



Article **Fitness and Hard Seededness of F**₂ and F₃ **Descendants of Hybridization between Herbicide-Resistant** *Glycine max* **and** *G. soja*

Rong Liang¹, Jia-Li Liu¹, Xue-Qin Ji¹, Kenneth M. Olsen², Sheng Qiang¹ and Xiao-Ling Song^{1,*}

- ¹ Weed Research Laboratory, College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China; 2020216029@stu.njau.edu.cn (R.L.); 2021116017@stu.njau.edu.cn (J.-L.L.); 2020116016@stu.njau.edu.cn (X.-Q.J.); wrl@njau.edu.cn (S.Q.)
- ² Department of Biology, Washington University in St. Louis, St. Louis, MO 63130, USA; kolsen@wustl.edu
- * Correspondence: sxl@njau.edu.cn

Abstract: The commercial cultivation of herbicide-resistant (HR) transgenic soybeans (Glycine max L. Merr.) raises great concern that transgenes may introgress into wild soybeans (Glycine soja Sieb. et Zucc.) via pollen-mediated gene flow, which could increase the ecological risks of transgenic weed populations and threaten the genetic diversity of wild soybean. To assess the fitness of hybrids derived from transgenic HR soybean and wild soybean, the F2 and F3 descendants of crosses of the HR soybean line T14R1251-70 and two wild soybeans (LNTL and JLBC, which were collected from LiaoNing TieLing and JiLin BaiCheng, respectively), were planted along with their parents in wasteland or farmland soil, with or without weed competition. The fitness of F_2 and F_3 was significantly increased compared to the wild soybeans under all test conditions, and they also showed a greater competitive ability against weeds. Seeds produced by F_2 and F_3 were superficially similar to wild soybeans in having a hard seed coat; however, closer morphological examination revealed that the hard-seededness was lower due to the seed coat structure, specifically the presence of thicker hourglass cells in seed coat layers and lower Ca content in palisade epidermis. Hybrid descendants containing the cp4-epsps HR allele were able to complete their life cycle and produce a large number of seeds in the test conditions, which suggests that they would be able to survive in the soil beyond a single growing season, germinate, and grow under suitable conditions. Our findings indicate that the hybrid descendants of HR soybean and wild soybean may pose potential ecological risks in regions of soybean cultivation where wild soybean occurs.

Keywords: wild soybean (*Glycine soja*); transgenic soybean; plant invasion; weed management; seed bank

1. Introduction

Genetically modified (GM) soybean (*Glycine max* Linn. Merr.) is one of the world's four most widely cultivated GM crops, with a planting area that increased from 500,000 hectares in 1996 to 91.9 million hectares in 2019, accounting for 48% of the global GM crop planting area [1,2]. Among GM soybean traits, herbicide resistance (HR) is the most important. In China, three of the four safety certificates for GM soybeans issued by the Ministry of Agriculture and Rural Affairs are for varieties with HR traits. Soybean's domestication origin is in eastern Asia, and one of the possible ecological risks posed by the commercial cultivation of HR soybeans in China is the potential for pollen-mediated gene flow to wild soybeans (*Glycine soja* Sieb. et Zucc.), creating GM hybrids whose descendants could persist indefinitely in the wild.

Wild soybean is the direct ancestor of cultivated soybean, and both *Glycine* species have the same chromosome number (2n = 40). Wild soybean, which occurs in all of China



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Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). and neighboring regions of eastern Asia, is of great value in studying the origin and evolution of soybean [3–5]. While both species are predominantly self-pollinating, occasional cross-pollination can lead to gene flow from cultivated soybean to wild soybean since there is no reproductive isolation between them [5–8]. Pollen flow and hybridization between HR soybean and wild soybean have been widely reported [4,9–11]. However, whether HR soybean genes can introgress into the wild population also depends on the fitness of hybrids and descendants. Fitness is considered to value the adaptation of individuals or populations with specific genotypes under different ecological conditions [12]. Wild soybean is characterized by high genetic diversity [3,13], which could make the hybrids difficult to control, and the hybrids could potentially contaminate wild germplasm resources. Therefore, before introducing widespread commercial planting of HR soybean in regions where wild soybean occurs, it is important to evaluate the multigenerational fitness of hybrid descendants resulting from gene flow from HR soybeans to wild soybeans.

Seeds of wild soybean are characterized by a hard, impermeable seed coat, a trait referred to as hardseededness [5,14,15]. Hard seededness is one of the dormancy traits of wild soybean that inhibit germination until favorable conditions appear [16]. In contrast, the seeds of cultivated soybean are protein rich and perishable, which prevents the domesticated species from overwintering and persisting outside of cultivation [17]. Previous studies have established that the hybrids of wild soybean (as the seed parent) and HR soybean (as the pollen donor) were more similar to wild soybeans in seed morphology due to the segregation distortion, and the hard seed coat of hybrids needed to be scarified to break dormancy [14,18]. Studies have also shown that hybrids can complete the entire life cycle and that their fitness in soybean fields is comparable to or higher than that of wild soybean [19–21]. However, it is unknown to what extent crop-wild hybrids and their descendants consistently show the hard seededness that would be required for survival and long-term persistence outside of cultivation.

In a previous work, we evaluated the sexual compatibility of 10 populations of wild soybean with HR soybeans [22]; for 9 of the F_1 created, we determined that the fitness of hybrids was significantly lower than that of the corresponding wild soybean parent [14]. However, that study did not examine fitness past the F_1 . In order to further explore the continuous impact of HR soybean gene flow on wild populations and the environment, the fitness of F_2 and F_3 derived from crosses of HR soybean line T14R1251-70 and two wild soybeans, LNTL and JLBC, which were collected from LiaoNing TieLing and JiLin BaiCheng, respectively, was investigated under two soil conditions and with or without weed competition in the current study. In addition, the hard seededness of hybrid seeds was assessed by observing the seed coat structure and determining the emergence rate after burying in different soil depths for different lengths of time. Our results on seed hardness and fitness of the F_2 and F_3 suggest that cultivation of HR soybean may pose risks for transgene escape to wild soybean and persistence of crop-wild hybrid descendants in the wild.

2. Results

2.1. Emergence Rate

For JLBC F₂, the mean emergence rate was 90.8%, which was significantly higher than the mean value of its wild parent grown in the same experiment (79.2%) (p < 0.05); no significant difference in mean emergence rates was observed for JLBC F₃ compared to its wild parent (Figure 1). In contrast, the mean emergence rate value of LNTL F₂ (77.5%) was significantly lower than that of its wild parent (88.1%) (p < 0.01); for LNTL F₃ and its wild parent, no significant difference was observed. Thus, variation in emergence rate differed in opposite directions at the F₂ between the two wild populations, and they were not consistent between the F₂ and F₃ generations for either population.



Figure 1. Emergence rate of F_2 , F_3 and its wild soybean JLBC and LNTL. Note: * and ** indicates significant difference (p < 0.05) and extremely significant difference (p < 0.01) between hybrid descendants and its wild soybean.

2.2. True Leaf and Cotyledon Size

For the JLBC F_2 and F_3 , both generations had statistically greater mean values of true leaf length than their wild soybean parent (15.8% and 13.2% longer, respectively). For JLBC F_3 only, true leaf width was statistically smaller than JLBC. Similarly, for the LNTL, F_2 and F_3 had significantly greater mean values of true leaf length compared to the LNTL (7.79% and 29.7% longer, respectively). However, the mean leaf width of the LNTL F_3 was also significantly greater than that of the wild parent.

No clear pattern was apparent for cotyledon size data. JLBC F_3 were significantly smaller than those of JLBC. Mean cotyledon width of LNTL F_2 was significantly smaller than that of LNTL, whereas for LNTL F_3 , the mean values of both cotyledon length and width were significantly greater than those of their wild soybean parent (Figure 2).



Figure 2. Size of cotyledon and true leaf of F_2 , F_3 and wild soybeans ((**A**): LNTL; (**B**): JLBC). Note: * and ** indicates significant difference (p < 0.05) and extremely significant difference (p < 0.01) of the same trait between hybrid descendants and its wild soybean.

There were differences in the mean plant height of JLBC, JLBC F_2 , and F_3 among the four planting conditions. The mean values of JLBC F_2 and F_3 were 3.9–11.7% higher than JLBC. There was no significant difference in plant height of LNTL or F_2 among the four planting conditions, and the mean plant height of LNTL and F_3 was significantly higher when pure planted in farmland soil than that in wasteland soil. Under the same planting conditions, the mean plant heights of F_2 were 16.70–20.30% higher and F_3 were 36.98–44.63% higher than those of LNTL, respectively (Figure 3).



Hybrid descendants and wild soybeans

Figure 3. Plant height of F_2 , F_3 , and wild soybean ((**A**): LNTL; (**B**): JLBC) under four planting conditions (the third trifoliolate leaf stage). Note: PW: pure planting in wasteland soil; PF: pure planting in farmland soil; MW: mixed planting with weeds in wasteland soil; MF: mixed planting in farmland; * and ** indicates significant difference (p < 0.05) and extremely significant difference (p < 0.01) between hybrid descendants and its wild soybean. Different lowercase letters indicate significant difference (p < 0.05) of hybrid descendants or wild soybeans among four planting conditions.

2.4. Aboveground Dry Biomass

The mean aboveground dry biomass of JLBC F_2 was higher than that of their wild parent under pure planting, and that of JLBC F_3 was higher in farmland soil. JLBC F_2 and F_3 had 1.99–3.71 times greater mean aboveground dry biomass than JLBC under the same planting condition. The mean aboveground dry biomass of LNTL F_2 under mixed planting in farmland soil was significantly higher than that under the other three conditions, and that of LNTL in the same year was not significantly different among planting conditions. The aboveground dry biomass of LNTL F_3 and LNTL was significantly higher under pure planting in farmland soil and significantly lower under mixed planting in farmland soil than those under the other two conditions. Under the same planting conditions, the mean aboveground dry biomass of LNTL F_2 and F_3 was significantly higher than that of LNTL; F_2 was 1.3–1.59 times higher, while F_3 was 1.59–1.77 times higher than LNTL (Figure 4A,B).



Figure 4. Aboveground dry biomass of F_2 , F_3 , wild soybeans (**A**,**B**) and weeds (**C**,**D**) under four planting condition. Note: PW: pure planting in wasteland soil; PF: pure planting in farmland soil; MW: mixed planting with weeds in wasteland soil; MF: mixed planting in farmland; * and ** indicates significant difference (p < 0.05) and extremely significant difference (p < 0.01) of the same trait between hybrid descendants and its wild soybean. Different lowercase letters indicate significant difference (p < 0.05) of hybrid descendants or wild soybeans among four planting conditions.

Aboveground dry biomass of weeds in farmland soil was always higher than that in wasteland soil (Figure 4C,D). There were no significant differences between the weed biomass with JLBC F_2 , F_3 , and JLBC. There was no significant difference between the weed biomass with LNTL F_2 and with LNTL, while that of LNTL F_3 was significantly lower than LNTL in both farmland and wasteland soil.

2.5. Vitro Pollen Germination Rate

The pollen germination rates of JLBC F_2 were higher when pure planted than when mixed planted, while those of JLBC F_3 were higher in farmland soil than in wasteland soil. The mean pollen germination rates of JLBC F_2 were higher than or comparable to JLBC, and those of JLBC F_3 were significantly lower than JLBC. The pollen germination rates of LNTL F_2 and its wild soybean had the same trend under four conditions, with the highest under mixed planting in farmland soil or comparable. That of LNTL F_3 and its wild soybean also had the same trend under four conditions, with the highest under pure

planting in farmland soil and the lowest under mixed planting in wasteland soil. Under the same planting conditions, the pollen germination rate of LNTL F_2 and F_3 was 7.49–15.08% lower than that of LNTL (Figure 5).



Hybrid descendants and wild soybeans

Figure 5. Vitro pollen germination rate of F_2 , F_3 , and wild soybeans ((**A**): LNTL; (**B**): JLBC) at 60 min under four planting condition. Note: PW: pure planting in wasteland soil; PF: pure planting in farmland soil; MW: mixed planting with weeds in wasteland soil; MF: mixed planting in farmland; * and ** indicates significant difference (p < 0.05) and extremely significant difference (p < 0.01) of the same trait between hybrid descendants and its wild soybean. Different lowercase letters indicate significant difference (p < 0.05) of hybrid descendants or wild soybeans among four planting conditions.

2.6. Pod and Filled Seed Number per Plant

Mean values for pod and filled seed number per plant of JLBC F_2 and JLBC were higher under pure planting than under mixed planting conditions, while mean values for JLBC F_3 and JLBC were higher in farmland soil than in wasteland soil. Pod and filled seed numbers per plant for JLBC F_2 and F_3 were 1.1–3.7 times higher than JLBC in all four conditions.

Mean values for pod and filled seed number per plant of LNTL F_2 and its wild soybean were significantly higher under mixed planting in farmland soil than in the other three planting conditions. In contrast, mean values for pod and filled seed number per plant of LNTL F_3 and LNTL were significantly higher under pure planting in farmland soil than in the other three conditions. Under the same planting conditions, the mean number of pods per plant of LNTL F_2 and F_3 was 8.46–24.28% higher than that of LNTL (Figure 6).



Hybrid descendants and wild soybeans

Figure 6. Pod number ((**A**): LNTL; (**B**): JLBC) and filled seed number ((**C**): LNTL; (**D**): JLBC) per plant of F_2 , F_3 and wild soybeans under four planting condition. Note: PW: pure planting in wasteland soil; PF: pure planting in farmland soil; MW: mixed planting with weeds in wasteland soil; MF: mixed planting in farmland; * and ** indicates significant difference (p < 0.05) and extremely significant difference (p < 0.01) of the same trait between hybrid descendants and its wild soybean. Different lowercase letters indicate significant difference (p < 0.05) of hybrid descendants or wild soybeans among four planting conditions.

2.7. 100-Seed Weight

The mean values of 100-seed weight for self-pollinated seeds of JLBC F_2 and F_3 were significantly lower than JLBC. JLBC F_3 under mixed planting in farmland soil had significantly lower mean values than under other planting conditions; the values for JLBC F_2 and F_3 under other planting conditions were similar. Under the same planting condition, the mean 100-seed weight values for self-pollinated seeds of LNTL F_2 and F_3 were significantly higher than those of LNTL, with mean values 1.56–1.92 times greater than those of the wild parent. The mean 100-seed weight of LNTL F_3 under pure planting in farmland soil was significantly higher than that of the other three conditions, while others were similar (Figure 7).



Figure 7. 100-seed weight of seeds of F_2 , F_3 , and wild soybean ((**A**): LNTL; (**B**): JLBC) under four planting conditions. Note: PW: pure planting in wasteland soil; PF: pure planting in farmland soil; MW: mixed planting with weeds in wasteland soil; MF: mixed planting in farmland; * and ** indicates significant difference (p < 0.05) and extremely significant difference (p < 0.01) of the same trait between hybrid descendants and its wild soybean. Different lowercase letters indicate significant difference (p < 0.05) of hybrid descendants or wild soybeans among four planting conditions.

2.8. Relative Composite Fitness

Taking wild soybean as the standard "1", the values of correspondingly F_2 and F_3 were valued as the relative composite fitness. The relative composite fitness of JLBC F_2 and F_3 was higher than that of JLBC under all four planting conditions, but not statistically significant. The relative composite fitness of JLBC F_2 among four planting conditions had no difference, while that of JLBC F_3 was higher under pure planting conditions or in farmland soil. The relative composite fitness of LNTL F_2 and F_3 was higher than that of LNTL under all four planting conditions, but the difference was not significant for F_2 , while it was significant for F_3 . There was no significant difference between LNTL F_2 and its wild parent among the four conditions, and both LNTL F_3 and LNTL had significantly higher

fitness when pure planted in farmland soil than under the other three conditions, while there was no significant difference among the three conditions (Figure 8).



Figure 8. Comparison of composite fitness between wild soybeans and F_2 , F_3 . Note: PW: pure planting in wasteland soil; PF: pure planting in farmland soil; MW: mixed planting with weeds in wasteland soil; MF: mixed planting in farmland; The dashed line represents the composite fitness of wild soybean as 1, * indicates significant difference (p < 0.05) of the same trait between hybrid descendants and its wild soybean.

2.9. Hard Seededness and Germination Rate

Self-pollinated seeds of LNTL F_2 and F_3 were used to conduct this experiment. The hard seededness rate of LNTL F_2 seeds was 89.50%, which was extremely significantly lower than that of LNTL (98.50%), and there was no significant difference between LNTL F_3 seeds and LNTL seeds. After scarification, there was no significant difference in germination rate between F_2/F_3 seeds and wild soybean seeds (Table 1).

Table 1. Hard seededness rate and germination rate with scarification of LNTL F₂, F₃ seeds.

Material	Hard Seededness Rate (%)	Germination Rate with Seed Scarification (%)
LNTL LNTL F ₂	98.50 ± 0.96 ** 89.50 ± 0.96	$\begin{array}{c} 94.44 \pm 0.93 \\ 93.86 \pm 0.53 \end{array}$
LNTL LNTL F ₃	$\begin{array}{c} 100\\ 96.50\pm0.02\end{array}$	$\begin{array}{c} 98.00 \pm 0.00 \\ 94.50 \pm 0.02 \end{array}$

Note: ** indicates extremely significant difference (p < 0.01) between hybrids and its wild soybean.

2.10. Seed Coat Structure

Self-pollinated seeds of LNTL F_2 were used to conduct this experiment. There are obvious pits on the surface of the HR soybean seed coat, and the shape of the pits is irregular (Figure 9A,B). There are three main types of pits: Shallow long pits, deep round pits, shallow round pits, and some pits with a crack width of 0.1–0.3 µm. The surface of the HR soybean has almost no attachment, and the stratum corneum is directly exposed to the outside. Both LNTL and its LNTL F_2 seed coat surface are covered by a thick layer of sediment, similar to a bulge at the basin margin, and the entire seed coat surface is honeycomb-shaped (Figure 9E,I); there are no cracks on the surface of wild soybean or F_2 seed coat. At the hila of wild soybean and F_2 , there is a middle dent and several multiple irregular cracks on both sides of the dent, with a width of 3–20 µm (Figure 9F,J). At the same time, no honeycomb-like sediment attachment was observed around the hila of LNTL and F_2 seeds.



Figure 9. SEM of seed coat structure of transgenic soybean, LNTL wild soybean and F₂ seeds. Note: (**A**) Seed coat surface of TS (×500); (**B**) depress and crack on seed coat surface of TS (×2000); (**C**,**D**) seed coat layers of TS (×250); (**E**) seed coat surface of LNTL F₂ (×500); (**F**) hilum surface of LNTL F₂ (×60); (**G**,**H**) seed coat layers of LNTL F₂ (×300); (**I**) seed coat surface of LNTL F₂ (×500); (**J**) hilum surface of LNTL (×60); (**K**,**L**) seed coat surface of LNTL (×300).

The seed coat structure of HR soybean, LNTL, and LNTL F_2 all contains four cell layers, followed by the palisade epidermis, hourglass cells, parenchyma, and aleurone layer; LNTL and F_2 seeds also have a stratum corneum over the seed coat. Among them, the aleurone layer has monolayer cells, which are not easy to recognize with SEM (Figure 9C,D,G,H,K,L).

The palisade epidermis of LNTL wild soybean was comparable to that of the F_2 seeds, and both were higher than that of HR soybean. The hard seededness rates of LNTL, F_2 , and HR soybean seeds decreased from 98.50%, 89.50%, and 1.00%, respectively. However, the proportion of palisade epidermis thickness in the seed coat decreases with the decrease in hard seededness rate. The thickness of hourglass cells and their proportion increased with the decrease in hardness rate. The parenchyma layers of LNTL and F_2 seeds were significantly thinner than those of HR soybean (Figure 10).



Figure 10. Relationship between hard seededness rate and thickness of seed coat layers of transgenic soybean, LNTL wild soybean and F_2 seeds. Note: Different lowercase letters indicate significant difference (p < 0.05) among hybrid descendants, wild soybean, and transgenic soybean.

2.11. Mineral Element in Seedcoat

Self-pollinated seeds of LNTL F_2 were used to conduct this experiment. The content of Ca in the seed coat palisade epidermis of LNTL F_2 seeds was significantly lower than

that of LNTL; however, there was no significant difference for other mineral elements that were measured (Figure 11).



Figure 11. Main mineral element content of seed coat palisade epidermis of LNTL wild soybean and F_2 seeds. Note: * indicates significant difference (p < 0.05) between hybrid descendants and wild soybean.

2.12. Seed Vitality in Soil

For self-pollinated seeds of both JLBC F_3 and JLBC, under both 3 cm and 10 cm of soil, the trend of natural emergence rate increased with time, and the emergence rate with seed scarification hardly changed over time. The emergence rate of JLBC F_3 seeds was higher with seed scarification and lower without seed scarification than JLBC, respectively (Figure 12A).



Figure 12. The emergence rate with or without scarification of seeds of JLBC F_2 (**A**), LNTL F_2 (**B**) and LNTL F_3 (**C**) after burying in 3 cm or 10 cm soil. Note: * and ** indicates significant difference (p < 0.05) and extremely significant difference (p < 0.01) between hybrid descendants and its wild soybean.

For self-pollinated seeds of both LNTL F_2 and LNTL, under both 3 cm and 10 cm soil, the trend of natural emergence rate increased with time, and the natural emergence rate of LNTL F_2 seeds after burying for 6 months was significantly higher than that of LNTL. After seed scarification, the trend of the natural emergence rate of both soybeans decreased with time, and the natural emergence rate of F_2 seeds after burying for 3 months was significantly higher than that of LNTL (Figure 12B).

For self-pollinated seeds of LNTL F_3 and LNTL, both under 3 cm and 10 cm of soil, the trend of natural emergence rate of both seeds increased with time and decreased after burying for 15 months. The natural emergence rate of LNTL F_3 seeds after burying was higher than that of LNTL but not significantly. The emergence rate with scarification of LNTL F_3 seeds was higher than that of LNTL but only significant at one time point (Figure 12C).

3. Discussion

3.1. Fitness of F₂, F₃ Compared with Parents

The F_2 and F_3 of this experiment were obtained by hybridizing HR soybeans (as the pollen donor) with wild soybeans (as the seed parent). The genetic difference between cultivated soybeans and wild soybeans derives from the domestication by humans of the wild species into the cultivated crop species [23–26]. In the domestication of legumes, selection favors enhanced aboveground traits, including greater seed size and palatability, reduced seed dormancy, and other desirable agronomic traits. The hybrids of cultivated soybean and wild soybean usually have a growth advantage over wild soybean [6,21]. In the context of crop improvement, hybridization of domestic soybeans (as the seed parent) and wild soybeans (as the pollen donor) can improve the resistance of hybrids and even promote the diversity of varieties [27,28]. However, if it is allowed to grow outside of cultivation, these same fitness advantages create potential ecological risks, particularly in regions of transgenic HR soybean cultivation; in this context, the advantages of hybrids do not bode well.

In our previous study, it was found that F_1 hybrids of HR soybean and wild soybeans, including LNTL and JLBC, had lower fitness than the wild soybean parents [14]. In this study, F_2 and F_3 of both LNTL and JLBC, regardless of soil conditions and whether there was weed competition, showed significantly elevated mean values relative to their wild parents for multiple fitness-related traits, including plant height, number of pods per plant, number of filled seeds per plant, filled seed weight per plant, aboveground dry biomass, and 100-seed weight. The mean composite fitness of LNTL F_3 under all four planting conditions was significantly higher than that of LNTL. As the generations increased, the adverse effects of hybridization are gradually eliminated through gene segregation and recombination [29,30]. The wild soybean LNTL and JLBC were collected at high latitudes, and in-field experiments were at lower latitudes. The fitness of wild soybeans was decreased due to the shorter photoperiod and other unsuitable environmental factors [31,32]. After receiving pollen of HR soybean adapted to the climate of the experimental location, F_2 and F_3 inherited adaptability to the local climate and environment, which ultimately led to the improvement of the survival competitiveness of the hybrid descendants.

It is worth noting that regardless of the planting conditions, LNTL F_2 and F_3 pollen viability was significantly higher than that of wild soybeans, while JLBC F_3 had lower pollen viability. Pollen activity reflects the quality of pollen, affects seed formation, and is an important indicator for valuing reproductive ability [33]. The probability of interspecific hybridization and the fertility of hybrid descendants depend largely on the homology of the genomes and the degree of homology, which determines the possibility of pairing and recombination between the chromosomes of the parents [34]. HR soybean and wild soybean both belong to *Glycine*, and they have the same chromosome number (2n = 40), but there are differences in chromosome behavior and the division cycle of meiosis [5,35]. Therefore, in meiosis, hybrid descendants would have abnormal chromosome behavior. And different

populations of wild soybean have varying degrees of chromosomal abnormalities, which also reflect the diversity of the germplasm resources of wild soybeans.

3.2. Effects of Soil Nutrition and Competition on Fitness of F₂, F₃

As the substrate for crops, there are various elements and substances that affect the development and reproduction of plants in the soil [36,37]. Unlike other crops, legumes have the ability to symbiotically fix nitrogen with nitrogen-fixing bacteria [38,39]. Therefore, the growth of soybean is not only affected by soil nutrition, especially nitrogen in the soil [40]. It was proven that the nitrogen-fixing capacity of cultivated soybean and wild soybean and the interaction mode with rhizosphere microorganisms are different [17,41,42].

In this experiment, soils from farmland and wasteland were used to plant the hybrids. The results showed that when there was no weed competition, except for LNTL F_2 , the fitness of JLBC F_2 , JLBC F_3 , LNTL F_3 , and their wild soybean under the farmland soil was significantly higher than that of the wasteland soil. The nitrogen form in soil may partly explain this anomalous difference in LNTL F₂. Nitrate nitrogen and ammonium nitrogen, which are called available nitrogen, are effective forms of nitrogen nutrients in soil and can be directly absorbed and utilized by roots [43,44]. For JLBC F₃ and LNTL F_2 , the content of available nitrogen was 10.71 mg/kg in wasteland soil and 23.59 mg/kg in farmland soil, both were not high enough for growth. At the same time, there was no significant difference in total nitrogen content between farmland soil and wasteland soil this year. This may explain the similar fitness of JLBC F_3 and LNTL F_2 and their wild soybeans in both wasteland and farmland soils. The restriction on the growth of JLBC F₃ and LNTL F_2 in wild soybean may be due to a lack of available phosphorus. Under limiting phosphorus, the uptake and utilization of nitrogen and other metabolic pathways will also be affected [45-48]. Therefore, when the nutrients were relatively abundant, the available nitrogen and available phosphorus, which were significantly different between farmland and wasteland soil, also had a significant impact on the growth of JLBC F_2 , LNTL F_3 , and wild soybean.

All hybrids have similar patterns in different soils to wild soybeans, suggesting that the utilization pattern of soil nutrients of hybrids and symbiotic nitrogen fixation are inherited from the seed producer, the wild soybean.

Weeds not only compete with crops for light [49,50] and nutrition in soil [51], but also change the environment and microorganisms of the rhizosphere through root exudates, which affects the growth of soybeans [52,53]. When there was weed competition, the number of pods and filled seeds per plant of JLBC F₃, LNTL F₂, and their wild soybean in farmland soil were significantly higher than those in wasteland soil, but the fitness of JLBC F_2 , LNTL F_3 and their wild soybean was exactly opposite. This difference came from differences in the nutrient content of the soil used in the three-year trial. Weed dry biomass can reflect the nutrient level of the soil. It can also be seen that LNTL wild soybeans are less competitive with weeds than LNTL F_3 . This increased competitiveness may come from the genes of the paternal HR soybeans [54]. Although the available nitrogen level in wasteland soil was relatively low, the nitrogen fixation ability of hybrids and wild soybeans could still maintain the nitrogen balance in the soil and the normal growth of plants. The number of pods and filled seeds per plant under LNTL F₃ mixed planting in wasteland soil was significantly higher than that of mixed planting in farmland soil. This phenomenon may also come from biological nitrification inhibition [55]. When there was weed competition, weeds, soybeans, soil nutrients, and the rhizosphere formed a complex interacting system [56–59]. The environment was changed to benefit the strong side, such as wild soybeans.

3.3. Seed Coat Structure and Seed Dormancy

Honeycomb epidermal attachments may be the first barrier to prevent the seed from absorbing water and expanding, and they are an important way for the seed to remain dormant. There is no obvious attachment on the surface of the seed coat of HR soybean, and the dormancy ability of crop seeds is almost completely lost. This attachment comes from the endocarp, known as bloom, and directly acts to change the gloss of the surface of the seed, reducing the chance that the seed will be found and eaten by animals [60,61]. Meanwhile, bloom has been proven to be related to seed oil content [62], and the change in soybean permeability in domestication was caused by human selection. The difference in bloom explained the difference in natural emergence rates between HR soybean and wild soybeans, but it still does not explain the difference between hybrid descendants and wild soybeans.

The emergence rate of seeds with scarification showed that there was no significant difference in seed viability between seeds of LNTL F₂, F₃, and wild soybean, which showed similar embryonic activity. The hard seededness of wild soybean ensures long-term seed dormancy. With time, buried seeds of all hybrids and wild soybeans were more likely to break dormancy, and embryonic activity decreased. Point mutations in *Gm*Hs1-1 cause the loss of hard seededness and this gene correlates with the content of calcium in the seed coat [63]. In the experiment, the calcium content in the seed coat of LNTL F₂ seeds was significantly lower than that of wild soybean, indicating that LNTL F₂ seeds partially inherited the soft seed coat of HR soybean, resulting in its hard seededness being weaker than wild soybean. However, some soybeans promote water absorption and break dormancy while maintaining the calcium content of the seed coat by cracking through the seed coat. This is the case with irregular cracks on the surface of HR soybeans seeds observed by SEM, but LNTL F_2 seeds did not have this character. The formation of such cracks may come from changes in the seed coat layers. The shape and number of hourglass cells are often thought to be strongly related to seed dormancy and viability [64,65]. Palisade epidermis and parenchyma of LNTL F_2 seeds were both similar to those of wild soybean, but the hourglass cells were significantly higher than those of wild soybean and lower than those of HR soybean. Hourglass cells are associated with the accumulation of various enzymes associated with water absorption and germination, such as catalase [66,67]. The difference in the hourglass cell layer could exactly explain the decline in hard seededness rate of LNTL F_2 seeds compared to wild soybean.

Therefore, the hybrid seeds of wild soybean and HR soybean reduced the hard seededness compared to wild soybeans through the thickening of hourglass cells and the reduction of calcium content in the palisade epidermis.

4. Materials and Methods

Herbicide-resistant transgenic soybeans T14R1251-70 were provided by the National Soybean Improvement Center of Nanjing Agricultural University. The HR soybean, containing one single-copy *cp4-epsps*, was obtained by Agrobacterium-mediated co-transformation of the receptor soybean NJR44-1, which is an elite line bred by the National Soybean Improvement Center of Nanjing Agricultural University. The HR soybean withstands 3600 g a.i. ha-1 41% glyphosate isopropylammonium AS (Roundup Ultra; Monsanto, St. Louis, MO, USA). Wild soybean populations were collected from Tieling, Liaoning Province, and Baicheng, Jilin Province. Crossed seeds were obtained by artificial hybridization of wild soybeans as the seed producer and HR soybeans as the pollen donor from 2016 to 2017 [22]. The crossed seeds were harvested from different seed producers individually and then stored at 4 °C until further use. Experiments were conducted in a greenhouse and net house at the Pailou Experimental Farm (32°20′ N, 118°37′ E), Nanjing Agricultural University, China, from 2018 to 2020.

4.1. Seed Treatment and Seeding

Scarify the seed coat of wild soybeans and hybrid descendants. Seeds were sown in a plastic cup with a hole at the bottom (a diameter of 7 cm and a height of 7.5 cm). The substrate for seeding was farmland soil and wasteland soil, as described in Table 2. Seedings were placed in a net chamber for normal water management, and all test materials were

	Soils	Organic Matter g/kg	Total Nitrogen g/kg	Total Phosphorus g/kg	Total Potassium g/kg	Available Phosphorus mg/kg	Available Nitrogen mg/kg
JLBC F ₂	Wasteland soil Farmland soil	$\begin{array}{c} 2.79 \pm 0.10 \\ 38.51 \pm 0.35 \ * \end{array}$	$\begin{array}{c} 0.37 \pm 0.01 \\ 2.20 \pm 0.03 \ * \end{array}$	$0.56 \pm 0.01 \\ 1.76 \pm 0.01 *$	$\begin{array}{c} 22.04 \pm 0.46 \\ 18.94 \pm 0.19 \end{array}$	$\begin{array}{c} 22.39 \pm 0.52 \\ 47.81 \pm 0.33 \ ^* \end{array}$	$\begin{array}{c} 44.15\pm 0.2 \\ 163.74\pm 0.54 \ ^* \end{array}$
JLBC F ₃ and LNTL F ₂	Wasteland soil Farmland soil	$\begin{array}{c} 4.82 \pm 0.22 \\ 9.74 \pm 0.81 \ ^* \end{array}$	$\begin{array}{c} 0.27 \pm 0.37 \\ 0.37 \pm 0.04 \end{array}$	$\begin{array}{c} 0.17 \pm 0.11 \\ 0.26 \pm 0.12 \ ^* \end{array}$	$\begin{array}{c} 9.79 \pm 0.09 \\ 10.07 \pm 0.10 \end{array}$	$0.1 \pm 0.03 \\ 1.68 \pm 0.31 *$	$\begin{array}{c} 10.71 \pm 1.25 \\ 23.59 \pm 2.61 \ ^{*} \end{array}$
LNTL F3	Wasteland soil Farmland soil	$\begin{array}{c} 7.78 \pm 0.40 \\ 11.19 \pm 1.50 \end{array}$	$\begin{array}{c} 0.72 \pm 0.02 \\ 1.06 \pm 0.11 \end{array}$	$\begin{array}{c} 0.25 \pm 0.01 \\ 0.36 \pm 0.07 \end{array}$	$\begin{array}{c} 20.94 \pm 0.42 \\ 21.10 \pm 0.48 \end{array}$	9.99 ± 0.86 28.21 ± 1.32 *	51.91 ± 1.38 145.41 ± 21.08 *

randomly placed in the net chamber and cultured under natural light and photoperiod, during which the temperature fluctuated between 20 and 38 °C.

Table 2. Son physicochemical properties per yea	Table 2. Soil	physicoche	mical pro	perties	per	year
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Note: * indicates significant difference between wasteland soil and farmland soil (p < 0.05).

4.2. Emergence Rate and Cotyledon, True Leaf Size

When the cotyledons of the plants are unearthed and completely green (about 2 weeks after sowing), the number of seedlings of soybean plants is counted. When the first compound leaf of the plant has formed and the leaves are wrinkled but not fully expanded, the longest and widest cotyledons and true leaves are determined using Vernier calipers; each single plant is a replicate, and 20 plants per material are randomly selected for measurement.

4.3. cp4-Epsps in Hybrids

After the first ternately compound leaf of the plant was unfolded, the *cp4-epsps* was detected by PCR with a specific primer (5'-GGCACAAGGGATACAAACC-3'; 5'-ACCGCCGAACATGAAGGAC-3'). Count the number of plants carrying resistance genes and plants without resistance genes, and use the chi-square test to verify whether the resistance of hybrid separation ratio results conform to Mendel's law of 3:1. The specific formula is as follows:

$$\chi^{2} = \frac{\left[|b \times A_{1} - a \times A_{2}| - (a+b)/2\right]^{2}}{a \times (A_{1} + A_{2})}$$
(1)

 χ^2 represents the chi-square value, such as $\chi^2 < 3.84$, that is, p > 0.05, indicating that the inheritance law of resistance genes in hybrids conforms to Mendel's law of inheritance; A_1 indicates the number of plants carrying resistance genes; A_2 indicates the number of plants that do not carry resistance genes; F_2 : a = 3, b = 1; F_3 : a = 5, b = 1.

4.4. Planting Conditions

Wasteland soil and farmland soil were collected at the Pailou base. Take three copies of the soil and entrust Nanjing Zhongding Biological Company to test the physical and chemical properties of the soil (Table 2).

Four planting conditions were set: Pure planting in wasteland soil (PW), pure planting in farmland soil (PF), mixed planting with weeds in wasteland soil (MW), and mixed planting with weeds in farmland (MF). For the emergence rate test, 60 plants with consistent growth of HR soybean, LNTL, JLBC, and hybrid descendants were selected, and 15 plants were transplanted under four planting conditions. Under single planting conditions, a pod (23 cm in diameter and 25 cm in height) with bamboo was set for LNTL, JLBC, and hybrid descendants growth. When mixed planting with weeds, *Setariaviridis* (L.) *Beauv*. 0.5 g, *Digitariasanguinalis* (L.) Scop. 0.5 g, *Echinochloacolona* (L.) Link. 0.5 g, and *Eleusine indica* (L.) Gaertn. 0.25 g were sown evenly in pots (52 cm diameter and 35 cm height).

4.5. Fitness Determination

Investigate fitness indicators during plant vegetative and reproductive periods. Emergence rate: Two weeks after sowing, count the number of all seedlings unearthed with green cotyledons; true leaf size: When the first compound leaf has been formed and the leaf is wrinkled but not fully expanded, the cotyledon length width and true leaf length width are measured by vernier calipers; plant height: At the third-ternately-compound stage, the length from the tip of the main stem to the cotyledon ring was measured; pollen vitality: Randomly collect flower buds on plants at full bloom period (flag petals are 1–2 mm higher than sepals), culture in vitro for 60 min, and count the number of germinated pollen under a microscope; aboveground dry biomass: After harvesting, the aboveground part of the plant is dried to a constant weight and weighed; number of pods per plant: After harvesting, the total number of pods per plant is counted and artificially threshed; number of filled seeds per plant: After harvesting, select the filled seeds from all single seeds (with regular shape, no depression, and no shrink), count the number, and weigh them; composite fitness: Taking wild soybean as the standard "1", the seedling emergence rate, cotyledon length \times cotyledon width + true leaf length \times true leaf width, plant height, aboveground dry biomass, pollen germination rate of 60 min, number of pods and filled seeds per plant, 100-seed weight to wild soybean were valued, and the composite relative fitness is the average of the values.

4.6. Seed Hard Seededness Rate and Scarified Emergence Rate

Fifty seeds were randomly selected from all the plants under the pure planting in soil with 4 repeats. The number of seeds that did not swell (seed size did not change) after 7 days of soaking in distilled water was counted. Hard seededness rate = number of unswollen seeds/total number of seeds × 100%. After the hard seededness rate is determined, scarify the seed coat of the remaining hard wild soybean and hybrid seed without damaging the embryo. Incubate the scarified seeds at a constant temperature of 25 °C for 7 days; count the germinating seeds with a radicle length twice that of the seed length. Emergence rate (%) = total number of germinated seeds / total number of seeds × 100%.

4.7. Seed Coat Structure and Elemental Content

Select 3 filled seeds with a complete seed coat from plants purely planted in farmland soil. Cut the seeds along the seed ridge corresponding to the center point of the seed hilum to avoid damage to the embryo. Stick the cut seeds on the sample stage; use a Hitachi-1010 ion sputterer to spray gold on the surface; use a Hitachi-SU8010 scanning electron microscope for observation and photography; and use an SEM accelerating voltage of 20 kV. Photoshop (version 21.1.2; Adobe Systems Incorporated, San Jose, CA, USA) was used to measure the thickness of each structure. The elemental content of the palisade layer of the seed coat was determined with an X-ray spectrometer (HORIBA).

4.8. The Seed Vitality under Soil

Eighty seeds were randomly selected from each of the 15 plants purely planted in farmland soil, and they were packed into nylon mesh bags with a 0.2 mm pore size and buried in the research base of Nanjing Agricultural University in December of that year, 3 cm and 10 cm deep from the soil surface. The number of seeds that had emergence, the emergence rate after scarifying the seed coat, and the number of ungerminated seeds checked for rot and mildew were recorded.

4.9. Data Analysis

All data are statistically analyzed using SPSS (SPSS 22.0). Duncan's multiple range test in the univariate ANOVA test was used to analyze the differences in fitness indexes of the same material under four planting conditions, the thickness of different cell layers of transgenic soybean, wild soybean, and hybrid descendant seed coat, and the proportion of total thickness. The independent sample T test was used to analyze the differences in fitness indexes and composite fitness, in hard seededness rate and emergence rate after nicking hard seed coat, and in mineral element content between wild soybean and hybrid, and the data were plotted with Prism GraphPad.

5. Conclusions

The fitness of the F_2 and F_3 of herbicide-resistant transgenic soybean line T14R1251-70 and wild soybean LNTL and JLBC was significantly increased under farmland and wasteland soil conditions, as well as with or without weed competition, and the competitiveness was significantly enhanced. Self-pollinated seeds produced by hybrid descendants were similar to wild soybeans with a hard seed coat but had a lower hard seededness rate due to the seed coat structure. The decrease in hard seededness was due to the thicker hourglass cells and the lower Ca content in the seed coat. Hybrid descendants containing modified gene *cp4-epsps* can complete life histories and produce a large number of seeds, which can persist in the soil for a long time, germinate, and grow under suitable conditions. So, the hybrid descendants of herbicide-resistant transgenic soybean and wild soybean have potential ecological risks.

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References

- ISAAA. Global Status of Commercialized Biotech/GM Crops in 2018: Biotech Crops Continue to Help Meet the Challenges of Increased Population and Climate Change; The International Service for the Acquisition of Agri-Biotech Applications (ISAAA): Ithaca, NY, USA, 2018; Volume 54.
- ISAAA. GM Approval Database. Available online: https://www.isaaa.org/gmapprovaldatabase/citations/default.asp (accessed on 17 August 2022).
- 3. Wang, K.J.; Li, X.H. Interspecific gene flow and the origin of semi-wild soybean revealed by capturing the natural occurrence of introgression between wild and cultivated soybean populations. *Plant Breed.* **2011**, *130*, 117–127. [CrossRef]
- 4. Kim, D.Y.; Heo, J.H.; Pack, I.S.; Park, J.-H.; Um, M.S.; Kim, H.J.; Park, K.W.; Nam, K.-H.; Oh, S.D.; Kim, J.K.; et al. Natural hybridization between transgenic and wild soybean genotypes. *Plant Biotechnol. Rep.* **2021**, *15*, 299–308. [CrossRef]
- 5. Wang, K.J.; Li, X.H. Genetic diversity and gene flow dynamics revealed in the rare mixed populations of wild soybean (*Glycine soja*) and semi-wild type (Glycine gracilis) in China. *Genet Resour. Crop Ev.* **2013**, *60*, 2303–2318. [CrossRef]
- Kitamoto, N.; Kaga, A.; Kuroda, Y.; Ohsawa, R. A model to predict the frequency of integration of fitness-related QTLs from cultivated to wild soybean. *Transgenic Res.* 2012, 21, 131–138. [CrossRef] [PubMed]
- Mizuguti, A.; Yoshimura, Y.; Matsuo, K. Flowering phenologies and natural hybridization of genetically modified and wild soybeans under field conditions. *Weed Biol. Manag.* 2009, *9*, 93–96. [CrossRef]
- 8. Nakayama, Y.; Yamaguchi, H. Natural hybridization in wild soybean (*Glycine max* ssp. *soja*) by pollen flow from cultivated soybean (*Glycine max* ssp. *max*) in a designed population. *Weed Biol. Manag.* **2002**, *2*, 25–30. [CrossRef]
- 9. Liu, B.; Xue, K.; Liu, L.-P.; Zhou, Y.; Han, J. Progress on the Gene Flow From Genetically Modified Soybeans to Wild Soybeans. J. Ecol. Rural Environ. 2020, 36, 833–841. [CrossRef]
- 10. Kan, G.-Z. Fitness of Hybrids between Wild Soybeans(*Glycine soja*) and the Glyphosateresistant Transgenic Soybean (*Glycine max*). *Soybean Sci.* **2015**, *34*, 177–184.
- 11. Kim, H.J.; Kim, D.Y.; Moon, Y.S.; Pack, I.S.; Park, K.W.; Chung, Y.S.; Kim, Y.J.; Nam, K.-H.; Kim, C.-G. Gene flow from herbicide resistant transgenic soybean to conventional soybean and wild soybean. *Appl. Biol. Chem.* **2019**, *62*, 54. [CrossRef]

- 12. Jenczewski, E.; Ronfort, J.; Chevre, A.-M. Crop-to-wild gene flow, introgression and possible fitness effects of transgenes. *Environ. Biosaf. Res.* 2003, *2*, 9–24. [CrossRef]
- 13. Wang, K.J.; Li, X.H. Phylogenetic relationships, interspecific hybridization and origin of some rare characters of wild soybean in the subgenus *Glycine soja* in China. *Genet Resour. Crop Evol.* **2012**, *59*, 1673–1685. [CrossRef]
- 14. Liu, J.Y.; Sheng, Z.W.; Hu, Y.Q.; Liu, Q.; Qiang, S.; Song, X.L.; Liu, B. Fitness of F1 hybrids between 10 maternal wild soybean populations and transgenic soybean. *Transgenic Res.* **2021**, *30*, 105–119. [CrossRef] [PubMed]
- 15. Baskin, C.C. Breaking physical dormancy in seeds-focussing on the lens. New Phytol. 2003, 158, 229–232. [CrossRef]
- Thiruppathi, D. Why so stubborn? MtKNOX4-regulated MtKCS12 manifests hardseededness. *Plant Physiol.* 2021, 186, 1367–1368. [CrossRef]
- 17. Liu, A.; Ku, Y.S.; Contador, C.A.; Lam, H.M. The Impacts of Domestication and Agricultural Practices on Legume Nutrient Acquisition Through Symbiosis With Rhizobia and Arbuscular Mycorrhizal Fungi. *Front. Genet.* **2020**, *11*, 583954. [CrossRef]
- Liang, R.; Ji, X.; Sheng, Z.; Liu, J.; Qiang, S.; Song, X. Fitness and Rhizobacteria of F2, F3 Hybrids of Herbicide-Tolerant Transgenic Soybean and Wild Soybean. *Plants* 2022, 11, 3184. [CrossRef]
- Guan, Z.J.; Zhang, P.F.; Wei, W.; Mi, X.C.; Kang, D.M.; Liu, B. Performance of hybrid progeny formed between genetically modified herbicide-tolerant soybean and its wild ancestor. *AoB Plants* 2015, 7, plv121. [CrossRef] [PubMed]
- Yook, M.J.; Park, H.R.; Zhang, C.J.; Lim, S.H.; Jeong, S.C.; Chung, Y.S.; Kim, D.S. Environmental risk assessment of glufosinateresistant soybean by pollen-mediated gene flow under field conditions in the region of the genetic origin. *Sci. Total Environ.* 2021, 762, 143073. [CrossRef] [PubMed]
- 21. Kuroda, Y.; Kaga, A.; Tomooka, N.; Yano, H.; Takada, Y.; Kato, S.; Vaughan, D. QTL affecting fitness of hybrids between wild and cultivated soybeans in experimental fields. *Ecol. Evol.* 2013, *3*, 2150–2168. [CrossRef]
- 22. Hu, Y.-Q.; Sheng, Z.-W.; Liu, J.-Y.; Liu, Q.; Qiang, S.; Song, X.-L.; Liu, B. Sexual compatibility of transgenic soybean and different wild soybean populations. *J. Integr. Agr.* 2022, *21*, 36–48. [CrossRef]
- 23. Wang, X.; Chen, L.; Ma, J. Genomic introgression through interspecific hybridization counteracts genetic bottleneck during soybean domestication. *Genome Biol.* 2019, 20, 22. [CrossRef]
- Kim, M.Y.; Van, K.; Kang, Y.J.; Kim, K.H.; Lee, S.H. Tracing soybean domestication history: From nucleotide to genome. *Breed Sci.* 2012, 61, 445–452. [CrossRef]
- 25. Sedivy, E.J.; Wu, F.; Hanzawa, Y. Soybean domestication: The origin, genetic architecture and molecular bases. *New Phytol.* **2017**, 214, 539–553. [CrossRef] [PubMed]
- Kim, M.S.; Lozano, R.; Kim, J.H.; Bae, D.N.; Kim, S.T.; Park, J.H.; Choi, M.S.; Kim, J.; Ok, H.C.; Park, S.K.; et al. The patterns of deleterious mutations during the domestication of soybean. *Nat. Commun.* 2021, 12, 97. [CrossRef] [PubMed]
- 27. Hu, X.W.; Zhang, R.; Wu, Y.P.; Baskin, C.C. Seedling tolerance to cotyledon removal varies with seed size: A case of five legume species. *Ecol. Evol.* 2017, 7, 5948–5955. [CrossRef]
- Xiong, Y.W.; Gong, Y.; Li, X.W.; Chen, P.; Ju, X.Y.; Zhang, C.M.; Yuan, B.; Lv, Z.P.; Xing, K.; Qin, S. Enhancement of growth and salt tolerance of tomato seedlings by a natural halotolerant actinobacterium *Glutamicibacter halophytocola* KLBMP 5180 isolated from a coastal halophyte. *Plant Soil* 2019, 445, 307–322. [CrossRef]
- Clo, J.; Ronfort, J.; Gay, L. Fitness consequences of hybridization in a predominantly selfing species: Insights into the role of dominance and epistatic incompatibilities. *Heredity* 2021, 127, 393–400. [CrossRef]
- Winn, A.A.; Elle, E.; Kalisz, S.; Cheptou, P.O.; Eckert, C.G.; Goodwillie, C.; Johnston, M.O.; Moeller, D.A.; Ree, R.H.; Sargent, R.D.; et al. Analysis of Inbreeding Depression in Mixed-Mating Plants Provides Evidence for Selective Interference and Stable Mixed Mating. *Evolution* 2011, 65, 3339–3359. [CrossRef]
- 31. Wang, Y.; Xu, C.; Sun, J.; Dong, L.; Li, M.; Liu, Y.; Wang, J.; Zhang, X.; Li, D.; Sun, J.; et al. GmRAV confers ecological adaptation through photoperiod control of flowering time and maturity in soybean. *Plant Physiol.* **2021**, *187*, 361–377. [CrossRef]
- Singh, R.K.; Bhatia, V.S.; Bhat, K.V.; Mohapatra, T.; Singh, N.K.; Bansal, K.C.; Koundal, K.R. SSR and AFLP based genetic diversity of soybean germplasm differing in photoperiod sensitivity. *Genet. Mol. Biol.* 2010, 33, 319–324. [CrossRef]
- Bai, Z.Y.; Ding, X.L.; Zhang, R.J.; Yang, Y.H.; Wei, B.G.; Yang, S.P.; Gai, J.Y. Transcriptome Analysis Reveals the Genes Related to Pollen Abortion in a Cytoplasmic Male-Sterile Soybean (*Glycine max* (L.) Merr.). *Int. J. Mol. Sci.* 2022, 23, 12227. [CrossRef] [PubMed]
- 34. Singh, R.J.; Hymowitz, T. The Genomic Relationships among 6 Wild Perennial Species of the Genus Glycine Subgenus Glycine Willd. *Theor. Appl. Genet.* **1985**, *71*, 221–230. [CrossRef] [PubMed]
- Singh, R.J.; Nelson, R.L. Intersubgeneric hybridization between *Glycine max* and G-tomentella: Production of F-1, amphidiploid, BC1, BC2, BC3, and fertile soybean plants. *Theor. Appl. Genet.* 2015, 128, 1117–1136. [CrossRef] [PubMed]
- Greenfield, L.M.; Hill, P.W.; Paterson, E.; Baggs, E.M.; Jones, D.L. Do plants use root-derived proteases to promote the uptake of soil organic nitrogen? *Plant Soil* 2020, 456, 355–367. [CrossRef]
- 37. Otlewska, A.; Migliore, M.; Dybka-Stepien, K.; Manfredini, A.; Struszczyk-Swita, K.; Napoli, R.; Bialkowska, A.; Canfora, L.; Pinzari, F. When Salt Meddles Between Plant, Soil, and Microorganisms. *Front. Plant Sci.* **2020**, *11*, 553087. [CrossRef]
- Benezech, C.; Doudement, M.; Gourion, B. Legumes tolerance to rhizobia is not always observed and not always deserved. *Cell Microbiol* 2020, 22, e13124. [CrossRef]

- Peix, A.; Ramírez-Bahena, M.H.; Velázquez, E.; Bedmar, E.J. Bacterial Associations with Legumes. Crit. Rev. Plant Sci. 2014, 34, 17–42. [CrossRef]
- AbdElgawad, H.; Abuelsoud, W.; Madany, M.M.Y.; Selim, S.; Zinta, G.; Mousa, A.S.M.; Hozzein, W.N. Actinomycetes Enrich Soil Rhizosphere and Improve Seed Quality as well as Productivity of Legumes by Boosting Nitrogen Availability and Metabolism. *Biomolecules* 2020, 10, 1675. [CrossRef]
- 41. Liu, J.; Yu, X.; Qin, Q.; Dinkins, R.D.; Zhu, H. The Impacts of Domestication and Breeding on Nitrogen Fixation Symbiosis in Legumes. *Front. Genet.* **2020**, *11*, 00973. [CrossRef]
- Zheng, Y.; Liang, J.; Zhao, D.L.; Meng, C.; Xu, Z.C.; Xie, Z.H.; Zhang, C.S. The Root Nodule Microbiome of Cultivated and Wild Halophytic Legumes Showed Similar Diversity but Distinct Community Structure in Yellow River Delta Saline Soils. *Microorganisms* 2020, *8*, 207. [CrossRef]
- Zhou, H.L.; Zhao, Q.; He, R.; Zhang, W.; Zhang, H.J.; Wang, H.Y.; Ao, X.; Yao, X.D.; Xie, F.T. Rapid Effect of Enriched Nitrogen on Soybean Nitrogen Uptake, Distribution, and Assimilation During Early Flowering Stage. *J. Soil Sci. Plant Nutr.* 2022, 22, 3798–3810. [CrossRef]
- 44. Oaks, A. Primary Nitrogen Assimilation in Higher-Plants and Its Regulation. Can. J. Bot. 1994, 72, 739–750. [CrossRef]
- 45. Li, R.; Chen, H.; Yang, Z.; Yuan, S. Research status of soybean symbiosis nitrogen fixation. Oil Crop Sci. 2020, 5, 6–10. [CrossRef]
- Wang, Q.; Ma, M.; Jiang, X.; Guan, D.; Wei, D.; Cao, F.; Kang, Y.; Chu, C.; Wu, S.; Li, J. Influence of 37 Years of Nitrogen and Phosphorus Fertilization on Composition of Rhizosphere Arbuscular Mycorrhizal Fungi Communities in Black Soil of Northeast China. Front. Microbiol. 2020, 11, 539669. [CrossRef]
- 47. Zhu, Q.; Wang, H.; Shan, Y.Z.; Ma, H.Y.; Wang, H.Y.; Xie, F.T.; Ao, X. Physiological Response of Phosphorus-Efficient and Inefficient Soybean Genotypes under Phosphorus-Deficiency. *Russ. J. Plant Physl*+ **2020**, *37*, 175–184. [CrossRef]
- 48. Ribet, J.; Drevon, J.J. Phosphorus Deficiency Increases the Acetylene-Induced Decline in Nitrogenase Activity in Soybean (Glycine-Max (L) Merr). J. Exp. Bot. 1995, 46, 1479–1486. [CrossRef]
- 49. Mckenzie-Gopsill, A.G.; Lee, E.; Lukens, L.; Swanton, C.J. Rapid and early changes in morphology and gene expression in soya bean seedlings emerging in the presence of neighbouring weeds. *Weed Res.* **2016**, *56*, 267–273. [CrossRef]
- 50. Wiles, L.J.; Wilkerson, G.G. Modeling Competition for Light between Soybean and Broadleaf Weeds. *Agr. Syst.* **1991**, *35*, 37–51. [CrossRef]
- 51. Chetan, F.; Rusu, T.; Chetan, C.; Urda, C.; Rezi, R.; Simon, A.; Bogdan, I. Influence of Soil Tillage Systems on the Yield and Weeds Infestation in the Soybean Crop. *Land* **2022**, *11*, 1708. [CrossRef]
- 52. Rockenbach, A.P.; Rizzardi, M.A. Competition at the soybean V6 stage affects root morphology and biochemical composition. *Plant Biol.* **2020**, *22*, 252–258. [CrossRef]
- 53. Wagner, A. Competition for nutrients increases invasion resistance during assembly of microbial communities. *Mol. Ecol.* 2022, 31, 4188–4203. [CrossRef] [PubMed]
- 54. Martinez-Romero, E.; Aguirre-Noyola, J.L.; Taco-Taype, N.; Martinez-Romero, J.; Zuniga-Davila, D. Plant microbiota modified by plant domestication. *Syst. Appl. Microbiol.* **2020**, *43*, 126106. [CrossRef] [PubMed]
- 55. Subbarao, G.V.; Rondon, M.; Ito, O.; Ishikawa, T.; Rao, I.M.; Nakahara, K.; Lascano, C.; Berry, W.L. Biological nitrification inhibition (BNI)—Is it a widespread phenomenon? *Plant Soil* **2007**, *294*, 5–18. [CrossRef]
- Gong, T.; Xin, X.F. Phyllosphere microbiota: Community dynamics and its interaction with plant hosts. J. Integr. Plant Biol. 2020, 63, 297–304. [CrossRef]
- 57. Gutierrez, A.; Grillo, M.A. Effects of Domestication on Plant-Microbiome Interactions. *Plant Cell Physiol.* **2022**, *63*, 1654–1666. [CrossRef] [PubMed]
- 58. Pantigoso, H.A.; Newberger, D.; Vivanco, J.M. The rhizosphere microbiome: Plant-microbial interactions for resource acquisition. *J. Appl. Microbiol.* **2022**, *133*, 2864–2876. [CrossRef]
- 59. Xia, H.Y.; Wang, Z.G.; Zhao, J.H.; Sun, J.H.; Bao, X.G.; Christie, P.; Zhang, F.S.; Li, L. Contribution of interspecific interactions and phosphorus application to sustainable and productive intercropping systems. *Field Crop Res.* **2013**, *154*, 53–64. [CrossRef]
- 60. Gijzen, M.; Weng, C.; Kuflu, K.; Woodrow, L.; Yu, K.; Poysa, V. Soybean seed lustre phenotype and surface protein cosegregate and map to linkage group E. *Genome* **2003**, *46*, 659–664. [CrossRef]
- 61. Qutob, D.; Ma, F.; Peterson, C.A.; Bernards, M.A.; Gijzen, M. Structural and permeability properties of the soybean seed coat. *Botany* **2008**, *86*, 219–227. [CrossRef]
- 62. Zhang, D.; Sun, L.; Li, S.; Wang, W.; Ding, Y.; Swarm, S.A.; Li, L.; Wang, X.; Tang, X.; Zhang, Z.; et al. Elevation of soybean seed oil content through selection for seed coat shininess. *Nat. Plants* **2018**, *4*, 30–35. [CrossRef]
- 63. Sun, L.; Miao, Z.; Cai, C.; Zhang, D.; Zhao, M.; Wu, Y.; Zhang, X.; Swarm, S.A.; Zhou, L.; Zhang, Z.J.; et al. GmHs1-1, encoding a calcineurin-like protein, controls hard-seededness in soybean. *Nat. Genet* **2015**, *47*, 939–943. [CrossRef]
- 64. Satya Srii, V.; Nagarajappa, N.; Vasudevan, S.N. Is seed coat structure at fault for altered permeability and imbibition injury in artificially aged soybean seeds? *Crop Sci.* 2022, 62, 1573–1583. [CrossRef]
- Kuchlan, M.K.; Dadlani, M.; Samuel, D.V.K. Seed Coat Properties and Longevity of Soybean Seeds. J. New Seeds 2010, 11, 239–249. [CrossRef]

- Zablatzka, L.; Balarynova, J.; Klcova, B.; Kopecky, P.; Smykal, P. Anatomy and Histochemistry of Seed Coat Development of Wild (*Pisum sativum* subsp. elatius (M. Bieb.) Asch. et Graebn. and Domesticated Pea (*Pisum sativum* subsp. *sativum* L.). *Int. J. Mol. Sci.* 2021, 22, 4602. [CrossRef] [PubMed]
- 67. Smykal, P.; Vernoud, V.; Blair, M.W.; Soukup, A.; Thompson, R.D. The role of the testa during development and in establishment of dormancy of the legume seed. *Front. Plant Sci.* 2014, *5*, 351. [CrossRef]

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