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RACHIS BRITTLENESS IN A HYBRID-PARENT BARLEY (*HORDEUM VULGARE*) BREEDING GERMPLASM WITH DIFFERENT COMBINATIONS AT THE *NON-BRITTLE RACHIS* GENES

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Short running tittle: Rachis brittleness in hybrid barley.

Short informative: We evaluated rachis fragility through a mechanical test and under natural conditions, in F_1 crosses with different compositions at the *btr* genes. We confirmed the grain retention problem that arises in hybrids when crossing barley lines with alternative mutations in the *btr* genes. Moreover, non-brittle hybrids (*btr1btr1Btr2Btr2*/*Btr1Btr1btr2btr2*) showed higher brittleness than inbreds, pointing at further genes affecting the trait.

Abstract

Two dominant, closely linked and complementary genes, *Btr1* and *Btr2* control rachis brittleness in barley. Recessive mutations in any of these genes turn the fragile rachis (brittle) into a tough rachis phenotype (non-brittle). The cross of parents with alternative mutations in the *btr* genes leads to a brittle F_1 hybrid that presents grain retention problems. We evaluated rachis fragility through a mechanical test and under natural conditions, in F_1 crosses with different compositions at the *btr* genes. Brittleness was significantly higher in *Btr1btr1Btr2btr2* crosses compared to hybrids and inbred parents carrying one of the mutations (*btr1btr1Btr2Btr2*/*Btr1Btr1btr2btr2*). This fact could jeopardize the efficient harvest of hybrids bearing alternative mutations, reducing the

choice of possible crosses for hybrid barley breeding, and hindering the exploitation of potential heterotic patterns. Furthermore, non-brittle hybrids showed higher brittleness than inbreds, suggesting the presence of other dominant factors affecting the trait. In conclusion, this work encourages a deeper study of the genetic control of the rachis brittleness trait and urges the consideration of rachis tenacity as a target for hybrid barley breeding.

Keywords
Brittle rachis – shattering – domestication – hybrid breeding – dominance.

1. Introduction

Increased agricultural production needed to meet food demand can only be achieved by "sustainable intensification" (Tilman, Balzer, Hill, & Befort, 2011) of existing croplands, by adopting yield-increasing technologies. In this context, hybrid barley is attracting growing interest as a way to increase productivity per unit area, due to its greater yield potential and yield stability compared to conventional varieties, especially under stress conditions (Longin et al., 2012; Mühleisen, Maurer, Stiewe, Bury, & Reif, 2013; Mühleisen, Piepho, Maurer, Longin, & Reif, 2014).

Hybrid barley is increasingly important in Europe, with a significant market share in Germany, France and the United Kingdom, where it covers between 10 and 25% of the acreage devoted to winter six-row feed barley (Longin et al., 2012). The increased productivity of hybrids is the result of the heterosis due to the presence of a number of genes in heterozygosis (Semel et al., 2006). Hybrid yield gain over inbred parental lines has been estimated at about 10% (Longin et al., 2012; Mühleisen et al., 2013). However, it is important to evaluate the possible deleterious phenotypes resulting from heterozygous genes that are fixed in the conventional varieties. This is the case of the loss of the natural grain dispersal system (Pourkheirandish et al., 2015), one of the most relevant events occurred during barley domestication.

Wild barley (*Hordeum vulgare* ssp. *spontaneum*) has a fragile rachis facilitating seed dissemination, whereas the tough rachis of cultivated barley (*Hordeum vulgare* ssp. *vulgare*) prevents spontaneous disarticulation of mature spikelets, ensuring an efficient harvest (Pankin & von Korff, 2017). Rachis brittleness is controlled by two dominant, closely linked and complementary genes, located on chromosome 3H, *Btr1* and *Btr2*, involved in the thinning and collapse of the cell walls under the rachis node (Ubisch,

1915). In addition to *Btr1* and *Btr2* genes, secondary QTLs for brittle rachis have been detected on chromosomes 5H and 7H (Komatsuda, Maxim, Senthil, & Mano, 2004). Independent recessive mutations in any of the *Btr* genes, *Non-brittle rachis 1* (*btr1*) or *Non-brittle rachis 2* (*btr2*), turn the fragile rachis (brittle) into the tough rachis phenotype (non-brittle). All cultivated barleys present a non-brittle genotype, carrying a mutation in one of these two genes (Pourkheirandish et al., 2015). There is a clear pattern in the geographical distribution of *btr* mutations among cultivated barleys. Barley grown in Europe essentially carries the *btr1* mutation, while *btr2* is more frequent in other world regions (Pourkheirandish et al., 2015). Recently, a new non-brittle causal mutation (*btr1b*) has been described in some landraces of Serbia and Greece (Civáň & Brown, 2017). Hence, the cross of parents with alternative mutations in the *Non-brittle rachis* genes (*btr1btr1Btr2Btr2* by *Btr1Btr1btr2btr2*) would lead to a F_1 hybrid (*Btr1btr1Btr2btr2*) which shows a fragile rachis and, thus, might present grain retention problems (Figure 1).

The aim of this study was to quantify the potential agronomic problem that could arise in F_1 crosses from a real breeding program, testing crosses with different compositions at the *Non-brittle rachis* genes, and to develop a repeatable phenotyping method that could be used routinely in barley breeding programs aiming at hybrid cultivars.

2. Materials and methods

2.1. Plant material

Twenty-three barley (*Hordeum vulgare* L.) F_1 crosses and their twenty-five parents from the Spanish National Public Barley Breeding Program (Gracia et al., 2012) were chosen to represent the three possible combinations at the *Non-brittle rachis* genes. Six crosses *btr1*x*btr1* (short for *btr1btr1Btr2Btr2* x *btr1btr1Btr2Btr2*), six crosses *btr2*x*btr2* (short for *Btr1Btr1btr2btr2* x *Btr1Btr1btr2btr2*), eleven crosses *btr1*x*btr2* (short for *btr1btr1Btr2Btr2* x *Btr1Btr1btr2btr2* or its reciprocal), together with eighteen *btr1* (short for *btr1btr1Btr2Btr2*) and seven *btr2* (short for *Btr1Btr1btr2btr2*) parental lines were selected (Table 1).

2.2. Experimental setup

Two experiments were conducted at the facilities of the EEAD-CSIC located in Zaragoza (41°43'N, 00°49'W), one in a greenhouse and another one in the field. The greenhouse experiment had two replicates, placed in separate cabinets. In the first replicate (block 1) all genotypes (23 F_1 crosses and 25 parents) were evaluated; in the second replicate (block 2) only the genotypes for which remaining F_1 seed was available were assessed. Also, a sample composed of 3 hybrids (one with each combination of the *Non-brittle rachis* genes) and their respective parents were grown under field conditions (Table 1).

Table 1. Selected F_1 crosses for rachis brittleness assessment, *Non-brittle rachis* genes genotype and presence in experiments.

Cross	Female genotype	Male genotype	Hybrid genotype	Block 1 GR	Block 2 GR	Field
CNE-106 x Esterel	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	+		
CNE-126 x Esterel	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	+		
02V017-Z10 x 93Z074-Z1	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	+		
02V017-Z10 x Lavinia	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	+	+	+
04Z001-Z107 x 93Z074-Z1	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	+		
02V017-Z10 x 97V115-Z7	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	*btr1btr1Btr2Btr2*	+	+	
CNE-73 x Cierzo	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	+		
CNE-75 x Cierzo	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	+		
CNE-89 x Cierzo	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	-	+	
CNE-123 x Cierzo	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	+	+	+
CNE-145 x Cierzo	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	+		
CNE-81 x Cierzo	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	*Btr1Btr1btr2btr2*	+		
CNE-6 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	+	+	
CNE-37 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	+	+	+
CNE-49 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	+	+	
CNE-58 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	+	+	
CNE-79 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	-	+	
CNE-98 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	+	+	
CNE-106 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	+	+	
CNE-110 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	+	+	
CNE-138 x Cierzo	*btr1btr1Btr2Btr2*	*Btr1Btr1btr2btr2*	*Btr1btr1Btr2btr2*	-	+	
CNE-135 x Plaisant	*Btr1Btr1btr2btr2*	*btr1btr1Btr2Btr2*	*Btr1btr1Btr2btr2*	-		
CNE-145 x Plaisant	*Btr1Btr1btr2btr2*	*btr1btr1Btr2Btr2*	*Btr1btr1Btr2btr2*	+		

"Block 1 GR" column indicates the first replicate of the greenhouse experiment; "Block 2 GR" column indicates the second replicate from the greenhouse experiment; "Field" column indicates the field nursery experiment. + Presence of that cross and respective parents in a certain experiment; - plant failure.

For the controlled conditions experiment, seven to ten seeds of each genotype were sown in paper-pot trays (block 1 on 24th Nov 2017, block 2 on 30th Jan 2018) and vernalized for 52 days in a cold chamber (4 – 8 ºC, 16-h light/8-h dark photoperiod). After the cold treatment (with the plants at the three-leaf stage), seven to ten plants of each genotype were transplanted to a 60x20x15 cm pot and transferred to a heated sunlit glasshouse (23°C day/18°C night). The transplant mix composition was 2 x 70 L bales of black peat, 1 bag of vermiculite type 3 (100 L) , 2.5 bags of sand (6 kg/bag) and 250 g of slow-release fertilizer Plantacote® 14-9-15 (SQM Vitas, Cádiz, Spain). In addition, plants were fertilized during jointing stage with 3 g/L of Fertipron 20-20-20 (Probelte, S.A., Murcia, Spain). The first block of the greenhouse experiment suffered a powdery mildew (*Blumeria graminis* f. sp. *hordei*) attack, which was controlled with fungicide Bayfidan® (Bayer Hispania, S.L., Barcelona, Spain). The second block was sprayed with fungicides Bayfidan® and Aviator® Xpro (Bayer Hispania, S.L., Barcelona, Spain) as a preventive measure. To avoid spatial effects, the positions of the pots were shuffled every week. Irrigation was applied daily. At maturation stage, the greenhouse temperature was risen to 33 ºC and irrigation was stopped.

Regarding the natural conditions experiment, between 24 and 35 seeds of each genotype were sown in paper-pot trays in 1st Dec 2017. Once emerged (18th Dec 2017), seedlings were transplanted to a field nursery.

2.3. Genotyping

Leaf tissue from individual plants of the parental lines and F_1 crosses was sampled, frozen in liquid nitrogen and homogenized (Mixer Mill model MM301, 140 Retsch). Genomic DNA was extracted using the NucleoSpin® Plant II protocol (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Delaware, USA). Finally, samples were diluted to a final concentration of 50 ng/µl using TE buffer.

Genotypes were checked using specific KASP™ markers (Table S1) for the *Non-brittle rachis* genes developed in-house (via LGC Genomics Ltd, Hertfordshire, UK), which detect the canonical mutations of *btr1* (1 nucleotide deletion), and *btr2* (11 nucleotides deletion). The assay mix preparation and PCR protocols were conducted according to

LGC Genomics protocols in an ABI7500 real-time PCR system (Applied Biosystems, Foster City, CA).

2.4. Phenotyping

For the greenhouse experiment, rachis brittleness was assessed through mechanical processing of spikes in an adapted threshing machine equipped with cooking grade silicone toothed rotor blades. Spikes were threshed for five seconds at 900 rpm. The threshed material was collected in a removable plastic tray. Rachis fragility, in percentage, was calculated as previously reported by Komatsuda et al. (2004), i.e., the percentage of rachis nodes disarticulated over the total number of rachis nodes in a spike, measured in five F_1 plants per genotype, using two spikes per plant, at two different times (2 and 4 weeks after ripening, determined as stage Z91 (Zadoks, Chang, & Konzak, 1974)) (Video S1).

In the field nursery, all the spikes from three to ten plants of each genotype were bagged with breathable and translucent bags (Fito Agrícola S.L., Castellón, Spain). Spontaneous spikelet disarticulation was measured, at three different times (two, three, and four weeks after Z91), through the counting of the number of disarticulated rachis nodes per number of spikes inside the bag.

In addition, the disarticulation scars from a representative sample of brittle and non-brittle spikes were evaluated with the aid of a Nikon SMZ 745 T stereomicroscope connected to a Nikon DS-Fi camera.

2.5. Statistical analysis

In order to satisfy the assumptions required for later analyses (i.e. normality distribution of residuals and homoscedasticity of variances), the variable percentage of brittleness was transformed using an arcsin $\sqrt{x}$ function, suitable for percentage data (Sokal & Rohlf, 1969). All statistical procedures were performed with the transformed data. However, actual percentages are presented in tables and figures, as their interpretation is more intuitive. Differences in rachis brittleness between genotypes, sampling times (two and four weeks after Z91) and blocks (block 1 and block 2) were evaluated using the analysis of variance (ANOVA) “type III” procedure for unbalanced designs in JMP

(SAS Institute, Cary, North Carolina, United States). The ANOVA model included genotype, sampling time, block and genotype by time interaction. Genotype, time and block were all considered fixed factors. The ten spikes sampled per genotype (five plants, two spikes from each) were considered replicates. The contrasts defined were: brittle *vs.* non-brittle types[1], hybrids *vs.* parents within non-brittle type[2], *btr1 vs. btr2* alleles[3], hybrids *btr1*x*btr1 vs. btr2*x*btr2*[4], and finally, parents *btr1 vs.* parents *btr2*[5]. The interactions of all these contrasts with time were also tested. Means were compared using least significant difference (LSD) test ($P<0.05$).

3. Results

3.1. Rachis brittleness differences between brittle and non-brittle types

The analysis of the phenotypic data showed significantly higher rachis brittleness in crosses bearing alternative mutations in the *Non-brittle rachis* genes (*Btr1btr1Btr2btr2*) compared to hybrids and inbred parents carrying one of the deletions conferring the non-brittle phenotype (*btr1btr1Btr2Btr2* or *Btr1Btr1btr2btr2*) in the mechanic test (Table 2, contrast "brittle *vs.* non-brittle").

Table 2. Effects of genotype, block, time, genotype by time interaction, and contrasts on rachis brittleness.

Source of Variation	*df*	*SS*	*MS*	*F*	*p-value*	
Genotype	44	18.540	0.42	29.7	2.44E-158	***
brittle *vs.* non-brittle[1]	1	10.950	11.00	771.1	9.29E-131	***
within brittle	9	0.375	0.04	2.9	1.90E-03	**
within non-brittle	34	4.544	0.13	9.4	9.81E-42	***
Hybrids (*btr*) *vs.* Parents[2]	1	0.217	0.22	15.3	9.79E-05	***
within Hybrids (*btr*)	11	0.720	0.07	4.6	6.72E-07	***
within Parents	22	3.611	0.16	11.6	5.44E-37	***
btr1 vs. btr2[3]	1	0.000	0.00	0.0	9.73E-01	
within *btr1*	22	2.138	0.10	6.8	1.07E-19	***
within *btr2*	11	2.426	0.22	15.5	1.30E-28	***
Hybrids *btr1 vs.* Hybrids *btr2*[4]	1	0.022	0.02	1.5	2.13E-01	
within Hybrids *btr1*	5	0.217	0.04	3.1	9.54E-03	**
within Hybrids *btr2*	5	0.534	0.11	7.5	5.76E-07	***
Parents *btr1 vs.* Parents *btr2*[5]	1	0.101	0.10	7.1	7.76E-03	**
within Parents *btr1*	16	1.844	0.12	8.1	9.76E-19	***
within Parents *btr2*	5	1.769	0.35	24.9	6.48E-24	***
Repetition	9	0.015	0.00	0.1	9.99E-01	
Block[6]	1	6.308	6.31	444.2	7.77E-84	***
Time[7]	1	2.336	2.34	164.5	2.50E-35	***
Genotype * Time	44	2.353	0.05	3.8	1.02E-14	***
brittle *vs.* non-brittle * Time[8]	1	0.510	0.51	35.9	2.76E-09	***
within brittle * Time	9	0.386	0.04	3.02	1.42E-03	**
within non-brittle * Time	34	1.916	0.06	4.0	4.67E-13	***
Hybrids (*btr*) *vs.* Parents * Time[9]	1	0.142	0.14	10.0	1.62E-03	**
within Hybrids (*btr*) * Time	11	0.196	0.02	1.2	2.46E-01	
within Parents * Time	22	1.051	0.05	3.4	2.76E-07	***
btr1 vs. btr2 * Time	1	0.003	0.00	0.2	6.46E-01	
within *btr1* * Time	22	1.146	0.05	3.7	2.65E-08	***
within *btr2* * Time	11	0.649	0.06	4.1	4.85E-06	***
Hybrids *btr1 vs*. Hybrids *btr2* * Time	1	0.002	0.00	0.1	7.08E-01	
within Hybrids *btr*1 * Time	5	0.153	0.03	2.2	5.66E-02	
within Hybrids *btr2* * Time	5	0.042	0.01	0.6	7.03E-01	
Parents *btr1 vs.* Parents *btr2* * Time	1	0.001	0.00	0.1	7.91E-01	
within Parents *btr1* * Time	16	0.557	0.04	2.4	1.17E-03	**
within Parents *btr2* * Time	5	0.456	0.09	6.4	6.69E-06	***
Residuals	1169	16.600	0.01			

df, degrees of freedom; *SS*, sum of squares; *MS*, mean squares; *F*, F- statistic; *P<0.05 **P<0.01 ***P<0.001. Superscript numbers designate each contrast and will be used throughout the text to facilitate tracking.

Considering the two sampling times (two and four weeks after maturation), two blocks, parents and hybrids, the overall percentage of rachis nodes disarticulated shown by brittle types was 55% *vs.* 17% of non-brittle types[1] (Figure 2-A). Moreover, there was a visual difference in the disarticulation scar morphology between types (Figure S1). For the brittle types, 80% of the rachis nodes disarticulated easily into individual triplets, leaving a smooth surface. On the contrary, non-brittle rachises remained almost intact after mechanical processing, even if most grains became separated from the floral axis. Whenever these broke, breaks were mostly harsh (65%), leaving a jagged surface.

Differences between blocks were detected, probably due to the fungal infection mentioned above. Rachis brittleness was distinctly higher in block 2 than in block 1[6]. This was probably influenced by the length and overall volume of the spikes. Plants in block 1 produced spikes of smaller size than plants in block 2. In addition, there were visible differences in grain filling between the blocks. It was optimum in the second block, whereas spikes in the first block presented many shrivelled grains. The spike size difference is clear from the comparison of the average number of triplets per spike between blocks (Table 3), i.e., spikes in block 1 showed, on average, 20% less internodes than in block 2.

Table 3. Number of rachis internodes per spike for each block and *btr* genotype class.

Number of rachis internodes				
	Block 1		Block 2	
btr genotype	N	Mean ± CI	N	Mean ± CI
Parents *btr1*	304	14.28 ± 0.39	240	18.09 ± 0.36
Parents *btr2*	100	13.13 ± 0.67	60	16.88 ± 0.47
Hybrids *btr1*x*btr1*	105	15.84 ± 0.59	40	20.08 ± 0.93
Hybrids *btr2*x*btr2*	94	14.40 ± 0.52	40	19.63 ± 1.00
Hybrids *btr1*x*btr2*	104	15.91 ± 0.62	180	18.08 ± 0.39
TOTAL	707	14.60 ± 0.25	560	18.21 ± 0.23

N, number of spikes assessed within each genotypic class and block; Mean ± CI, mean of the number of rachis internodes ± 95 % confidence interval.

Despite dissimilarities in the range of values, the division between brittle and non-brittle types was clear in both data sets. Brittle types presented significantly higher rachis fragility values than non-brittle types[1] (Table 4). It is clear that the block had an effect on rachis brittleness[6], but the trends were consistent, as indicated by a positive correlation (r = 0.61, in both sampling times) between rachis fragility scores shown by common genotypes assessed in both blocks.

Table 4. Rachis brittleness for levels of brittle-type and block factors.

Rachis brittleness (%)						
	Block 1			Block 2		
Brittle-type	N	Mean ± CI	Groups †	N	Mean ± CI	Groups †
Brittle	104	28.15 ± 4.27		180	70.45 ± 1.98	
Non-brittle	603	12.62 ± 1.07		380	23.86 ± 1.41	
TOTAL[1]	707	14.91 ± 2.14	b	560	38.83 ± 1.18	a

N, number of spikes assessed within each factor level; Mean ± CI, rachis fragility mean (in percentage) ± 95 % confidence interval. † Means in the same row followed by the same letter were not different at P<0.05.

3.2. Rachis brittleness differences within non-brittle types

Significant differences in rachis fragility were found within non-brittle genotypes, in the controlled conditions experiment. Non-brittle hybrids (*btr1*x*btr1* and *btr2*x*btr2*) presented a significantly higher percentage of rachis brittleness (two sampling times averaged) than parents (inbred lines *btr1* and *btr2*)[2] (Figure 2-B).

No gene-specific effect on rachis brittleness was detected when comparing genotypes carrying the *btr1* mutation with genotypes bearing the *btr2* mutation[3], regardless of whether they were hybrids or parents. Furthermore, no significant differences in rachis fragility were found between hybrids *btr1*x*btr1* and *btr2*x*btr2*[4]. However, the effect of the *btr* gene on the percentage of rachis nodes disarticulated was significant when contrasting parents *btr1 vs. btr2*[5] (Table 5), though the size of the effect (1.24%) was probably too low to bear agronomic relevance.

Table 5. Rachis brittleness means (in percentage) ± 95% confidence intervals, averaged for two sampling times, for the genotypic contrasts considered.

Rachis brittleness (%)			
Contrast	N	Mean ± CI	Groups †
btr1 allele *vs. btr2* allele[3]			
btr1	689	16.96 ± 1.03	a
btr2	294	17.00 ± 1.91	a
Hybrids (*btr1*) *vs.* Hybrids (*btr2*)[4]			
*btr1*x*btr1*	145	18.08 ± 2.28	a
*btr2*x*btr2*	134	18.89 ± 2.82	a
Parents *btr1 vs.* Parents *btr2*[5]			
Parents *btr1*	544	16.65 ± 1.16	a
Parents *btr2*	160	15.41 ± 2.59	b
Time[7]			
2w	647	20.82 ± 1.77	b
4w	620	30.35 ± 1.91	a

2w, two weeks post-maturation; 4w, four weeks post-maturation; N, number of spikes assessed within each level; Mean ± CI. † Means followed by the same letter in this column were not significantly different at $P<0.05$ in the analysis of variance.

Regarding within groups variation, genotypes homozygous for *btr2* (both hybrids and parents) showed higher variability in brittleness than genotypes homozygous for *btr1* (F-test $_{\text{within } btr2 \text{ vs. within } btr1} = 2.27$, $P=0.049$).

3.3. Effect of time post-maturation on rachis fragility

We tested the influence of time after maturation on rachis brittleness. When considering the overall means for the whole set of genotypes, the percentage of rachis nodes disarticulated four weeks after maturation was significantly higher than after two weeks[7] (Table 5).

Furthermore, we found a significant interaction between brittle-type and time[8]. Both types increased their fragility with time. This notwithstanding, the increase in the percentage of disarticulated nodes over time for the brittle types doubled that of non-brittle types (Figure 3-A). The contrast of the interaction of hybrids (non-brittle only) against parents by time was significant[9]. While hybrids (*btr*) and parents presented similar rachis fragility at the two weeks sampling, rachis brittleness at the four weeks

sampling increased 15% for the non-brittle hybrids and only 5% for the parents (Figure 3-B).

Finally, we tested the interaction of *Non-brittle rachis* genes as a whole (*btr1 vs. btr2*) with time, and of hybrids (*btr*) and parents, independently (Table 2). We found no significant interaction with time for any of these contrasts.

3.4. Spontaneous disarticulation under natural conditions

Spontaneous spikelet disarticulation was assessed in the field nursery for three hybrids (*btr1*x*btr2*, *btr1*x*btr1* and *btr2*x*btr2*) and their parents. The weather during spike maturation was stormy and windy; therefore, the conditions were favourable for spike breakage.

We found significant differences in spontaneous disarticulation for brittle and non-brittle types (Table 6). Spikes of non-brittle plants were all intact, regardless of the time passed after maturation. On the contrary, we found broken spikes for some of the brittle type plants bagged starting from 3 weeks after Z91 (Figure 4). The fragments found were both big pieces and individual triplets. Because breakage occurs in the rachis, a single breakage results in total loss of the rest of the spike above that point. Moreover, spontaneous disarticulation rose with time in the brittle types.

Table 6. Effects of genotype, time, genotype by time interaction, and contrasts on spontaneous disarticulation (the ratio of number of rachis nodes disarticulated to number of spikes inside the bag) in the field nursery.

Source of Variation	*df*	*SS*	*MS*	*F*	*p-value*	
Genotype	7	0.824	0.118	6.78	3.02E-06	***
Brittle *vs.* non-brittle	1	0.819	0.819	47.14	1.57E-09	***
Time	2	0.11	0.055	3.16	4.79E-02	*
Repetition	8	0.104	0.013	0.75	6.49E-01	
Genotype*Time	14	0.879	0.063	3.61	1.40E-04	***
Residuals	76	1.32	0.017			

df, degrees of freedom; *SS*, Sum of squares; *MS*, Mean squares; *F*, F- statistic. *, **, *** factors significant at P<0.05, P<0.01, and P<0.001, respectively.

4. Discussion

Rachis brittleness has been thoroughly studied for the understanding of barley origin and domestication process (Pourkheirandish et al., 2015; Zohary, 1999). However, limited attention has been paid to the consequences this trait could have on current agriculture because it was not identified as a major problem until the recent development of hybrid cultivars.

We assessed rachis fragility in F_1 hybrids and parents from a breeding program, with different compositions at the *Non-brittle rachis* genes, both under controlled and field conditions. Moreover, we developed a fully standardized protocol for rachis brittleness assessment that could replace other operator-dependent methods (Komatsuda & Mano, 2002; Nalam, Vales, Watson, Kianian, & Riera-Lizarazu, 2006; Watanabe, Fujii, Kato, Ban, & Martinek, 2006). A previously published method also made use of an electrical thresher (Jiang et al., 2014), but the blade modification and the optimization of time of operation of our study allow the replication of the method with complete reliability. To the best of our knowledge, this is one of few studies based on the possible impact of this trait on plant breeding.

4.1. Brittle rachis could limit the range of potential crosses for the development of barley hybrids

Rachis fragility was significantly higher in crosses bearing alternative mutations compared to genotypes carrying one of the deletions conferring the non-brittle phenotype, both in the mechanic test and under natural conditions. These results agree with those obtained by Komatsuda et al. (2004), when evaluating rachis brittleness in F_1 plants from testcrosses between lines from a biparental population and two testers (one *btr1*, and the other *btr2*), as well as with those reported by Pourkheirandish et al. (2015) in test hybrids of cultivars from a world core collection. The latter study reported an average rachis fragility of 54.99% in brittle hybrids contrasting with 7.92% in non-brittle hybrids. Our analysis shows similar overall means for the brittle types (54.96%) and slightly higher values for the non-brittle types (16.97%), being this difference probably due to the higher aggressiveness of our phenotyping approach.

Furthermore, the morphology of the disarticulation scars between brittle (smooth) and non-brittle types (jagged), coincided with that already reported by Pourkheirandish et al.

(2015) between a brittle wild barley accession (OUH602) and its non-brittle mutant (M96-1), as well as with difference between wild (*H. vulgare* ssp. *spontaneum*) and domesticated (*H. vulgare* ssp. *vulgare*) barley archeological remnants (Zohary, Hopf, & Weiss, 2013).

Finally, not only could we observe higher rachis breakage in the brittle types through mechanical test, but also spontaneous rachis disarticulation in a *Btr1btr1Btr2btr2* genotype when grown under natural conditions in the field nursery, but only four weeks after maturity (Z91). Senthil and Komatsuda (2005) detected no differences in rachis brittleness between greenhouse and field conditions, suggesting rainfall and temperature have no significant effects on rachis fragility. We only analyzed one brittle hybrid under field conditions and, therefore, we cannot calculate a correlation with the experiment under controlled conditions.

Therefore, rachis fragility in hybrids derived from crosses of lines bearing alternative mutations in the *Non-brittle rachis* genes could jeopardize the efficient harvest of this type of hybrids and its acceptance in the market. This fact could reduce the choice of possible crosses for hybrid barley breeding.

4.2. The exploitation of certain potential heterotic patterns could be hampered by rachis brittleness

The success of hybrid barley breeding requires defining good heterotic patterns. Barley genetic diversity has not yet been explored from the point of view of finding heterotic patterns (Longin et al., 2012). However, considering that hybrid vigor is the result of the cross of genetically distinct germplasm groups (Melchinger & Gumber, 1998), and that barley genetic differentiation has a geographic basis (Morrell, Lundy, & Clegg, 2003; Muñoz-Amatriaín et al., 2014; Pasam et al., 2014; Poets, Fang, Clegg, & Morrell, 2015; Russell et al., 2016), promising heterotic patterns between geographically isolated populations could arise (Melchinger & Gumber, 1998).

There is an overlap between the geographical distribution of the *Non-brittle rachis* genes mutations and the geographical differentiation of barley. For instance, several authors have reported the genetic divergence of western and eastern barleys (Milner et al., 2018; Morrell & Clegg, 2007; Morrell, Gonzales, Meyer, & Clegg, 2014; Poets et al., 2015; Saisho & Purugganan, 2007), and their intercross may give rise to promising

genetic combinations. However, 'Occidental' barley lines mostly bear the *btr1* mutation, while the 'Oriental' lines mainly carry the *btr2* mutation (Komatsuda et al., 2004; Pankin & von Korff, 2017; Pourkheirandish et al., 2015; Saisho & Purugganan, 2007). Therefore, if a promising combination between predominantly *btr1* and *btr2* carrying pools was found, its exploitation could be prevented by the risk of grain loss when crossing barley lines with alternative mutations in the *Non-brittle rachis* genes.

4.3. Differences in rachis fragility within non-brittle rachis types could indicate a more complex genetic control of the rachis brittleness trait

The degree of rachis toughness in the non-brittle group can be variable (Åberg & Wiebe, 1948). We also found rachis fragility variation within non-brittle types. On the one hand, non-brittle hybrids (*btr1*x*btr1* and *btr2*x*btr2*) showed higher percentage of rachis disarticulation than inbred parents. Although brittle rachis is well explained by a two complementary gene model, the existence of further genetic factors involved in the control of this trait cannot be ruled out (Smith, 1951).

Several mechanisms involved in grain dispersal in the *Poaceae* probably coexist in barley. The brittle rachis character is specific to species within the *Triticeae* tribe, species that produce a spike-shape inflorescence (Avni et al., 2017; Chen, Yen, & Yang, 1998; Li & Gill, 2006; Nalam et al., 2006; Pourkheirandish et al., 2018). Intermediate seed dispersal mechanisms have also been described, as the "weak rachis", characterized for one or two rachis breaks, resulting in the loss of a spike segment (Kaufmann & Shebeski, 1954). Brittle rachis, weak rachis and grain shattering (breakage of grains above the glumes within the rachilla (Sakuma, Salomon, & Komatsuda, 2011)) have all been reported as dominant in barley (Kandemir, Yildirim, Kudrna, Hayes, & Kleinhofs, 2004). Schiemann (1921), concluded that, in addition to the brittleness factors B and R (now the genes *Btr1* and *Btr2*) of wild barley, at least another brittleness factor acting in the same direction existed. Kandemir, Kudrna, Ullrich, and Kleinhofs (2000) mapped a major QTL for weak rachis, *Hst-3*, on the short arm of chromosome 3H. Nonetheless, it was the QTL analyses performed by Komatsuda and Mano (2002) and Komatsuda et al. (2004) which represented a major step forward in the study of the genetic control of rachis brittleness. According to this latter analysis, non-brittle rachis of oriental lines would be controlled by the major gene

btr2 on chromosome 3H and two additional QTLs on chromosomes 5HL and 7H. An unlinked inhibitor gene, designated D, was suggested for the QTL on chromosome 7H, preventing rachis fragility in its *dd* condition. Later, the dense spike 1 (*dsp1*) gene (Taketa, Yuo, Sakurai, Miyake, & Ichii, 2011) was identified as the candidate gene behind this QTL. It reduces spike internode length (increasing spike density), and is correlated with a lower degree of rachis fragility compared with normal (or lax) spikes (Takahashi & Yamamoto, 1949). Lastly, Kandemir et al. (2004) also supported the complex inheritance of the rachis brittleness trait, concluding that there must be at least five genes involved. Besides *btr1* and *btr2* genes, and the D locus reported by Komatsuda and Mano (2002), two additional dominant factors affected brittleness, with the alleles for higher brittleness occurring in the *btr2* gene pool.

All these reports indicate that rachis brittleness is controlled by several genes interacting with each other to control the trait. Two of them are major genes, *btr1* and *btr2*. The hypothesis of additional dominant genetic factors described above agrees with the difference we found between non-brittle hybrids and inbred parents.

We found a broader dispersion range of brittleness values in the *btr2*x*btr2* hybrids (0 – 63%) in contrast to the *btr1*x*btr1* hybrids (0 – 50%). Pourkheirandish et al. (2015) reported similar results when assessing rachis fragility in F_1 plants derived from the cross of 274 cultivars from a world core collection and two testers (one *btr1* and one *btr2*). Their results also showed higher dispersion in the *btr2*x*btr2* hybrids (5 – 37%) compared to the *btr1*x*btr1* genotypes (2 – 17%). We used the test of homogeneity of variances of Bartlett to assess the heteroscedasticity between the *btr1* and *btr2* pools, both in Pourkheirandish et al.'s and in our own data. In both cases, variation within *btr2* non-brittle hybrids was significantly larger than within *btr1*x*btr1* hybrids. Likewise, in our results, rachis brittleness variation was significantly higher within all *btr2* genotypes (hybrids plus parents) compared to *btr1* genotypes. Again, this finding supports the existence of further genetic factors related to the control of rachis fragility with higher prevalence in the *btr2* pool.

4.4. Rachis brittleness changes over time and the response is higher in hybrid genotypes

Rachis fragility increased with time post-maturation. Nonetheless, the effect of time was higher on brittle types than on non-brittle ones, both under controlled conditions and in the field nursery test. This dissimilarity over time was presumably not identified before because previous surveys did not consider time after maturation as a factor (Komatsuda & Mano, 2002; Komatsuda et al., 2004). However, this increase in rachis brittleness with time is in agreement with the increase of smooth scars observed by Snir and Weiss (2014) for several wild barleys, due to the gradual collapse of the thin cell walls around the 'constriction groove', detected in the brittle-types rachis nodes (Pourkheirandish et al., 2015).

Furthermore, we also found a significant effect of time on rachis brittleness in non-brittle hybrids compared to inbred parents (7.6% less brittleness for inbreds 4 weeks after maturation), once again, indicating possible additional dominant genes involved in the control of the trait. This effect could be linked to the specific *btr2* parents used in this study. In fact, Kandemir et al. (2004) suggested that dominant alleles at additional loci affecting brittleness (besides *btr* genes) might confer rachis fragility in hybrids and not in inbred lines.

We do not know whether this effect could lead to spike loss in production fields and, therefore, potential agronomic losses for hybrid barley, and is something that deserves further investigation. However, we observed no spike breakage in non-brittle hybrids in the field evaluation and, therefore, we cannot support a non-brittle hybrid disadvantage with field data.

5. Conclusions and further prospects

Rachis brittleness in hybrids from parents carrying alternative mutations in the *Non-brittle rachis* genes was significantly higher in relation to the rest of genotypes, confirming an actual risk of seed loss in hybrid cultivars with this particular gene combination. Therefore, the search of heterotic patterns for hybrid barley will have to take into account the *btr* genotype of the components of each heterotic group. This situation reduces the choice of possible crosses for hybrid barley breeding, and should

be ammended through pre-breeding approaches. Moreover, the higher percentage of rachis nodes disarticulated in non-brittle hybrids (*btr1*x*btr1* or *btr2*x*btr2*) compared to parents, indicates the existence of further dominant genetic factors involved in the control of the rachis brittleness trait, whose effect increases with time. This effect, however, was small, and we do not know if these differences in non-brittle genotypes will result in yield penalties in the field. The possible agronomic consequences should be assessed accordingly. The phenotyping method here described will facilitate screening for differences in rachis brittleness in cereals.

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Conflict of interest statement

The authors declare no conflict of interest. This research has been partially funded by Syngenta Crop Protection AG, which is involved in hybrid barley breeding, as stated in the acknowledgments section of the manuscript.

Author contributions

EI, AC, and MG conceived this work. MG and EI obtained the plant material. AP developed the phenotyping machine. AC developed the *btr* markers. AC and MF performed the laboratory work. MF and AP carried out the data collection. EI and MF performed the statistical analyses. MF, AC, and EI drafted the document. All the authors read and approved the manuscript.

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Figure legends

Figure 1. Diagram of the *non-brittle rachis* genotype and phenotype of a potential barley cross and resultant hybrid. In the figure, the *non-brittle rachis* genotype of a possible barley cross between inbred lines bearing alternative mutations and its resultant hybrid are represented. The *Btr1* and *Btr2* genes are hypothesized to act as receptor and ligand (Pourkheirandish et al., 2015). Gene products are depicted following graphical representation of Haberer and Mayer (2015).

Figure 2. Contrasts on rachis fragility between (A) brittle and non-brittle types[1], and (B) between hybrids (*btr*) and parents[2]. "Brittle" includes all hybrids *btr1*x*btr2*; "non-brittle" comprises hybrids *btr1*x*btr1*, hybrids *btr2*x*btr2*, parents *btr1* and parents *btr2*. "Hybrids (*btr*)" includes hybrids *btr1*x*btr1* and hybrids *btr2*x*btr2*; "parents" comprises parents *btr1* and parents *btr2*. Raw measurement data points (left), probability density distribution (right), and mean ± 95 % confidence interval of rachis fragility in percentage are represented for each genotypic class within each contrast. Means are averaged for the two sampling times. Asterisks indicate significantly different group means at $P<0.05$ according to the contrast performed for the overall ANOVA with transformed data.

Figure 3. Genotype by time interaction. (A) Brittle-type by time interaction[8], in which "brittle" includes all hybrids *btr1*x*btr2*; "non-brittle" comprises hybrids *btr1*x*btr1*, hybrids *btr2*x*btr2*, parents *btr1* and parents *btr2;* and (B) hybrids (*btr*) *versus* parents by time interaction[9], in which "hybrids (*btr*)" includes hybrids *btr1*x*btr1* and hybrids *btr2*x*btr2*; "parents" comprises parents *btr1* and parents *btr2*. In both panels, raw measurement data points (left), boxplots with medians and interquartile range (center), mean ± 95% confidence interval of rachis fragility in percentage and probability density distribution (right), are represented for each genotypic class, 2 and 4 weeks after maturation. Points with different letter are significantly different at $P<0.05$ according to means separation by LSD.

Figure 4. Spontaneous disarticulation on brittle and non-brittle types assessed 2, 3 and 4 weeks after ripening in field conditions. Spontaneous disarticulation was measured as the number of disarticulated nodes per number of spikes inside a plant bagged. "Brittle" includes all hybrids *btr1*x*btr2*; "non-brittle" comprises hybrids *btr1*x*btr1*, hybrids *btr2*x*btr2*, parents *btr1* and parents *btr2*. Error bars represent 95 % confidence interval. Bars with different letter are significantly different at $P<0.05$ according to the contrast performed for the overall ANOVA with transformed data.

Table legends and footnotes

Table 1. Selected F_1 crosses for rachis brittleness assessment, *Non-brittle rachis* genes genotype and presence in experiments.

"Block 1 GR" column indicates the first replicate of the greenhouse experiment; "Block 2 GR" column indicates the second replicate from the greenhouse experiment; "Field" column indicates the field nursery experiment. + Presence of that cross and respective parents in a certain experiment; - plant failure.

Table 2. Effects of genotype, block, time, genotype by time interaction, and contrasts on rachis brittleness.

df, degrees of freedom; *SS*, sum of squares; *MS*, mean squares; *F*, F- statistic; *P<0.05 **P<0.01 ***P<0.001. Superscript numbers designate each contrast and will be used throughout the text to facilitate tracking.

Table 3. Number of rachis internodes per spike for each block and *btr* genotype class.

N, number of spikes assessed within each genotypic class and block; Mean ± CI, mean of the number of rachis internodes ± 95 % confidence interval.

Table 4. Rachis brittleness for levels of brittle-type and block factors.

N, number of spikes assessed within each factor level; Mean ± CI, rachis fragility mean (in percentage) ± 95 % confidence interval. † Means in the same row followed by the same letter were not different at P<0.05.

Table 5. Rachis brittleness means (in percentage) ± 95% confidence intervals, averaged for two sampling times, for the genotypic contrasts considered.

2w, two weeks post-maturation; 4w, four weeks post-maturation; N, number of spikes assessed within each level; Mean ± CI. † Means followed by the same letter in this column were not significantly different at P<0.05 in the analysis of variance.

Table 6. Effects of genotype, time, genotype by time interaction, and contrasts on spontaneous disarticulation (the ratio of number of rachis nodes disarticulated to number of spikes inside the bag) in the field nursery.

df, degrees of freedom; *SS*, Sum of squares; *MS*, Mean squares; *F*, F- statistic. *, **, *** factors significant at P<0.05, P<0.01, and P<0.001, respectively.