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# Effect of Gibberellin Responsive Reduced Height Allele *Rht13* on Agronomic Traits in Spring Bread Wheat in Field Experiment in Non-Black Soil Zone

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Received: 13 May 2020; Accepted: 26 June 2020; Published: 28 June 2020


**Abstract:** The introduction of gibberellin-responsive reduced height (GAR *Rht*) alleles is a promising tool for breeding semi-dwarf, high-input varieties of wheat. We have compared recombinant isogenic lines $F_{2:3}$ carrying dwarfing allele *Rht13* and without dwarfing alleles, obtained from the cross of isogenic lines and selected from $F_2$ using molecular markers. After phenotyping and statistical analysis, we found that the *Rht13* allele reduces total plant height by 13.0 cm (17.4%), while the proportions between the internodes in tall and short plants remain similar. The greatest decrease in length in plants with *Rht13* in comparison to wild-type plants is observed for the second internode (5.3 cm, or 31.9%). Due to the presence of *Rht13*, semi-dwarf plants, compared to the wild type, had a higher grain number per main spike, grain number per spikelet and higher number of productive tillers, and a slightly higher harvest index, although thousand grain weight and grain weight in the main spike were lower. Our results indicate the possibility of using *Rht13* in the breeding of wheat varieties without dramatic negative effects on yield and plant development.

**Keywords:** bread wheat; dwarfing alleles; gibberellins responsive height reducing; agronomic traits; marker assisted breeding

## 1. Introduction

Wheat is one of the most important crops in the world. According to FAO, the share of wheat in global cereal production in 2019 was 28%, with an increase of 4.2% compared to 2018. The Russian Federation ranks third in the world in wheat production after China and India. Wheat grain is one of the main sources of carbohydrates, proteins, vitamins, and mineral elements. Since its domestication, wheat has been widely used as a staple food crop as well as a raw material for the industry and animal husbandry [1].

A large increase in wheat yield during the Green revolution was achieved in part because of breeding new dwarf varieties that are more resistant to lodging. This was made possible due to the discovery of reduced height (*Rht*) genes. To date, about 24 genes responsible for reducing the height of wheat are known [2]. Besides different effect on the plant height, they vary by the effect on other agronomically important traits such as harvest index (HI), biomass production (BM), grain yield (GY),

thousand grain weight (TGW), grain number per spike (GNS), grain weight per spike (GWS), number of fertile tillers (FT), etc. Gibberellin-insensitive (GAI) *Rht-B1b* and *Rht-D1b* genes are widely used in breeding programs to obtain dwarf plants, increase yields and increase the harvest index, but they also reduce the coleoptile length and reduce germination strength [3–6]. Gibberellin-responsive (GAR) *Rht* genes, such as *Rht4*, *Rht5*, *Rht8*, *Rht12*, *Rht13*, *Rht24* and *Rht25*, have the potential for use in a breeding programs; they reduce plant height without affecting seedling vigor as they have little effect on coleoptile length which is important for seedling emergence [7–10]. In Russia, the assortment of GAR *Rht* alleles is limited to *Rht-8c* especially in Southern Regions [11]. Among other GAR *Rht* dwarfing alleles, only *Rht13* was reported to decrease plant height without drastic reduction of yield under conditions of the Non-Black Soil Zone [12].

The *Rht13* gene reduces plant heights in the range from 12% to 41% that exceeds the effect on the *Rht8* gene [3,7,8,13,14]. At the same time, it is noted that with a decrease in the overall height of plants, the proportions of internodes also change; however, in various experiments the strongest decrease was shown by different internodes [3,7,15]. The coleoptiles of *Rht13* plants are no shorter than those of the wild type and may be longer [3,8,15]. Moreover, *Rht13* increases the number of productive ears without affecting the spike fertility that results in higher plant biomass [7]. In some studies, plants carrying the *Rht13* gene have been reported to have reduced thousand-kernel weight [4,10,11], in others, however, no reduction was observed [3,8,15]. Wheat plants with the *Rht13* gene have a higher photosynthesis rate and slower leaf aging under conditions of low nitrogen content and higher relative water content in the leaves under conditions of osmotic stress [16,17].

Non-Black Soil Zone (Nechernozemye) is one of the most economically active regions of the Russian Federation, which requires its own wheat production to ensure food security. The climatic conditions of the Non-Black Soil Zone are characterized by extreme events in recent years, such as spring droughts with heavy rains during heading and ripening. So, on the one hand, it is necessary to ensure a sufficiently deep seeding of seeds into the soil for access to soil moisture. On the other hand, plants lodging during the rainy season should be prevented. Weather conditions in the Non-Black Soil Zone favors the formation of soil crust that negatively affects the seedling vigor and thus the use of dwarfing alleles with neutral or little impact on this trait is required. Thus, the introduction of GAR-dwarfing genes into wheat varieties adapted for the Non-Chernozem region is a relevant task.

The effect of the *Rht13* gene on the height and yield parameters observed in different studies varies. Therefore, the effect of this gene significantly depends on the growing conditions. The purpose of this study is to evaluate the effect of the *Rht13* gene on plant height and other agronomically important wheat traits in the conditions of the Non-Chernozem region under field experiment conditions.

## 2. Materials and Methods

### *2.1. Plant Material*

The $F_2$ population of spring common wheat segregating for *Rht13* was developed by crossing lines-analogues LAN5 and LAN13. These parental lines were produced by backcrossing the germplasm source of *Rht5* and *Rht13* with the spring form of Mironovskaya 808 [12]. The parental plants were hybridized and the $F_1$ hybrids were grown in a greenhouse. $F_2$ segregating population was grown under field conditions in 2018. Each individual plant of parental lines, $F_1$ hybrids and $F_2$ population, as well as the analyzed plants from $F_{2:3}$ families, were analyzed for the allelic state of *Rht13* and *Rht5* using microsatellite markers linked to the target genes; $F_{2:3}$ plants homozygous for *rht5* and homozygous for either *Rht13* or *rht13* were used for the analysis (see the following section on molecular analysis).

### *2.2. Molecular Analysis*

Genomic DNA was extracted from leaves using a CTAB method [18]. PCR was performed in a 25 µL reaction volume, containing 70 mM Tris–HCl buffer (pH 8.6), 16.6 mM $(NH4)_2SO_4$, 2.5 mM $MgCl_2$,

0.2 mM of each dNTP, 10%v/v dimethyl sulfoxide, 0.3 µM forward and reverse primers (Syntol Ltd., Moscow, Russia), 1.25 U of Colored Taq-polymerase (Sileks Ltd., Moscow, Russia) and 100 ng of template DNA. The identification of *Rht13* and *Rht5* was performed using SSR markers linked with the genes of interest, *Xwms*577 and *Xbarc*102, respectively, as described in [15] with the corresponding PCR conditions. The PCR reaction was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The PCR products were separated in a 1.5% agarose gel in TBE buffer using GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA) as a molecular weight marker, and stained with ethidium bromide for subsequent visualization in Gel Doc XR+ (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

### 2.3. Field Experiment

The field experiment was performed in the Field Experimental Station, Russian State Agrarian University—Moscow Timiryazev Agricultural Academy, Moscow (55°50′ N, 37°33′ E, hereinafter—Moscow) in 2019. Sowing was performed on 23 April 2019 using a breeding cassette drill SKS-6-10 with the following parameters: length of plot, 1 m; width of plot, 90 cm; width between the rows, 30 cm (Moscow); distance between the plots, 50 cm. Each row represented the family, i.e., the progeny of an individual $F_2$ plant homozygous for either *Rht13* or *rht13* allele. Overall, 14 families homozygous for *Rht13* and 21 families homozygous for *rht13* were grown. Individual plants were labelled and one leaf from each plant was used for DNA extraction to determine the allelic state of *Rht13* and *Rht5* (see section on molecular analysis). The plots were treated with pesticides to control pests, the weeds were removed manually. Plants of each family were harvested manually at complete ripeness; the last plants were harvested on 02 August 2019. The spikes were threshed using a spike thresher MKS-1M (MZOK Company, Moscow, Russia). Weather conditions in the period from late April to late June were characterized by higher temperatures (on average by 3.5 °C) and lower rain precipitation (on average by 7.4 mm) than annual average values registered for many years for the Moscow region (Figure 1a). From late June to early August a decrease in temperature (lower than annual average by 3.1 °C) was accompanied by rare but heavy rains (saw-toothed wave in Figure 1b).

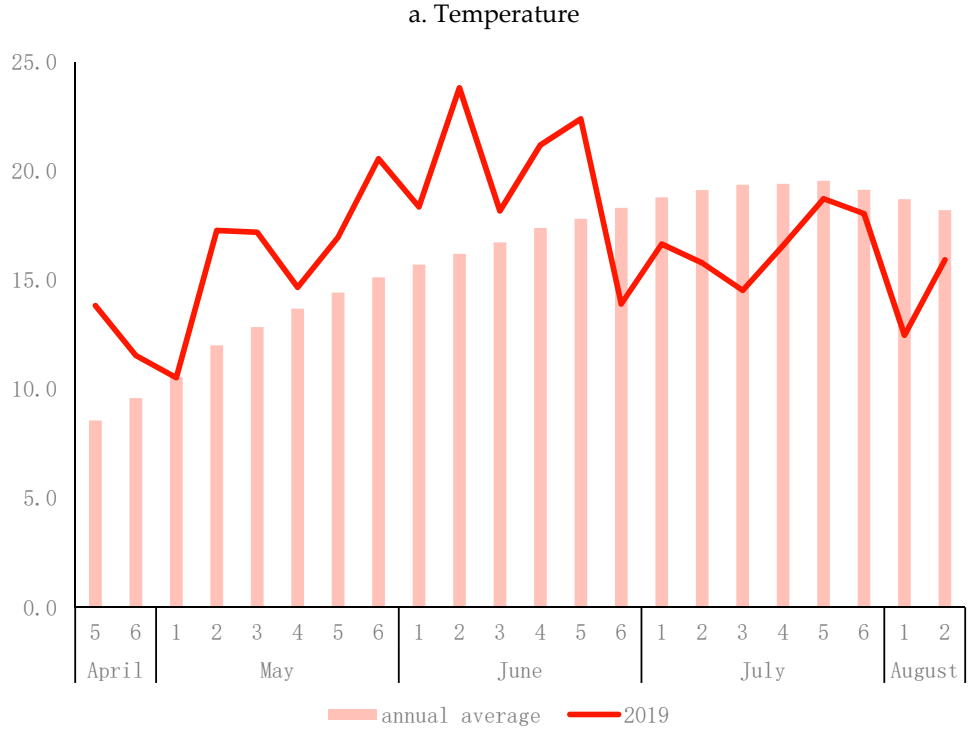


**Figure 1.** *Cont.*

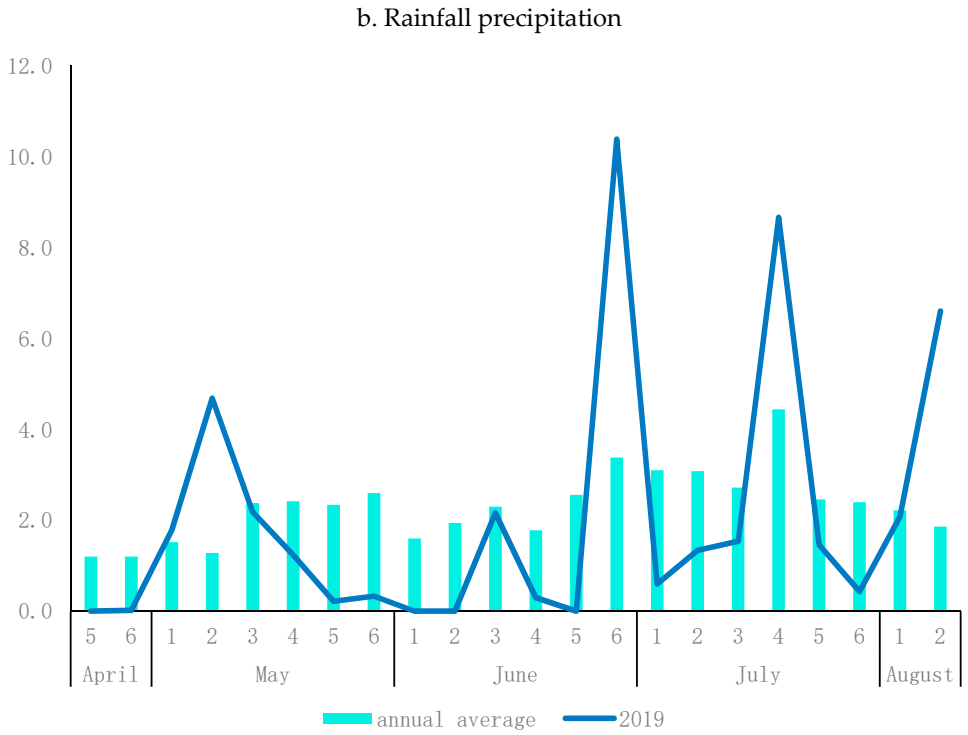


**Figure 1.** Temperature (**a**) and rain precipitation (**b**) as registered in 2019 (shown as graph) and annual average as a result of multi-year observations (shown as diagram).

### 2.4. Phenotyping

In total, 384 $F_{2:3}$ plants were analyzed, 239 plants with *rht13rht13* genotype and 145 plants with *Rht13Rht13* genotype. The following traits were measured in the main shoot for each individual plant (not less than 10 plants per each family were chosen randomly from the row): plant height (PH, cm), length of three upper and two lower internodes (cm), internode number (IN), main spike length (MSL, cm), spikelet number per main spike (SN), main spike weight (MSW, g), main culm weight (MCW, g), grain weight per main spike (GWS, g), and grain number per main spike (GN). The number of fertile tillers (FT) as well as grain weight in side tillers (GWT) were registered for each plant. The following parameters were calculated for each individual plant: spike compactness (SC, number of spikelets per 10 cm of main spike length), main shoot biomass (BM, sum of MSW and MCW, g), grain number per spikelet (GNS, GN divided by SN), thousand grain weight (TGW, thousandfold GWS divided by GNS, g), harvest index (HI, GWS divided by BM), and grain weight per shoot (GWSH, the sum of GWS and GWT divided by FT). The contribution of a given internode to a decrease in total plant height was calculated as a decrease in a given internode divided by a decrease in total plant (%). The heading and anthesis and dates were recorded for each family when not less than 80% of the plants came to a particular phase. The seeds were counted with the use of Seed Counter Application [19].

### 2.5. Statistical Analysis

For each measured trait we calculated the mean value for each genotype, *Rht13Rht13* and *rht13rht13*. The effect of *Rht13* on the studied traits was estimated as the difference (contrast) between the trait value for plants homozygous for dwarfing alleles *Rht13* and neutral wild type allele *rht13*, while the relative effect was calculated as the relation of this difference to the mean trait value of *rht13rht13* plants (%). The statistical evaluation of the data was carried out by one-way ANOVA analyses. The comparisons between means were detected using a least significant differences (Duncan) test at the level of significance of 0.01 and 0.05 (for heading time). Statistical analysis was performed using statistical software R [20].

## 3. Results

### *3.1. Anatomy of the Main Stem*

The presence of the *Rht13* gene significantly affected the height of plants and led to a shortening of all shoot internodes. The total height of plants with the *Rht13* gene was 13.0 cm (17.4%, $p < 0.05$) lower than the height of wild-type plants (Figure 2, Table 1). The number of internodes varied from 4 to 6, but most plants of both genotypes had five internodes. In absolute values, the greatest difference was observed in the length of the first and second upper internodes, the average decrease in length was 4.1 cm and 5.3 cm, respectively. The percentual difference in the first and second internodes between the *Rht13*-carriers and wild-type plants was 14.4% and 31.9%, correspondingly. The contribution of the first and second internodes to a decrease in total plant height was 32% and 41%, respectively (Table S1). The proportional length of each internode relative to the total length of the straw slightly changed: the peduncle became relatively longer, the second and third a little shorter (Table 1, Figure 2).

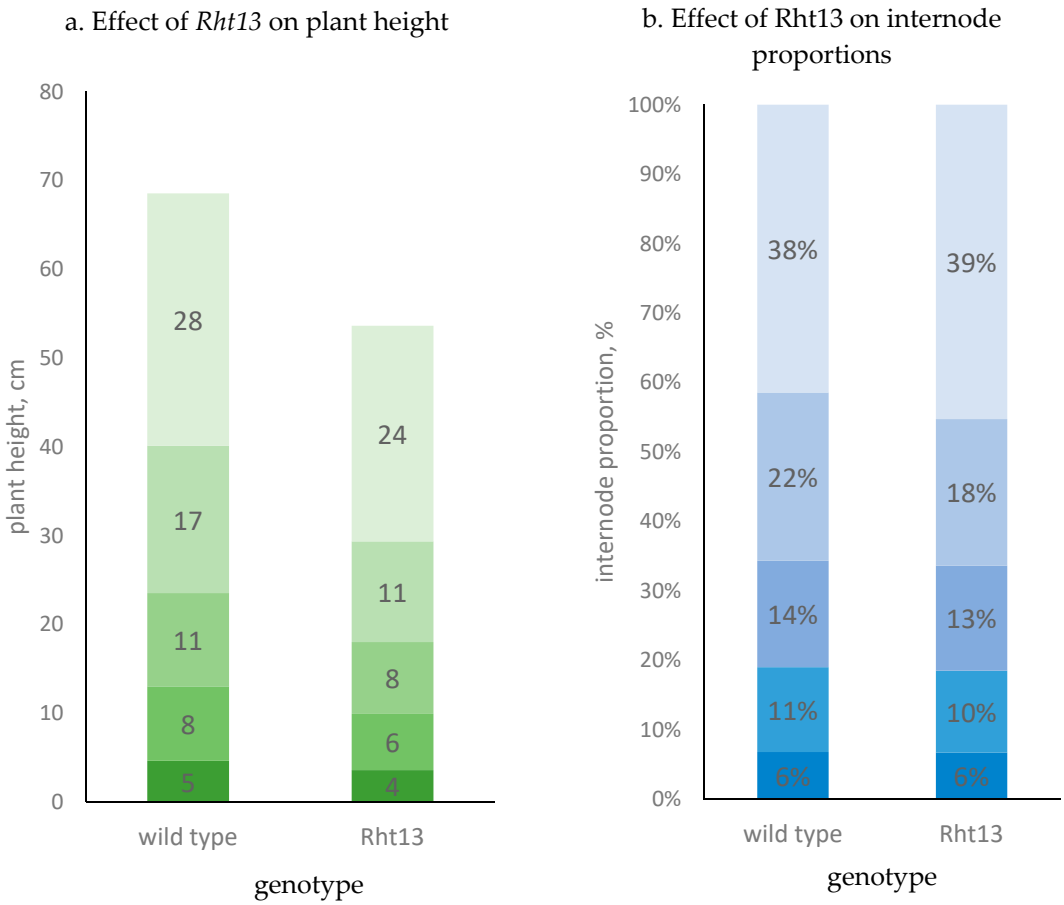


**Figure 2.** Effect of reduced height gene (*Rht13*) on plant height, internode length (**a**) and internode proportions (internode length related to plant height, (**b**) in common wheat families $F_{2:3}$ LAN5/LAN13 homozygous for *Rht13* (wild type allele) and *Rht13* (dwarfing allele). Each color shade corresponds to different internodes. The sum of proportions in Figure 2b is not equivalent to 100% as a few plants had six internodes and some had four internodes.

**Table 1.** Mean values of biometric traits measured and calculated in common wheat families $F_{2:3}$ LAN5/LAN13 homozygous for *rht13* (wild type allele) and *Rht13* (dwarfing allele).

| Trait | Allele | Mean ± Standard Deviation | Contrast *Rht13Rht13* vs. *rht13rht13* (%) |
|---|---|---|---|
| Plant height (PH), cm | *rht13* | 74.9 ± 6.0 | −13.0 (−17.4%) * |
| | *Rht13* | 61.9 ± 4.6 | |
| 1st upper internode (peduncle), cm | *rht13* | 28.4 ± 4.2 | −4.1 (−14.4%) * |
| | *Rht13* | 24.3 ± 3.5 | |
| 2nd upper internode, cm | *rht13* | 16.6 ± 2.1 | −5.3 (−31.9%) * |
| | *Rht13* | 11.3 ± 1.7 | |
| 3rd upper internode, cm | *rht13* | 10.5 ± 1.1 | −2.4 (−22.9%) * |
| | *Rht13* | 8.1 ± 1.3 | |
| 2nd lower internode, cm | *rht13* | 8.4 ± 1.5 | −2.1 (−25.0%) * |
| | *Rht13* | 6.3 ± 1.3 | |
| 1st lower internode, cm | *rht13* | 4.7 ± 1.9 | −1.1 (23.4%) * |
| | *Rht13* | 3.6 ± 1.5 | |
| Internode number | *rht13* | 4.7 ± 0.5a | 0.2 (4.3%) |
| | *Rht13* | 4.9 ± 0.4b | |
| Grain number per main spike (GN), pcs. | *rht13* | 28.9 ± 8.0 | 1.3 (4.5%) |
| | *Rht13* | 30.2 ± 7.9 | |
| Grain number per spikelet (GNS), pcs. | *rht13* | 1.9 ± 0.5 | 0.1 (5.3%) |
| | *Rht13* | 2.0 ± 0.5 | |
| Main spike length (MSL), cm | *rht13* | 9.0 ± 1.0 | 0.2 (2.2%) |
| | *Rht13* | 9.2 ± 1.0 | |
| Spikelet number per spike (SN), pcs. | *rht13* | 15.2 ± 1.4 | −0.2 (−1.3%) |
| | *Rht13* | 15.0 ± 1.3 | |
| Spike compactness (SC) | *rht13* | 16.9 ± 1.4 | −0.4 (−2.5%) |
| | *Rht13* | 16.5 ± 1.4 | |
| Thousand grain weight (TGW), g | *rht13* | 52.1 ± 3.6 | −5.1 (−9.8%) * |
| | *Rht13* | 47.0 ± 4.8 | |
| Grain weight per main spike (GWS), g | *rht13* | 1.5 ± 0.4 | −0.1 (−6.7%) * |
| | *Rht13* | 1.4 ± 0.4 | |
| Number of fertile tillers per plant (FT), pcs. | *rht13* | 2.5 ± 1.3 | 0.4 (16.0%) * |
| | *Rht13* | 2.9 ± 1.5 | |
| Grain weight in side tillers (GWT), g | *rht13* | 1.87 ± 1.14 | 0.01 (0.5%) |
| | *Rht13* | 1.86 ± 1.20 | |
| Grain weight per shoot (GWSH), g | *rht13* | 1.18 ± 0.42 | −0.13 (11.0%) * |
| | *Rht13* | 1.05 ± 0.35 | |
| Main culm weight (MCW), g | *rht13* | 0.74 ± 0.17 | −0.12 (−16.2%) * |
| | *Rht13* | 0.62 ± 0.15 | |
| Main shoot biomass (BM), g | *rht13* | 2.6 ± 0.6 | −0.2 (−7.7%) * |
| | *Rht13* | 2.4 ± 0.6 | |
| Harvest index (HI) | *rht13* | 0.556 ± 0.085 | 0.016 (2.9%) * |
| | *Rht13* | 0.572 ± 0.082 | |
| Days to 80%-heading | *rht13* | 50.8 ± 1.9 | −1.5 (−3.0%) |
| | *Rht13* | 49.3 ± 2.7 | |
| Days to 80%-anthesis | *rht13* | 52.1 ± 1.8 | −1.6 (−3.1%) |
| | *Rht13* | 50.5 ± 2.1 | |

* Contrasts designated with asterisks showed significant differences at $p < 0.05$.

### 3.2. Spike Anatomy and Productivity

The grain number per spike in semi-dwarf lines was higher by an average of 1.3 grains (4.5%) compared with tall plants (wild type allele). This is due, first of all, to an increase in grain number per spikelet by 5.3% (Figure 3, Table 1). At the same time, in the *Rht13* lines were observed (1) a slight increase in the length of the main spike, (2) a decrease in the number of spikelets and, (3) as a consequence, a slight decrease in the compactness of the main spike. In plants with *Rht13*, compared

to wild-type plants, thousand grain weight was lower by 5.1 g (9.8%), and grain weight per main spike was 0.1 g (6.7%) (Figure 3, Table 1). Plants with *Rht13* had a higher number of fertile tillers compared to wild-type plants by 0.4 pcs. (16.0%). Grain weight in side tillers was specifically the same in both genotypes, but since *Rht13* plants had more fertile tillers than tall plants, grain weight per shoot decreased by 11.0% (Figure 3, Table 1).

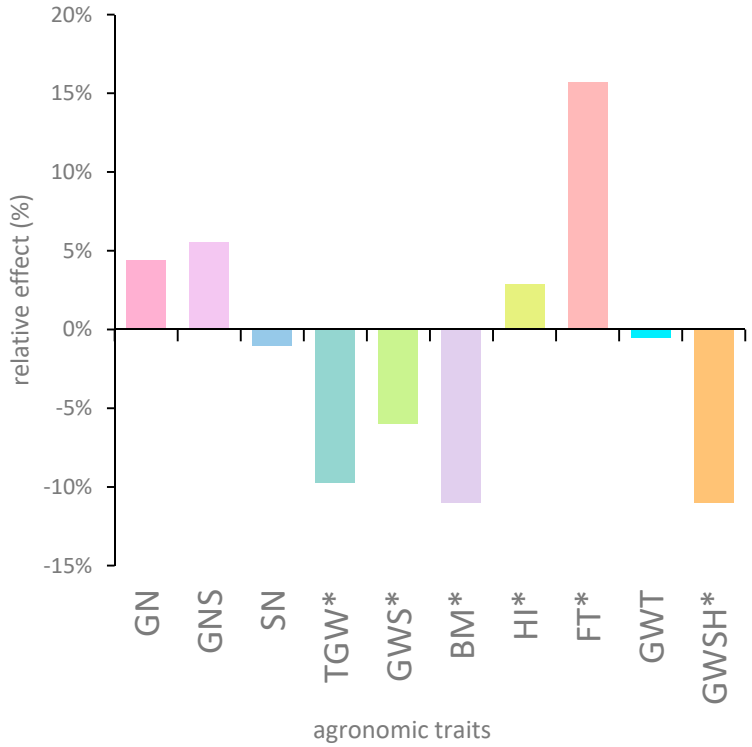


**Figure 3.** The relative effect of the *Rht13* gene on main shoot productivity (%) compared to plants carrying the wild type allele. GN, grain number per spike; GNS, grain number per spikelet; SN, spikelet number; TGW, thousand grain weight; GWS, grain weight in spike; BM, main shoot biomass; HI, harvest index; FT, number of fertile tillers; GWT, grain weight in tillers; GWSH, grain weight per shoot. Statistically significant differences at $p < 0.05$ are shown with asterisks (*).

### 3.3. Harvest Index and Plant Development

In plants with *Rht13*, a decrease in the culm weight of the main shoot by 0.12 g (16.2%) and the shoot itself by 0.2 g (7.7%) was observed (Table 1). Harvest index was 0.556 for the wild-type and 0.572 for semi-dwarf plants, which is a difference of 2.9% ($p < 0.05$) (Figure 3, Table 1). On average, complete heading and anthesis in plants with *Rht13* occurred 1–2 days earlier (Table 1). Plants, homozygous for *Rht13* did not differ significantly in height from wild-type plants at booting; the significant difference of 5.2 cm (11.0%) was registered when 50% of plants came to heading and increased to 13.0 cm (17.4%) at maturity (Figure 4).

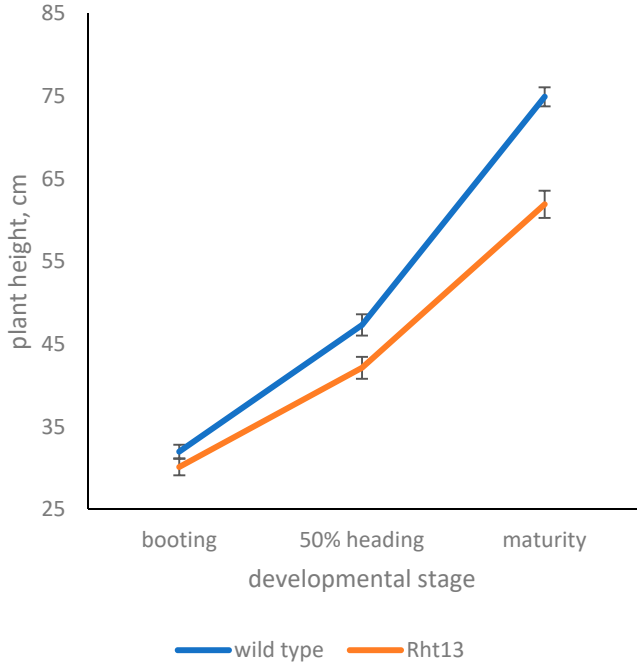


**Figure 4.** Plant height at booting, anthesis and maturity stages in plants homozygous for *rht13* (wild type) and dwarfing *Rht13* alleles. Bars show standard errors.

## 4. Discussion

Currently, breeding programs use mainly a limited set of GAI dwarfing genes, mainly *Rht-B1b* and *Rht-D1b*, and the effect of GAR genes on the important traits, such as thousand grain weight, seed number per spikelet, biomass production, etc., remain poorly understood [21]. This work was carried out with the aim of studying the effect of the GAR *Rht13* gene on the morphological and important agronomic traits of bread wheat grown in the central part of the Non-Black Soil Zone of Russia.

The phenotypic manifestation of a gene can vary significantly depending on changes in the growing conditions. Here, we showed that the decrease in height caused by the *Rht13* gene was 17.4%, which is consistent with the results in the study where *Rht13* decreased plant height by 17.1% in $F_{2:3}$ and by 16.4% in $F_{3:4}$ families with an average decrease of 16.7% [3]. However, in other studies the plant reduction due to *Rht13* varied from 12% to 44% [7,8,12,14,15] (Table S1). Therefore, on average the agronomic performance of the dwarfing *Rht13* allele under the conditions of the Non-Black Soil Zone has no more negative effects on agronomic traits than *Rht-B1b*, *Rht-D1b*, and *Rht-B1p* GAI genes [5,8,22–25]. We demonstrated slight changes in proportions between internodes (Figure 2b). Most published studies on the effects of *Rht13* have shown that *Rht13* changes the proportions of internodes, although the particular internode which proportion is altered is different [3,7,12,13,15]. The contribution of different internodes to a decrease due to *Rht13* in total plant height differs in various studies [4,8–10]. In our study, the maximum fraction of the decrease occurred in the second internode (41%), the parameter that also varied in different published results [3,7,13,15] (Table S1).

Grain weight per spike is a reflection of average grain weight (thousand grain weight is used as a proxy for this), the number of grains in a spike and spikelet number. Previously published studies suggest that *Rht13* has a neutral or negative effect on thousand grain weight. A number of studies have shown a decrease in thousand grain weight due to *Rht13* ranging from 7% to 23% [3,7,8,14] (Table S1), although in other experiments the effect of *Rht13* on this trait was insignificant [12,13,15]. In the study of Wang et al. [13], a decrease in grain number per spike ranged from 17% to 20%, in the study of Loskutova [12] it increased by 16%, while in other papers no effect of *Rht13* on this trait is reported [3,8,15]. In our study, the presence of *Rht13* reduced thousand grain weight by 9.8%, however,

it increased seed number per spikelet and, as a result, seed number per spike. The introduction of the alleles of genes and quantitative trait loci associated with higher thousand grain weight could improve this drawback in the future [26,27].

Plant productivity is determined by the number of fertile tillers; the effect of *Rht13* with regard to this is unclear. In the studies of Wang et al., [3,13], *Rht13* did not significantly affect the number of productive stems, which against the background of a decrease in spike productivity, led to a drop in yield from the plant. In the works of Rebetzke et al. [7,8], in contrast, *Rht13* plants had more ears per unit area compared to wild type and, as a consequence, a yield increase of 18–21% was recorded (Table S1). In our study, plants with the *Rht13* gene had a higher number of fertile tillers, which could potentially increase the productivity of the plant as a whole. Weather conditions in late June–early August in the Non-Black Soil Zone are usually characterized by intensive rains (Figure 1b), that may result in lodging, pre-harvest sprouting and yield losses. Therefore, in comparison to tall plants, plants with *Rht13* with shorter and more side tillers could have benefits that may rescue the final yield.

The harvest index is a complex function of the mass of grains and biomass of the harvested plant. In a study by Rebetzke et al. (2012) [8], it is higher grain weight that resulted in an increase in the total biomass and, consequently, in harvest index by 7% in plants with *Rht13*. In studies of Wang et al. [3,13], due to the productivity of semi-dwarf plants with *Rht13*, the biomass decreased by 25–28%, however, the harvest index did not change significantly. In our study, a moderate increase in the harvest index (2.9%) is observed, primarily due to a decrease in the culm weight.

In our experiments, *Rht13* did not alter the heading and anthesis dates; the same was observed in the experiments in Rebetzke et al. [7,8]. We found that the significant differences in plant height became noted at 50% heading. Maddocks (2008) found that the significant difference in height was notable few days before the end of booting [15]; Drouyer et al. (2008) observed the growth slowing of *Rht13* NILs from the beginning of ear emergence [28]. Therefore, generally, *Rht13* lines tend to slow its growth after booting without delay in maturity date. Therefore, *Rht13* fastens the reach of the final height by the plants that may have some advantages as a way to escape unfavorable conditions after heading and to start redistributing assimilates earlier.

The decrease in *Rht13* plants of such important traits as thousand seed weight, grain weight per spike and grain weight in side tillers can be partially compensated by an increase in other valuable traits, such as higher number of seeds per spike and productive tillers. The semi-dwarf phenotype would enable higher sowing rates and higher plant density.

## 5. Conclusions

In conclusion, the results of our study demonstrate the prospect and possibility of using the *Rht13* gene in wheat breeding programs for the Non-Black Soil Zone as a gene that reduces the plant height with an increase in seed number per spike and the number of productive tillers. The semi-dwarf phenotype would also help to tolerate lodging under unfavorable rainy weather conditions in the Non-Black Soil Zone. Additional studies of germination and grain yield per unit area will allow the evaluation of the advantage of *Rht13* in wheat breeding for the particular region. In addition, our results can be used in planning studies on the interaction of *Rht13* and widely used dwarf genes such as *Rht-B1b* and *Rht-D1b* and their other allelic variants.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2073-4395/10/7/927/s1, Table S1. The comparison of height reduction and agronomic performance for *Rht13* in common wheat as revealed in different studies.

**Author Contributions:** Conceptualization, M.G.D.; methodology, M.G.D.; software, P.Y.K.; validation A.Y.K.; formal analysis, P.Y.K., S.Y.S., M.V., and A.Y.K.; investigation, S.Y.S., M.V., and A.Y.K.; resources, G.I.K.; data curation, M.G.D.; writing—original draft preparation, P.Y.K and, S.Y.S.; writing—review and editing, M.G.D., G.I.K.; visualization, P.Y.K.; supervision, M.G.D.; project administration, M.G.D. and G.I.K.; funding acquisition, M.G.D. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the Russian Science Foundation, grant number 17-76-20023

**Acknowledgments:** The authors acknowledge Dmitry Yu. Litvinov for the assistance in the manuscript translation, Evgeny V. Zuev (Head of Department of Wheat Genetic Resources, N.I.Vavilov Institute of Plant Genetic Resources (VIR)) for seeds of the parental lines, Mikhail S. Bazhenov for the assistance in plant material development, Alexey Ermolaev for the assistance in statistical analysis.

**Conflicts of Interest:** The authors declare no conflict of interest.

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