established. Cultivated beets carry similar combinations of their *BTC1*, *BvBBX19*, *BvFT1,* and *BvFT2* haplotypes while sea beets displayed a much higher heterogeneity. These results demonstrate that haplotype variations of flowering time regulator genes are main drivers of the adaptive evolution of *Beta* species and the domestication of cultivated beet.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Phenotypic Analysis

Beta accessions were selected based on geographical diversity and expected bolting characteristics (annual and biennial;

TABLE 1 | Plant material used in this study.

Variety	Species name	Seed code	Geographical Origin	Latitude (0°'N)
Wild beet	*B. vulgaris* ssp. *maritima*	080287	Ireland	53.0
		080461	Denmark	56.0
		080468	Egypt	27.0
		080437	Pakistan	31.0
		080418	India	21.0
		100539	Germany	51.0
		991971	Greece	39.0
		080260	Netherlands	52.0
		930034	Spain	40.0
		112787	France	46.0
		112823	Great Britain	54.0
		080538	Great Britain	54.0
Sugar beet	*B. vulgaris ssp. vulgaris*	090023	Germany	51.0
		930176	Germany	51.0
		130333	Germany	51.0
		100043	Germany	51.0
		001684	Germany	51.0
		080394	Iran	32.0
		930181	USA	45.0
		080384	Turkey	39.0
		091645	Germany	51.0
Fodder beet	*B. vulgaris ssp. vulgaris*	080281	Germany	51.0
		080313	Greece	39.0
		080396	Iran	32.0
Red table beet	*B. vulgaris ssp. vulgaris*	092312	Russia	60.0
		080339	France	45.5
Leaf beet	*B. vulgaris ssp.vulgaris*	080238	Iraq	33.0
		081845	China	35.0
		092459	Italy	42.0

Table 1). Seeds were sown in 9 cm^2 pots and plants were grown and phenotyped in a climate chamber with 10 plants per accession under different experimental conditions: 22 h of light, 20°C [experiment 1 (E1)], 16 h of light, 20°C [experiment 2 (E2)], and 22 h of light, 20°C interrupted by a cold treatment at 4°C for 3 months [experiment 3 (E3)]. Plants were watered every second day. In experiment 3 plants were fertilized twice, after 119 days directly before vernalization as well as after 210 days directly after vernalization with PERIMOR. The light intensity was held at 315 µmol $m^{-2}s^{-1}$ and the humidity was about 70%. Bolting (BBCH 51) and flowering (BBCH 60) was recorded according to Meier et al., 1993). Without vernalization, 16 weeks after sowing, plants were classified as annual (bolting) or biennial (non-bolting; experiment 1 and 2). Plants which did not bolt 16 weeks after vernalization were classified as "never bolting" (experiment 3).

Molecular Analysis

The coding region of the flowering time genes *BvBBX19*, *BTC1, BvFT1,* and *BvFT2* was amplified by PCR. Primers and PCR conditions are listed in **Supplementary Tables 1**, **2**. *In silico* prediction of the coding gene structures of *BTC1*, *BvBBX19*, *BvFT1,* and *BvFT2* and primer positions are indicated in **Supplementary Figure 1**. DNA was isolated from leaves using the CTAB method (Rogers and Bendich, 1985) with slight modifications. PCRs were performed for single plants and PCR products of all plants of the same accession were diluted to an equal concentration and pooled. Sanger sequencing of all pools was performed at the Institute of Clinical Molecular Biology (IKMB, CAU Kiel). Sequence analysis was done with the CLC Main Workbench 6.9 (CLC bio, Aarhus, Denmark) and the DNASTAR Lasergene SeqMan Pro (DNASTAR Inc., Madison, USA) program packages. Allelic haplotypes were defined by aligning obtained sequences of the amplified fragments and checking for single nucleotide polymorphisms (SNPs) and insertion/deletion polymorphisms. Pooled sequences were blasted against the beet reference sequence (KWS2320Refseq0.9) (Dohm et al., 2013) using the BLASTN function of the CLC Main Workbench 6.9. All SNP positions were numbered beginning with the translation start site. The evaluation of SNPs and their positions was performed according to the IUPAC code (Johnson, 2010; **Supplementary Table 3**). Polymorphisms were categorized as synonymous (no impact on the amino acid sequence) or non-synonymous.

Statistical Analysis

The software R (R Development Core Team, 2015) was used for statistical analysis. The data evaluation started with the definition of an appropriate statistical mixed model (Laird and Ware, 1982). The data were assumed to be normally distributed and to be heteroscedastic due to the different levels of environments (experiments) and latitude. These assumptions are based on a graphical residual analysis. The statistical model included a pseudo factor (Schaarschmidt and Vaas, 2009), consisting of the actual factors experiment (E1, E2, E3), latitude (21°N-60°N) and varieties (sea beet, sugar beet, table beet, fodder beet, and leaf beet). This pseudo factor was necessary because the actual factors are not orthogonal. The genotype was regarded as a random factor. Based on this model, multiple contrast tests (Bretz et al., 2011) were conducted in order to compare the several levels of (i) variety, (ii) latitude, and (iii) experiment, respectively. Moreover, a further statistical model was established using latitude and experiment as covariates instead of the pseudo factor. On the basis of this model, an analysis of covariance (ANCOVA) was conducted (Cochran, 1957), resulting in (three) different linear regression functions with the same slope.

RESULTS

Large Phenotypic Variation for Flowering Time in Species of the Genus *Beta*

We chose 29 accessions from different geographical origin to represent the genetic diversity of the species *B. vulgaris* (**Table 1**). Of each accession, 10 plants were grown in a climate chamber

under three different environmental conditions. The onset of bolting was assessed as beginning of elongation of the main stem (BBCH51) after Meier et al., 1993). In experiment 1 and 2, plants were held under 22 and 16 h of light, respectively. In the third experiment, the same day/night regime as in experiment 1 was applied but biennial accessions were subjected to another 12 weeks of cold treatment (4°C). We uncovered annual and biennial bolting behavior in both wild and cultivated accessions (**Figure 1**, **Supplementary Table 4**).

There was a clear tendency for earlier bolting before vernalization under 22 h of light (experiment 1 and 3). On average, annual plants bolted 10 days earlier as when grown under 16 h of light. Six accessions were classified as annual (or segregating for annual and biennial) under 22 h of light while they behaved as biennials under 16 h of light (**Figures 2A–C**, **Supplementary Table 4**). The earliest accession, 080437 from Pakistan (31°N), bolted 19 days after sowing when grown under 22 h of light. Under 16 h of light it bolted after 27 days. The earliest accession under 16 h of light was 080468 from Egypt (27°N), which bolted 23 days after sowing. Contrary, 080538 from Great Britain (54°N) was the latest accession under 22 h of light which bolted 69 days after sowing. Interestingly, in experiment 3 only seven out of 10 plants bolted before vernalization, but the remaining three bolted after vernalization. When grown under 16 h of light, accession 080538 performed a biennial life cycle. There was considerable phenotypic variation within accessions under the same experimental conditions. The sea beet accessions 080260 (52°N), 080538 (54°N), 100539 (51°N), and 112787 (46°N) from northern latitudes segregated into annual and biennial plants (22 h of light). Similarly, the cultivated beet accessions 080384 (39°N), 080394 (32°N), and 080396 (39°N) from southern latitudes segregated for bolting under 22 h light (**Supplementary Table 4**).

We reasoned that the phenological development of *Beta* genotypes depends on latitude of origin. To test this hypothesis, an analysis of covariances (Cochran, 1957) with data from experiment 1–3 was conducted. This analysis revealed three different linear regression functions with the same slope (**Figure 3**), suggesting that accessions from southern latitudes of origin flowered earlier than those from northern latitudes. As all regression functions revealed the same slope, we concluded that all environments exert a similar effect of latitude on days to bolting. Additionally, our data show that accessions from northern latitudes exhibited a tendency toward biennial bolting (**Supplementary Table 4**). Surprisingly, not all cultivated beets displayed a biennial behavior. The leaf beet accession 080238 from Iraq (33°N) revealed an annual life cycle under all experimental conditions (without vernalization). Similar as some sea beet accessions, it bolted earlier under 22 h light (35 days), than under 16 h light (50 days). Moreover, the sugar beet accession 080384 from Turkey (39°N) segregated for bolting and non-bolting before vernalization, while bolting plants had a strong tendency toward early flowering in all experiments. After vernalization, sugar and fodder beets bolted within 26–61 days. However, one leaf beet accession from Italy (42°N) and one table beet accession from Russia (60°N) segregated into bolting and "never bolting" after vernalization. Also, the sea beet accessions 080287, 080461, 112823, and 112787 from Ireland (53°N), Denmark (56°N), Great Britain (54°N), and

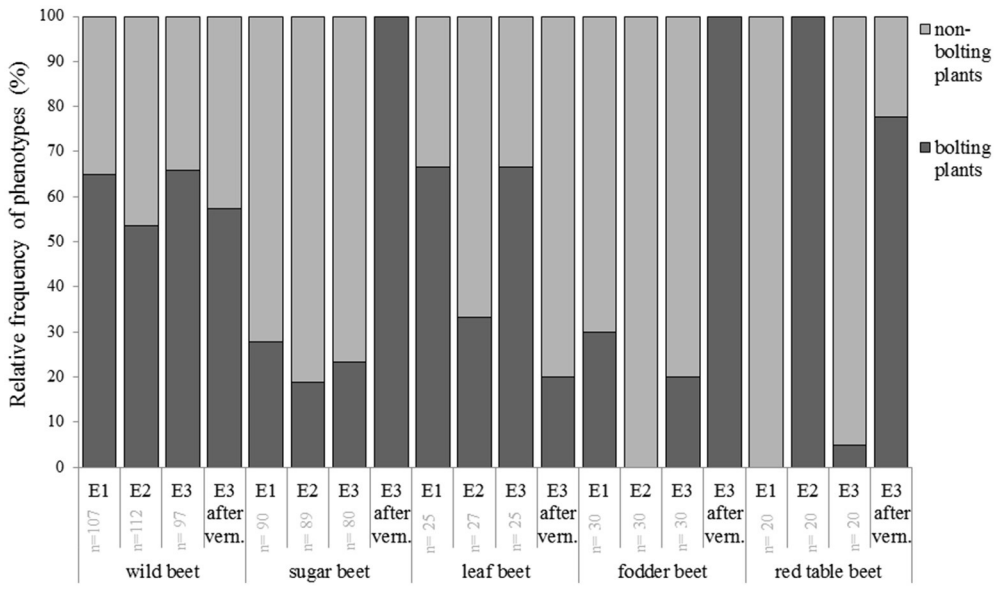


FIGURE 1 | Phenological development of cultivated beet under three different environments (experiment 1–3). Bolting was determined as the beginning of shoot elongation (BBCH51; Meier et al., 1993). Plants were grown in pods in a climate chamber and kept under three different LD conditions: 22 h light, 20°C (E1), 16 h light, 20°C (E2), 22 h light, 20°C, interrupted by 12 weeks of 4°C (E3). The number of cultivars used (*n*) is indicated in light gray. In E1 and E2 "non-bolting" means that plants did not bolt until 16 weeks after sowing. In E3 "non-bolting" means that plants did not bolt until the end of the experiment (16 weeks after vernalization).

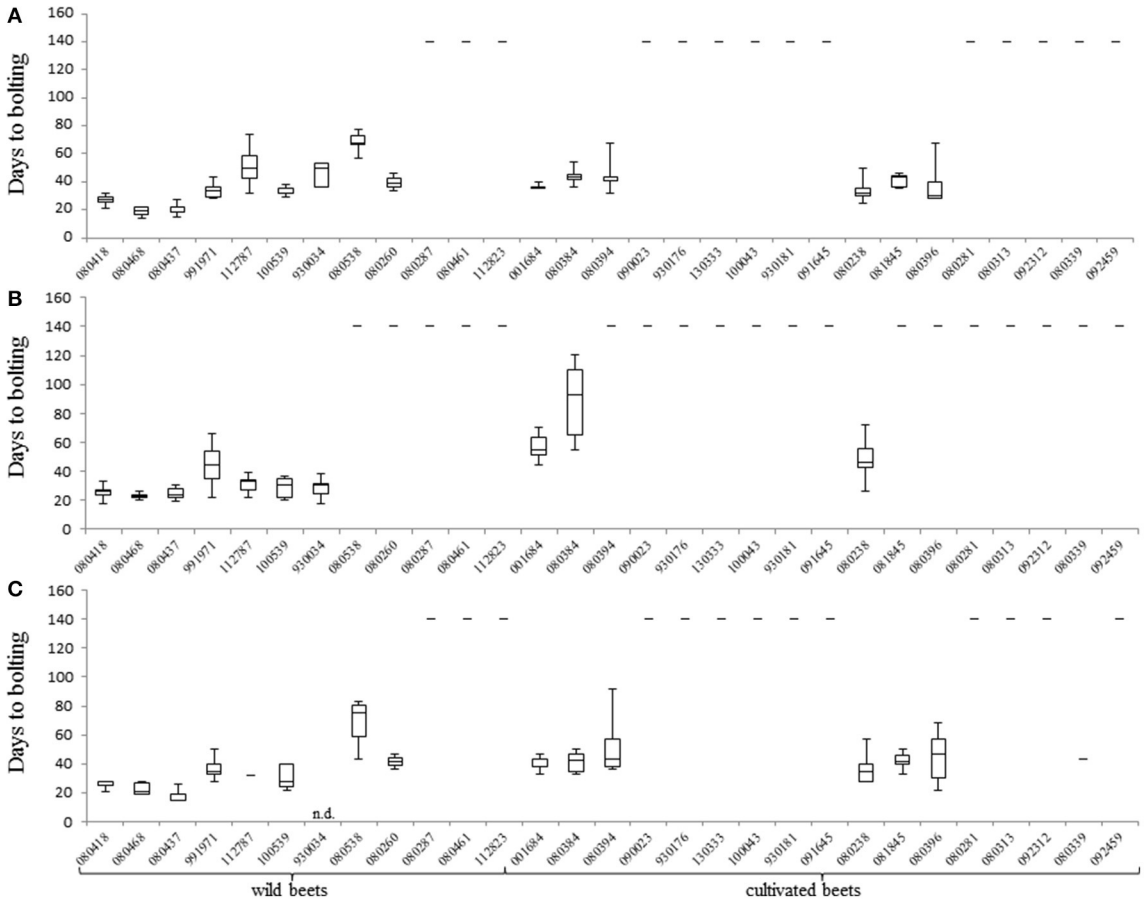


FIGURE 2 | Bolting time measurements for all accessions under different environmental conditions. **(A)** Days to bolting (DTB) of plants of accessions that bolted before vernalization when held in experiment 1 (22 h light/2 h dark). **(B)** DTB of plants of accessions when held in experiment 2 (16 h light/8 h dark). **(C)** DTB of plants of accessions when held in experiment 3 (22 h light/2 h dark). Bolting was determined as DTB after sowing without vernalization. Plants were grown in pods in a climate chamber and kept under LD conditions at 20°C, 315 μmol $m^{-2}s^{-1}$ and 70% humidity. Plants are separated by wild beet accessions and cultivated beet accessions. For plants of accessions that did not bolt without vernalization the value of days to bolting was set to 140 days. Error bars represent the standard error of the mean (SEM).

France (46°N) exhibited a tendency toward "never bolting" (**Supplementary Table 4**).

Haplotype Variation of Four Major Flowering Time Regulators

Next, we aimed to link sequence variations and phenological development. For haplotyping, the coding regions of *BTC1*, *BvBBX19*, *BvFT1,* and *BvFT2* were sequenced because they had been identified as major constituents of the bolting time regulatory pathway in beet (Pin et al., 2010, 2012; Dally et al., 2014). We sequenced pooled PCR products from single plants of an accession grown in experiment 1. If a pooled DNA sample turned out to be a mixture of different sequences or if segregation into bolting and non-bolting plants was detected, single plants were sequenced.

First, we sequenced the *BTC1* coding region (2,367 bp) for each accession and compared it to the reference sequence (Pin et al., 2012). Twenty-five out of 27 SNPs have already been described by Pin et al. (2012) whereas two additional polymorphisms (exon 8 nt2 and exon 9 nt29) turned out to be new (**Table 2**). Four polymorphic nucleotides are located within the sequence encoding the CCT- and the REC-domain (two in each domain). One of these (exon 3, nt351) represents a non-synonymous mutation from Asparagine to Lysine (Pin et al., 2012). In total, 1 different *BTC1* haplotypes were identified among 29 accessions. Of these, three haplotypes have been unknown so far ($BTC1_l$, $BTC1_m$, $BTC1_n$), the remaining eight haplotypes have already been described by Pin et al. (2012) (**Table 2**). As expected, most of the cultivated (biennial) beet accessions carried the $btc1_a$ haplotype (**Supplementary Tables 5**, **9**), which has already been attributed as "biennial" *btc1* haplotype ($btc1_{a-c}$; Pin et al., 2012).

Second, we sequenced the coding region of the *BvBBX19* gene (588bp). Sequence variation was much lower as observed for *BTC1*. We identified one non-synonymous and three synonymous polymorphisms. As expected, none of the accession carried the EMS mutations which had been published by Dally et al. (2014). Interestingly, only one synonymous SNP was located

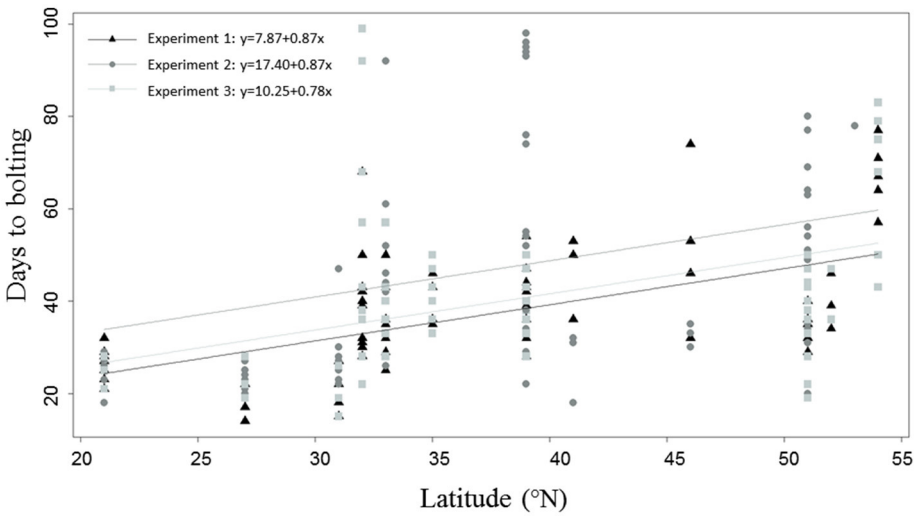


FIGURE 3 | Days to bolting on a latitudinal cline (°N). Bolting plants from experiment 1 are depicted as black triangles. Bolting plants from experiment 2 are depicted as dark gray, filled circles and bolting plants from experiment 3 are depicted as light gray, filled boxes. The statistical model included experiment and latitude as covariates and an analysis of covariances (ANCOVA) was conducted, resulting in different linear regression functions for all environments with the same slope. Plants were grown in pods in a climate chamber and kept under LD conditions (either 16 h of light/8 h dark, 20°C (experiment 2) or 22 h of light/2 h dark (experiment 1 and 3), 20°C, 315 μmol $m^{-2}s^{-1}$ and 70% humidity). Non-bolting plants are not included in the analysis.

within the region coding for the B-Box-domains. Taken together, all polymorphisms gave rise to seven haplotypes ($BvBBX19_{a-g}$) across all accessions analyzed in this study (**Table 3**). The non-synonymous SNP was present only in two haplotypes ($BvBBX19_{f,g}$). These haplotypes occurred in all leaf beet- and several sea beet accessions, but only in one fodder beet- and one sugar beet accession (**Supplementary Table 6**). Intriguingly, all accessions with the $BvBBX19_f$or $BvBBX19_g$haplotypes originate from southern latitudes (21–42°N).

Third, we analyzed the coding region of the floral repressor *BvFT1* (540 bp). In total, we found one synonymous as well as four non-synonymous SNPs. Additionally, an insertion of 3 bp in exon 1 was identified which occurred only in two accessions. These polymorphisms could be assembled to eight haplotypes ($BvFT1_{a-h}$; **Table 4**). Four of the five polymorphisms are located outside the PEBP -domain region. Only two accessions house a polymorphism within the PEBP coding region (exon 4 nt57, haplotype $BvFT1_c$; **Supplementary Table 7**), indicating a high conservation of this domain.

Fourth, the *BvFT2* gene was studied and two non-synonymous polymorphisms giving rise to four haplotypes ($BvFT2_{a-d}$; **Table 5**) were identified. One SNP is located within the PEBP-domain region (exon 4 nt39). Remarkably, those haplotypes with the PEBP domain mutation ($BvFT2_b$ and $BvFT2_c$) were only present in sea beet accessions from northern latitudes (39–56°N). In contrast, $BvFT2_a$ and $BvFT2_d$ are highly abundant in cultivated *Beta* accessions (**Supplementary Table 8**).

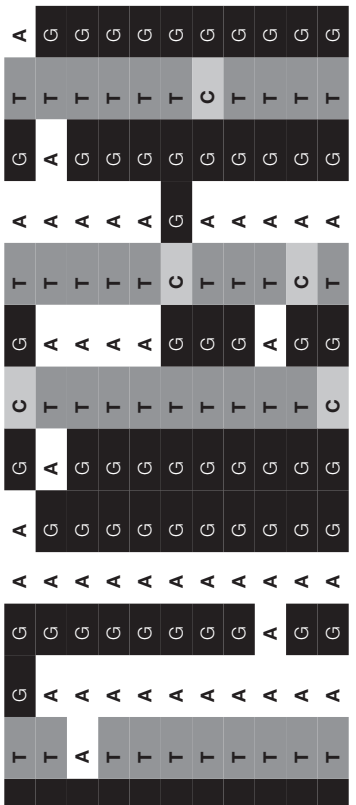
Relation between Haplotype Variation and Life Cycle Regime

We anticipated a link between haplotype variation and life cycle regime which in turn depends on the geographical origin of an accession. First, we looked for a reciprocal relation between haplotypes and phenological development under long day conditions (experiment 1; **Supplementary Table 9**). As a general rule, cultivated beets which mainly exhibited a biennial life cycle displayed low genetic variation. In the majority, they carried similar combinations of their *BTC1*, *BvBBX19*, *BvFT1,* and *BvFT2* haplotypes. Most cultivated sugar beet accessions (090023, 130333, 091645, 100043, and 930176) revealed the "biennial" $btc1_a$ and $BvBBX19_a$ haplotypes, respectively. Moreover, seven out of nine sugar beet accessions, as well as both red table beet accessions displayed either the $BvFT1_a$ and $BvFT2_a$ or the $BvFT1_a$ and $BvFT2_d$ haplotype combination. Sea beets displayed a much higher heterogeneity (between and within accessions) regarding their *BvBBX19*, *BvFT1,* and *BvFT2* haplotypes whereas most accessions were fixed for only one *BTC1* haplotype (exceptions: 991971, 080538, 081845), despite a high sequence variation within this gene across all accessions. Noteworthy, the new $BTC1_m$ haplotype only occurred in sea beet accessions from higher latitudes (21.0-39.0°N).

DISCUSSION

We examined 29 *Beta* accessions from different geographical origins including wild and cultivated beets for phenotypic plasticity under different photoperiodic conditions. Further, the haplotypes of four flowering time regulators, *BTC1*, *BvBBX19*, *BvFT1,* and *BvFT2* were analyzed to uncover the relationship between haplotype variation and life cycle adaptation. We found a general southward shift toward earlier flowering. Plants from northern latitudes flowered considerably later or did not flower at all, pointing at a strong coherence of life cycle and geographical origin. Besides, our data revealed a high

TABLE 2 | Haplotype studies with the *BTC1* gene.



Twenty-seven SNPs were assembled to 11 haplotypes. The coding sequence of BTC1 was sequenced from all plants of the B. vulgaris panel. The position of the SNPs is given relative to its translation start site according to the regarding exons. The different nucleotides are indicated by different colors. Asteriks represent non-synonymous SNPs.

TABLE 3 | Haplotype variation within the *BvBBX19* gene.

Exon	**2**		**4**	
SNP position/Haplotype	69	45	59	231
$BvBBX19_a$	**A**	G	**C**	**T**
$BvBBX19_b$	**A**	G	**C**	**C**
$BvBBX19_c$	G	G	**C**	**T**
$BvBBX19_d$	G	**A**	**C**	**C**
$BvBBX19_e$	G	**A**	**C**	**T**
$BvBBX19_f$	G	**A**	**T**	**C**
$BvBBX19_g$	G	**A**	**T**	**T**
Non-syn. SNP			*	

Four SNPs were assembled to 7 haplotypes. The coding sequence of BvBBX19 was sequenced from all plants of the B. vulgaris panel. The position of the SNPs is given relative to its translation start site according to the regarding exons. The different nucleotides are indicated by different shading. Asteriks represent non-synonymous SNPs.

TABLE 4 | Haplotype variationç within the *BvFT1* gene.

Exon	**1**			**4**		**ATT insertion in exon 1 btw. n111 and 112**
SNP position/Haplotype	11	20	70	72	57	
$BvFT1_a$	G	**C**	**C**	**C**	**C**	
$BvFT1_b$	G	**C**	G	**C**	**C**	
$BvFT1_c$	G	**C**	G	**C**	**T**	
$BvFT1_d$	G	**T**	G	**C**	**C**	
$BvFT1_e$	G	**T**	**C**	**C**	**C**	
$BvFT1_f$	**T**	**T**	**C**	**T**	**C**	X
$BvFT1_g$	G	**T**	**C**	**T**	**C**	
$BvFT1_h$	G	**C**	**C**	**T**	**C**	
Non-syn. SNP	*	*	*		*	

Five SNPs were assembled to 8 haplotypes. The coding sequence of BvFT1 was sequenced from all plants of the B. vulgaris panel. The position of the SNPs is given relative to its translation start site according to the regarding exons. The different nucleotides are indicated by different colors. Asteriks represent non-synonymous SNPs.

conservation of the important protein domains (CCT-, REC-, BBX-, and PEBP) for all genes emphasizing their evolutionary relevance for life cycle adaptation in *Beta* species. Withal, haplotype analysis of *BvBBX19*, *BvFT1,* and *BvFT2* displayed only a few polymorphisms when compared with the high SNP frequency in *BTC1*. While most cultivated beets carried similar haplotype combinations of *BTC1*, *BvBBX19*, *BvFT1,* and *BvFT2*, sea beets displayed much higher heterogeneity. Our findings display several new haplotypes of beet's major floral regulators and connect these to different life cycle regimes.

To warrant evolutionary success of a flowering plant, the adaptation to different climates concomitant with life cycle control is of utmost importance. There are several environmental factors which impact phenotypic plasticity of a flowering plant, such as temperature and photoperiod (Andrés and Coupland, 2012). Species of the genus *Beta* have evolved different life cycles over the years allowing adaptation to a broad spectrum of latitudes. Besides annuality, beets have evolved biennial and

TABLE 5 | Haplotype variation within the *BvFT2* gene.

Exon	1	4
SNP position/Haplotype	82	39
$BvFT2_a$	A	G
$BvFT2_b$	A	A
$BvFT2_c$	C	A
$BvFT2_d$	C	G
Non-syn. SNP	*	*

Two SNPs were assembled to 4 haplotypes. The coding sequence of BvFT2 was sequenced from all plants of the B. vulgaris panel. The position of the SNPs is given relative to its translation start site according to the regarding exons. The different nucleotides are indicated by different colors. Asteriks represent non-synonymous SNPs.

perennial life cycles (Hautekèete et al., 2002), which makes this species an ideal species to study life cycle adaptation. However, prior to this study there was only scant knowledge about the relationship between plant phenology and geographical origin in correlation with genetic diversity of known flowering time genes. What are possible explanations for this relationship particularly with regard to vernalization requirement?

For sea beets (*B. vulgaris* spp. *maritima*) a genetically based latitudinal gradient for flowering time along Western European coasts has been shown, together with heritability for flowering time and vernalization requirement. Van Dijk et al. (1997) and Boudry et al. (2002) have demonstrated that differences in life cycle due to vernalization requirement seem to be an adaptive response to season length and spring temperatures in particular latitudes. Van Dijk et al. (1997) sampled seeds from 93 sea beet populations situated along a latitudinal cline around the French coast and in adjacent regions and examined flowering behavior and the relationship between latitude and vernalization requirement under greenhouse conditions. They found that the more southern the origin of plants is the less vernalization is required. While the frequency of flowering phenotypes without vernalization requirement is very high in coastal and inland populations around the Mediterranean, most plants from the Atlantic coast are not able to flower without vernalization. The authors suggested that flowering time in southern parts is controlled by warm temperatures and day length, whereas northern populations have a strong requirement for vernalization. The general importance of vernalization requirement was further emphasized by Van Dijk (2009) who showed that a shortened cold period leads to a complete inhibition of flowering which can be compensated by an artificial increase of photoperiod. Similar observations were obtained by Boudry et al. (2002), who investigated a smaller number of sea beet populations but a higher number of plants along a similar north-south cline of France as Van Dijk et al. (1997). The authors applied several cold regimes to plants of different age over 3 years in the glasshouse and in the field and found that plants from northern origins exhibit a greater requirement for vernalization. Intriguingly they detected that the northernmost population exhibited a lower reproductive success than other populations and thus seemed to be not well adapted. They hypothesized that these findings may underlie inbreeding depression and a lack of genetic variability in small rather isolated populations and that the evolutionary equilibrium has not been reached since the last ice age.

The general aspects of early flowering in wild population have been reviewed by Charnov and Schaffer (1973) who hypothesized that selection for earlier flowering and therewith reproduction underlies a decreased probability of survival. In case of sea beets, a high mortality pressure often occurs especially for inland populations in disturbed environments along roadsides or in sunflower fields, where they are under heavy selective pressure (Boudry et al., 1993). These so-called weed beets are a result of accidental cross-fertilization between ruderal wild beets and crop lineages, mainly found close to seed production fields. The selection for early flowering and a short life history based on high mortality pressure has been suggested for weed beet populations in sugar beet production areas (Van Dijk and Desplanque, 1999).

Even though several studies covered a higher number of populations and plants compared to our study, their observations mainly focus on sea beets from the Mediterranean and the Atlantic coast of France. Our *Beta* panel, by contrast, displays a broader range of genotypes from different geographical origin and it includes several cultivated beets. We detected a southward shift toward earlier flowering and a complete absence of vernalization requirement of *Beta* accessions from southern latitudes which is in line with previous studies from Boudry et al. (2002) and Van Dijk et al. (1997). Our findings show that *Beta* accessions from northern hemispheres flowered later or did not flower at all, hinting at a major effect of vernalization requirement. By contrast, accessions from southern latitudes flowered earlier, especially wild beets. This result is in line with a general observation that in southern latitudes flowering is mediated mainly by spring temperatures, while in northern latitudes winter chilling is a limiting factor (Tooke and Battey, 2010). In our study, three cultivated accessions from southern latitudes (080384, 39°N; 080394, 32°N; and 080396, 39°N) segregated into annual and biennial plants which can be explained by cross-fertilization with sea beets that suffered high mortality pressure as demonstrated by Boudry et al. (1993) and Van Dijk and Desplanque (1999). The very early flowering phenotypes we observed for some accessions from southern latitudes could be the result of southern climate conditions or environmental instability as discussed by Hautekèete et al. (2009).

How can the phenological development of *Beta* genotypes be explained by genetic variation? In a changing climate, early flowering will be selected for in long day plants (Van Dijk and Hautekèete, 2007). The direct effect of climate change on phenology in sea beets was recently demonstrated by Van Dijk and Hautekèete (2014). They sampled seeds from 73 sea beet populations on Mediterranean and European Atlantic coasts in 2 different years (1989 and 2009) and grew the plants under greenhouse conditions. As a result of natural selection within 20 years, the southern populations shifted toward later flowering, whereas the northern populations flowered earlier. The authors conclude that their findings are based on genetic changes in sensitivity to environmental cues, such as increased temperature over the years. Thus, evidence for genetic change imparting flowering phenology has been given, but the

genetic reasons remained in the dark. Today we know that vernalization requirement is a key component of flowering time regulation.

In *A. thaliana*, the MADS-box gene *Flowering Locus C* (*FLC*) plays a central role in regulating vernalization response (Michaels and Amasino, 1999; Sheldon et al., 2000). In *B. vulgaris*, a vernalization-responsive *FLC* homolog, *FLC-LIKE 1* (*BvFL1*) has been identified and a conserved function as a floral repressor was suggested after genetic complementation in Arabidopsis (Reeves et al., 2007). Hébrard et al. (2013) detected RNA methylation of the *BvFL1* mRNA after vernalization which seemed to indicate its role in vernalization response. However, a recent study clearly demonstrated that RNAi-mediated down-regulation of *BvFL1* did not reveal any major effect on bolting without or after vernalization. Moreover, over-expression of *BvFL1* only led to a 1 week delay in bolting after vernalization, suggesting that *BvFL1* is not a major regulator of vernalization response in beet (Vogt et al., 2014).

Evidently, vernalization requirement is under control of the bolting locus *B* (Abegg, 1936; Van Dijk et al., 1997; Boudry et al., 2002; Van Dijk, 2009; Pin et al., 2012). Pin et al. (2012) had cloned the *BTC1* bolting gene from the *B* locus and demonstrated for the first time that natural allelic variation of a single gene impacts life cycle variation of beet. The authors suggested that life cycle adaptation results from haplotype diversity of *BTC1* which alters the plant's response to long day conditions. Interestingly, only one of the non-synonymous SNPs (exon 3, nt351) was located within the sequence encoding for the CCT-domain. This polymorphism was only present in cultivated beets which carry the $btc1_a$ haplotype, indicating a potential target during domestication. In our study eleven *BTC1* haplotypes from which three were unknown to date were identified, suggesting a high genetic diversity of the chosen material. Our findings are in line with those of Pin et al. (2012) in a manner that "annual" *BTC1* haplotypes primarily occurred in sea beets from southern regions, whereas "biennial" *btc1* haplotypes were mainly found in cultivated beets from northern regions. Interestingly, we identified two sea beet accessions from northern latitudes (080287 from Ireland and 080461 from Denmark) which showed a biennial life cycle under all experimental conditions although these genotypes carried an "annual" *BTC1* haplotype.

Apart from *BTC1*, three more genes (*BvBBX19*, *BvFT1,* and *BvFT2*) are major flowering time regulators associated with life cycle adaptation in beet (Pin et al., 2010; Dally et al., 2014). For *BvBBX19,* it was shown that polymorphisms derived from EMS mutagenesis turned an annual to a biennial beet (Dally et al., 2014). In our study we focused on natural variation within these flowering time genes. We detected four polymorphisms within the coding region of *BvBBX19* resulting in seven haplotypes. One SNPs (exon 4, nt59) results in a non-synonymous mutation, while another one (exon 2, nt69) causes a synonymous mutation within the B-Box coding domain. The haplotypes $BvBBX19_{f-g}$, harboring the non-synonymous mutation, were found exclusively in accessions from southern latitudes. Interestingly, these haplotypes appear in both, wild and cultivated beets and, with one exception, all of these accessions segregated for bolting when grow under 22 h of light. The remaining two SNPs resulted in synonymous mutations which are located outside the B-Box coding domain.

Recently, *BvFT2* was proposed as a candidate gene for seasonal bolting time at the *SBT-4* locus (Tränkner et al., 2017). We identified two and five polymorphisms within *BvFT2* and *BvFT1*, respectively. Interestingly, for each gene we found one non-synonymous SNP within the PEBP-domain coding sequence. *BvFT2* haplotypes which carried this SNP ($BvFT2_b$ and $BvFT2_c$) were only present in sea beets from northern latitudes (39–56°N) which primarily exhibited a biennial life cycle. In addition, the $BvFT1_f$ haplotype, which displayed a 3bp insertion in the coding region of the PEBP domain, was only identified in the two southernmost sea beet accessions (080437 and 080418) from Pakistan and India which exhibited very early bolting phenotypes. We hypothesize that this insertion may impair the repressing function of *BvFT1* in these plants, thus enabling early flowering. Overall, *BvFT2* displayed the highest sequence conservation which underpins its importance as a floral integrator. This is in line with other studies where *FT* functional orthologs that induce flowering are highly conserved in diverse species, such as the rice *FT* ortholog *Heading date3* (*Hd3a*; Tamaki et al., 2007), *SINGLE FLOWER TRUSS* (*SFT*) from tomato (Lifschitz et al., 2006) or *CENTRORADIALIS8* (*ZCN8*) from *Zea mays* (Lazakis et al., 2011; Meng et al., 2011).

In conclusion, our findings show that geographical origin impacts life cycle adaptation of *Beta* genotypes. We found that vernalization requirement is absent in sea beet accessions from southern latitudes. A comparison of sequence variation of main flowering time genes between wild and cultivated beets exhibited a general tendency for increased sequence heterogeneity in sea beets. This can be explained by domestication and breeding which resulted in reduced genetic variation within these genes, indicative for selective sweeps. The $BvFT1_f$ haplotype which was found in the two southernmost sea beet accessions is of great interest for further studies, because it may represent an example for evolutionary genetic change to enable a short life history due to high mortality pressure in disturbed areas as suggested by Van Dijk and Desplanque (1999). Moreover, the new *BvFT2* and *BvBBX19* haplotypes may serve as novel resource for beet breeding to broaden the variation for bolting resistance even after winter which is necessary to breed winter beets (Jung and Müller, 2009).

AUTHOR CONTRIBUTIONS

NH: planned, conducted, and analyzed all experiments and drafted and wrote the manuscript; MH: helped with statistical analyses; ND: participated in designing the study and helped to draft the manuscript; CJ: participated in the design of the study and revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.02211/full#supplementary-material

Supplementary Figure 1 | *In silico* prediction of the coding gene structures of *BTC1*, *BvBBX19*, *BvFT1,* and *BvFT2*. Exons are depicted by cross-striped boxes. Conserved regions encoding for protein domains (REC., CCT-, BB1-, BB2-, and PEPB-domain) are indicated below the exonic structures. 3′- and 5′ UTRs are indicated by arrows above the exons. Identified non-synonymous polymorphisms are indicated by red arrows and synonymous polymorphisms are indicated by blue arrows.

Supplementary Table 1 | List of primer combinations and PCR conditions used for sequence analysis.

Supplementary Table 2 | List of markers including primer sequences used in this study for PCR and SANGER sequencing.

Supplementary Table 3 | IUPAC code for incomplete nucleic acid specification (Johnson, 2010).

Supplementary Table 4 | Phenotypic data from three different experiments. Plants were classified as annual (bolting within 16 weeks after sowing) or biennial (bolting only after vernalization). Plants that did not bolt 16 weeks after vernalization were classified as never bolting. Plants were grown and phenotyped in a climate chamber and kept under LD conditions with a light intensity of 900 µE.

Supplementary Table 5 | *BTC1* haplotypes of all plants from the *Beta* panel. The coding region was sequenced. The position of the SNPs is given relative to the translation start site according to the regarding exons. For each accession, PCR products of 10 plants were pooled and sequenced for haplotype analysis. In case of sequence heterogeneity, all single plants were sequenced and haplotypes were assembled which could result in more than one haplotypes per accession. The nomenclature of polymorphisms was given according to the IUPAC code (Johnson, 2010).

Supplementary Table 6 | *BvBBX19* haplotypes of all plants from the *Beta* panel. The coding region was sequenced. The position of the SNPs is given relative to the translation start site according to the regarding exons. For each accession, PCR products of 10 plants were pooled and sequenced for haplotype analysis. In case of sequence heterogeneity, all single plants were sequenced and haplotypes were assembled which could result in more than one haplotypes per accession.

Supplementary Table 7 | *BvFT1* haplotypes of all plants from the *Beta* panel. The coding region was sequenced. The position of the SNPs is given relative to the translation start site according to the regarding exons. For each accession, PCR products of 10 plants were pooled and sequenced for haplotype analysis. In case of sequence heterogeneity, all single plants were sequenced and haplotypes were assembled which could result in more than one haplotypes per accession.

Supplementary Table 8 | *BvFT2* haplotypes of all plants from the *Beta* panel. The coding region was sequenced. The position of the SNPs is given relative to the translation start site according to the regarding exons. For each accession, PCR products of 10 plants were pooled and sequenced for haplotype analysis. In case of sequence heterogeneity, all single plants were sequenced and haplotypes were assembled which could result in more than one haplotypes per accession.

Supplementary Table 9 | Complete list of all haplotype combinations and phenotypic data. The table also comprises information on growth type and latitude of origin. Rows shaded in light gray indicate accessions which were non-bolting without vernalization under all environments. Cells shaded in dark gray indicate accessions which revealed a non-bolting phenotype under 16 h of light (experiment 2) but an annual phenotype or mixed phenotype (annual + biennial) under 22 h light (experiment 1+3) before vernalization.

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Innovative Approaches to Evaluate Sugar Beet Responses to Changes in Sulfate Availability

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In this study, a system based on omics profiling was set-up for sugar beet (*Beta vulgaris* L. subsp. *vulgaris*) evaluation after changes in sulfate availability. Seedlings were grown on sulfate-deprived Hoagland solution. Six days after germination, 100 μM $MgSO_4$ was added to the solution. Root samples were collected 36 h after treatments. WinRHIZO root-scanning approach was used for the automated image analysis of plant root morphology. Inductively Coupled Plasma Spectrometry (ICP-OES) and quadrupole-time-of-flight mass spectrometry (Q-TOF) were used for ionomic and metabolic analysis, respectively. Nanofluidic real-time PCR (OpenArray system) was used for molecular profiling. OpenArray chips were designed with TaqMan probes for 53 sugar beet genes putatively involved in sulfate nutrition. At morphological level treated seedlings showed significantly higher values ($P < 0.01$) than untreated plants for root traits related to soil exploration and nutrient uptake, such as total root length, fine roots length and root tips number. ICP-OES, Q-TOF and transcriptomic data revealed changes due to sulfate availability in sugar beet samples. Two key results are highlighted in sulfate-supplied roots and leaves. Firstly, high expression levels of auxin efflux carrier component 1 (PIN) and 5-phosphoribosyl-anthranilate, precursor of tryptophan and auxin synthesis, were observed in roots. Secondly, high levels of 2-Cys peroxiredoxin BAS1, chloroplastic, thioredoxin reductase (NADPH) and cysteine synthase, chloroplastic/chromoplastic, *O*-acetylserine sulfhydrylase, involved in protection against oxidative stress and cysteine synthase activity, respectively, were observed in leaves. Based on our findings, the combination of evaluated omics approaches could become a key system for the evaluation of the nutritional status of sugar beet under different nutrient availability conditions.

Keywords: sugar beet yield, nutritional stress, sulfate availability, omics profiling, high-throughput qPCR profiling

INTRODUCTION

Sugar beet (*Beta vulgaris* L. subsp. *vulgaris*) is an important crop that satisfies 25% of world sugar demand with a total production of 269 Mt (FAO, 2013). In the Mediterranean area, global climate change has led to a decrease of about 1 t ha^{-1} in sugar production due to water shortage and low nutrient availability (Barbanti et al., 2007). Particularly, in modern day agriculture, sulfur deficiency has become a major constraint, since its availability in the soil is gradually decreasing (Lewandowska and Sirko, 2008).

In sugar beet, sulfur is essential for protein synthesis and to keep the presence of amino and sulfur-containing compounds balanced (Hoffmann et al., 2004). S-demand and S-removal of sugar beet is 30 and 5 kg ha^{-1}, respectively (Haneklaus et al., 1998). Critical leaf concentration for S-deficiency, which results in yield depression of 5%, was estimated as 3 mg g^{-1} S (d.w.) by Haneklaus et al. (2007). An unbalanced proportion between N and S, due to low S concentration, results in alfa-amino N accumulation leading to a lower sugar beet technical quality and decreasing root storage capacity (Burba, 1996). Thomas et al. (2000) reported a N/S threshold value of 20:1 in the shoot for yield reduction, whereas Haneklaus and Schnug (1996) found a lower N/S ratio of 14 as limiting value in sugar beet.

Genes related to sulfate response, able to transmit external signals and trigger adaptive changes, are well known and studied. Plants can adapt to the environment in a highly coordinated and dynamic manner (Giehl et al., 2014). This consists of multiple organization levels and links genes through different molecular pathways.

An approach to investigate the effect of changes in sulfur availability on plant composition concerns the application and integration of advanced omics technologies (Huang and Salt, 2016). The omics technologies are specifically utilized to describe the global profiling of biological matrices. Combinations of these techniques have been applied and served as high-throughput screening to allow the identification of potential specific biomarkers (Yuan et al., 2008).

Many studies attribute a fundamental role to the plant root system in the competition for survival in natural environments and the greatest selection pressure is for the acquisition of elements of soil fertility (water and nutrients), which is strictly dependent on root morpho-physiology (Gruber et al., 2013). The characterization of root phenomics is therefore essential to understand how trait variations are attributable to genotype and environmental factors (Cichy et al., 2009).

Changing metabolic homeostasis due to environmental stresses triggers the production of different proteins that could restore a new homeostasis. An integration of ionomic and metabolomic approaches could give a comprehensive assessment to understand which metabolites are involved in responses to a specific environment (Atkinson and Urwin, 2012). A specific stress response can be identified by specific metabolic fingerprinting (Shulaev et al., 2008). Moreover, target metabolite analysis combined with a dynamic gene expression profile is used to elucidate gene-to-gene and metabolite-to-gene networks through which plants coordinately modulate their responses to nutritional stresses.

In this study, a method based on the combination of different omics technologies was set-up for sugar beet (*Beta vulgaris* L. subsp. *vulgaris*) evaluation to achieve a holistic view of plant response to changes in sulfate availability. Root morphology, plant ionome and metabolome, together with gene expression profiling, were analyzed in leaves and roots of sulfate-deprived and supplied plants. In particular, we evaluated the capacity of the proposed omics techniques to depict complex plant–sulfate interactions.

MATERIALS AND METHODS

Plant Material

The plant material used in this study was the sugar beet hybrid "Shannon" provided by Lion Seeds Co., Ltd. (Maldon, United Kingdom). It is a diploid hybrid obtained from a cross between a multigerm pollinator resistant to rhizomania (Rizor-Holly source) and a susceptible monogerm male-sterile.

Growing Conditions

Seeds were surface-sterilized by dipping in 76% ethanol for 5 min and rinsed three times in distilled water. Seeds were germinated on distilled water-moistened filter paper in a growth chamber in the dark for 48 h at a temperature of 25°C. After germination, seedlings were transplanted to 35-liter plastic tanks containing a sulfate-deprived Hoagland solution. The tanks were placed in a growth chamber at 25/18°C and 70/90% relative humidity with a 14/10 h light/dark cycle (PPFD above shoot: 300 $\mu E\ m^{-2}\ s^{-1}$) and nutrient solution was replaced daily. Six days after germination, 100 μM of $MgSO_4$ was added to the solution. On the eighth day, fresh leaves and roots were harvested and stored at −80°C for further analysis.

Ionomic Analysis

Leaf samples were digested with concentrated HNO_3 in a microwave system. The elements concentration was determined by inductively coupled plasma ICP-OES, Ciros Vision EOP (Spectro A. I. GmbH, Germany). Elements were quantified using certified multi-element standards. Sulfate was extracted in 20 cm^3 of Millipore water by incubation at 70°C for 30 min. The extract was centrifuged at 20,000 *g* for 30 min, and the supernatant filtered through a 0.45 μm filter unit. Sulfate content was determined by ICP-OES. This procedure was previously adopted by Stevanato et al. (2015).

Root Morphological Analysis

Root morphological parameters (total root length, surface area and total number of tips) were determined by computerized scanning (STD 1600, Regent Instruments, Quebec, QC, Canada) and analyzed using WinRHIZO software (Regent Instruments).

Metabolomic Analysis

An untargeted screening was conducted as previously set up (Rouphael et al., 2016). Briefly, compounds were comminuted using Ultra-Turrax and extracted in 70% methanol (added

with 1% HCOOH), then analyzed using a quadrupole-time-of-flight mass spectrometer coupled to an UHPLC chromatographic system (UHPLC/Q-TOF). A 1290 UHPLC system was used coupled to a 6550 quadrupole-time-of-flight mass spectrometer and equipped with a Jet Stream ESI ionization system (all from Agilent Technologies, Palo Alto, CA, United States). Reverse-phase chromatographic separation was achieved using a Knauer BlueOrchid C18 column (100 mm × 2 mm i.d., 1.8 μm) and a mixture of water (proteomic grade, VWR, Milan, Italy) and methanol (LCMS grade, VWR, Milan, Italy) as mobile phase. Acquisition was performed in positive SCAN mode (100–1200 m/z) and compounds were then identified using accurate mass and isotopic pattern (isotopic spacing and isotopic ratio) against the database exported from PlantCyc[1]. Metabolomic data were interpreted using Agilent Mass Profiler Professional B.12.06. Compounds were filtered by abundance and frequency (area of >5000 counts and detection in 100% of samples in at least one condition, respectively), normalized at the 75th percentile and baselined to the median of each compound in all samples. Unsupervised hierarchical cluster analysis was then conducted using the fold-change heat-map and setting the similarity measure as Euclidean and Wards as linkage rule. Partial least squares discriminant analysis (PLS-DA, N-fold validation with $N = 4$) was also performed, and variables loadings, used to build the class prediction model, plotted according to their weight within the latent vectors. Compounds with the highest scores on the first and second latent vectors were exported from the covariance structures in the PLS-DA hyperspace. The identification of differential metabolites was finally investigated by combining analysis of variance ($P < 0.001$, Bonferroni multiple testing correction) and fold-change analysis (cut-off > 5) into Volcano Plots.

Transcriptomics Analysis

Total RNA was extracted from 100 mg of root tissues using a EuroGold TriFastTM kit (Euroclone, Italy) following the manufacturer's recommendations. RNA was quantified with a Qubit Fluorometer (Invitrogen, Carlsbad, CA, United States) using a Qubit RNA HS Assay Kit. One microgram of total RNA was reverse transcribed using the FastGene 55-Scriptase (Nippon Genetics, Japan) in a total volume of 20 μl following the manufacturer's recommendations. The cDNAs were used to analyze the expression level of 53 genes related to nutritional status in sugar beet (Barone et al., 2017). Real-time PCR experiments were conducted in a final volume of 5 μl containing 2.5 μl of 2× TaqMan Open Array master mix (Life Technologies, United States), and 2.5 μl of cDNA. Real-time PCR was performed on the QuantStudio 12K Flex Real-Time PCR System (Life Technologies, United States) using the following thermocycler program: 10 min pre-incubation at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. The sequences of the primers and TaqMan probes designed for the Real-time PCR experiments are reported as Supplementary Material S1.

[1] pmn.plantcyc.org

The comparative *C*t method was used to analyze the genes relative expression (Livak and Schmittgen, 2001). Data were normalized against the average transcript abundance of three housekeeping genes (*Tubulin, Bv2_037220_rayf; GAPDH, Bv5_107870_ygnn; Histone H3, Bv6_127000_pera*). The fold change in expression of genes was calculated using the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t$ = (C_t target gene − average C_t reference genes)$_{treatment}$ − (C_t target gene − average C_t reference genes)$_{control}$. All data are the means of three biological replicates, each one composed of three technical replicates ±SE of one representative experiment. The C_t method was used to quantify the relative gene expression levels and the results expressed as $2^{-\Delta Ct}$, where ΔC_t = (C_t of reference gene − C_t of target gene).

Data Analysis

A completely randomized experimental design was adopted with five replications per treatment and 60 seedlings per replicate. All data were subjected to the normality test (Kolmogorov–Smirnov) and homogeneity of variance (Levene-Median). A factorial ANOVA was conducted using the statistical software package Statistica v. 13.0 (Dell Inc., United States) to investigate the effect of different treatments, tissues, genes and their interactions.

RESULTS

The accumulation dynamics of sulfate was evaluated for a period of 36 h in deprived control plants (−S) and supplied plants (+S) (**Figure 1**). Sulfate content increased strongly in leaves of treated

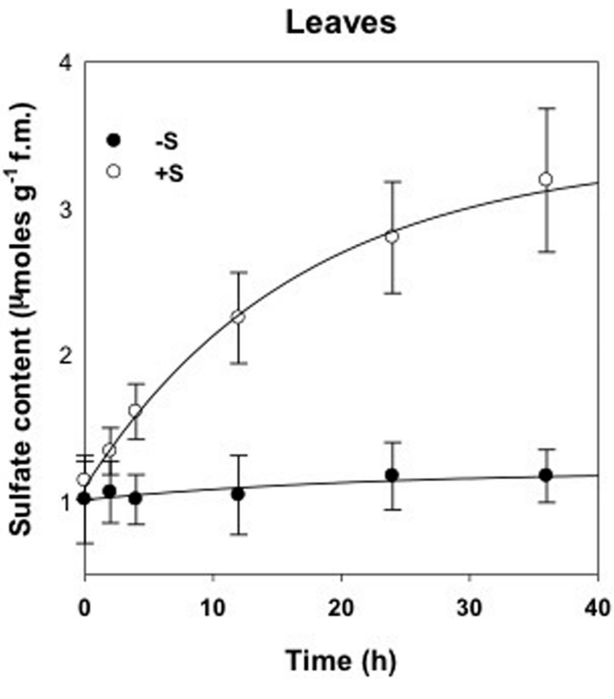

FIGURE 1 | Sulfate content in leaves of deprived control plants (−S) and supplied with 100 μM of $MgSO_4$ for 36 h (+S).

plants and, as expected, remained mostly stable in deprived plants. After 36 h of treatment with 100 μM of $MgSO_4$, the sulfate contents of leaves were significantly higher ($P < 0.01$) than those of the deprived plants.

Leaves were analyzed with ICP-OES in order to reveal the effect of changes in sulfate availability on the ionome profile (**Table 1**). No significant increases were detected in the elements concentration in response to S nutrition, except for S and Mg. S concentration significantly increased ($P < 0.01$) in leaves (+162%) in treated compared to untreated plants. A significant difference ($P < 0.05$) in Mg concentration was observed in leaves of treated plants, which showed a 4.5% increase. Samples within treatments showed a clear separation as detected by the principal component analysis (PCA) (**Figure 2**). Factors 1 and 2 explained 38.27% and 24.61% of the total variation, respectively. Factor 1 is related to the variation of S and Mg in leaves ionome.

To evaluate the sulfate treatment effects on root apparatus, we studied three different parameters: total root length, surface area and number of tips. As represented in **Figure 3**, plants grown in 100 μM of sulfate solution show significantly higher values ($P < 0.01$) for all the parameters analyzed than deprived plants. In addition, the data obtained reveal that the mechanism actuated by deprived plants, in response to an additional 36 h of sulfate deprivation, is expressed in a significant increase ($P < 0.01$) in the number of root tips.

A total of 2,400 metabolites were identified via UHPLC/Q-TOF. The unsupervised cluster analysis was performed on the dataset to better focus on the effect of the sulfate treatment (**Figure 4**). The results showed that the two treatments were properly grouped in both roots and leaves, thus indicating the presence of a chemical signature from the treatment in the metabolomics dataset. The output of PLS-DA, shown in **Figure 5**, consistently indicated a good discrimination between treatments on the basis of their metabolic profile. Indeed, the class prediction model gave good accuracies for both tissues and treatments (overall accuracy of 100%). The compounds selected from the Volcano analysis of plants treated with 100 μM of $MgSO_4$ for 36 h (using a fold change cut off >5 and a *p*-value of 0.001) are reported as Supplementary Material S2. Most of the differential compounds identified were free amino acids such as tryptophan, proline, lysine, glutamate, glutamine and cysteine. The Volcano analysis also revealed high levels of *O*-acetyl-*L*-serine, Jasmonic acid and 12-hydroxy-jasmonoyl-L-isoleucine in leaves.

The expression level of the 53 sulfate-related genes was evaluated in deprived and supplied leaves and roots. The ANOVA showed a significant effect of treatments ($P < 0.01$) and tissues ($P < 0.01$), as well as genes ($P < 0.01$) (**Table 2**). Relative expression of 53 genes in leaves (**Figure 6**) was significantly higher compared to roots (**Figure 7**) of treated and untreated plants. Moreover, gene expression analysis revealed that the highest relative quantity is shown in supplied roots by Flavonol sulfotransferase-like (AIY90PI, Bv6_137840_uaap), 28 kDa ribonucleoprotein, chloroplastic (AIT970M, Bv7u_180460_dcmt) and Glutamate/leucine/phenylalanine/valine dehydrogenases (AIWR4C1, Bv3_057000_nenr). A sixfold increase of the gene auxin efflux carrier component 1 (AI1RW1X, Bv3_065290_srwc) was detected in roots compared to leaves. Supplied leaves have a high relative quantity of the same three genes highly expressed in roots. In particular, Flavonol sulfotransferase-like showed a onefold increase compared to roots. However, supplied leaves are subjected to a significant expression of other genes: Aspartic proteinase-like protein 1 (AIVI56U, Bv_24910_jato), 3-ketoacyl-CoA synthase 17 (AIMSIZA, Bv7_156890_eowm) and cysteine synthase, chloro plastic/chromoplastic, O-acetylserine sulfhydrylase (AIGJR36, Bv1_004580_xnrs). Gene coding for thioredoxin reductase (NADPH) (AIKAMMV, Bv3_063630_mpup) and 2-Cys peroxiredoxin BAS1, chloroplastic (AII1OGN, Bv7_157460_rcod) were twofold and onefold more expressed in leaves than roots, respectively.

TABLE 1 | Leaf concentration of mineral elements of deprived control plants (−S) and supplied plants with 100 μM of $MgSO_4$ for 36 h (+S).

Samples Treatment	Leaf	
	+S	−S
Elements		
Al	9.6 ± 1.6	7.7 ± 3.0
B	17.3 ± 1.0	16.1 ± 0.9
Ba	6.6 ± 1.0	6.5 ± 0.7
Ca	2929.8 ± 534.3	3375.7 ± 207.8
Cd	0.3 ± 0.0	0.3 ± 0.0
Cr	0.4 ± 0.1	0.4 ± 0.1
Cu	12.9 ± 0.8	16.7 ± 4.4
Fe	152.7 ± 7.3	137.7 ± 10.4
K	75147.6 ± 5225.3	72134.2 ± 1699.0
Mg	4969.2 ± 532.0	4086.2 ± 169.1*
Mn	91.8 ± 7.9	88.0 ± 3.6
Na	841.1 ± 84.9	781.2 ± 73.9
P	9153.2 ± 635.2	10387.9 ± 382.2
S	2599.7 ± 261.3	994.0 ± 40.3**
Si	20.7 ± 1.6	16.9 ± 1.3
Zn	42.7 ± 6.8	45.9 ± 4.2

*The concentration of elements (mg kg^{-1} DW) is expressed as mean ±SE of three biological replicates and each replicate consisted of sixty seedlings. Significant difference between treatments was determined by ANOVA and marked as *P < 0.05 and **P < 0.01.*

DISCUSSION

Many studies have focused their attention on explaining plant nutritional stress using an integrated omics approach (Saito and Matsuda, 2010). In particular, sulfur starvation has been extensively studied for the model plant *Arabidopsis* (Nikiforova et al., 2005, 2006). In this work, sulfur shortage, one of the main sugar beet nutritional deficiencies that causes significant sugar yield losses, is dissected by means of omics technologies to set up a method able to detect and describe this nutritional limitation. The 53 genes used in this study were selected on the basis of a previous experiment of RNA-seq analysis and validated for their involvement

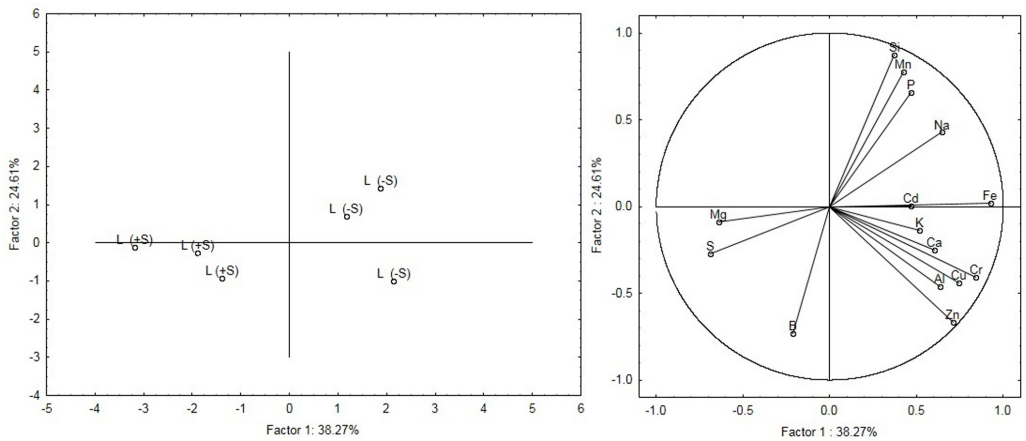

FIGURE 2 | Principal components analysis (PCA) of the leaves ionome. The figure on the Left shows the modification of the leaves ionome as a function of the nutritional regime (S-deficiency and S-sufficiency). The figure on the Right shows the relationship between variables and principal components and also highlights relationships between the variables themselves.

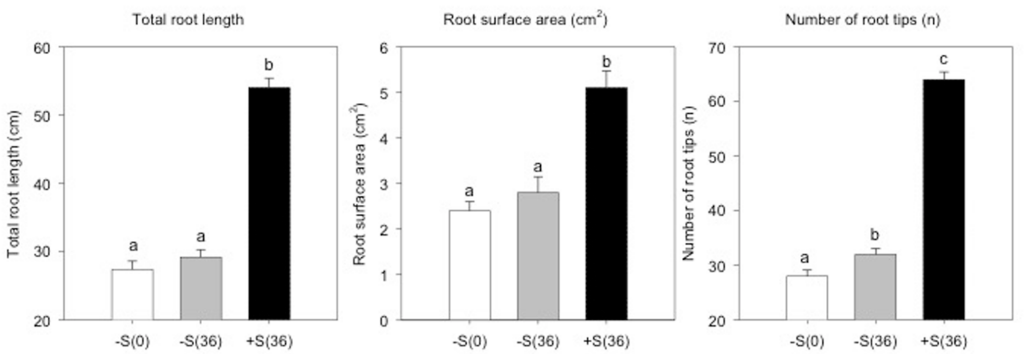

FIGURE 3 | Total root length, root surface area (cm^2) and number of root tips of sulfur deprived plants (–S(0)), sulfur deprived plants after 36 h (–S(36)) and sulfur supplied plants after 36 h (+S(36)).

in plant responses to nutritional changes (Barone et al., 2017). The product of these genes plays important roles in many biological processes, such as biosynthesis, response to stress, cellular amino acid metabolism, transport, and sulfur compound metabolism. Changes in root morphology, plant ionome, metabolome and gene expression profile, in leaves and roots of sulfate-deprived and supplied sugar beet plants, highlighted the presence of potential biomarkers involved in sulfate nutrition. These biomarkers are mainly related to auxin synthesis, plant protection, amino acid and cysteine biosynthesis.

Root morphology is closely related to plant S uptake efficiency, especially the root length, surface area and number of tips (Stevanato et al., 2015). These parameters show a significant difference in plants treated with 100 μM of $MgSO_4$ for 36 h compared to sulfur deprived plants. This has also been observed in the soil, since sulfur is mobile and present in the deeper soil profile and stimulates plants to rapidly elongate the primary root (Zhao et al., 2014). From a transcriptomics and metabolomics point of view, this is reflected in a high expression of auxin efflux carrier component 1 (PIN) and in the up regulation of 5-phosphoribosyl-anthranilate, precursor of tryptophan and auxin synthesis. The root morphological analysis also revealed a significant increase in the number of root tips in plants maintained at sulfur deficiency for 36 h. Root tips have a fundamental role in nutrients acquisition and the perception of nutritional stress (López-Bucio et al., 2003). A previous study on *Arabidopsis* reported that sulfate-deprived plants have a larger number of root tips and fine roots, increasing the root/shoot ratio (Gläser et al., 2014). In addition, Zhao et al. (2014) observed that sulfate deprivation stimulated cell division activity and root tip expansion.

Plants grown without sulfur show different mineral composition from plants grown with 100 μM of $MgSO_4$ as highlighted by ICP-OES analysis. The leaf sulfur content of the treated plants was much lower than the threshold value below which significant production losses were observed by Haneklaus et al. (2007). PCA analysis of mineral elements revealed that leaves and root samples belonged to separate

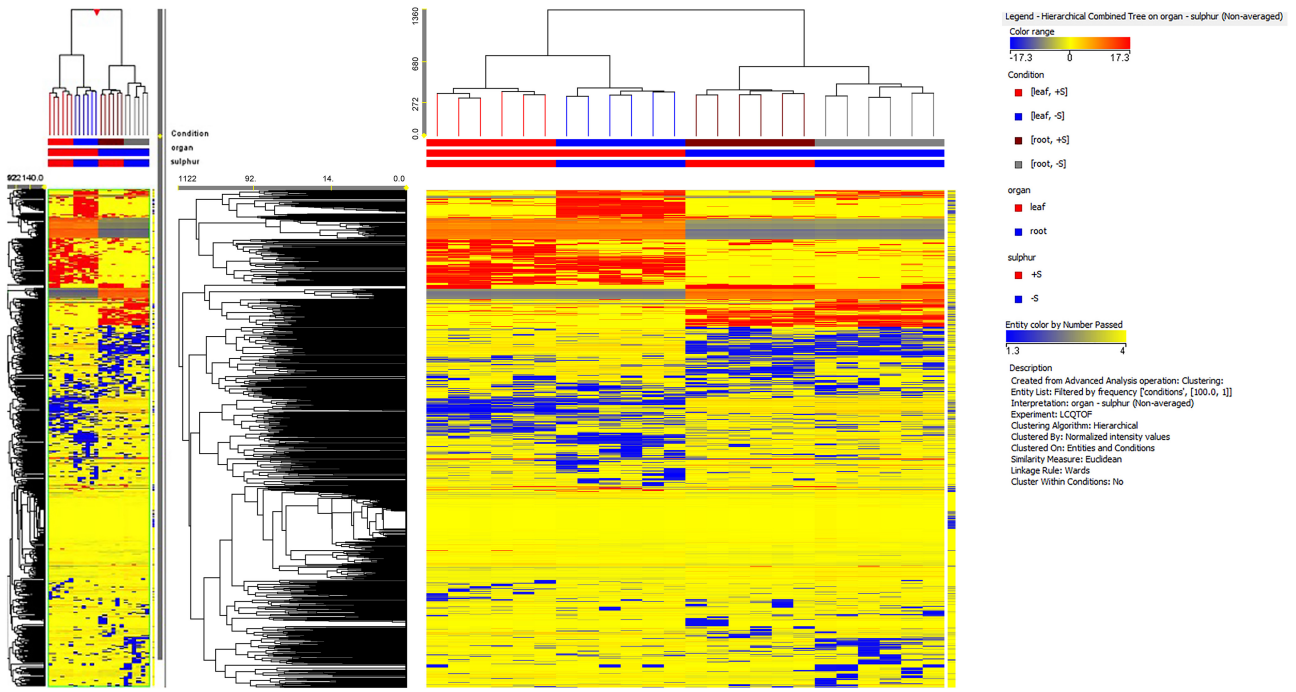

FIGURE 4 | Not averaged unsupervised cluster analysis in sugar beet roots and leaves of supplied and deprived plants (similarity: Euclidean; linkage rule: Ward). Compound intensity was used to build up heat maps, on the basis of which the clusters were generated.

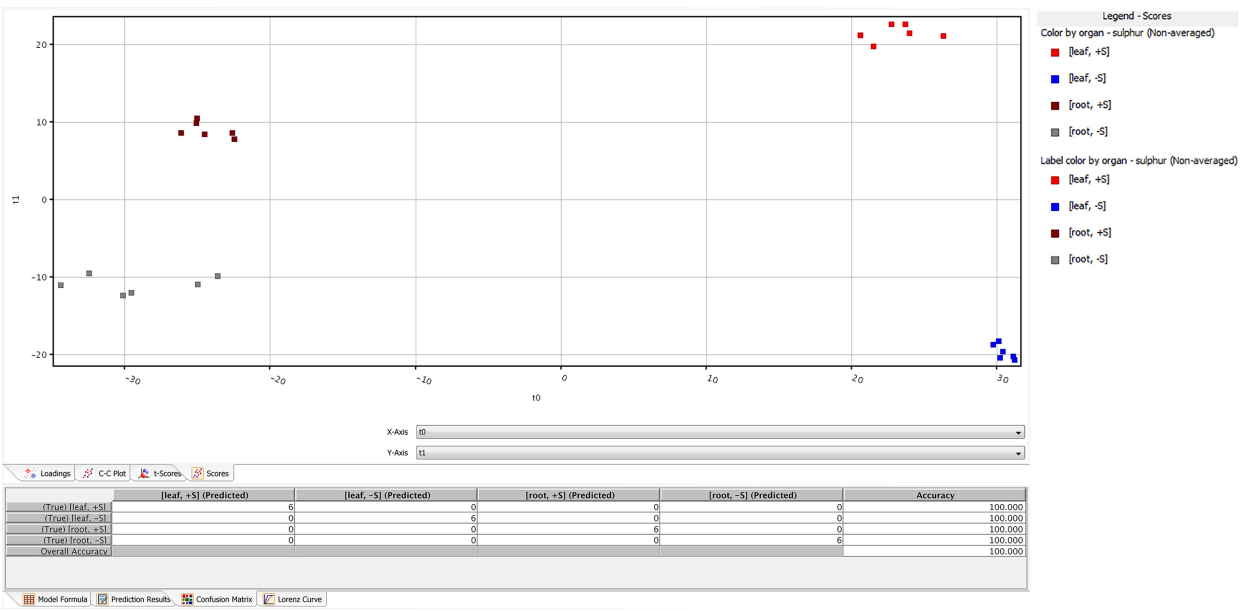

FIGURE 5 | Partial least squares discriminant analysis (PLS-DA) conducted from the UHPLC-/QTOF metabolite profiling in sugar beet roots and leaves of supplied and deprived plants. Samples distribution in the hyperspace of the class prediction model is provided in the Upper, while the compounds loading plot is provided in the Lower one.

clusters corresponding to their nutritional regime (S-deficiency and S-sufficiency). Plants treated with sulfur show higher Mg levels than S-deprived ones, demonstrating that these nutrients are correlated with each other (Dietz, 1989). Sulfur is essential for chlorophyll formation and magnesium is the central core of the chlorophyll molecule. In sugar beet, a decrease of chlorophyll under sulfur deficiency has already been described by Thomas et al. (2000).

TABLE 2 | Analysis of variance (ANOVA) showing the effect of different treatments, tissues and genes (*$P < 0.05$; **$P < 0.01$, factorial ANOVA test) on the expression of 53 sugar beet genes putatively involved in sulfate nutrition.

Effect	*df*	*SS*	*MS*	*F*	*P*
Treatment	1	1690	1690	152.9	**
Tissue	1	9431	9431	853.2	**
Gene	52	3124	57	5.1	*

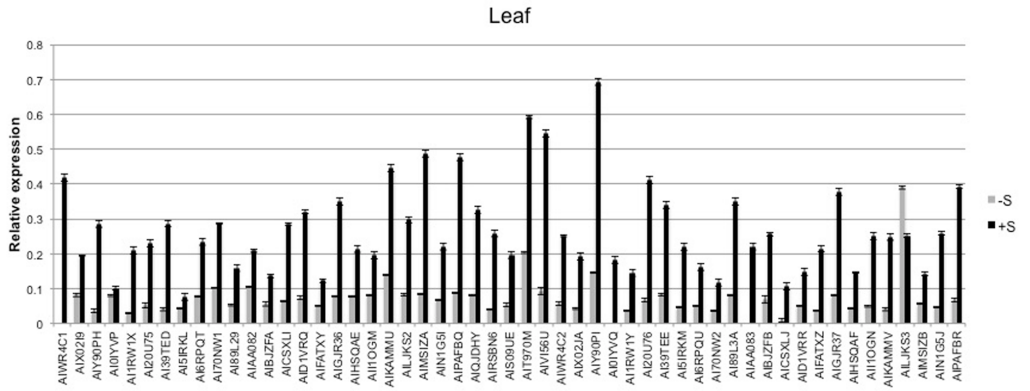

FIGURE 6 | Average relative expression of the 53 sulfur related gene in deprived and supplied leaves. The C_t method was used to quantify the relative gene expression levels and the results expressed as $2^{-\Delta Ct}$, where $\Delta C_t = (C_t \text{ of reference gene } - C_t \text{ of target gene})$.

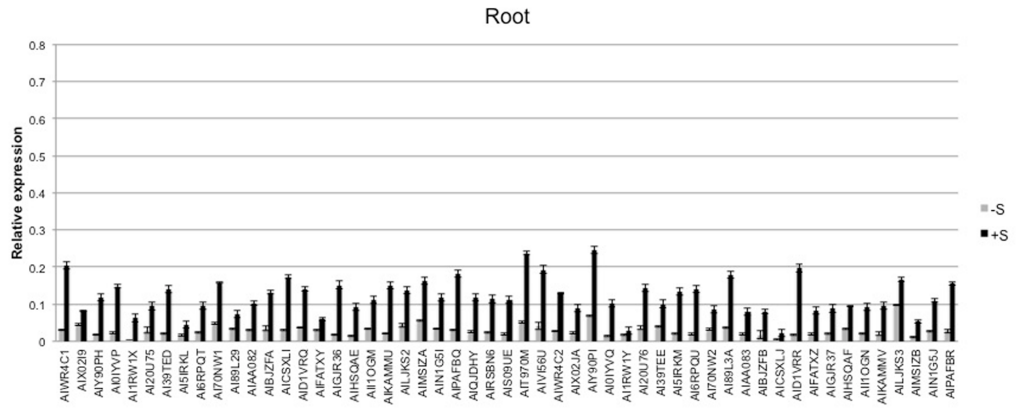

FIGURE 7 | Average relative expression of the 53 sulfur related genes in deprived and supplied roots. The C_t method was used to quantify the relative gene expression levels and the results expressed as $2^{-\Delta Ct}$, where $\Delta C_t = (C_t \text{ of reference gene } - C_t \text{ of target gene})$.

Many genes highly expressed in the leaves supplied with 100 μM of $MgSO_4$ are involved in stress response, in particular 2-Cys peroxiredoxin BAS1, chloroplastic and thioredoxin reductase (NADPH). The first one is an antioxidant enzyme that has an important role in cell protection against oxidative stress and is involved in protecting photosynthesis (Dietz et al., 2002). The second is involved in regulation of chlorophyll biosynthetic process and in the removal of superoxide radicals. Furthermore, the enhanced defense activity was also observed at metabolic levels with an up regulation of Jasmonic acid and 12-hydroxy-jasmonoyl-L-isoleucine in leaves, which have a crucial role in mediating plant stress response.

The presence of sulfur in the form of cysteine residues is essential for the conformation and stability of proteins. Transcriptomics analysis shows an over expression of cysteine synthase, chloroplastic/chromoplastic, *O*-acetylserine sulfhydrylase in supplied leaves that turn into an up regulation of *O*-acetyl-*L*-serine, glutamate and glutamine. *O*-acetyl-*L*-serine is a direct precursor of cysteine biosynthesis and is hence crucial for sulfur assimilation, while glutamate and glutamine are involved in the biosynthetic pathway of glutathione (Hirai et al., 2003). Change in the oxidation state of Cys residues and its thiol group promotes the response to change in redox environments and enable an organism to adapt to stress conditions (Montrichard et al., 2009). Plants grown with 100 μM

of $MgSO_4$ started to up regulate the synthesis of cysteine and several free amino acids, as detected by gene expression (high level Glutamate/leucine/phenylalanine/valine dehydrogenases and Flavonol sulfotransferase-like) and Volcano analysis, and to activate the protein synthesis pathway (Durenkamp et al., 2007). Furthermore, the accumulation of both glutamate and glutamine suggests an increase in activity of the GS-GOGAT cycle, likely to support nitrogen assimilation and protein synthesis. Similar results were also found studying the response to sulfur stress in *Brassica napus* (Zhang et al., 2015).

CONCLUSION

Our approach was able to identify and highlight the main determinants of sugar beet response to changes in sulfate availability. The combination of ionomics, morphological, metabolomics and molecular approaches appeared to be a particularly valuable system for the evaluation of sugar beet nutritional status under different nutrient availability conditions.

AUTHOR CONTRIBUTIONS

PS, CB, and GB wrote the main manuscript and performed the OA analysis. VM and GM developed the panel of TaqMan assays for the OA analysis. MC and MB conducted the ionomic analysis. LL performed the metabolomic analysis. VB, DP, and AB conducted the morphological analysis. GC, AS, and SN coordinated data collection and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

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Genetic and Genomic Tools to Asssist Sugar Beet Improvement: The Value of the Crop Wild Relatives

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Sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) is one of the most important European crops for both food and sugar production. Crop improvement has been developed to enhance productivity, sugar content or other breeder's desirable traits. The introgression of traits from Crop Wild Relatives (CWR) has been done essentially for lessening biotic stresses constraints, namely using *Beta* and *Patellifolia* species which exhibit disease resistance characteristics. Several studies have addressed crop-to-wild gene flow, yet, for breeding programs genetic variability associated with agronomically important traits remains unexplored regarding abiotic factors. To accomplish such association from phenotype-to-genotype, screening for wild relatives occurring in habitats where selective pressures are in play (i.e., populations in salt marshes for salinity tolerance; populations subjected to pathogen attacks and likely evolved resistance to pathogens) are the most appropriate streamline to identify causal genetic information. By selecting sugar beet CWR species based on genomic tools, rather than random variations, is a promising but still seldom explored route toward the development of improved crops. In this perspective, a viable streamline for sugar beet improvement is proposed through the use of different genomic tools by recurring to sugar beet CWRs and focusing on agronomic traits associated with abiotic stress tolerance. Overall, identification of genomic and epigenomic landscapes associated to adaptive ecotypes, along with the cytogenetic and habitat characterization of sugar beet CWR, will enable to identify potential hotspots for agrobiodiversity of sugar beet crop improvement toward abiotic stress tolerance.

Keywords: *Beta*, *Patellifolia*, crop wild relatives, crop breeding pool, next-generation sequencing

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CWR FOR SUGAR BEET IMPROVEMENT: CURRENT STATUS AND PROSPECTS

Sugar beet (*Beta vulgaris* L. ssp. *vulgaris*, cultivar group Sugar Beet) is one of the most important crops, being within the 2013 top 10 world commodities, with Europe contributing with 68% production (Food Agricultural Organization of the United Nations, 2016). The sugar beet accounts for 20% of the global sugar production. The genus also includes the cultivar groups Fodder Beet,

Garden Beet, and Leaf Beet (Lange et al., 1999). The origin and domestication of sugar beet have been comprehensively reviewed in Biancardi et al. (2012). As one of the youngest crops, sugar beet breeding pool was considered narrow, limiting breeding progress (Bosemark, 1979). Since then, *B. vulgaris* L. ssp. *maritima* L. (Arcang.) was extensively used as a source of resistance gene (Biancardi et al., 2012) to specifically complement the breeding pool. At the global market sugar beet competes with sugar cane. In order to stay competitive an incremental breeding progress is no longer sufficient. Rather, a performance leap is required as targeted by the French AKER project (AKER, 2017). To this end, today's sugar beet breeding pools need to be purposefully broadened by incorporating alleles from wild species which may have gone lost during the domestication history of cultivated beets or have never existed in the breeding pool due to crossing barriers between the wild ancestor of cultivated beets and related wild species (Frese et al., 2001).

A taxonomic system allowing reliable inferences on the phylogeny, the geographic spread of species during evolution and the today's genetic relationships between taxa facilitates the identification of genetic resources suitable for base broadening programs. The taxonomic inconsistencies within the subfamily Betoideae have been reported (e.g., Ford-Lloyd, 2005; Hohmann et al., 2006) repeatedly (see **Table 1**). While the taxonomy of *Beta* section *Beta* is settled, uncertainties still exists with respect to section *Corollinae* and the genus *Patellifolia* (former *Beta* section *Procumbentes*) (Frese, 2010; Frese et al., 2017).

The widespread use of genetically uniform crop varieties has caused agricultural crops to lose some of the genetic diversity present in their wild progenitors. CWR offer important sources of useful agronomic traits, including: intermediate C3-C4 photosynthetic activity; tolerance for cold, salt and drought conditions, and nutraceutical characteristics, i.e., plant-based compounds with health-protective roles. (Zhang et al., 2017). Selection almost inevitably causes unintentional loss of genetic diversity in the breeding pools. However, as long as breeding pools can be replenished by introgression or incorporation of genetic diversity contained in wild species, the genetic diversity in breeding programs can be kept in balance as was discussed by Ordon et al. (2005). Since loss of genetic diversity in breeding pools as well as genetic erosion in CWR within their natural habitats are both slow and long-term processes, the connection between breeding progress in crop species and the need for effective conservation programs for CWR tends to be overlooked. Clearly, without CWR conservation programs operative within the next 10 years future breeding progress will be at risk. Genetic and genomic tools already support planning of CWR conservation and effective breeding programs as exemplified by Andrello et al. (2017). Their investigations provided insight into the geographic patterns of genetic diversity, which is not only relevant for CWR conservation planning but also contributed to the understanding of statistical relations between genetic markers and environmental variables.

The West Mediterranean Region encloses a number of undisturbed habitats (e.g., cliff coasts, and salt marshes) that holds some of the most important CWR of sugar beet, namely the sea beet (*B. vulgaris* ssp. *maritima*) and other endemic species within *Beta* (*B. macrocarpa* and *B. patula*), as well as *Patellifolia* (*P. patellaris*, *P. procumbens*, *P. webbiana*). Considering the available yet unexplored wild germplasm from *Beta* and *Patellifolia* species occurring in this region, their potential for supplementing sugar beet breeding pool is easily recognized, mainly due to their occurrence in habitats of extreme conditions. Generally, crop yield reduction is a consequence of increasingly abiotic stresses (Mickelbart et al., 2015), which is a major limiting factor in plant growth. Indeed, drought is expected to cause salinization of 50% of all arable lands by 2050 (Ashraf and Wu, 1994). Although sugar beet breeding programs have already allowed the introgression of genes related to disease resistance from wild *Beta* and *Patellifolia* species (e.g., Munerati, 1932; Gidner et al., 2005), through marker-assisted crossing (Francis and Luterbacher, 2003), work on abiotic tolerance still remains underdeveloped. Therefore, the genetic characterization of traits responsible for the adaptation of wild populations to saline and/or hot and dry habitats should be a viable step toward raising the level of abiotic stress tolerance in sugar beet breeding pools.

In this perspective, we pointed out that some genetic and genomic tools are presently available for screening for trait variation. In this way wild species of *Beta* and *Patellifolia* could be explored to uncover novel variation in functional traits associated to adaptive capacity under abiotic stresses.

ESTABLISHING THE RELATEDNESS BETWEEN CROPS AND CWR

To ascertain the degree of relatedness between CWRs and crops, several schemes have been proposed. Harlan and de Wet (1971) suggested an informal classification system and assigned species to the primary (GP1), secondary (GP2) and tertiary (GP3) genepool using the strength of crossing barriers between the crop species and wild species as criteria. The tertiary genepool describes the extreme outer limit of the potential genepool of a crop. If information on the reproductive isolation is lacking the "Taxon Group concept" of Maxted et al. (2006) can be applied and the taxonomic hierarchy be used to assess the relatedness between the crop and wild species potentially suited as gene donors. More recently, Vincent et al. (2013) defined the "provisional gene pool concept" (PGP) as *to be used when there is no formally published gene pool concept and when taxonomic treatments lacked subgeneric information, but there is published crossability evidence between the crop and related taxa*. Thus, determination of genetic diversity allied to taxonomy is of major interest when using CWR, as both genetic distance and species classification can be assigned.

In the face of environmental changes likely resulting in a dramatic loss of CWR in Europe (Aguirre-Gutiérrez et al., 2017), the understanding of the relationships among taxa of agronomically important crops is not of marginal interest (Knapp et al., 2013). It is of crucial importance as breeders should be enabled to capture genetic diversity present in CWR before they get lost and to maintain the transferred valuable traits in breeding pools.

TABLE 1 | Classifications of the subfamily Betoideae (Amaranthaceae).

	Classifications of the subfamily Betoideae				Distribution of *Beta* and *Patellifolia* species*
Tribe	**Ulrich (1934)**	**Ford-Lloyd (2005)**	**Hohmann et al. (2006)**	**Romeiras et al. (2016)**	
Beteae	***Beta***	***Beta***	***Beta***	***Beta***	
	sect. *Corollinae*	sect. *Beta*	sect. *Beta*	sect. *Beta*	Western Mediterranean region and Macaronesian archipelagos *B. vulgaris* ssp. *maritima* (L.) Arcang—Mediterranean coasts (Iberian Peninsula and North Africa), Azores, and Madeira *B. macrocarpa* Guss.—Iberian Peninsula, North Africa and Canaries *B. patula* Aiton —Madeira (endemic) *B. vulgaris* ssp. *vulgaris*—cultivated
	sect. *Nanae* sect. *Procumbentes*	sect. *Corollinae* sect. *Nanae*	sect. *Corollinae* (incl. sect. *Nanae*)	sect. *Corollinae* (incl. sect. *Nanae*)	Eastern Mediterranean region and Southwestern Asia *B. corolliflora* Zosimovic ex Buttler *B. intermedia* Bunge ex Boiss. *B. lomatogona* Fisch. and C.A.Mey. *B. macrorhiza* Steven *B. trigyna* Waldst & Kit. *B. nana* Boiss and Heldr.
	sect. *Vulgares*	sect. *Procumbentes*		*Patellifolia*	Western Mediterranean region and in Macaronesian archipelagos *P. procumbens* (C.Sm.) A.J.Scott, Ford-Lloyd and J. T.Williams—Madeira, Canaries and Cabo Verde (endemic) *P. webbiana* (Moq.) A.J.Scott, Ford-Lloyd and J. T.Williams—Canaries (endemic) *P. patellaris* (Moq.) A.J.Scott, Ford-Lloyd and J. T. Williams—Iberian Peninsula, Italy, North Africa, Madeira, Canary and Cabo Verde
Hablitzieae	*Acroglochin*	*Acroglochin*	*Patellifolia*		
	Aphanisma	*Aphanisma*	*Aphanisma*	*Aphanisma*	
	Hablitzia	*Hablitzia*	*Hablitzia*	*Hablitzia*	
	Oreobliton	*Oreobliton*	*Oreobliton*	*Oreobliton*	

*For details see Romeiras et al. (2016)

Classification within Betoideae has been frequently altered, which challenges the assignment of CWR taxa to a gene pool. Recently, a phylogeny reconstruction of this subfamily (Romeiras et al., 2016), pointed out that *Patellifolia*, formerly included in *Beta* section *Procumbentes*, should be a separate genus, supporting that genetic divergence is responsible for the crossing difficulties faced in breeding programs. An early diversification between *Beta* (GP1, GP2) and *Patellifolia* (GP3) is postulated, and within GP1 and GP2 an ecological divergence between West and East Mediterranean *Beta* species was identified (Romeiras et al., 2016). Also, Frese et al. (2017) assessed genetic diversity in *P. patellaris* revealing that occurrences from Portugal are genetically different from the Spanish ones (Andrello et al., 2017), thus highlighting that Portuguese populations may harbor a different genetic variation that could be associated to the restricted coastal areas where they occur. Several genetic diversity studies in sea beet (e.g., Leys et al., 2014; Andrello et al., 2016) showed a distribution of genetic diversity according to ecogeographical ranges and, recently, discrimination in Portuguese populations from dissimilar habitats were accomplished (Ribeiro et al., 2016). Overall, studies with sea beet populations occurring in West Mediterranean Region point out to a clinal gradient, thus promoting adaptive radiation into ecoclines of populations from GP1 species (Monteiro et al., 2013). The main outcomes of phylogenetic and genetic diversity studies suggest that agronomically important traits associated to abiotic stress reside in wild species of the GP1 and GP3 and could be used to broaden the genetic basis of sugar beet.

AGRIGENOMICS: CWR AS IMPORTANT SOURCES

Before CWR can be used in any plant-breeding program, only if genetic variation in traits of interest for breeders is evaluated they are included as genetic resources. The field of Agrigenomics is in the focus of a technological revolution caused by the emergence of high-throughput DNA sequencing technologies. Recent studies highlight the importance of prospecting CWRs

and crops with the current advances in genome sequencing (Bevan et al., 2017). For example, a 43% reduction in genetic diversity in modern maize lines was reported when compared to their progenitor populations (Wright et al., 2005); sequencing of 31 wild and cultivated soybean genomes identified higher allelic diversity in wild accessions (Lam et al., 2010). Altogether, these studies indicate a loss of genetic diversity caused by a genetic bottleneck during the domestication process. The completion of the sugar beet genome sequencing provided genomic resources to support molecular breeding (Dohm et al., 2014). However, identification of agronomic traits linked to adaptive phenotypic capacity, through assessment of *in situ* CWR populations that occur under abiotic stresses, should be an important follow up for finding "new genetic variation" which may benefit sugar beet breeding (**Figure 1**).

Recovering the Diversity Lost by Domestication

Through the relationship between genetic factors and phenotypes, a genomics-assisted breeding will be possible to assist onto the sustainable production at global food needs. Thus, identifying adaptive variation from neutral mutations is an important feature toward the understanding of the molecular basis of heritable phenotypic traits. Rather than genome sequencing alone, the reduction of the complexity of a genome by the Genotyping by sequencing (GBS), allows a high-throughput sequencing approach of multiplexed samples that associates genome-wide molecular marker discovery and genotyping (see He et al., 2014). Large-scale discovery of single nucleotide polymorphisms (SNPs) through restriction enzymes -site associated DNA (RAD) sequencing, have been successfully applied in crop genetics for ascertain markers linked to disease resistance (e.g., Talukder et al., 2014).

A recent study used 200 naturally occurring sea beets, to identify the sugar beet resistance gene *Rz2* with a modified version of mapping-by-sequencing (Capistrano-Gossmann et al., 2017). This study features the prominent potential of CWR for rapid discovery of causal genes relevant for crop improvement. Several studies in wild and domesticated crops using GBS tools underpinned the potential of wild populations from diverse agro-climatic regions for genetic enhancement of adaptive traits in crop gene pools (e.g., Bajaj et al., 2015) and distinct traits of cultivated and wild accessions associated to domestication process (e.g., Yang et al., 2016; Marrano et al., 2017). By using a whole genome SNPs approach on wild relatives of crops it would be possible to identify naturally selected trait-regulating genomic targets/functional allelic variants associated to adaptive capacity for genetic enhancement of cultivated gene pools. Specifically, by identifying signatures of selection in *Beta* and *Patellifolia* species that occur in ecotypes under drought/salt conditions, genomic information behind adaptive capacity could be assessed.

Epialleles as Fingerprinting of CWR

As sessile organisms, plants develop several mechanisms to cope with abiotic and biotic stresses. Besides heritable phenotypic variation within a species, phenotypic plasticity is considered one of the major means by which plants can cope with environmental factor variability (Boyko and Kovalchuk, 2011; Zheng et al., 2017). Epigenetic modifications are thought to play a particularly important role in fluctuating environments (Kooke et al., 2015), in contrast to DNA sequence variation. Epigenetics refers to meiotically or mitotically heritable variations of phenotypic traits caused by genetic modifications, especially DNA methylation (Ekblom and Galindo, 2011). Epigenetic variations with stability over multiple generations have been reported in processes of local adaptation (Dubin et al., 2015). In plant systems, epigenetic inheritance is well documented (Taudt et al., 2016), and epigenomic variation at a locus can be treated as a quantitative trait. Long-term exposure to abiotic and biotic conditions shape distinct heritable epigenetic landscapes (e.g., Feil and Fraga, 2012), thus major differences in epigenetic landscapes are expected when comparing distinct ecotypes (Flatscher et al., 2012). As epigenetic variation can be environmentally induced, this source of natural variation in ecologically relevant traits may be subjected to selection (Latzel et al., 2013). Former studies identified epigenetic variation as being responsible for phenotypic plasticity in mangrove individuals [*Laguncularia racemosa* (L.) C.F.Gaertn] occurring in distinct habitats (Lira-Medeiros et al., 2010). Despite the importance on characterizing epigenetic landscapes across ecological ranges (Rodríguez López and Wilkinson, 2015), little information is available outside of model organisms (Fortes and Gallusci, 2017), and particularly in natural populations. The potential of epigenetics to play a role in crop improvement is growing, namely by the identification/selection of epialleles (Springer, 2013). New sequencing tools as bisulfite-converted RADseq (BsRADseq), an approach to quantify the level of DNA methylation differentiation across multiple individuals (Trucchi et al., 2016), allow an epigenomic screening in natural populations. Large-scale epigenetic surveys will allow comparison of epigenetic variation in natural *Beta* and *Patellifolia* species, occurring in extreme habitats, and their association to phenotypic variation could be addressed. In this context, investigating the extent of epigenetic divergence from natural *Beta* and *Patellifolia* populations that thrive in different ecological conditions, would allow to determine the heritable epigenetic landscapes shaped by abiotic conditions. As such, epialleles identified would be an innovative tool useful as an epi-fingerprinting for selecting resilient sugar beet genotypes, which can better cope with environmentally challenging conditions. The development of new breeding strategies that could incorporate epigenomic information is a major challenge. Epigenome editing tools as CRISPR/Cas9 have been considered a promising tool for targeted epigenetic-marker breeding strategies by selecting agronomical desirable quantitative traits (Thakore et al., 2016).

Cytogenomics

In breeding programs, the importance of interspecific hybridization and polyploidy has long been widely acknowledged (Mason, 2016). Crops can cross-pollinate with their related wild species and exchange chromosome segments by homoeologous recombination. Such hybrids are most often sterile, but chromosome doubling (either spontaneous or instantaneously, originating allopolyploids) or the fixation of viable recombinant

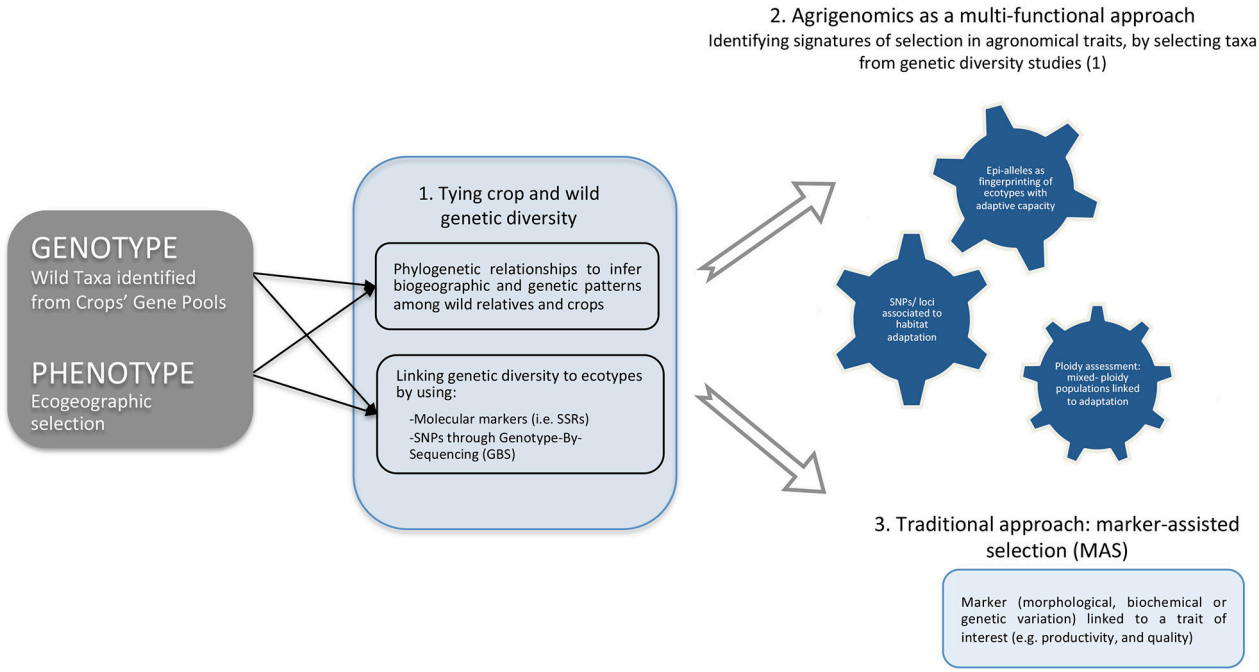

FIGURE 1 | Genetic and genomic tools streamline toward a proposed genomic-assisted breeding strategy using wild relatives. Genotype and phenotype interactions, through identifying wild taxa from crop's gene pools and by selecting taxa occurring in different ecogeographic, should be considered the first step prior to any genetic and/or genomic prospection. From genetic studies (1), the determination of the genetic diversity between crop and wild relatives are the key to assess the relatedness of wild taxa with the crop itself, either by phylogenetics or by assessing genetic diversity with ecological ranges using high-resolution power molecular markers, e.g., microsatellites (SSRs) or SNPs through Genotype-By-Sequencing (GBS) approaches. The agrigenomics approach (2) is hereby proposed as multi-functional method to identify signatures of selection in agronomical traits, by selecting taxa from genetic diversity studies (1), rather than using neutral markers with are not subjected to selection. Thus, by selecting SNPs/Epi-alleles associated to adaptive capacity of extreme habitats in wild taxa along ploidy assessment, it will be possible to detect genetic variation potential on adaptive ecotypes on wild relatives of crops. Particularly, agronomic traits can be disclosed from genes/function/epigenomics/ploidy assessments toward the utilization in future crop improvement as a genomics-assisted breeding approach. Conversely, the traditional approach (3) only allow to incorporate a marker (morphological, biochemical or genetic variation) linked to a trait of interest (e.g., productivity, and quality) using marker-assisted introgression, thus not taking into consideration the complete genomic panorama need to understand the adaptive capacity of a plant that could be transferable effectively to a crop.

chromosome sets (homoploidy) can help to overcome hybridization barriers, obtain sterile cultivars and restore fertility in hybrids (Rieseberg and Carney, 1998). Polyploidy can also contribute to enhanced pest resistance (Heijbroek et al., 1983) and stress tolerance (Colmer et al., 2006) and/or enhanced crop vigor (Nassar et al., 2008). In sugar beet, polyploid breeding was also used to increase crop yield (Jusubov, 1967; Xuan et al., 2009). These allopolyploid or homoploid forms can constitute important bridges and gene reservoirs for subsequent gene flow back to their diploid progenitors (Benavente et al., 2008).

Nowadays, different cytogenomic techniques, from classical cytogenetic methods, cytomolecular approaches (including different fluorescent *in situ* hybridization—FISH), such as the use of different types of DNA probes, from repeated DNA sequences and BAC clones to microdissected chromosomes, pachytene spreads, extended DNA fibers, among others—(Benavente et al., 2008) to flow cytometry can be used to study genomes. These techniques enable to distinguish genomes, identify specific regions in the chromosomes, and/or detect chromosome doubling.

In the *Beta-Patellifolia* species several cytogenetic studies have been developed. The section *Beta* has been described as cytogenetically uniform, mostly harboring diploid species. However, the detection of tetraploid individuals of *B. macrocarpa* in wild populations from the Canary Islands (Buttler, 1977), clearly revealed the need for wide geographical studies that could attest the cytogenetic diversity within the wild *Beta*. Indeed, Castro et al. (2013) revealed a cytogenetically diverse scenario. The authors analyzed several wild *Beta* populations across mainland Portugal and islands, and although most of the studied populations were diploid, they also discovered novel cytogenetic diversity. In particular, both diploid and tetraploid individuals were found in one population of *B. vulgaris* ssp. *maritima*, and *B. macrocarpa* revealed even more diversity with two populations harboring two or three cytotypes, including diploids and tetraploids, and/or hexaploids, the later described for the first time (Castro et al., 2013). These populations bearing cytogenetic diversity are of major importance for conservation and genetic resources management programs. The tetraploid *Beta macrocarpa* has been suggested to have an allopolyploid origin, resulting from hybridization between *B. vulgaris* ssp. *maritima* and diploid *B. macrocarpa* (Villain et al., 2009). Interestingly, previous works in Californian populations have documented the occurrence of hybridization between *B. vulgaris*

and *B. macrocarpa*, showing introgression of *B. vulgaris* alleles into the later species (Bartsch and Ellstrand, 1999). The genus *Patellifolia* currently recognize three species, but still presents several taxonomic problems that need to be solved. Species boundaries have been questioned by several authors. For example, the diploid species were observed hybridizing spontaneously in natural populations and could form fertile offspring (Szota, 1964/1971; cited in Jassem, 1992), raising questions on if they should be treated as variants of the same species. Later, Wagner et al. (1989) also questioned if the diploids *P. procumbens* and *P. webbiana* were distinct species. The genus *Patellifolia* also revealed to have cytogenetic diversity. Giménez and Cueto (2009) studied *P. patellaris* from Andalucía and described it as a species having both diploid and tetraploid individuals. Recent analyses (unpublished data) confirmed these results, with *P. patellaris* being mainly tetraploid, while *P. procumbens* and *P. webbiana* being diploid. However, the cytogenetic diversity in certain regions/taxa was higher than expected: the ploidy of *P. patellaris* was variable with diploids being found in southeastern Spain and mainland Portugal. Also, in Tenerife, *P. patellaris* and *P. procumbens* co-occurred and seemed to cross and form a hybrid swarm, as supported by the occurrence of diploid, triploid and tetraploid plants and by the high morphological diversity. These results indicate that cryptic diversity and interspecific hybridization generates novel genetic variation within the genus, which benefits species survival as it may broaden the adaptive potential and also generate genetic variants of interest to plant breeding. The possible presence of cryptic diversity may also explain why the delineation of the three species is a challenge to genetic resources collectors and genebank curators.

Considering the importance of CWR for supplementing crops gene pool, species conservation actions in geographical regions encompassing mixed-ploidy populations, as recently reported in the *Beta-Patellifolia* species complex (Castro et al., 2013; unpublished data) could benefit plant breeding.

FINAL REMARKS

In conclusion, we presented how the application of genomic tools could help uncovering new traits in CWR and how such diversity can be disclosed using high-throughput methodologies to identify new genomic information for breeding application. Such innovative tools will provide crucial genetic/epigenetic/cytogenetic elements to breeding programs. From identification to breeding application is a challenging step and will likely benefit from the emergence of genomics-breeding approach that is still in its infancy.

AUTHOR CONTRIBUTIONS

FM and MR conceived the manuscript. All the authors improved upon successive versions.

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ORIGINAL RESEARCH
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Closing the Yield Gap of Sugar Beet in the Netherlands—A Joint Effort

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The reform of the European Union's sugar regime caused potential decreasing beet prices. Therefore, the Speeding Up Sugar Yield (SUSY) project was initiated. At the start, a 3 × 15 target was formulated: in 2015 the national average sugar yield in the Netherlands equals 15 t/ha (60% of the sugar beet potential) and the total variable costs 15 euro/t sugar beet, aspiring a saving on total variable costs and a strong increase in sugar yield. Based on their average sugar yield in 2000-2004, 26 pairs of "type top" (high yielding) and "type average" (average yielding) growers were selected from all sugar beet growing regions in the Netherlands. On the fields of those farmers, all measures of sugar beet cultivation were investigated, including cost calculation and recording phytopathological, agronomical and soil characteristics in 2006 and 2007. Although there was no significant difference in total variable costs, the "type top" growers yielded significantly 20% more sugar in each year compared to the "type average" growers. Therefore, the most profitable strategy for the growers is maximizing sugar yield and optimizing costs. The difference in sugar yield between growers could be explained by pests and diseases (50%), weed control (30%), soil structure (25%) and sowing date (14%), all interacting with each other. The SUSY-project revealed the effect of the grower's management on sugar yield. As a follow up for the SUSY-project, a growers' guide "Suikerbietsignalen" was published, Best Practice study groups of growers were formed and trainings and workshops were given and field days organized. Further, the benchmarking and feedback on the crop management recordings and the extension on variety choice, sowing performance, foliar fungi control and harvest losses were intensified. On the research part, a resistance breaking strain of the Beet Necrotic Yellow Vein Virus (BNYVV) and a new foliar fungus, *Stemphylium beticola*, were identified and options for control were tested, and implemented in growers practices. The joint efforts of sugar industry, sugar beet research and growers resulted in a raise in sugar yield from 10.6 t/ha in 2002-2006 to 13.8 t/ha in 2012-2016.

Keywords: sugar beet, yield potential, grower's management, pests and diseases, soil structure, harvest losses, agronomy, extension

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INTRODUCTION

Historically, the share in farmers income from the sugar beet crop was relatively high (Berkhout and Berkum, 2005). In those years, the sugar regime of the European Union (EU) guaranteed minimum sugar beet prices for quota beet and cause a relative stable income compared to other crops of which the prices are fluctuating within and between years, like carrots, onions and potatoes (Berkhout and Bruchem, 2005, 2010; Vrolijk et al., 2009). With the sugar market reform of 2006 the European

Union lowered the guaranteed sugar beet price for farmers from 43.63 euro/t sugar beet (EC, 2001; Zeddies, 2006) to 26.29 euro/t from 2009 onwards (EC, 2006), which is decrease of a 39.7%. This causes a dramatic drop in farmers' income when the costs remain on a similar level. After the sugar marketing year 2016/2017 the system of sugar quotas is abolished (EP and EC. Regulation 1308, 2013) with a high price volatility in a free market as an expected result.

A study on the inputs of sugar beet production in the Netherlands called Low Input Sustainable Sugar Yield (LISSY), identified possibilities to save up to 20% of the total variable costs (Pauwels, 2006). To keep the profitability of the sugar beet crop on the level of before 2006, an increase in yield is needed because the savings on the total variable costs could not compensate the sugar beet price drop. Early research estimated the potential sugar yield in the Netherlands at 23 t sugar/ha (De Wit, 1953), while the average sugar yield realized by growers in the period 2002–2006 was 10.6 t/ha only 46% of the estimated potential (Van Swaaij, 2007). Large differences in yield levels between growers in the same region, with the same production circumstances like soil and climate, are reported frequently (Agrarische Dienst, 2007). This phenomenon is not restricted to sugar beet production in the Netherlands, it's found in Sweden (the 4T project), Germany and the United Kingdom (Blomquist et al., 2003; Fuchs et al., 2008; Limb and Atkin, 2010). Also for other crops large differences in yield levels among growers are reported as well (Lobell et al., 2009). Although large differences exist, it seems that in many cases the average yield of other crops is close to 80% of the crops potential in that region (Lobell et al., 2009). This unexploited yield gap in sugar beet cultivation and the possibilities of high price volatility in future, was the reason for the IRS (Institute of Sugar Beet Research, The Netherlands) to initiate a chain of research and knowledge transfer in Dutch sugar beet production. The basic idea was that stable high yields at farm level is the best strategy to compensate for high price volatility. This chain approach included research (SUSY-project) and knowledge exchange by extension via Best Practice Groups, field days and trainings of harvester drivers, crop specialists and crop advisors. At the start of the SUSY-project a 3 × 15 target was formulated: in 2015 the national average sugar yield in the Netherlands equals 15 t/ha (60% of the sugar beet potential) and the total variable costs 15 euro/t sugar beet, aspiring a saving on total variable costs and a strong increase in sugar yield.

MATERIALS AND METHODS

SUSY-Project

The SUSY-project (Speeding Up Sugar Yield) studied the difference in sugar yield of growers in a pairwise comparison (Hanse, 2011). Growers were selected based on their sugar yields in the period 2000–2004. A grower with high yields ("type top") and a grower with average yields ("type average") which were neighbors formed a pair in the study. Both growers of a pair encountered the same production prerequisites: soil and climate. The pairwise comparison comprised 26 pairs (52 growers). A "type top" grower was defined as a grower with sugar yields in the period 2000-2004, on average and in each single year above the 75% quartile of the region where the farm was situated. A "type average" grower was defined as a grower with sugar yields among the 50% quartile in that region in the same period. Within a formed pair, the yield level of a "type top" and a "type average" grower differed at least 1.5 ton sugar per hectare based on the 5 years average between those two growers (Hanse, 2011).

From the participating growers data on parameters of soil physics, soil fertility, soil health, rainfall, drilling (date, depth, distance), field establishment, canopy closure, pests and diseases, nutrient uptake, yield, and quality, harvest losses and exact field size (GPS) were collected in 2006 and 2007. Next to that, the growers recorded all agronomic measurements, including application dates, prices, type and amounts of consumables etc. In 2008 only the exact field size, harvest losses and yield data was recorded next to the agronomic measurements. The SUSY-study, the measurements, recorded data and statistical methods are described in more detail in Hanse et al. (2010, 2011a,b). The yields of the participating growers from 2016 were taken and compared with their yields of 2006 without correction for harvest losses. The obtained data were analyzed using GenStat, 18th edition (VSN International Ltd.). To analyse the effect of grower, location and their interactions, linear mixed models were used. The pair rank number, region and the interaction of both, were used as random terms (random model) to analyse the "type top" and "type average" growers within a pair directly with each other.

Best Practice Groups

In the period 2007–2010, 37 Best Practice groups were formed. The groups consisted of 13 farmers on average (smallest group 9 and biggest 18) which followed a voluntary 2-years' program under the supervision of a crop specialist of the Agricultural Department of the sugar industry. The crop specialists were trained for the supervision of the Best Practice groups. The aim of the Best Practice groups was to exchange and deepen knowledge and experience between the participating farmers. Five meetings were held annually and after the first meeting the topics for a year were selected, which were prepared by a subgroup of three to four group members. Meetings were held on the farm of one of the participating farmers with a field visit to discuss on sugar beet growing. At the end of the 2 years' period each Best Practice Group formulated tips for sugar beet growers. The first results of the SUSY-project and the first Best Practice group meetings among daily practice of the crop specialists of Suiker Unie brought up the idea to produce a practical guide Sugar Beet Signals (In 't Hout and Maassen, 2008).

Field Days

From the results generated by the SUSY-project and discussed in the Best Practice groups, topics for field days were selected. Fifteen field days were organized from 2007 onwards on locations within sugar beet growing areas, one field day per year moving from area to area in subsequent years, finally covering the whole of the Netherlands. The field days were organized with a guided tour for the visitors. A tour had multiple topics explained by an expert in circa 10–15 min with 5 min for questions from the audience and lasted in total for 1 to 2 h.

Integrated Management of Foliar Fungi

To raise the awareness of sugar beet growers of foliar fungi and how to recognize them, the project "Integrated management of foliar fungi in sugar beet" was initiated (fund of the Ministry of Agriculture of the Netherlands). The goal of this project (2006-March 2008) was communication and knowledge transfer on foliar fungi in sugar beet. At the end of the project the impact was monitored by a telephone inquiry before the start of the project (autumn 2005) and after the project (spring 2008).

Harvester Driver Training and Workshops Diagnostics of Pests and Diseases

The results of the SUSY-project gave also rise to the idea to train harvester drivers with the aim to minimize harvest losses. In 2009 and 2011 a harvester driver training was held. The training lasted for 1 day with an introduction on harvest and harvest quality of sugar beets and minimizing harvest losses. In the afternoon the group of drivers divided in subgroups based on the brand of harvester they were working with daily. Technicians of each manufacturer, which also participated in the introduction, explained for their machine the possible adjustments for adapting the machine to the circumstances in the field and minimizing harvest losses. The effect of making adjustments was real time tested on an available sugar beet field for each brand of machine separately.

The importance of pests and diseases on sugar yield found in the SUSY-project (Hanse et al., 2011a) initiated a series of workshops on the recognition or diagnosis of pests and diseases. The aim was to increase the ability of crop specialists and crop advisors to recognize pests and diseases in sugar beet. Workshops were typically setup with a short introduction on the importance of the right diagnosis of pests and diseases for all participants, following with the task to diagnose 20–40 randomized samples obtained from the IRS Diagnostic Service (Raaijmakers et al., 2014). During the provided time of 1 h IRS diagnostic specialists helped the participants with pointing at symptoms and showing out the subtle difference, without directly diagnose the concerning sample. After 1 h, the answers were provided and questions on the samples were answered centrally. Also more detailed information for the management of the pests and diseases concerning were provided. In 2012 and 2013 one workshop with the topic "Recognition of foliar fungi" was held in Bergen op Zoom (at the IRS facilities) and Valthermond (at the facilities of the local research farm of Wageningen University & Research), respectively. A workshop with the topic "Early season diagnostics" was held in Bergen op Zoom (2014) and Valthermond (2015). A workshop with the topic "Late season diagnostics" was held in Rolde (at the facilities of the local research farm of Wageningen University & Research; 2014) and Bergen op Zoom (2015).

Development of Sugar Yield in the Netherlands

The average sugar yields in the Netherlands of 1950–2016 were analyzed with non-linear and split-line regressions to estimate the effect of the total chain approach on sugar yield and identify the breakpoint in time. For the regression analyses, the statistical package GenStat, 18th edition (VSN International Ltd.) was used. The effect of breeding on sugar yield level for the period 2006–2016 was analyzed using the variety choice and the yield data of the official variety trials in the Netherlands using the same methodology as described by Rijk et al. (2013).

RESULTS

SUSY-Project

The sugar yields of "type top" growers were significantly 20% higher in comparison to the "type average" growers, but the total variable costs did not differ significantly between both grower types (**Table 1**). The sugar yield differences between growers were explained by pests and diseases (50%), weed control (30%), soil structure (25%), and sowing date (14%), all interacting with each other (Hanse, 2011). Within the category of pests and diseases on the clay soils *Heterodera schachttii* and BNYVV infestation levels were found to be important variates explaining sugar yield levels, and on sandy soils the number of fungicide applications, *Aphonomyces cochlioides* and *Heterodera betae* infestation levels (Hanse et al., 2011a). Harvest losses were initially recorded to correct the sugar factory delivered yield into grown yield on a participating field. They were found surprisingly high during the project. Total harvest losses (whole beet losses, losses due to root tip breakage and too deep topping), were on average 2.9 t/ha, minimum 0.45 t/ha and maximum 9.1 t/ha (Hanse and Tijink, 2010). Those variates became topics of further research and extension in the Netherlands, especially in harvester driver training. The sugar yields without harvest losses of the participants of the SUSY-project, 10 years after the project are shown in **Table 2**. The significant difference in sugar yield level between "type top" and "type average" growers disappeared in the 10 years after the start of the project. Although the average sugar yield is 1 t/ha higher for the "type average" in 2016, this difference is not significant ($P = 0.586$). This is due to the large variation in the 2016 yield data caused by extreme rainfall in early summer (June and July) in the South East causing low yield or even crop failures. With regard to the national yield level, both "type top" and "type average" are yielding at the 75% quartile level in 2016. **Table 2** also shows the national sugar yield level for the average and 75% quartile. The national yield level of 2016 is 22% higher compared to the yield level of 2006. On the national level, the difference between the 50% quartile and 75% quartile in 2006 and 2016 is comparable (12.8 and 13.1%, respectively).

Best Practice Groups

Almost 500 growers participated in the Best Practice study groups. At the end of 2010, when the last started Best Practice groups finished the first years' period, the tips formulated by each Best Practice group were listed to 15 tips in total and printed on the back side of each paper of a block note. Block notes were distributed to each sugar beet grower visiting the regional winter meetings or study groups (**Table 3**).

TABLE 1 | Influence of grower type on yield and costs in Dutch sugar beet production; SUSY-project, 2006–2008.

Grower	Root yield (t/ha)[a]	Sugar content (%)	Sugar yield (t/ha)[a]	Revenues (euro/ha)	Total variable costs (euro/ha)[b]
type top	78.1	17.21	13.4	3,099	1,416
type average	66.7	17.01	11.4	2,618	1,356
LSD 5%	2.89	0.22	0.51	128.8	73.35
P	≤0.001	≤0.05	≤0.001	≤0.001	n.s.

Data from Hanse et al. (2010).
[a]Yield not corrected for harvest losses.
[b]Costs mentioned exclude the fixed costs e.g. tenancy for the field and the overhead of the farm. The overhead encloses profit margin, costs of sugar quota, assurances for crop and grower, buildings, maintenance of fields, field and ditch edges.
n.s. means not significant.

TABLE 2 | Sugar yield in 2006 and 2016 of "type top" and "type average" growers participating the SUSY-project in the Netherlands.

Grower	SUSY-project				The Netherlands	
	Number		Sugar yield (t/ha)		Sugar yield (t/ha)	
	2006	2016	2006	2016	2006	2016
"type top"	26	23	12.8	15.2	12.5	15.3
"type average"	26	22	10.9	16.2	10.9	13.3
LSD 5%			0.80	6.99		
P			<0.001	0.586		

Field Days

Fifteen field days were organized across the sugar beet growing area's in the Netherlands (**Table 4**). On average 414 sugar beet growers visited the field days, implying that in each region large numbers of sugar beet growers got informed by the topics identified in het SUSY-project and the Best Practice groups.

Integrated Management of Foliar Fungi

Within this project an interactive map to visualize the regional warnings when foliar fungi were found in the different sugar beet growing regions, was developed and made online accessible. This interactive map was visited 8,712 times from 1 October 2005 till 17 March 2008. To improve the recognition of the foliar fungi in sugar beet and provide information on the fungi species and their management, a special website was developed in which the interactive map was incorporated as well. In the period from 1 October 2005 till 17 March 2008 this website received 13,042 visits. The website and interactive map remains online and are accessible via www.irs.nl/bladschimmel. The inquiries before and after the project revealed that sugar beet growers became more aware of the foliar fungi. In 2005, 42% of the growers applied fungicides against foliar fungi and in 2007 79% of the growers. The increased attention of foliar fungi management in the extension resulted in more attention of growers for this topic. Also the recognition of foliar fungi and timing of applications was improved after the project (**Table 5**).

TABLE 3 | Fifteen tips for a high sugar yield from sugar beet growers participating the Best Practice groups.

Number	Tip
1	grow your sugar beet conscious for the highest profit, review critically every handling and watch how colleagues are doing it
2	have a wide as possible crop rotation and take care for the right soil pH
3	use an acreage as low as possible to fulfill contract obligations
4	beet cyst nematode tolerant varieties pays back quickly, already from a low infestation level
5	cherish your soil, the reward is a high yield
6	when the soil is dry enough, sow as soon as possible
7	conduct soil treatments preferably in a single pass
8	choose the lowest tire pressure from the table; low tire pressure saves soil structure, fuel and time
9	fertilization with nitrogen, phosphorus and potassium can often be more economical
10	ask a colleague grower why he is doing things, listen to his arguments, don't judge too quickly and try to get benefit out of it for yourself
11	be keen on weeds and spray on seedlings, prevent hardening of weeds
12	be alert for foliar fungi and perform the first fungicide application on time (first infection at that field)
13	harvest what is grown, pay attention to top, tip and whole beet losses; topping 1 mm to deep means 1% of nett root loss!
14	store beets dry, cool and frost free. A fleece cover will keep your beets dry
15	stay informed on what is going on and register for the free e-mail service of IRS (www.irs.nl)

Harvester Driver Training and Workshops Diagnostics of Pests and Diseases

At the harvester training day of 2009, 30 drivers participated and in 2011, 40. This training has since 2012 a follow up with Harvest Checks by the crop specialists of the sugar industry. At the workshop for the recognition of foliar fungi, 55 crop specialists and crop advisors participated from the south of the Netherlands. In 2013, the same workshop had 40 participants in the north. The workshop in early season diagnostics had 58 participants in Bergen op Zoom (2014) and 37 in Valthermond (2015). The late season diagnostics workshop had 49 participants in Rolde (2014) and 60 in Bergen op Zoom (2015).

Development of Sugar Yield in the Netherlands

The average sugar yield in the Netherlands from 1950 to 2016 is shown in **Figure 1**. The split-line regression identified a break in the trend after the year 2000. In the first period from 1950 till the breakpoint the yearly sugar yield increase was 0.06 t/year (0.9%) and in the period after the breakpoint 0.33 t/year (3.4%). The effect of breeding was estimated as 1.0% in the period 2006-2016. In that period the use of resistant varieties as a tool to circumvent damage by pests and diseases increased. In 2006 the

TABLE 4 | Field days on sugar beet growing organized in the Netherlands (2007–2017).

Year	Location	Demonstration	Topics in guided tour	Growers
2007 (October)	Colijnsplaat (southwest NL)	- harvest and topping - tyre pressure and fuel consumption	- beet cyst nematode management - verticillium wilt - green manure crops - diagnostics of pests and diseases - soil management - control of foliar fungi	400
2008 (October)	Valthermond (northeast NL)	- harvest and topping - tyre pressure and fuel consumption	- beet cyst nematode management - control of foliar fungi - fertilization - yellow spots (*Stemphylium beticola*)	550
2009 (June)	Valthermond (northeast NL)	- volunteer potato control - mechanical weed control	- soil treatments and seed bed preparation - optimal Nitrogen rate - cleaning spraying equipment - chemical weed control - variety choice	500
2009 (September)	Vredepeel (southeast NL)	tyre pressure and fuel consumption	- harvest and topping - control of foliar fungi - sugar beet as energy crop - fertilization and water quality - soil management - maize for biogas - trichodorid nematodes - rhizoctonia tolerant variety choice	450
2010 (October)	Lelystad (central NL)	Beet Europe 2010; demonstration of 10 sugar beet harvesters by manufacturers with independent test 2 days before	- storage after harvest - variety choice - green manure crops - nitrogen application rate - control of foliar fungi - effect of worn out drilling disks on crop uniformity	1,200
2011 (June)	Munnekezijl (north NL)	spray technique (drift reduction)	- variety choice - nitrogen application techniques - manganese fertilization - effect of worn out drilling disks on crop uniformity - symptoms of herbicide damage	400
2011 (September)	Wijnandsrade (south NL)	- tyre pressure - soil compaction - soil treatment	- spray technique - storage after harvest - beet cyst nematode management - soil profile	350
2013 (June)	Valthermond (northeast NL)	none	- variety choice - leaf miner control - control of *Stemphylium beticola*	200
2014 (August)	Valthermond (northeast NL)	none	- optimal soil pH for sugar beet in a rotation with a high share (33–50%) of potatoes - soil treatment - variety choice - control of *Stemphylium beticola*	250
2015 (February)	Dronten (central NL)	precision sowing machines	- effect of grower on emergence and uniform crop stand - seedbed preparation - soil treatment and adjustment of equipment - GPS usage	180

(Continued)

TABLE 4 | Continued

Year	Location	Demonstration	Topics in guided tour	Growers
2015 (June)	Valthermond (northeast NL)	none	- variety choice - control of *Stemphylium beticola* - optimal soil pH for sugar beet in a rotation with a high share of potatoes (33-50%) - diagnostics of pests and diseases	200
2015 (July)	Vredepeel (southeast NL)	spray technique (drift reduction)	- increasing the humus content in the soil - mechanical weed control - diagnostics of pest and diseases - control of foliar fungi - rhizoctonia tolerant variety choice - nitrogen and phosphorus application	300
2016 (June)	Lelystad (central NL)	tyre pressure and soil compaction	- long term phosphorus application - liquid fertilizers - weed control with 75% drift reduction nozzles - spray application and drift reduction - diagnosis of pests and diseases - variety choice	350
2016 (September)	Wijnandsrade (south NL)	harvest quality	harvest quality	140
2017 (August)	Westmaas (southwest NL)	none	- harvest quality - soil treatment and soil structure - liquid fertilizers - green manure crops - tyre pressure at 100 kPa - drones for crop inspection - diagnosis of pests and diseases - variety choice	750

TABLE 5 | Results extension project "Integrated management of foliar fungi" (2006-2008).

Activity	Number	Growers reached	Remarks
regional winter meetings	66	8,500	management of foliar fungi topic in program
regional demostration strips	13	1,100	
publications in growers magazine	10	All (14,000)	
internet articles	32	9,800 visits via e-mail notifications	inspired 45 articles in agricultural press

share of rhizomania resistant varieties in the Netherlands was 97%, in 2016 100%. The share of rhizomania resistant varieties with rhizoctonia tolerance increased from 17% in 2006 to 26% in 2016. Also the share of rhizomania resistant varieties with beet cyst nematode tolerance increased from 2% in 2006 to 41% in 2016. Next to that in 2016, 1.2% of the acreage was grown with a rhizomania resistant variety which combines the rhizoctonia and beet cyst nematode tolerance (triple resistance). In 2016, 17% of the rhizomania resistant varieties had two major resistance genes ($Rz1 + Rz2$). In 2006 this two last categories of varieties were not available on the national variety list.

DISCUSSION

To keep the sugar beet crop profitable in the Netherlands, the sugar yield level is extremely important. The SUSY-study showed that there was no relation between total variable costs and sugar yield level. The conclusion was drawn that the most profitable strategy for sugar beet growers, preparing for future uncertainties in a market with high price volatility, is the maximizing sugar yield with simultaneously optimizing costs (Hanse et al., 2010). The SUSY-project also found a huge impact of the grower on sugar yield level. Also other studies underlined the importance of the grower's management for the sugar yield level (Fuchs et al., 2008; Trimpler et al., 2017). The effect a grower has on the sugar yield level was the motivation to organize the Best Practice groups, field days, the project on foliar fungi, the trainings of harvester drivers and the workshops on diagnostics of pests and diseases. The central topics for the field days arose or were underlined from the results of the SUSY-project. An example of a topic that arose from the SUSY-project are the harvest losses initially intended to measure in order to correct the sugar factory delivered yield to field grown yield. Already after the first year of the SUSY-project it showed up as a factor with a quick win and potential to improve due to the measured variance among growers on similar soil types (Hanse and Tijink, 2010). The management of foliar diseases is an example of

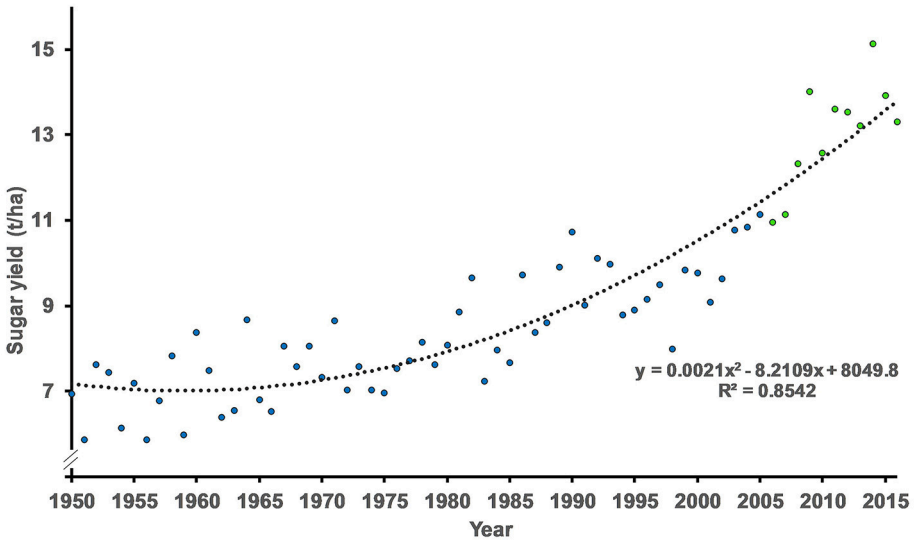

FIGURE 1 | The average sugar yield in the Netherland (1950–2016). The period 1950–2005 is indicated in blue and had a yearly sugar yield increase of 0.9%. The period 2006–2016 is indicated in green and had a sugar yield increase of 3.4% a year.

a topic underlined by the SUSY-project. The importance for sugar beet production was addressed just before the start of the SUSY-project by Vereijssen et al. (2007) with the project on integrated management of foliar fungi as a follow up. That latest, extension based, project resulted in almost a doubling of growers applying fungicides to protect their sugar beets from foliar fungi (increase of 42 to 79% in 2 years' time). However, the yellow leaf spots appearing from 2007 onwards caused new research on foliar diseases resulting in the identification of *Stemphylium beticola*, a new foliar fungus in sugar beet (Hanse et al., 2015; Crous et al., 2016; Woudenberg et al., 2017). The management of *S. beticola* became an important topic at field days and the recognition and diagnosis at the workshops on diagnosis of pests and diseases. These workshops were also organized for the diagnosis of most common pests and diseases in sugar beet growing by crop advisors and crop experts, since pests and diseases explained a large part of the difference in sugar yield of "type top" and "type average" growers in the SUSY-project. Despite crop protection measures applied the participating growers lost 24% of their sugar yield to pests and diseases (Hanse et al., 2011a). This result is quite similar to the estimated losses to pest and diseases in sugar beet worldwide (Oerke and Dehne, 2004). Therefore, also new research (and subsequent extension) was generated on the management of *Heterodera betae* (Raaijmakers, 2014). This nematode species was known to be present, but the SUSY-project pointed out the impact on sugar yield on sandy soils, urging for options of control. At the first sight, the impact of rhizomania on sugar yield levels on clay soils was curious, since the whole sugar beet acreage was sown with rhizomania resistant varieties from 2007 onwards. Further investigation showed that on fields with rhizomania symptoms in a *Rz*1 resistant variety a resistant breaking P25 tetrad (AYPR) of the Beet Necrotic Yellow Vein Virus (BNYVV) A-type occurred (Bornemann et al., 2015). The spread of this tetrad type caused an increase in the share of *Rz*1*Rz*2 rhizomania resistant varieties. Finally, the results in the SUSY-project on the white beet cyst nematode, *Heterodera schachtii*, caused more extension on the choice of the right variety, with a shift to a share of 41% nematode tolerant varieties in 2016. The annual increase in sugar yield showed a clear discontinue trend and raised from 0.9 to 3.4% after the breakpoint. One explanation of the yield increase in sugar beet is the genetic improvement by breeding. Studies on the breeding progress estimate a 0.7–2.0% yearly increase of sugar yield based on variety trials (Scott and Jaggard, 2000; Zimmermann and Zeddies, 2000; Märländer et al., 2003; Koch, 2006). In field research with stored seeds which were tested under equal agronomical and climatological conditions a breeding progress of 0.9% was found (Loel et al., 2014), while different resistance traits against pathogens were not included (Loel et al., 2014). The resistance against pathogens is an essential part of the breeding progress (Jansen and Stibbe, 2007). Compared with potatoes and cereals, having a linear yield increase, the yield increase of sugar beet is convex, showing a larger effect in yield increase than breeding progress could explain (Rijk et al., 2013). Analysis of the yield gap of sugar beet producing countries showed that the Netherlands had the highest increase in sugar yield (Jaggard et al., 2012). This study also suggest an effect of agronomy (or management) in the sugar yield increase, while the breeding effort for all countries is similar. It also revealed that progress in yield in variety trials and in practise developed parallel in the Netherlands. Despite changes in weather growers in the Netherlands were able to achieve the same speed of progress in yield increase; in most other countries the yield gap between variety trials and delivered beet was increasing. Analysis of the variety choice and the yield data of the official variety trials in the period 2006–2016 in the Netherlands showed

that breeding was responsible for a 1.0% average yield increase per year. The remaining increase in sugar yield is mainly due to the management of the grower, interacting with the weather conditions encountering on his fields. The effect of climate change on sugar yield level in the Netherlands is unclear. Positive effects on sugar beet yield might be reduced by negative effects, resulting in a very small or even zero effect (Van Oort et al., 2012). A crop model simulation by Reidsma et al. (2015) found substantial effects of climate change (increasing temperature and annual rainfall) on sugar beet yields. However, the factor management was set to zero for sugar beets in this study. The analyses by Rijk et al. (2013) could not disentangle environment and management. There might also be an influence of grower's management on the impact of climate change on crop yield, for instance: "type top" growers had a higher rooting depth and potentially suffer less from the longer periods of drought and had a better soil structure below plowing depth as well, giving the field more capacity in case of excessive rainfall (Hanse et al., 2011b). The development of the sugar yield in the Netherlands shows a clear discontinue trend. This is due to the effect growers can have on yield once they make the right choice on the right time before and during the season. The whole integrated extension effort described in this manuscript supported the growers in their management. The effect of the grower is once more underlined by the results of the "type average" growers in the SUSY-project, 10 years after the project the difference in yield level with the "type top" growers is vanished, thus raising the average yield level. This could be due to more attention to the crop and solving some of the management issues by the "type average" growers. The difference of the 50 and 75% quartile of the national sugar yield of 12.8 and 13.1% in 2006 and 2016 respectively, indicates that there is still potential left among all sugar beet growers in the Netherlands for a further future yield increase.

AUTHOR CONTRIBUTIONS

There was an equal contribution of the authors to this manuscript. All authors participated in discussing the focus, data to select, analyse and present this data in tables, describing results and putting it to discussion.

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New Generation of Resistant Sugar Beet Varieties for Advanced Integrated Management of Cercospora Leaf Spot in Central Europe

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Cercospora leaf spot (CLS) epidemics in sugar beet have been increasing in recent years causing higher use of fungicides. Concomitantly, the availability of effective fungicides is at risk because of resistance development in the fungus, the lack of new active ingredients as well as restrictive approval practices. A key option for an integrated management of CLS is cultivation of resistant varieties. Because of the yield penalty in resistant varieties, acceptance in commercial practice so far has been low. The aim of our study was to characterize recent sugar beet varieties registered in Germany in terms of resistance and tolerance to CLS and their value for integrated pest management. The genetic basis of CLS resistance in varieties is protected by intellectual property rights even after variety registration and not open to the public due to economic competition. To gain reliable data for cultivation, varieties have to be tested for their resistance traits under field conditions at varying levels of infection with *Cercospora beticola*. In collaboration with variety related stakeholders, 15 sugar beet varieties were tested in 49 field trials in Germany from 2014 to 2016 for their yield response to CLS. The trials were set up in a split-plot design with and without infection (i.e., with and without fungicide). The classification of varietal reaction to CLS is based on symptomatic leaf area (susceptibility) and the resulting relative yield loss (tolerance). Since the relation between both parameters varied among varieties, it was used as an additional parameter to describe tolerance. On this basis, three groups of varieties were identified. They can be characterized as a susceptible, a resistant and a presumably tolerant cluster. A comparison of the data with an older dataset originating from 2009 to 2011 revealed that yield performance of recent varieties with resistance to *C. beticola* caught up with susceptible varieties due to breeding progress. They showed no yield penalty in the absence of the disease and better economic performance than susceptible varieties. It is assumed that these varieties will allow a substantial reduction of fungicide use for an advanced integrated pest management under central European conditions.

Keywords: *Cercospora beticola*, sugar beet, variety trials, resistance, breeding progress, sugar beet yield, yield penalty, economic performance

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INTRODUCTION

Cercospora leaf spot (CLS) caused by the fungus *Cercospora beticola* Sacc. is the most widespread and most damaging foliar disease in sugar beet (*Beta vulgaris* L.) worldwide (Skaracis et al., 2010). Yield losses up to 50% and inferior processing quality caused by CLS have been reported (Wolf et al., 1998; Rossi et al., 2000). In recent years, 60–90% of the German sugar beet area was infested by *C. beticola*, whereas other pathogens (*Erysiphe betae*, *Uromyces betae,* and *Ramularia beticola*) occurring on less than 20% had a significantly lower economic importance (Brendler et al., 2008; Vasel et al., 2013).

The area infested with CLS has steadily enlarged from the southern and western part to the north and east of Germany (Buhre et al., 2014). Model calculations for different regions forecast even more favorable conditions for the fungus in the future resulting in an earlier occurrence of CLS, and increasing use of fungicides is discussed (Richerzhagen et al., 2011; Kremer et al., 2016). This development contrasts with the public request to reduce pesticide use and with the principles of integrated pest management. They are implemented by European law, stating pesticide use to be reduced to the necessary minimum (EU, 2009). To meet this demand, infection threshold values for fungicide application were developed (Wolf and Verreet, 2002; Lang, 2005) and field monitoring as well as forecasting models are employed to derive site specific control strategies (Racca et al., 2004). In the past, one fungicide application was sufficient in most cases to control CLS under German conditions, but three necessary applications have been reported as well (Buhre et al., 2014; Roßberg et al., 2017).

The widespread use of fungicides and the consequent selection pressure on *C. beticola* caused the development of resistances against fungicides with different modes of action (Varrelmann and Märländer, 2017). Already in the 1970s and 1980s, resistance against benzimidazoles was observed in southern Europe and the United States. Benzimidazole fungicides were mainly replaced by triazoles and strobilurins, which in turn led to a shift in the sensitivity of *C. populations* to triazoles and to resistance against strobilurines as summarized by Karaoglanidis and Ioannidis (2010).

The development of fungicide resistances underlines the necessity of an integrated management of CLS relying on other means beyond fungicides. A key factor is breeding for resistance against *C. beticola* in sugar beet. Varietal resistance against pathogens often comes along with a yield penalty in the absence of the disease (Brown, 2002). This was also found in several studies with sugar beet (e.g., Miller et al., 1994; Mittler et al., 2004; Kaiser et al., 2010; Gummert et al., 2015). Breeding of resistant varieties with high yield performance even without or under low infection pressure is crucial for acceptance in commercial practice. Whereas resistance describes the quality to hinder the development of a pathogen, the ability to produce high yield even under severe infection is called tolerance (Agrios, 2005). The resistance to CLS in sugar beet is quantitatively inherited and based on at least 4 to 5 major resistance genes and thus expressed gradually (Smith and Gaskill, 1970; Weiland and Koch, 2004). The genetic basis of CLS resistance in varieties is protected by intellectual property rights even after registration, i.e., not open to the public due to economic competition. To gain reliable data for cultivation, varieties have to be tested for their resistance traits under field conditions. In Germany, sugar beet varieties are tested in nationwide trials with two fungicide levels. The plots are either non-treated or fungicides are applied repeatedly to keep the crop as healthy as possible for a *ceteris paribus* comparison of varietal performance with and without foliar diseases (Ossenkop et al., 2005).

To describe the varieties according to their reaction toward CLS, two parameters are used. The first one is the infection of the leaves with CLS based on a grading of disease severity (DS) before harvest in the level without fungicide (BSA, 2000). It indicates the level of resistance/susceptibility toward CLS. The second parameter is yield loss caused by CLS. It is calculated as the relative difference in white sugar yield (WSY) between the non-treated and healthy fungicide levels and is supposed to describe the tolerance toward CLS (Ossenkop et al., 2005). As resistance against CLS maintains photosynthetic leaf area and thereby reduces yield loss (Rossi et al., 2000), it has been a matter of discussion, whether reduced yield loss can be attributed to tolerance traits or not (Ossenkop et al., 2005; Kaiser et al., 2010). Consequently, it has to be evaluated in more detail how CLS resistance and tolerance are connected in sugar beet varieties.

The aim of the present study was (i) to identify parameters to characterize resistance and tolerance toward CLS in sugar beet varieties, (ii) to distinguish variety groups according to their reaction toward CLS, (iii) to assess whether the yield penalty in resistant varieties has changed in recent years, and (iv) to describe consequences for beet cultivation and integrated pest management.

MATERIALS AND METHODS

Field Trials

The data originated from national variety trials with sugarbeet in Germany. Two 3-year datasets from 2014–2016 (trial series 1) and 2009–2011 (trial series 2) at 45 and 49 environments (i.e., location × year), respectively, were analyzed. The 49 trials in series 2 were part of a bigger dataset analyzed earlier by Gummert et al. (2015). All trials were run according to the official guidelines for the implementation of agricultural variety trials (BSA, 2000). Sugar beets were sown between beginning of March and end of April in three-row plots of 10.8–12.0 m^2. As plant density may cause unintended variance in root yield, the plots were manually thinned after field emergence to a density of 80,000–90,000 plants ha^{-1}.

The trial setup was a randomized split-plot design with two replications. The main-plot factor was fungicide strategy and the subplot factor was variety. The two fungicide strategies included a treatment without fungicide application ('non-treated') and a treatment with fungicide application aiming to keep the sugar beets as healthy as possible ('healthy'). This setup allows the comparison of variety performance with and without leaf diseases. Fungicide application started at the onset of first symptoms of foliar diseases in the susceptible varieties and

was repeated if symptoms recurred. The last application was timed to comply with the pre-harvest interval (21–35 days depending on the product) at the earliest possible harvest date. The fungicides applied were chosen site-specifically. They mainly belonged to the groups of triazoles and strobilurines. Even though fungicides were sprayed regularly, foliar diseases could occasionally occur. As the disease level remained rather low, the plots were considered as healthy (Gummert et al., 2015).

The trials were harvested between mid-September and beginning of November. Root yield and quality were determined at the local sugar factories. The beets were weighed after washing and processed to beet brei. The brei samples were analyzed for sucrose, potassium, sodium, and amino-nitrogen with automatic beet laboratory systems (Venema Installations, Eeemshaven, Netherlands or Anton Paar OptoTec GmbH, Seelze, Germany) according to standardized procedures (Hoffmann, 2006). WSY as the key indicator of variety performance was calculated from root yield and quality parameters according to German standard equations (Märländer et al., 2003).

Varieties

In trial series 1 and 2, 15 and 13 varieties were tested, respectively, which represented the varieties available for cultivation in Germany (**Table 1**). Each variety was tested at all environments within one series. Variety ratings for susceptibility to CLS according to the German variety list (BSA, 2011/2016) ranged from 3 to 5 in series 1 and from 2 to 5 in series 2. Tolerance to foliar diseases was calculated as the difference of relative WSY between the healthy and non-treated levels, i.e., the yield loss due to foliar diseases (IfZ, 2011, 2016). The larger the negative value, the less tolerant the variety was.

Disease Severity and Classification of Environments

The occurrence of *C. beticola* and other foliar pathogens (*R. beticola, E. betae, U. betae*) was regularly assessed in all trials. DS of each foliar disease was rated by plot on a 1–9 scale (1: no infection, 9: very high infection) at least twice between canopy closure and harvest according to BSA (2000). CLS was the predominant foliar disease in both trial series (data not shown). The CLS rating with the greatest differentiation among varieties (DS_{end}) was used for further data analyses (BSA, 2000; Gummert et al., 2015). This was with few exceptions the rating before harvest.

Environments were assigned to levels of infection according to mean DS_{end} of CLS in all varieties in the level without fungicide. Gummert et al. (2015) concluded that two groups of infection levels are sufficient to evaluate variety performance due to marginal differences between environments without or low to medium infection. Environments with $DS_{end} < 5$ were thus summarized in one group with no/low infection and $DS_{end} \geq 5$ was regarded as high infection (**Table 2**). This was in line with studies by Uphoff (2011) and Hoberg et al. (2015). Environments without CLS but with other foliar diseases were excluded from the dataset. Environments with CLS and further foliar diseases were also excluded if a fungicide effect was found which was related to high ratings of other foliar diseases than CLS.

Statistical Evaluation

Statistical analysis was carried out with SAS Desktop-Version 9.4 (SAS Institute, Inc., Cary, NC, United States). The MIXED procedure was applied for ANOVA of WSY with *post hoc* Tukey-Test and estimation of variance components. To describe the relation of DS_{end} and relative loss of WSY, Spearman's rank correlation coefficient was calculated with the CORR procedure and regression analysis and calculation of residuals was made with the REG procedure.

DS_{end} and relative loss of WSY were used as cluster-building variables in a cluster analysis. The aim was grouping of the varieties, i.e., to reveal groups with high similarities within and as many differences as possible between clusters. The SAS procedure DISTANCE was used to calculate Euclidian distances for the distance matrix. For cluster generation, the average linkage method was used with the procedure CLUSTER considering the mean distances between the members of two different clusters. The resulting differences between clusters were visualized in a dendrogram. Distances from 0.0 to 0.1 were considered to show very high analogy, from 0.1 to 0.3 high, from 0.3 to 0.5 average, and from 0.5 to 0.7 low analogy between the groups. No analogy was assumed for distances $\geq$0.7 (Hoberg et al., 2015).

Economical Evaluation

Economic performance of the different variety clusters was assessed with management accounting using (a) yield and quality data from the 2014–2016 field trials, (b) beet prizes 2017 in 1-year contract of Nordzucker (2016), (c) input data for seeds, fertilizers and plant protection products from a farm survey in Germany in 2012–2014 (Stockfisch et al., 2013), (d) mean costs of seeds at the sugar companies Südzucker AG (BISZ, 2017), Nordzucker AG (Ewers, personal communication) and Pfeifer & Langen GmbH & Co. KG (LIZ, 2017), mean costs for fertilizers in Lower Saxony January–March 2017 (Landwirtschaftskammer Niedersachsen, 2017) and mean costs for plant protection products at agricultural dealers (AGRAVIS Raiffeisen AG, Münster and Hanover, and BayWa AG, Munich), (e) farm business management data bases (KTBL, 2017; Uppenkamp and Nacke, 2017) to estimate labor and machinery costs of fungicide application based on the aforementioned German farm survey. The number of fungicide applications according to the threshold system (Wolf and Verreet, 2002; Lang, 2005) was assumed according to variety cluster and disease pressure (**Table 3**).

RESULTS

White Sugar Yield

White sugar yield in the 2014–2016 trials was significantly influenced by environment, fungicide level, variety and their interactions (**Table 4**). Fungicide level and environment had the strongest influence whereas the effect of variety was much smaller and on a similar level with the environment × fungicide interaction. All further interactions were of minor relevance.

TABLE 1 | Sugar beet varieties tested in national variety trials in Germany 2014–2016 and 2009–2011.

Test period	Variety	ID	Release	Susceptibility	Tolerance
2014–2016	1	1665	2006	4	−4.3
	2	1991	2010	4	−4.9
	3	2056	2011	4	−5.9
	4	2059	2011	5	−7.5
	5	2097	2011	3	−5.9
	6	2148	2012	4	−5.9
	7	2155	2012	4	−7.3
	8	2158	2012	4	−6.7
	9	2192	2012	3	−5.3
	10	2197	2012	4	−5.2
	11	2257	2013	5	−7.6
	12	2301	2013	4	−6.6
	13	2306	2013	4	−5.2
	14	2309	2013	3	−4.8
	15	2313	2013	5	−7.8
2009–2011	1	1665	2006	4	−4.0
	101	1409	2003	4	−3.2
	102	1492	2004	3	−3.6
	103	1560	2005	4	−5.1
	104	1632	2006	4	−5.0
	105	1648	2006	3	−3.8
	106	1718	2007	4	−5.9
	107	1748	2007	5	−3.9
	108	1779	2008	4	−4.3
	109	1802	2008	2	−5.2
	110	1806	2008	4	−4.9
	111	1824	2008	3	−2.5
	112	1830	2008	4	−5.3

Identification number (ID), year of release and rating of susceptibility to Cercospora leaf spot (CLS) (susceptibility) according to German Federal Plant Variety Office (BSA, 2011/2016): 1 = absent/very low to 9 = very high. Tolerance to foliar diseases (tolerance): relative loss of white sugar yield (WSY) between treatments with and without fungicide; 100 = mean of standard varieties in the level with fungicides (BSA, 2000; IfZ, 2011, 2016).

The estimation of variance components for the different levels of CLS infection and fungicide confirmed the dominant influence of environment on WSY (**Table 5**). With increasing disease pressure, the effect of variety significantly increased from 1.6% under healthy conditions at low CLS infection to 4.0% in the non-treated level at high infection. Similarly, the environment × variety interaction increased from 0.0 to 3.8%.

Under low infection, mean WSY across varieties was 15.59 t ha^{-1} in the non-treated and 16.12 t ha^{-1} in the healthy level (**Figure 1A**). The difference between the two fungicide levels ranged from 0.24 to 0.86 t ha^{-1} among varieties. Changes in the variety ranking between non-treated and healthy were relatively small. Under high infection, mean WSY was 15.72 t ha^{-1} in the

TABLE 2 | Classification of environments according to mean disease severity (DS) of Cercospora leaf spot (15 varieties in 2014–2016, 13 varieties in 2009–2010) without fungicide application.

Year	Disease severity of Cercospora leaf spot	
	Low (<5)	High (≥5)
	No. of environments	
2014	10	5
2015	11	4
2016	9	6
2014–2016	30	15
2009	5	6
2010	20	2
2011	11	5
2009–2011	36	13

National variety trials in Germany, DS rating according to BSA (2000).

TABLE 3 | Number of fungicide applications according to the threshold system (Wolf and Verreet, 2002; Lang, 2005) in sugar beet varieties susceptible, tolerant, and resistant to *Cercospora beticola* at environments with disease severity (DS) <5 (low) and DS ≥5 (high); rating according to BSA (2000).

Variety type	Disease severity of CLS	
	Low	High
Susceptible (A)	1	3
Tolerant (B)	1	3
Resistant (C)	1	2

TABLE 4 | Analysis of variance for factors influencing white sugar yield of 15 sugar beet varieties tested at 45 environments in Germany 2014–2016.

Effect	DF	Sum of squares	Mean square	*F*-Value
Environment	44	13049.4	296.6	584.0 ***
Fungicide	1	585.3	585.3	1152.5 ***
Variety	14	276.6	19.8	38.9 ***
Environment × fungicide	44	481.4	10.9	21.6 ***
Environment × variety	616	752.9	1.2	2.4 ***
Fungicide × variety	14	25.2	1,8	3.5 ***
Environment × fungicide × variety	616	321.8	0.5	1.0 n.s.
Replication (environment)	45	204.6	4.5	9.0 ***
Error	1305	662.7	0.5	
Corrected sum	2699	16359.9		

*CV, coefficient of variance; DF, degrees of freedom; ***$P \leq 0.001$; n.s., not significant.*

TABLE 5 | Estimation of variance components (%) for factors influencing white sugar yield of sugar beet at low and high disease severity of Cercospora leaf spot and two fungicide levels (non-treated/healthy); 15 varieties tested at 45 environments in Germany 2014–2016.

	Low infection (*n* = 30)		High infection (*n* = 15)	
Fungicide level	**Healthy**	**Non-treated**	**Healthy**	**Non-treated**
Environment	87.6 a	87.3 a	81.7 a	80.4 a
Variety	1.6 b	1.8 ab	2.0 ab	4.0 a
Environment × variety	0.0 c	0.9 b	4.5 a	3.8 a
Error	11.0 a	9.1 b	11.8 a	11.8 a

Different letters indicate significant differences within each row (Tukey-Test, $P \leq 0.05$).

non-treated and 17.44 t ha^{-1} in the healthy level (**Figure 1B**). The varietal difference between both levels was 1.04–2.52 t ha^{-1}, i.e., the range was wider than under low infection causing greater changes in the variety ranking between fungicide levels. These changes were greatest in varieties 1 and 2, which ranked lower in the healthy than in the non-treated level, and varieties 8, 11, and 12 reacting vice versa. Comparing the levels of CLS infection, even greater changes in variety ranking occurred. Varieties 9 and 11, e.g., were placed 13th and 6th under low infection and 3rd and 15th under high infection (non-treated). By contrast, other varieties showed high yield stability, namely varieties 13 and 14.

Disease Loss Relation

In the 2014–2016 trials, mean DS_{end} in the non-treated fungicide level varied from 1.0 to 8.3 among environments covering almost the whole 1–9 scale (**Figure 2**). The corresponding loss in WSY ranged between −2 and 21% and significantly increased with increasing DS_{end}.

The disease loss relation for the different varieties was separately assessed for low and high CLS infection (**Figure 3**). Under low infection with DS_{end} ranging from 2.2–3.3, yield loss was 1.3–5.1% (**Figure 3A**). Many varieties differed significantly inDS_{end}. Significant differences in relative yield loss only occurred between variety 1 and varieties 4 and 15. Under high infection, DS_{end} was 4.9–7.4 (**Figure 3B**). The corresponding yield loss ranged from 7.4 to 13.5% WSY being significantly lower in varieties 2 and 10 than in variety 11. In the 2009–2011 trials, a closer disease loss relation under high infection was found than in 2014–2016 (**Figure 4**). DS_{end} ranged from 4.5 to 7.0 among varieties. Yield loss was 3.5–9.8% and thus lower than in 2014–2016.

Variety Grouping

Based on DS_{end} of CLS and relative loss of WSY as cluster-building variables, in trial series 1 (2014–2016), three groups of varieties (clusters A, B, C) were distinguished at an average distance of 0.7 between clusters (**Figure 5**). Average distances within clusters A, B and C were 0.51, 0.40, and 0.55. For series 2 (2009–2011), three clusters (a, b, c) were identified as well (not shown).

Significant differences between the variety clusters were determined in both trial series (**Table 6**). In the 2014–2016 trials, clusters A and C differed in all traits except for WSY in either fungicide level under low infection (**Table 6A**), cluster B was intermediate. Under high infection, the ranking for WSY was C > B > A in the non-treated and C > B = A in the healthy level with relative loss of WSY being considerably higher (8.2–11.9%) than under low infection (2.3–4.1%). In the 2009–2011 trials, differences between the clusters were less distinct (**Table 6B**). Relative loss of WSY was 2.3–3.1% under low infection and 3.9–8.8% under high infection and thus lower than in series 1.

Economic Performance (Trial Series 1)

Under low infection, mean revenue less direct and operating costs was almost identical for the three clusters A, B, and C (range of 18 Euro ha^{-1}; **Figure 6**). Nevertheless, among all varieties, revenue less direct and operating costs of the most profitable and the least profitable variety differed by more than € 150 ha^{-1}.

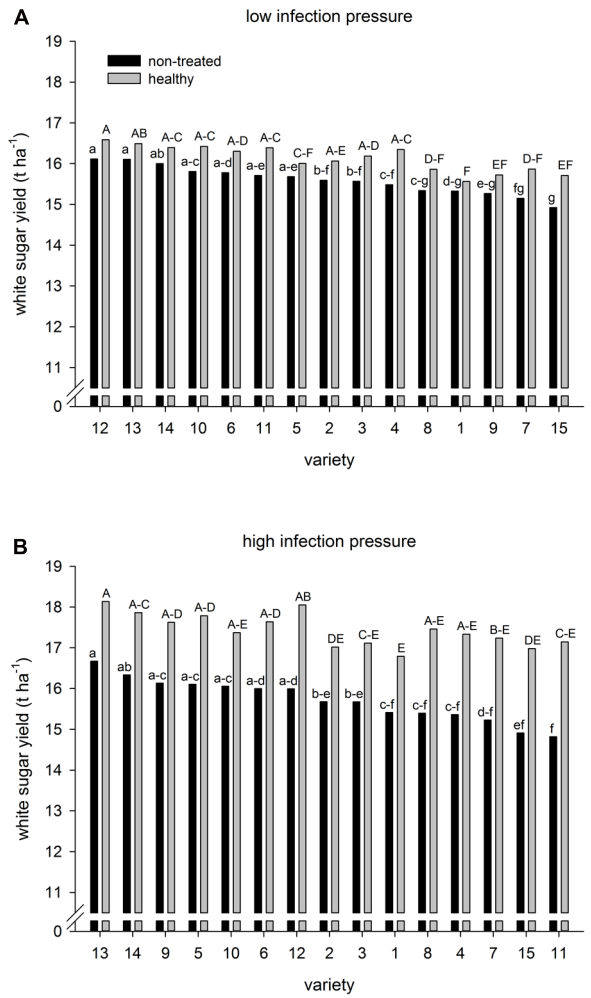

FIGURE 1 | White sugar yield of sugar beet varieties at environments with **(A)** low and **(B)** high infection with *Cercospora beticola* at two fungicide levels (non-treated and healthy). 30 and 15 environments in Germany, 2014–2016. Different lower case letters indicate significant differences in the non-treated level; different upper case letters indicate significant difference in the healthy level (Tukey-Test, $P \leq 0.05$).

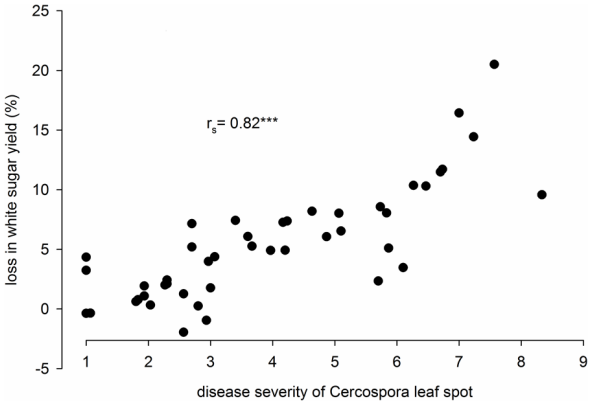

FIGURE 2 | Disease severity (DS) of Cercospora leaf spot and relative loss in white sugar yield (WSY) in 45 environments in Germany, 2014–2016; mean of 15 varieties. Relative loss in white sugar yield (WSY) is the yield difference between healthy and non-treated fungicide levels as percentage of WSY in the healthy level. DS was rated in the non-treated plots on a 1–9 scale (1: no infection, 9: very high infection; BSA, 2000); r_s = Spearman's rank correlation coefficient, $^{***}P \leq 0.001$.

However, under high infection, resistant varieties were on average relatively more profitable than tolerant or susceptible varieties. The economic advantage was € 162 ha^{-1} and € 152 ha^{-1}, respectively.

DISCUSSION

The aim of the present study was to identify groups of sugar beet varieties with varying resistance and/or tolerance to CLS within the most recent set of varieties available in Germany. To evaluate breeding progress in resistant varieties and to identify options for an advanced management, the results of 45 national field trials conducted in 2014–2016 (trial series 1) were compared to an older dataset with 49 trials in 2009–2011 (trial series 2).

Factors Affecting White Sugar Yield

The split-plot design of the field trials with the main factor fungicide made it possible to distinguish between natural infection with CLS and virtually disease free conditions achieved by frequent fungicide application (Ossenkop et al., 2005). Fungicide application had by far the highest influence on WSY of all factors under study, which emphasizes the importance of controlling fungal diseases. The effect of variety was much lower than the effect of environment, as it has been demonstrated before (e.g., Gummert et al., 2015; Hoberg et al., 2015). The environment × fungicide interaction was on a similar level as variety due to the varying severity of CLS infection among environments. Thus, the mean difference between healthy and non-treated conditions was 0.54 t ha^{-1} WSY under low infection, and 1.68 t ha^{-1} under high infection in 2014–2016. Mean WSY was highest in the healthy level under high infection. In Germany, the most severe Cercospora epidemics usually occur in the south where climatic conditions favor the growth of the fungus (Vasel et al., 2013; Gummert et al., 2015). At the same time, WSY is highest in the southern regions where spring temperatures allow early sowing and water supply in summer is high (Kenter et al., 2006; Fuchs et al., 2008). The differences in disease pressure are thus to some extend confounded with regional yield differences, but we do not assume an interaction between regional yield level and fungicide effect.

Estimation of variance components at the two levels of CLS infection under healthy and non-treated conditions confirmed the high environmental effect, which explained >80% of the variance in WSY. Its proportion of variance did not significantly change with increasing pressure of CLS whereas the effect of variety and the environment × variety interaction increased, albeit on a much lower level (<5%). This points to the changes in variety ranking between the levels of infection and fungicide

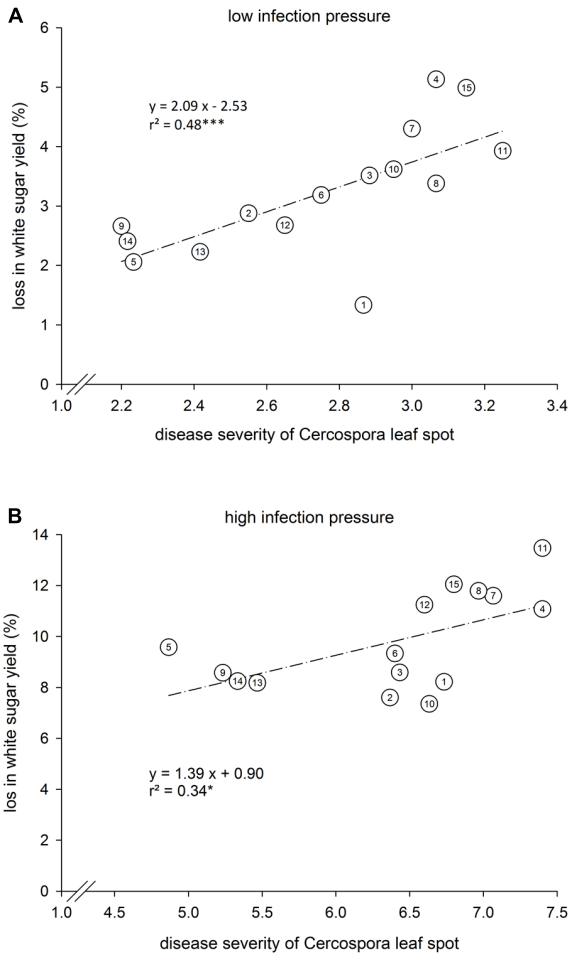

FIGURE 3 | Disease severity of Cercospora leaf spot (CLS) and relative loss of white sugar yield (WSY) in 15 sugar beet varieties tested at **(A)** 30 environments with low infection with CLS and **(B)** 15 environments with high infection with CLS; Germany, 2014–2016 under high infection in the non-treated level. Relative loss in WSY is the yield difference between healthy and non-treated fungicide levels as percentage of WSY in the healthy level. DS was rated in the non-treated plots on a 1–9 scale (1: no infection, 9: very high infection; BSA, 2000). $^{*}P \leq 0.05$ and $^{***}P \leq 0.001$.

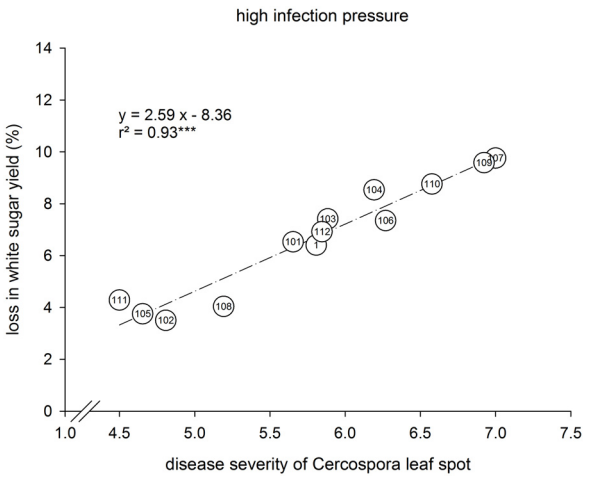

FIGURE 4 | Disease severity of Cercospora leaf spot (CLS) and relative loss of white sugar yield (WSY) in 13 sugar beet varieties tested at 13 environments with high infection with CLS; Germany, 2009–2011. Relative loss in WSY is the yield difference between healthy and non-treated fungicide levels as percentage of WSY in the healthy level. DS was rated in the non-treated plots on a 1–9 scale (1: no infection, 9: very high infection; BSA, 2000). $^{***}P \leq 0.05$ and 0.001.

use and is an indication of varying resistance and/or tolerance to CLS. This is in line with results by Gummert et al. (2015).

Occurrence and Impact of CLS

Heavy CLS infection is necessary to identify resistant varieties, but it does not occur regularly under German climatic conditions (Kaiser and Varrelmann, 2009). Due to the high number of field trials in our study, the whole scale of DS of CLS was covered. High infection occurred in 15 out of 45 trials in the 2014–2016 series, which was sufficient to distinguish different types of varieties (see section "Variety Groups and Yield Performance").

The loss in WSY caused by CLS increased significantly with increasing level of CLS infection. It increased more rapidly at environments where DS_{end} was above 5, which is presumably due to the non-linear connection of symptomatic leaf area in percent and DS_{end} grading according to the BSA (2000) guidelines. This rating scale was implemented in variety trials for practical reasons to assess resistance in numerous varieties aiming at better discrimination under low infection (BSA, 2000), but it impairs statistical evaluation. In future studies, DS in percent should thus be recorded for a higher accuracy of the regression analysis.

Furthermore, the yield effect of CLS not only depends on the severity, but also on onset time and progress of the epidemics (Wolf and Verreet, 2009). A parameter of the disease progress like the area under the disease progress curve (Shaner and Finney, 1977) thus seems more appropriate to estimate yield losses than single ratings of the disease. Disease progress was not assessed in the present study because the effort for its determination is too high in the high number of official variety trials.

Variety Groups and Yield Performance

It has been discussed before how resistance and tolerance against CLS in sugar beet varieties can be distinguished (Kaiser et al., 2010). Susceptible varieties express higher DS and loose more photosynthetic leaf area than resistant ones and thus suffer higher relative losses of WSY (Rossi et al., 2000). Ossenkop et al. (2005) thus proposed DS of CLS and relative loss of WSY as describing parameters. In the present study, both DS_{end} and yield loss in dataset 1 varied among the tested varieties indicating varying susceptibility to CLS. This effect was more distinct under high than under low CLS infection confirming the demand for high infection levels for variety characterization (Kaiser and Varrelmann, 2009). The connection of DS_{end} and yield loss was proven at both levels of infection. It was very consistent among the varieties under low infection where merely variety 1 deviated from the regression line. In relation to its DS_{end}, it suffered a low relative loss of WSY. Under high infection, several varieties

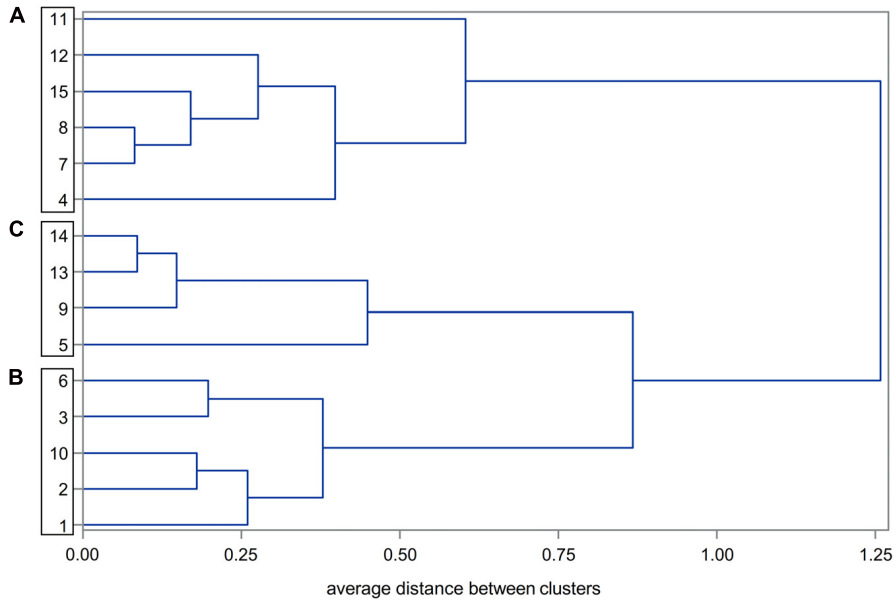

FIGURE 5 | Dendrogram of sugar beet varieties obtained through average linkage cluster analysis based on disease severity of Cercospora leaf spot and relative loss of white sugar yield (WSY). 15 varieties tested at 15 environments in Germany, 2014–2016 under high infection in the non-treated level. Relative loss in WSY is the yield difference between healthy and non-treated fungicide levels as percentage of WSY in the healthy level. **(A–C)** Denote clusters with an average distance of 0.7.

TABLE 6 | Different traits of three clusters (for details see **Figure 5**) of sugar beet varieties tested at **(A)** 45 environments in Germany, 2014–2016 and **(B)** 49 environments in Germany, 2009–2011.

(A)

Set 1 (45 environments, 15 varieties)		**Cluster A**	**Cluster B**	**Cluster C**
	Susceptibility to CLS	4.5 a	4.0 a	3.3 b
	Tolerance to foliar diseases	−7.3 a	−5.2 b	−5.3 b
Low infection level (*n* = 30)	Disease severity of CLS	3.0 a	2.7 a	2.2 b
	Relative loss of WSY (%)	4.1 a	2.9 b	2.3 b
	WSY non-treated	15.5	15.6	15.8
	WSY healthy	16.1	16.1	16.2
High infection level (*n* = 15)	Disease severity of CLS	7.0 a	6.5 b	5.2 c
	Relative loss of WSY (%)	11.9 a	8.2 b	8.6 b
	WSY non-treated (t ha^{-1})	15.3 c	15.8 b	16.3 a
	WSY healthy (t ha^{-1})	17.4 b	17.2 b	17.9 a

(B)

Set 2 (49 environments, 13 varieties)		**Cluster a**	**Cluster b**	**Cluster c**
	Susceptibility to CLS	3.8	4.0	3.3
	Tolerance to foliar diseases	−5.0	−4.4	−3.6
Low infection level (*n* = 36)	Disease severity of CLS	3.0 a	2.6 b	2.2 c
	Relative loss of WSY (%)	3.1	2.7	2.3
	WSY non-treated	14.4	14.3	14.0
	WSY healthy	14.9	14.8	14.3
High infection level (*n* = 13)	Disease severity of CLS	6.6 a	5.8 b	4.8 c
	Relative loss of WSY (%)	8.8 a	6.8 b	3.9 c
	WSY non-treated (t ha^{-1})	15.3	15.4	15.8
	WSY healthy (t ha^{-1})	16.8	16.6	16.4

Susceptibility to Cercospora leaf spot (CLS) and tolerance to foliar diseases according to BSA (2011/2016) and IfZ (2011, 2016). Different letters indicate significant differences within each row (Tukey-Test, $P \leq 0.05$). WSY, white sugar yield.

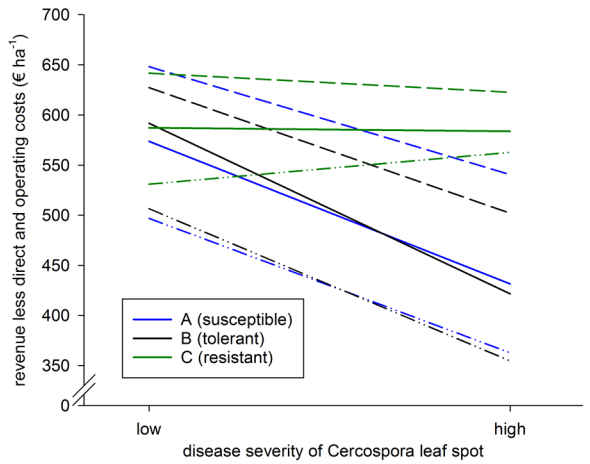

FIGURE 6 | Revenue less direct and operating costs of sugar beet varieties tested in 30 environments with low and 15 environments with high infection with CLS; Germany, 2014–2016. Connecting lines were added to illustrate changes in relative excellence. Susceptible **(A)**, tolerant **(B)**, and resistant **(C)** varieties were clustered according to disease severity of *C. beticola* and yield reaction to the disease. For details see text. Highest and lowest yielding varieties within each group are indicated by dashed and dash-dotted lines, respectively.

deviated from the regression, i.e., the residuals were larger. In certain varieties (5, 11), yield loss was higher than expected according to DS_{end}, in others it was lower (1, 2, 10) pointing to differences in tolerance/sensitivity.

Focusing on the comparative description of single varieties as also done in previous studies (Ossenkop et al., 2005; Kaiser et al., 2010; Gummert et al., 2015) may nevertheless bias the description of resistance or tolerance to CLS by variety traits that are not regarded (e.g., other resistances/tolerances). To avoid this drawback, we carried out a cluster analysis. Cluster A with highest DS_{end} and highest yield loss accordingly had the highest susceptibility to CLS. Cluster C was distinctly less susceptible with lower DS_{end} and lower yield loss, i.e., the varieties within this cluster expressed resistance traits. Cluster B was intermediate. Despite higher DS_{end} than in cluster C, yield loss was lower than expected according to the regression. This effect points to tolerance traits and supports the assumption of Ossenkop et al. (2005) that resistance and tolerance can be distinguished in sugar beet varieties. Cluster B is thus referred to as tolerant.

In the 2009–2011 trials (trial series 2), the cluster analysis resulted in three clusters with different DS_{end} and yield loss as well. By contrast to series 1, there was hardly any deviation from the regression between both parameters under high infection. It is thus concluded that the varieties within this older set represent different degrees of susceptibility/resistance, but there was no intermediate cluster like cluster B in the more recent set. Under low infection, the relative loss in WSY was similar in both trial series (data not shown). Under high infection, it was greater in 2014–2016 than in 2009–2011. Both DS_{end} and yield loss under high infection were similar for cluster B in trial series 1 (referred to as tolerant) and cluster a in trial series 2 (susceptible). Because of the quantitative inheritance of CLS resistance, there are no sharp borderlines between the variety groups. The differences between the datasets are probably due to the different varieties tested and to more severe CLS epidemics in 2014–2016 than in 2009–2011. At highly infested environments, mean DS_{end} was 6.4 in 2014–2016 and 5.8 in 2009–2011 (data not shown). Variety 1, which was the only one tested in both series also showed a higher DS_{end} under high infection in the more recent trial series than in the older one (6.7 vs. 5.8) and a higher relative yield loss (8.2 vs. 6.7%).

The comparison of both datasets is limited by the fact that they origin from different years. For a comparison of older and newer varieties, they should ideally be grown in the same trials (Loel et al., 2014), but this is not possible for a high number of trial sites and varieties. Nevertheless, the data show clearly that the yield penalty of resistant varieties under low infection has disappeared in the varieties currently on the German market. Even regular fungicide applications did not improve the relative competiveness of the susceptible varieties. This supports the assumption by Gummert et al. (2015) that a new generation of resistant varieties is able to catch up with susceptible ones under low infection.

Economic Performance

In our study, revenue varied by up to € 242 among varieties, direct costs by up to € 52 and operating cost by up to € 12 (data not shown). Variety was thus the key factor for revenue less direct and operating costs as an indicator of economic performance and explains why beet growers choose varieties according to their yield performance (Manthey and Ladewig, 2009).

Under low infection, revenue less direct and operating costs of the variety clusters was close. The greatest difference among varieties was ca. € 150 with both varieties belonging to the susceptible cluster. Under low infection, variety reaction to CLS is thus of lower importance than yield potential. Under high infection, all resistant varieties reached higher revenue less direct and operating costs than susceptible and tolerant ones. Beet growers should thus choose resistant varieties for two reasons: first, tolerant and susceptible varieties show higher yield losses even with fungicide application as also shown by Mittler et al. (2004). Second, resistant varieties usually reach the threshold for fungicide application later than tolerant and susceptible ones. This extends the period for fungicide application and may permit to save at least one spraying (Wolf et al., 1998; Kaiser et al., 2007). Due to higher revenues and the assumption that one fungicide application can be skipped, economic advantage of the resistant varieties was € 152 and € 162 compared to susceptible and tolerant varieties, respectively, which yielded similarly when fungicide was applied.

The difference in revenue less direct and operating costs among fungicide levels indicates whether fungicide application makes economic sense. This is the case when the extra earnings are beyond the fungicide application costs (Lang, 2005). It has to be considered that the yield data for this comparison originate from field trials with the aim to keep the fungicide treated plots as healthy as possible. The cost calculation, however, was based on fungicide applications according to the threshold

system. Revenue of fungicide application could thus have been overestimated or costs underestimated, respectively (Kaiser et al., 2007). Under high infection, the mean difference of extra earnings and extra cost was € 163 and is thus most likely economical, even if a certain inaccuracy is supposed.

Consequences for Integrated Pest Management

White sugar yield was mainly influenced by environment and fungicide treatment. Variety had a minor effect, but it increased at environments with high infection of CLS. Beet growers can hardly influence the environmental conditions driving CLS epidemics such as temperature and humidity, but they determine variety and fungicide strategy. The aim of integrated pest management is to reduce fungicide use and to control fungal diseases by other means as far as possible (EU, 2009). Our results indicate that sugar beet with resistance traits toward *C. beticola* can be one of these means. The current resistant varieties caught up with susceptible ones under low disease pressure and there is thus no longer reason to prefer susceptible varieties and to rely on fungicide applications when CLS might occur. This offers opportunities to increase eco efficiency of sugar beet production in terms of fungicide use (Wießner et al., 2010).

By contrast to the resistant varieties, tolerant varieties had no economic advantage over susceptible ones. Under high infection, WSY was the same in the susceptible and tolerant variety clusters when fungicide was applied. Furthermore, it is unlikely that beet growers will skip a fungicide application on tolerant varieties due to the high DS they express. Following the current threshold system for fungicide application (Wolf and Verreet, 2002; Lang, 2005), the tolerant varieties will thus not contribute to the reduction of fungicide use and they will not increase revenue less direct and operating costs compared to susceptible ones under high infection either. Further studies have thus to assess the importance of resistant/tolerant varieties for integrated pest management in terms of variety specific control thresholds, treatment index (i.e., intensity of fungicide use; Sattler et al., 2007) and management of fungicide resistance.

Gummert et al. (2015) demonstrated that omitting the final fungicide application of two or three applications following the threshold system (Wolf and Verreet, 2002; Lang, 2005) had no effect on WSY independently of the variety type. They pointed out that this advantage has to be weighed against the risk of increasing inoculum potential and stronger epidemics in the following year (Pringas and Märländer, 2004; Khan et al., 2008). Even if the necessary cropping interval of 2–3 years (Windels et al., 1998) is kept, this may concern neighboring fields. Resistant varieties, which delay epidemic development and reduce spore yield (Weiland and Koch, 2004), might nevertheless reduce inoculum potential as well. This has to be assessed in further studies. Moreover, as reduced efficacy of fungicides in relation to their mode of action (Varrelmann and Märländer, 2017) is increasingly observed in commercial practice in central Europe (e.g., Kempl, 2017; Zellner, 2017), resistant varieties may contribute to inhibit this development by reduced fungicide application.

CONCLUSION

The older resistant varieties tested in 2009–2011 yielded 2–4% lower than susceptible ones under low infection or healthy conditions. By contrast, the newer resistant varieties tested in 2014–2016 yielded higher than susceptible ones under high infection and showed no yield penalty under low infection or healthy conditions. It can thus be assumed that this new generation of resistant varieties will gain acceptance among growers. It has to be studied in more detail, but there is a realistic chance that these varieties will require less fungicide application than susceptible ones. Resistant varieties will thus enhance both economic and ecological efficiency of sugar beet production, especially under high infection of CLS.

AUTHOR CONTRIBUTIONS

BM and CK planned and supervised the study. JV and CK analyzed the field data. JV and CH made the economical evaluation of the data. CK and JV wrote the manuscript with support from BM and CH.

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Crop Rotational Effects on Yield Formation in Current Sugar Beet Production – Results From a Farm Survey and Field Trials

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In Europe, the framework for sugar beet (*Beta vulgaris* L.) production was subject to considerable changes and for the future it is expected that sugar beet cultivation might concentrate around the sugar factories for economic reasons. Based on data from a national sugar beet farmers' survey and multi-year crop rotation trials, the effects of cropping interval (number of years in between two subsequent sugar beet crops) and of preceding crops on sugar yield were elucidated under current Central European management conditions. The dominating sugar beet cropping interval was ≥4 years in the farm survey with pronounced differences between regions. However, the cropping intervals 2, 3, and ≥4 years did not affect the sugar yield. Therefore, significant differences in sugar yield between regions were assumed to be caused by multiple interactions between year, site, and farmers' skills. Throughout Germany, the dominating preceding crops in sugar beet cultivation were winter wheat (*Triticum aestivum* L.) and winter barley (*Hordeum vulgare* L.). In the field trials, the sugar yield was 5% higher after pea (*Pisum sativum* L.) compared to maize (*Zea mays* L.) as preceding crop, while differences between the preceding crops pea and winter wheat, and wheat and maize were not significant. Repeated measurements of canopy development and leaf color during the growing season revealed a higher N-availability after pea as preceding crop. However, decreased growth after maize was not completely compensated for by high N-fertilizer doses. Overall, the causes for the differences in sugar yield between the preceding crops remained open. The results do not support concerns about substantial yield losses in sugar beet production due to a reduction in the cropping interval from 3 to 2 years. Nevertheless, short rotations with maize and sugar beet might increase the risk of *Rhizoctonia solani* crown and root rot infestation. Leguminous crops such as pea offer the potential for higher sugar beet yield with lower N-fertilizer doses.

Keywords: crop rotation, cropping interval, preceding crop, nitrogen, sugar yield

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INTRODUCTION

Negative impacts of extended use of pesticides have fostered public criticism and the need for alternative measures to control weeds, pests, and diseases in agricultural crop production while simultaneously a growing world population has to be fed. In order to meet both goals, the concept of ecological intensification was developed (Godfray et al., 2010; Petersen and Snapp, 2015).

Cultivation of annual crops in well-designed sequence with other species instead of continuous cropping or short rotations can help to control specific pathogens in arable crops and reduce the need for pesticide use to ensure high and stable yields (Coulter et al., 2011). Frequent cultivation of sugar beet (*Beta vulgaris* L.) on one field is known to stimulate infestation by soil-borne pests and diseases such as beet cyst nematode (*Heterodera schachtii* Schmidt) or black root rot (*Aphanomyces cochlioides* Drechsler) which can cause substantial yield losses (Schäufele and Winner, 1989; Hauer et al., 2016). To minimize such negative impacts, sugar beet is traditionally not grown in monoculture but in rotations with cropping intervals, here defined as the number of years in between two subsequent beet crops on the same field, of two or more years (Märländer et al., 2003).

In addition to phytopathological aspects, which are often linked to the survival of pests on crop residues, crop rotational effects are known to include a wide range of impacts related to e.g., nutrients supplied by the direct and/or previous preceding crops (Smith et al., 2008). Further, soil structural conditions affected by the rooting properties of the preceding crops plus measures taken to manage the preceding crops (soil tillage and machinery use) contribute to preceding crop effects (Ball et al., 2005; Munkholm et al., 2013). Bennett et al. (2012) reported various examples for yield responses of crops grown in short rotation or monoculture compared to diverse rotations and identified numerous biotic and abiotic factors as potential causing agents for the yield decline observed in short rotations/monoculture. Nevertheless, they also stated that evidence for the precise contribution of single factors or factor combinations is often missing due to the complexity of field experiments, but need to be clarified in future research. Finally it is worth to mention that residues from herbicides applied to a preceding crop can cause injury to a future crop (Stipičević et al., 2015; Cornelius and Bradley, 2017) and thus, may contribute to crop rotational effects.

Concerning sugar beet, current knowledge on crop rotational effects derives from field trials conducted in the 1960s up to the 1980s in Northwest and Central Europe as comprehensively summarized by Götze (2017). More recently, Götze et al. (2017) reported crop rotation effects on the stability of beet yield and quality. In these studies, almost all experimental sites were characterized by a moderate to high beet cyst nematode infestation level and the cultivation of a susceptible beet variety. Such a combination does, however, not match the current situation in agricultural practice, because choosing a beet cyst nematode tolerant or resistant variety would be highly preferable under infested conditions; tolerant and resistant varieties respond with substantially lower yield decline compared to susceptible varieties (Hauer et al., 2016). Only Draycott et al. (1978) and Liste et al. (1990) evaluated crop rotation effects on sugar beet yield in a long-term trial conducted on a soil without beet cyst nematode infestation; these trials were ceased in 1976 and 1989, respectively, and thus, cropping conditions were not comparable to current sugar beet cultivation with regard to the preceding crops included and the crop management applied (variety, nutrient supply, and crop protection). Hao et al. (2001) compared two 4-year crop rotations including two times spring wheat, one legume and one sugar beet crop, with beets grown after either legume or wheat, under irrigated conditions in the continental climate of North America. Overall, very limited knowledge exists on preceding crop and cropping interval effects under current central European soil, climatic and management conditions.

In Europe, the legal and economic framework for sugar beet production was subject to considerable changes in the past decade (2005/06: reduction in minimum beet price and sugar production quota; 2016/17: abolition of price and sugar quota restrictions), resulting in a decline in the area cultivated with sugar beet after 2005 followed by a re-increase after 2015 (EUROSTAT, 2017). This increase in cropping area exclusively took place on farms located in traditional growing regions with an existing infrastructure for beet production and processing. In Germany, such changes came along with the emergence of silage maize (*Zea mays* L.) used for biogas feedstock production (Jacobs et al., 2014) and leguminous crops such as field pea (*Pisum sativum* L.) grown on ecological focus areas (DirektZahlDurchfV, 2014). Nevertheless, in 2010–2015 winter wheat (*Triticum aestivum* L.) and winter barley (*Hordeum vulgare* L.) were still the most common preceding crops for sugar beet (60 and 20%, respectively), and sugar beet were most frequently grown with cropping intervals of 2 or 3 years (32 and 26%, respectively; Trimpler and Stockfisch, 2017). But in future, sugar beet production will likely need to face periods of low beet price and thus, concentrate close to sugar factories in order to minimize transportation costs (Isermeyer et al., 2005), thereby shortening the beet cropping interval even if shorter intervals may cause lower yield.

In order to provide information on crop rotational effects on sugar beet performance under current Central European management conditions, data from a national sugar beet farmers' survey were evaluated to answer questions concerning: (i) What is the dominating cropping interval in Germany today? and (ii) How does sugar yield respond to decreasing cropping intervals of $\geq$4, 3, and 2 years under soil and climatic conditions prevailing in Germany? Further, crop rotation trials were conducted on highly productive sites in Lower Saxony and Southern Bavaria, Germany, to answer the research questions: (iii) How do field pea and maize compared to winter wheat as reference preceding crop affect sugar beet yield? and (iv) Does a high compared to a low nitrogen (N) supply level for sugar beet modify preceding crop effects? Overall, our study aimed to evaluate crop rotational effects in the context of a sustainable development of sugar beet cultivation in Central Europe.

MATERIALS AND METHODS

Farm Survey

The survey included 2148 sugar beet fields in Germany and was carried out in seven seasons (2010–2016). The data were collected through a questionnaire sent to more than 300 sugar beet farmers per year, each providing information from his biggest sugar beet field. The farms were distributed throughout all growing regions of Germany according to the area under sugar beet. Farms were randomly picked to represent a range of farm and field

sizes, crop rotations and specialties. The questionnaire provided some general information about the farm and the management practices concerning the largest sugar beet field. These included crop rotations, pesticide use, mineral and organic fertilization, sowing and harvest dates, and taproot yield plus sugar content of sugar beet, which were used to calculate the sugar yield (Trimpler et al., 2017). Taproot yield and sugar content were derived from the growers' records received from the sugar factory. In order to consider fundamental differences in productivity between sites, the farmers were asked for the field evaluation index (German "Ackerzahl"; BodSchätzG, 2007) of their field which is provided by national inventories. The field evaluation index describes the soil's quality together with natural conditions of the site. It includes soil texture, rootability, and field slope plus influences of climate and other factors, and ranges from about 20 (low quality for cropland) to 120 (highest quality).

Results are presented either for all farms in Germany or aggregated in sub-groups for the regions North (Lower Saxony and Schleswig-Holstein), East (Mecklenburg-Western Pomerania, Brandenburg, Saxony-Anhalt, Saxony, Thuringia), South (Bavaria, Hesse, Baden-Württemberg, Rhineland-Palatinate), and West (North Rhine-Westphalia) according to Trimpler et al. (2017).

Field Trials

Crop Rotation Trial at Harste

In 2006, a crop rotation trial was established at Harste near Göttingen, Lower Saxony, Germany (51°36′23.5″N, 9°51′55.8″E), on silty loam Luvisol soil (IUSS Working Group WRB, 2006; topsoil 0–30 cm: clay 100 g kg^{-1}, silt 760 g kg^{-1}; organic C 13 g kg^{-1}; pH ($CaCl_2$) 7.2; Mg ($CaCl_2$) 96 mg kg^{-1}; P, K (CAL) 75, 122 mg kg^{-1}, respectively). The climate was characterized by a 30-year (1981−2010) mean annual rainfall of 651 mm and a mean annual temperature of 9.2°C (Deutscher Wetterdienst [DWD], 2015). Beet cyst nematode infestation on the experimental field measured in spring 2005 was below 400 eggs and juveniles kg^{-1} of soil.

The field experiment included eight crop rotations, out of which three with sugar beet were included in this study: (1) winter wheat − winter wheat − white mustard (*Sinapis alba* L.) cover crop − sugar beet; (2) winter wheat − mustard cover crop − maize − sugar beet; (3) winter wheat − winter rapeseed − winter wheat − winter wheat − phacelia cover crop (*Phacelia tanacetifolia* L.) − field pea − white mustard cover crop − sugar beet. This allowed to compare the effects of winter wheat, maize, and field pea as preceding crops on subsequent sugar beet growth and yield. In the rotations (1) and (3), such effects included the impact of mustard cover crop that was grown in autumn between preceding crop harvest and subsequent sugar beet. Maize was grown either as corn (2006−2009) or silage maize (2010−2016). For sugar beet cultivated in 2011−2013, the amount of mineral N-fertilizer was varied as a second factor in doses of 0, 40(2011)/60(2012, 2013), 80/90, and 120 kg N ha^{-1}, subsequently addressed as N0, N1, N2, and N3, respectively. For this purpose, the main plots (220 m^2) were split up into four sub-plots (55 m^2), resulting in a split-plot design with the preceding crop on main level and the N-fertilizer dose plot on sub-plot level. Each crop rotation element was present in the trial each year with three replicates arranged in complete blocks. Within replicates, six incomplete blocks with four out of the eight crop rotations were combined.

Primary soil tillage was conducted with a cultivator to 15−20 cm depth with two exceptions: (i) in autumn 2006−2009 after grain maize plots were moldboard ploughed to 15−20 cm depth after harvest to incorporate maize straw; (ii) in summer/autumn 2015 all plots were moldboard plowed to 25 cm depth. Sugar beet sowing was performed after seedbed preparation between late March and mid-April with placement of pelleted seeds in rows 45 cm apart and at 7.7 cm in-row distance. At 6-8-leaf-stage of plants in May, crops were manually singled to a final stand of approximately 23 cm in-row distance resulting in a plant population of 9−10 plants m^{-2}. The sugar beet varieties cultivated were tolerant against beet necrotic yellow vein virus ("Rhizomania") and beet cyst nematodes: Lucata (2007−2008), Beretta (2009−2010), Belladonna KWS (2011−2014), Lisanna KWS (2015−2016) (Federal Plant Variety Office [FPVO], 2017). The mustard cover crop grown was beet cyst nematode resistant and non-winter hard. Crop management including pesticide use followed the recommendations of the regional extension service of the federal state of Lower Saxony partially adapted according to the personal experience of the technician responsible for the trial. Weeds and leaf spot diseases were effectively controlled by pesticides. Main crop and cover crop residues were left in the field.

For all crops, the mineral N-fertilizer dose was derived according to the concept of a mineral N target value ("Sollwert"), taking into account (i) anticipated differences in N-mineralization due to the specific preceding crop and cover crop cultivation (140 kg N ha^{-1} after pea and wheat; 160 kg N ha^{-1} after maize), and (ii) the soil mineral N-content (N_{min}, 0−90 cm depth) measured in March each year (Landwirtschaftskammer Niedersachsen-Geschäftsbereich Landwirtschaft, 2010). The N-fertilizer doses applied to sugar beet ranged across years between 0−85 kg N ha^{-1} (mean 45 kg N ha^{-1}), 60−100 kg N ha^{-1} (mean 75 kg N ha^{-1}) and 90−135 kg N ha^{-1} (mean 112 kg N ha^{-1}) after pea, wheat and maize, respectively. The N-fertilizer was broadcasted immediately after sowing either as calcium−ammonium−nitrate granules (2011−2013) or ammonium−nitrate−urea solution. Cover crops were supplied with 50 kg N ha^{-1}.

In addition to the March sampling date, N_{min} was determined in the N0 plots in May and June 2011−2013. Seven cores per plot were mixed to a composite sample. Soil N_{min} (NH_4^+ and NO_3^-) was extracted from a sub-sample of 100 g soil by 250 ml of 0.0125 molar $CaCl_2$ solution and analyzed colorimetrically with a Continuous-Flow-Analyzer (Skalar Analytical BV, SFAS 5100, Netherlands).

In 2007−2010 and 2014−2016, sugar beet yield was determined at the end of September on a core area of 12.9 m^2 per plot (2 adjacent rows each 14 m long) by an experimental sugar beet harvester after manual topping. In 2011−2013 the harvest plot size was 10.8 m^2 (4 rows 6 m long). In the lab, beet taproots were washed to determine beet fresh weight, and processed

to brei, out of which a sub-sample was shock-frozen and stored at −18°C until sugar analysis according to International Commission for Uniform Methods of Sugar Analysis [ICUMSA] (2007). Sugar yield was calculated from taproot yield and sugar content.

In 2011−2013, early growth of sugar beet was established by harvesting the surplus plants removed at singling to the final stand in May. On a surface of 8.8 m^2 per N-fertilizer sub-plot entire plants (excluding fibrous roots) were manually removed from the soil by hand, counted, washed in the lab and dried to constant weight at 105°C. Dry matter yield per plant was used to calculate dry matter yield per ha at a plant population of 9 plants m^{-2}. Around mid-June and mid-July leaf area index (LAI) was measured in N-fertilizer sub-plots with the LAI-2200 (LI-COR, Lincoln, NE, United States) according to the protocol of Röver and Koch (1995). The concentration of chlorophyll in young, almost fully expanded sugar beet leaves was determined with the Yara N-Tester (YARA, Germany), which operates similar to the SPAD meter (MINOLTA, Japan).

Crop Rotation Trial at Aiterhofen

In 2010, a crop rotation trial was established at Aiterhofen near Straubing, Bavaria (48°51′06.5″N, 12°37′58.5″E) on silty loam Luvisol soil [IUSS Working Group WRB, 2006; topsoil 0−45 cm: clay 667 g kg^{-1}, silt 76 g kg^{-1}; organic C 10 g kg^{-1}; pH ($CaCl_2$) 7.3; Mg ($CaCl_2$) 106 mg kg^{-1}; P, K (CAL) 172, 134 mg kg^{-1}, respectively]. The climate of this site was characterized by a 30-year (1981−2010) mean annual rainfall of 757 mm and a temperature of 8.6°C (Deutscher Wetterdienst [DWD], 2015).

This experiment included four crop rotations, out of which two were included in this study: (1) winter wheat − winter wheat − white mustard cover crop − sugar beet; (2) winter wheat − white mustard cover crop − silage maize − sugar beet for comparing the effects of winter wheat and silage maize as preceding crops on subsequent sugar beet. Each crop rotation element was present in the trial each year with four replicates arranged in complete blocks (plot size 420 m^2). Primary tillage was performed as conservation tillage in autumn, using a cultivator at a soil depth of 18 cm. For seedbed preparation in spring a rotary harrow was used. Sugar beet sowing date varied between mid-March and mid-April among years (row width 50 cm, 6 cm in-row distance). In May crops were manually singled to a final stand of approximately 24 cm in-row distance resulting in a plant population of 9 plants m^{-2}.

The sugar beet varieties grown were Rhizomania tolerant and beet cyst nematode tolerant: Deborah KWS (2011−2014), Isabella KWS (2015) (Federal Plant Variety Office [FPVO], 2017). The N-fertilizer doses for sugar beet were uniform among preceding crops within each year, but varied among years from 100 to 135 kg N ha^{-1}. Beet cyst nematode resistant white mustard cover crop grown after wheat harvest was supplied with 40 kg N ha^{-1}. For all crops N-fertilizer was sprayed shortly before or after sowing as ammonium−nitrate−urea solution. Crop management including pesticide use followed the recommendations of the regional extension service of the federal state of Bavaria partially modified according to the personal experience of the technician responsible for the trial. Main crop and catch crop residues were left in the field.

In 2011−2015, sugar beet yield was manually determined around mid-October on a core area of 12 m^2 per plot (3 adjacent rows each 8 m long). Further processing and analyses to establish the sugar yield followed the protocol as described previously (section "Crop Rotation Trial at Harste").

Statistical Analyses

For the analysis of sugar yield 2010−2016 from the farm survey, the model for the analysis of variance included the effects cropping interval, region and year. The unbalanced data set included 2148 observations and passed the normality tests. Therefore, a three way analysis of variance was performed with the General Linear Model procedure. The analysis of variance was repeated including the field evaluation index as covariate. The *F*-values of the main effects and their interactions were considered significant for $p \leq 0.001$. All average values from the farm survey are presented as median values. All statistical analyses were conducted with the software package SAS Version 9.4 (SAS Institute Inc., Cary, NC, United States).

For the analysis of sugar yield in 2007−2016 of the Harste experiment, the statistical model included the following effects: preceding crop, year, their interaction (all fixed); year nested within replicate, block, plot (all random). For the evaluation of total plant dry matter yield in May, LAI, N-Tester and sugar yield data of 2011−2013 the model was: preceding crop, N-fertilizer dose (N0−N3), year, and their interactions (all fixed); interaction of preceding crop and replicate nested within year (random). Soil N_{min} data in 2011−2013 were calculated with the effects: preceding crop, year, their interaction (fixed); year nested within replicate (random). Sugar yield in 2011−2015 of the Aiterhofen experiment was evaluated with the model: preceding crop, year, its interaction (fixed); year nested within replicate (random).

Analyses of variance were conducted with the MIXED procedure after having checked the data residues for normal distribution with the UNIVARIATE procedure. If not normally distributed, data were square root transformed before analysis of variance. Comparisons of mean values were performed with Tukey's LSD test at $p \leq 0.05$. Tables and figures display re-transformed data when applicable.

RESULTS

Cropping Intervals and Their Effects on Sugar Yield (Farm Survey)

The farm survey revealed that winter cereals, namely winter wheat or winter barley, were grown before sugar beet on more than 80% of the fields surveyed in the years 2010−2016. Winter wheat as preceding crop was cultivated on 57% of all fields, ranging between 55 and 60% throughout the years. Winter barley as preceding crop grew on 24% of the fields and varied from 22 to 26%. In order to eliminate effects from unusual preceding crops on sugar yield, the following analysis focused on sugar beet fields with the preceding crops winter wheat or winter barley.

The sugar beet cropping interval dominating was ≥4 years (**Figure 1**). In the region North, however, the most frequent cropping interval for almost half of all fields was 2 years (not shown). In contrast, over 70% of all fields in the region East showed cropping intervals of ≥4 years. The cropping intervals in the regions West and South were more evenly distributed.

The median sugar yield in the survey was 13.8 Mg ha^{-1}. The variables region and year had a significant influence on the sugar yield ($p \leq 0.001$), which ranged between 12 and 15 Mg ha^{-1} in the regions North, East, South, and West (**Table 1**, year not shown). No significant effects were observed for the variable cropping interval or the interactions between cropping interval and year, cropping interval and region and cropping interval, region and year (not shown). Across regions, increasing the cropping interval from 2 to 3 years or ≥4 years did not increase the sugar yield. It tended to decrease from cropping interval 2 to 3 years in regions North and South, while in regions East and West a slight increase occurred with increasing cropping interval.

Subsequently, the data set was examined for influences of the field evaluation index on sugar yield (**Table 1**). The average field evaluation index was 69 and ranged from 73 to 60 between regions. All regions showed lower field evaluation indices for fields with cropping intervals of ≥4 years compared to fields with cropping intervals of 2 or 3 years. Consequently a correlation between field evaluation index and sugar yield was assumed and the field evaluation index was included as covariate in the analysis of variance. The field evaluation index turned out to be a significant covariate ($p < 0.001$) but neither the variable cropping interval nor the interaction of cropping interval with field evaluation index were significant (not shown).

TABLE 1 | Median of sugar yield (n = 2148) and field evaluation index (n = 2121) according to sugar beet cropping intervals and regions in Germany.

Cropping interval (years)	2	3	≥4	All cropping intervals
	Sugar yield (Mg ha^{-1})			
Average	14.4	13.9	13.4	13.8
Regions:				
North	14.2	13.4	13.8	13.9
East	12.0	12.3	12.4	12.4
South	14.4	14.2	14.4	14.4
West	14.4	15.6	15.1	14.9
	Field evaluation index			
Average	73	70	62	69
Regions:				
North	78	68	55	72
East	67	72	57	60
South	70	68	66	68
West	75	75	70	73

Only fields with the preceding crops winter wheat or winter barley were selected from the farm survey sugar beet cultivation Germany 2010–2016.

Preceding Crop Effects on Yield Formation (Field Trials)

At Harste, the sugar yield was significantly affected by the factors year (not shown) and preceding crop: sugar yield was higher after pea compared to maize, and intermediate after wheat as preceding crop (**Figure 2**). Similarly, the yield was not different after wheat compared to maize at Aiterhofen, and the effect of the year was significant (not shown). The interaction between year and preceding crop was not significant at Harste, but significant at Aiterhofen (not shown). Nevertheless, there was not a single year in which sugar yield was significantly different after the two preceding crops tested here (not shown). Differences in sugar yield were primarily due to differences in taproot yield and not sugar content at both sites (not shown).

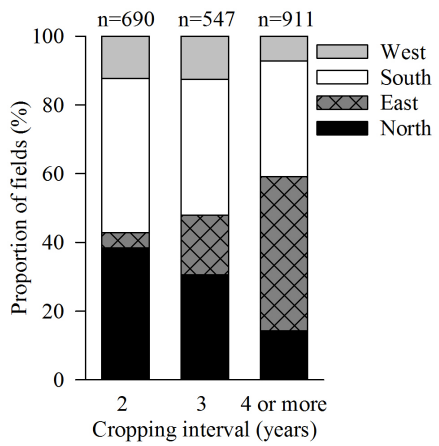

FIGURE 1 | Distribution of fields with different cropping intervals throughout regions in Germany. Only fields with the preceding crops winter wheat or winter barley selected from the farm survey of sugar beet cultivation in Germany 2010–2016 are presented here (n = 2148).

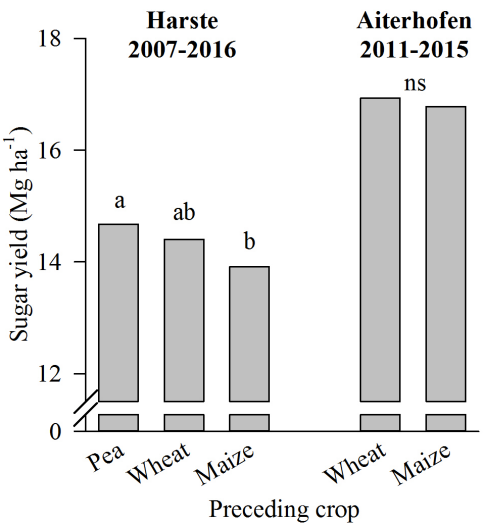

FIGURE 2 | Preceding crop effect on sugar yield in the crop rotation trials at Harste in 2007–2016 and Aiterhofen in 2011–2015 (mean of field replicates and years). Different letters indicate significant differences at $p \leq 0.05$ according to Tukey's LSD test, ns = not significant.

On average of the years 2011−2013 with altered N-fertilizer treatments at Harste, the soil N_{min} in the unfertilized plots doubled from March to May and decreased again until June in all treatments (**Figure 3**). For all sampling dates, the interaction of the factors year and preceding crop was significant. This was due to significant differences among the preceding crops in 2011 and 2013, but a lack of effect in 2012 (not shown). Thus, the significantly higher mean value after pea compared to wheat and wheat compared to maize (March), and pea compared to wheat and maize (May) was due to differences occurring in 2011 and 2013 (**Figure 3**). At sampling in June, the slightly higher 3-year average of soil N_{min} in unfertilized sugar beet plots after pea was caused by a significantly higher value in 2013 only.

Total plant dry matter yield in May was significantly affected by all main effects and interactions (**Table 2**). The interaction of year, preceding crop and N-dose was significant due to a strong yield increase with increasing N-dose after maize in 2011, while in the other combinations of year and preceding crop the N-dose had no significant effect (not shown). Across years, there was a yield increase from N0 to N2 after pea (significant) and wheat (not significant) as preceding crops, while after maize yield increased significantly from N0 to N3 (**Figure 4**). Nevertheless, this increase compensated just incompletely for the lower yield after maize compared to the other preceding crops across all N-fertilizer levels; such differences among preceding crops occurred in 2011 and 2012, but not in 2013 (not shown).

The development of the sugar beet canopy, measured as the LAI, and the N-supply of sugar beet leaves, measured as the N-Tester value, in June and July were significantly affected by the factors year, preceding crop and N-fertilizer dose (**Table 2**). In addition, the interaction of year and preceding crop (LAI June and LAI July) was significant, which was due to a higher LAI value after pea compared to wheat and maize in 2011, while in 2012 and 2013 LAI was equal after pea and wheat but higher compared to maize (not shown). Further, year and N-dose interacted significantly for LAI June, N-Tester June, and N-Tester July (**Table 2**). For LAI June, N3 caused higher values compared to N0 in 2011; in 2012 the difference between N2 and N0 was significant, and in 2013 values of N1 to N3 were higher than those of N0 (not shown). In June, N1 to N3 caused significantly higher N-Tester values than N0 in 2011 while in 2012 a similar but insignificant trend as in 2011 was obvious, and in 2013 the N-dose had no effect on the N-Tester values (not shown). In July, the N-Tester value of N-fertilizer dose N1 was significantly higher than of N0, and higher with N3 compared to N2; in 2012 only, N0 caused significantly lower values compared to N1−N3, and in 2013 the N-dose had no effect on the N-Tester value in July (not shown). Overall, despite such manifold interactions the N-doses N1 and N2 caused a substantial increase in LAI and N-Tester value at both measuring dates compared to the respective lower N-dose, while N3 did not further increase values compared to N2 in most combinations of year and preceding crop, and as the mean across years and preceding crops. Further, across years and N-fertilizer doses LAI and N-Tester values were highest after pea, intermediate after wheat and lowest after maize (**Figure 5**).

Sugar yield in autumn was significantly affected by all main effects and interactions (**Table 2**). The interaction of year, preceding crop and N-dose was due to a significant yield increase from N0 to N2 (and N3) after maize in each year of the study period, while after pea, the N-fertilizer dose did not reveal a significant effect in any year (not shown). In contrast, after wheat as preceding crop increasing the N-dose increased the sugar yield in 2011 and 2013 (N0−N2), but not in 2012 (not shown). On average across years, increasing the N-dose from N0 to N1 significantly increased sugar yield after all preceding crops. However, after maize only, a further yield increase was obtained when increasing the N-dose from N1 to N2 (**Figure 6**); increasing the N-dose from N2 to N3 caused no further yield increase and even at the highest N-dose yield remained lower after maize compared to pea.

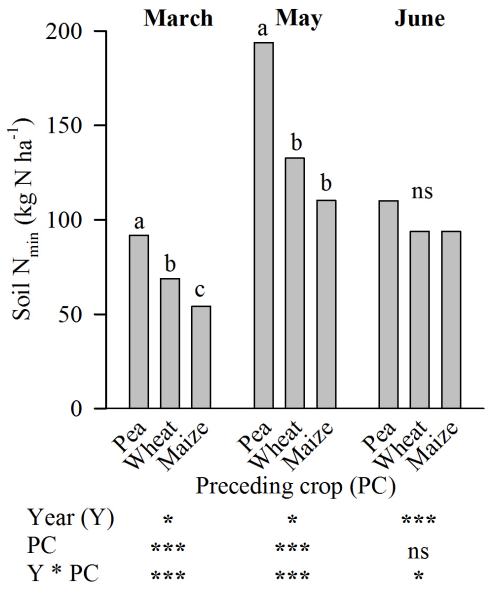

FIGURE 3 | Preceding crop effect on the soil mineral N-content (N_{min}, 0–90 cm soil depth) at three dates in the crop rotation trial at Harste (mean of field replicates and years 2011–2013). Different letters indicate significant differences at $p \leq 0.05$ according to Tukey's LSD test for individual sampling dates, ns = not significant. The table below the figure shows the results of the analysis of variance. Significance of *F*-values was displayed with $*p < 0.05$ and $***p < 0.001$.

DISCUSSION

Future development of the legal and economic framework in Europe for sugar beet production and crop production in general might cause considerable changes in crop rotations with sugar beet. Farm survey data and results from crop rotation trials were analyzed for effects of cropping intervals, preceding crops, and the potential of elevated mineral N-supply for leveling out the yield decline observed after maize as preceding crop.

Data from the farm survey revealed the current situation concerning preceding crops and cropping intervals in sugar beet cultivation in Germany. We expected a major effect on sugar beet yield by the specific crop that was grown directly prior to sugar

TABLE 2 | Significance of *F*-values for the effects of year (2011–2013), preceding crop (pea, wheat, maize), N-fertilizer dose (N0–N3, for details c.f. section "Crop Rotation Trial at Harste") and its interactions on parameters of sugar beet growth measured during the growing season and sugar yield in autumn in the crop rotation trial at Harste (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$), DF = degrees of freedom.

Effect	*DF*	Total plant dry matter yield May	LAI June	LAI July	N-Tester June	N-Tester July	Sugar yield autumn
Year (Y)	2	***	***	***	**	**	***
preceding crop (PC)	2	***	***	***	***	***	***
N-dose (N)	3	***	***	***	**	***	***
Y*PC	4	***	***	*	ns	ns	***
Y*N	6	**	***	ns	***	***	**
PC*N	6	*	ns	ns	ns	ns	***
Y*PC*N	12	**	ns	ns	ns	ns	*

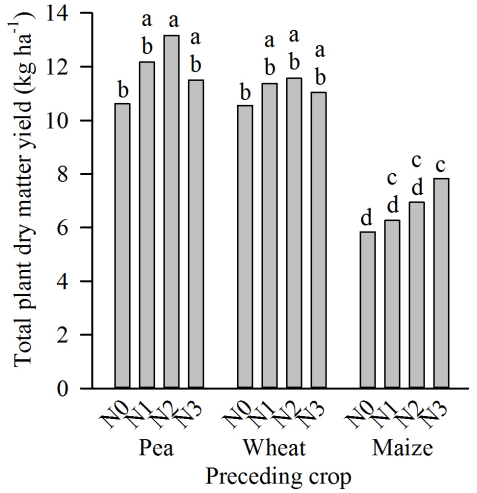

FIGURE 4 | Interaction between preceding crop and N-fertilizer dose (N0–N3, for details c.f. section "Crop Rotation Trial at Harste") on total plant dry matter yield in May in the crop rotation trial at Harste (mean of field replicates and years 2011–2013). Different letters indicate significant differences at $p \leq 0.05$ according to Tukey's LSD test.

beet. Therefore, we concentrated our evaluation of cropping interval effects on fields with winter wheat or winter barley before sugar beet, being the most frequent preceding crops before sugar beet in Germany. Simultaneously, these crops were represented in the farm survey with a frequency high enough to allow for the formation of sub-groups such as preceding crop intervals. Further, sugar beet growth and yield is the result of a multitude of impacts including genetic, environmental, and management factors (van Ittersum et al., 2013). Thus, the yield produced on any individual field presented in our farm survey data set is the result of a specific combination of such influencing factors. In addition, factors such as weather conditions, soil properties, pest and disease occurrence might differ regionally and correlate with the cropping interval. Therefore, we evaluated cropping interval effects within regions and anticipated a yield increase from 2 to 3 and 3 to ≥4 years of cropping interval. This expectation, however, was not confirmed neither for the nationwide data nor for the four regional data sets. In addition, the lack of difference in sugar yield between 2 to ≥4 years cropping intervals in the farm survey data was not attributed to a bias with soil fertility as assessed by including the field evaluation index as covariate in the statistical analyses.

Further, the farm survey showed that differences in sugar yield between regions were larger than differences between cropping intervals. A significant influence of the year was determined in a previous evaluation of sugar yield data from the farm survey for the years 2010–2014 (Trimpler et al., 2017). By means of a principal component analysis, the combination of site (soil type and field evaluation index), weather (year), and management specific (N-fertilization, pesticide use intensity) variables proved to influence the sugar yield significantly. Nevertheless, only 37% of the variance of the data could be explained by these variables, underlining the complexity of influencing factors and their interactions for cropping systems in real farm situations as stated by Bennett et al. (2012).

The lack of a significant effect of the cropping interval of 2, 3, and ≥4 years in our farm survey data is presumably explainable by the increased use of nematode tolerant varieties. Tolerance describes a limited yield decline compared to a susceptible plant (Müller, 1989). In 2011, up to 30% of all sugar beet varieties grown by farmers had a tolerance toward beet cyst nematode infestation in some regions (Buhre et al., 2014). Until 2016, the proportion of nematode tolerant varieties steadily increased to more than 30% of the whole sugar beet area in Germany. It is further supposed that nematode tolerant varieties are preferably grown on fields with an elevated beet cyst nematode infestation. For those fields, one cause for a reduced sugar yield in short rotations is eliminated. Besides, nematodes are known to reduce root yield depending on the environmental situation (temperature and water availability) of the particular growing season (Hauer et al., 2016), which causes additional variation in the yield of sugar beet grown on nematode infested fields.

The cropping interval effects obtained from the farm survey data are confirmed by field trial results, although information on cropping interval effects is available only from older field trials, which just partly reflect the conditions of current sugar beet cultivation. In a long-term trial located in Central Germany, Liste et al. (1990) found no difference in taproot yield among 1, 2, and 3 years cropping intervals on a site without beet cyst nematode infestation, while the 0 year cropping interval caused 14% yield

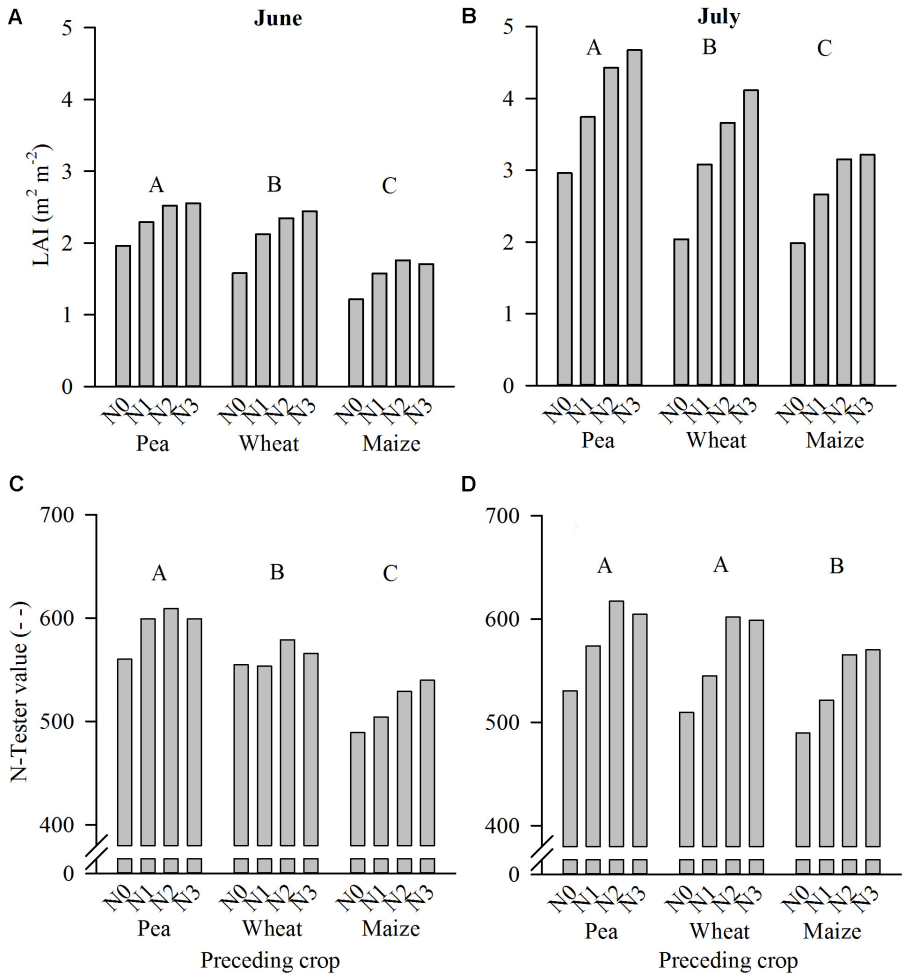

FIGURE 5 | Interaction between preceding crop and N-fertilizer dose (N0–N3, for details c.f. section "Crop Rotation Trial at Harste") on leaf area index (LAI; **A,B**) and N-Tester value **(C,D)** in June and July in the crop rotation trial at Harste (mean of field replicates and years 2011–2013). Different capital letters above column groups indicate significant differences between preceding crops across N-fertilizer doses and years at $p \leq 0.05$ according to Tukey's LSD test.

decline, and 4 years of cropping interval increased taproot yield by 4% compared to 1−3 years cropping intervals. In contrast, at another site highly infested with beet cyst nematodes the yield of the nematode susceptible variety cultivated continuously increased by 35% from 0 to 4 years of cropping interval on average from 1974 to 1989 (Liste et al., 1990). However, the yield increase due to increasing cropping intervals in the relevant range of 2−3 and 3−4 years accounted for an increase in yield of less than 4 and 7%, respectively. Deumelandt et al. (2010) found a white sugar yield increase of 2 and 6% for the same type of comparison in the same trial but for the years 1991−2006, while Götze et al. (2017) reported an increase of 7 and 1% on average of the years 2002−2016. Overall, the size of the yield increase due to increasing the cropping interval from 2 to 4 years was relatively low even under beet cyst nematode infested conditions.

In addition to cropping interval effects, crop rotational effects mainly derive from the influence of the immediate preceding crop on the growth of the subsequent one (Hao et al., 2001). In our field trials conducted on highly productive sites, sugar yield in autumn was significantly higher by about 5% after pea compared to maize as preceding crop at Harste, while differences between the preceding crops pea and winter wheat (Harste), or wheat and maize were only small (Aiterhofen). Nevertheless, sugar yield tended to be higher after wheat than after maize at both sites in the long-term average. Regarding the higher yield of sugar beet after pea compared to maize at Harste it has to be acknowledged that the cropping interval for sugar beet simultaneously differed with 5 years in the rotation with pea compared to 2 years in the maize rotation. Thus, we cannot exclude that the wider cropping interval might have contributed to the higher yield after pea. However, taking into account the lack of cropping interval effects in the farm survey data and only small effects in several field trials as discussed above (Liste et al., 1990; Deumelandt et al., 2010; Götze et al., 2017) we hypothesize that the cropping interval effect was negligible at Harste.

Possible negative influences of maize as preceding crop before sugar beet may include effects of poor soil structure due to heavy machinery and frequent passes during harvest under wet soil

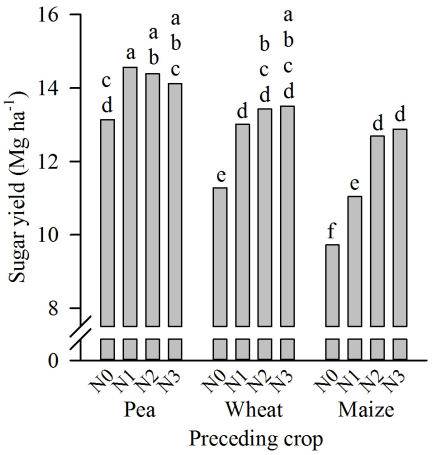

FIGURE 6 | Interaction between preceding crop and N-fertilizer dose (N0–N3, for details c.f. section "Crop Rotation Trial at Harste") on sugar yield in autumn in the crop rotation trial at Harste (mean of field replicates and years 2011–2013). Different letters indicate significant differences at $p \leq 0.05$ according to Tukey's LSD test.

FIGURE 7 | Sugar beet plant losses caused by Rhizoctonia crown and root rot infestation in the fourth cycle of the crop rotation winter wheat – white mustard cover crop – maize – sugar beet in the Harste crop rotation trial, September 01, 2017. Diseased patches occurred adjacent to healthy plants in two out of three replicate plots.

conditions (Chamen et al., 1992). However, the machinery used in our field trials was smaller and lighter than the machines used on farmers' fields and a severe impact on soil structure can be excluded. Nonetheless, the N-availability for sugar beet which had maize as preceding crop was obviously reduced as indicated by lower N-Tester values, which did not reach the level of those sugar beet cultivated after winter wheat or pea even at the highest N-doses given at Harste in 2011—2013. In addition, the sugar beet grown after pea yielded higher compared to maize as preceding crop in the other years of investigation, even though the N-fertilizer dose was substantially lower after pea. Therefore, we suggested that other effects than the N-availability were additionally limiting plant growth and yield performance of sugar beet grown after maize as preceding crop. These effects were variable among years, indicating that temperature and precipitation or soil moisture during spring and early summer (Kenter et al., 2006) as well as pathogens, such as *Heterodera schachtii* or *Rhizoctonia solani* Kühn (Anees et al., 2010; Hauer et al., 2016), are interacting effects ruling the conditions for sugar beet growth and yield.

Although we did not observe any symptom of Rhizoctonia infestation in the susceptible beet variety grown in our trials in 2007—2016, we cannot exclude a low level infestation by this disease causing some sugar yield reduction when sugar beet was grown after maize. Maize is a host for the soil-borne fungus *Rhizoctonia solani*, anastomosis group 2-2IIIb, the causing agent of Rhizoctonia crown and root rot in sugar beet. In other studies, a high frequency of host crops was shown to increase infestation level (Buhre et al., 2009; Kluth and Varrelmann, 2010). At Harste, in 2017 an increasing risk of Rhizoctonia infestation became obvious in the fourth rotational cycle of the wheat — white mustard cover crop — maize — sugar beet rotation, when Rhizoctonia occurred in several sugar beet plots for the first time (**Figure 7**).

In addition, residues of the herbicides applied in maize as preceding crop before sugar beet might have caused toxic effects and thus growth reduction in the subsequent sugar beet. Although maize herbicides were chosen for high compatibility with sugar beet, elevated concentrations of terbuthylazin and desethylterbuthylazin were detected in selected topsoil samples from plots with maize compared to wheat grown before sugar beet at Harste in summer 2012 (not shown). Residual herbicide and/or Rhizoctonia effects, however, could not explain the positive impact of pea compared to wheat as preceding crop. For the rotation including pea with a 5-year cropping interval for sugar beet, one might hypothesize that the overall infestation pressure exerted by other beet specific soil-borne pests and diseases, such as *Phoma betae* and *Aphanomyces cochlioides*, was lower as when grown in 2-year intervals even if we never observed related disease symptoms. Overall, the causes for the differences in sugar yield namely between pea and maize as preceding crops remain open for the Harste trial up to date. Similarly, Bennett et al. (2012) summarize in their review of a broad range of studies in various crops that in addition to plant pathogens numerous biotic and abiotic factors were supposed as being involved in yield decline caused by cultivation in short rotations or monoculture; but due to the complex nature of cropping systems evidence for the specific significance of single factors or factor combinations is usually lacking.

The farm survey revealed that the current situation concerning preceding crops in sugar beet cultivation is rather uniform throughout Germany. Winter cereals as dominating preceding crops are supplemented by winter wheat as the succeeding crop after sugar beet on more than 75% of the fields (Trimpler and Stockfisch, 2017). Stein and Steinmann (2018) reported similar results for crop sequences including sugar beet for Lower Saxony during the years 2005—2011. Provided that a crop sequence on one field correlates with the crops grown on neighboring fields in the same year, the cropping interval for sugar beet provides an estimation of the cropping density for sugar beet within one region. If a larger acreage of sugar beet was

grown within a region this could increase the disease pressure, especially leaf spot diseases and virus diseases transmitted by aphids. Decreases in yield stability or overall lower yield could result from this concentration effect (Lin, 2011) and need intensive monitoring. Contrastingly, our study demonstrates that introducing leguminous crops into cereal dominated crop rotations offers the potential for increasing the yield of subsequent sugar beet. Simultaneously, it allows for a reduced N-fertilizer input, which contributes to lower greenhouse gas emissions of sugar beet. Similar positive effects on yield and N-fertilizer requirement were reported recently when replacing sunflower by pea in a wheat − sorghum − sunflower rotation under Mediterranean climate (Plaza-Bonilla et al., 2017).

CONCLUSION

For future changes in sugar beet production and steadily increasing demands on the sustainable development of crop cultivation, our data set from the farm survey did not support the expectation that shorter sugar beet cropping intervals are to cause dramatic yield losses in sugar beet. As long as growers do not violate fundamental crop rotation rules, the yield seems to rely more on the influences of year (weather) and management. A preceding crop different to the 'classic' winter cereal is not expected to lead to drastic changes in sugar yield as found in our field trials. Anyway, we showed a trend that pea as preceding crop offers the opportunity for gaining some yield increase with a lower amount of N-fertilizer, which may contribute to lower greenhouse gas emission of sugar beet production. Although the Harste field trial provides data from a period of 10 years, specific preceding crop effects, which have not been detected up to now, might start to appear in future. In short rotations with sugar beet and maize, Rhizoctonia infestation might become a serious threat for sugar beet production.

AUTHOR CONTRIBUTIONS

H-JK developed the basic concept of the field trials, conducted the respective statistical data evaluation and developed most of the figures and tables, and wrote substantial parts of the text. AJ was responsible for the coordination, data collection and evaluation of the field trials, and contributed to the interpretation of the field trial results and the text of the manuscript. NS was responsible for developing the farm survey, arrangement of the corresponding table and figure, and wrote parts of the manuscript. KT and NS analyzed the survey data including the respective statistical calculations and interpreted the results.

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Yield Potential of Sugar Beet – Have We Hit the Ceiling?

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The yield of sugar beet has continuously increased in the past decades. The question arises, whether this progress will continue in the future. A key factor for increasing yield potential of the crop is breeding progress. It was related to a shift in assimilate partitioning in the plant toward more storage carbohydrates (sucrose), whereas structural carbohydrates (leaves, cell wall compounds) unintendedly declined. The yield potential of sugar beet was estimated at 24 t sugar ha^{-1}. For maximum yield, sufficient growth factors have to be available and the crop has to be able to fully utilize them. In sugar beet, limitations result from the lacking coincidence of maximum irradiation rates and full canopy cover, sink strength for carbon assimilation and high water demand, which cannot be met by rainfall alone. After harvest, sugar losses during storage occur. The paper discusses options for a further increase in yield potential, like autumn sowing of sugar beet, increasing sink strength and related constraints. It is prospected that yield increase by further widening the ratio of storage and structural carbohydrates will come to its natural limit as a certain cell wall stability is necessary. New challenges caused by climate change and by prolonged processing campaigns will occur. Thus breeding for improved pathogen resistance and storage properties will be even more important for successful sugar beet production than a further increase in yield potential itself.

Keywords: sugar beet, yield potential, assimilate partitioning, sink limitation, water supply, storage losses, cambial rings

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YIELD INCREASE IN THE PAST

A high yield potential of agricultural crops is crucial for an efficient use of the available arable land. Yield potential is defined as the yield of a genotype grown in an environment to which it is adapted, without any limitations in water or nutrients or damage by pests, diseases, weeds, or other stresses (Evans and Fischer, 1999). For sugar beet, the yield potential has not been analyzed yet. In the past decades, sugar beet varieties have shown an annual increase in sugar yield by 1.5% (Märländer et al., 2003; Jaggard et al., 2010). This was partly due to increased spring temperature (Jaggard et al., 2007) and improved management practices. About 50% of the increase in yield and quality (0.9% a^{-1} for sugar yield) were achieved by breeding progress (Hoffmann and Loel, 2015), reflecting an increase of the yield potential.

When a high yield level has been achieved, breeding progress is essential for future yield improvements because increases achieved by improving technology, e.g., optimizing fertilizer use and crop protection, cannot be repeated (Jaggard et al., 2010). This begs the question about the extent to which breeding can increase the yield potential and which effect might arise from the expected climate change. The aim of the paper is thus to point out the perspectives for further

improvement of sugar beet yield by analyzing the genetic potential as well as limiting factors apart from the effects of pests and disease.

SHIFT IN ASSIMILATE PARTITIONING

Presuming that weather conditions cannot be changed, the genetic potential of a crop is the key factor for the potential yield. In order to assess whether the observed yield increase of sugar beet varieties will progress in the future, its physiological basis has to be analyzed. For this purpose, Loel et al. (2014) compared 17 old and new varieties. They found that the speed of leaf formation and the number of expanded cambial rings in the storage root had not changed in the registration period from 1964 to 2003. Hence, the cause of yield progress is neither increasing light interception and source activity (leaves), nor rising sink capacity (storage root).

Instead, breeding has obviously shifted assimilate partitioning within the plant (**Figure 1**). The total biomass produced by a sugar beet plant is partitioned into root and leaf dry matter (DM). The root DM consists mainly of sugar, which is targeted by breeding, and of all the non-sugar compounds including the molassigenic substances (mainly K, Na, amino acids) and the cell wall compounds (the marc, which forms the beet pulp) (Hoffmann et al., 2005; Hoffmann, 2010a). The ratios of marc to sugar found in experiments from 2000 to 2002 and from 2012 to 2014 clearly show a general shift toward less structural carbohydrates (leaves, cell wall compounds) and more storage carbohydrates (sugar). Hence, sugar yield was evidently increased on the expense of leaf dry matter and cell wall compounds in the storage root, so that the marc content of sugar beet varieties today is much lower than in the past (Hoffmann et al., 2005; Kenter and Hoffmann, 2009).

This process has continued further and marc content of current varieties is often below 4% (Hoffmann and Schnepel, 2016), whereas the sugar content did not change noticeably. Sugar and marc content of sugar beet varieties are nevertheless always closely related, which can be explained by an optimal cell volume for sugar storage (Milford, 1973; Kenter and Hoffmann, 2009). Hoffmann (2010a) confirmed a linear relation between sugar and marc in two sets of sugar beet varieties tested in the 1980ies and in 2006, but the regression line had shifted toward a lower marc:sugar ratio and a lower level of marc content in the more recent varieties.

This change in dry matter composition caused by a shift in assimilate partitioning is an unintended side-effect of the breeding progress so far. It is not clear yet, whether it forms the functional basis for the increase in yield potential and will therefore continue with further yield increase. Moreover, the question arises at which point the reduction in cell wall compounds will become limiting.

ESTIMATION OF THE YIELD POTENTIAL

In the absence of pests and diseases, the realization of the genetic potential of sugar beet depends on the regional weather

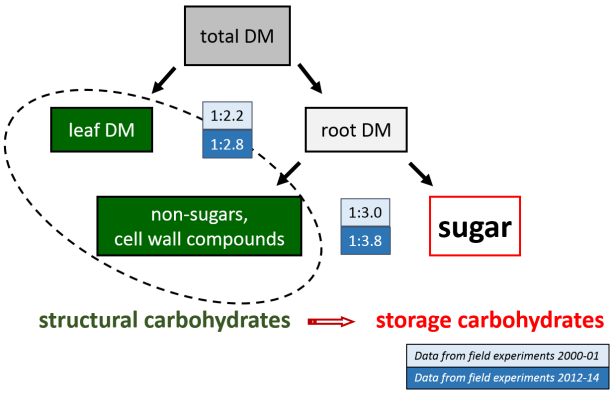

FIGURE 1 | Shift in assimilate partitioning in sugar beet due to breeding progress based on data from 27 field experiments in Germany, 2000–2001 and six field experiments with three sowing dates and two varieties in 2012–2014. DM, dry matter.

conditions. The growth of the storage root of sugar beet has no specific growth stages and accordingly no phase of maturation (Meier et al., 1993). Consequently, sugar yield increases with the length of the growing period, i.e., the number of days between sowing and harvest, and thus intercepted radiation (Scott et al., 1973). From this relation, the yield potential can be estimated.

Assuming average weather conditions, early sowing and late harvest, the maximum light interception of sugar beet is about 2,200 MJ photosynthetic active radiation (PAR) per ha during the growing season in Germany (Hoffmann and Kluge-Severin, 2010). The intercepted light is converted into biomass at 1.4 g DM per MJ PAR [radiation use efficiency (RUE); Monteith, 1977; Hoffmann and Kluge-Severin, 2010] to 1.8 g DM per MJ PAR (Werker and Jaggard, 1998; Qi et al., 2005). Assuming a high radiation interception (2,200 MJ ha^{-1}) and very optimistic RUE (2.2 g MJ^{-1}), potential sugar yield is 24 t ha^{-1}. This value is close to earlier results by De Wit (1967) based on theoretical assumptions for assimilation and weather conditions.

Kenter et al. (2006) used data from a large series of field trials to calculate the potential yield of sugar beet. The maximum growth rates, which sugar beet had achieved under various environmental conditions in Germany, were summed up over the growing period. According to this calculation, the potential yield of sugar beet is 42 t of total DM ha^{-1} with 24 t of sugar ha^{-1}, i.e., the different approaches give the same result.

UTILIZATION OF GROWTH FACTORS

Light Interception

A prerequisite for high yields is the coincidence of complete canopy cover with periods of high radiation in spring/early summer (Scott and Jaggard, 1978). It is thus expected that the cultivation of autumn sown beet could greatly increase yield by better synchronization of irradiance and canopy cover (Jaggard and Werker, 1999; Hoffmann and Kluge-Severin, 2010, 2011). Currently, autumn sowing of sugar beet is restricted because

of lacking bolting resistance, but also, and probably of similar importance, because of the insufficient frost hardiness of sugar beet (Kirchhoff et al., 2012; Reinsdorf and Koch, 2013; Loel and Hoffmann, 2014, 2015). Therefore, the yield benefit from autumn sown beets can only be calculated theoretically (Jaggard and Werker, 1999; Hoffmann and Kluge-Severin, 2011). To get more knowledge about the maximum yield of long growing sugar beet, Schnepel and Hoffmann (2016a) conducted a pot experiment in the greenhouse where vernalisation was prevented. The sugar yield increased continuously with time, reaching about 500 g of sugar per plant after 800 days of growing. A crop with 100,000 plants per ha could thus obtain around 50 t of sugar per ha which can basically be assumed as the potential yield of sugar beet. However, this will not be possible within one growing season. It is a question of efficiency whether one crop produces more biomass in a prolonged growing period than subsequent crops in a rotation, even when risks of pests and diseases are neglected.

Within the current system of cultivation, an early sowing date is essential for high yield. To fully benefit from early spring sowing, the plants have to emerge quickly even under low temperature conditions. Furthermore, the crop has to accelerate canopy closure compared to plants sown at the normal date. Therefore, adapted sugar beet varieties need to have a lower minimum temperature for emergence and leaf formation (<3°C; Milford et al., 1985) and higher growth rates at temperatures below 10°C. Furthermore, the vernalisation requirement should be higher and the bolting sensitivity of the varieties lower than today (Milford et al., 2010) to ensure yield formation in a vegetative phase. Therefore, in future it will be important to select for sugar beet varieties, which are adapted in their yield formation process to low temperatures (high cold tolerance and bolting resistance).

CO_2 Assimilation

It has been demonstrated that rising atmospheric CO_2 concentrations enhance sugar beet growth through higher assimilation rates (Demmers-Derks et al., 1998; Manderscheid et al., 2010). Yield increase in the past can thus partly be attributed to increasing CO_2 in the atmosphere. However, Manderscheid et al. (2010) showed that white sugar yield increased by only 10–15%, when CO_2 concentration was elevated from 375 to 550 ppm as forecasted for the middle of the 21st century, and thus less than expected from theory. This low response provides evidence for a sink limitation of beet growth.

Sink limitation (except for the phase of incomplete canopy closure in late spring) is further emphasized by results obtained under drought stress (Mäck and Hoffmann, 2006), where sugar accumulated in the leaves, resulting in a feed-back inhibition to assimilation, presumably due to lacking storage capacity in the root (Hoffmann, 2010b, 2014). Also Schnepel and Hoffmann (2016a) observed a decline in the rate of photosynthesis with increasing sugar concentration in the beet. It can thus be concluded that for a further increase in yield potential and to fully exploit rising atmospheric CO_2, in particular the sink capacity of sugar beet has to be enhanced.

LIMITATIONS – ACTUAL YIELD

The actual yield is always lower than the potential one, because weather conditions are usually not optimal and management operations, headlands and many other factors restrict sugar beet yield in commercial fields (Trimpler et al., 2017). The gap between attainable yield measured in official variety testings and the actual yield at farmers' fields amounts to more than 30% in some countries (Jaggard et al., 2012). In the following, some important physiological factors are discussed which will limit the actual sugar beet yield in future.

Water Supply

Even in years with favorable conditions for sugar beet growth, water shortage may occur during the summer months, when ambient temperature, water saturation deficit and thus transpiration demand are high. Therefore, yield reductions resulting from water shortage generally occur, in particular on light soils, and climate change is expected to fortify this effect in the future (Schindler et al., 2007; Okom et al., 2017).

With increasing level of yield and dry matter production, the probability of water limitation will increase as well. Assuming a transpiration coefficient of about 200 L of water to produce 1 kg of dry matter (Ehlers and Goss, 2003; Hoffmann, 2014), the production of 24 t of sugar ha^{-1}, which equals a total DM of about 42 t ha^{-1} and a root DM of 31 t ha^{-1}, will require more than 8.000 L water (800 mm). In the traditional sugar beet cultivation areas, this demand can hardly be met by rainfall alone and consequently, yield formation will always be restricted to a certain extent. Without additional water supply by irrigation, the potential yield can never be approached, in particular not if breeding achieves further increase and in a changing climate.

Therefore, varieties are needed, which are drought tolerant and respond with a lower yield reduction to insufficient water supply, not only to secure higher yields, but also for a higher yield stability under various environmental conditions. The water demand can be reduced when less DM is partitioned into leaf DM, leaving a higher percentage of assimilates for the storage of sugar. In pot experiments it has been shown that sugar beet can achieve very high yields with a much lower leaf DM than is usually produced in the field (Hoffmann, 2014). Furthermore,

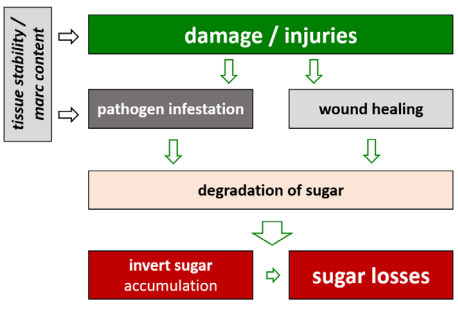

FIGURE 2 | Effect of damages of sugar beet roots on sugar losses during storage.

a smaller leaf apparatus was associated with lower transpiration rates at constant yield (Hoffmann, 2014). A possible way to increase water use efficiency and produce more DM from the available water ("more crop per drop"; Blum, 2005, 2009) could thus be the reduction of the often luxurious canopy of sugar beet, but this might conflict with the aim to accelerate early leaf growth and might also increase weed competition.

Storage Losses

Apart from limitations due to unfavorable weather conditions, further reductions in harvested sugar yield occur before the roots are processed in the factory. During storage sugar is cleaved to provide energy for life-sustaining processes of the sugar beet plant (Klotz and Finger, 2004). As the processing campaigns in the sugar factories are currently being extended, varieties are needed which can retain the assimilated sugar during the storage period. Among other factors such as damage during harvest operations, the genotype has an effect on the storability of sugar beet (Schnepel and Hoffmann, 2016b). Interestingly, there is evidence that varieties with lower marc content show higher sugar losses and invert sugar accumulation during storage. This seems to be the consequence of a higher susceptibility toward damage during harvest operations and toward the subsequent infestation with mold and rots during storage (**Figure 2**; Hoffmann and Schnepel, 2016; Schnepel and Hoffmann, 2016b).

HIT THE CEILING?

The essential question concerning future progress is whether the success of continuously increasing the yield level can be continued. If a further increase in potential sugar yield will be related to a further decline in cell wall compounds (marc content), problems will probably arise. The proportion of cell wall compounds will approach a natural limit, because a certain cell wall stability is needed to counterbalance the turgor pressure in the cell, but also to form a barrier to mechanical strain as well as pathogen attacks. As marc content and root yield are usually negatively correlated (Hoffmann et al., 2005), varieties with the highest yield will most likely not show best storability. Hence, for efficient sugar beet production in the future, a differentiation between genotypes with either highest yield potential or best storage properties will be necessary.

A further increase in yield potential will probably be based on rising root yield, as seen in the past (Märländer et al., 2003, 2017), while increasing sugar content is not very likely to contribute largely to higher sugar yields. The uptake of sucrose to parenchymal cells in the storage root results from the membrane transport of solutes. As it is inhibited by increasing cell turgor as determinant of sink strength because of the inhibition of the plasma membrane ATPase (Wyse et al., 1986), the increase of the sugar content is limited.

Greenhouse experiments have shown that sugar beet plants can obtain very high storage root yields with little leaf dry matter (Hoffmann, 2010b; Schnepel and Hoffmann, 2016a). As plants usually feature a higher leaf area index than required for assimilation, a reduction of the leaf dry matter after canopy closure might contribute to a further improvement of root yield. But, this shift in assimilate partitioning also requires an increased sink strength of the storage root.

Milford (1973) hypothesized that a possible way to increase sink strength would be a higher number and the complete development of all cambial rings in the storage root. However, sugar beet with an extended growing period (>300 days) neither formed a higher number of cambial rings nor became the outer cambial rings fully developed (Schnepel and Hoffmann, 2016a). Root yield constantly increased due to the development of the inner 5 to 6 rings as also found by Loel et al. (2014) in the comparison of old and new varieties. This is underlined by transcript analyses by Bellin et al. (2007), who reported a spatial gradient from the inner to the outer root zone in sugar beet. Cells in the outer cambial rings remained small and undeveloped, so that mature beets simultaneously contained transcripts typical of innermost sucrose-rich cells and of differentiating sucrose-poor cells in the outer parts. Hence, in contrast to former assumptions, the sink strength of the storage root of sugar beet seems not to be determined solely by cambial ring formation.

As there is currently no strategy available to increase sink strength in sugar beet, efforts should focus on exploiting the full potential of about 24 t ha^{-1} of sugar, which current varieties have already achieved in single field trials (IfZ, unpublished data). Due to the aforementioned limitations for yield formation, this cannot be realized in all environments, but there is still potential for further agronomic improvement. Laidig et al. (2014) demonstrated that in addition to the genetic improvement and in contrast to other crops, sugar beet shows a high increase in agronomic performance.

Nevertheless, future challenges will grow. Climate change will not only increase the risk of drought stress, but also the infestation pressure of pests and diseases (Juroszek and von Tiedemann, 2013; Kremer et al., 2016). Moreover, the availability of crop protection active ingredients is decreasing due to resistance development (Varrelmann and Märländer, 2017) and restrictive approval practices. In addition, prolonged processing campaigns will cause losses by both earlier harvest and longer storage periods (Kenter and Hoffmann, 2007). Hence, in the future breeding is expected to contribute more to successful sugar beet growing by improving pathogen resistance and storage properties of the beet than by increasing the yield potential itself.

AUTHOR CONTRIBUTIONS

CH and CK: conceptualized the paper; CH: wrote the manuscript; CK: edited and completed it.

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Identifying Quantitative Trait Loci (QTLs) and Developing Diagnostic Markers Linked to Orange Rust Resistance in Sugarcane (*Saccharum* spp.)

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Sugarcane (*Saccharum* spp.) is an important economic crop, contributing up to 80% of table sugar used in the world and has become a promising feedstock for biofuel production. Sugarcane production has been threatened by many diseases, and fungicide applications for disease control have been opted out for sustainable agriculture. Orange rust is one of the major diseases impacting sugarcane production worldwide. Identifying quantitative trait loci (QTLs) and developing diagnostic markers are valuable for breeding programs to expedite release of superior sugarcane cultivars for disease control. In this study, an F_1 segregating population derived from a cross between two hybrid sugarcane clones, CP95-1039 and CP88-1762, was evaluated for orange rust resistance in replicated trails. Three QTLs controlling orange rust resistance in sugarcane (qORR109, qORR4 and qORR102) were identified for the first time ever, which can explain 58, 12 and 8% of the phenotypic variation, separately. We also characterized 1,574 sugarcane putative resistance (*R*) genes. These sugarcane putative *R* genes and simple sequence repeats in the QTL intervals were further used to develop diagnostic markers for marker-assisted selection of orange rust resistance. A PCR-based *Resistance* gene-derived maker, G1 was developed, which showed significant association with orange rust resistance. The putative QTLs and marker developed in this study can be effectively utilized in sugarcane breeding programs to facilitate the selection process, thus contributing to the sustainable agriculture for orange rust disease control.

Keywords: orange rust disease, sugarcane, *Saccharum* spp., quantitative trait locus (QTL), marker-assisted selection (MAS)

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INTRODUCTION

Sugarcane (*Saccharum* spp.) is one of the most important economic crops, cultivated on ∼27.1 million hectares in over 100 countries with a worldwide harvest of 1.9 billion metric tons and a gross production value of $81.5 billion (FAO, 2013, 2014). As the most important sugar resource, sugarcane contributes up to 80% of sugar production in the world. Additionally, sugarcane accounts for ∼60% of global bio-ethanol production, with an energy output-to-input ratio five

times higher than that of maize (Waclawovsky et al., 2010; Dahlquist, 2013). Nevertheless, like many other crops, sugarcane production has been vulnerable by many diseases.

Orange rust, caused by *Puccinia kuehnii*, was first reported in Australia more than 100 years ago; however, this disease only began to draw attention due to its devastating epidemic in 2000 on a major Australian sugarcane cultivar Q124 (Braithwaite et al., 2009). Orange rust was first identified in the Western Hemisphere, specifically in Florida, United States, in 2007 (Comstock et al., 2008) and shortly after in Guatemala (Ovalle et al., 2008), Mexico, El Salvador, Panama (Flores et al., 2009), Costa Rica, Nicaragua (Chavarria et al., 2009), Brazil (Barbasso et al., 2010), Colombia (Cadavid et al., 2012), and recently Ecuador (Garcés et al., 2014). The disease immediately became an emerging threat to sugarcane production and breeding programs since its discovery (Comstock et al., 2008, 2010), which can cause up to 50% cane yield loss in its susceptible hosts (Magarey et al., 2011).

In the United States, Florida is the top sugarcane producer for sugar production with ∼28.0 million tons of cane and a gross value of $909.7 million annually (FAO, 2014). The majority of commercial cultivars grown in Florida and the parental germplasm used in the sugarcane breeding program are susceptible to the orange rust (Comstock et al., 2010), marking this disease a major concern in sugarcane industry. Application of fungicides has proven to increase sugar production from 7.9 to 26% on CP80-1743 in commercial fields (Comstock et al., 2010). However, fungicide treatments can lead to input cost increasing as well as potential environmental problems, which is against the principles advocated in sustainable agriculture (Lichtfouse et al., 2009). Therefore, developing and utilizing resistant cultivars is undoubtedly the favorable approach for sugarcane breeders and farmers for the disease control.

Screening for disease resistant sugarcane breeding materials can be performed by evaluating plant reactions both in the field and/or in greenhouse after rust spore inoculation artificially. The phenotype-based selection is direct and efficient when plants are inoculated with viable inoculum at the right growth stages in an environment favoring disease development. Unfortunately, selection based on phenotyping is time-consuming, labor-intensive, and environment-dependent due to requirement of large space, inoculation process, and possible escapes if favorable conditions or plant developmental stages are not achieved during inoculation. Marker-assisted selection (MAS) of resistant materials is a desirable alternative method, in which resistant individuals can be selected in the laboratory in less than 24 h by testing the presence of molecular markers linked to disease resistance. The MAS depends on reliable markers that are tightly linked to genes or genomic regions controlling disease resistance through quantitative trait loci (QTL) mapping and association study. Though sugarcane is a complex auto-polyploid species with large genome size, several disease resistance loci have been identified, such as *Bru*1 (Daugrois et al., 1996) and *Bru*2 (Raboin et al., 2006) for brown rust resistance, four DNA markers for pachymetra root rot and leaf scald resistance, five for Fiji leaf gall resistance, and 11 for smut resistance (Wei et al., 2006), a major QTL for yellow spot resistance (Aljanabi et al., 2007), a major quantitative trait allele for the *Sugarcane yellow leaf* resistance (Costet et al., 2012b) were identified in sugarcane. Once identified and validated, these molecular markers could be used in MAS to quickly identify and select desirable resistant resources or progeny, though no disease resistance gene has been cloned in sugarcane yet. For example, since the discovery of *Bru*1 and the identification of its co-segregating markers (R12H16 and 9O20-F4) (Costet et al., 2012a), the diagnostic markers have widely been used in sugarcane breeding programs. In Florida sugarcane breeding program, the markers for *Bru*1 are being utilized to evaluate brown rust resistance in sugarcane germplasm and hybrid clones (Glynn et al., 2013). It was estimated that 27% of the clones used for crossing (a total of 1027) contained *Bru*1, and the frequency of *Bru*1 in sugarcane clones increased from 15% (1975–1985) to 47% (2002–2012) after brown rust was introduced to Florida.

Although reliable molecular markers are of great value for controlling disease effectively in a way fitting the goals of sustainable agriculture, so far none has been reported to be linked to orange rust resistance in sugarcane. The objectives of this study were (1) to phenotypically evaluate the orange rust resistance reaction in a mapping population; (2) to map QTLs controlling orange rust resistance in sugarcane; and (3) to develop diagnostic markers for MAS of orange rust resistance in sugarcane. The putative QTLs and markers developed in this study can be effectively utilized in sugarcane breeding programs to expedite release of resistant cultivars, thus contributing to sustainable agriculture for orange rust disease control.

MATERIALS AND METHODS

Plant Materials

The sugarcane mapping population comprised of 173 F_1 progeny that were derived from a cross between sugarcane clones CP95-1039 and CP88-1762, which were developed by the United States Department of Agriculture (USDA), Agricultural Research Service (ARS) Sugarcane Field Station at Canal Point, FL, United States. Individuals of the progeny were clonally propagated for leaf sampling and phenotype evaluation. The whole population was genotyped using genotyping by sequencing (GBS), and sequence variations were called according to Yang et al. (2017a). The GBS libraries and sequencing were performed at the Institute of Genomic Diversity, Cornell University following the optimized protocol (Elshire et al., 2011) with a rare cutting restriction enzyme *Pst*I (CTGCAG) for library construction and a 96-plex (95 DNA samples and one negative control) for sequencing on the Illumina HiSeq 2000 platform (Yang et al., 2017b). The cleaned reads were aligned to sorghum genome v3.0 (Paterson et al., 2009) for calling single nucleotide polymorphisms (SNPs) using seven different SNP callers described by Yang et al. (2017b). Only the single dose markers were used for genetic map construction using Joinmap 4.0 (Van Ooijen, 2006). Two high density genetic maps were constructed with one for CP95-1039 including 2,453 markers and a total length of 4224.4 cM and the other for CP88-1762 including 2,154 markers and a total length of 4373.2 cM (Yang et al., 2017b).

For validating the diagnostic marker developed in this study, a diversity panel with 165 sugarcane clones derived from multiple crosses in multiple years (Supplementary Table S1) was used.

Evaluation of Orange Rust Resistance

The whole F_1 mapping population along with the two parental clones, CP95-1039 and CP88-1762, were used in orange rust resistance evaluation. CP95-1039 is resistant to orange rust, whereas CP88-1762 is susceptible to the disease (Supplementary Figure S1). Rust resistance evaluation of this population was conducted using artificial inoculation methods under both field and greenhouse conditions.

The inoculum preparation and inoculation were performed following the protocol described by Sood et al. (2009). Briefly, sugarcane orange rust urediniospores were collected by vacuuming the abaxial side of symptomatic leaves since *P. kuehnii* is biotrophic and difficult to culture in medium. A large number of leaves diagnosed with orange rust disease symptoms from multiple highly susceptible cultivars were used to collect enough inoculum for this experiment. Freshly collected urediniospores were used as inoculum or stored at −70°C. Rust spores were suspended in sterile distilled water containing 0.1% (V/V) Tween-20 and 0.002% 1-nonanol with an adjusted concentration of 10^4 urediniospores/ml.

For the field experiment, seedlings from the mapping population grown in the greenhouse were transplanted in the field on August 2010 for population establishment at the USDA-ARS Sugarcane Field Station, Canal Point, FL, United States. The planting canes were trimmed above the soil in February 2011 for first ratoon regrowth. The inoculum was applied to three stalks of each genotype when the sugarcane seedlings were 2 months old after transplanting in October 2010, and when the regrown ratoon plants were 2 and 3 months old after cutting in April 2011 and May 2011, respectively. To infect plants, one-third of the tips of the uppermost leaves were cut off as a marker, and a 0.5-ml aliquot of the spore suspension was deposited inside the leaf whorl of the marked stalks. Three weeks post-inoculation, any rust signs or symptoms were recorded from the newly emerged leaf following a 0–4 scale (Sood et al., 2009). A score of 0 indicated that the plants were asymptomatic. A score of 1 corresponded to the presence of some chlorotic flecks without any pathogen spores on the leaves. A score of 2 indicated that leaves had some brown discoloration, but no pustules or sporulation. A score of 3 corresponded to the presence of ≤10 pustules and sporulation on the leaves. A score of 4 corresponded to >10 pustules with massive sporulation. The inoculation on plant cane was scored one time, while each inoculation on ratoons was scored by two researchers independently. Five observations were conducted after artificial inoculation in the field; one was on the plant cane and the other four were on ratoons.

For the greenhouse experiment, individuals of the mapping population were clonally propagated with four plants per sugarcane clone in 46 cm × 38 cm × 10 cm flats filled with Miracle-Gro® Potting Mix (Scotts Miracle-Gro® Company, Marysville, OH, United States). Scott's Osmocote Classic 14-14-14 fertilizer (Scotts-Sierra, Marysville, OH, United States) and daily irrigation were applied to maintain healthy plants. Each flat contained four genotypes, with four stalks for each genotype. The inoculum (prepared in the same way as described above) was applied to three healthy stalks (4 months old) with a complete leaf whorl developed. Three weeks after inoculation, any rust signs or symptoms were scored from the newly emerged leaf following the 0 (no disease present) to 4 (highly susceptible) scale (Sood et al., 2009). Artificial inoculation experiments were conducted under greenhouse conditions in March 2014, November 2014 and May 2015, respectively. The first greenhouse inoculation experiment was performed on plant cane, whereas the other two inoculation experiments were on ratoons. The inoculation and scoring of rust symptoms in the greenhouse were the same as described above. Therefore, three observations were conducted after artificial inoculation in the greenhouse.

Data Analysis and QTL Mapping

A pairwise Pearson correlation among scores of eight observations of orange rust resistance was calculated using the 'Hmisc' package in R3.0.2 (R Development Core Team, 2013; Harrell, 2018). Analysis of variance was performed using the 'nlme' package in R (Pinheiro et al., 2009). Broad sense heritability (H^2) was estimated using the formula below:

$$H^2 = \frac{\delta_g^2}{\delta_g^2 + \delta_\varepsilon^2}$$

where δ_g^2 and $\delta_\varepsilon^2$are the genetic variance of progeny lines, and the error variance, respectively.

The eight observations of orange rust resistance were manually checked for 'escape' inoculation (progeny with a score of two or more after inoculation were considered as 'susceptible,' and then only observations with scores higher than two were used to calculate average scores), and average scores were used for QTL analysis. A pseudo-testcross strategy was employed for QTL analysis using WinQTLCart 2.5 (Wang et al., 2012). Composite interval mapping (CIM) was performed using forward and backward stepwise regressions to select markers as cofactors with 10 cM window size and 1 cM walking speed in WinQTLCart 2.5 (Wang et al., 2012). The QTL nomenclature was used according to McCouch (2008).

Identification of Sugarcane Putative *R* Genes

Five sugarcane transcript sequence databases (**Table 1**), including a total of two million sugarcane transcripts, were used to identify sugarcane transcripts having homologous genes in the sorghum genome. Redundant transcripts were removed by using cdhit/4.6 (sequence identity threshold 0.95) to reduce subsequently computational and manual efforts (Li and Godzik, 2006; Fu et al., 2012). The non-redundant sugarcane transcripts and their corresponding sorghum orthologs (Paterson et al., 2009) were identified and verified by using reciprocal BLASTN. The sugarcane transcripts with corresponding sorghum orthologs were used for subsequent analysis.

Sugarcane putative *R* genes were identified by two complementary methods. First, BLASTN of sugarcane transcripts with corresponding sorghum orthologs was performed against

TABLE 1 | Sugarcane transcript database sources used for identification of sugarcane putative resistance genes.

	Number of sequences	Sum of nucleotides	Average length
NCBI UniGene[1]	220,997	136,000,000	615
SOGI UniGene[2]	490,240	361,700,000	738
In-house RNA-Seq dataset 1	102,944	69,590,000	676
In-house RNA-Seq dataset 2	1,080,060	441,200,000	408
In-house RNA-Seq dataset 3	101,080	73,280,000	725
Total	1,995,321	1,081,770,000	542
Unique transcripts	700,194	345,243,774	493

[1] *ftp://ftp.ncbi.nlm.nih.gov/repository/UniGene/Saccharum_officinarum/.*
[2] *ftp://occams.dfci.harvard.edu/pub/bio/tgi/data/Saccharum_officinarum/.*

known plant *R* genes, which included 112 reference resistance genes with experimental support from the Plant Resistance Genes database (PRGdb)[1] (Sanseverino et al., 2013). Second, InterProScan 5.0 was used to characterize sugarcane nucleotide-binding site (NBS)–leucine-rich repeat (LRR) genes from the sugarcane transcripts with corresponding sorghum orthologs following the procedures described previously (Jones et al., 2014; Yang and Wang, 2016). All bioinformatics analyses were performed locally at the University of Florida High Performance Computing Center using command lines.

Development of PCR-Based Markers for Orange Rust Resistance Selection

Sugarcane simple sequence repeats (SSRs) sequences were collected from published literatures and aligned to sorghum genome 3.0 (Liu et al., unpublished data). Primers for putative *R* genes were designed using Primer3.[2] Primers across introns based on the sorghum gene models were selected. Only primers uniquely aligned to the sorghum genome and aligned to sugarcane transcripts with no more than two mismatches were selected for synthesis (Invitrogen, Carlsbad, CA, United States). All the SSRs and gene markers were first screened using two parental clones, CP95-1039 (resistant to orange rust) and CP88-1762 (susceptible to orange rust). A touchdown PCR program was used as described by Li et al. (2011) for amplification. In brief, the reaction mixture was incubated at 95°C for 5 min, then five cycles of 60 s of denaturing at 96°C, 5 min of annealing at 68°C with a decrease of 2°C in each subsequent cycle, and 1 min of extension at 72°C; For another five cycles, the annealing temperature started at 58°C for 2 min with a decrease of 2°C for each subsequent cycle; PCR continued through an additional 25 cycles of 60 s at 96°C, 1 min at 50°C, and 1 min at 72°C with a final extension at 72°C for 5 min. PCR products were run on a 1% agarose gel in a horizontal electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA, United States). Polymorphic markers were used to genotype the mapping population of 173 F_1 progeny derived from the cross between CP95-1039 and CP88-1762. Candidate diagnostic markers (PCR-based) were further genotyped in a diversity panel with 165 sugarcane clones derived from multiple crosses in multiple years (Supplementary Table S1). These 165 sugarcane clones were also screened phenotypically for orange rust resistance in the field following the protocol described above in the evaluation of orange rust resistance section. The Student *t*-tests of each marker were conducted for mean orange rust scores by using functions in Excel 2010 (Microsoft Corp., Redmond, WA, United States).

Based on artificial inoculation data, progeny with scores of 0, 1, and 2, that were not sporulating, were categorized as phenotypic 'resistant,' and progeny with scores of 3 and 4, that were sporulating, were categorized as phenotypic 'susceptible.' The 173 progeny were considered as 'resistant' or 'susceptible' based on sporulation for marker evaluation. Based on the closest flanking SNP markers to the significant QTLs, each individual in the whole population was characterized as genotypic 'resistant' or genotypic 'susceptible.' Selection accuracy was defined as the percentage of individuals which were both phenotypic and genotypic resistant among the total number of genotypic resistant individuals. Selection efficiency was defined as the percentage of individuals which were both phenotypic and genotypic resistant among the total number of phenotypic resistant individuals in the mapping population. Selection accuracy and selection efficiency were calculated following formula:

Selection accuracy = (the number of both phenotypic and genotypic resistant individuals/the number of genotypic resistant individuals) * 100.

Selection efficiency = (the number of both phenotypic and genotypic resistant individuals/the number of phenotypic resistant individuals in the mapping population) * 100.

RESULTS

Phenotypic Data Analysis

Orange rust resistance reactions of the mapping population showed a nearly normal distribution (**Figure 1**), supporting orange rust resistance segregating in this bi-parental mapping population is controlled by multiple genes/alleles. Orange rust resistance reactions of artificial inoculations were highly correlated ($P < 0.0001$) with an average coefficient of 0.64 (**Table 2**). Heritability of orange rust resistance was 0.67, indicating that the disease resistance in the mapping population was largely controlled by genetic factors. In this study, the mean scores of the eight rust resistance observations were used for subsequent QTL analysis, which can reduce discrepancy caused by experimental errors in single artificial inoculations.

QTL Analysis

High density genetic maps of both parental clones (Yang et al., 2017b) were used for detecting QTLs employing WinQTLCart 2.5 (Wang et al., 2012). Three QTLs controlling orange rust resistance in sugarcane, qORR109, qORR4 and qORR102, were detected in the mapping population (**Figure 2** and **Table 3**). Of the three QTLs, one was located on linkage group (LG) 109 of the resistant parent CP95-1039 map, explaining 58% of the phenotypic variance with a logarithm of odds (LOD) of 19.3. The

[1] http://prgdb.org
[2] http://biotools.umassmed.edu/bioapps/primer3_www.cgi

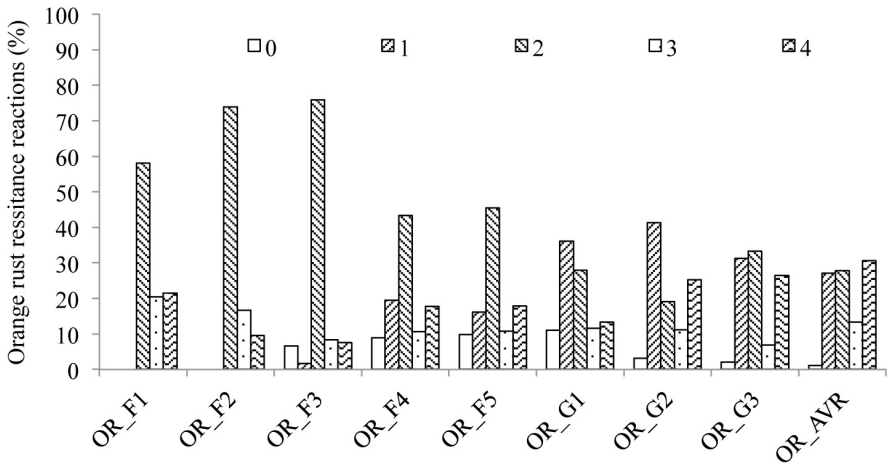

FIGURE 1 | Distribution of orange rust (OR) resistance reactions in the mapping population across multiple trials observations. F, Field; G, Greenhouse; AVR, average.

TABLE 2 | Pearson's correlation coefficients among rust resistance reactions on the F_1 population derived from the cross CP95-1039 × CP88-1762.

	OR_F2	OR_F3	OR_F4	OR_F5	OR_G1	OR_G2	OR_G3	OR_mean
OR_F1	0.74***	0.66***	0.71***	0.69***	0.69***	0.47***	0.61***	0.76***
OR_F2		0.77***	0.79***	0.77***	0.60***	0.47***	0.56***	0.77***
OR_F3			0.70***	0.68***	0.57***	0.37***	0.47***	0.62***
OR_F4				0.93***	0.65***	0.33**	0.50***	0.79***
OR_F5					0.65***	0.33**	0.47***	0.78***
OR_G1						0.60***	0.65***	0.74***
OR_G2							0.55***	0.73***
OR_G3								0.75***

***P < 0.001; ***P < 0.0001; F, Field; G, Greenhouse.*

other two QTLs were located on LG 4 and LG 102 of the CP88-1762 map, explaining 12 and 8% of the phenotypic variance with a LOD of 5.4 and 3.4, respectively. Of the three QTLs, heterozygous genotypes of qORR109 and qORR102 positively contributed to orange rust resistance, whereas that of QTL qORR4 showed a negative contribution (**Table 3**). The three nearest markers, 2SNP350, 5SNPUN3354 and 3SNP3092, were 2 cM, 0.2 cM and 2.2 cM away from their corresponding QTL peak, qORR109, qORR4 and qORR102, respectively.

To further study the genetic effect of the three QTLs, the phenotypic variation was dissected for each QTL and their combination (**Table 4**). Progeny containing the three susceptible QTL intervals based on the nearest marker alleles (Homozygote for QTL qORR109 and qORR102, and heterozygote for QTL qORR4) had a mean disease resistance score of 3.62, which served as the baseline for calculating orange rust disease reduction in the mapping population. With a single QTL, the disease severity can be reduced by 37.8 and 46.8% for QTLs qORR4 and qORR102, respectively. Since only one progeny existed in this category of single QTL qORR109 with heterozygote, the calculation of its contribution to rust resistance was ignored. With a combination of two QTLs, the disease severity could be reduced by 45.9, 34.3, and 19.6%, separately (**Table 4**). The combination of the three positive QTLs (Heterozygote for QTL qORR109 and qORR102, and homozygote for QTL qORR4) could reduce the disease severity by 56.4%.

Sugarcane SSRs and Putative *R* Genes

Out of 6,149 available SSR primers recruited from a literature search, a total of 1,095 sugarcane SSR primer pairs were uniquely aligned to the sorghum reference genome (Liu et al., unpublished data). Of all the mapped SSRs, 96 were found in the vicinity of three orange rust resistance QTL intervals and were close to the nearest markers of the QTLs according to the sorghum genome (Supplementary Table S2).

A custom-made sugarcane transcript database was formed including 700,194 sequences after removing redundant sequences. In total, 24,665 sugarcane transcripts were identified with orthologs in the sorghum genome. To identify sugarcane putative resistance genes, only the transcripts with orthologs in sorghum were utilized for subsequent analysis for a collinear comparison and application of the QTL intervals based on the sorghum genome. Out of the 24,665 sugarcane transcripts, 1,574 were characterized as putative *R* genes (Supplementary Table S3). Transcript sequences, corresponding sorghum orthologs, and reference *R* genes for sugarcane putative *R* genes were deposited in Supplementary Table S3.

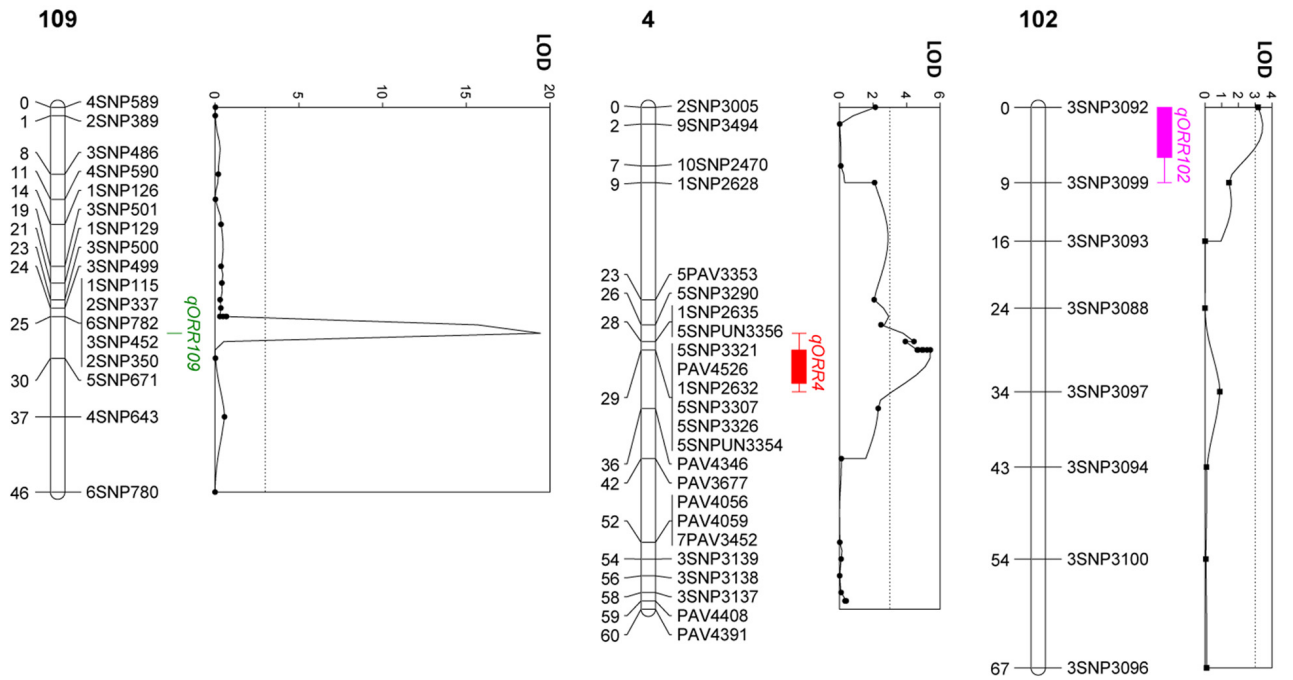

FIGURE 2 | Logarithm of odds (LOD) score plots of the quantitative trait loci (QTLs) for sugarcane orange rust resistance in the regions harboring the three identified QTL clusters.

TABLE 3 | Quantitative trait loci (QTL) associated with orange rust resistance of the F_1 population derived from the cross CP95-1039 × CP88-1762.

Trait	QTL	LGs[a]	Position (cM)	LOD	Additive effect	PVE (%)[b]	Marker[c]	Distance (cM)[d]
Orange rust	qORR109	P1LG109	27.3	19.3	−2.07	58	2SNP350	2
Orange rust	qORR4	P2LG4	29.5	5.4	0.9	12	5SNPUN3354	0.2
Orange rust	qORR102	P2LG102	2.2	3.4	−0.67	8	3SNP3092	2.2

[a]LG, Linkage group. [b]PVE, Phenotypic variance explained. [c]Nearest marker from the QTL peak. [d]Distance of nearest marker from the respective QTL peak.

TABLE 4 | Phenotypic effects of quantitative trait loci (QTL) allele combinations on orange rust resistance in the F_1 population from the cross CP95-1039 × CP88-1762.

qORR109,qORR4,qORR102[a]	Marker genotype[b]	No of progeny	Disease resistance (se)[c]	Reduction in disease severity (%)[d]
−,+,−	Ho.,He.,Ho.	12	3.62 ± 0.19	NA
+,+,−	He.,He.,Ho.	1	3.75	NA
−,−,−	Ho.,Ho.,Ho.	12	2.25 ± 0.29	37.8
−,+,+	Ho., He.,He.	29	2.91 ± 0.20	19.6
+,−,−	He.,Ho.,Ho.	49	1.96 ± 0.15	45.9
+,+,+	He.,He.,He.	12	2.38 ± 0.35	34.3
−,−,+	Ho.,Ho.,He.	19	1.93 ± 0.23	46.7
+,−,+	He.,Ho.,He.	18	1.58 ± 0.13	56.4

[a]Symbols "+"and "−"represent presence and absence of corresponding QTL, respectively. [b]Genotypes of the nearest marker for corresponding qORR109, qORR4, and qORR102. He., Heterozygous genotype. Ho., Homozygous genotype. [c]Se, standard error. [d]Reduction in disease severity compared with the mean severity of 12 progeny carrying three susceptible alleles.

Developing Diagnostic Markers for MAS of Orange Rust Resistance in Sugarcane

Sugarcane SSRs in vicinity of the three orange rust resistance QTL intervals were used for diagnostic marker development. Of the 96 SSRs, only two showed robust polymorphisms between the two parental clones, CP95-1039 and CP88-1762. In addition, 61 gene-derived primer pairs crossing introns of 18 sugarcane putative *R* gene sequences were designed (Supplementary Table S2). Of the

TABLE 5 | Probability (*P*) value PCR-based markers on orange rust resistance.

	QTLs (% Exp.)	*P*-value for *t*-test	
		CP95-1039 × CP88-1762	Diversity CP clones
G1	qORR109 (58)	<0.001	0.044
M16	qORR4 (12)	0.011	0.092

61 pairs of *R* gene-derived primers, three showed polymorphisms between the two parental clones. The five polymorphic markers (two SSRs and three gene-derived) were first genotyped in the F_1 mapping population. Interestingly, two gene-derived markers, G1 ($P < 0.001$) and M16 ($P < 0.05$), were significantly associated with orange rust resistance based on single marker analysis (**Table 5** and Supplementary Figure S2). The candidate diagnostic markers were further validated by genotyping 165 diverse sugarcane clones (Supplementary Table S1). The result further confirmed that G1 maker was associated with orange rust resistance ($P < 0.05$), while M16 was weakly associated. Based on the F_1 mapping population, selection accuracy of G1 marker for orange rust resistance was 65.8% and the selection efficiency was 85.6%.

DISCUSSION

Previously restricted to Asia, and the Pacific regions, sugarcane orange rust has also been detected in multiple countries in the western hemisphere (Comstock et al., 2008; Ovalle et al., 2008; Chavarria et al., 2009; Flores et al., 2009; Barbasso et al., 2010; Cadavid et al., 2012; Garcés et al., 2014). The outbreak of this disease could result in devastating economic loss, with an estimation of Aus$150–210 million in 2000 in Australia (Braithwaite et al., 2009). In Florida, orange rust has gained full attention due to its sudden emergence and negative impact on sugarcane production. In one crop season, economic losses associated with orange rust were estimated at $40 million (Dixon et al., 2010). It continues to be a serious disease for growers for several reasons. From a chemical perspective, optimal fungicide selection, and the rate, timing, and frequency of applications to prevent or control this newly emerged disease are unknown. Though several fungicides (e.g., strobilurin class fungicides and triazole class fungicides) have recently been registered and used to manage orange rust, more research is needed to fine-tune control recommendations that are economically reasonable (Rott et al., 2014). Biologically, orange rust pathogen releases a high density of spores into the air, and tolerates high temperatures, which allows the disease to develop throughout most of the sugarcane growing season and production areas, assuming infection conditions are suitable (Rott et al., 2014). Finally, the use of resistant host plants is currently limited because 77.5% of commercial cultivars grown in Florida and 41% of parental germplasm are susceptible to orange rust (Comstock et al., 2010). Unfortunately, it takes 12–14 years to release a new sugarcane cultivar with disease resistance, and even longer to propagate the cultivar for commercial production.

Compared to fungicide treatment, developing and using resistant cultivars is undoubtedly the thumbs-up approach for disease control because of a low input cost and healthy environment for sustainable sugarcane production. With reliable diagnostic markers developed, MAS would make this approach achievable for sugarcane breeding programs by quickly identifying resistant germplasm for crossing and selecting resistance progeny. In this study, we evaluated an F_1 segregating population following a pseudo-testcross strategy for orange rust resistance QTL identification. Since the sugarcane is an outcrossing species and the parental clones for the F_1 population were heterozygous, the F_1 population was segregating genetically in the way similar as the backcross population of self-pollinating species if only the loci with homozygous genotype in one parent and heterozygous genotype in the other parent were considered. With multiple evaluation of the phenotype and high stringent genotypes of this segregating F_1 population, we identified three QTLs controlling orange rust resistance for the first time, which can explain 58, 12, and 8% of the phenotypic variation, separately. Moreover, a PCR-based *R* gene-derived maker, G1 was developed, which showed significant association with orange rust resistance. The results of this research would be valuable for expediting release of resistant cultivars, thus contributing to the sustainable agriculture for orange rust disease control.

QTL Analysis for Orange Rust Resistance

By performing QTL analysis using high density genetic maps and the mean scores of the eight rust resistance evaluations, for the first time we identified three QTLs controlling orange rust resistance. It's noteworthy that GBS empowered QTL identification in sugarcane in this study. We used multiple SNP callers to maximize the detection of single dose markers, applied a robust filtering process on raw SNPs for each individual sample to improve SNP calling, and exploited a high LOD ($\geq$10) in genetic map construction to avoid confusing linkage from different homoeologs (Yang et al., 2017b), which provide high density and reliable genetic maps for QTL analysis in this study. The three QTLs combined together could reduce 56.4% of disease severity compared with progeny containing the three susceptible QTL alleles (**Table 3**), indicating pyramiding these positive QTL alleles into sugarcane cultivars through MAS could effectively reduce the impact of the disease. So far, we have no evidence on whether these QTLs were linked to other disease resistance or agronomically important traits yet. The strategy for pyramiding multiple genes to improve disease resistance has been discussed in bean (Kelly et al., 1995), wheat (Liu et al., 2000), and rice (Singh et al., 2001; Jiang et al., 2004). Interestingly, two QTLs, qORR4 and qORR102, were identified from the same susceptible parental clone, CP88-1762, suggesting breeding materials that have robust agronomic traits but are susceptible to the disease should not directly be eliminated from sugarcane breeding programs as they may contain valuable minor resistance QTL alleles.

Although the molecular mechanisms of pathogen recognition and resistance to orange rust disease remain unclear, the resistance characterized in the current mapping population is

less likely to be a single pathogen-race specific resistance since a mixture of orange rust spores collected from multiple highly susceptible cultivars from multiple fields were used to screen for resistance. Therefore, most likely a broad-based and durable resistance was identified within the segregating population. Although no genetic diversity has been reported in *P. kuehnii* so far, however, a change of orange rust pathogenicity has most likely been occurred in Florida. Earlier, major sugarcane cultivars CP88-1762 and CP89-2143 had been symptomless or resistant before 2010–2011, however, recently exhibited severe disease symptoms, suggesting a change in *P. kuehnii* pathogenicity (Comstock et al., 2010; Philippe Rott, Personal Communication). The resistance genes identified in the mapping population of this study may recognize multiple elicitors from orange rust pathogens, including pathogen-associated molecular patterns and/or weak effectors. Subsequent defense responses in sugarcane can be triggered to reduce the damage with contributing effects from each resistance gene. Since spores were artificially injected in the leaf, so lesions were also observed on leaves in the resistant type, but no sporulation was observed even after 4 weeks of infection (Supplementary Figure S1). The resistance in this mapping population may belong to a general non-specific resistance, in which sporulation of the fungus is inhibited to a low level to reduce the disease spread and severity (Kiraly et al., 2007). Therefore, this resistance should be fully exploited for breeding resistant sugarcane cultivars.

Development of Diagnostic Markers for MAS of Orange Rust Resistance in Sugarcane

Release of orange rust resistant cultivars could be expedited by quickly identifying parental resistant germplasm and selecting resistant progeny through MAS using reliable molecular markers, especially PCR-based markers, which are easy to run for breeders without relying on fancy equipment and complicated procedure. In this study, we constructed a sugarcane putative *R* gene database (Supplementary Table S3) by bioinformatics analysis of several transcript databases. A total of 1,574 sugarcane putative *R* genes were annotated as NBS-LRR genes. Information on these putative *R* genes, with corresponding sorghum gene models, corresponding reference *R* genes, annotation, sugarcane transcript sequences (Supplementary Table S3) will be an important source to sugarcane research community for sugarcane disease resistance genes mining, genetic map construction, and PCR-based marker development.

In this study, we designed 61 *R* gene-derived primers according to the orange rust QTL intervals and the sugarcane putative *R* database. Out of the 61 primers, three (4.9%) showed polymorphisms with different fragment sizes between the two parental clones, indicating this strategy of crossing introns could functionally generate markers for subsequent analysis. Even more interestingly, after single marker analysis in the bi-parental mapping population and the diversity sugarcane clones, G1 marker was significantly linked to orange rust resistance, while M16 showed weak association (**Table 5**). We also screened 96 SSR markers; however, none of them was linked to orange rust resistance. Lack of linkage between the two polymorphic SSRs to orange rust resistance could be due to that the marker were far away from the QTL; or most likely that the marker linked with QTLs in a repulsion phase. The sugarcane genome is complex, with up to 12 sets of homo(eo)logs. A single locus may have multiple alleles and/or alleles with different dosages. Theoretically, only markers in coupling linkage with target QTLs contributing alleles can be used for MAS. Therefore, a successful rate of identifying PCR-based markers in the expected linkage is relatively low using this strategy.

Furthermore, we found that the target gene of G1 marker, *Sobic.002G166150*, is homologous to *RFO1*, a dominant *Arabidopsis* disease resistance gene, which encoded a wall-associated receptor-like kinase and conferred resistance to a broad spectrum of *Fusarium* races (Diener and Ausubel, 2005), further indicating that the resistance QTLs identified in this study carried durable instead of race-specific resistant genes. In this study, we used a mixture of orange rust spores collected from infected leaf of multiple sugarcane cultivars as inoculum (hard to culture this pathogen in medium). Thus the inoculum was most likely a mixture of multiple races, though the *Puccinia* race variation was not reported in North America yet. If the inoculum contained multiple races (we speculated that multiple races of *Puccinia* existed due to observation of the changing of host resistance over years; Comstock et al., 2010; Philippe Rott, Personal Communication), then resistance to the most popular orange rust pathogen races would be detected in our experiments. In another word, the QTLs we identified should harbor durable orange rust resistance gene(s) instead of a single race-specific resistance gene. The resistance provided by *RFO1* is quantitative and stronger resistance achieved if combined with other loci (additive effect). We also observed the same patterns in our results (**Table 4**). However, further experiments are needed to confirm whether sugarcane ortholog of *Sobic.002G166150* is the gene contributing to the orange rust resistance in sugarcane, and to elucidate the interactions among the three QTLs in sugarcane.

CONCLUSION

QTL analysis revealed three QTLs controlling orange rust resistance, which can explain 58, 12, and 8% of the phenotypic variation, respectively. The three QTLs together can reduce 56.4% of disease severity compared with progeny containing the three susceptible QTL alleles. This is the first time that QTLs controlling orange rust resistance in sugarcane have been reported. To develop diagnostic markers for orange rust resistance, we identified 1,574 sugarcane putative *R* genes and aligned publicly available sugarcane SSRs to the sorghum genome. Out of 61 gene-derived markers and 96 SSRs screened, five were polymorphic with different fragment sizes between the two parental clones. The G1 marker was significantly linked to orange rust resistance based on single marker analysis. The putative QTLs and marker developed in this study can be utilized

in sugarcane breeding programs to utilize MAS of orange rust resistance in sugarcane.

AUTHOR CONTRIBUTIONS

JW conceived and designed the experiments. XY, SS, MI, and SM performed the experiments. XY performed the data analysis. XY and JW drafted the manuscript. XY, JW, SS, SM, EH, MI, and JC revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00350/full#supplementary-material

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Long Term Management of Rhizomania Disease—Insight Into the Changes of the *Beet necrotic yellow vein virus* RNA-3 Observed Under Resistant and Non-resistant Sugar Beet Fields

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Rhizomania disease, caused by the *Beet necrotic yellow vein virus* (BNYVV), is considered as one of the major constraints for sugar beet production, worldwide. As a result of the introgression of major resistance genes (*Holly*, *Rz2*) in commercially available sugar beet varieties, the virus has endured strong selection pressure since the 90s'. Understanding the virus response and diversity to sugar beet resistance is a key factor for a sustainable management of only few resistance genes. Here we report rhizomania surveys conducted in a rhizomania hot spot, the Pithiviers area (France) during a 4-year period and complementary to the study of Schirmer et al. (2005). The study aimed at evaluating the intra- and inter-field BNYVV diversity in response to different sources of resistance and over the growing season. To follow rhizomania development over the sugar beet growing season, extensive field samplings combined with field assays were performed in this study. The evolution of the BNYVV diversity was assessed at intra- and inter-field levels, with sugar beet cultivars containing different resistance genes (*Rz1*, *Rz1* + *Heterodera schachtii* resistance and *Rz1Rz2*). Intra-field diversity was analyzed at the beginning and the end of the growing season of each field. From more than one thousand field samples, the simultaneous presence of the different A, B and P types of BNYVV was confirmed, with 21 variants identified at positions 67–70 of the p25 tetrad. The first variant, AYHR, was found most commonly followed by SYHG. Numerous mixed infections (9.93% of the samples), mostly of B-type with P-type, have also been evidenced. Different tetrads associated with the A- or B-type were also found with a fifth RNA-genome component known to allow more aggressiveness to BNYVV on sugar beet roots. Cultivars with *Rz1+Rz2* resistant genes showed few root symptoms even if the BNYVV titre was quite high according to the BNYVV type present. The virus infectious potential in the soil at the end of the growing season with such cultivars was also lower despite a wider diversity at the BNYVV RNA3 sequence level. *Rz1+Rz2* cultivars also exhibited a lower presence of *Beet soil-borne virus* (BSBV), a *P. betae*-transmitted *Pomovirus*. Cultivars with *Rz1* and nematode (*N*) resistance genes cultivated

in field infected with nematodes showed lower BNYVV titre than those with *Rz1* or *Rz1+Rz2* cultivars. Overall, the population structure of BNYVV in France is shown to be different from that previously evidenced in different world areas. Implications for long-term management of the resistance to rhizomania is discussed.

Keywords: BNYVV, soil-borne virus, sugar beet, *Rz1* (*Holly*), *Rz2*, rhizomania, *Polymyxa betae*, nematode

INTRODUCTION

Rhizomania is one of the most challenging diseases of the sugar beet plant because of the difficulty in maintaining sustainable plant resistance sources against the causal virus, the *Beet necrotic yellow vein virus* (BNYVV), a soil-borne virus transmitted by the plasmodiophorid *Polymyxa betae* (Biancardi and Tamada, 2016). Since its discovery in (1952), the disease characterized by a constriction of the taproot with a proliferation of lateral rootlets was named accordingly "root madness" or rhizomania (rizomania) (Biancardi et al., 2002). Rhizomania is now widespread in most sugar beet-growing countries (Koenig et al., 2008; Chiba et al., 2011). Other soil-borne viruses also share the same vector and are frequently found in association with rhizomania: *Beet soil-borne virus* (BSBV) (Meunier et al., 2003), *Beet virus Q* (BVQ) (Crutzen et al., 2009), two viruses belonging to the genus *Pomovirus*, and *Beet soil-borne mosaic virus* (BSBMV) (Mahmood and Rush, 1999; Ratti et al., 2009), another *Benyvirus*. *Beet black scorch virus* (BBSV) (Mehrvar and Bragard, 2009) was also found associated with sugar beet exhibiting rhizomania symptoms in the USA, Iran and Inner Mongolia of China, but is considered to be transmitted in the soil to host roots by the Chytrid vector *Olpidium brassicae* (Weiland et al., 2007).

The BNYVV has a multipartite genome comprising either four or five positive sense single stranded RNAs (Tamada, 1999). The virus is able to function with the RNA1 and RNA2 molecules only for virus infection. Indeed, only these two RNAs are required for virus propagation in leaves of *Chenopodium quinoa* (Quillet et al., 1989), encoding proteins involved in viral replication, encapsidation, transmission by *P. betae* and cell-to-cell movement (Richards and Tamada, 1992; Peltier et al., 2008). The RNA3 is needed for systemic movement of the BNYVV in *Beta* species (Flobinus et al., 2016). The *p25* gene located on RNA3 was reported to enhance pathogenicity as well as general fitness and acts as an avirulence gene (*avr* gene) in resistant genotypes (Pferdmenges, 2007; Chiba et al., 2011). The *p31* gene on RNA4 is involved in transmission by the vector *P. betae*, pathogenicity and the suppression of post-trancriptional gene silencing (Guilley et al., 2009). Sometimes, the virus is also associated with an additional RNA5, which is known to enhance both symptom developments in sugar beets and aggressiveness of the virus (Tamada et al., 1996). This RNA codes the protein p26, which also contains a transcriptional activation domain (Covelli et al., 2009).

Although the variability of the BNYVV genome is considered limited in comparison with other plant viruses, a set of four consecutive amino acids, or "tetrad," has been linked to a strong positive selection pressure on the *p25* gene (Meunier et al., 2003; Schirmer et al., 2005). This tetrad was linked with resistance-breaking (RB) events, i.e. with the emergence of symptoms in cultivars considered tolerant or resistant (Acosta-Leal and Rush, 2007; Liu and Lewellen, 2007; Koenig et al., 2008, 2009a; Acosta-Leal et al., 2010).

Since 1994, based initially on RFLP studies, three types (named A, B and P) have been described within the BNYVV species and used to differentiate isolates (Kruse et al., 1994; Koenig et al., 1997). Later on, an additional J type was proposed for Asian strains with an additional RNA5 (Schirmer et al., 2005). In 2011, based on an extensive comparison of 73 isolates of worldwide origin, Chiba et al. (2011) and Biancardi and Tamada (2016) proposed phylogenetic evolutionary pathways for BNYVV populations, with up to eight different groups with different geographical distributions.

The presence of a fifth RNA molecule, associated with the viral genome, was reported in several places in Europe, such as northern France, the UK, and Germany. The P-type was found in Pithiviers in France (Koenig et al., 1997), Kazakhstan (Koenig and Lennefors, 2000), and the UK (Ward et al., 2007) while the J-type was recorded in China and Japan (Tamada et al., 1989; Kiguchi et al., 1996). An RNA5-containing BNYVV genotype closely resembling the Chinese isolate Har4 was identified in Germany (Koenig et al., 2008). In general, no RNA5 has been associated with the A-type or the B-type (Peltier et al., 2008). In Iran, the tetrad SYHG associated with the P-type has been found without a fifth RNA in fields with severe symptoms (Mehrvar et al., 2009).

Rhizomania is primarily managed through the use of the *Rz1* gene, which confers partial resistance (McGrann et al., 2009). The *Rz1* source of resistance was initially found from *Beta vulgaris* (Holly source in California), but was broadly introgressed into sugar beet varieties (Gidner et al., 2005; Stevanato et al., 2015). It is now commonly used throughout most sugar beet-producing areas worldwide. The partial resistance of sugar beet cultivars is linked to a restriction of virus multiplication and translocation in taproots rather than in rootlets (Scholten et al., 1994; Tamada et al., 1999; Chiba et al., 2008).

The Pithiviers area, south of Paris, France was the first place where BNYVV (P-type) with the RNA5 was found and linked with a higher aggressiveness (Pferdmenges, 2007). It was also an area of intense breeding efforts with *Rz1* cultivars. In intensively cropped areas, a resistance breakdown to rhizomania began in cultivars with either *Rz1* or *Rz1+Rz2* due to virus evolution. It is commonly thought that such a problem is due to the emergence of resistance-breaking (RB) viral strains

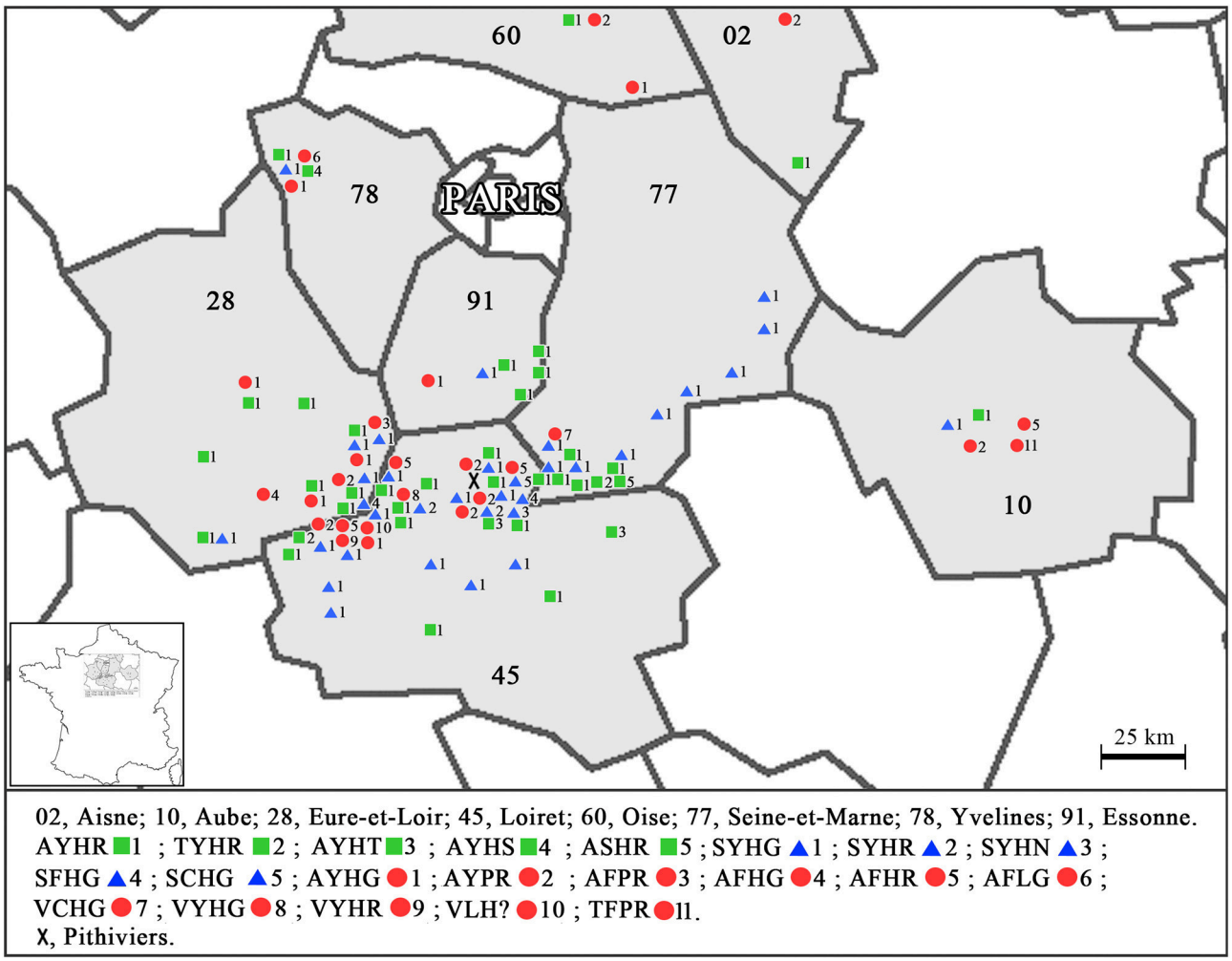

FIGURE 1 | Distribution of *Beet necrotic yellow vein virus* (BNYVV) and the associated tetrads in the Pithiviers region of France. The figure shows all the tetrad diversity of BNYVV and their dispersion around Pithiviers (X). The sequences represented by a square are associated with the B-type, those represented by a circle are associated with the A-type and those represented by a triangle are associated with the BNYVV P-type.

(Liu et al., 2005; Pferdmenges and Varrelmann, 2009). Moreover, the genetic erosion in the sugar beet due to the loss of minor genes may also play a role in the loss of resistance against the virus (Kingsnorth et al., 2002; Lennefors, 2006; Asher et al., 2009) or act synergistically to favor the emergence of such RB BNYVV isolates. The emergence of resistant-breaking isolates has now been reported in sugar beet-growing areas in Asia (Chiba et al., 2011), Europe (Bornemann and Varrelmann, 2013) and in most US production regions (Acosta-Leal and Rush, 2007), but only in Minnesota and California have these been documented to affect production at a field level.

Despite the relative diversity of BNYVV strains, mixed infections have only rarely been reported (Koenig et al., 2009a). Ratti et al. (2006) detected only a single A/B type infection out of 72 European samples. Evidence for reassortments have been reported in the UK (Ward et al., 2007), France (Koenig et al., 2009a), and localized areas in Asia (Li et al., 2008; Chiba et al., 2011).

Here we report rhizomania surveys conducted in the Pithiviers area (near Paris) during a 4-year period, complementary to the study of Schirmer et al. (2005), based on extensive field sampling and testing, combined with field assays set up to follow rhizomania development over the sugar beet growing season. The proposed study aims at evaluating the intra- and inter-field BNYVV diversity in response to different sources of resistance and over the growing season.

The diversity in 27 fields and with different sugar beet cultivars (three cultivars per field) was assessed. Different field situations around the Pithiviers area were studied at the beginning and at the end of the growing season. The different field situations are infected with nematode or not, infected with strong rhizomania and associated *Pomoviruses* or not, with high or low levels

TABLE 1 | Locations where field assays were conducted in France and plant materials.

Year	Region	Field code	Variety code	Variety	Resistance type	Seed company
FIELD ASSAYS						
Year1	Boynes 1	F1	V1	Ludwinia	*Rz1rz1* & *Rz2rz2*	KWS
			V3	Sophia	*Rz1rz1*	KWS
			V5	Python	*Rz1rz1*	SES Vanderhave
	Boynes 2	F2	V1	Ludwinia	*Rz1rz1* & *Rz2rz2*	KWS
			V5	Python	*Rz1rz1*	SES Vanderhave
			V3	Sophia	*Rz1rz1*	KWS
	Teillay-le-Gaudin 1	F3	V8	Bering	*Rz1rz1* & *Rnematode*	Strübe
			V9	Annouschka	*Rz1rz1* & *Rnematode*	KWS
			V4	Julietta	*Rz1rz1* & *Rnematode*	KWS
	Sougy 1	F4	V4	Julietta	*Rz1rz1* & *Rnematode*	KWS
			V9	Annouschka	*Rz1rz1* & *Rnematode*	KWS
			V8	Bering	*Rz1rz1* & *Rnematode*	Strübe
	Dambron 1	F5	V4	Julietta	*Rz1rz1* & *Rnematode*	KWS
			V8	Bering	*Rz1rz1* & *Rnematode*	Strübe
			V13	Fiorenza	*Rz1rz1* & *Rnematode*	KWS
	Poupry 1	F6	V9	Annouschka	*Rz1rz1* & *Rnematode*	KWS
			V4	Julietta	*Rz1rz1* & *Rnematode*	KWS
			V8	Bering	*Rz1rz1* & *Rnematode*	Strübe
	Mondreville 1	F7	V1	Ludwinia	*Rz1rz1* & *Rz2rz2*	KWS
			V3	Sophia	*Rz1rz1*	KWS
			V5	Python	*Rz1rz1*	SES Vanderhave
	Chenou 1	F8	V5	Python	*Rz1rz1*	SES Vanderhave
			V1	Ludwinia	*Rz1rz1* & *Rz2rz2*	KWS
			V3	Sophia	*Rz1rz1*	KWS
Year2	Boynes 3	F9	V1	Ludwinia	*Rz1rz1* & *Rz2rz2*	KWS
			V5	Python	*Rz1rz1*	SES Vanderhave
			V3	Sophia	*Rz1rz1*	KWS
	Yèvres-la-Ville 1	F10	V3	Sophia	*Rz1rz1*	KWS
			V1	Ludwinia	*Rz1rz1* & *Rz2rz2*	KWS
			V5	Python	*Rz1rz1*	SES Vanderhave
	Teillay-le-Gaudin 2	F11	V4	Julietta	*Rz1rz1* & *Rnematode*	KWS
			V8	Bering	*Rz1rz1* & *Rnematode*	Strübe
			V10	Bison	*Rz1rz1* & *Rnematode*	SES Vanderhave
	Sougy 2	F12	V10	Bison	*Rz1rz1* & *Rnematode*	SES Vanderhave
			V4	Julietta	*Rz1rz1* & *Rnematode*	KWS
			V8	Bering	*Rz1rz1* & *Rnematode*	Strübe
	Dambron 2	F13	V12	Adriana	*Rz1rz1* & *Rnematode*	KWS
			V4	Julietta	*Rz1rz1* & *Rnematode*	KWS
			V8	Bering	*Rz1rz1* & *Rnematode*	Strübe
	Poupry 2	F14	V4	Julietta	*Rz1rz1* & *Rnematode*	KWS
			V8	Bering	*Rz1rz1* & *Rnematode*	Strübe
			V10	Bison	*Rz1rz1* & *Rnematode*	SES Vanderhave
	Corbeilles-en-Gatinais	F15	V3	Sophia	*Rz1rz1*	KWS
			V5	Python	*Rz1rz1*	SES Vanderhave
			V1	Ludwinia	*Rz1rz1* & *Rz2rz2*	KWS
	Mondreville 2	F16	V1	Ludwinia	*Rz1rz1* & *Rz2rz2*	KWS
			V3	Sophia	*Rz1rz1*	KWS
			V5	Python	*Rz1rz1*	SES Vanderhave
	Chenou 2	F17	V3	Sophia	*Rz1rz1*	KWS
			V1	Ludwinia	*Rz1rz1* & *Rz2rz2*	KWS
			V5	Python	*Rz1rz1*	SES Vanderhave

(Continued)

TABLE 1 | Continued

Year	Region	Field code	Variety code	Variety	Resistance type	Seed company
FIELD ASSAYS						
	Gironville 1	F18	V5	Python	*Rz1rz1*	SES Vanderhave
			V3	Sophia	*Rz1rz1*	KWS
			V1	Ludwinia	*Rz1rz1* & *Rz2rz2*	KWS
Year3	Boynes 4	F19	V2	Britta	*Rz1rz1* & *Rz2rz2*	KWS
			V5	Python	*Rz1rz1*	SES Vanderhave
			V3	Sophia	*Rz1rz1*	KWS
	Yèvres-le-Chatel 1	F20	V7	Rosalinda	*Rz1rz1*	KWS
			V6	Magellan	*Rz1rz1*	SES Vanderhave
			V2	Britta	*Rz1rz1* & *Rz2rz2*	KWS
	Teillay-le-Gaudin 3	F21	V11	Baobab	*Rz1rz1* & *Rnematode*	SES Vanderhave
			V4	Julietta	*Rz1rz1* & *Rnematode*	KWS
			V10	Bison	*Rz1rz1* & *Rnematode*	SES Vanderhave
	Baigneaux	F22	V11	Baobab	*Rz1rz1* & *Rnematode*	SES Vanderhave
			V4	Julietta	*Rz1rz1* & *Rnematode*	KWS
			V10	Bison	*Rz1rz1* & *Rnematode*	SES Vanderhave
	Dambron 1	F5	V4	Julietta	*Rz1rz1* & *Rnematode*	KWS
			V11	Baobab	*Rz1rz1* & *Rnematode*	SES Vanderhave
			V10	Bison	*Rz1rz1* & *Rnematode*	SES Vanderhave
	Poupry 3	F23	V11	Baobab	*Rz1rz1* & *Rnematode*	SES Vanderhave
			V4	Julietta	*Rz1rz1* & *Rnematode*	KWS
			V10	Bison	*Rz1rz1* & *Rnematode*	SES Vanderhave
	Yèvres-le-Chatel 2	F24	V3	Sophia	*Rz1rz1*	KWS
			V5	Python	*Rz1rz1*	SES Vanderhave
			V2	Britta	*Rz1rz1* & *Rz2rz2*	KWS
	Mondreville 3	F25	V5	Python	*Rz1rz1*	SES Vanderhave
			V3	Sophia	*Rz1rz1*	KWS
			V2	Britta	*Rz1rz1* & *Rz2rz2*	KWS
	Moisancelle-en-Gatinais	F26	V5	Python	*Rz1rz1*	SES Vanderhave
			V3	Sophia	*Rz1rz1*	KWS
			V2	Britta	*Rz1rz1* & *Rz2rz2*	KWS
	Gironville 2	F27	V5	Python	*Rz1rz1*	SES Vanderhave
			V2	Britta	*Rz1rz1* & *Rz2rz2*	KWS
			V3	Sophia	*Rz1rz1*	KWS

of virus and vector infectious potential, with several types of soil, with different pH of soils, with different methods of irrigation, from sugar beet production basin or not, with different previous crop grown, with different numbers of years before last beet culture. Additional samples were analyzed and information about the cultivars and the resistance profile is given. Different indicators linked to the disease were used such as the infectious potential through the Most Probable Number (MPN) of the vector and BNYVV in the soil (not shown); the presence of BNYVV, BVQ, and BSBV detected by reverse transcriptase polymerase chain reaction (RT-PCR); the BNYVV p25 amino acid tetrad, and finally, the titre of the virus in each sample by enzyme-linked immunosorbent assay (ELISA). The impact of the disease has also been measured in the field by evaluating the root symptoms severity (RS) of sugar beet root symptoms.

MATERIALS AND METHODS

Field Assays and Plant Materials

Eight to ten field assays were conducted each year. Two French regions were studied in particular, "Loiret (with Pithiviers)" and "Seine-et-Marne," but other regions such as "Aisne," "Eure-et-Loire," "Aube," "Oise," "Yvelines," and "Essonne" were also investigated (**Figure 1**).

A total of 13 commercial cultivars (mixture of seed lots) with different resistance types were used in field assays. A frequent problem was the difficulty to get information from the breeder company regarding the homozygosity or heterozygosity of the major resistance genes in order to assess their impact on the BNYVV level or the presence of other associated viruses in plants. The cultivars were coded and are presented in **Table 1**. The field assays were also coded. Each field was different from the others

TABLE 2 | Plant material from farmer's fields.

Year	Region	Variety code	Variety	Resistance type	Seed company
FARMER'S FIELDS (PATCHES)					
Year0	Mérouville & Sougy & Ruan & Bricy & Baigneaux & Trinay & Bucy-le-Roi & Bondaroy	V4	Julietta	*Rz1rz1* & *Rnematode*	KWS
	Chézy-sur-Marne	V5	Python	*Rz1rz1*	SES Vanderhave
	Dadonville	V17	Sporta	*Rz1rz1*	Syngenta
	Boutigny-sur-Essonnes & Saint-Pierre	V18	Ardan	*Rz1rz1*	Florimond Desprez
	Chézy-sur-Marne & Aufferville	V19	Jetta	*Rz1rz1*	Ringot Betteraves
	Mérouville & Bondaroy	V20	Zanzibar	*Rz1rz1*	SES Vanderhave
	Ramoulu	V21	Galactica	*Rz1rz1*	KWS
	Thiersanville	V22	Nordika	*Rz1rz1*	KWS
	Bondaroy	V23	Cheyenne	*Rz1rz1*	SES Vanderhave
	Bondaroy	V24	Danube	*Rz1rz1*	Florimond Desprez
	Sougy	V25	Narcos	*Rz1rz1*	Florimond Desprez
	Bondaroy	V26	Carissima	*Rz1rz1*	Betaseed
	Bondaroy	V27	Rigel	*Rz1rz1*	Betaseed
	Bondaroy	V28	Zoulou	*Rz1rz1*	Ringot Maribo
	Bondaroy	V29	Emilia	*Rz1rz1*	KWS
	Bondaroy	V30	Othello	*Rz1rz1*	Ringo Maribo
	Bondaroy	V31	Harmonia	*Rz1rz1*	Hilleshog NK
	Bucy-le-Roi & Autroche & Saint-Pierre	V32	Annalisa	*Rz1rz1* & *Rnematode*	KWS
	Bondaroy	V33	Encarta	Susceptible	Syngenta
	Bondaroy	V35	Harmonia	*Rz1rz1*	Betaseed
Year1	Yèvres-la-Ville & Bondaroy	V1	Ludwinia	*Rz1rz1* & *Rz2rz2*	KWS
	Yèvres-la-Ville & Bondaroy	V2	Britta	*Rz1rz1* & *Rz2rz2*	KWS
	Montargis & Yèvre-la-Ville & Bondaroy	V5	Python	*Rz1rz1*	SES Vanderhave
	Yèvres-la-Ville & Bondaroy	V6	Magellan	*Rz1rz1*	SES Vanderhave
	Gidy & Ruan	V8	Bering	*Rz1rz1* & *Rnematode*	Strübe
	Yèvres-la-Ville & Bondaroy	V16	Cetus	*Rz1rz1*	Deleplanque
	Yèvres-la-Ville & Bondaroy	V33	Encarta	Susceptible	Syngenta
	Yèvres-la-Ville & Bondaroy	V34	Magistral	*Rz1rz1*	SES Vanderhave
	Yèvres-la-Ville & Bondaroy	V35	Harmonia	*Rz1rz1*	Betaseed
	Yèvres-la-Ville & Bondaroy	V36	Deborah	*Rz1rz1*	KWS
	Yèvres-la-Ville & Bondaroy	V37	Antoinetta	*Rz1rz1*	KWS
Year2	Chezy	V1	Ludwinia	*Rz1rz1* & *Rz2rz2*	KWS
	Sougy	V4	Julietta	*Rz1rz1* & *Rnematode*	KWS
	Neuville-au-bois	V6	Magellan	*Rz1rz1*	SES Vanderhave
	Sougy	V10	Bison	*Rz1rz1* & *Rnematode*	SES Vanderhave
	Mayvillers	V12	Adriana	*Rz1rz1* & *Rnematode*	KWS
	Poupry	V14	Massima	*Rz1rz1* & *Rnematode*	Betaseed
	Laon	V15	Unknown	*Rz1rz1*	Unknown
	Baigneaux	V16	Cetus	*Rz1rz1*	Deleplanque
	Chezy	V28	Zoulou	*Rz1rz1*	Syngenta
	Chezy	V30	Othello	*Rz1rz1*	Ringot Maribo
	Pierrefonds	V33	Encarta	Susceptible	Syngenta
	Voué	V39	Unknown	*Rz1rz1*	Unknown

(Continued)

TABLE 2 | Continued

Year	Region	Variety code	Variety	Resistance type	Seed company
FARMER'S FIELDS (PATCHES)					
Year3	Mérouville	V2	Britta	*Rz1rz1* & *Rz2rz2*	KWS
	Teillay-le-Gaudin	V4	Julietta	*Rz1rz1* & *Rnematode*	KWS
	Sougy	V6	Magellan	*Rz1rz1*	SES Vanderhave
	Janville	V7	Rosalinda	*Rz1rz1*	KWS
	Sougy	V38	Belino	*Rz1rz1*	Florimond Desprez

except for the field coded F5 in Dambron 1 which is the same in year 1 and year 3. For each assay, the field was subdivided into three varietal bands and each band was subdivided into four zones. There were consequently 12 zones per field. The fields containing cyst nematodes were planted with rhizomania and nematode resistant cultivars. The minimum and maximum numbers of BCN (beet cyst nematode) eggs/larvae in 100 g of soil at initial egg density (pi) and at final egg density (pf) were the following from the set of nematode fields: the minimum pi measured was 0/0 and the maximum pi was 98/318 for the eggs/larvae, respectively; the minimum pf measured was 0/0 and the maximum pf was 85/558 for the eggs/larvae, respectively. In year 1 and year 3, there were mixed samples (one mixed sample consisted of five or six different roots) per zone pooled for the analysis. In year 2, only a single root was analyzed per zone to observe the diversity in a single root. **Table 2** shows the cultivars in the diseased fields (between year 0 and year 3) that were analyzed in patches of diseased plants with symptoms. In a 150 km^2 area corresponding to locations with severe rhizomania, targeted BNYVV genes in samples were analyzed by RT-PCR and sequencing. A total of 1058 samples were analyzed over 4 years. The root symptoms severity percentage for the root symptoms evaluation was supplied by ITB for each cultivar/plot in the field. This root symptoms severity score takes into account the percentage frequency of visible symptoms on 100 beets in this plot and the percentage of roots displaying a strong root symptom (level 2: strong symptoms, level 1: medium symptoms, level 0: no symptoms) evaluating the heterogeneity of the attack. Strong root symptom displays a heavily BNYVV infected plant displaying typical severe Rhizomania symptoms (a small "T-like" taproot with brownish vasculature and dark brown lateral roots).

Total RNA Extraction

Each rootlet was lyophilized (Lyophilisator Heto PowerDry PL6000-55 Thermo by Thermo Scientific, France) and then homogenized. The total RNA was extracted from 100 mg of homogenized root powder from each sample using the RNeasy extraction kit (Qiagen, Hilden, Germany) or similar technique.

RT-PCR Detection of the BNYVV Virus

A duplex RT-PCR assay was used for the detection of RNA3 and RNA5 of BNYVV. For the RNA3, the primer pair 5′-CAGTTTATGATTTAGGGCACA-3′/5′-ATCATCATCA ACACCGTCAG-3′ was used to amplify the *p25* gene by RT-PCR. For the RNA5, the primer pair 5′-ATGTTTGTTGGTCCC CCGCT-3′/5′-CGAGCCCGTAAACACCGCAT-3′ was used to amplify the *p26* gene.

Sequencing

Before sequencing, the PCR products were purified by the MSB® Spin PCRapace Kit (Invitek GmbH, Berlin, Germany). The nucleotide sequences of the PCR products from the *p25* gene were obtained using an ABI377 Sequencer-Genetic Analyser and the "Big Dye Terminator Cycle Sequencing Kit" (Applied Biosystems). The sequences were analyzed by a Blastn search in NCBI. The Clustal W program of the Genetic Computer Group (Devereux et al., 1984) at EMBL-EBI was used to obtain multiple sequences alignment. The Expasy Proteomics Server tools (http://www.expasy.org/) were used to translate nucleotide in amino acids and the Invitrogen Vector NTI Advance 10 program (Life Technology) was used to manage the data. Each RNA3 type was characterized by SNPs (single nucleotide polymorphisms) analysis as biological markers and compared with French sequence references of each BNYVV type (A-type, AF197545; B-type, M36894; P-type, DQ682454; J-type, NC_003516).

Quantification of BNYVV

Double antibody sandwich ELISA (DAS-ELISA) using the DSMZ kit (Leibniz Institute, Braunschweig, Germany) was carried out using polyclonal antibodies raised against BNYVV as described by Pferdmenges (2007) and following the manufacturers' instructions. The root samples coming from the field were normalized by their weight (100 mg) for the ELISA analysis. The ELISA reading was carried out at an absorbance of 405 nm. The ELISA results of the root samples were compared to a 2-fold dilution series of a positive control for the generation of a standard curve ($R^2 = 0.99$). The positive control was a solution of purified BNYVV particles with a starting protein concentration of 2380 ng/ml. The ELISA values of the 2-fold dilution series of the positive control after 1 h of incubation at 37°C in the dark were 2.58, 2.09, 1.77, 1.45, 1.06, 0.80, 0.55, 0.39, 0.25, 0.17, 0.11, 0.06 for a BNYVV concentration of 2380, 1190, 595, 297.50, 148.75, 74.38, 37.19, 18.59, 9.30, 4.65, 2.32, 1.16 ng virus protein/ml buffer, respectively. The BNYVV detection limit (0 ng/ ml) resulted from a mean of tested healthy controls plus three times the standard error. Plants with estimated virus concentrations below 4 ng/ml were considered to be free of the virus as described by Paul et al. (1994).

The reason for the choice of ELISA instead of real-time RT-PCR is mostly for the robustness of the technology on samples

TABLE 3 | Tetrad diversity observed in the Pithiviers region in France based on 482 isolates of *Beet necrotic yellow vein virus* (BNYVV) collected from year 0 to year 3.

BNYVV infection type	BNYVV type according to RNA3	Data from (Schirmer et al., 2005)		This study			
		RNA3	RNA5	RNA3	RNA5	Freq. (%)	Found in varieties (this study)
DIVERSITY IN FRANCE							
Single	B-type	**AYHR** $_4$	+	**AYHR** $_4$	+	26.45	V1, V3, **V4**, V5, V6, **V7**, **V8**, **V9**, **V10**, **V12**, **V14**, V16, V18, V21, V23, V25, V26, V28, **V32**, V35
		AYHR $_4$	−	**AYHR** $_4$	−	25.05	V1, V3, **V4**, V5, V6, **V8**, **V9**, **V10**, **V12**, V18, V19, V20, V21, V22, V30, (V33)
				TYHR	−	0.43	**V8**
				AYHR $_4$ - TYHR	+	0.43	V1, **V4**
				AYHR $_4$ - AYHT	−	0.21	V5
	P-type	**SYHG** $_4$	+	**SYHG** $_4$	+	19.01	V1, V2, V3, **V4**, V5, V6, **V9**, V16, V19, V20, V22, V25, V26, V28, (V33), V34, V36, V37
				SYHG $_4$	−	1.51	V1, **V4**, V5
				SYHR	+	0.21	V1
				SYHG $_4$ - SYHR	+	0.21	V35
	A-type			**AYPR** $_5$	+	3.46	**V4**, V5, V6, **V7**, **V10**, V17, V24, V27, V29, V30, V31
				AYPR $_5$	−	1.08	V5, **V10**
		AFHR $_{2,4}$	−	**AFHR** $_{2,4}$	+	0.43	V20
				VCHG $_{1,3}$	−	0.43	V1
				AYHG $_{2,4}$	−	0.21	**V4**
				AFPR	−	0.21	V20
				VYHR	+	0.21	**V4**
				VLH? $_1$ - AFHR	+	0.21	**V4**
		ALHG	+	ND	ND	ND	Unknown
		AHHG	−	ND	ND	ND	Unknown
Mixed	B-/P-type			**AYHR** $_4$ - **SYHG** $_4$	+	9.93	V1, V2, V3, **V4**, V6, **V7**, **V8**, **V9**, **V10**, (V33), V34, V37, V38
				AYHR $_4$ - **SYHG** $_5$	−	3.89	**V4**, **V10**
				AYHR $_4$ - **SYHG** $_4$ -TYHR	+	0.21	**V4**
	B-/A-type			**AYHR** $_4$ - **AYPR** $_5$	+	2.16	V1, V6, **V10**, **V12**
				AYHR $_4$ - **AYPR** $_5$	−	0.65	**V4**, **V10**
				AYHR $_4$ - **AYHG** $_{2,4}$	+	0.21	**V8**
				AYHR $_4$ - **AYHG** $_{2,5}$	−	0.65	V3, V5
				AYHR $_4$ - **AFHG** $_{2,4}$	−	0.21	**V10**
				AYHR $_4$ - **VCHG** $_{1,3}$	−	0.21	V1
				AYHR $_4$ - **AYPR** $_5$ -TYHR	+	0.21	V1
				AYHR $_4$ - **AYHG** $_{2,4}$ - **AYPR** $_5$	−	0.21	**V10**
	P-/A-type			**SYHG** $_4$ - AFHR	+	0.21	V20
				SYHG $_4$ - TFPR	−	0.21	V39
				SYHG $_4$ - **AYPR** $_5$ - AFHR	−	0.21	V39
	B-/P-/A-type			**AYHR** $_4$ - **SYHG** $_4$ - **AYPR** $_5$	+	0.65	V6, **V10**
				AYHR $_4$ - **SYHG** $_4$ - **AYPR** $_5$	−	0.43	**V10**

Tetrads observed also by other authors: 1. Acosta-Leal and Rush, 2007; 2. Chiba et al., 2008; 3. Koenig et al., 2009b; 4. Chiba et al., 2011; 5. Bornemann and Varrelmann, 2013. Tetrads associated with resistance-breaking (RB) in Rz1 cultivars are in bold and tetrads associated with RB in wild beet B. vulgaris subsp. maritima lines are under-lined. The frequencies established are based on the 482 sequences. For the tetrad VLH?, ? means that we weren't able to identify with certitude this amino acid because there were a lot of chromatographic peaks in the position 70 when direct sequencing was made. The cultivar in parentheses corresponds to a susceptible cultivar. The under-lined cultivars correspond to Rz1Rz2 cultivars. The cultivars in bold correspond to Rz1 cultivars with nematode resistance and the others are Rz1 cultivars without the nematode resistance. ND means that we didn't found the tetrad in our study. The presence of RNA2 was also tested and was present for 63.73% of the samples. The occurrence of RNA5 with other tetrads than SYHG represents 33.77% of the samples. The total of mixed BNYVV types represents 20.25% of the samples and the total of single BNYVV types represents 79.75%.

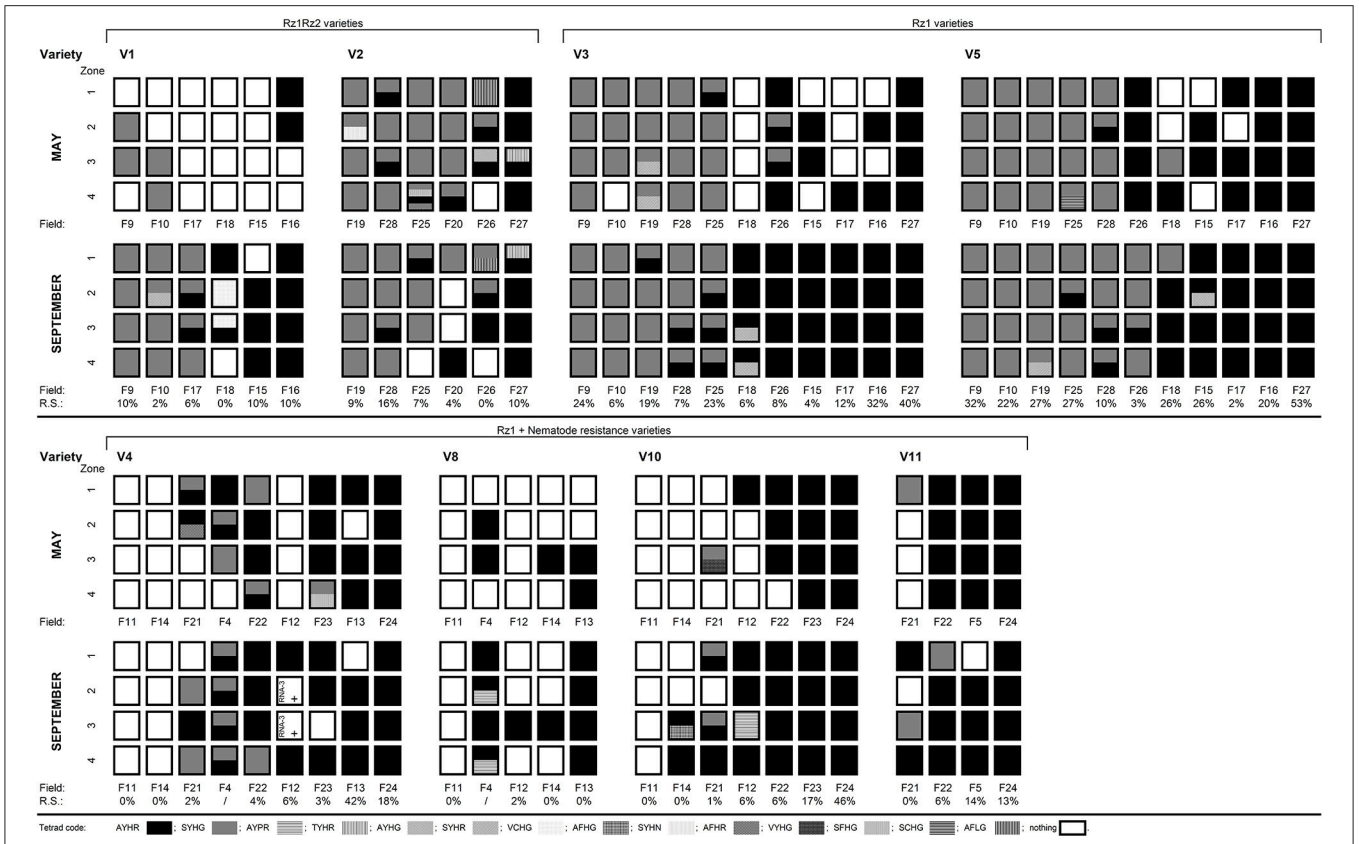

FIGURE 2 | Tetrad diversity for *Beet necrotic yellow vein virus* (BNYVV) isolates by cultivar between year 1 and year 3 for samples collected from sugar beet fields in May and September in the region of Pithiviers of France. Each field was subdivided in four zones and three varietal bands. V1 and V2 were *Rz1rz1Rz2rz2* tolerant cultivars. Cultivars V3 and V5 to V7 were *Rz1rz1* tolerant cultivars. V4 and V8 to V13 were *Rz1rz1* tolerant cultivars that also included tolerance to *Heterodera schachtii*. R.S. = root symptoms severity (%) observed in September is represented by R.S based on the observation of 2 times 50 roots in each cultivar band noted with frequencies of a scale ranking the level of root symptoms (0, no symptoms; 1, average root symptom between 0 and 50%; 2, strong root symptom between 50 and 100%). /, no information.

that had to be collected from different fields, over long distances, moved and stored before processing and measuring. ELISA is offering a rather robust quantitative and comparable measure, and has been used as a standard method (e.g., Pferdmenges et al., 2009), when compared to other methodologies also used for quantification purposes (Stevanato et al., 2016). ELISA also allows a rapid quantification of the BNYVV coat protein, while real-time RT-PCR would have focused on a targeted RNA molecule from the BNYVV genome (Harju et al., 2005; Acosta-Leal et al., 2008), despite the possibility of variation between each RNA molecules (Harju et al., 2005).

RESULTS

Diversity of the BNYVV p25 Tetrads in the French Pithiviers Area

The types of p25 amino acid tetrads were evaluated over a 4-year period. The frequency of the tetrads and their location in the Pithiviers area were determined (**Table 3**; **Figure 1**, respectively). A total of 1,058 samples were evaluated and 835 were found positive for BNYVV. Amongst these BNYVV-infected samples, 482 isolates were evaluated from individual sugar beet roots (**Table 3**) and 353 isolates were evaluated from a composite sample made of a mix of sugar beet roots collected from a single zone in the field.

Amongst these 835 BNYVV positive samples, few mutations resulting in substitution of alanine with valine residue in the tetrad area of the RNA3 (0.65% of the total of 482 isolates from individual sugar beet roots) were detected. In a single sugar beet root, single infections (presence of only one BNYVV RNA3 type and only one tetrad motif) were observed in 80% of the samples. The other 20% represented mixed infection, meaning P-/B-type, B-/A-type, P-/A-type, or B-/A-/P-type infection (**Table 3**). In this study, 56% of the positive samples (frequency occurrence based on 835 positively infected samples) had the AYHR tetrad and a B-type RNA3 and 32% had the SYHG tetrad and a P-type RNA3 (**Table 3**; **Figure 1**). The A-type AHHG, and ALHG tetrads were not found in the root samples. Conversely, tetrads SYHG, AYHR, AYPR, AFHG, AFHR, VCHG, and VLHG linked previously with resistance-breaking events were found (**Table 3**). The highest tetrad diversity was found when the sugar beet cultivars with both the *Rz1* and *Rz2* resistance genes were infected with BNYVV

even though the root symptoms severity was the lowest in these cultivars.

Large Co-occurrence of BNYVV A-Type or B-Type RNA3 With the Fifth RNA (P-Type)

Table 3 also shows a lot of single infections of BNYVV with a B-type (27% of the 835 positive samples) or A-type (4%) tetrads/RNA3 associated with a P-type RNA5. These particular strains with a fifth RNA account for 34% of the 482 individual sugar beet root samples with B-type AYHR being the most frequently found and then A-type AYPR in the p25. Mixed BNYVV A-/B-type infections with a fifth RNA were also observed in a single sugar beet root.

Changes Over a Single Growing Season

A striking feature of the field mapping is the diversity of virus types and tetrads observed at field-level (**Figure 2**). Along with such diversity, changes observed between the situations early in the growing season and later on are also evident. BNYVV was detected in September in 53 zones where the virus had not been detected in May despite a systematic sampling procedure. New specific tetrads not found in May in an infected field zone were observed in September (**Figure 2**). Conversely, to a lesser extent, specific tetrads found in May were not observed in September. Overall, more tetrads were detectable at the end of the growing season. The number of zones per field where the virus was present also increased in September, but sometimes with different tetrads/types of virus.

The BNYVV P-Type Titre Is Higher in Plant

Based on the 20 field trials, BNYVV with a P-type RNA3 showed a higher virus content in plants (mean ELISA value of 1.75 for *Rz1rz1Rz2rz2* and 1.81 for *Rz1rz1*) than BNYVV with B-type RNA3 (mean ELISA value of 0.42, 0.49 and 0.51 for *Rz1rz1Rz2rz2, Rz1rz1,* and *Rz1rz1+N*, respectively) or mixed-type RNA3 (mean ELISA value of 0.63, 1.04 and 0.46 for *Rz1rz1Rz2rz2, Rz1rz1,* and *Rz1rz1+N*, respectively) in the cultivars (**Table 4**).

Statistically, the highest ELISA value was measured in *Rz1rz1Rz2rz2* and *Rz1rz1* cultivars when infected with the P-type and then in *Rz1rz1* cultivars when infected with the mixed-type. The intermediate ELISA value was measured in *Rz1rz1Rz2rz2* cultivars when infected with the B-type or the mixed-type. The lowest ELISA value was measured in *Rz1rz1* cultivars when infected with the B-type and in the *Rz1rz1+N* cultivars when infected with the mixed-type or the B-type (**Table 4**).

Moreover, at the end of the growing season, there were statistically no significant differences (Fisher's LSD, homogenous group, alpha = 0.05) between the ELISA value of the B-type tetrads with the RNA5 or without RNA5 (data not shown). However, the frequency of the B-type RNA3 in the field (52%) was higher than the frequency of the P-type RNA3 (21%), the frequency of the A-type RNA3 (6%) and the one of the mixed-type RNA3 (20%) (**Table 3**; **Figure 2**).

Mixed BNYVV Types

Mixed infections with various BNYVV types were observed in the collected sugar beet taproot. In the field between year 1 and year 3, the tetrads AYHR and SYHG were the ones that were most frequently found in mixed infections. All three RNA3 types (A-type, B-type, P-type) could also be found in some individual samples (**Table 3**).

When there was mixed-RNA3 B- & P-type infection, the recorded root symptoms severity (RS) was the lowest in *Rz1rz1Rz2rz2* (mean RS: 5.62%) and *Rz1rz1+N* (rhizomania and nematode resistance) (mean RS: 2.71%) cultivars. It was also the lowest compared to single type infection in all kinds of cultivars (**Table 5**). The RS of the *Rz1rz1Rz2rz2* (mean RS: 9.5%) cultivars infected with the P-type alone, the RS of the *Rz1rz1Rz2rz2* (mean RS: 10%) cultivars and the RS of the *Rz1rz1+N* (mean RS: 12.85%) cultivars infected with the B-type alone are statistically similar. This group has an intermediate RS that is significantly different from the previous group and the next one. The highest RS was measured in *Rz1rz1* cultivars whatever the kind of infection (P-type alone, B-type alone or mixed-type infection). The range of the mean RS is for this group between 17.4% for the mixed-type infection and 21.37% for the single type infection.

The P-type alone was not detected from individual roots in fields infested with nematodes, but was detected in mixed infections (**Tables 4**, **5**; **Figure 2**). Generally, the P-type alone was less present in the fields with nematodes.

Overall, cultivars infected with the AYHR tetrad without RNA5 showed the lowest mean RS (11%) followed by cultivars infected either by mixed AYHR & SYHG tetrads with RNA5 (12%) or by the SYHG tetrad alone with RNA5 (15%). The highest mean RS (16%) was measured in cultivars infected by strains showing the tetrad AYHR with RNA5 (**Table 6**). There were statistically significant differences between these three groups, independently of the cultivar.

DISCUSSION

The BNYVV diversity in the Pithiviers region, south of Paris in France, was found to be very different from other resistance-breaking associated epidemic foci. The Pithiviers region has been repeatedly described as an intense sugar beet breeding area (De Biaggi et al., 2003). In line with Schirmer et al. (2005) who previously pointed out the presence of various BNYVV types and tetrads, this study emphasizes the increasing diversity of the virus both at the area and field levels, thereby underlining the complexity of the disease. During a single growing season, independently of the cultivar background, different p25 tetrads appeared and others disappeared. There was as such a tetrad selection through the cultivar background. Before 2005, three types and five tetrads (four hypervariable amino acids in the ARN3-coded p25) known in the area, based on 40 isolates, were detected: the B-type AYHR tetrad (54%), followed by P-type SYHG tetrad (32%) and A-type AFHR (3%), AHHG (3%), and ALHG tetrad (6%) (Schirmer et al., 2005). In contrast, a much higher diversity (21 tetrads in total from 3 BNYVV types) was assessed in this study (**Table 3**; **Figure 1**).

TABLE 4 | Enzyme linked immunosorbent assay (ELISA) values for *Beet necrotic yellow vein virus* resistance cultivars grown in France.

Resistance background	BNYVV RNA3 type	Mean ELISA ± *SD*	*n*	Fisher's LSD**
Rz1rz1Rz2rz2	P	1.75 ± 0.15	3	c
	B	0.42 ± 0.29	2	ab
	Type mixture	0.63 ± 0.57	8	ab
*Rz1rz1 without N	P	1.81 ± 0.74	8	c
	B	0.49 ± 0.24	8	a
	Type mixture	1.04 ± 0.63	10	b
*Rz1rz1 with N	P	Not observed	0	
	B	0.51 ± 0.23	13	a
	Type mixture	0.46 ± 0.14	7	a

*The average ELISA values are represented as the average optical density of the four zones (three replicates per zone minus twice the blank) more or less a standard deviation (SD). The average of the average ELISA values is then calculated for each type of resistance. There is a statistically significant difference between the comparisons among the three different categories of means at P = 0.05 when F obs> F-test (Fisher's LSD test, homogeneous group, alpha = 0.05). *Rz1rz1 cultivars without N (nematode resistance); Rz1rz1 cultivars with N (nematode resistance). **Means followed by same letter are not significantly different according to Fischer's LSD test (P = 0.05).*

TABLE 5 | Root symptoms severity for *Beet necrotic yellow vein virus* resistance cultivars grown in France.

Resistance background	BNYVV RNA3 type	Mean root symptoms severity ± *SD* (%)	*n*	Fisher's LSD**
Rz1rz1Rz2rz2	/	7 ± 4.80	12	a
*Rz1rz1 without N	/	19.12 ± 12.82	24	b
*Rz1rz1 with N	/	9.30 ± 13.19	20	a
Rz1rz1Rz2rz2	P	9.5 ± 0.71	2	ab
*Rz1rz1 without N	P	19 ± 9.34	6	a
*Rz1rz1 with N	P	Not observed	0	/
Rz1rz1Rz2rz2	B	10 ± 0	2	ab
*Rz1rz1 without N	B	21.37 ± 18.54	8	a
*Rz1rz1 with N	B	12.85 ± 15.27	13	ab
Rz1rz1Rz2rz2	Type mixture	5.62 ± 5.45	8	b
*Rz1rz1 without N	Type mixture	17.4 ± 9.81	10	a
*Rz1rz1 with N	Type mixture	2.71 ± 2.63	7	b

*There is a statistically significant difference between the comparisons among the three different categories of means at P = 0.05 when F obs> F test (Fisher's LSD test, homogeneous group, alpha = 0.05). *Rz1rz1 cultivars without N (nematode resistance); Rz1rz1 cultivars with N (nematode resistance). **Means followed by same letter are not significantly different according to Fischer's LSD test (P = 0.05).*

TABLE 6 | Mean root symptoms severity values for BNYVV tetrads with or without RNA5 independently of the cultivar.

BNYVV RNA3 tetrad	RNA5	Mean root symptoms severity ± *SD* (%)	*n*	Fisher's LSD*
AYHR	No	11.27 ± 12.62	51	a
AYHR-SYHG mixed	Yes	11.58 ± 9.35	24	ab
SYHG	Yes	15.03 ± 9.25	58	ab
AYHR	Yes	16.33 ± 15.87	61	b

*There is statistically a significant difference between the comparisons among the different categories of means at P = 0.05 when F obs> F-test (Fisher's LSD test, homogeneous group, alpha = 0.05). *Means followed by same letter are not significantly different according to Fischer's LSD test (P = 0.05).*

The Situation in Pithiviers Is Different

The presence of A-, B-, and P-type BNYVV in the Pithiviers area is quite unique. This situation is distinct from that of other sugar beet production regions: the strong selection pressure created by the widespread use of resistant cultivars, associated with an intense breeding area (De Biaggi et al., 2003), seems to favor the occurrence of parallel mutations in individual virus populations, calling into question how long *Rz1* resistance will be useful (Acosta-Leal et al., 2010; Bornemann and Varrelmann, 2013). Chiba et al. (2011) have proposed an evolutionary pathway reconstructing the spread and the occurrence of resistance breaking isolates of BNYVV in different sugar beet-growing areas (VCHG, VLHG, ALHG, AHHG, ACHG, AFHG, SYHG, AQHG, AYRV tetrads, a hypervariable region in the p25). The resistant-breaking (RB) selection can be found in association with a single mutation in the tetrad (Bornemann and Varrelmann, 2013) and also with other mutations along the *p25* (Klein et al., 2007). Usually, both the former wild type (WT) and new resistant-breaking (RB) tetrads are geographically localized. In the USA, an RB zone without a fifth RNA is linked with a single mutation in *p25* (Acosta-Leal et al., 2010).

Rhizomania in France around Pithiviers is different from other regions with resistant-breaking strains in the USA, Europe (Acosta-Leal and Rush, 2007; McGrann et al., 2009), and Asia (Chiba et al., 2011), in that much of the known world virus diversity can be found in only a 150 km^2 production area. Nine recognized and putative (SYHG, AYHG, AFHG, AFHR, AYPR, VLHG, VCHG, TYHR, and AYHR) *Rz1*- and/or *Rz2*-RB BNYVV isolates have been documented in the area. Furthermore, the simultaneous presence of at least three BNYVV types along with evidence for reassortments (Koenig et al., 2008) between isolates highlights the need to manage available sources of resistance as efficiently as possible. Peltier and colleagues also found RNA5 associated with European BNYVV A-, B-, and SYHG P-types (Peltier et al., 2008). Despite reduced symptoms, our data showed that *Rz1+Rz2* cultivars did not offer a total protection each time, with both an increase in the number of plots infected by the virus between the beginning and the end of the sugar beet growing season, and sometimes the appearance of new tetrad variants. Consequently, there is a risk for the emergence of new *Rz1+Rz2* breaking strains. So far no other resistance genes than *Rz1* and *Rz2* have been discovered, and their use should be managed. Before reaching such conclusion, it would have been useful to ascertain the allelic status of *Rz1*, *Rz2* and nematode resistance sources of the different sugar beet varieties used in this study.

Spatial Diversity at Regional and Field Level

Besides these observations, the high frequencies (years 1–3) of BNYVV P-type (SYHG tetrad) or BNYVV B-type (AYHR tetrad) were striking when compared to the very low frequencies

measured for known BNYVV A-type RB isolates with the tetrads VCHG or others. Based on our field-based trials, it was possible to determine that the diversity was found not only at the regional level but also at the field-scale level, and both at the beginning and at the end of the growing season. Using deep sequencing and bioassays, Bornemann and Varrelmann found that sugar beet genotypes induce a strong selective effect on the accumulation of different p25 tetrad variants. RB tetrad mutations are selected with a loss of relative fitness, with the exception of P-type (Bornemann and Varrelmann, 2013). Some genotype would select for the development of viral strains and other genotype would support the development of other unrelated specific viral strains. The same phenomenon was observed in our study but at the field level. The diversity observed in the Pithiviers area could be a reflection of the major breeding and rhizomania resistance research effort that has taken place in the area since the 1970s (De Biaggi et al., 2003).

In general, a tetrad diversity was observed intra-field (within the same field) and a cultivar resistance difference between the three cultivars in the same field was also observed regarding these tetrads. Schirmer et al. found only little tetrad diversity in France. The A-, B-, and P-type were also found in their study, but only the tetrad SYHG, AYHR, AFHR, AHHG, and ALHG was described (**Table 3**) (Schirmer et al., 2005). Variability is a key factor for RNA virus pathogenicity, where adaptation to changing situations serves to preserve genetic robustness and maintain fitness despite the presence of mutations in the genome (Schirmer et al., 2005). According to Bornemann and Varrelmann, different sugar beet genotypes would support the development of divergent viral populations (Bornemann and Varrelmann, 2013). In the present study, we found higher tetrad diversity in each type (**Figure 1**). The tetrad AYPR was also associated with clear resistance-breaking events. Several tetrads usually associated with the A-type or the B-type were also found with the fifth RNA.

Higher Accumulation of BNYVV P-Type

In the present study, the P-type associated RNA5 was detected in 52% of the 835 BNYVV positive samples. The relationship between the tetrad SYHG, the virus aggressiveness and the sugar beet susceptibility is still not fully clear. By ELISA, BNYVV P-type accumulates at much higher levels in resistant, tolerant and susceptible sugar beet cultivars than BNYVV B-type. This was also observed by Heijbroek et al. where the percentage of plants in which the virus reached only a low concentration was much lower in P-type than in A- or B-type infections (Heijbroek et al., 1999). Therefore, one could speculate that the P-type move faster in the plant than the B-type. Surprisingly, such quantification and root symptoms severity measures indicate a competition between both types, based on the measures on both single and mixed infection within an individual beet. The BNYVV mixed infection level in a single sugar beet was lower than in P-type single infection level or B-type single infection level. It seems that there is a modulation of the different BNYVV RNAs in the plant according to the type present. The tetrad SYHG was also linked to a very high level of ELISA value and consequently CP content. However, the tetrad AYHR was linked to a lower content of CP in the plant but higher in case of mixed infections. The competition between B- & P-type is less evident when comparing the ELISA data rather than the RS.

RNA5 Preferentially Associated With RNA3 B-Type or A-Type

Ratti et al. (2009) showed that BSBMV RNA3 can be replicated and encapsidated when co-inoculated with BNYVV RNA1 and 2 in *Beta macrocarpa*. Long-distance movement was observed indicating that BSBMV RNA-3 could substitute BNYVV RNA-3 for systemic spread, even though the p29 encoded by BSBMV RNA-3 is much closer to the BNYVV RNA-5-encoded p26 than to BNYVV RNA-3-encoded p25. In this study, mixed infections of tetrads were related to the A-type and/or B-type and/or P-type of BNYVV RNA3 in individual sugar beet taproot. Approximately 20% had a fifth RNA with a tetrad associated to the A-type and/or B-type RNA3 while the P-type RNA3 was associated with the P-type RNA5 for 100% most of the time. This indicates a stronger presence of A-type or B-type associated with P-type RNA5 than previously indicated (Koenig et al., 2009a). Based on Ratti et al. (2009)'s results one could then speculate that the BNYVV mixed type infection could also occur in a single plant. Meunier et al. (2005) suggested earlier that a recombination event had taken place within the RNA2 between the BNYVV B-type and A-type. In 2009, Koenig et al. also suggested a BNYVV genome reassortment in several resistant sugar beet cultivars with strong rhizomania symptoms (Koenig et al., 2009a). The RNA5 was usually detected either with A-type RNAs 1–4 or with a mixture of B-type and P-type RNAs. However the distinction between mixed BNYVV type infection and reassortment is not easy to formally demonstrate. In Spain, particular tetrads (VCHG) linked to the BNYVV A-type were responsible for resistance-breaking and the fifth RNA was not found (Koenig et al., 2005; Schirmer et al., 2005). In Italy, only the A-type without the fifth RNA was found (Koenig and Lennefors, 2000).

Rz1+N Cultivars Grown in Field Infected With Nematodes Showed Lower Virus Content

In 1995, an experimental hybrid (obtained by different pollinators crossed with the same monogerm CMS) with R22 (Lewellen and Wrona, 1997; Lewellen, 2000) was grown in an Imperial Valley test under rhizomania conditions in comparison to "Rhizosen" (*Rz1* Holly Hybrids cultivar) and a rhizomania susceptible commercial cultivar "HH41." R22 was developed from a cross between a single sugar beet line (C37) and 60 sea beet accessions (Lewellen, 1992). R22 and R22 hybrids seemed to express higher resistance to rhizomania than that conditioned solely by *Rz1* (Biancardi et al., 2012). It was unclear whether this higher resistance was due to improved resistance to rhizomania or to some other pest or disease (Biancardi et al., 2012). When *Rz1+N* cultivars were grown in field infected with nematodes, the ELISA values were lower than within *Rz1* or *Rz1Rz2* cultivars (**Table 3**). This was observed in fields infected either with the BNYVV B-type or with a mixed-type. Surprisingly, the

different fields infected with nematodes showed almost no P-type in the different cultivars, especially the more rhizomania resistant ones (**Figure 2**). Moreover, the root symptoms severity in *Rz1+N* cultivars was at the same level as *Rz1Rz2* cultivars independently of the BNYVV type observed (**Table 5**). Those results suggest that the concomitance of the nematodes in the soil and the presence of nematode resistance in the *Rz1* cultivar allow a drastic reduction of the rhizomania titre in the plant during the growing season. One could speculate either that the interaction between the nematode and the nematode resistance gene stimulate the SAR in the plant or other resistant mechanism enhancing performance and/or disease resistance against the BNYVV or that the nematode in the soil is in competition with the viruliferous *P. betae*. Incidentally, this last hypothesis was observed in the virus MPN results (data not shown). Over the sugar beet growing season, the viruliferous *P. betae* multiplied less under the *Rz1+N* cultivars than under the *Rz1* or *Rz1Rz2* cultivars. Moreover, the *P. betae* themselves or other pathogens (bacteria, nematode) could be involved in the stimulation of the plant defense mechanism, reducing infection of sugar beets by *P. betae* as Desoignies et al. (2013) showed with lipopeptides of *Bacillus amylolequifaciens*.

ACCESSION NUMBER

The partial BNYVV RNA3 sequences in this study have been deposited in GenBank. GenBank accession numbers for partial RNA sequences are MG839229 to MG839249 for the BNYVV RNA3-coded p25 of isolates AYHR, TYHR, ASHR, AYHT, AYHS, SYHG, SYHR, SYHN, SFHG, SCHG, AYPR, AFPR, TFPR, AYHG, AFHG, AFHR, AFLG, VCHG, VYHG, VYHR, VLHX, respectively.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work, and approved it for publication. YG and CB conceived the study. YG conducted the assays. All authors analyzed the data, and wrote the manuscript. All authors have read and approved the final manuscript.

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Effect of Sugar Beet Genotype, Planting and Harvesting Dates and Their Interaction on Sugar Yield

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Climate changes are affecting the plant production, including sugar beet growing especially in the southern and central parts of the Europe. Modifying the sowing and harvesting dates are one of the most often used adaptations in sugar beet cultivation. The aim of this study was to assess the interactions between planting date and sugar beet genotypes for different harvest dates with recommendation for duration of vegetation period for specific hybrids in order to achieve the best performance and to evaluate influence of climatic factors on sugar yield. Three-way analysis of variance and AMMI (Additive main effect and multiple interactions) analysis were performed to investigate interaction between main factors. Analysis of variance revealed that genotypes (G), planting date (PD), harvest date (HD) and interaction G × PD significantly affected sugar yield in 2016. In 2017 genotypes, planting date, harvest date and G x PD interaction significantly affected sugar yield on probability level of 1%, while PD × HD interaction had significant effect on probability level of 5%. Results of AMMI analysis enabled discrimination of genotypes with the highest level of stability in certain planting dates. Hybrids with combined yield and sugar content (NZ type) should have the advantage in earlier planting dates compared to of sugar beet hybrids with higher sugar content (Z type). However, in shortened vegetation period Z type hybrids are more stable and with better sugar yield results. Results of our study suggest that delaying the harvest date decreases differences between sugar yields obtained from hybrids sown in different planting dates. Major factors in the study affecting sugar yield were growing degree days, insolation and number of days from planting to harvest.

Keywords: planting date, harvest date, environment, interaction, AMMI, genotype, sugar yield

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INTRODUCTION

Trends of high average temperatures, with increased frequency of droughts, are affecting plant production throughout the Europe, but southern and central parts of the continent are especially endangered (Schär et al., 2004; Spinoni et al., 2015). Temperate regions of Pannonian plain and countries such as Hungary, Serbia, Croatia, and Romania are likely to be strongly affected by climate changes followed by summer heat waves and droughts during the vegetation, without possibilities for effectively shifting crop cultivation to other parts of the year (Olesen et al., 2011). A wide range of adaptations in agricultural practice (irrigation, intercropping, mineral nutrition etc.) are used in many European regions to minimize the negative impacts of climate change on crop production. According to White et al. (2011) adjusting the sowing date is by far the most frequently investigated

climate change adaptation option. Yield potential of many crops is highly influenced by sowing date since it determines the length of vegetation period and the amount of captured radiation (Van Ittersum and Rabbinge, 1997).

Plant growth, development and, finally, yield are the result of genetic composition, the environmental effects and the interaction of these two factors. Phenomenon of the genotype by environment interaction (GEI) is always present in the crop production causing genotypes to have different results and ranks in various environmental conditions (Ndhlela et al., 2014). Environments differ in the amount and quality of inputs and stimuli that they convey to plants including, e.g., the amount of water, nutrients or incoming radiation (Malosetti et al., 2013). Often GEI is associated and explained with genetically terms of adaptation and stability (Dimitrijević and Petrović, 2000; Das et al., 2010). Various statistical methods such as regression analysis, nonparametric statistics and multivariate models are used for investigation and interpretation of this phenomenon and evaluation of different genotypes (Gauch et al., 2008). Additive main effect and multiple interactions (AMMI) is one of the most used methods for interpretation of GEI data. AMMI associates the analysis of variance (ANOVA) with principal component analysis (PCA) in one method (Gauch, 2013). In final phase AMMI removes the additive effect from interaction by ANOVA and then analyses interaction structure using PCA method.

Sugar beet is the main sugar producing crop in the Europe, and since it has been grown in the wide range of environmental conditions, successful management and production of the crop often represent a challenge for breeders and farmers (Jaggard et al., 2007; Hergert, 2010). Choosing the sugar beet hybrid with high yield potential is important as well as good adapted agronomic measures and practices, synchronized with requirements and needs of the plant (Đulaković et al., 2015). Commercially, the sugar beets most important trait is sugar yield (Bosemark, 2006), which is strongly influenced by environment and highly correlated to root yield and sugar content (Powers et al., 1963; Schneider et al., 2002; Hoffmann et al., 2009).

Various types of sugar beet hybrids, developed by many seed companies, are present in the southern and central parts of the Europe do not have the same requirements and reactions to the local environmental effects. In the Serbia are usually grown two types of hybrids: Z type, with high levels of sugar content, intended for early harvest; and NZ type, with balanced root yield and sugar content, designed for medium and late harvest (Ludecke, 1953; Bosemark, 2006). Sowing period of sugar beet in the Serbia starts in the middle of March ends in April and might last for 45 days. During recent years campaigns of harvesting and processing beet roots were often prolonged from the end of August to the beginning of December and lasted for approximately 120 days.

Considering the long period from sugar beet sowing to harvest, the aim of this study was to: (i) detect the interactions between planting date and hybrids for two harvest dates; (ii) recommend sugar beet hybrids with the best performance for the specific vegetation period as useful tool for increasing the sugar yield; and (iii) to determine the effect of environmental variables on sugar yield.

TABLE 1 | Sugar beet hybrids used in trials.

Hybrid	Company	Type of hybrid*	Year of registration	Harvest time recommendation
Tibor	Strube	Z	2004	Early/medium
Tajfun	Maribo	Z	2008	Early/medium
Tesla	Strube	Z	2016	Early/medium
Beetle	SES van der Have	NZ	2016	Medium/late
Koala	SES van der Have	Z	2013	Early/medium
Grandiosa KWS	KWS	NZ	2016	Medium
Eduarda KWS	KWS	NZ	2014	Harvest flex
Leopolda KWS	KWS	ZN	2014	Early/medium
Vandana KWS	KWS	NZ	2016	Medium

**Z type, hybrid with high levels of sugar content; NZ type, hybrid with balanced root yield and sugar content.*

MATERIALS AND METHODS

Plant Material

The hybrids included in the study have been selected in order to obtain a high diversity regarding yield and quality properties. The sugar beet hybrids chosen for the first year of study were (i) newly registered hybrids with the best results from 2016 registration trials organized by the Ministry of Agriculture, Republic of Serbia (Tesla, Grandiosa, Beetle) and (ii) hybrids with high market share (Tibor, Tajfun). Since newly registered hybrids were not introduced on larger acreages, in second year of the field trials were tested hybrids registered in the last five years, with high market share in 2017 (Koala, Eduarda, Leopolda, Vandana). The hybrids were developed by different seed companies and belonged to Z and NZ type (**Table 1**).

Field Trials

The field trials were carried out at the fields of Institute of field and vegetable crops, Novi Sad (IFVCNS), at the location Rimski Šančevi (45°20′N, 19°51′E) during two successive years (2016 and 2017). Experiment was organized in the randomized complete block design (RCBD) with four replications. Basic plot size was 20 m^2, with four rows 10 m long and row spacing 0.5 m. Soil type was chernozem with characteristics presented in the **Table 2**. Sowing was performed by seed drills on four different planting dates (PD) (**Table 3**) with the distance of 0.09 m in row and 0.5 m between the rows. After the development of the second pair of leaves, the seedlings were singled out to a final, recommended crop density of 100,000 plants/ha. Standard agricultural practices for sugar beet growing were applied during the vegetation period. Roots were harvested manually on two harvest dates (HD) (**Table 3**). Combinations of different planting and harvest dates were considered as different trial environments (**Table 3**). The root yield (RY) was determined by measuring the weight of roots from two middle rows and recalculating it as t/ha. Root samples were analyzed in the Laboratory for sugar beet root quality testing of at IFVCNS. Sugar content (SC) was measured according to polarimetric method. Sugar yield (SY) was calculated following the equitation: $SY = RY \times SC$.

TABLE 2 | Soil characteristics in 2016 and 2017.

Year	Humus (%)	pH		P_2O_5 (mg/100g)	K_2O (mg/100g)
		H_2O	nKCL		
2016	2.57	7.23	8.17	30.6	30.9
2017	2.34	6.92	7.82	20.6	29.5

TABLE 3 | Combinations of different planting and harvest dates named as trial environments.

Planting date	Harvest date		Year
	13. September	28. October	
18.03.	En1-1-16	En1-2-16	2016
25.03.	En2-1-16	En2-2-16	
31.03.	En3-1-16	En3-2-16	
13.04.	En4-1-16	En4-2-16	
	22. September	**7. November**	
25.03.	En1-1-17	En1-2-17	2017
01.04.	En2-1-17	En2-2-17	
10.04.	En3-1-17	En3-2-17	
18.04.	En4-1-17	En4-2-17	

TABLE 4 | Summary of environmental variables for trial environments.

Environment	DNo	GDD (°C)	Insolation (h)	Precipitation (mm)	Temperature (°C)		
					Tmn	Tmx	Tma
En1-1-16	179	2,755.3	1,499.7	380.2	11.83	25.82	18.37
En2-1-16	172	2,725.0	1,461.4	354.4	12.19	26.37	18.82
En3-1-16	166	2,689.3	1,427.8	348.4	12.54	26.78	19.18
En4-1-16	153	2,524.2	1,323.3	346.2	12.87	27.14	19.48
En1-2-16	224	3,195.7	1,718.2	461.8	10.87	24.75	17.31
En2-2-16	217	3,165.4	1,679.9	436.0	11.14	25.15	17.63
En3-2-16	211	3,129.7	1,646.3	430.0	11.38	25.43	17.88
En4-2-16	198	2,964.6	1,541.8	427.8	11.56	25.62	18.02
En1-1-17	181	2,954.2	1,748.2	299.2	11.46	27.45	19.26
En2-1-17	175	2,902.7	1,692.0	299.2	11.83	27.82	19.62
En3-1-17	165	2,816.2	1,626.0	297.0	12.14	28.26	20.00
En4-1-17	157	2,743.1	1,566.0	293.6	12.54	28.69	20.41
En1-2-17	227	3,354.4	2,020.2	335.2	10.25	25.91	17.75
En2-2-17	221	3,302.9	1,964.0	335.2	10.51	26.15	17.98
En3-2-17	211	3,216.4	1,898.0	333.0	10.69	26.43	18.21
En4-2-17	203	3,143.3	1,838.0	329.6	10.94	26.68	18.45

GDD, growing degree days; Tmn, minimum temperature; Tmx, maximum temperature; Tma, mean average temperature.

Environmental Conditions

Data on daily maximum and minimum temperatures, rainfall and insolation were obtained from meteorological station located less than 1 km away from the experimental plots. The number of days (DNo) was calculated from planting to harvest date. Thermal time (growing degree-days, GDD) was calculated by summing the daily values of mean temperatures minus the threshold value of 3°C (Milford et al., 1985), from the planting to the harvest date. Weather conditions in the years 2016 and 2017 differed especially in the precipitation, average temperatures and insolation (**Table 4**, Supplementary Table 1). In 2016 the amount of rainfall and its distribution were close to the sugar beet monthly requirements (Vučić, 1992). In 2017 severe summer drought and high temperatures had large negative impact on sugar beet crop. In 2016, the first autumn frosts were recorded on October 6 for a period of 3 days, while the appearance of frost in 2017 was not recorded before the second harvest date (Republički hidrometeorološki zavod RepublikaSrbija, 2017).

Data Analysis

Factorial ANOVA for sugar yield data was computed using Statistica 13 software package (Dell Inc, 2015, StatSoft, Tulsa, OK, USA) and Duncan's multiple range tests for detection of statistically significant differences. Factors genotype, planting date, harvest date were assumed fixed. Values of $P \leq 0.05$ were considered significant. GEI data were analyzed using computing environment (R Development Core Team, 2013). AMMI analysis was completed using Excel Biplot Macros (Lipkovich and Smith, 2002). Pearson correlation coefficients between environmental data and sugar yield were calculated. To identify the environmental variables discriminating between different lengths of growing season, principal component analysis (PCA) was performed on the correlation matrix, calculated from the mean values for each growing season (R Development Core Team, 2013).

RESULTS

In both years of research newly registered hybrids showed better performance compared to old hybrids (**Tables 5A,B**). The highest average sugar yield had hybrids Tesla, Grandiosa and Beetle in 2016, while best performing hybrids in 2017 were Eduarda, Koala and Vandana. Delayed harvest date increased sugar yield in 2016 and 2017. Regardless of the different HD, the third PD resulted in the highest sugar yield, while the latest PD had the lowest yield in first year of research. In 2017 the highest sugar yield was recorded for second HD, while the first HD had the lowest yield.

According to three-way factorial ANOVA genotypes, PD, HD and G x PD interaction significantly affected sugar yield in 2016 (**Table 6**). PD accounted for 88.22% of total sum squares, while genotypes and G × PD interaction accounted for 5.04 and 3.97%, respectively. In 2017 genotypes, PD, HD, and G × PD interaction affected sugar yield on probability level of 1%, while PD × HD interaction had effect on probability level of 5%. HD effects participated in total variance with 55.51%, PD 16.08%, G × PD interaction 13.54%, genotypes 6.28%, while PD × HD interaction accounted for 3.7% of total sum squares.

The AMMI ANOVA (**Table 7A**) showed that in 2016 genotypes and PD had significant effects for both HD, but interactions were significant only for the first HD. In 2017

TABLE 5 | Sugar yield of tested sugar beet hybrids in trial environments in 2016 (A) and 2017 (B).

Environments	Sugar yield (t/ha)					Average
	Tibor	Tajfun	Tesla	Grandiosa	Beetle	
(A)						
En1-1-16	8.89 ± 0.20	9.56 ± 0.05	9.96 ± 0.51	10.82 ± 0.78	9.49 ± 0.51	9.74^{b}
En2-1-16	7.74 ± 0.32	9.00 ± 0.71	9.80 ± 0.14	8.67 ± 0.28	10.68 ± 0.91	9.18^{b}
En3-1-16	10.2 ± 0.21	10.27 ± 0.28	10.61 ± 0.19	10.75 ± 0.41	10.42 ± 0.48	10.45^{a}
En4-1-16	5.61 ± 0.37	5.12 ± 0.25	5.58 ± 0.38	4.49 ± 0.45	5.53 ± 0.26	5.27^{c}
Average	8.11	8.49	8.99	8.68	9.03	8.66^{b}
En1-2-16	9.64 ± 0.34	9.23 ± 0.40	10.97 ± 0.86	11.57 ± 0.75	11.27 ± 0.94	10.54^{a}
En2-2-16	7.66 ± 0.48	8.56 ± 0.86	9.44 ± 0.47	9.82 ± 0.84	10.36 ± 0.52	9.17^{b}
En3-2-16	9.85 ± 0.38	10.39 ± 0.48	11.06 ± 1.06	11.12 ± 0.67	10.95 ± 0.87	10.67^{a}
En4-2-16	5.53 ± 0.33	4.97 ± 0.24	6.43 ± 0.49	5.64 ± 0.46	5.83 ± 0.24	5.68^{c}
Average	8.17	8.28	9.47	9.54	9.60	9.01^{a}
Mean	8.14^{b}	8.39^{b}	9.23^{a}	9.11^{a}	9.32^{a}	

Environments	Sugar yield (t/ha)						Average
	Tibor	Tajfun	Eduarda	Koala	Leopolda	Vandana	
(B)							
En1-1-17	9.59 ± 0.46	9.01 ± 0.49	10.23 ± 0.71	10.05 ± 0.79	7.98 ± 0.56	10.21 ± 0.24	9.51^{b}
En2-1-17	7.46 ± 0.32	8.36 ± 0.63	8.38 ± 0.18	8.40 ± 0.34	7.76 ± 0.57	7.73 ± 0.61	8.02^{c}
En3-1-17	8.50 ± 0.24	6.87 ± 0.39	7.91 ± 0.71	9.01 ± 0.93	7.47 ± 0.52	7.94 ± 0.32	7.95^{c}
En4-1-17	7.90 ± 0.22	8.19 ± 0.08	6.95 ± 0.22	8.05 ± 0.41	8.48 ± 0.48	7.75 ± 0.49	7.89^{c}
Average	8.36	8.11	8.37	8.88	7.92	8.41	8.34^{b}
En1-2-17	10.39 ± 0.88	10.01 ± 0.15	11.72 ± 0.20	11.87 ± 0.74	9.09 ± 0.49	12.66 ± 0.96	10.96^{a}
En2-2-17	9.06 ± 0.73	9.41 ± 1.00	9.58 ± 0.58	9.94 ± 0.50	9.90 ± 0.68	9.40 ± 0.67	9.55^{b}
En3-2-17	10.89 ± 0.38	10.10 ± 0.44	10.29 ± 0.62	10.44 ± 0.67	10.79 ± 0.32	10.04 ± 0.46	10.43^{a}
En4-2-17	10.28 ± 0.74	9.84 ± 0.49	10.16 ± 0.51	11.76 ± 0.60	10.23 ± 0.39	10.45 ± 0.31	10.45^{a}
Average	10.15	9.84	10.44	11.01	10.00	10.64	10.34^{a}
Mean	9.26^{ab}	8.97^{b}	9.40^{ab}	9.94^{a}	8.96^{b}	9.52^{ab}	

Values followed by the same letter(s) are not significantly different (Duncan's Multiple Range test at $p < 0.05$).

sugar yield in both HD were influenced by PD and G × PD interaction, while genotypes effects were significant only for second HD (**Table 7B**). Contribution of G x PD interaction varied from 3.89% in 2016, to 46.87% in 2017. PD had the greatest contribution to total variation in 2016. In 2017, PD had the greatest contribution to total variation in the first HD, while for the second HD the interaction G x PD contributed the most. Effect of genotype increased in second HD for both years.

In 2016 IPCA axes were not significant for the second HD, so AMMI biplots were made only for first HD. The AMMI 1 biplot indicate that hybrid Beetle had the best performance (9.03 t ha^{-1}) while Tibor (8.11 t ha^{-1}) had the lowest sugar yield among the PD (**Figure 1**). The most stable sugar beet genotypes in 2016 were Z type varieties Tajfun, Tesla and Tibor. The tested hybrids had the highest sugar yield on the third PD, while their lowest performance was on the fourth PD.

AMMI 2 biplot indicated that certain hybrids had the potential for the best performance for the specific PDs (**Figure 2**). The hybrid Beetle showed the best performance in second PD, Grandiosa in the first PD, Tajfun and Tesla in the third PD. The close position of Tajfun and Tesla indicates that both hybrids would perform best in the similar environmental conditions.

According to the AMMI 1 biplot of the first HD in 2017 the best performance had Z type hybrid Koala (8.88 t ha^{-1}), with small interaction score and relatively good level of stability, while the lowest sugar yield had Leopolda (7.92 t ha^{-1}) (**Figure 3**). Beside Koala, stable sugar beet genotype for the first HD in 2017 was Z type hybrid Tibor. The tested hybrids had the highest performance on the first PD while the lowest results were recorded for fourth PD. Placement of both these planting dates indicated low level of stability for sugar yield. According to the positions of PD2 and PD3, although they were under-average environments they were more stable.

In 2017, Koala was again the best performing hybrid (11.01 t ha^{-1}) for the second HD, while the Tajfun had the lowest performance (9.84 t ha^{-1}) (**Figure 4**). The highest stability showed Z type hybrids Tibor, Tajfun and Koala. The tested hybrids had the highest sugar yield on the first PD, while their lowest performance was on the second PD.

TABLE 6 | Summary ANOVA for sugar yield in 2016 and 2017.

Source of variation	2016					2017				
	SS	df	MS	F	% of SS	SS	df	MS	F	% of SS
G	36.8	4	9.2	7.75**	5.04	21.84	5	4.37	3.58**	6.28
PD	643.9	3	214.66	180.90**	88.22	55.89	3	18.63	15.28**	16.08
HD	4.98	1	4.98	4.20*	0.68	192.9	1	192.9	158.2**	55.51
G × PD	28.9	12	2.41	2.03*	3.97	47.0	15	3.14	2.57**	13.54
G × HD	5.71	4	1.43	1.2	0.78	1.59	5	0.32	0.26	0.46
PD × HD	3.46	3	1.15	0.97	0.47	12.86	3	4.29	3.52*	3.7
G × PD × HD	6.06	12	0.5	0.43	0.83	15.37	15	1.02	0.84	4.42
Error	142.4	120	1.19			175.61	144	1.22		

*SS, sum of squares; MS, mean squares; df, degrees of freedom; G, genotype; PD, planting date; HD, harvest date. *,** indicate the significance levels of P < 0.05 and P < 0.01.*

TABLE 7 | Additive main effect and multiplicative interaction (AMMI) analysis of variance (ANOVA) for sugar yield in 2016 (A) and 2017 (B).

Source of variation	df	1st HD				2nd HD			
		SS	MS	*F*-value	% of SS	SS	MS	*F*-value	% of SS
(A)									
Total	79	402	5.09			465.3	5.89		
Treatments	19	355.7	18.72	23.62**		369.2	19.43	14.17	
Genotypes	4	9.2	2.31	2.91*	2.59	33.3	8.32	6.07**	13.52
PD	3	323.1	107.69	156.72**	90.83	324.4	108.13	42.86**	82.59
Block	12	8.2	0.69	0.87		30.3	2.52	1.84	
G x PD	12	23.4	1.95	2.46*	6.58	11.6	0.96	0.70	3.89
IPCA 1	6	13.9	2.32	2.92*		8.1	1.35	0.99	
IPCA 2	4	9.5	2.38	3.00*		2.8	0.7	0.51	
Residuals	2	0	0.01	0.02		0.7	0.33	0.24	
Error	48	38.1	0.79			65.8	1.37		
(B)									
Total	95	151.33	1.593			178.88	1.883		
Treatments	23	79.91	3.474	3.5**		74.69	3.247	2.57**	
Genotypes	5	8.4	1.681	1.69	10.51	15.03	3.005	2.37*	20.12
PD	3	44.1	14.698	14.86**	55.19	24.66	8.22	3.49*	33.01
Block	12	11.87	0.989	1.00		28.26	2.355	1.86	
G x PD	15	27.41	1.827	1.84*	34.30	35.01	2.334	1.84*	46.87
IPCA 1	7	18.46	2.637	2.66*		29.57	4.224	3.34**	
IPCA 2	5	7.18	1.436	1.45		3.58	0.717	0.57	
Residuals	3	1.78	0.592	0.60		1.85	0.617	0.49	
Error	60	59.56	0.993			75.93	1.266		

*IPCA, interaction principal component axis; HD, harvest date; PD, planting date; df, degrees of freedom; SS, sum of squares; MS, mean squares. *,** indicate the significance levels of P < 0.05 and P < 0.01.*

To identify the combination of variables that better explained the environmental variation, we conducted principal component analysis (PCA) on the mean values of the environmental variables (**Figure 5**). The first two axes of the PCA accounted for 91.5% of the total variance, indicating that the most of the information held in the data could be summarized by projecting the points on the plain determined by these two axes. The first principal component (PC1) accounted for 66.5% of the expressed variation. PC1 was related to all environmental variables and sugar yield, with minimal effect of precipitation. Increases in PC1 were related to number of days, growing degree days, sugar yield and insolation. The negative direction of PC1 was related to minimum, maximum and mean average temperatures. The second principal component (PC2) accounted for 25% of the expressed variation. Increases in PC2 were related to insolation and average maximum temperature. The negative direction of PC2 was related to precipitation.

The points corresponding to the each environment were ploted in the **Figure 5**. The first group of environments in the upper right part of the figure, representing the 2017 second

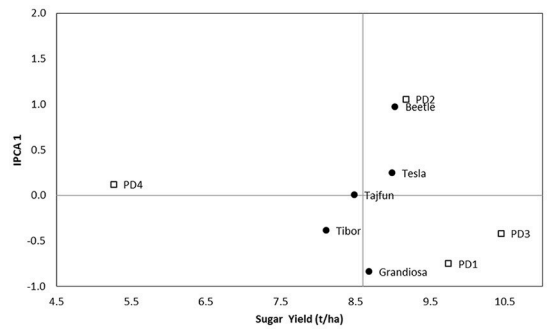

FIGURE 1 | AMMI 1 biplot for sugar yield showing hybrids (black dots) and PD (squares) plotted against their IPCA1 scores in 2016 first HD.

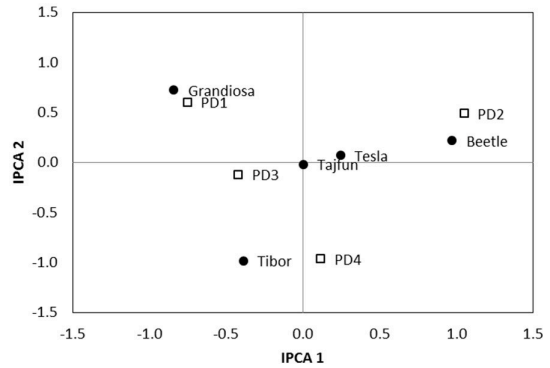

FIGURE 2 | AMMI 2 biplot for sugar yield showing the interaction of IPCA2 against IPCA1 scores of five hybrids (black dots) across four PD (squares) in 2016.

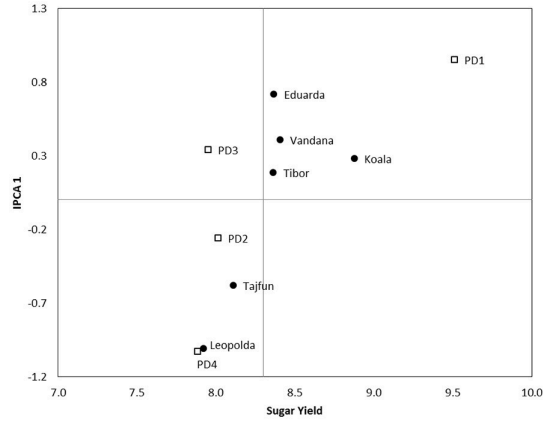

FIGURE 3 | AMMI 1 biplot for sugar yield showing hybrids (black dots) and PD (squares) plotted against their IPCA1 scores in 2017 first HD.

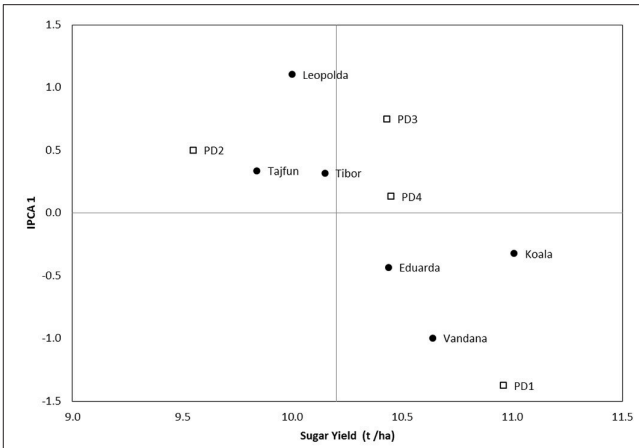

FIGURE 4 | AMMI 1 biplot for sugar yield showing hybrids (black dots) and PD (squares) plotted against their IPCA1 scores in 2017 second HD.

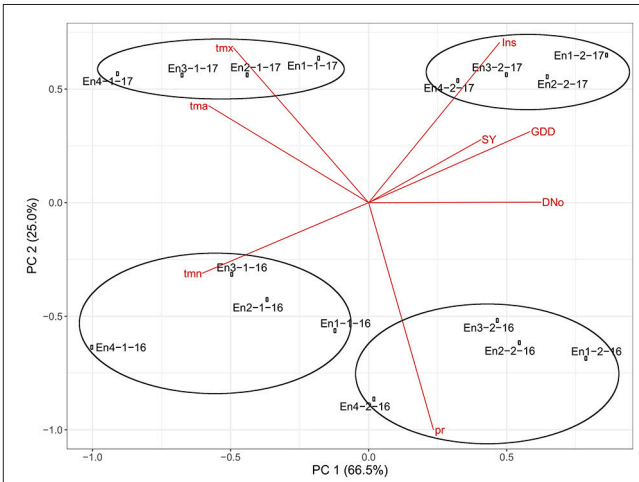

FIGURE 5 | Plot of the principal component analysis (PCA) with eigenvectors for the environmental variables and eigenvalues for the environments in the trials. (DNo, number of days; GDD, growing degree days; Ins, insolation; pr, precipitation; tmn, average minimum temperature; tmx, average maximum temperature; tma, mean average temperature and SY, sugar yield).

HD, indicates that they were characterized by large amount of insolation and GDD which agrees with the data shown in **Table 4**. The points corresponding to 2017 first HD were located in the left upper part of the plot and characterized with higher maximum average and daily average temperatures. The points belonging to the third and fourth groups were located in the lower part of the figure representing the environments of 2016 with higher minimal temperatures and larger precipitation which is in agreement with their meteorological background shown in **Table 4**.

Correlations between environmental variables and sugar yield are represented in the **Figure 6**. Sugar yield was positively correlated with GDD (0.58), Ins (0.56), and DNo (0.56), while negative correlation was detected only with Tmn (−0.59). The cumulative variables, DNo, GDD, and Ins were positively correlated. Temperature variables were also positively correlated with each other, but were in the negative correlation with DNo, GDD and Ins. Precipitation was negative correlated with Tmx

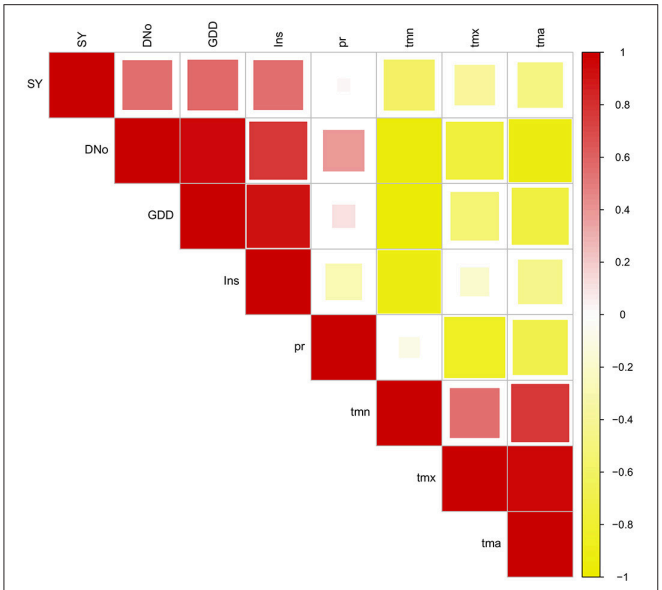

FIGURE 6 | Pearson's correlation coefficients between environmental variables and sugar yield (DNo, number of days; GDD, growing degree days; Ins, insolation; pr, precipitation; tmn, average minimum temperature; tmx, average maximum temperature; tma, mean average temperature).

and Tma. These findings comply with the results of principal component analysis, presented in **Figure 5**.

DISCUSSION

In the study, performance of sugar beet hybrids through vegetation periods of different duration (different planting and harvest dates), were investigated, using the sugar yield as the main evaluation criterion. Previous investigations considering sugar beet cultivation (Jozefyová et al., 2003; Öztürk et al., 2008; Filipović et al., 2009; Hoffmann and Kluge-Severin, 2011; Bu et al., 2016) indicated that the earlier planting dates and later root harvest can be advantageous. Considering the difference in genetic potential, as well as the moment of technological maturity of sugar beet hybrids, our aim was to determine if there were interactions between the genotype and combination of different planting and harvesting dates.

For these purposes NZ and Z type sugar beet hybrids were tested in 16 different environments. In both years significant effect of the genotype, PD, HD, and G × PD interaction on sugar yield were recorded. Ratios between variances for these effects in the first year were similar to those obtained in study of Hoffmann et al. (2009), probably because the environmental conditions for sugar beet production were similar to those in Western Europe. In 2017 the ratio of the effects was quite different—the genotype effect remained similar, PD effect decreased, while effect of HD increased. Interaction G × PD increased and PD × HD became significant. In our opinion, probably because the changes in the variances of investigated effects are result of different environmental conditions in 2017, characterized with hot and dry summer, typical for the Pannonian plane. It is likely that increased genotype x environment interaction was mostly due to different reaction of tested hybrids to water deficit (Pidgeon et al., 2006) Similarly to previous studies of Wolf (1995), Bloch and Hoffmann (2005), and Ćurčić et al. (2012), there was no interaction between genotypes and HD, indicating that in autumn different sugar beet hybrids have very similar root development.

The ANOVA for the AMMI model showed that interaction G × PD was significant and twice as large as the genotype effect, which is in compliance with the research of Srivastava et al. (2008). Significant effect of G × E interaction in sugar beet field trials was recorded in many studies (Moradi et al., 2012; Hoberg et al., 2015; Al Jbawi et al., 2017). However, in the studies by Hoberg et al. (2016) and Shao et al. (2015), environment had predominant effect on sugar yield, while the effect of genotype x environment interaction had no significance. Campbell and Kern (1982) and Trimpler et al. (2017) concluded that among the numerous significant factors, year effect had the greatest influence on sugar beet production. In the studies of Sklenar et al. (2000) and Ćirić et al. (2017) G × E interaction was significant, but not the factor with the strongest effect on yield.

According to IPCA-1 biplots the genotypes and environments with high coordinates on IPCA-1 contributed to a greater extent to the G × E interaction while the genotypes and environments with IPCA-1 coordinates close to origin have little contribution in this interaction effect (Crossa et al., 1990). It could be concluded that in both years of research Z type hybrids were more stable and therefore less contributed to the interaction comparing to NZ type hybrids. The AMMI 2 biplot enabled connection of the specific genotypes and environments based on the G × E interaction scores. The grouping of the genotypes and the environments in the same quadrant indicated positive association between them. NZ type hybrids showed better adaptation to earlier PD, while Z type hybrids showed better reaction to third and fourth PD.

Although other factors such as soil condition could induce variability between environments, the results of the PCA showed that 91.5% of the environmental variation was explained by the environmental variables considered in the study. Since climate factors determine where and how plants grow, environmental variables (temperature, solar radiation, precipitation etc.) were used for description of environment as in Xu (2016). Weather conditions during the trial differed greatly. The first year had sufficient amount and good distribution of rainfall, while 2017 was characterized by extreme drought and exceptionally high temperatures in especially during July and August. Also, the absence of precipitation and lower temperatures in April 2017 resulted in lower number of plants per unit area.

The weather conditions between HD in tested years were different. Beside the frost appearance in 2016, the main difference was the insolation. In 2017 there was 50 h more of insolation between the HD than in 2016. This was probably one of the main reasons why sugar yield in 2017 increased by 2 t ha^{-1} between harvest dates, while in the same period in 2016, yield was increased only by 0.35 t ha^{-1}. In the research of Kenter et al. (2006), there was positive correlation between root

yield and solar radiation in the autumn, 175–200 days after planting.

To quantify influence of environmental variables on sugar beet hybrid performance, they were correlated to sugar yield. Although precipitation is often regarded as a major factor affecting sugar beet growth (Jaggard et al., 1998) in our study it was not significant for sugar yield, similarly to results of Kenter et al. (2006). There were positive correlations between GDD, Ins and DNo, which was in accordance to the research of Schnepel and Hoffmann (2016).

Considering the changing environmental conditions, as well as the introduction of new sugar beet hybrids in the production, research on the genotype and the planting date interaction for different harvest dates could provide the answer to the question which hybrids to grow under such conditions. The obtained results can help sugar factories to increase the total sugar yield per unit area, by recommending sugar beet hybrids for individual planting dates, with advanced planning of sugar beet harvest. Results of AMMI analysis in this study enabled discrimination of hybrids with the highest level of stability in certain planting dates. Priority for earlier planting dates should be given to NZ type of sugar beet hybrids. On the other hand, Z type sugar beet hybrids were more stable and achieved better results during shorter vegetation period. Our results suggest that by delaying the harvest, differences between sugar yield from different planting dates decrease and sugar yields from later harvesting dates are on the same level regardless of the planting date.

AUTHOR CONTRIBUTIONS

ZC and MC designed and performed experiment, collected data, prepared the manuscript. NN and KT-A supervised the project, participated in preparation of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01041/full#supplementary-material

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