# A rapid and efficient in vivo inoculation method for introducing tree stem canker pathogens onto leaves, suitable for large-scale assessment of resistance in poplar breeding progeny

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## Abstract

Hybrid breeding is the most direct and efficient method of controlling and managing tree diseases. However, "in vitro stem inoculation" can not be used for rapid, efficient, and low-cost screening of resistant clones in the early stage of stem canker infection. Therefore, we inoculated stem canker pathogens on poplar leaves to evaluate the resistance of hybrid clones in the poplar-*Valsa sordida* pathosystem. "In vivo leaf inoculation" showed that: 1) *V. sordida* induces extended necrotic lesions and conidia on leaves. 2) The upper leaves exhibited higher resistance than the middle leaves. 3) The shading conditions induced more severe symptoms on leaves than the lighting conditions. 4) The susceptibility of poplar leaves to juvenile mycelium was higher than old mycelium's. 5) The resistance of 48 poplar clones against *V. sordida* was distributed normally. 6) The efficacy of "in vivo leaf inoculation" was consistent with "in vitro stem inoculation". In summary, "in vivo leaf inoculation" has the advantages of a wide application range, simple operation, short experimental process, and host safety. This method is of great significance for poplar breeding, the study of pathology and molecular biology, pathogenic differentiation, and sporulation of fungal canker pathogens.

**Keywords:** poplar hybrid breeding, tree diseases, stem canker, in vivo leaf inoculation, *Valsa sordida*

## 1. Introduction

Poplar species are always affected by different kinds of pests, especially two stem canker pathogens: *Botryosphaeria dothidea* (*B. dothidea*) and *Valsa sordida* (*V. sordida*) canker. These are the two major forest diseases in China (Zhang & Luo, 2003), which can ruin all the seedlings

in poplar plantations or severely damage mature poplar forests. Hybridization breeding is the most direct and efficient pathway to manage and control forestry diseases.

For breeding resistant clones to plant disease, accurately and quickly getting the phenotypic characteristics, including disease incidence, onset time, area of the necrotic, and disease index, has become a critical pathological problem affecting breeding efficiency. There are three methods, field investigation assays, callus inoculation (Zhang *et al.*, 1989) and in vitro stem segment inoculation method, to obtain the disease prototype for the breeding of poplar canker; however, the In vitro stem segment inoculation is the most commonly used method (Chen *et al.*, 1985; Shi *et al.*, 2014; Zhang *et al.*, 2017). It used eight to ten stem segments 30-40 cm long as inoculation materials. Each stem segment was inoculated with 5 points, observing the incidence rate and recorded every five days within 60 days of inoculation, and the disease index was calculated. Thus, the cost of breeding time and land, as well as the cost of labor and expenditure that accompanied the former, will be huge.

During the growing season, abundant leaves were continuously produced on trees. Then, should we carry out an "in vivo leave inoculation" to detect the host resistance to stem canker disease? The first and most crucial step of the disease onset is the invasion and colonization of pathogens in host tissues. Therefore, whether the stem canker pathogens reside in poplar leaves has become the prerequisite for this method. *B. dothidea* not only induces canker disease on stems and branches of plants but also causes latent infection as endophytic pathogens or lives in leaves, fruits, and other places (Reviewed by Marsberg *et al.*, 2017). The excised leaves of *Malus spectabilis* were used to detect the pathogenicity of the mutant isolates of *B. dothidea* (Zheng *et al.*, 2017). The mycelium and conidial suspension solution of *V. ceratosperma* (stem canker pathogen of apple trees) induced disease symptoms on the excised leaves, shoots, and fruits of apple trees (Wei *et al.*, 2010; Xie, 2019). In addition, *Septoria musiva* Peck, an important fungal pathogen of hybrid poplars in the north-central and northeastern regions of North America, causes a leaf spot and stem canker disease of hybrid poplars (review by Feau *et al.*, 2010; Dunnell & LeBoldus, 2017). Based on the above research, we speculated that leaf disease may be induced by inoculation of stem canker pathogens. In addition, Zhang *et al.* (2018) proposed inoculating rice blight pathogens with isolated leaves to detect pathogenicity. Inoculation of isolated leaves and shoots of *V. ceratosperma* can be used as an accurate and rapid method to evaluate the pathogenicity of stem canker pathogen *V. ceratosperma* in apple trees. However, no specific method for disease resistance detection was proposed in this paper, and it has not been applied to cultivating disease-resistant varieties in tree trunks (Wei *et al.,* 2010). As described above, acquiring pathotypes and selecting resistance clones are still technical problems in the hybrid breeding of poplar stem canker diseases.

This study proposed a novel method for the resistance detection of poplar clones based on the in vivo leaf inoculation of stem canker pathogens. Then, using the poplar/*V. sordida* disease

system, the effects of the leaf development stage (leaf position on the branch), light condition of leaves, and cultured time of the fungal pathogen were assayed. The differentiations of plant resistance to *V. sordida* were assayed in a small, randomly selected population of hybrid poplar clones and then were verified using stem segment inoculation methods. Finally, the diversity of pathogenicity to poplar in some *B. dothidea* isolates was also detected in this study. It is expected to develop a rapid, effective, and low-cost method for the inoculation of stem canker pathogens and a new strategy of hybrid breeding for the canker diseases of poplars and other tree species.

## 2. Materials and methods

### 2.1. Fungal and plant materials

The fungi used in this study are poplar canker pathogen *V. sordida* isolate CZC and 13 poplar blister canker (*B. dothidea*) isolates (Table 1). All fungal isolates were deposited in the plant physiology laboratory at the Institute of Ecology Conservation and Restoration, Chinese Forestry Academy (CAF), Beijing. The pathogenic isolates were grown on PDA medium (20% potato, 2% dextrose, 2% agar; pH 6.0) at 28℃ in dark conditions for 4 or 7 days after inoculation, and then cut into square mycelium plugs; the side length was about 1.0-1.2 cm.

One or 2-year-old poplar saplings of *Populus euramericana* cv. 'Bofeng 3' (Huang *et al.*, 2014), 6-year-old *P. alba var. pyramidalis* saplings, and 2-year-old saplings of the hybrid clones (*P. deltoids*×*P. deltoids*, 48 genotypes) were used in this study. All poplar saplings were cultivated in the experimental field of CAF, with no pest infestation on leaves and stem, and well irrigated during the experiments. The day before inoculation, 3-4 mature and neighbored leaves were washed using sterilized water and then sterilized with 75% alcohol (vol/vol) (Figure 1-A1). After drying, all sterilized leaves were marked for inoculation.

### 2.2. Inoculation methods

Two or four square mycelium plugs were first placed on the selected leaves upper surface according to the size of leaves, about 1-3 cm apart from the central veins, and avoiding the secondary veins (Figure 1-A2). The mycelium faced the upper surface of the poplar leaves. One leaf of each poplar genotype near the pathogen-inoculated leaves was inoculated by 2% WA (water agar) square plugs as the control. After the pathogen inoculation, the leaves were carefully covered and sealed with 5 cm wide household scotch tape to fix and prevent the water loss of the mycelium or WA plugs (Figure 1-A3). Then, the leaves were pierced into 5 points from the lower surface with a needle, at the center of the inoculated mycelium or WA square plugs or just at the corners of the square (about 1-2mm away from the corners) (Figure 1-A4). After inoculation, the sealed condition of the scotch tape and the development of disease symptoms that appeared on the inoculated leaves were observed and recorded daily. Remove the household

scotch tape after three days (Figure 1-A5). All the inoculated leaves were picked from poplar branches or twigs five days after inoculation (DAI), placed in dark plastic bags, and stored at 4°C. After the experiment, all picked leaves were immediately taken into the laboratory, and scotch tapes, mycelium, and WA plugs were carefully removed from the leaves. Then, all leaves were photoed or scanned as JPEG images, and the images were at least 1500 pixels. The necrotic spots around the prick sites on the leaves were identified, and their area was calculated automatically in the software ImageJ (https://imagej.nih.gov/ij/) or was manual adjusted if necessary. The results were kept in Microsoft EXCEL.

In this study, the lesion shape, color, areas, and whether the hypha-like structure formed around the prick sites inoculated by pathogen or WA were set as the determining criterion for leaf disease. After pathogen inoculation, the leaf necrotic lesions were always extended, the necrotic area was more than 2.0 $mm^2$; the lesion color was always grey or dark; the hypha-like structure was consistently observed, or sometimes, the pycnidia-like structure was observed on the surface of the necrotic spot. On the contrary, after WA inoculation, the leaf necrotic lesions were always brown; the lesion area was average of 1.5 $mm^2$, not extended, and no hypha-like structure was observed on the leaves. For simplicity, the necrotic areas on the inoculated leaves, $A = 2.0\ mm^2$, were set as the quantitative criteria to determine the occurrence of leaf disease (Table 2). If $A > 2.0\ mm^2$, the prick sites are diseased, or vice versa. The disease index of each poplar clone was calculated according to the criteria and the ranking level of disease development (Table 2).

In this study, the disease incidence and disease index was calculated using the following formula:

Formula 1: Disease incidence rate (IR) = Number of the diseased prick sites/Total Number of the efficient prick sites × 100%.

Formula 2: Disease index (DI) = ∑ (Number of prick sites by level × Values of severity at all levels)/(Value of the highest severity level × Total Number of the efficient prick sites) × 100.

### 2.3. Comparison of the resistance to canker pathogen among the different stages of leaves

The square mycelium plugs of *V. sordida* isolate CZC, which were cultivated in darkness for seven days, were inoculated on the 5-7$^{th}$ newly matured leaves (from the top to the bottom of the poplar saplings, the same applies from now on) and 18-20$^{th}$ mature leaves on the same saplings of *P. euramericana* cv. 'Bofeng 3'. The inoculation method was described in 2.2; two square mycelium plugs inoculated every leaf, and three biological replicates (saplings) were used. In total, 120 pathogen-inoculated prick sites were created, 60 for the upper and 60 for the lower leaves. One additional poplar sapling was inoculated by WA plugs as the control and a total of 40 prick sites were created.

139 **2.4. Comparison of the resistance between lighting and shading leaves**

140 The lighting and shading leaves on the different branches of 6-year-old *P. alba var.*
141 *pyramidalis* were inoculated by *V. sordida* isolate CZC (cultivated in the darkness at 28°C for
142 seven days). Besides the light condition, the branches' location and base diameter were identical.
143 Two square pathogen mycelium plugs inoculated the 5-7$^{th}$ newly matured leaves. Two branches
144 (shading and lighting) of three poplar replicates were inoculated in this experiment. The
145 inoculation method is described in 2.2. Therefore, 120 pathogen-inoculated prick sites were
146 created: 60 for the lighting and 60 for the shading groups. In addition, two shading and lighting
147 leaves were also inoculated by WA plugs as the control.

148 **2.5. Comparison of the pathogenicity of canker pathogen with different cultured time**

149 To detect the effect of the culture time of the pathogen on its pathogenicity, *V. sordida*
150 isolates CZC cultivated in darkness for 4 and 7 days were inoculated on the leaves of *P.*
151 *euramericana* cv. 'Bofeng 3'. In this experiment, six poplar saplings were divided into two
152 groups: one inoculated by a 4-days-cultured pathogen (D4) and another inoculated by a 7-days-
153 cultured pathogen (D7). Two square pathogen mycelium plugs inoculated the 5-7$^{th}$ newly
154 matured leaves. The inoculation method is described in 2.2. Finally, 60 pathogen-inoculated
155 prick sites were created for group 4 days, 60 for group 7 days, and 30 prick sites as the control.

156 **2.6. In vivo assessment of resistance to *Valsa* canker in hybrid poplar clones using leaf**
157 **inoculation method**

158 The *Valsa* pathogen isolate CZC was cultured on a PDA medium in the darkness at 28°C
159 for seven days. The 5-7$^{th}$ newly matured leaves, located on one or two hybrid clones, were
160 inoculated by two or four square pathogen mycelium plugs. Three leaves were inoculated with
161 mycelium plugs, and one leaf was inoculated with WA plugs. After all 48 hybrid clones were
162 inoculated, needles immediately pricked the leaves. Finally, pathogen plugs inoculated a total of
163 30 or 60 prick sites, and WA plugs inoculated 30 prick sites as the control. The detailed
164 inoculation method was the same as the methods described in 2.2. The disease symptoms were
165 observed and photoed five days after inoculation (5 DAI). According to the symptoms and
166 necrotic areas around the prick sites determine the disease incidence and index.

167 **2.7. In vitro validation of poplar resistance to *Valsa* canker pathogen using stem segments**

168 To validate the effectiveness of the leaf inoculation method in assessing popular resistance
169 to stem canker pathogen, the response of 15 *P. deltoids*×*P. deltoids* hybrid clones (5
170 resistance/high resistance, five non-resistance/non-susceptible, and five susceptible/high

susceptible to canker pathogen determined by in vivo leaf inoculation in Section 2.7) were detected using an excised stem segment inoculation method. This study used ten dormant poplar stem segments (2-3 cm in diameter, 25-30 cm in length) derived from the base region (50-60 cm) of the 1-year-old branches of the selected poplar clones. Before inoculation, the 25-30 cm long poplar segments were all washed with sterilized water and then sterilized with 75% alcohol, and the upper end of the segments using plant callus ointment (a commercial product promoting the regeneration of callus around the wound) after dry (Figure 1B-1). The segments were soaked in water for three days at room temperature and then inoculated with *V. sordida* isolate CZC (cultured in 2% PDA at 28°C for seven days) (Figure 1B-2). Zhang (2017) described the inoculation method, each segment was inoculated with five mycelium plugs (0.5 cm in diameter) every 3-4 cm per inoculation site from the beginning site (10 cm height from the base end of the segment) to the upper back. Every inoculation site was pricked using a needle as a regular triangle (the side length is about 1-2mm). After inoculation, all inoculation sites were covered by household PE film to preserve moisture, and then all inoculated stem segments were cultured in water. Ten segments were inoculated in this study, and two were mock inoculated.

The disease development around the inoculation sites was observed and recorded according to whether the necrotic produced every five days; the necrotic sites were marked, and the total number of disease sites of every poplar clone was also recorded until 40 days after inoculation (Yang *et al.*, 1989; Shi *et al.*, 2014). According to the time the disease symptoms appeared, the disease severity of each poplar clone was ranked (Table S1) (Yang *et al.*, 1989; Zhang *et al.*, 1989), and the disease index of each poplar clone according to the formula (2). At the last observation, every lesion's longitudinal extension length (a, mm) and the horizontal length (b, mm) were measured. Because the lesion shapes were mainly subelliptic, the necrotic area of disease spots was calculated according to the elliptic area formula ($A = \mathrm{a\,b}\,\pi/4$). The severity of the disease was ranked according to the criterion in Table S2, and the disease index was calculated according to the formula (2).

### 2.8. Determination of pathogenicity of stem canker pathogens using in vivo leaf inoculation method

The pathogenicity of a total of 12 *B. dothidea* isolates, which came from different regions and different, was determined in this study using the leaf inoculation method. All fungal isolates were inoculated on the 5-7$^{th}$ newly matured leaves of 13 different twigs of one 2-year-old poplar 'Bofeng 3' hybrid clone. Each isolate inoculated three leaves, and two or four square mycelium plugs were used. Therefore, 30 or 60 pathogen-inoculated prick sites were created after needle piercing. Moreover, WA plugs inoculated two other twigs or just needle piercing as the two control treatments, Ctrl-1 and Ctrl-2, respectively. The leaf inoculation method was described in 2.2. Five days after inoculation, the symptoms were observed, photoed, and then automatically

identified using ImageJ software, which was adjusted in the handle if necessary. The disease incidence, area of necrotic spots, and disease index were determined using results derived from ImageJ software.

### 2.9. Data statistics

In this study, the t-test, ANOVA (analysis of variance), and LSD (least significant difference) methods were used to test the significance of the necrotic area; the chi-square method was used to test the importance of the disease incidence rate, and the Shapiro-Wilk method was used to test the normal distribution of the numbers of the poplar clones in different resistance levels.

## 3. Results

### 3.1. Stem canker pathogen *Valsa sordida* induced necrotic symptoms on poplar leaves.

In this study, the control (WA inoculation) and *V. sordida* inoculation induced necrotic spots around the prick sites on the poplar leaves at 5 DAI (Figure 2A-B). All the control necrotic spots are unextended; their average areas are 1.5± 0.5 $mm^2$ (Figure 2A, 2C-F), while the pathogen inoculation can produce extended necrotic spots on poplar leaves (Figure 2B, 2G-K). No hyphen structure was observed in the stereomicroscope on the surfaces of the control poplars. In contrast, hyphen structures were observed in the pathogen-inoculated poplars (Figure 2G-I), even some pycnidium structures (Figure 2J-K), and some extruded milky white spores were observed on the leaves of some hybrid poplar clones B246 (Figure 2K), B73, B14, B18, B76, B181 and B1. In some severed diseased poplar clones, such as clone B246, almost all five necrotic spots overlapped. Based on the average area size of the necrotic spots (NA) in the control poplar leaves, NA > 2.0$mm^2$ was set as the quantity criterion of the disease onset on the pathogen-inoculated poplar leaves (Table 2).

### 3.2. The influence of leaf ontogeny on disease severity

This study shows 5-7$^{th}$ newly matured leaves (upper leaves) and 18-20$^{th}$ mature leaves (lower leaves) of *P. euramericana* cv. 'Bofeng 3' was used to detect the influence of leaf ontogeny on disease severity. As shown in Figure 2, five days after inoculation (DAI), the disease severity of the upper leaves is significantly lower than the lower leaves (chi-square test, $n = 60$, $p < 0.05$), the percentage of disease incidence (P) of the upper and lower leaves are 28.33% and 70.00%, respectively. After discarding the undiseased inoculation points, results showed that the disease area of the necrotic spots of the upper leaves is also significantly lower than that of

238 lower leaves (2.49± 0.13 (n = 17) vs 2.94± 0.13 $mm^2$ (n = 42); t-test, $p < 0.05$). Moreover, the
239 disease index (DI) of the upper and lower leaves are 5.67 and 14.67, respectively (Figure 3A).

240 **3.3. The influence of light condition of leaves on disease severity**

241 In this study, the 5-7$^{th}$ leaves grew on three lighting branches (were exposed to the sun all
242 day) and on three shading branches (were not exposed to direct sunlight) of the same two-year-
243 old saplings were used to compare the influence of light conditions of leaves on disease severity.
244 Results showed no differences in the disease incidence rate between the lighting and shading
245 leaves ($IR_{lighting}$ 58.33% vs. $IR_{shading}$ 55.00%, respectively; chi-square test, n = 60, $p > 0.05$).
246 However, results suggested that shading promoted the disease severity in poplar leaves: the
247 average necrotic area of the lighting and shading leaves are 3.87± 0.29 (n = 35) and 7.40± 1.20
248 $mm^2$ (n = 33), respectively (t-test, $p < 0.05$). In addition, the disease index of lighting leaves
249 ($DI_{lighting}$) is 18.00, while the disease index of shading leaves ($DI_{shading}$) is 26.00 (Figure 3B).

250 **3.4. The culture time of fungal inoculum altered the disease severity on leaves.**

251 Results showed that the culture time of mycelium inoculums also affected the disease
252 development in poplar leaves. Their disease incidence rate induced by 4-day-cultured *V. sordida*
253 mycelium ($IR_{4day}$) is 83.33%, significantly $IR_{7day}$ (65.00%) (chi-square test, n = 60, $p < 0.05$),
254 suggesting that the juvenile pathogen inoculums had with higher pathogenicity than the older.
255 After discarding the undiseased inoculation points, results showed that the necrotic area induced
256 by 4-day-cultured mycelium plugs ($NA_{4day}$ = 8.51± 0.57 $mm^2$, n = 50) is significantly higher than
257 that caused by 7-day-cultured mycelium plugs ($NA_{7day}$ = 3.37± 0.20$mm^2$, n = 39) (t-test, p <
258 0.05). Moreover, the disease indexes, $DI_{4day}$ and $DI_{7day}$, are 50.67 and 13.33, respectively (Figure
259 3C).

260 **3.5. In vivo inoculation of stem canker pathogen *Valsa sordida* on leaves revealed the**
261 **resistance diversity in poplar hybrid clones.**

262 In this study, every hybrid clone produced 60 prick sites, and all 48 clones produced 2,880
263 sites; after discarding the inefficient inoculation sites (the mycelium plugs moved away or dried
264 in 1-3 DAI), a total of 2,715 efficient inoculation data were obtained occupying 94.27% of all
265 schemed inoculation dataset. Taking the criteria of the necrotic area (NA) > 2.0 $mm^2$ as the
266 determining criteria for the occurrence of disease on poplar leaves, a total of 2,145 inoculation
267 sites exhibited definite necrotic features, indicating that the authentic leaf disease incidence in 40
268 poplar hybrid clones 79.00%.
269 Results revealed high resistance diversity to the *Valsa* canker pathogen among 48 poplar
270 hybrid clones using a novel leaf inoculation method. As shown in Table S3, the disease

incidence rate in 48 poplar clones varied from 31.67% (poplar clone B133) to 100.00 (B1 and B104) (Figure 4A), the size of necrotic area varied from 2.72± 0.13mm$^2$ (B168) to 15.28± 1.26mm$^2$ (B14) (Figure 4B), and the disease index ranged from 6.67 (B168) to 83.67 (B246 and B135) (Figure 4C).

This study used the Shapiro-Wilk model to test the distribution of the poplar numbers in different resistance levels. Though the p-value in the Shapiro-Wilk test did not support a normal distribution, the absolute kurtosis of the normality test is 1.05 (<10.0). The absolute skewness is 0.44 (3.0), and the resistance among these 48 poplar clones is generally accepted as a normal distribution (Kline *et al.*, 2023) and supported in the P-P plot analysis (Figure 5).

According to the disease index, all 48 poplar hybrid clones were divided into seven disease resistance levels, from the high resistance group to the very high susceptible group (Figure 4C). For example, hybrid B133 was identified as a high resistance (HR) clone, hybrid B168, and other seven clones were identified as resistance (R) clones, and hybrid B135, B246, and B104 were susceptible (S) to *Valsa* canker pathogen CZC, using these leaf disease severity assessment methods (Table S3).

### 3.6. In vitro stem segment inoculation verified the effectiveness of the leaf inoculation method.

As shown in Table 3 and Table S4, the disease incidence rate (IR) of 15 poplar hybrid clones varied from 18.00% (B133) to 98.00% (B246), the $DI_{stem\text{-}time}$ varied from 15.71 (B133) to 92.86 (B246). The $DI_{stem\text{-}area}$ of 15 poplar hybrid clones varied from 5.00 (B133) to 78.00 (B246) (Figure 6A-C). The values and ranking of $DI_{leaf\text{-}area}$, $IR_{stem}$, $DI_{stem\text{-}time}$, and $DI_{stem\text{-}area}$ were compared to assess the consistency of the leaf and stem inoculation methods. As shown in Table 3 and Figure 6D, besides clone B145 in the R/HR group and clone B76 in the S/LS group, the resistance rankings of the other eight poplar clones that were assessed by leaf and stem inoculation method are the same, in particular, the ranking of the highest resistance clone B133 and lowest resistance clone B246 were identically in these two methods. Therefore, the results suggested an alternative pathway, the leaf inoculation method, for acquiring pathotypes and assessing resistance clones in tree pathological research and poplar breeding.

### 3.7. In vivo leaf inoculation revealed the diversity of pathogenicity in stem canker pathogen *B. dothidea.*

Results showed that the pathogenicity of 12 *B. dothidea* isolates on poplar hybrid 'Bofeng 3' varied hugely. *B. dothidea* isolate SD47 induced the highest necrotic area in poplar leaves (6.57±0.41mm$^2$), while the size of necrotic area induced by isolate SD60 and CZ1218 are 4.53± 0.68 and 3.20± 0.19 mm$^2$, respectively. Though there was no significant difference between

305 isolate SD47 and SD60, and also between isolate SD60 and CZ1218, the necrotic area of isolate
306 SD47 was significantly higher than isolate CZ1218 (ANOVA, $p<0.05$) (Figure 7). In addition,
307 the sizes of the necrotic area of 9 isolates (CZ1070c, CZ1070b, CZ1010, CZ1055, etc.) were
308 smaller than 2.0 $mm^2$, the quantitative criteria to determine disease occurrence in leaf in this
309 study indicated these nine isolates had with low pathogenicity on poplar hybrid 'Bofeng 3' (Table
310 4). These results suggested that the leaf inoculation method can be used to identify pathogen
311 pathogenicity.

312 **4. Discussion**

313 Selection based on phenotypic is the crucial step of hybrid breeding. Due to their large
314 crown, slow growth, and long maturity period characteristics in tree species, phenotypic
315 acquisition of tree stem canker diseases always requires more time, space, and human resources
316 than herb diseases and tree leaf diseases. Because the necrotic spots are dispersed around the
317 surface of the branches at different heights, the phenotypic acquisition of stem canker diseases is
318 more tedious, complicated, and complex than leaf diseases.
319 Studies illustrated that stem canker pathogens can infect host leaves or live in leaves as
320 endophytes (Marsberg *et al.*, 2017; Feau *et al.*, 2010; LeBoldus *et al.*, 2010; Dunnell &
321 LeBoldus, 2017; Zheng *et al.*, 2017; Wei *et al.*, 2010), provided a feasible pathway to detect the
322 resistance of stem canker disease through leaf inoculation method. However, the consistency of
323 the leaf inoculation method with the stem inoculation method remains controversial. Wei *et al.*
324 (2010) and this study in *Valsa* canker disease illustrated the consistency between these two
325 methods, and the leaf inoculation methods can be used to select resistance clones or the assays of
326 pathogenicity differentiation in the pathogen population. However, there was no correlation in
327 the disease severity between leaf spot and stem canker in the poplar-*Septoria* pathosystem
328 (Dunnell & LeBoldus, 2017).
329 The disease severity after leaf inoculation is directly related to the resistance of leaves. The
330 correlation between the leaf age (and the leaf position on branches) and resistance has been
331 observed for more than 100 years (reviewed by Gusberti *et al.*, 2013). This pattern of increasing
332 resistance with tissue age is called ontogenic or age-related resistance and can be specified as
333 leaf-stage resistance in leaf tissues (Asalf *et al.,* 2014; Dunnell & LeBoldus, 2017; Xu *et al.*,
334 2018). In physiological research, since the active and stable physiological performance, the
335 newly matured leaves at the top of the branches or saplings (always 5-7$^{th}$ leaves) were taken as
336 the ideal materials used in the study of photosynthesis, chlorophyll fluorescence, stomatal
337 movement, water status, physiology and biochemistry, and also gene expression regulation of
338 tree species (Zhao *et al.*, 2017; Xing *et al.*, 2019; Li *et al.*, 2019; Shen *et al.*, 2023; Li *et al.*,
339 2024). In powdery mildew of strawberry and *Malus-Venturia* interactions, the leaf resistance
340 increased rapidly from the first unfurled and expanding leaf to lower leaves, and the resistance

reached the top at the 5-6th matured expanding leaves (Asalf *et al.*, 2014; Gessler & Stumm, 1984; MacHardy *et al.*, 1996), in *Malus*-*V. ceratosperma* system, the inoculated leaves were described as "fully extended at the top of the stems" (Wei *et al.*, 2010). This study illustrated that the resistance of 5-7th leaves (always newly matured) is significantly higher than the 18-20th leaves (32-35 leaves grow on the 1-year-old saplings). Moreover, this study illustrated that the resistance results from the 5-7th leaves were consistent with those from the excised stem segments (Table 3, Figure 6D). However, besides the differences in disease type (*Valsa* canker vs. *Septoria* canker) and inoculation method (wounding mycelium vs. non-wounding spore inoculation), the difference in the leaf position (5-6th newly matured leaves vs. the five middle leaves in the branches of 3 or 4-month-old hybrid poplar clones) might be the main reason for the inconsistency between the leaf and stem resistance inpoplar-*Septoria* pathosystem (Dunnell & LeBoldus, 2017). Based on the above analysis, the top 5-7 leaves were suggested as the optimal inoculation materials for poplar leaf inoculation in assessing and selecting the resistance clones in poplar breeding.

Solar UVB radiation is a positive modulator of plant defense (Ballaré, 2014). Evidence from several plant-pathogen systems indicates that shading increases infection by a range of pathogens (Roberts & Paul, 2006). In addition, high population density (which is usually accompanied by reduced light exposure) has been shown to markedly increase the severity of plant disease in natural and agricultural systems (Augspurger & Kelly, 1984; Bell *et al.*, 2006; Burdon & Chilvers, 1982; Jurke & Fernando, 2008). In this study, we use the leaf inoculation method in the resistance detection of perennial poplars or the pathogenicity detection of different pathogen isolates using the leaves on the various branches of the same poplar tree. The light conditions of different leaves are remarkably different, especially for the backlit and light-oriented leaves. Then, can the difference be detected among these leaves? The current study provided a positive answer to the above question: The susceptibility to the pathogen CZC significantly increased in the shading leaves in hybrid poplar "Bofeng 3" (Figure 3B). Shading is a regular method to promote the disease onset in a controlled environment. For example, in the hybrid-Septoria system, LeBoldus and his colleagues covered and sealed the black plastic garbage bags (with two pieces of moist paper towel) for 48 h to promote the germination and invasion of spore inoculants and the appearance of disease symptoms (LeBoldus *et al.*, 2010; Dunnell & LeBoldus, 2017). In this study, the leaf inoculation experiment showed that the smaller size of disease necrotic areas was detected on the lighting leaves (Figure 3B). Moreover, another study showed that high density (or shading) induced more severe canker disease symptoms on the truck of 6-year-old *P. alba var. pyramidalis* (Shen *et al.*, 2024). Therefore, in the assays of the poplar resistance or the pathogen's pathogenicity using the leaf inoculation method, the poplar leaves with an identical light condition must be carefully considered.

In the Petri plate, the morphology, colors, and growth rate of the colony and mycelium of the pathogens varied during the culture period. Since plants can exhibit age-related resistance, such as leaf-stage resistance, pathogens will likely have some culture of age-related pathogenicity. For example, the pathogenicity and infection rate of the conidia of *Dactylaria higginsii*, which were harvested from 15 days of culturing on PDA, was higher than that of the conidia harvested from other culture ages (Kadir *et al.*, 2011). Conidiospores of the three entomogenous fungi (*Metarhizium anisopliae*, *Paecilomyces fumoso-roseus,* and *Verticillium lecanii*) harvested from young (2–3d old) Petri plate cultures germinated more rapidly than those taken from older cultures. However, conidia of *Beauveria bassiana* all germinated at the same rates, irrespective of culture and age (Richard *et al.*, 1994). This study illustrated that the pathogenicity of the young culture (4 days old in PDA medium) of *V. sordida* isolate CZC to poplars was higher than that of the old culture (7 days old). However, some fungal isolates cannot grow over a 9 cm diameter Petri dish in 7 days; for example, some *V. sordida* isolates, to harvest more mycelium inoculants, the culture conditions of canker pathogens suggested as follows: cultured on PDA medium, at 28°C, in dark condition, for seven days.

Some researchers considered that the most aggressive or virulent strains of a pathogen that causes the disease should be used in the selection and assays of plant germplasm and breeding lines (Balendres *et al.*, 2019). This selection strategy may be beneficial to the funding of the authentic highest resistance clones in the breeding population; however, it also may be a disaster for the whole breeding program for the disease or even the death of all offspring, or it has no breeding significance for the other poor production phenotypes. As shown in Figure 5, the numbers of poplar clones in 7 different pathogenicity levels were near normal distribution, suggesting that the pathogen *V. sordida* CZC is the optimal test strain for the current poplar hybrid population.

If another isolate of *V. sordida* was adopted, whose pathogenicity is higher than that of *V. sordida* CZC, then the number of these 48 poplar clones might be right skewed distributed (almost clones susceptible to the pathogen), and if a weak pathogenicity isolate was adopted, the number of poplar clones might exhibit a left-skewed distribution. Therefore, the optimal pathogen strains must preselected in a new breeding program before final resistance assays. The side length of the square, 1.0-1.2 cm, is an experience value based on our preliminary experiment; if resistance assays of the 48 poplar clones were against a high pathogenicity strain, larger mycelium plugs might be an optimal option. Finally, besides three isolates of of *B. dothidea*, the other nine fungal isolates exhibited no or very low pathogenicity to hybrid poplar "Bofeng 3". The reason for this phenomenon might come from the high disease-resistance characteristics of the hybrid poplar. Then, the pre-selection of poplar clones, which have an appropriate resistance level, is needed in the assays of large-scale pathogen populations.

This study illustrated that poplar stems canker pathogen *V. sordida* can induce lesion symptoms on leaf tissue using a leaf inoculation method. The efficiency of this method in hybrid breeding was validated using a small poplar hybrid population in this study. The process is simple and easy to operate; using this method, a team of three people can complete the inoculation of more than 200 clones in a day. For the rapid development of disease symptoms, the pathotypes can acquired in 3-5 days after inoculation; then, breeders can acquire the pathotypes of a large-scale population (1,000 genotypes or much more) in one month. For short periods of disease stresses (removed from the stems at 3-5 days after inoculation), the pathogen did not or less affected the growth and physiology of the inoculated poplars and then did not or less change the other phenotypes (such as the heights, stem diameter and mass. Therefore, the breeders can quickly achieve multi-objective breeding of canker disease and fast growth, high yield, etc.

In short, compared to the time-consuming and high-cost in vitro stem segment inoculation method, the in vivo leaf inoculation method is fast, efficient, low-cost, and high-throughput, suitable for the large-scale resistance assays of hybrid poplar clones, can be used in the seedling selection of resistance clones in the breeding of stem canker diseases (and some leaf diseases) of poplar or other tree species. Combined with the new breeding technology, such as genomic selection, the breeding resources can excavate deeply for the new resistance-related genes, gene loci, or gene modules (Du *et al.* 2023). In addition, this leaf inoculation method can be used in the large-scale detection of pathogenicity, and the differentiation assays of poplar and tree stem canker or leaf pathogens.

**Author Contributions:** Conceptualization, J.Z. and B.Z.; methodology, J.Z. and B.Z.; software, Z.L. and Y.S.; validation, Z.L., Y.S., W.S. and Y.F.; formal analysis, Z.L., Y.S., W.S. and Y.F.; investigation, Z.L., Y.F. and Y.S.; resources, B.Z. and J.Z.; data curation, Z.L, Y.S. and J.F..; writing—original draft preparation, Z.L. and J.Z.; writing—review and editing, J.Z., Y.Z., Z.L., H.L. and L.P.; visualization, Z.L. and W.S.; supervision, X.S.; project administration, J.Z. and B.Z.; funding acquisition, J.Z. All authors have read and agreed to the published version of the manuscript.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Data availability**

Data will be made available on request.

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545 # Figures:

546 **Figure 1:** The schematic diagram of the leaf inoculation method provided in this study (A). This
547 study provided a schematic diagram of the stem segments' inoculation method. (B).

548 **Figure 2:** Using a novel leaf inoculation method, the necrotic symptoms caused by pathogen
549 *Valsa sordida* isolate CZC on the poplar leaves. In the WA medium inoculated poplar leaf, only
550 unextended spots (1.5± 0.5 $mm^2$) were observed (A); *V. sordida* inoculated the poplar leaf isolate
551 CZC, some extended necrotic spots (more than 2.0 $mm^2$) were observed (B); the poplar leaves
552 and the necrotic spots induced by *V. sordida* isolate CZC (A-B); the necrotic spots on the PDA
553 inoculated leaves (C-F); the necrotic spots on the PDA inoculated leaves (G-I); the formation of
554 fungal hyphen and pycnidium structure on the leaves of high susceptible hybrid clone B246 at
555 five days after inoculation by *V. sordida* isolate CZC (J-K).

556 **Figure 3:** The effects of leaf position (A), leaf light condition (B), and culture age of the
557 mycelium inoculants (C) on the leaf disease severity inoculated by poplar stem canker *Valsa*
558 *sordida* isolate CZC, using the leaf inoculation method.

559 **Figure 4:** The disease incidence rate (A), necrotic area (B), and disease index (C) of the leaves
560 of 48 poplar hybrid clones were inoculated by poplar stem canker *Valsa sordida* isolate CZC
561 using the leaf inoculation method.

562 **Figure 5:** The leaf inoculation method revealed that the resistance of the 48 hybrid poplar clones
563 is generally normally distributed. The standard distribution model (A) and the P-P plot of 48
564 hybrid poplar clones (B).

565 **Figure 6:** The disease incidence rate (A), disease index of onset time (B), disease index of onset
566 area (C), and two-dimensional representation of $DI_{stem\text{-}time}$ and $DI_{leaf\text{-}area}$ (D) of the stems of 15
567 poplar hybrid clones were inoculated by poplar stem canker *Valsa sordida* isolate CZC using the
568 stem inoculation method.

569 **Figure 7:** The necrotic area in the inoculated leaves of "Bofeng 3" poplar against 12
570 *Botryophaeria dothidea* isolates.

571 # Tables:

572 **Table 1:** The hosts and locations of fungal isolates used in this study.

573 **Table 2:** Severity grading of necrotic symptoms induced by canker pathogen on leaves.

574 **Table 3:** The disease index of 15 poplar hybrid clones was detected using in vivo leaf
575 inoculation and in vitro stem segments inoculation (the ranking values are based on the results of
576 the leaf inoculation method).

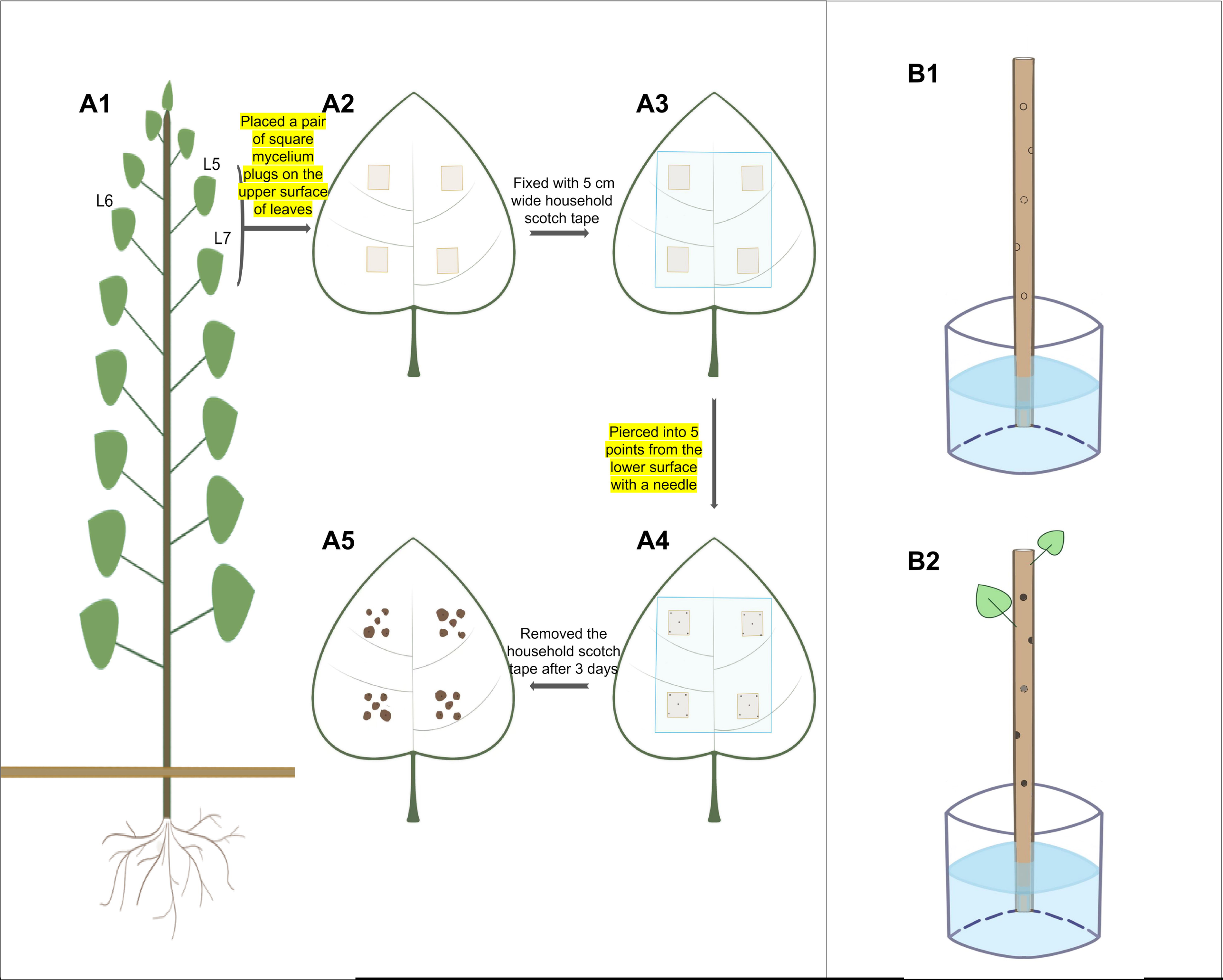
577 **Table 4:** The necrotic areas in the inoculated leaves of "Bofeng 3" poplar against 12
578 *Botryophaeria dothidea* isolates.

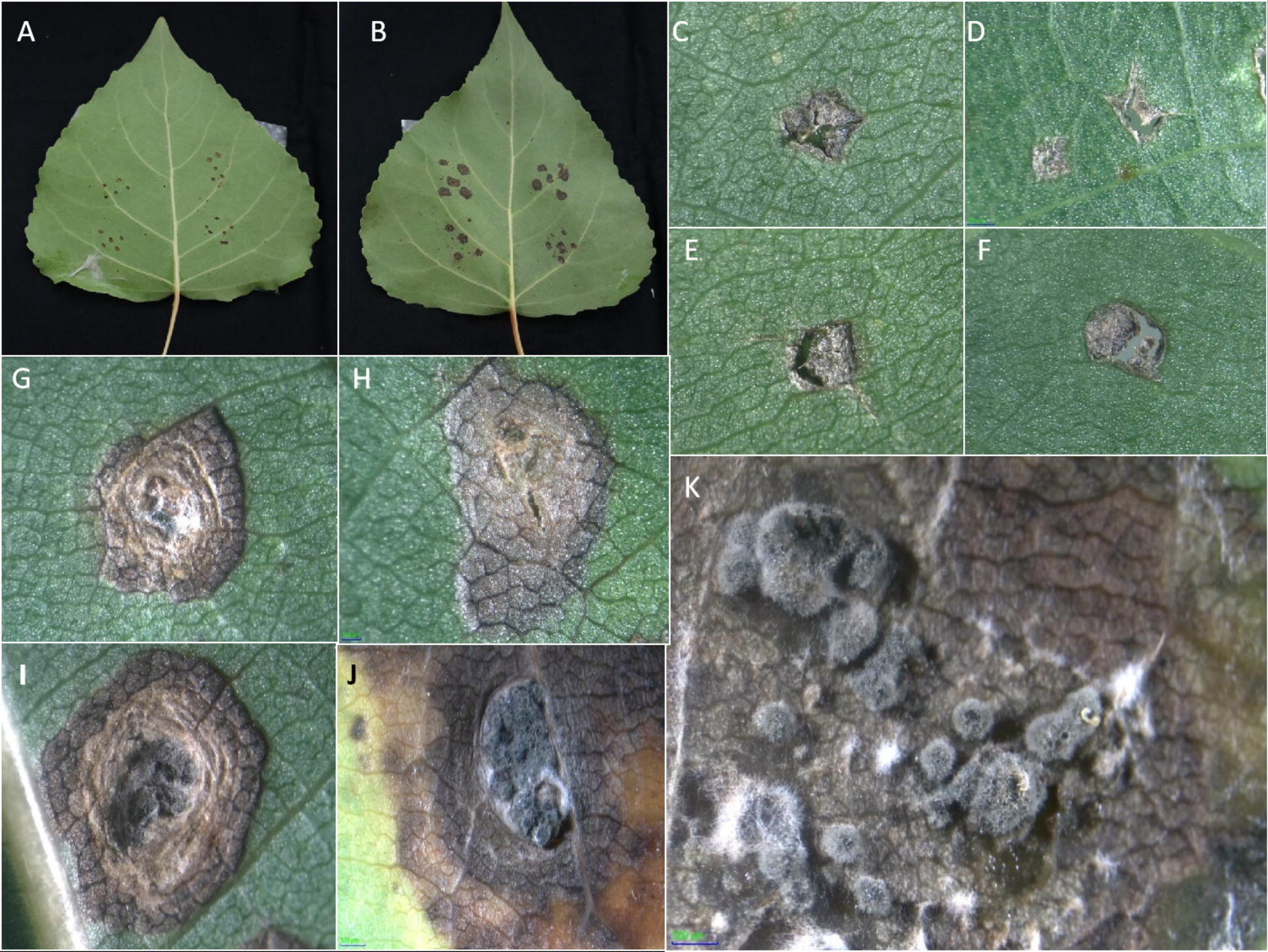
579 **Table S1:** Severity grading criteria for disease onset time in poplar stems.

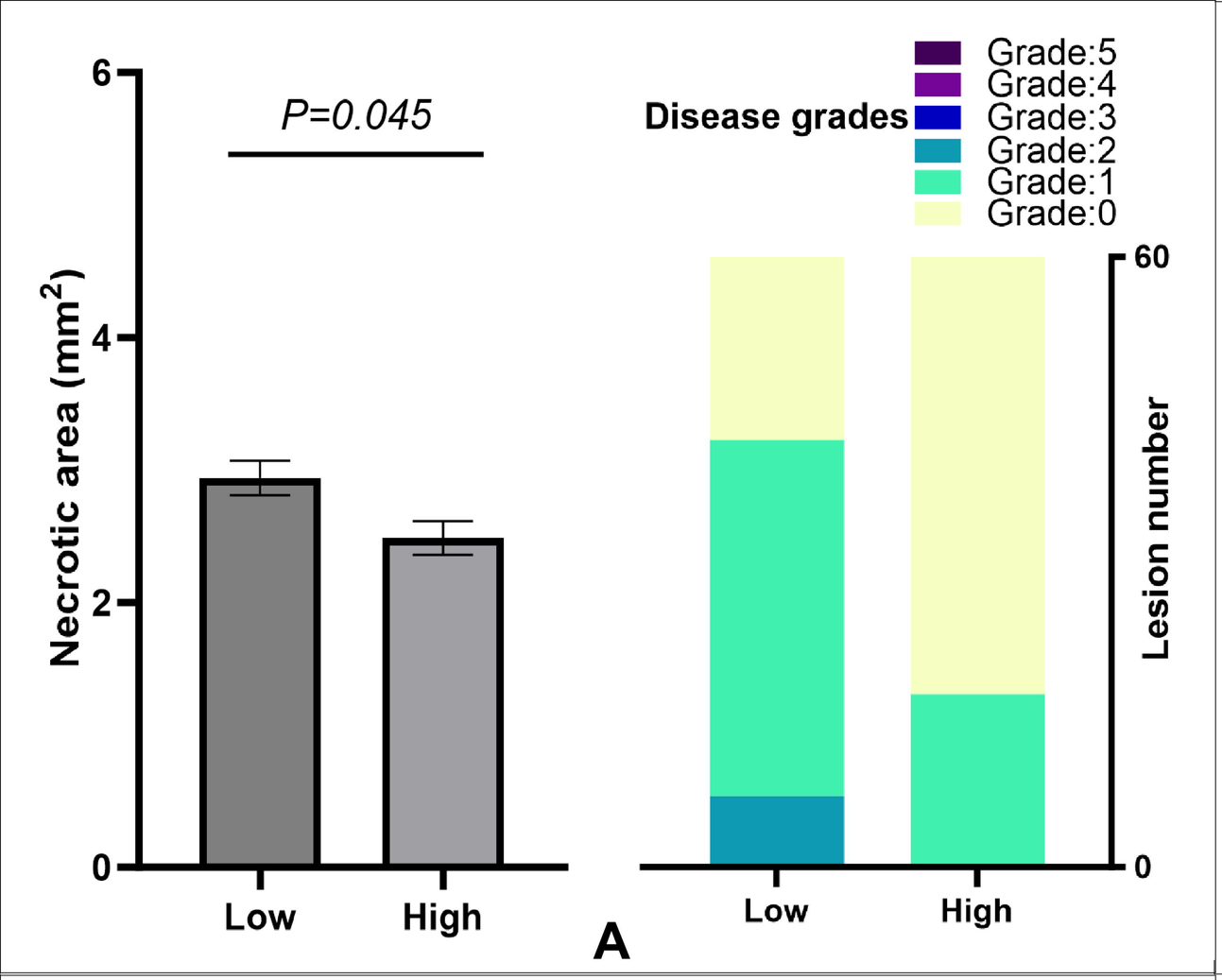
580 **Table S2:** Severity grading criteria for disease onset area in poplar stems.

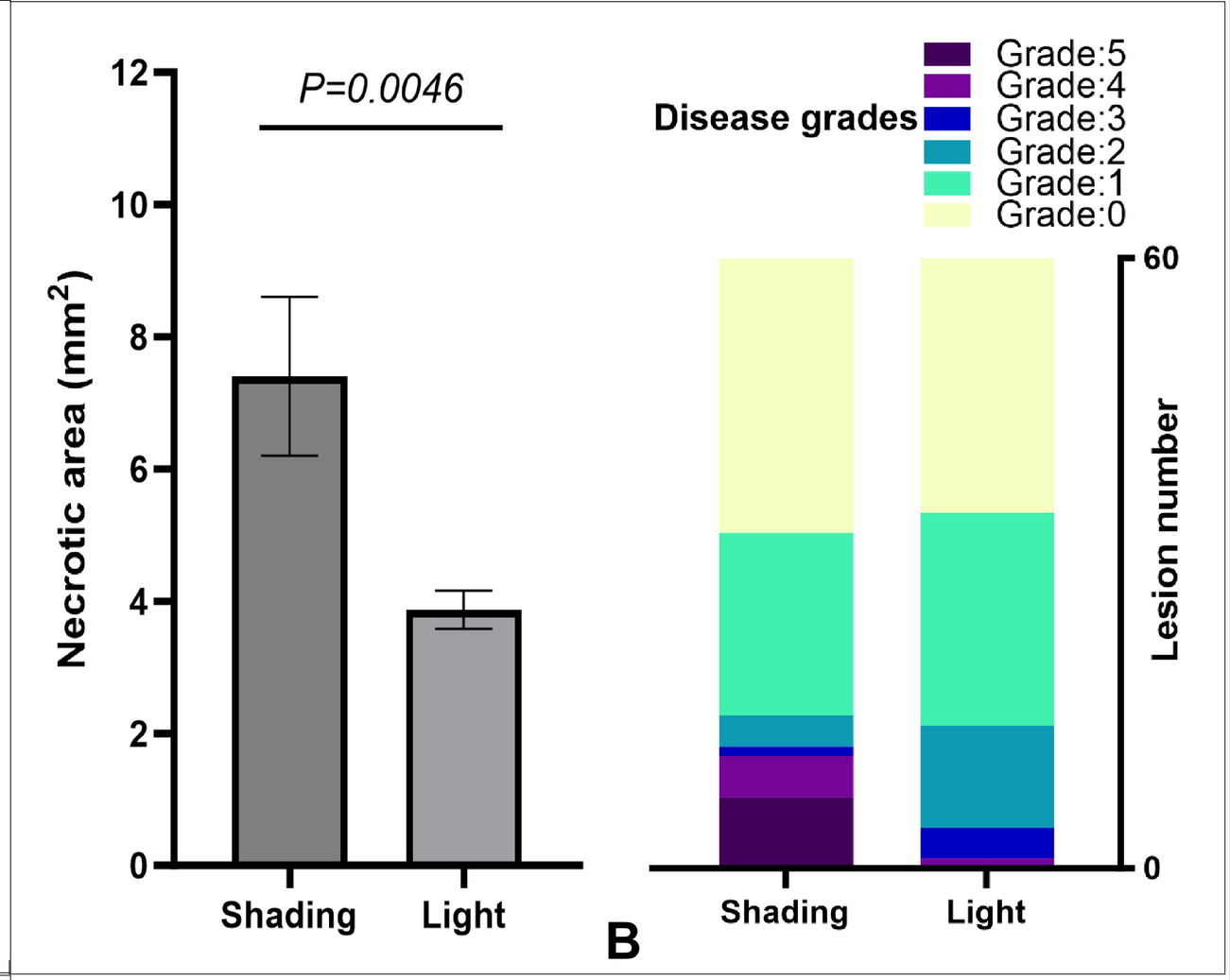
581 **Table S3:** The leaf necrotic areas of the 48 hybrid poplar clones inoculated by *Valsa sordida*
582 isolate CZC.

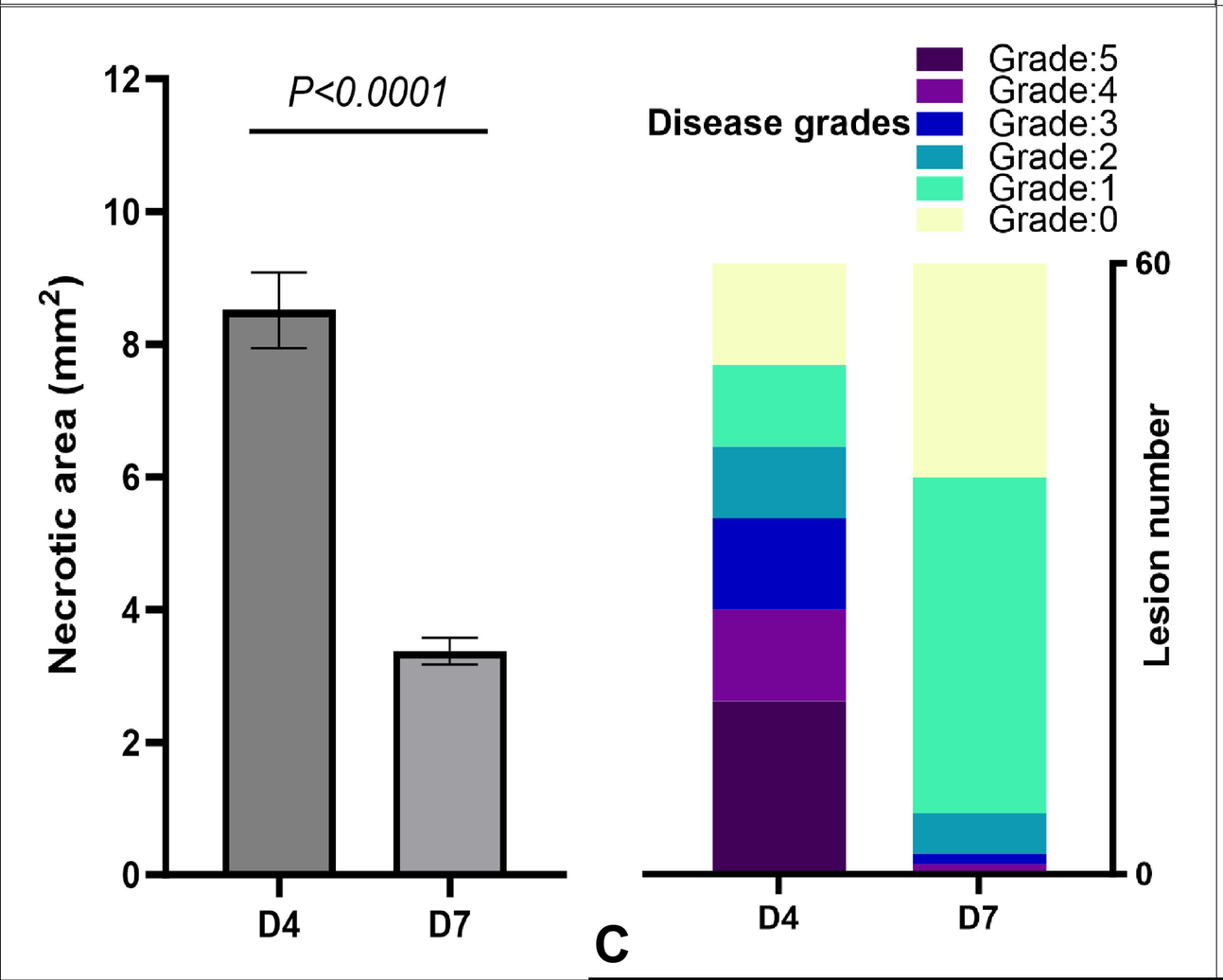
583 **Table S4:** The number of diseased poplars stem segments at the different time points (days after
584 inoculation) during the experiments.

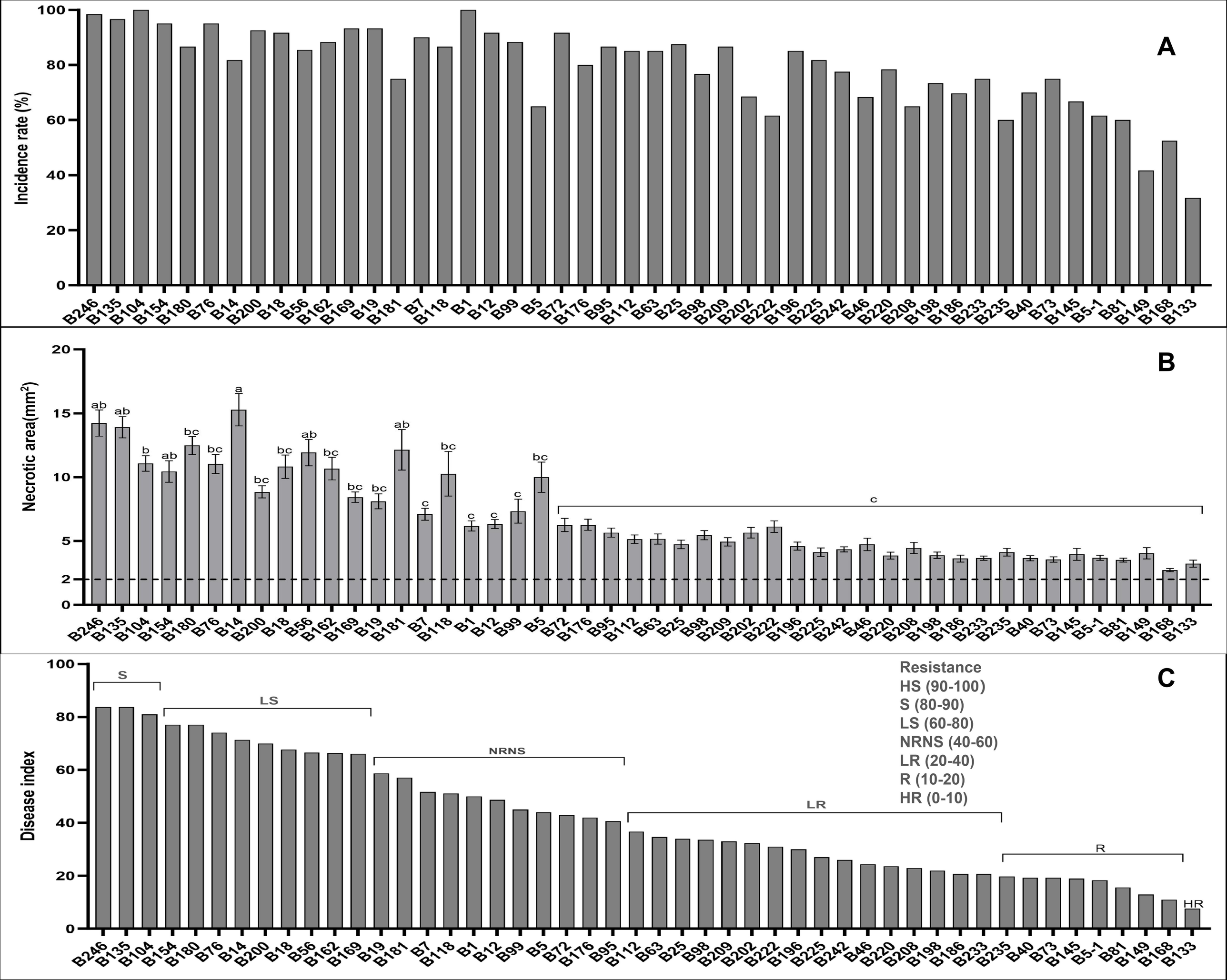

A1
L5
L6
L7
A2
Placed a pair of square mycelium plugs on the upper surface of leaves
A3
Fixed with 5 cm wide household scotch tape
Pierced into 5 points from the lower surface with a needle
A4
Removed the household scotch tape after 3 days
A5
B1
B2

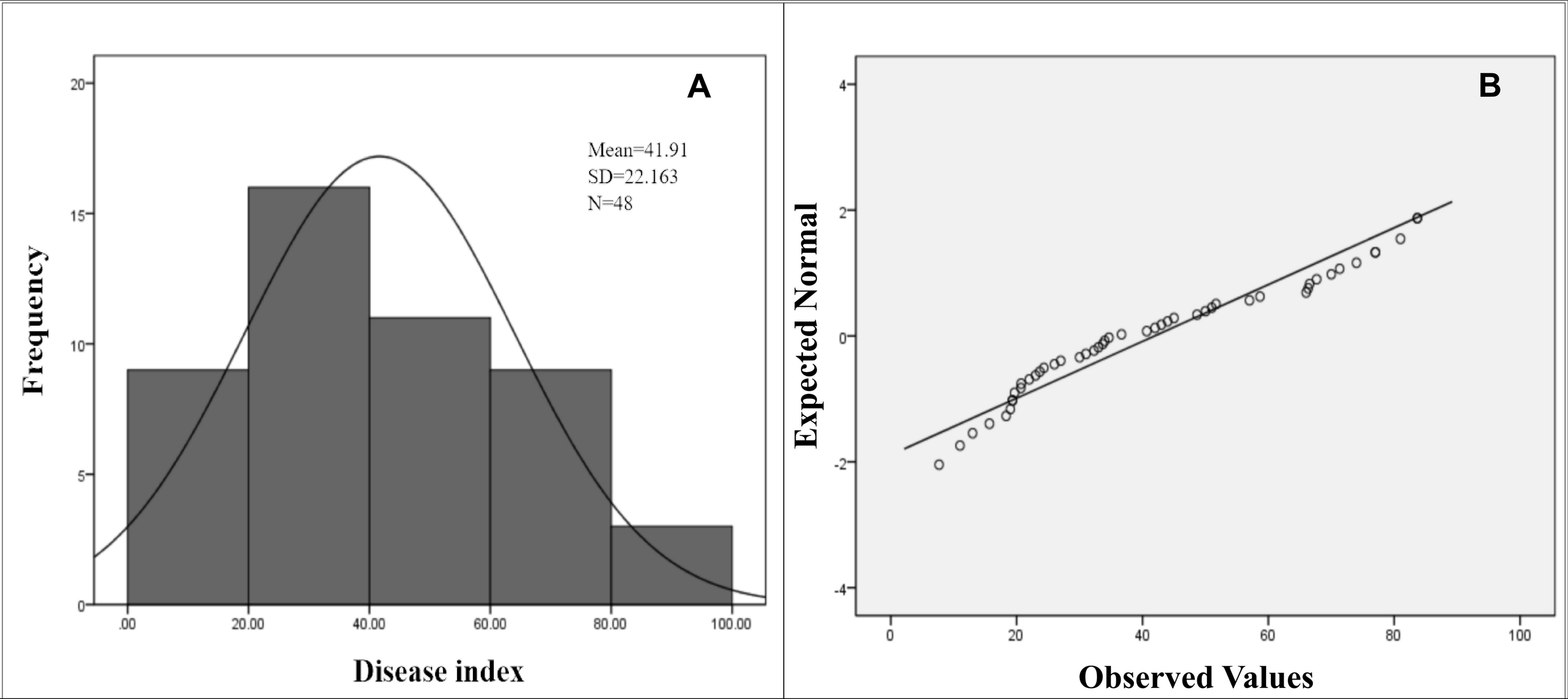

A
B
C
D
E
F
G
H
I
J
K

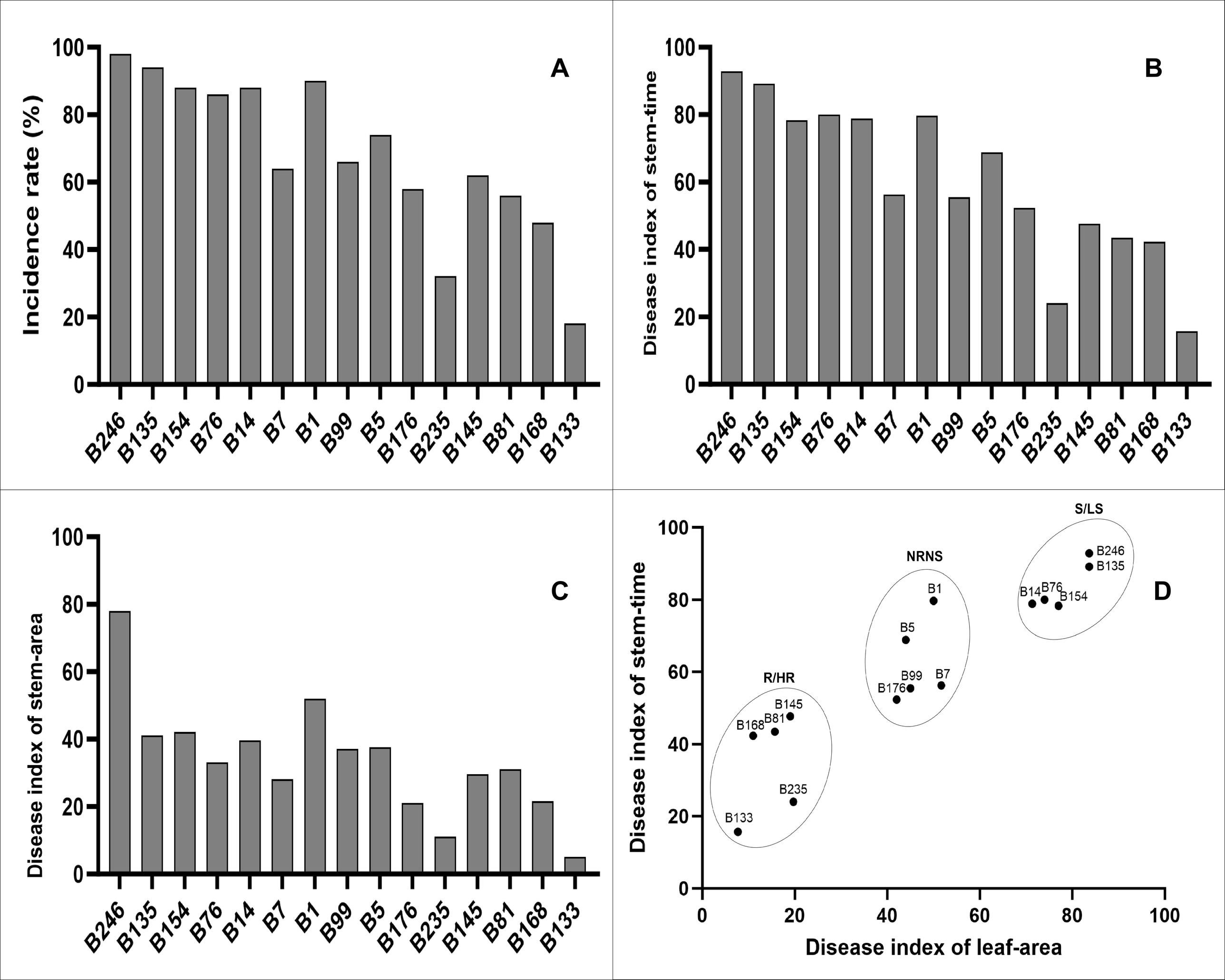

P=0.045
Disease grades
Grade:5
Grade:4
Grade:3
Grade:2
Grade:1
Grade:0
Necrotic area (mm2)
Lesion number
Low
High
A

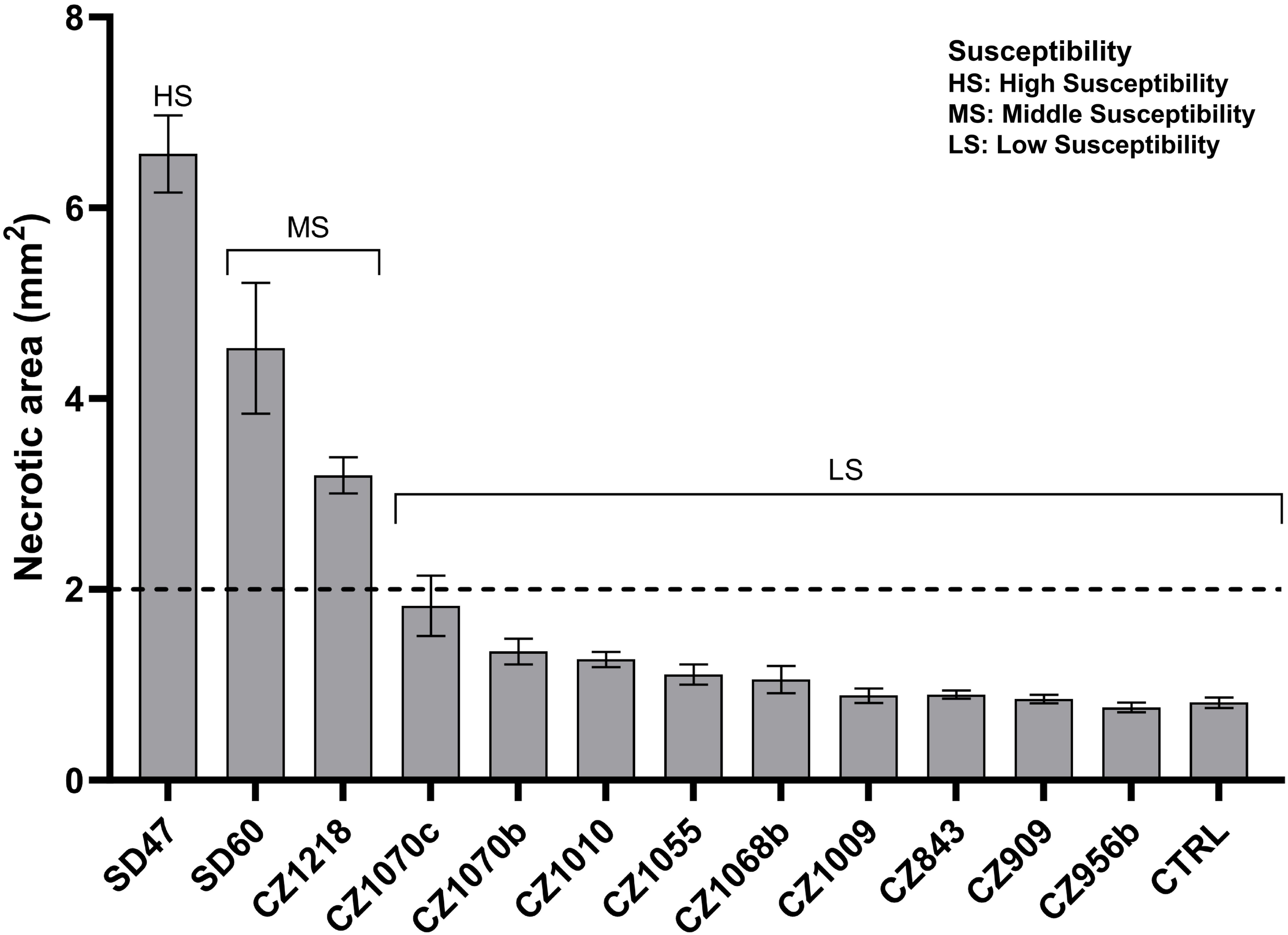

P=0.0046
Disease grades
Grade:5
Grade:4
Grade:3
Grade:2
Grade:1
Grade:0
Necrotic area (mm2)
Lesion number
Shading
Light
B


P<0.0001
Disease grades
Grade:5
Grade:4
Grade:3
Grade:2
Grade:1
Grade:0
Necrotic area (mm2)
Lesion number
D4
D7
C


A
Incidence rate (%)
0
20
40
60
80
100
B
Necrotic area(mm²)
0
2
5
10
15
20
ab
ab
b
ab
bc
bc
a
bc
bc
bc
bc
bc
ab
c
bc
c
c
c
bc
c
C
Disease index
0
20
40
60
80
100
S
LS
NRNS
LR
R
HR
Resistance
HS (90-100)
S (80-90)
LS (60-80)
NRNS (40-60)
LR (20-40)
R (10-20)
HR (0-10)
B246 B135 B104 B154 B180 B76 B14 B200 B18 B56 B162 B169 B19 B181 B7 B118 B1 B12 B99 B5 B72 B176 B95 B112 B63 B25 B98 B209 B202 B222 B196 B225 B242 B46 B220 B208 B198 B186 B233 B235 B40 B73 B145 B5-1 B81 B149 B168 B133


A
Mean=41.91
SD=22.163
N=48
Frequency
20
15
10
5
0
.00
20.00
40.00
60.00
80.00
100.00
Disease index
B
Expected Normal
4
2
0
-2
-4
0
20
40
60
80
100
Observed Values


A
Incidence rate (%)
0
20
40
60
80
100
B246
B135
B154
B76
B14
B7
B1
B99
B5
B176
B235
B145
B81
B168
B133
B
Disease index of stem-time
C
Disease index of stem-area
D
Disease index of leaf-area
S/LS
NRNS
R/HR


Susceptibility
HS: High Susceptibility
MS: Middle Susceptibility
LS: Low Susceptibility
Necrotic area (mm2)
8
6
4
2
0
HS
MS
LS
SD47
SD60
CZ1218
CZ1070c
CZ1070b
CZ1010
CZ1055
CZ1068b
CZ1009
CZ843
CZ909
CZ956b
CTRL