Development and Application of an in Vitro Method to Evaluate Anthracnose Resistance in Soybean Germplasm

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Abstract

Background:

Anthracnose caused by *Colletotrichum truncatum* is a major fungal disease of soybean, especially vegetable soybean (edamame). Studies of this disease have mainly focused on resistance evaluation, but the primary methods used—*in vivo* inoculation of pods or plants under greenhouse or field conditions—have limitations with respect to accuracy, stability, scale, and environmental safety.

Result:

In this study, we developed a method for inoculating pods *in vitro* that entails soaking in a mycelial suspension. We optimized the crucial components, including the mycelial suspension concentration (40 to 60 mg mL^{-1}), maturity of sampled pods (15 days after flowering), and post-inoculation incubation period (5 days). Application of the mycelial suspension by spraying rather than soaking improved the efficiency of inoculation and made large-scale evaluation possible. Using this method, we evaluated 589 soybean germplasm resources [275 cultivars (C), 233 landraces (L), and 81 wild-type accessions (W)]. This screening resulted 37 highly resistant (25 C, 11 L, and 1 W), 148 resistant (100 C, 43 L, and 5 W), 210 moderately susceptible (116 C, 77 L, and 17 W), 91 susceptible (23 C, 52 L, and 16 W), and 103 highly susceptible (11 C, 50 L, and 42 W) materials.

Conclusions:

The resistance levels of soybean germplasm resources were effectively distinguished by the method we developed. We thus identified many resistant cultivars but only a few resistant landraces and wild-type germplasm resources. Our results will greatly aid future research on soybean anthracnose resistance, including gene discovery, elucidation of molecular mechanisms, and the breeding of resistant cultivars.

Background

Anthracnose, caused by *Colletotrichum truncatum* (*C. truncatum*), is a major fungal disease of soybean (*Glycine max* (L.) Merrill) [1]. All aboveground parts of soybean plants can be infected at any stage of development by *C. truncatum*. During early stage of infection, typical distinctive symptoms of anthracnose on soybean are irregularly shaped brown blotches on cotyledons, stems, petioles, leaves, and pods, which gradually develop into dark, depressed, irregular lesions. As the disease progresses, rolling of leaves, premature defoliation, necrosis of laminar veins and pods, and seed abortion may be observed. Moreover, infected seeds often become discolored and can die pre- or post-emergence damping off [2, 3, 4, 5, 6].

Soybean anthracnose is more prevalent in subtropical and tropical regions, such as southern growing areas of China and the USA as well as northern Argentina, Brazil, Thailand, and India [2]. Estimated yield losses attributable to anthracnose are significant. In the top eight soybean-producing countries

(Argentina, Bolivia, Brazil, Canada, China, India, Paraguay, and the USA), losses of 25.4 million tons have been reported, including a record 16.6 million tons in China in 2006 [7]. Anthracnose is an especially serious problem in vegetable soybean (edamame) production. Given that fresh pods are the final product, their commodity value is greatly reduced as soon as disease blotches or lesions develop on the pods. The resulting economic loss per unit area is higher than that experienced in traditional soybean production. China is the largest producer and exporter of vegetable soybean worldwide, with a cultivated area of 1.0×10^5 to 1.5×10^5 hm^2 and a yield of 5.0×10^5 tons per year [8]. Vegetable soybean production in China, however, is concentrated in the southeastern coastal area, where anthracnose frequently occurs because of the warm, humid climate. For example, in Zhejiang Province, the anthracnose incidence of soybean plants is about 50% and that of soybean pods is about 30% in typical fields which may rise to more than 90% and more than 50%, respectively, in severely infected fields[9, 10].

Current methods used to manage the disease and limit pathogen spread during soybean production include rapid diagnosis, biological control, planting of pathogen-free seeds, and chemical control [5, 11, 12, 13]. These methods are economically costly and/or ecologically unsound; in the case of vegetable soybean, food safety issues are also a concern. The use of disease-resistant cultivars is therefore the best option. The screening of soybean germplasm for anthracnose resistance is a crucial step in the breeding of resistant cultivars. Currently, researchers have evaluated the anthracnose resistance of modern soybean cultivars from the USA, China, Brazil, and India. Although some resistant sources have been identified, no immune ones have been reported [3, 14, 15, 16].

The methods used to evaluate anthracnose resistance have differed in several respects, such as the means of disease introduction (natural disease and artificial inoculation), inoculum type (conidia and mycelia), incubation conditions (greenhouse and field), and disease severity scoring (visual assessment of inoculated plants and pods) [14, 16, 17, 18,19, 20] . All of these methods have limitations. For example, several years of repetition are required to obtain accurate results, with the outcome thus strongly influenced by environmental factors. The establishment of a dedicated area, which is time consuming and labor intensive, may be required to avoid affecting other planting schedules. A rapid (enabling multiple repeat assessment in one planting season), stable and accurate (not affected by environmental factors) evaluation method is thus needed.

In this study, we developed a rapid, reliable and precise method for the evaluation of soybean anthracnose resistance. We then applied this method to evaluate the anthracnose resistance of various Chinese soybean germplasm resources mainly collected from across the Huanghuai region, and south of Changjiang river [10, 21], including cultivars, landraces, and wild-type accessions, and identified superior resistant sources. Our findings provide a foundation for the discovery of resistance genes, elucidation of the associated molecular mechanisms, and the breeding of resistant cultivars.

Materials And Methods

Soybean planting and pods sampling

All soybean germplasm used in this study was planted at the experimental farm of the Zhejiang Academy of Agricultural Science, Jiaxing,China. To minimize the risk of soybean diseases and insect pests in the field, we selected a field where legumes have not been planted for 8 years to plant our soybean germplasms. Each germplasm resource was planted in three 1.3 m ´ 2.0 m plots, with inter-row spacing of 0.4 m and within-row plant spacing of 0.3 m.

Pods from healthy soybean plants in the field were collected and quickly placed in an incubator. Ice packs were used to maintain the temperature within the incubator at about 4°C. The pods were separated from the ice packs with absorbable cotton to protect the pods from freezing injury. The sampled pods were transferred to the laboratory and washed with sterile water.

Fungal isolate and Preparation of inoculum

A fungal isolate (CT5) was obtained from infected soybean pods collected in Longyou, Zhejiang Province, China (28.91797° N, 119.220583° E). The isolate was identified as *C. truncatum* on the basis of its morphological characteristics and DNA sequence (GenBank accession: MW301345) [15]. The isolate was cultivated on potato dextrose agar (PDA) at 25°C under dark conditions.

The mycelial suspension used as inoculum was prepared from isolate CT5 as follows. Ten 5-mm-diameter mycelial disks from an actively growing culture of isolate CT5 on PDA were added to 200 mL of sterilized potato dextrose broth (PDB) in 500-mL flasks. Each mycelial solution was incubated for 4 days in an incubator with shaking at 120 rpm at 25 °C under dark conditions and then filtered through sterilized gauze. The mycelial pellets was rinsed with sterilized water at least six times to remove as much residual PDB as possible, squeezed to minimize the water content, and weighed. The pellets was re-suspended in sterilized water, fragmented in a blender at low speed for 20 s, and diluted with sterilized water to the required final concentration (50 mg mL^{-1}). All steps were completed on an ultra-clean workbench.

Determination of the optimal mycelial suspension concentration

Pods of consistent maturity sampled from 'Zhexian No. 9' (ZX9) and Nanhua Black Bean (NBB), which are susceptible to anthracnose during cultivation, were inoculated with mycelial suspension at five concentrations (20, 30, 40, 50, and 60 mg mL^{-1}) as follows. The pods were rinsed with sterilized water to remove debris and then inoculated with mycelial suspension by spraying with a hand-held watering can until run-off. Inoculated pods were transferred onto filter papers in 15-cm Petri dishes containing 3 mL sterilized water. The Petri dishes were then placed in an incubator at 25 °C under 14-h dark/10-h light conditions. Two to six days post-inoculation (DPI), the area covered by disease blotches was continuously scanned and calculated with a LA-S plant analysis system (Wseen, Shenzhen, China). Three repetitions were performed and each repetition comprised 15 pods.

Determination of the optimal pod sampling period

Pods were sampled from ZX9 and NBB at four stages: (I) ca. 5 days after flowering (DAF), when the pods were small and soft; (II) ca. 15 DAF, when the pods were fully grown and no longer soft but were still flat because the seeds had not yet begun to protrude; (III) ca. 25 DAF, when the pods were half filled; and (IV) ca. 40 DAF, when the pods were completely filled, which is the harvest stage for vegetable soybean. The sampled pods were inoculated by spraying with a mycelial suspension (50 mg mL^{-1}). The other inoculation and incubation steps were the same as described above. Three repetitions were performed and each repetition comprised 15 pods.

Determination of the optimal post-inoculation incubation duration

Pods (type II) sampled from 86 soybean germplasm resources were sprayed with a mycelial suspension (50 mg mL^{-1}) prepared from mycelium incubated for 5 days. After 4, 5, and 6 days of incubation, blotchy regions were scanned and their areas calculated. All other inoculation and incubation steps were performed as described above.

Disease severity was scored on a scale of 0 to 5 based on the proportion of the pod surface covered by blotches as follows: 0, no visible blotches; 1 to 5, blotches covering 1.0% to 10.0% (1), 10.1% to 35.0% (2), 35.1% to 65.0% (3), 65.1% to 90.0% (4), and 90.1% to 100% (5) of the pod. The anthracnose resistance level of each germplasm resource was defined according to its calculated disease index (DI), i.e., $\{[(n_1 \times 1) + (n_2 \times 2) + (n_3 \times 3) + ... + (n_n \times n) / [N \times (n_1 + n_2 + n_3 + ... + n_n)]\} \times 100$, where $n_1 ... n_n$ is the number of pods with a respective disease severity score, and N is the highest disease severity score (Li et al., 2009). Six levels of anthracnose resistance were defined: immune (IM), DI = 0; highly resistant (HR), DI = 0.01 to 15.00; resistant (R), DI = 15.01 to 35.00; moderately susceptible (MS), DI = 35.01 to 65.00; susceptible (S), DI = 65.01 to 85.00; and highly susceptible (HS), DI = 85.01 to 100.

Comparison of spray vs. soak inoculations

We first tested the effect of soaking duration on disease severity. Type-II pods sampled from ZX9 were placed in a flask containing 500 mL of mycelial suspension, gently stirred for 5, 10, 15, 20, or 25 s, and removed. After draining off excess mycelial suspension, inoculated pods were transferred to filter paper in 15-cm Petri dishes containing 3 mL sterilized water. The Petri dishes were then placed in an incubator at 25°C under 14-h dark/10-h light conditions. Following 2 to 6 days of incubation, disease blotches were scanned and their areas calculated using the LA-S plant analysis system (Wseen, Shenzhen, China).

Next, type-II pods sampled from 10 randomly selectedsoybean accessions were inoculated by spraying or by 10 s of soaking as described above. The inoculated pods were transferred to filter paper in 15-cm Petri dishes containing 3 mL sterilized water. The Petri dishes were then placed in an incubator at 25°C under 14-h dark/10-h light conditions. Disease blotches were scanned and their areas calculated after 3 to 6 days of incubation using the LA-S plant analysis system (Wseen, Shenzhen, China).

Evaluation of soybean germplasm resources for resistance to anthracnose

The 589 soybean germplasm resources [275 cultivars (C), 233 landraces (L), and 81 wild-type accessions (W)] were planted at the experimental farm of the Zhejiang Academy of Agricultural Science in the summer of 2019. Given the large number of soybean germplasm resources, resistance evaluation could not be completed at one time; The duration of post-inoculation incubation may differ by several hours among multiple resistance evaluations. Thus, ZX9, which showed stable disease progression and severity in multiple experiments, was chosen as a control. Seed of ZX9 were sown in stages, at intervals of 5 days, and five sowing times in total. Each resistance evaluation included ZX9. The same disease severity for ZX9 was observed in the multiple resistance evaluations.

Anthracnose resistance was evaluated in type-II pods inoculated with 50 mg mL^{-1} mycelial suspension using our newly developed *in vitro* method. The mycelial suspension was prepared from mycelium incubated for 4 days, and the inoculation was performed by soaking for 10 s. All other inoculation, incubation, scanning, and calculation steps were the same as described above. Then the inoculated pods were transferred to filter paper in 15-cm Petri dishes containing 3 mL sterilized water. The Petri dishes were then placed in an incubator at 25 °C under 14-h dark/10-h light conditions. Disease blotches were scanned and their areas calculated after about 5 days of incubation using the LA-S plant analysis system (Wseen, Shenzhen, China). Each germplasm resource was evaluated in three replicates and each planting plot was treated as one replicate.

Data analysis

Data analyses were conducted using SPSS Statistics 22.0 software. The homogeneity of variance for the proportion of the pod surface covered by blotches or DI values among repeated experiments was tested using Bartlett's test with replications as random effects. All differences between pairs were considered significant at $P \leq 0.05$ based on a two-tailed t-test. Pearson correlations coefficients were calculated to evaluate the relationship between soak and spray inoculations.

Results

Determination of the optimal mycelial suspension concentration

Compared with conidial suspensions, mycelial suspensions are more infectious and easier to prepare [14]. For simplicity and to maximize disease pressure, we therefore used a mycelial suspension as the inoculum in this study. As shown in Figure 1, the severity of anthracnose disease on pods of ZX9 slightly increased as the mycelial suspension concentration was increased. No significant differences were observed within the range of 30 to 50 mg mL^{-1} over the entire incubation period (except at 3 DPI). In cultivar NBB, increasing the concentration of the mycelial suspension had no significant effect on disease severity over the entire incubation period. The only significant differences observed were those between 20 and 60 mg mL^{-1} and 6 DPI; in this case, the difference in the areas covered by blotches was less than 10% (Fig. 1).

Our results thus indicate that a concentration above 30 to 50 mg mL^{-1} is sufficient to ensure that the mycelial concentration has no significant effect on disease severity. This conclusion is consistent with the findings of a previous study [14]. provided the concentration of the mycelial suspension attains the required threshold for full infection of soybean plants or pods, it has no significant effect on anthracnose disease severity. Within the above threshold range, the mycelial suspension concentration can thus be flexibly selected according to the needs of the specific experiment.

Determination of the optimal maturity stage for sampling pods

In contrast to previously reported inoculation methods, we used an *in vitro* pod inoculation approach. To determine the optimal maturity stage for sampling pods, we examined the relationship between pod maturity and disease severity. We observed significant differences in disease severity among pods of different maturities, with the most rapid disease progression occurring in younger pods, especially those of type-I. At 2 DPI, obvious blotches were visible on type-I pods, whereas pods at other stages of maturity had only a few small blotches. At 4 DPI, almost 100% of the surface of type-I pods was covered with blotches; in contrast, 6 days of incubation was required before older pods reached this state (Fig. 2). Our results corroborate the finding that pods, consistent with other soybean plant tissues, are more susceptible to anthracnose when young [14, 19]. Choosing young pods can save incubation time and improve efficiency and is conducive for the screening of superior disease-resistant germplasm resources. The use of very young pods is not appropriate, however, for large-scale resistance evaluation, as the shorter post-inoculation incubation period would shorten the time available for scanning blotchy areas without introducing large errors. Type-I pods were thus not suitable for sampling. The disease progression of type-II pods was much slower than that of type-I pods but slightly faster than type-III and type-IV pods. The most important consideration was that the visual and tactile characteristics of type-II pods were obvious, thus improving sampling efficiency and reducing errors.

Determination of the optimal post-inoculation incubation time

None of the 86 tested germplasm resources exhibited immunity to soybean anthracnose. After inoculation with mycelial suspension followed by incubation for 4 days, the majority of accessions (44; 51.16%) were classified as resistant (16 HR and 28 R). After incubation for 6 days following inoculation, susceptible germplasm resources (58; 67.44%) predominated, with 20 classified as HS and 38 as S (38). When a post-inoculation incubation period of 5 days was used, the distribution of resistance levels was relatively reasonable and close to a normal distribution (Fig. 3). We therefore considered a 5-day incubation time to be optimal.

Comparison of inoculation by spraying vs. soaking

During our evaluation of the optimal post-inoculation incubation time, we found that spray-based inoculation was time consuming. To save time during inoculation, we thus investigated the utility of inoculation by soaking as a replacement for spraying.

We first tested the effect of soaking duration on disease severity. Soaking for 5 s appeared to be sufficient to fully inoculate pods, and increasing the soaking period from 5 s to 30 s had no significant effect on disease severity (Fig. 4). When we compared inoculation by spraying with inoculation by soaking, the high Pearson correlation coefficients (*r*-values) and all *p*-values were greater than 0.05, which indicated that the disease progression and severity were consistent between the two methods (Fig. 5). Inoculation by soaking can therefore be used instead of spray inoculation to improve inoculation efficiency and facilitate large-scale evaluation of anthracnose resistance.

Evaluation of soybean germplasm resources for anthracnose resistance

The homogeneity of variance for DI values among the germplasm resources was significant ($P < 0.001$). This finding indicated that the resistance levels of soybean germplasm resources were effectively distinguished. The distribution of resistance levels of all 589 germplasm resources was close to a normal distribution. Our screening revealed 37 HR (25 C, 11 L, and 1 W), 148 R (100 C, 43 L, and 5 W), 210 MS (116 C, 77 L, and 17 W), 91 S (23 C, 52 L, and 16 W), and 103 HS (11 C, 50 L, and 42 W) individuals, whereas no germplasm resources exhibited immunity to anthracnose. Among cultivars, the proportion of HR and R materials (125; 45.45%) was much larger than that of S and HS accessions (34; 12.36%). In contrast, the proportion of HR and R accessions (6; 7.41%) among wild-type materials was much smaller than that of wild-type S and HS accessions (58; 71.60%), and only one wild-type HR germplasm resource was identified (Fig. 6, Supplemental Excel file 1). Among landraces, the distribution of resistance levels was relatively close to a normal distribution. The resistance level and habitat of each germplasm resource is presented in Figure 7 and Supplemental Excel file 2. Soybean germplasm resources from different habitats exhibited no obvious differences in resistance to anthracnose.

Discussion

In this study, we developed a rapid, stable, accurate, and safe method for evaluation of soybean resistance to anthracnose. Unlike previous methods that are based on *in vivo* inoculation and incubation under controlled laboratory conditions [14, 16, 17, 19, 20, 22], our method involves *in vitro* inoculation and incubation under controlled laboratory conditions. The advantages of *in vitro* inoculation and incubation are as follows: stability and accuracy (fewer environmental effects), speed and efficiency (ability to conduct multiple, repeated large-scale resistance evaluations), and safety (will not cause pathogen accumulation in the test field). *In vitro* inoculation is not a novel method. Given the above-mentioned advantages, *in vitro* inoculation has been widely used in large-scale resistance evaluations of plant resistance and studies of the molecular mechanisms against diseases such as rice blast [23], *Marssonina* apple blotch [24], wheat Fusarium head blight [25], and apple Alternaria blotch [26].

Another outstanding feature of our proposed method is the administration of inoculum by soaking instead of spraying, which greatly saves inoculation time and improves efficiency. We found that the two inoculation methods gave highly consistent results in terms of disease progression and severity, thus demonstrating that either means of application allows mycelium to fully adhere to the pods. However,

although suitable for *in vitro* inoculation, however, inoculation by soaking may not be applicable for *in vivo* use.

The method developed in this study creates favorable conditions for the occurrence of anthracnose and the establishment of a strong screening pressure; as a consequence, this approach is beneficial for distinguishing differences in the resistance of different germplasm resources and for identifying materials having superior resistance. Although offering many advantages, our method is limited to the planting season. To fully exploit available time and space, the exploration of methods that are completely laboratory based, such as the use of soybean seedlings for resistance evaluation, is required.

In this study, we used our proposed method to evaluate the anthracnose resistance of 589 soybean germplasm resources. The overall distribution of resistance levels was close to a normal distribution, which indicates that our method is feasible. We identified many resistant cultivars that can be applied for the resistance improvement of vegetable soybeans. Because high-yield soybean cultivars, which are relatively few in number and genetically similar, dominate the production of soybean, an autogamous species, the genetic diversity of modern soybean cultivars has declined to an alarmingly low level [27, 28]. Although many resistant cultivars are known, their resistance may thus be due to only a few resistance genes. Many rare alleles that are likely to benefit future soybean improvement are present in wild types and Asian landraces [29]. To our surprise, however, only a few of the landraces and wild-type accessions in our study exhibited resistance. In the long run, the discovery of more resistance genes will therefore require resistance evaluation of landraces and wild types on an expanded scale.

Conclusions

This study has yielded a new, rapid, reliable and safe method for soybean anthracnose resistance evaluation. We used this method to evaluate the resistance of 589 soybean germplasm resources and identified many resistant accessions. Our work will be of importance in future research on soybean anthracnose, such as resistance gene discovery, elucidation of resistance molecular mechanisms, and resistance breeding. Although offering many advantages, our method is limited to the planting season. The exploration of completely laboratory-based methods, such as the use of soybean seedlings for resistance evaluation, is needed to maximize available time and space.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

Not applicable

Acknowledgments

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Founding

Authors' contributions

XY and XJ collected and planted the soybean germplasm resources; LZ and LF conducted the experiments; LZ, HJ and QY analyzed the data; LZ and FY designed the experiment and wrote the manuscript.

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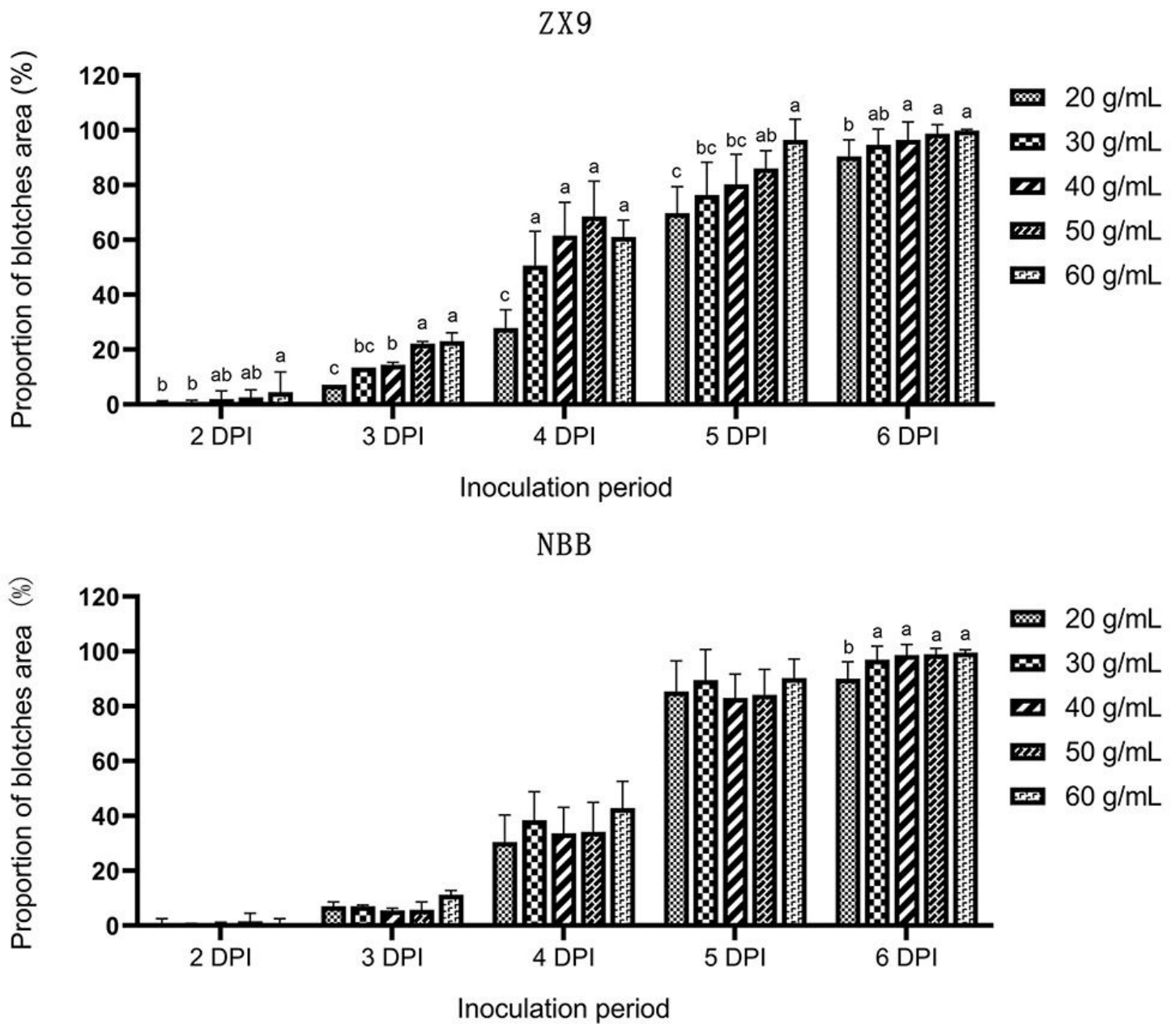
Figures

Figure 1

Proportion of pod surface area covered with blotches 2 to 6 days after inoculation of pods of Zhexian No. 9 (ZX9) and Nanhua Black Bean (NBB) soybean with different concentrations of mycelial suspension. Different letters above columns indicate significant differences (P = 0.05) based on a two-tailed t-test.
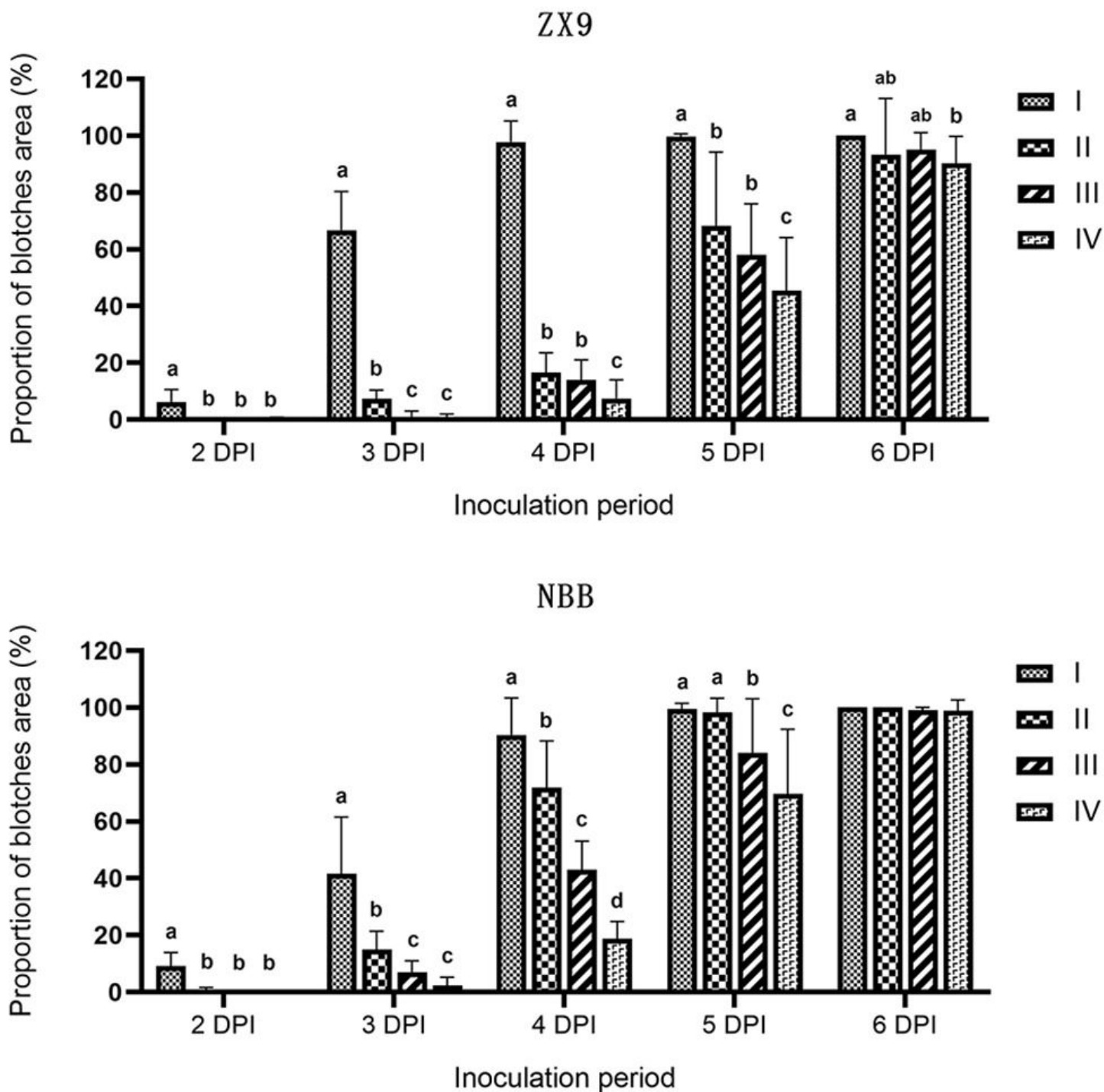
Figure 2

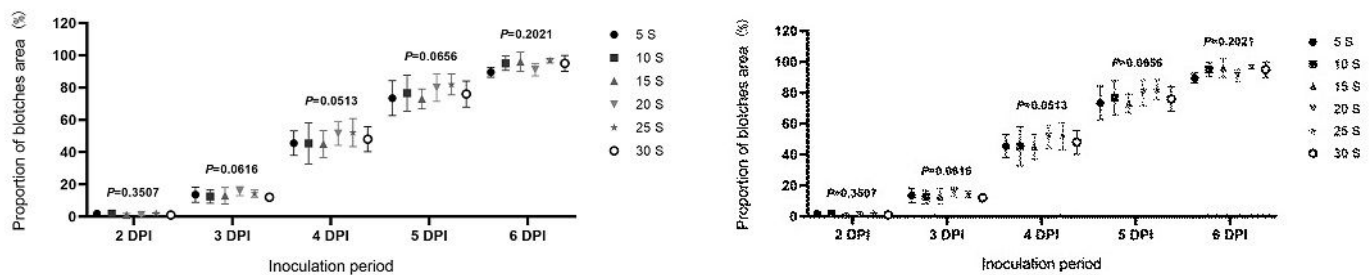Proportion of pod surface area covered with blotches 2 to 6 days after inoculation of pods of different maturity of 'Zhexian No. 9' (ZX9) and Nanhua Black Bean (NBB) soybean inoculated with mycelial suspension. I, II, III, and IV represent four different stages of maturity, ordered from youngest to oldest. Different letters above columns indicate significant differences (P = 0.05) based on a two-tailed t-test.

Figure 3

Number of soybean accessions at each level of soybean anthracnose resistance at three different post-inoculation incubation times. HR, highly resistant; R, resistant; MS, moderately susceptible; S, susceptible; HS, highly susceptible.

Figure 4

(A) Proportion of pod surface area covered by blotches 2 to 6 days after inoculation of pods of 'Zhexian No. 9' (ZX9) soybean with mycelial suspension administered by soaking for 5, 10, 15, 20, 25, and 30 s.
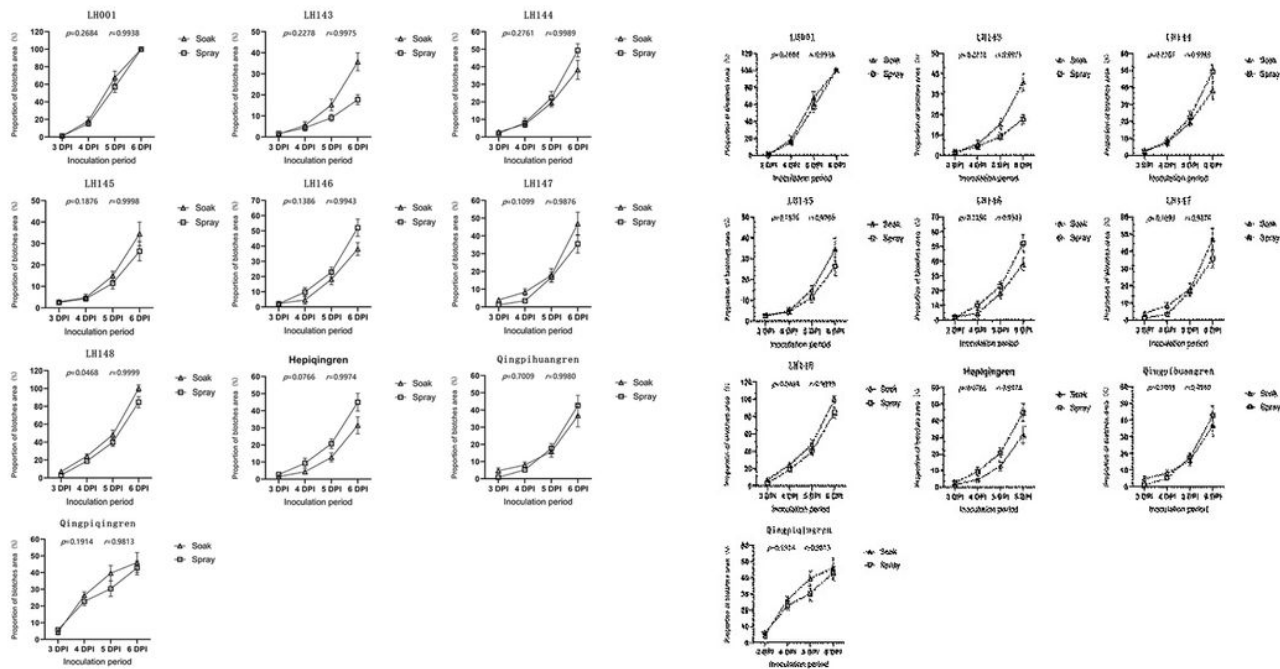

Figure 5

Proportion of pod surface area covered by blotches 2 to 6 days after inoculation of pods from 10 soybean accessions with mycelial suspension administered by soaking or spraying. In each plot, the correlation coefficient (r) and significance of differences (p-value) between the two methods for each accessions are shown.
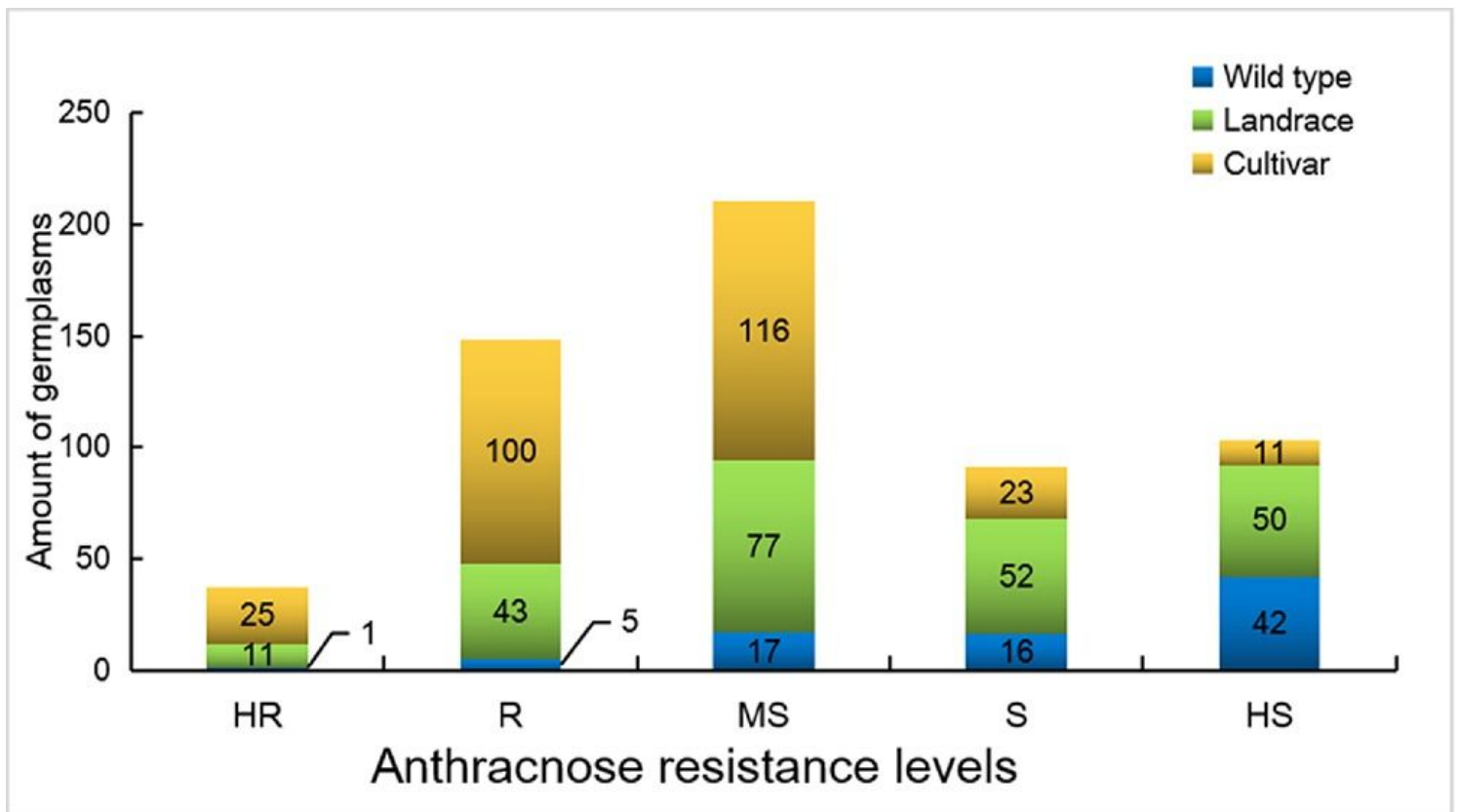
Figure 6

Distribution of 589 soybean germplasm accessions according to the level of soybean anthracnose resistance. HR, highly resistant; R, highly resistant; MS, moderately susceptible; S, susceptible; HS, highly susceptible.
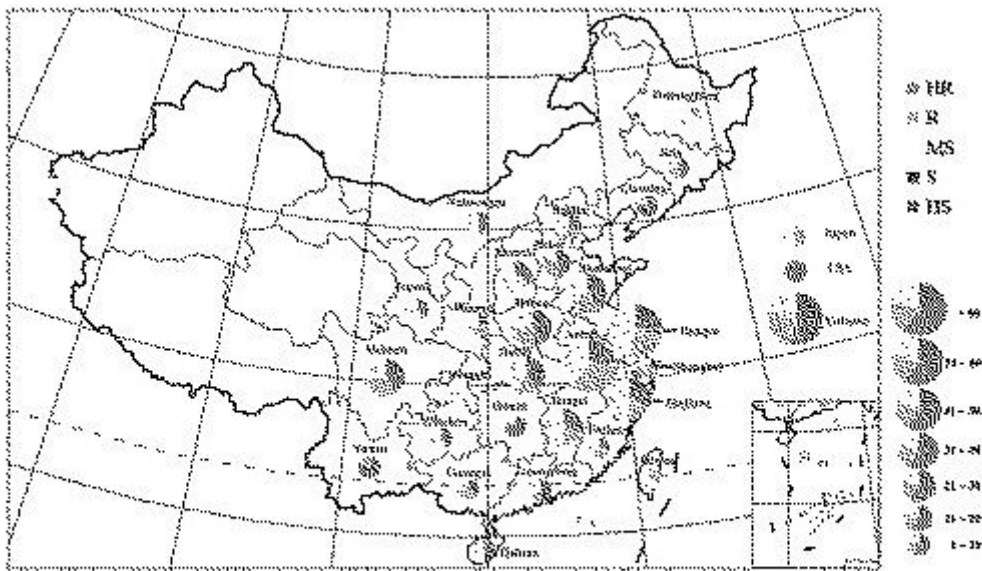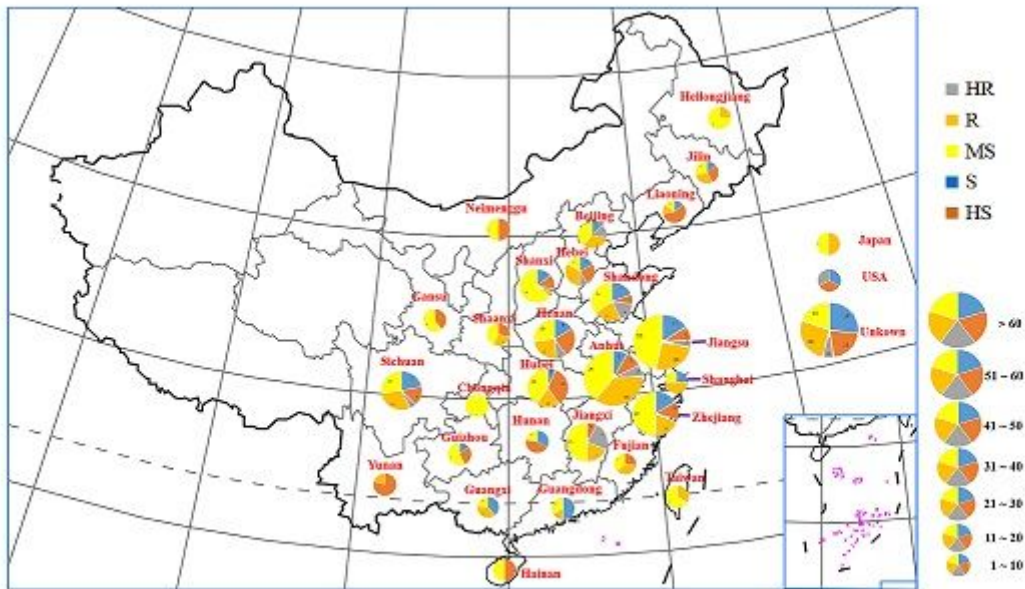
Figure 7

Geographic distribution of soybean germplasm resources and levels of anthracnose resistance. HR, highly resistant; R, resistant; MS, moderately susceptible; S, susceptible; HS, highly susceptible. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Supplementary Files

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- SupplementaryExcel1..xlsx
- SupplementaryExcel2..xlsx