

Green Revolution dwarfing *Rht* genes negatively affected wheat floral traits related to cross-pollination efficiency

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SUMMARY

Hybrid breeding is a promising strategy to quickly improve wheat yield and stability. Due to the usefulness of the *Rht* 'Green Revolution' dwarfing alleles, it is important to gain a better understanding of their impact on traits related to hybrid development. Traits associated with cross-pollination efficiency were studied using Near Isogenic Lines carrying the different sets of alleles in *Rht* genes: *Rht1* (semi-dwarf), *Rht2* (semi-dwarf), *Rht1 + 2* (dwarf), *Rht3* (extreme dwarf), *Rht2 + 3* (extreme dwarf), and *rht* (tall) during four growing seasons. Results showed that the extreme dwarfing alleles *Rht2 + 3*, *Rht3*, and *Rht1 + 2* presented the greatest effects in all the traits analyzed. Plant height showed reductions up to 64% (*Rht2 + 3*) compared to *rht*. Decreases up to 20.2% in anther length and 33% in filament length (*Rht2 + 3*) were observed. Anthers extrusion decreased from 40% (*rht*) to 20% (*Rht1* and *Rht2*), 11% (*Rht3*), 8.3% (*Rht1 + 2*), and 6.5% (*Rht2 + 3*). Positive correlations were detected between plant height and anther extrusion, anther, and anther filament lengths, suggesting the negative effect of dwarfing alleles. Moreover, the magnitude of these negative impacts depends on the combination of the alleles: *Rht2 + 3* > *Rht3*/*Rht1 + 2* > *Rht2*/*Rht1* > *rht* (tall). Reductions were consistent across genotypes and environments with interactions due to magnitude effects. Our results indicate that *Rht* alleles are involved in multiple traits of interest for hybrid wheat production and the need to select alternative sources for reduced height/lodging resistance for hybrid breeding programs.

Keywords: *Triticum aestivum* L., Green Revolution, dwarfing genes, grain yield, hybrid seed, reduced height; yield potential; elite pollinators.

INTRODUCTION

Hybrid wheat breeding is a promising strategy to increase grain yield and stability. With yield improvements ranging from 10 to 25% as well as enhanced resistance against biotic and abiotic stresses, the development of new hybrids is gaining attention among breeding programs worldwide (Boeven et al., 2016; Garst et al., 2023; Longin et al., 2012, 2013; Matuschke et al., 2007; Okada & Whitford, 2019; Sade et al., 2022). In the face of quickly declining arable land expansion and increases in the incidence of abiotic/biotic stresses within climate change, a next wave of increase in potential yield and global production of wheat is needed (Gornicki & Faris, 2014). Thus, the

identification of factors affecting the development of hybrid wheat is of paramount importance in order to accelerate the rate of genetic gain of the crop.

Even though hybrid wheat breeding possesses great potential, the identification of lines with favorable male floral characteristics required for hybrid seed production is currently severely limited by wheat's naturally low ability to cross-pollinate (Boeven et al., 2016; Würschum et al., 2018; Zajaczkowska et al., 2021; Zohary, 1967). These factors and consequently the high costs associated with hybrid seed production explain the difficulty for their development and establishment in the market (<1% of market share), whose interest began 60 years ago with the discovery of male

sterility and restoration systems (Gornicki & Faris, 2014; Whitford et al., 2013). Diverse characteristics are required for the male parent ideotype in order to increase the opportunity for cross-pollination such as high anther extrusion (AE), long anther length (AL) containing abundant pollen that can be easily dispersed and taller than the female parent (Denisow et al., 2022; Whitford et al., 2013). In addition, large spikes with well-spaced spikelets would facilitate the ease of flower opening and pollen dispersal. Moreover, AE and dehiscence of male anthers synchronously with female stigma exertion and receptivity are also of key importance. This large number of characteristics implies a current limitation in genetic resources for elite pollinators (Langer et al., 2014; Nguyen et al., 2015; Okada et al., 2019), which suggests the need for extended studies to detect genetic factors affecting the development of parental lines useful for hybrid wheat programs.

The deployment of *Reduced height (Rht)* semi-dwarfing genes (*Rht-B1* and *Rht-D1*) was a crucial component of the 'Green Revolution' for improving yield potential due to an increased harvest index, lodging resistance, and fertilizer use efficiency (Casebow et al., 2016; Pearce et al., 2011). Due to these factors, more than 70% of wheat cultivars grown globally nowadays incorporate at least one of these semi-dwarfing genes (Boeven et al., 2016; Buerstmayr & Buerstmayr, 2016; Casebow et al., 2016; Evans, 1998; Lu et al., 2013; Miedaner et al., 2022). Currently, 25 major dwarfing genes known as *Rht* genes have been reported (*Rht1-Rht25*) (McIntosh et al., 2017; Würschum et al., 2017).

The widely used GA-insensitive dwarfing genes are homologs located on chromosomes 4BS (*Rht-B1*) and 4DS (*Rht-D1*), encoding DELLA proteins, recognized as negative regulators of the gibberellin acid (GA) signal transduction pathway (Borrill et al., 2022; Pearce et al., 2011; Peng et al., 1999; Van De Velde et al., 2021). These proteins act as repressors of plant growth and are degraded in the presence of GA in the wild-types (*Rht-B1a* and *Rht-D1a*), whereas the mutant alleles *Rht-B1b* (also designated as *Rht1*) and *Rht-D1b* (*Rht2*) have a reduced sensitivity to GA resulting in decreased plant height while improving the translocation of assimilates. Moreover, a wide variety of pleiotropic effects related to DELLA proteins have been reported. Diverse roles such as cell elongation, seed germination or the transition to flowering, pollen development, and biotic/abiotic stress tolerance are also affected (Tiaz & Zeiger, 2015; Wilhelm et al., 2013). In drought or heat-stressed environments, varieties carrying dwarfing alleles lose their advantage due to decreased seedling emergence caused by reduced coleoptile length (Borrill et al., 2022; Flintham et al., 1997). Different *Rht* allele combinations can also be used to achieve a wider range of plant height and agronomic phenotypes and are also linked with reductions in seed size and protein content (Casebow et al., 2016; Jobson et al., 2019) and alpha-

amylase activity (Van De Velde et al., 2017). Although the increases in crop yield through the use of the Green Revolution *Rht* genes have been widely documented, the impact of these genes on floral traits related to hybrid development has only been partially addressed showing some contradictions.

According to Boeven et al. (2016), genotypes carrying *Rht* semi-dwarfing genes might lack good male floral characteristics needed for hybrid seed production. Some investigations exploring genome-wide association studies (GWAS) and biparental mapping populations (He, Lillemo, et al., 2016; He, Singh, et al., 2016; Muqaddasi et al., 2016; Muqaddasi, Brassac, et al., 2017; Muqaddasi, Pillen, et al., 2017; Okada et al., 2019) reported contrasting results regarding the effect of semi-dwarfing alleles (*Rht1* and *Rht2*) on anther extrusion (AE). For their part, the effect of extreme dwarfing alleles such as *Rht3* and *Rht2 + 3* or *Rht1 + 2* on this trait has not been reported so far.

The anther length (AL) has also shown a significant positive correlation with AE, pollen grain number per anther, and pollen mass when the addition of rye chromosome 4R was assessed in wheat (Nguyen et al., 2015). Moreover, Langer et al. (2014) observed a positive association between plant height (PH) and pollen mass in wheat, while Beri and Anand (1971) reported that increased PH had a positive effect on pollen grain shed outside the floret. Some authors using bi-parental mapping populations under controlled conditions suggested that different *Rht-B1* and photoperiod response (*Ppd-1*) alleles combinations can be used for selecting a suite of pollinator traits for successful hybrid wheat seed production (Okada et al., 2019). However, more studies assessing the effect of several *Rht* alleles under different environmental field conditions are necessary to confirm this association.

Due to the importance of *Rht* dwarfing alleles in breeding programs, it is vital to explore how these alleles affect those traits related to the development of hybrid wheat seed production. Furthermore, prior experiments exploring the effects of separate semi-dwarfing alleles on these traits have been conducted in growth chambers, which may not be representative of field conditions. In addition, the influence of these genes on wheat pollinator traits has been evaluated only in biparental populations or using a genome-wide association study (GWAS) (Okada et al., 2019), without considering the effect of each *Rht* gene and their combinations in different genotypic backgrounds.

In this work, we intend to explore the influence on floral and agronomic traits with relevance for hybrid breeding of different combinations of the Green Revolution *Rht* genes using near-isogenic lines (NILs), which are very precise and useful populations available to study the effects of specific genes. The purpose of this research is to assess the effect of the *Rht* alleles on floral and agronomic traits under different field conditions. We found that the

dwarfing *Rht* genes negatively influenced the floral traits related to pollination efficiency, with consistent effects across environments.

RESULTS

With the exception of DTA, the floral and agronomical traits evaluated were highly significantly influenced by the environments, alleles, and genotypes as well as the double (*Environment* × *Allele*, *Allele* × *Genotype*; *Environment* × *Genotype*) and triple interactions involved (*Environment* × *Allele* × *Genotype*) (Table 1). The mean values of single

variables are shown in Table 2. Correlations among cross-pollination-related traits are presented in Figure 1.

Significant ($P \leq 0.001$) and positive correlations were detected between plant height and anther extrusion (0.69), anther length (0.49), and anther filament length (0.62). Anther extrusion also showed a positive correlation with anther length (0.31), anther filament length (0.38), and DTA (0.36) and a weak negative correlation with spike length and number of spikelets per spike (Figure 1).

As a mean, significant differences among the four environments and genotypes were reported for all the

Table 1 Means square and *P*-value (ANOVA) of different pollinator traits in four wheat genotypes carrying six different *Rht* alleles in four environments

Source of variation	D.F.	AE	AL	AFL	PH	DTA	SpkL	Spikl/Spk
Environment (E)	3	5372***	5.15***	11.06**	2312.0***	4700 ***	134.5***	88.64***
Error A	6	39.25	0.008	0.59	9.49	5.33	0.21	1.74
Allele (ALL)	5	7117***	2.83***	47.9***	41316***	2.02 ns	4.96***	7.26***
E × ALL	15	601.7***	0.12***	3.16***	42.17***	1.84 ns	1.48***	3.65***
Error B	40	47.34	0.015	0.33	4.57	2.98	0.12	0.39
Genotype (G)	3	13315***	0.37***	13.9***	8999.3***	25.03***	24.85***	23.21***
E × G	9	774.0***	0.18***	6.24***	74.16***	11.36***	3.34***	3.04***
ALL × G	15	668.1***	0.064***	1.56***	547.7***	3.15 ns	1.80***	2.83***
E × ALL × G	45	146.7***	0.047***	0.93***	35.77***	1.85 ns	1.13***	1.82***
Error C	144	45.66	0.009	0.24	9.35	2.63	0.249	0.47
Total	287							

AE, anther extrusion; AFL, anther filament length; AL, anther length; DTA, days to anthesis; ns, non-significative; PH, plant height; Spikl/Spk, spikelets per spike; SpkL, spike length.

*** $P < 0.001$ and ** $P < 0.05$.

Table 2 Mean values of different pollinator traits in four wheat genotypes carrying six different *Rht* alleles in four environments

Source of variation	AE (%)	AL (mm)	AFL (mm)	PH (cm)	DTA	SpkL (cm)	Spikl/Spk
Environment							
2019-IPK	21.29 b	3.15 b	7.51 a	84.45 a	150.1 b	11.67 b	19.31 b
2021-IPK	27.16 a	3.32 a	6.75 b	75.44 b	158.7 a	9.94 c	19.27 b
2021-ARG	7.06 d	3.17 b	6.66 b	70.94 c	139 c	12.40 a	21.43 a
2022-IPK	14.80 c	2.70 c	6.80 b	75.44 b	148.4 d	9.54 d	19.08 b
LSD	2.56	0.04	0.31	1.26	0.94	0.19	0.54
Allele							
<i>rht</i> (tall)	39.37 a	3.49 a	8.55 a	118.7 a	149.4	10.42 d	19.12 d
<i>Rht1</i>	19.95 b	3.19 b	7.16 b	94.04 b	148.8	10.73 c	19.50 c
<i>Rht2</i>	20.44 b	3.11 c	7.38 b	91.39 c	149	10.85 bc	19.88 b
<i>Rht1</i> + 2	8.32 cd	2.97 d	6.32 c	60.49 d	149.1	11.40 a	19.93 b
<i>Rht3</i>	10.86 c	2.96 d	6.48 c	51.98 e	149.2	10.91 b	20.12 a
<i>Rht2</i> + 3	6.54 d	2.74 e	5.69 d	42.79 f	149	11.00 b	20.09 ab
LSD	2.84	0.05	0.24	0.88	ns	0.15	0.26
Genotype							
April Bearded	37.63 a	2.99 c	7.15 b	92.01 a	149.4 a	10.72 b	20.25 a
Bersee	12.74 b	3.06 b	6.42 d	76.57 b	149.4 a	10.87 b	19.60 b
Maris Huntsman	7.36 c	3.13 a	7.41 a	65.90 d	148.4 b	10.27 c	20.20 a
Maris Widgeon	12.58 b	2.78 d	6.74 c	71.78 c	148.8 b	11.68 a	19.05 c
LSD	2.23	0.03	0.16	1.00	0.53	0.16	0.23

Means followed by the same letter within the same factor and trait are not statistically different (LSD $P \leq 0.05$).

AE, anther extrusion; AFL, anther filament length; AL, anther length; DTA, days to anthesis; ns, non-significative; PH, plant height; Spikl/Spk, spikelets per spike; SpkL, spike length.

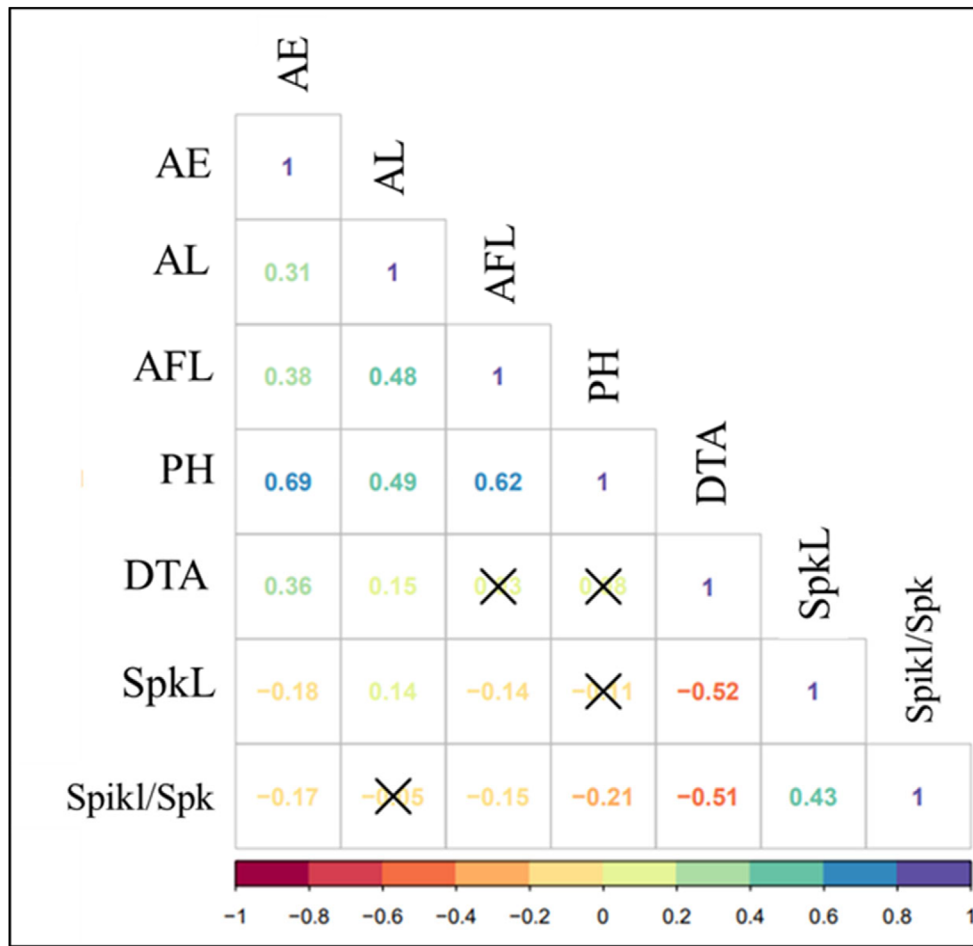


Figure 1. Correlations among cross-pollination ability traits in the 24 NILs across the four environments explored. The color reflects the strength of the correlation. Non-significant correlations at $P < 0.05$ are expressed using crosses. AE, anther extrusion; AFL, anther filament length; AL, anther length; DTA, days to anthesis; PH, plant height; Spikl/Spk, spikelets per spike; SpkL, spike length.

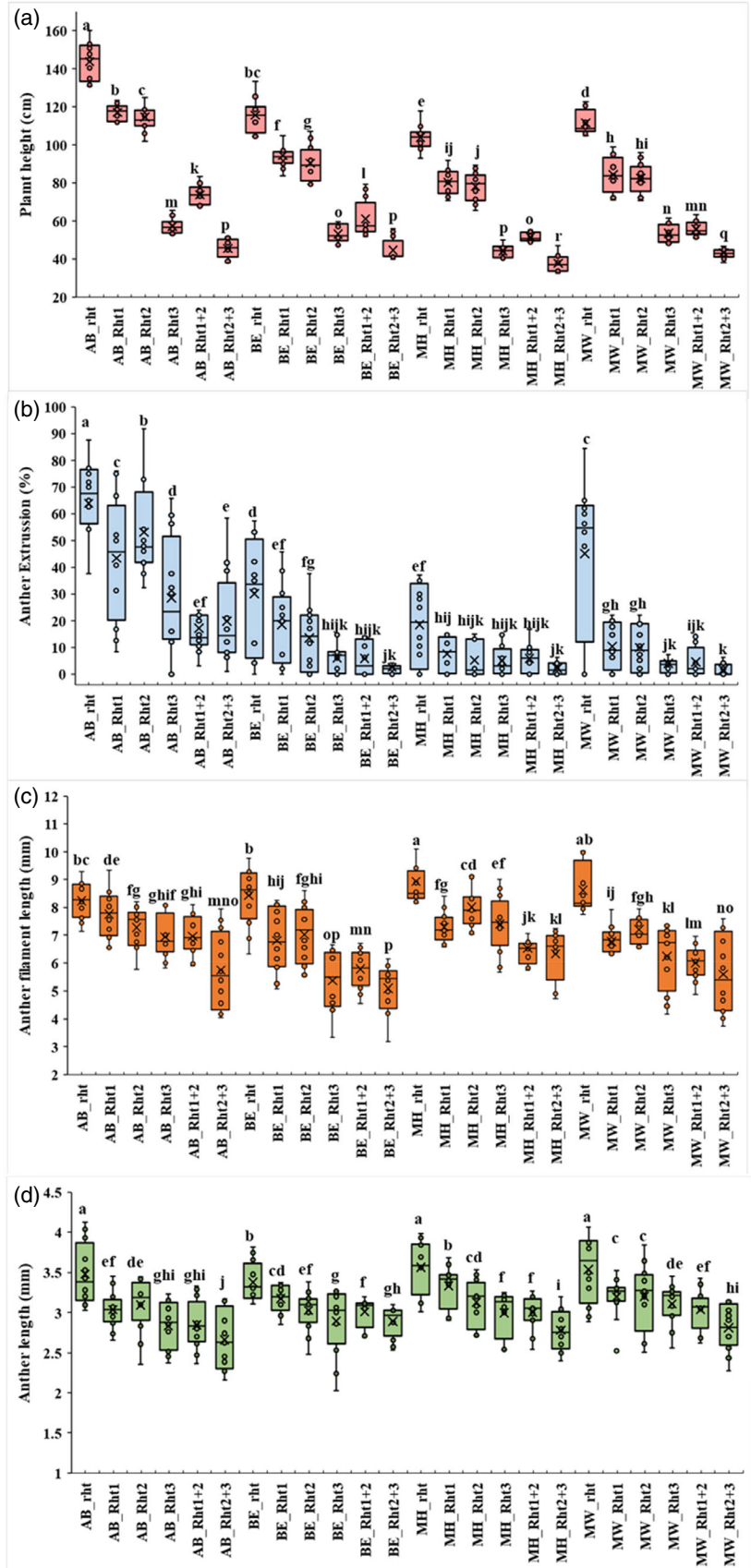
pollinator traits (Table 2). Under Argentinian field conditions, lower values for AE, AFL, PH, and DTA were reported, while higher values were detected for SpkL and Spikl/Spk compared to the three environments explored in Germany (Table 2; Figures S1 and S2). Compared to *rht* (tall), the extreme dwarfing allele (*Rht 2 + 3*) reduces anther extrusion from 39.37% to 6.54%; anther length (-21.5%), anther filament length (-34.2%) and plant height (-64%), showing similar reductions for *Rht3* and *Rht1 + 2*. For their part, intermediate responses for the semi-dwarfing *Rht1* and *Rht2* were reported for all the traits. Moreover, lower values for spike length and spikelets per spike were detected for *rht* (tall). Regarding the genotypic differences, April Bearded showed higher AE, PH, and Spikl/spk. For their part, Maris Huntsmann presented longer anthers (AL) and AFL and lower AE. Intermediate values were reported for Bersee (Table 2).

Highly significant effects of the different *Rht* dwarfing alleles among the genotypes on traits related to cross-

pollination ability were found (Figures 2–4). For all the floral and agronomical traits, important differences between the four cultivars as well as a differential magnitude of the response among the alleles evaluated within the same genotype were reported. The effects of the *Rht* dwarfing mutations on anther traits, including extrusion were consistent with their strength, the stronger the dwarfing allele the greater the reduction compared to *rht* (tall). In spite of the significant double and triple interactions, this behavior was significant and consistent across the different genetic backgrounds and environments, although the magnitude of the response varied.

Plant height showed significant differences between the NILs among the four genetic backgrounds (Figure 2a). Reductions in the PH of 18.3–26% were documented for *Rht1* and *Rht2* compared to the wild-type allele (*rht*). For their part, decreases of 47–50.4% and 52–60.2% were documented when *Rht1 + 2* and *Rht3* were compared to *rht* (tall), respectively. Extreme effects were reported when

Figure 2. Mean values for (a) plant height (cm), (b) anther extrusion (%), (c) anther filament length (mm), and (d) anther length (mm) for the *Allele* × *Genotype* interaction in 24 winter wheat NILs across four environments. Genotypes: AB (April Bearded), BE (Bersee), MH (Maris Huntsman), and MW (Maris Widgeon). Rht alleles: *rht* (tall), *Rht1* (Semi-dwarf), *Rht2* (Semi-dwarf), *Rht3* (extreme dwarf), *Rht1 + 2* (dwarf), *Rht2 + 3* (extreme dwarf). Data points of the four environments explored, error bar, outliers, median, and mean values (crosses) are shown. Means followed by the same letter are not statistically different (LSD $P \leq 0.05$).



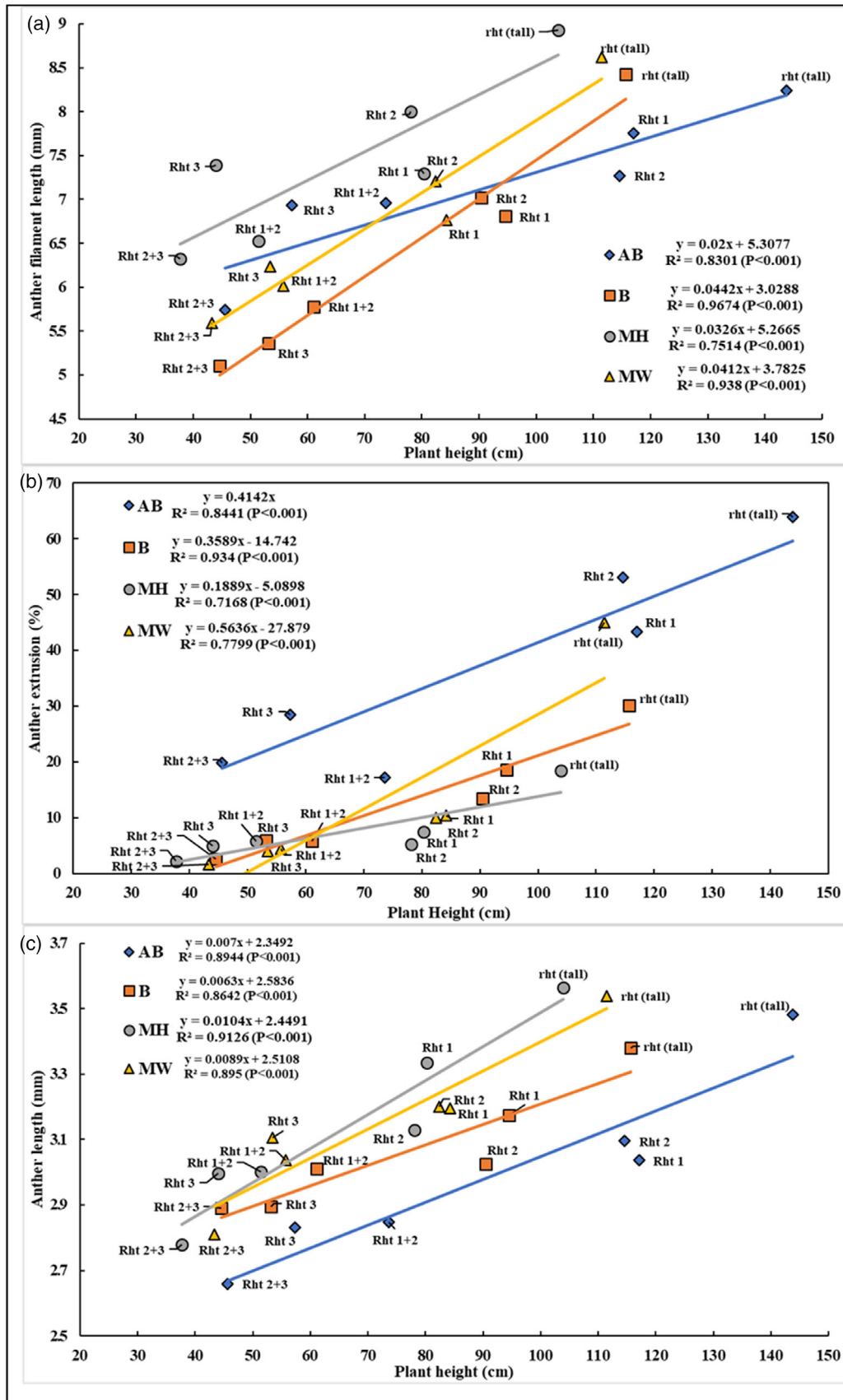


Figure 3. Linear regression between plant height (cm) and (a) anther extrusion (%), (b) anther filament length (mm), and (c) anther length (mm). Points represent the means of the interaction *Allele* × *Genotype* interaction in four winter wheat genotypes carrying six different combinations of *Rht* dwarfing genes across four environments. Genotypes: ◆ AB (April Bearded); ■ B (Bersee); ● MH (Maris Huntsman); ▲ MW (Maris Widgeon). *Rht* alleles: *rht* (tall), *Rht1* (Semi-dwarf), *Rht2* (Semi-dwarf), *Rht1* + 2 (dwarf), *Rht3* (dwarf), *Rht2* + 3 (extreme dwarf).

rht (tall) lines were contrasted with the extreme dwarf double mutant *Rht2* + 3, showing reductions of 68.3% (April Bearded), 61.5% (Bersee), 63.7% (Maris Huntsman), and 61.2% (Maris Widgeon). When the different alleles were evaluated across the four environments, the order of the negative effect of dwarfism genes on PH was maintained, but differences were detected in the final height reached, with lower values in the ARG21 environment (Figure S1a).

For anther extrusion, important reductions were found in the four genotypes when extreme dwarfing alleles (e.g., *Rht2* + 3, *Rht1* + 2, and *Rht3*) were compared to *rht* (tall) (Figure 2b). AE values ranging from 63.9% to 19.8% (April Bearded), 30% to 2.6% (Bersee), 18.5% to 2.1% (Maris Huntsman), and 45% to 1.6% were found when *rht* (tall) were contrasted with the extreme dwarfing lines *Rht2* + 3. Lower reductions were reported for the commercial semi-dwarf alleles (*Rht1* and *Rht2*). In addition, important differences among genotypes and environments were documented. The *Allele* × *Environment* interaction showed lower values and less differences among *Rht* alleles in the environment explored in Argentina (Figure S1b).

For their part, decreases in AFL up to 30.3% for April Bearded, 39.4% (Bersee), 29.1% (Maris Huntsman), and 35.2% (Maris Widgeon) were reported when *rht* (tall) were compared with *Rht2* + 3. NILs carrying semi-dwarfing alleles (*Rht1* and *Rht2*) showed AFL reduction ranging 6 to 21% compared to *rht* (tall) (Figure 2c). Differences among the same *Rht* level among different environments explain the significant differences in the *Allele* × *Environment* interaction (Figure S1d).

The anther length also exhibited significant decreases in NILs carrying extreme dwarfing alleles while semi-dwarfing alleles (*Rht1*, *Rht2*) presented an intermediate response. Reductions up to 23.6% (April Bearded), 14.5% (Bersee), 22% (Maris Huntsman), and 20.6% (Maris Widgeon) were documented when *rht* (tall) were contrasted with lines carrying the extreme dwarf *Rht2* + 3 (Figure 2d). As was reported for the other pollinator traits, the significant *Allele* × *Environment* interaction was explained due to differences in the same *Rht* alleles among the four environments, while the order of the negative effects exerted by the dwarfing alleles was maintained (Figure S1c). Linear regression between PH and AE, AFL, and AL showed a strong association ($P \leq 0.001$) in the four genetic backgrounds and environments tested (Figure 3a–c; Figure S2).

For spike length, increases for Maris Huntsman and Maris Widgeon were detected when dwarfing and extreme dwarfing alleles were compared with *rht* (tall) (Figure 4a). In this sense, spike length increases between 5.8% and

22% were reported for M. Huntsman and 3.2–12.1% (M. Widgeon) when dwarfing alleles were compared with *rht* (tall). For the *Allele* × *Environment* interaction, higher spike length as well as a greater increase for dwarfing alleles were reported in the ARG21 environment (Figure S3a).

The number of spikelets per spike also showed a tendency to increase in NILs carrying dwarfing and extreme dwarfing alleles (Figure 4b). Compared to the *rht* (tall), rises of 8.2% for April Bearded, 4.2% (Bersee), 9% (Maris Huntsman), and 6.9% for Maris Widgeon were detected. The ARG21 trial showed higher spikelets number and higher increases for severe dwarfing alleles (*Rht3* and *Rht1* + 2) compared to environments explored in Germany (Figure S3b).

For their part, even the environment explored in Argentina showed a shorter crop cycle than in Germany, no differences were reported for days to anthesis among alleles, *Alleles* × *Genotypes* and *Environment* × *Alleles* × *Genotypes* interactions (Table 1, Figure 4c).

Effect of dwarfing genes on grain yield-related parameters

The grains per square meter and thousand kernel weight showed highly significant differences for all the factors and the interactions evaluated (Table S1). For both variables, the experiment performed in Argentina showed lower values (up to –32.4% for TKW and –31.1% for GN) compared to those conducted in Germany. As a mean, genotypes carrying *rht* (tall) showed higher TKW values compared to the semi-dwarfing *Rht1* and *Rht2* alleles (–13.1%) while reductions up to 23.2% were reported for NILs carrying severe dwarfing *Rht3*, *Rht1* + 2 and *Rht2* + 3 (Table S2). The grain number was increased by *Rht2* (+25.6%), *Rht1* (+19.7%), *Rht1* + 2 (+15.1%), and *Rht3* (+6.7%) compared to *rht* (tall). Only the extreme dwarf *Rht2* + 3 showed decreases in GN (–24.8%) compared to *rht* (tall). Regarding the genotypic variability, the TKW ranged between 36.58 g (Maris Widgeon) to 25.44 g for April Bearded, while the GN ranged from 36 969 (Bersee) to 24 056 grains per square meter for Maris Widgeon (Table S2).

The *Allele* × *Genotype* interaction for GN and TKW is shown in Figure 5(a,b). For both variables, important genotypic variations and differential percentage responses due to different allele combinations within the same genotype were detected. For GN, all the genotypes showed higher values for the semi-dwarfing Green Revolution allele *Rht2*, while the extreme dwarf *Rht2* + 3 presented lower values. This response was similar in the four environments tested (Figure S4a). Compared to the *rht* (tall), increases of 41.2%

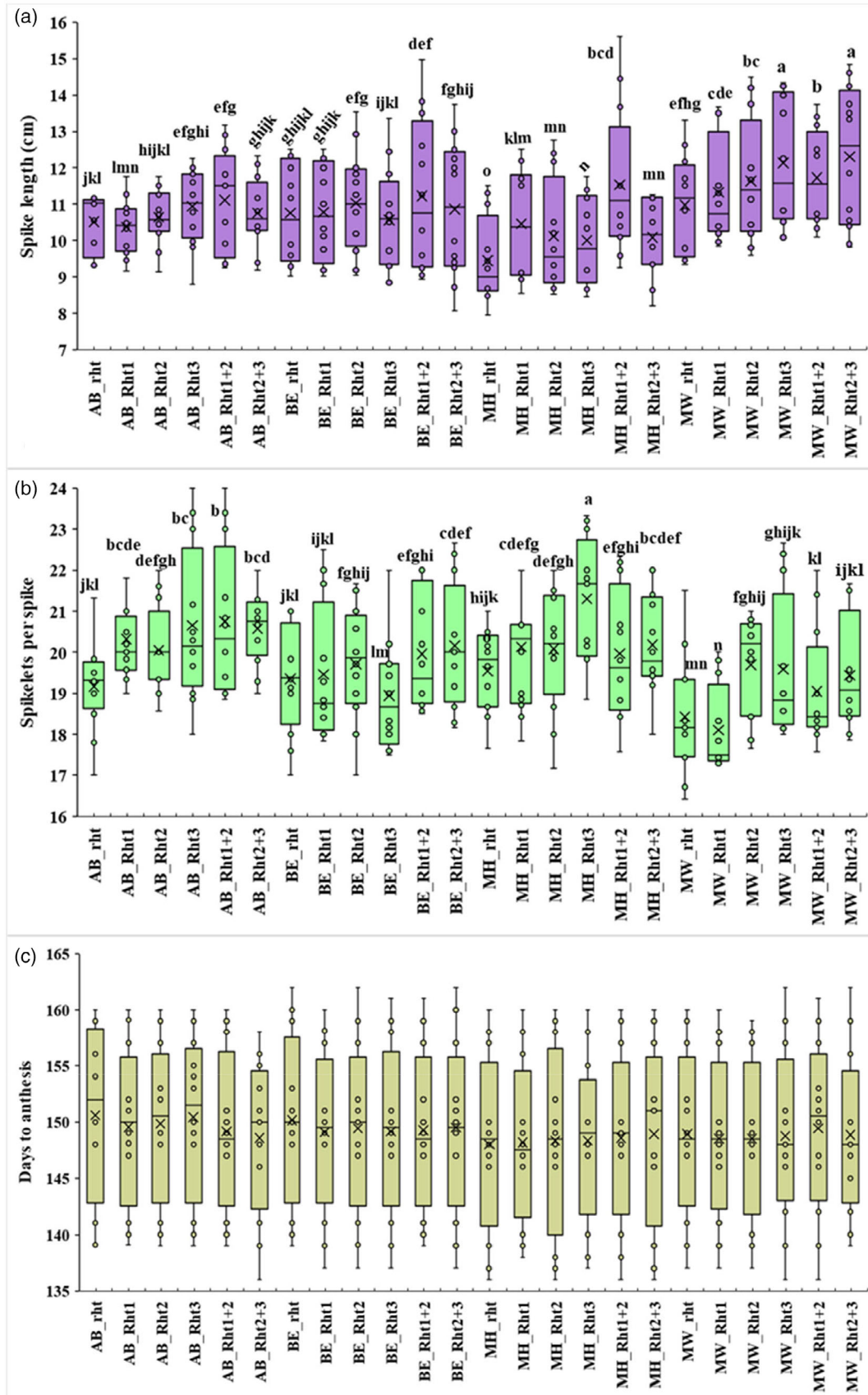


Figure 4. Mean values for (a) spike length, (b) spikelets per spike, and (c) days to anthesis for the *Allele* × *Genotype* interaction in 24 winter wheat NILs across four environments. Genotypes: AB (April Bearded), BE (Bersee), MH (Maris Huntsman), and MW (Maris Widgeon). Rht alleles: *rht* (tall), *Rht1* (Semi-dwarf), *Rht2* (Semi-dwarf), *Rht3* (extreme dwarf), *Rht1* + 2 (dwarf), and *Rht2* + 3 (extreme dwarf). Data points of the four environments explored, error bars, outliers, median, and mean values (crosses) are shown. Means followed by the same letter are not statistically different (LSD $P \leq 0.05$).

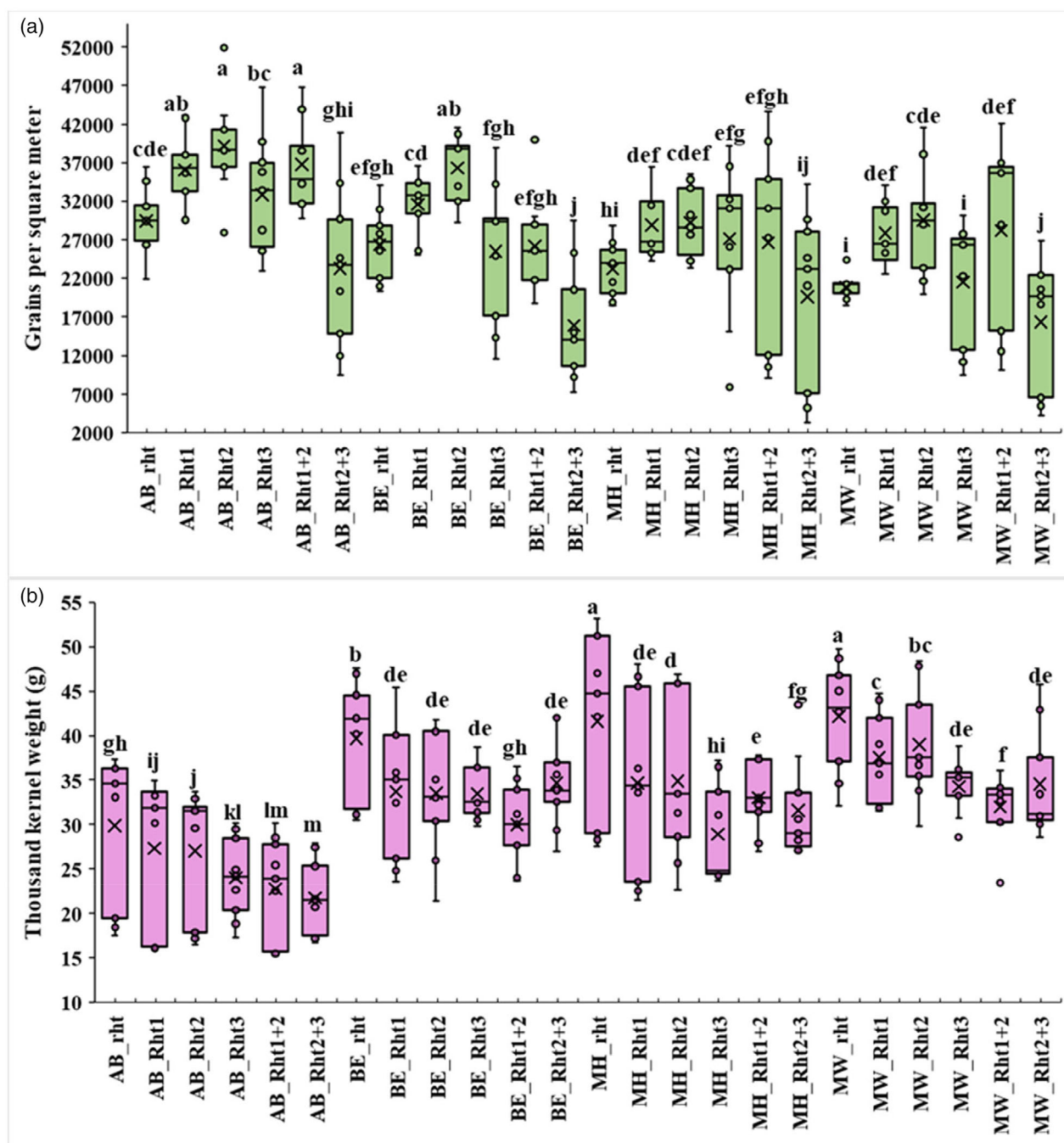


Figure 5. Mean values for (a) grains per square meter and (b) thousand kernel weight for the *Allele* × *Genotype* interaction in 24 winter wheat NILs across four environments. Genotypes: AB (April Bearded), BE (Bersee), MH (Maris Huntsman), and MW (Maris Widgeon). Rht alleles: *rht* (tall), *Rht1* (Semi-dwarf), *Rht2* (Semi-dwarf), *Rht3* (extreme dwarf), *Rht1* + 2 (dwarf), *Rht2* + 3 (extreme dwarf). Data points of the four environments explored, error bars, outliers, median, and mean values (crosses) are shown. Means followed by the same letter are not statistically different (LSD $P \leq 0.05$).

(Maris Widgeon), 37.5% (Bersee), 33% (April Bearded), and 26.1% (Maris Huntsman) were reported for *Rht2*. The *Rht1* also showed high GN values and generally did not show

significant differences with *Rht2* (except for Bersee). For TKW, the degree of dwarfism was highly correlated with the reductions in this variable. In this sense, all the

genotypes showed higher TKW values for NILs carrying the *rht* (tall) with important genotypic differences: April Bearded (29.84 g), Bersee (39.66 g), Maris Hunstmann (41.6 g), and Maris Widgeon (42.2 g), while *Rht1* and *Rht2* presented intermediate values in all the genotypes. The NILs carrying extreme dwarfing alleles showed reductions in TKW up to -30.5% for Maris Hunstmann, -27.1% for April Bearded, and *ca.* -24.3% for Bersee and Maris Widgeon compared to the *rht* (tall).

For their part, the *Allele* \times *Environment* interaction presented lower values for GN and TKW under the Argentinian environment. For GN, higher reductions due to extreme dwarfing alleles were reported for the ARG21 environment compared to field experiments conducted in Germany (IPK21 and IPK22). Moreover, no differences were reported for TKW among *Rht* alleles in ARG21 while reductions were reported for semi-dwarfing and dwarf alleles for IPK21 and IPK22 (Figure S4a,b).

DISCUSSION

Although the development of hybrid wheat has been identified as a fundamental tool for increasing yield potential and tolerance to biotic/abiotic stresses, a scarcity of elite pollinators has been reported (Langer et al., 2014; Nguyen et al., 2015; Okada et al., 2019). The redesigning of floral architecture has been identified as a crucial factor in promoting cross-pollination and ensuring a high level of pollen availability for wheat hybrid seed production (Thompson & Hake, 2009; Whitford et al., 2013). This fact implies the need for new studies to identify the factors influencing the development of germplasm useful for hybrid wheat programs. In this work, a novel approach was reported to evaluate the effect of different combinations of the most widely deployed *Rht1* and *Rht2* Green Revolution semi-dwarfing alleles as well as the extreme dwarfing *Rht3*, *Rht2 + 3*, and *Rht1 + 2* on floral and agronomic traits related to cross-pollination ability for further importance in hybrid wheat seed development.

The discovery of *Rht* genes played a crucial role in the development of modern high-yielding wheat varieties due to their capacity to allocate more energy to grain production (Borrill et al., 2022). With regards to the impact of the *Rht1* and *Rht2* mutations on plant height, the effects observed were consistent with those reported by Flintham et al. (1997) and Youssefian et al. (1992) explained by reductions in internode and peduncle length. These observations highlighted the strength of the dwarfing genes, which are presumably dependent on their effects inhibiting gibberellin signaling (Peng et al., 1999).

Regarding spike morphological traits, we reported a trend of increases for both spike length and spikelets per spike for dwarfing alleles explained by an increased portioning of photosynthates to the developing ear and higher floret fertility leading to a high harvest index, as suggested

for the semi-dwarfing *Rht1* and *Rht2* (Miralles et al., 1998; Slafer et al., 2023; Ukozehasi et al., 2022; Youssefian et al., 1992). In concordance with Börner et al. (1993) and Buerstmayr and Buerstmayr (2016) non-significant effects of the *Rht* alleles assessed were detected for flowering time, however, other authors suggested contrasting responses and strong *Environment* \times *Allele* \times *Genotype* effect for this traits (Ukozehasi et al., 2022).

The positive role of Green Revolution genes *Rht1* and *Rht2* that lead to higher grain yield potential has been extensively reported worldwide (Flintham et al., 1997). In agreement with our results, Sherman et al. (2014) and Tang et al. (2021) indicate that the semi-dwarfed plants carrying these genes are associated with an increase in productive tillers and grains per spike, leading to higher GN and grain yield. Nonetheless, their effect using NILs including double mutant lines (*Rht1 + 2* and *Rht2 + 3*) in different environments has been studied to a lesser extent (Börner et al., 1993). However, Austin (1999) suggested that in severe dwarfism alleles, reductions in grain yield were evident related to a low aboveground biomass accumulation explained by reduced leaf area index and consequently reduced light interception and radiation use efficiency. In our experiments and coinciding with Nagel et al. (2013), strong reductions in thousand kernel weight were detected for lines carrying dwarf and severe dwarfism alleles (*Rht3*, *Rht1 + 2*, and *Rht2 + 3*) compared to *rht* (tall). Nonetheless, Börner et al. (1993) reported that the increases in GN for *Rht1/Rht2* were sufficient to compensate for the reductions in TKW and resulted in higher grain yields compared to *rht* (tall). In our experiments, also those reductions were compensated in some cases by significant increases in GN as for the mutant line *Rht1 + 2* in the April Bearded background, the *Rht3* in the Maris Huntsman background, and the *Rht1/Rht2* in the Maris Widgeon background. The lower yield potential (10.2 tn ha^{-1} versus 6.4 tn ha^{-1}) plus limitation in soil and climatic conditions in the ARG21 environment explained the lower GN and TKW values obtained (Figure S4) (Global Yield Gap and Water Productivity Atlas, 2023).

Due to the wheat's cleistogamous nature, it is necessary to increase anther filament length and, consequently, AE to ensure sufficient pollen to pollinate the female lines. Whitford et al. (2013) also suggest that high AE in female lines will also increase the cross-fertilization rates due to a positive association with the receptivity of the stigmas. In the same sense, Murai et al. (2002) suggested that well-spaced spikes carrying large lodicules, a soft lemma, and palea would facilitate the wide opening of each floret. Environmental and genotypic variability here reported for male floral traits under field conditions were partially documented for AE (Boeven et al., 2016; Garst et al., 2023; Langer et al., 2014; Muqaddasi et al., 2016; Muqaddasi, Brassac, et al., 2017; Muqaddasi, Pillen, et al., 2017; Sade

et al., 2022), AL (Komaki & Tsunewaki, 1981; Nguyen et al., 2015; Song et al., 2018), and AFL (Beri & Anand, 1971; Denisow et al., 2022). Our results showed that the degree of dwarfism produced by the different combinations of *Rht* genes resulted in significant reductions in parameters of strategic importance for the development of elite pollinators needed for hybrid wheat breeding programs. The magnitude of these detrimental effects was closely related to the degree of dwarfism of the alleles: *Rht2* + 3/*Rht3* (severe dwarfing) > *Rht1* + 2 (dwarf) > *Rht2/Rht1* ('Green Revolution' semi-dwarfs) > *rht* (tall). In this sense, average reductions in traits of interest for parental lines of -48% (AE), AL (-24%), AFL (-39%), and PH (-68%) were reported for NILs carrying extreme dwarfing alleles. These adverse responses can be confirmed by the correlations between PH and AE, AL and AFL across the different genotypes and environments explored (Figures 1 and 3; Figure S2). In line with this, previous reports have suggested positive associations between PH and AL with floral traits related to cross-pollination efficiency such as AE, pollen grain number per anther, and pollen mass (Nguyen et al., 2015). Moreover, Langer et al. (2014) observed a positive association between PH and pollen mass, while Beri and Anand (1971) observed that increased PH and AFL had a positive effect on pollen grain shed. Denisow et al. (2022) reported that AE played a key role in cross-pollination ability showing a close relation with long anther filaments. For their part, Sage and De Isturiz (1974) evaluating two spring wheat genotypes under greenhouse conditions described that PH was moderately positively correlated with the AFL, which could explain the poor AE and pollen shedding capacity of wheat lines carrying dwarfism alleles. However, the response of different *Rht* genes and their combinations tested under multiple field environments using NILs in four genetic backgrounds, as explored in our study, has not been reported up to now.

Contrasting results regarding the effect of *Rht* genes in floral traits such as AE have been reported. Some authors describe that *Rht-B1/Rht-D1* loci have a medium effect on this trait, with the semi-dwarfing alleles (*Rht-B1b/Rht-D1b*) reducing AE (Boeven et al., 2016; He, Singh, et al., 2016; Muqaddasi, Pillen, et al., 2017), while others reported no significant association of this loci with AE (He, Lillemo, et al., 2016; Lu et al., 2013; Muqaddasi et al., 2016; Muqaddasi, Brassac, et al., 2017). These responses could be partly explained due to a low number of *Rht-B1b/Rht-D1b* lines evaluated in the association panel (Muqaddasi et al., 2016; Muqaddasi, Brassac, et al., 2017), or can also be interpreted as *Rht-B1/Rht-D1* loci affecting AE only in certain accessions. Recent reports documented increases in Fusarium head blight susceptibility in semi-dwarf NILs carrying *Rht-B1b/Rht-D1b* alleles, explained by increases in anther retention and reduced plant height (Buerstmayr & Buerstmayr, 2022). This response had been

associated due to an overlap between significant QTLs (-*QFhb.caas-4BS* and *QFhb.caas-4DS*) related to anther retention and fusarium resistance with the *Rht1* loci (Buerstmayr & Buerstmayr, 2016; He, Lillemo, et al., 2016; He, Singh, et al., 2016; Lu et al., 2013; Xu et al., 2019; Zhu et al., 2021). According to Okada et al. (2019), the effect of the *Rht-B1* locus on AE appears to be genotype-dependent and is supported by data derived from several mapping populations and some NILs evaluated under greenhouse conditions. Our results showed that AE was reduced by semi-dwarfing (*Rht1* and *Rht2*) and greatly diminished by extreme dwarfing alleles (*Rht3*, *Rht2* + 3, *Rht1* + 2) which in three of four genotypes assessed (Bersee, Maris Huntsmann, and Maris Widgeon) led to complete retention of anthers (<2.6% of AE). The degree of this response was genotype and environment-dependent. The effect of the different dwarfing alleles reducing AE was clearer under high-yield potential environments like those explored under German conditions (Figures S1b and S2b) due to longer photoperiod and optimal temperatures that increase pollination duration compared to Argentinian conditions (Garst et al., 2023; Jones et al., 2017).

Interestingly, Okada et al. (2019) evaluating two F_2 mapping populations under controlled conditions, proposed that *Rht-B1* and *Ppd-1* are major effecting loci for AE and particular allele combinations can be used for selecting a suite of pollinator traits for successful hybrid wheat seed production. For their part, a reduced pollen mass, as well as low AE, have been reported in lines carrying the semi-dwarfing allele *Rht-D1b* (Langer et al., 2014). However, these effects might also be attributed to other closely linked genes in the chromosomal region of *Rht-D1* (Li et al., 2012). The observed effects of *Rht-D1* are likely attributable to the GA-insensitivity of the dwarfing allele, due to their effect on cell division and/or elongation and their possible influence on male fertility and stamen development (Mutasa-Göttgens & Hedden, 2009). Likewise, *Arabidopsis* GA-deficient mutants showed shorter stamen filament length likely due to reduced cell elongation as well as pollen normal development (Cheng et al., 2004). Hence, it has been speculated that semi-dwarf and dwarf wheat lines also have shorter AFL and consequently reduced AE due to reduced cell elongation, resulting in a poor male floral trait performance as reported in our work (Figures 2 and 3; Figures S1b-d and S2a-c).

Our results indicated that the tall wild-type *rht* (tall-*Rht-B1a/Rht-D1a*) showed positive effects on AE, AL, and AFL, traits closely related to enhancing the cross-pollination ratio (Figures 1-3; Figures S1-S3). Other floral attributes including long stigmatic hairs, that are entirely extruded and receptive for extended periods would enhance the probability of cross-pollination. Most importantly, it is crucial that the female plant is either male-sterile or self-incompatible, to prevent self-pollination and ensure

cross-fertilization (Whitford et al., 2013). These characteristics added to a higher PH are required for elite pollinators in hybrid breeding programs. Previous efforts (Boeven et al., 2016; Würschum et al., 2018) suggested that the positive effects of wild-type *Rht-D1a* allele (tall phenotype) on floral traits are rather beneficial for hybrid wheat breeding since male lines are desired to present high AE, AL, and should be taller than the females. These authors also suggested that, while *Rht-D1a* could be used as elite pollinators, the semi-dwarf *Rht2* could be fixed in the female pool, and due to the semi-dominant inheritance of this allele, the resulting hybrids would possess the desired plant height reduction (Boeven et al., 2016; Börner et al., 1996; Zhao et al., 2014). The exploration of modern Green Revolution *Rht* genes such as *Rht24b* has shown the potentiality of decreasing plant height without penalties in grain yield (Tian et al., 2022) while increasing anther extrusion and resistance against Fusarium head blight compared to the Green Revolution semi-dwarf allele *Rht2* (Akohe et al., 2022; Miedaner et al., 2022; Würschum et al., 2017).

The negative impact of *Rht* genes on flowering traits should be balanced with respect to the beneficial impact on yield components in hybrid wheat breeding. It should be considered that the floral traits studied are only important for the male parent. In addition, many hybrid combinations will have heterosis showing at least higher yields than the average of the parents, so the appropriate combinations must be made between parents with *Rht* alleles to increase yield, without significant negative effects on the floral characteristics of the male parent. Our study analyzing six NILs in four genetic backgrounds under multi-environmental conditions indicates that *Rht* alleles are involved in multiple traits (PH, AE, AL, AFL, spikelets/spike, and spike length) of interest for hybrid wheat production and suggests the utility of broad genetic sources for reduced plant height/lodging resistance for future breeding programs.

EXPERIMENTAL PROCEDURES

Growing conditions

The experiments were performed through three growing seasons (2018/2019, 2020/2021, and 2021/2022) at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany (11°16' LE; 51°49' LN) and during 2021 at the Julio Hirschhorn Experimental Station, Faculty of Agricultural and Forestry Sciences, National University of La Plata, Argentina (34°52' LS; 57°58' LW).

Plant material an experimental design

Four sets of NILs carrying the alleles *Rht1* (Rht-B1b/Rht-D1a), *Rht2* (Rht-B1a/Rht-D1b), *Rht3* (Rht-B1c/Rht-D1a), *Rht1 + 2* (Rht-B1b/Rht-D1b), *Rht2 + 3* (Rht-B1c/Rht-D1b), and *rht* (tall; Rht-B1a/Rht-D1a) in the genetic backgrounds of the winter wheat varieties 'April Bearded', 'Bersee', 'Maris Huntsman', and 'Maris Widgeon' were

investigated (Börner et al., 1993). Genotypes were grown in a split-split plot design with three blocks in the four environments (IPK19, ARG21, IPK21, and IPK22). Environments were the main plots, the *Rht* alleles showing different dwarfism degrees were the subplots and the genotypes were the sub-subplots. Alleles and genotypes were considered fixed effects whereas environments and blocks were considered random effects. Each plot comprised 1.2 m² (1 m long by 1.2 m wide), containing six rows (Methods S1).

Evaluations and statistical analysis

Seven male pollinator traits associated with cross-pollination efficiency were measured.

(a) Flowering date: The date was recorded as the number of days to anther exertion from 50% of the main spikes per plot (Langer et al., 2014).

(b) Plant height: Plant height was measured from the ground to the tip of the spikes (excluding awns) at the early dough stage (GS82, Zadoks et al., 1974).

(c) Anther extrusion (AE): The assessment was based on the method described by Atashi-Rang and Lucken (1978), Langer et al. (2014), Muqaddasi et al. (2016), and Muqaddasi, Brassac, et al. (2017). Anther extrusion was scored 7 days after flowering on ten randomly selected main spikes per plot. Of the six central spikelets of a spike, the number of anthers that remained in the two lateral florets was counted. Lateral wheat florets contain three anthers resulting in a maximum of 36 anthers per counted spike (i.e., 3 anthers per floret × 2 florets per spikelet × 6 spikelets per spike). Consequently, the number of extruded anthers was derived by subtracting the number of counted anthers that remained in the florets from 36. Data were expressed as % of anther extrusion.

(d) Spikelets per spike and (e) spike length: The number of spikelets was counted on ten randomly selected main spikes per plot and averaged (Boeven et al., 2016). Spike length was measured from the base of the rachis to the tip of the terminal spikelet excluding the awns (Zhai et al., 2016).

(f) Anther length (AL) and (g) anther filament length (AFL) were measured by the following procedure: anthers from primary and secondary florets of two spikelets located at the middle of the spike were collected at Zadoks GS59 (full heading stage). Anthers images were taken using a stereo dissecting microscope equipped with a digital camera (NIKON SMZ25 stereomicroscope) (Methods S2 and S3). AL and AFL were then measured directly from the image as a length of polygonal line segments drawn through the center of the anther. Five spikes per plot and ten anthers collected for each spike sample were recorded (Okada et al., 2019).

(h) Grain yield parameters: At the ripening stage (GS95), the grain number per square meter (GN) was calculated by counting the spikes contained in 2 m central rows and threshing and counting the grains in 20 spikes per plot. In addition, thousand kernel weight (TKW) was measured using the MARVIN Digital Seed Analyzer (MARVITECH GmbH, Wittenburg, Germany) (Schierenbeck et al., 2021).

Statistical analysis of the four environments evaluated was analyzed by ANOVA for split-split plot designs using Genstat Release 18 (Goedhart, 2016). The least significant differences (LSD) were reported at the 5% level of confidence (**P* < 0.05). Treatment variances were homogeneous according to the Bartlett test and residuals fitted normal distributions according to the Shapiro-Wilks test (Snedecor & Cochran, 1983). Summary statistics and correlations were calculated using the web-based statistical analysis MVApp v2.0 (Julkowska et al., 2019), <https://mvapp.kaust.edu.sa/>.

AUTHOR CONTRIBUTIONS

MS and AB designed the research. MS, AMA, and EL analyzed data with help from MRS and EGA. AB provided genotypic resources. MS wrote the manuscript with contributions from all co-authors.

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CONFLICT OF INTEREST

No conflict of interest declared.

DATA AVAILABILITY STATEMENT

The data produced and analyzed for this study are available from the corresponding author on request.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Mean values for (a) plant height (cm), (b) anther extrusion (%), (c) anther filament length (mm), and (d) anther length (mm) for the *Allele* × *Environment* interaction. Environments: IPK19 (IPK Germany, 2019); ARG21 (Argentina, 2021), IPK21 (IPK Germany, 2021) and IPK22 (IPK Germany, 2022). *Rht* alleles: *rht* (tall), *Rht1* (Semi-dwarf), *Rht2* (Semi-dwarf), *Rht3* (extreme dwarf), *Rht1* + 2 (dwarf), and *Rht2* + 3 (extreme dwarf). Data points of the four environments explored, error bars, outliers, median, and mean values (crosses) are shown. Means followed by the same letter are not statistically different (LSD $P \leq 0.05$).

Figure S2. Linear regression between plant height (cm) and (a) anther filament length (mm), (b) anther extrusion (%), and (c) anther length (mm). Points represent the means of the interaction *Allele* × *Environments* interaction using four winter wheat genotypes carrying six different combinations of *Rht* dwarfing genes. Environments: ●IPK19 (IPK Germany, 2019); ◆ARG21 (Argentina, 2021); ■IPK21 (IPK Germany, 2021); ▲IPK22 (IPK Germany, 2022). *Rht* alleles: *rht* (tall), *Rht1* (Semi-dwarf), *Rht2* (Semi-dwarf), *Rht1* + 2 (dwarf), *Rht3* (dwarf), and *Rht2* + 3 (extreme dwarf).

Figure S3. Mean values for (a) spike length and (b) spikelets per spike for the *Allele* × *Environment* interaction. Environments: IPK19 (IPK Germany, 2019), ARG21 (Argentina, 2021), IPK21 (IPK Germany, 2021), and IPK22 (IPK Germany, 2022). *Rht* alleles: *rht* (tall), *Rht1* (Semi-dwarf), *Rht2* (Semi-dwarf), *Rht3* (extreme dwarf), *Rht1* + 2 (dwarf), and *Rht2* + 3 (extreme dwarf). Data points of the different NILs across the four environments explored, error bars, outliers, median, and mean values (crosses) are shown. Means

followed by the same letter are not statistically different (LSD $P \leq 0.05$).

Figure S4. Mean values for (a) grains per square meter and (b) thousand kernel weight for the *Allele* × *Environment* interaction. Environments: ARG21 (Argentina, 2021), IPK21 (IPK Germany, 2021) and IPK22 (IPK Germany, 2022). *Rht* alleles: *rht* (tall), *Rht1* (Semi-dwarf), *Rht2* (Semi-dwarf), *Rht3* (extreme dwarf), *Rht1* + 2 (dwarf), and *Rht2* + 3 (extreme dwarf). Data points of the different NILs across the three environments explored, error bars, outliers, median, and mean values (crosses) are shown. Means followed by the same letter are not statistically different (LSD $P \leq 0.05$).

Methods S1. Field trials at heading (GS59) and dough stage (GS82)

Methods S2. Comparison between *rht* (tall) and *Rht2* + 3 (extreme dwarf) for anther (AL) and anther filament (AFL) lengths in the four genotypes evaluated. Images were taken using a stereo dissecting microscope equipped with a digital camera (NIKON SMZ25 stereomicroscope).

Methods S3. Evaluation of (a) anthesis date; (b) anther extrusion (AE); (c) anther length (AL); and anther filament (AFL) lengths.

Table S1. Means square and *P*-value (ANOVA) of thousand kernel weight and grain number per square meter in four wheat genotypes carrying six different *Rht* alleles.

Table S2. Mean values of thousand kernel weight and grain number per square meter in four wheat genotypes carrying six different *Rht* alleles.

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