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Large-Scale Evaluation of Indonesian Elite Maize Breeding Lines for Resistance Against Bacterial Stalk Rot Caused by *Dickeya zeae*

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ABSTRACT

Bacterial stalk rot is one of the important diseases in maize caused by *Dickeya zeae*. Infection of this disease can lead to a considerable amount of loss in yield, with up to 98 percent loss of yield. The use of resistant materials is the most effective approach to managing bacterial stalk rot in maize. This study evaluates a large-scale phenotypic screening of 624 maize lines against bacterial stalk rot, divided into two groups based on a heterotic pool. These lines are used in a commercial breeding program in Indonesia. This study develops a stabbing method with a large gauge hypodermic needle, allowing for scalability in delivering inoculum while performing large-scale line evaluation. The result from ANOVA reveals a significant ($P<0.05$) effect of lines, day after infection, and interaction between day after infection and group. Group two displays fewer resistant lines compared to group one. This work presents a method for large-scale line evaluation for resistance against bacterial stalk rot, where the information obtained can be used in industrial breeding programs for routine material screening during the development of new lines or hybrids and for genomic studies of bacterial stalk rot resistance.

INTRODUCTION

Bacterial stalk rot has become one of the most important diseases affecting maize cultivation in the world, including Indonesia. Bacterial stalk rot, caused by *Dickeya zeae*, is an economically important disease that has the potential to reduce crop yields by 21 to 98.8% (Kumar et al., 2017; Muis et al., 2022). This disease is a major disease in tropical and subtropical maize planting areas (van der Wolf et al., 2021; Zhu et al., 2021). Studies have also reported the extent of distribution of bacterial stalk rot disease in different countries, such as Turkey (Caplik et al., 2022), Korea (Myung et al., 2010), Mexico (Martinez-Cisneros et al., 2014), and Indonesia (Suriani et al., 2021). In Indonesia itself, *D. zeae* infection was first reported as early as 2020 (Aeny et al., 2020) but with pineapple as the host plant, while *D. zeae* infection was first reported in maize in 2021 (Suriani et al., 2021).

High temperatures and high humidity, followed by heavy rainfall, are favorable environments for *D. zeae* (Jackson-Ziems et al., 2014; Kumar et al., 2017). Moreover, maize plants grown in flooding-prone areas have a higher risk of infection than in well-drained soil (Freije & Wise, 2016). These environments can support the physiological and metabolic activity of *D. zeae* bacterium, providing a favorable environment for bacteria to grow (Kumar et al., 2017). Disease management includes management strategies in agronomy such

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as chemical application (bactericides), optimum fertilizer to support health and optimum defense, and crop rotation to reduce the bacteria inoculum in the soil by alternating the planting with crops that are not the host of bacteria stalk rot (Jackson-Ziems et al., 2014; Osdaghi, 2022; R. Singh et al., 2020). Resistance hybrids are also another way to manage bacterial stalk rot infection. Resistance hybrids can be obtained through the breeding of bacterial stalk rot-resistant lines. Therefore, line evaluation for resistance against bacterial stalk rot is needed to develop lines and hybrids that are resistant against bacterial stalk rot infection.

In Indonesia, the first reported evaluation of maize lines against bacterial stalk rot was first reported by Suriani et al. (2023) using S1 lines. However, as of the current time, no large-scale evaluation of maize lines for resistance against bacterial stalk rot in the field has been reported. Performing a phenotypic screening against bacterial stalk rot infection at a large scale in the field poses challenges in balancing effectiveness and efficiency, scalability, and the quality of the data generated. Effectiveness and efficiency can be achieved through an inoculation method that can be implemented in a large field while maintaining labor efficiency and cost. The scalability of the approach used will allow the handling of a growing number of materials to be tested and larger fields. This scalability will be beneficial in the high-throughput phenotyping and phenomics era shortly. The scale of the trials must not sacrifice the quality of the phenotyping data, producing consistent results irrespective of the trials' size.

This study evaluates many maize lines commonly used in breeding programs and commercial combinations to assess the resistance level against bacterial stalk rot. This information is needed as the basis for constructing future breeding strategies. This study also develops an inoculation method that allows us to perform large-scale phenotyping to assess the resistance level to bacterial stalk rot in elite maize breeding lines in Indonesia. Further, this study assesses the performance of the stabbing method in inducing bacterial stalk rot infection, including variability within the susceptible and resistant check lines and variability between the two groups used in the maize breeding program.

The research aims to evaluate a phenotypic screening of 624 maize lines against bacterial stalk rot to assess the level of their resistance.

MATERIALS AND METHODS

Material

A total of 624 elite maize inbred lines were collected from major commercial maize breeding programs in Indonesia and divided into two groups. Group 1 consisted of 331 lines, and Group 2 consisted of 293 lines.

Experimental Plot

The experimental plot used a check-plot design with three replications. Each replication was planted with a plot size of 5.2 m x 70 cm with a planting distance of 20 cm, with each plot consisting of 26 plants. Every 40 entries, a resistant and susceptible line was planted as a check. A total of 624 lines were planted along with one resistant and one susceptible line as check. The experiment was performed at PT Syngenta Indonesia R&D Site located at Papar, Kediri Regency, East Java Province, Indonesia (7° 42' 25.092'' S, 112° 6' 52.614'' E) with the total size of the experimental plot was more or less 1.5 hectare (ha). This experiment was performed during the wet season beginning in January 2022. During the vegetative stage, pesticides and fungicides were applied to the trial plot to control for infection caused by pathogens other than *D. zeae*.

Inoculation with *D. zeae*

D. zeae was isolated from maize plants in the field showing symptoms of bacterial stalk rot, and inoculum was isolated and cultured to obtain a pure culture of *D. zeae*. The *D. zeae* inoculum was diluted to reach a final 1 x 10^8 CFU/ml concentration. Inoculation was performed 49 days after planting using a stabbing method modified from the inoculation method, which has been used in previous studies (Baer, 2022; Canama & Hautea, 2011). Stabbing of maize plants was performed using a hypodermic needle with an 18 gauge (18G) needle with a length of 15 mm and an outer diameter of 1.2 mm (18G x ½). The type of needle used in this study has a metal hub at its base (Fig. 1a) and is commonly used for veterinary purposes. Before injection, the needle was attached to a syringe and dipped into the inoculum with the syringe plunger pressed down to the bottom of the syringe barrel (Fig. 1a and b). A little droplet

was allowed to form at the tip of the needle (Fig. 1c). Injection was performed on the stem as near as possible to the base of the stem (Fig. 1d). The Falcon tube containing the inoculum was flipped after the inoculation of plants in every four plots to homogenize the inoculum.

Observation and Disease Incidence Level

Observation was performed 5 days after infection (DAI), 10 DAI, and 15 DAI. The observation counted the number of infected plants showing the symptoms of bacterial stalk rot infection. The bacterial stalk rot disease incidence (DI) score was calculated as follows (Equation 1):

$$Disease\ Incidence\ (\%) = \frac{Number\ of\ infected\ plants}{Number\ of\ plants\ in\ each\ plot}\ x\ 100\% \quad .....1)$$

Each line's bacterial stalk rot incidence score became the germplasm evaluation metric. The bacterial stock rot incidence score was classified into 5 resistance levels. They are R (resistance), MR (Moderate Resistance), MS (Moderate Susceptible), S (Susceptible), and HS (High Susceptible) (Table 1).

Table 1. Disease incidence score classification

Bacterial stalk rot incidence score	Resistance classification
0-10% DI (Disease Incidence)	Resistant
11-25% DI	Moderate Resistant
26 – 50% DI	Moderate Susceptible
51 – 75% DI	Susceptible
76 – 100% DI	Highly Susceptible

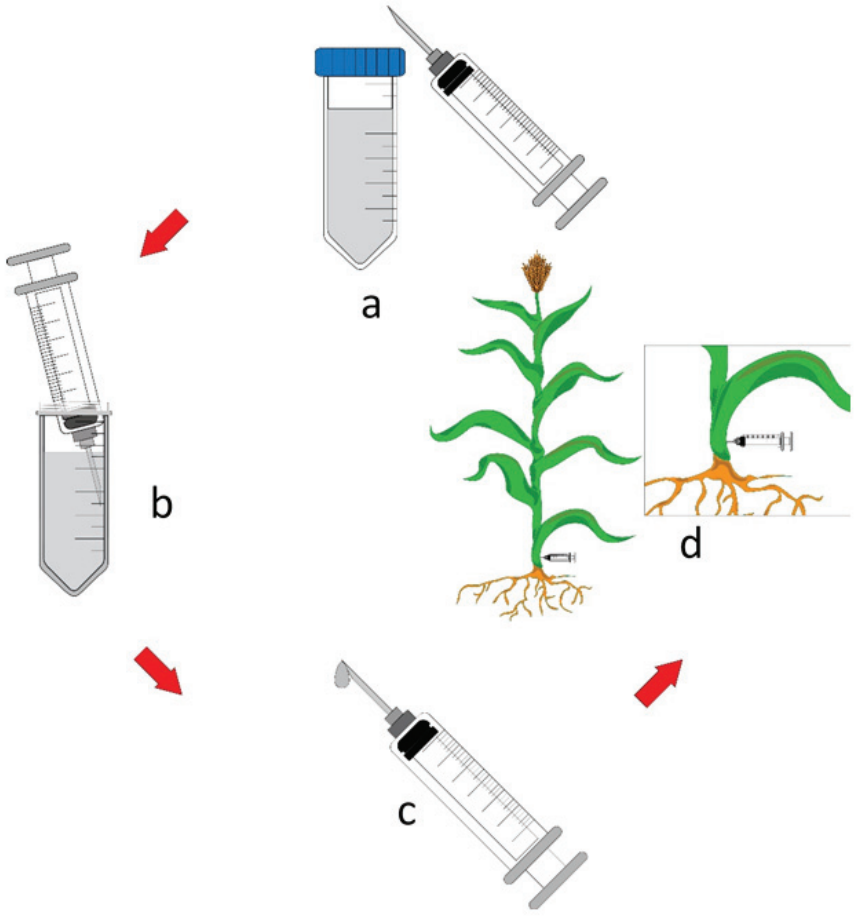


Fig. 1. Inoculation of maize plants with *D. zeae* inoculum. (a) The syringe used for inoculation with the plunger set at the lowest position and the inoculum in a 50 ml falcon tube. (b) The syringe was dipped into the falcon tube containing inoculum prior to inoculation. (c) The syringe prior to inoculation with the small amount of inoculum hanging from the tip of the needle. (d). The inoculum was delivered by stabbing the maize stem as close as possible to the base of the stem.

Data Analysis

Statistical analysis was done to determine the relationship between the evaluated maize lines ("line"), sampling time ("dai"), and interaction between sampling time ("dai") and group ("group") as independent variables with bacterial stock rot incidence score ("bsr") as a dependent variable using linear regression analyses. The regression was done using the lm function, and ANOVA analysis was done using the ANOVA function provided in the R statistical language (R Core Team, 2021). The "emmeans" package (Lenth et al., 2022) in R statistical language was used to perform a pairwise comparison between the different levels in the interaction between sampling time and pooling group.

RESULTS AND DISCUSSION

Reliability of Stabbing Method for *D. zeae* Inoculation

This study evaluates the reliability of the stabbing method to deliver *D. zeae* inoculum into maize plants and asses the resistance level of lines that were used in maize breeding programs in Indonesia. A consistency check was performed on both the stabbing method's performance and the *D. zeae* inoculum by inoculation and observation in one resistant and one susceptible line. These two lines were planted every 40 entries to better understand the response's uniformity across the entire experimental field.

Based on the observation, the resistance check line displayed fewer bacterial stalk rot infections. In contrast, the susceptible check line displayed a high number of infections. This pattern is consistent across the multiple check plots distributed across the experimental plot, as shown by the clear separation between the distribution of DI scores as early as 5 DAI (Fig. 2). Two-tailed t-tests between the resistant and susceptible produce a significant difference between the two check lines' DI score (p-value = 1.854e-05) at 5 DAI. The difference between resistant and susceptible check lines DI scores was consistent even at 10 DAI (two-tailed t-test, p-value = 2.457e-13) and 15 DAI (two-tailed t-test, p-value < 2.2e-16). In terms of the distribution of DI score from each of the replicates for resistant and susceptible check lines, the resistant check lines produced a narrower distribution of DI score. In contrast, susceptible check lines are more spread apart, with the DI score at 10 DAI displaying the largest variability compared to other DAI within the susceptible check lines (Fig. 2).

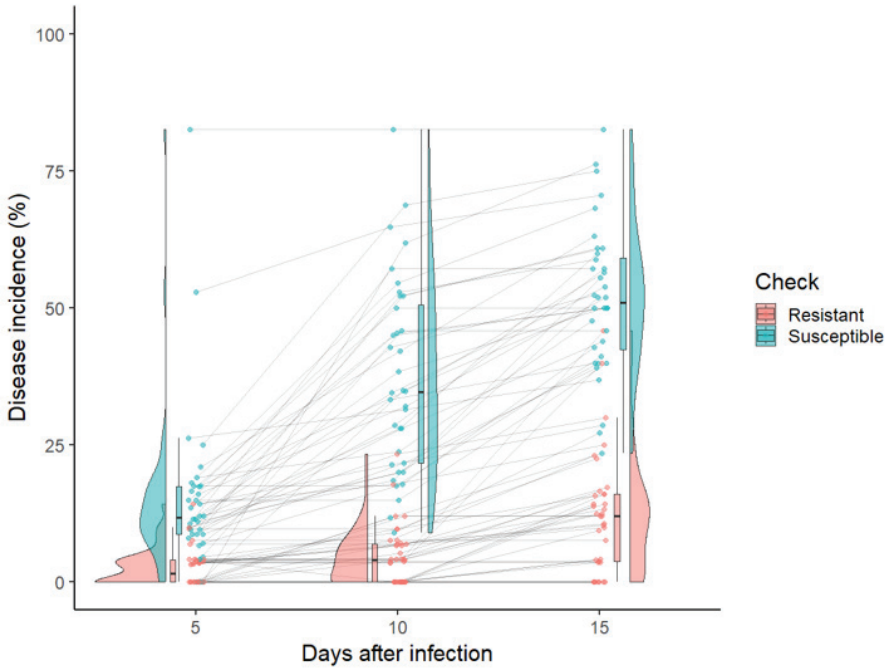


Fig. 2. Resistance and susceptible check lines consistency at 5, 10, and 15 DAI. Grey lines connect the DI score from the same observation plot across the three different DAI.

A key step in evaluating the efficacy of the stabbing method in delivering *D. zeae* for bacterial stalk rot screening is to observe the performance and stability of lines that were previously known to be susceptible or resistant as a benchmark. The susceptible line's infection status indicates that the inoculum injected into each plant is active and able to induce disease response. In contrast, the consistency of the disease progression across multiple resistant and susceptible check plots was used as an indicator of the uniformity of the field across the experiment. This data consistency is necessary because a large-scale screening experiment poses a unique challenge in maintaining variability since uniformity of the response is imperative for high-quality data.

Several common challenges in performing large-scale line evaluation are field uniformity, the number of entries to manage, the selected mode of inoculum delivery, the time needed to deliver the inoculum into the plant using the selected mode of delivery, and the amount of time needed to perform the observation. The present study addressed the abovementioned challenges when performing an assay on 624 elite maize breeding lines for resistance against *D. zeae*. Artificial inoculation in the form of syringe-mediated stabbing was used to deliver bacterial inoculum into the plant tissue. Using a syringe needle allows for targeted delivery of the inoculum that would mount a tolerance response that is easy to evaluate. The challenge of ensuring uniformity of responses is reflected in the variability of the distribution of DI scores in both the resistance and susceptible check lines across the three different DAI, which can be observed from the distribution of DI scores of resistant and susceptible lines from different replicated plots (Fig. 2).

Several methods of delivering bacterial inoculum into plant tissue have been used in other studies. Ahamad et al. (2015) reported the four most common methods used for delivering bacterial inoculum in studies of resistance against *E. chrysanthemi*/*D. zeae*: (1) hypodermic syringe inoculation, where 5 ml of 2 x 10^8 CFU/ml of bacterial inoculum was injected into the basal portion of the maize stalk, (2) root inoculation, in which excised root tips were soaked and kept in bacterial cell suspension for several hours, followed by replanting, (3) leaf whorl inoculation, where bacterial inoculum was poured in the leaf whorl, (4) scissor leaf inoculation, where the leaf was first cut with scissors and then dipped into the bacterial cell suspension.

Out of the four methods discussed in Ahamad et al. (2015), the hypodermic syringe inoculation was reported to be the most effective in inducing tolerance response and plant death, followed by the root inoculation method. A similar study on bacterial stalk rot by Suriani et al. (2023) used a similar approach, albeit with a smaller amount of inoculum, 1 ml of 1 x 10^8 CFU/ml. Another study on bacterial stalk rot in maize by Canama & Hautea (2011) used a different approach in delivering the inoculum by stabbing, but the mode of delivery was not described.

A similar study on *D. zeae* tolerance response in sorghum has been reported by Singh & Singh (2016) using four different methods: (1) leaf whorl inoculation method, (2) stem injection method, (3) root tip cut and dip method, and (4) toothpick method. Singh & Singh (2016) reported that the stem injection method performed the best in delivering bacterial inoculum.

The main goal of Ahamad et al. (2015) was to study the best method of delivering inoculum. In their study, a high amount of inoculum was delivered in each method, with approximately 5 ml of inoculum delivered with the hypodermic needle injection, resulting in 100 percent infection 6-7 days after injection. In Singh & Singh (2016), the inoculum was delivered using a hypodermic needle with the size of 21G, but no information on the volume of inoculum delivered was reported. This is different from the approach reported by Canama & Hautea (2011), where they applied the use of a stab-inoculator with no mention of the volume of inoculate injected.

This study modified the hypodermic syringe inoculation used in Ahamad et al. (2015). The amount of inoculum delivered for each plant in this study was less than the amount reported in Ahamad et al. (2015) and Suriani et al. (2023), addressing the scalability issue when performing assay at a larger scale. A needle with an 18G size with the syringe plunger is used at its lowest position (Fig. 1a). The size of the needle used in this study is larger compared to the one used by Singh & Singh (2016), which corresponds to a more sturdier needle. The syringe plunger is set to its lowest position to ensure that the volume of bacterial inoculum delivered is

exactly the amount retained inside the needle bore plus a small amount in the form of a droplet remaining at the tip of the needle and coating the needle (Fig. 1c). In comparison, Canama & Hautea (2011) used a stab-inoculator previously dipped in bacterial inoculum, suggesting that the amount of inoculum delivered was whatever amount of bacterial inoculum coating the stab-inoculator.

Given that the needle length was 15 mm, the needle would only puncture the outer skin of the stem and stem tissue but would not pass through the whole stem. Pulling the needle from the stem creates a vacuum that helps transfer all the inoculum inside the needle bore from the droplet at the tip of the needle and any inoculum on the outer wall of the needle into the stem tissue. It is found that even with the low inoculum volume used in this study compared to that of Ahamad et al. (2015) and Suriani et al. (2023), this study can induce bacterial stalk rot infection in maize as shown by the performance of the resistant and susceptible check lines (Fig. 2). It is beneficial since the low amount of inoculum needed for each injection allows us to perform the study with a higher number of plant observations.

Another important factor considered when choosing the stabbing method in the present study was its environmental safety advantage. The stabbing method was environmentally safer than drenching bacterial inoculum directly into the soil at the base of the plant. The drenching process can introduce the bacterial inoculum into the soil at a high concentration, which can then leach to adjacent fields. The leaching of bacterial stalk rot inoculum with the help of water is the main reason bacterial stalk rot infection is at its highest during the wet season. The low volume of inoculum needed, combined with the safety offered by the stabbing method with a syringe needle, became the main reason for using the stabbing method in this study.

The ability to perform large-scale phenotypic screening for disease resistance can have a lot of potential since it can be performed as part of a routine evaluation of maize lines and hybrids in all stages of the breeding program. In addition, a large-scale phenotypic screening can provide data to support future studies in the field of genomics and phenomics that involve high throughput phenotyping, genomic-wide association study, fine mapping of traits of interest, and genomic selection

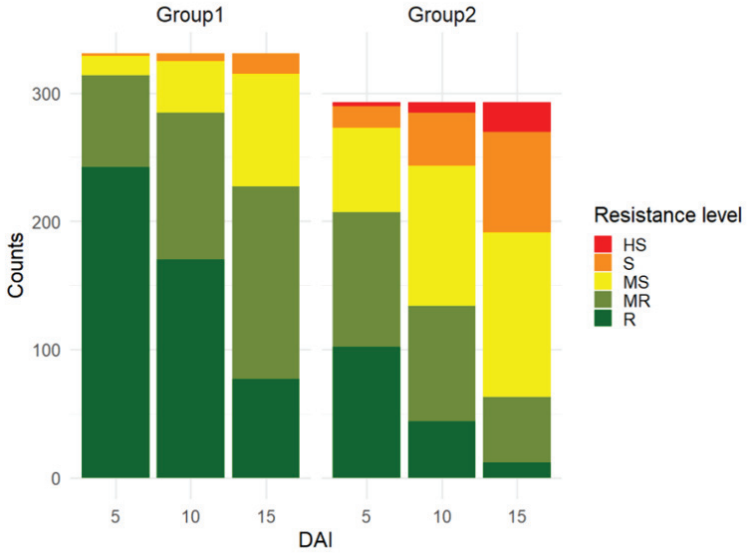
Response of Different Maize Lines Against *D. zeae* Infection

Phenotypic observation of bacterial stalk rot infection was performed at 5 DAI with follow-up observation at 10 and 15 DAI. Some early symptoms of bacterial stalk rot infection observed in this study are premature withering of leaves followed by leaf sheath discoloration (Fig. 3a). This was then followed by browning of the leaves (Fig. 3b). The infection also can cause the stalk to become twisted and toppled over (Fig. 3c). Sometimes, the first internode directly above the soil completely decayed and becomes a soft mass of disintegrated tissue (Fig. 3d). In more severe cases, the infected plant will emit a foul odor. If any of the symptoms described above were observed, then the plant was reported as infected. Data observation was terminated at 15 DAI following Shekhar & Kumar (2012).

The symptoms of infection in maize plants in *D. zeae* have been discussed before (Fig. 3) (Freije & Wise, 2016; Hooda et al., 2018; Jackson-Ziems et al., 2014; Kumar et al., 2017). One unique characteristic of bacterial stalk rot is the emission of a foul odor in decaying tissue. The progression of the disease will eventually affect the ears of plants infected with this disease. The ear will become water-soaked, turning slimy and later drying up. The plants can also produce undeveloped ears with many rotting grains and a covering of slime, or the infected ear completely rots (cob rot) and does not bear any seed. If not managed, those symptoms can lead to a considerable yield loss for the grower because infected maize plants can die at any stage, with the maturity stage having the worst impact on yield.

Based on the phenotyping for bacterial stalk rot resistance, this study can readily observe bacterial stalk rot infection in both groups at 5 DAI. Group 1 contains more lines with R and MR resistance levels at 5 DAI compared to group 2, and group 1 showed a smaller number of lines with MS and S resistance levels compared to group 2 (Fig. 4). As the observation progressed into 10 and 15 DAI, the number of lines with R and MR resistance levels decreased rapidly in group 2 compared to group 1, with more lines shifting to MS and S levels and even into HS in group 2. At 15 DAI, the level of infection ranged from R to S for group 1, while for group 2, the level of infection ranged from R to HS.

Fig. 3. Phenotypic response of maize lines against *D. zeae*, (a) premature withering and leaf sheath discoloration, (b) browning of the leaves, (c) twisted stems and toppled over plants, and (d) toppled over and uprooted plants as the results of severe *D. zeae* infection.



Remarks: R = Resistant, MR = Moderate Resistant, MS = Moderate Susceptible, S= Susceptible, HS = Highly Susceptible; DAI = days after infection

Fig. 4. Distribution of lines at each level of bacterial stalk rot resistance in group 1 and 2 at 5, 10, and 15 DAI.

Further breakdown of the number of lines in each of the different levels of resistance between the three different DAI reveals a difference in the resistance trends of lines in group 1 compared to group 2. In group 1, the number of lines displaying R resistance level at 5 DAI was 243 lines, declining to 78 lines as the infection progressed to 15 DAI. This declining trend was accompanied by an increased trend in the number of lines with lower resistance levels, such as MR, MS, and S, from 71, 15, and 2 lines to 149, 89, and 15, respectively (Table 2).

However, this is different from the observation in group 2, where the declining trends occur at a rapid rate and not only in lines displaying the R level of resistance but also at the MR level of resistance from 102 and 105 lines at 5 DAI into 12 and 51 lines at 15 DAI, respectively. The observation of group 2 was found that there is an increase in the number of lines that fall into the MS, S, and HS resistance, with MS, S, and HS levels of resistance increasing from 66, 17, and 3 at 5 DAI into 128, 79, and 23 at 15 DAI, respectively (Table 2).

Table 2. Total line number each category level of resistance in 5, 10, 15 DAI and infection trends each resistance level in 5, 10, 15 DAI.

Group	Level	Total Lines 5 DAI	Total Lines 10 DAI	Total Lines 15 DAI	Infection trends
1	R	243	171	78	
	MR	71	115	149	
	MS	15	39	89	
	S	2	6	15	
	HS	0	0	0	
2	R	102	44	12	
	MR	105	90	51	
	MS	66	109	128	
	S	17	42	79	
	HS	3	8	23	

Remarks: R = Resistant, MR = Moderate Resistant, MS = Moderate Susceptible, S= Susceptible, HS = Highly Susceptible; DAI = days after infection

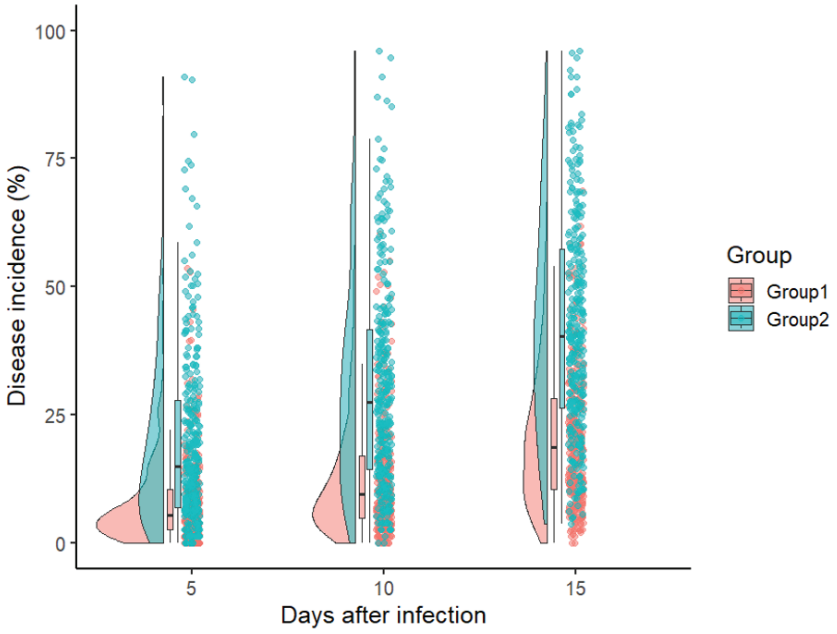


Fig. 5. Distribution of DI score of 624 maize lines divided into group 1 and 2 at 5, 10, and 15 DAI

The ANOVA analysis assessed the difference in DI score between lines, DAI, and DAI based on group status. Significant differences were found among the maize lines used in this study, among the different DAI observations, and DAI by group (p-value < 0.05) (Table 3). It includes the interaction between DAI and group to assess the response of the maize lines as a group as the infection progressed across the three different DAI. Based on ANOVA analysis, most of the variation in DI score can be attributed to the performance of lines and the progression of bacterial stalk rot disease across DAI. Although significant, the interaction between DAI and group only contributes a small fraction of the observed variation in DI score (Table 3). Pairwise comparison between different combinations of DAI and group reveals significant differences in DI scores between different DAI within and between groups (p-value < 0.05) (Table 4 and Fig. 5).

ANOVA analysis on the maize breeding lines' response to this study reported a statistically significant difference observed among genotypes, within the disease response at different DAI, and the interaction of different DAI and groups. The statistically significant difference in disease response between DAI can be explained by the disease progression in maize lines, resulting in a shift of disease response to a higher level of infection. When looking at the interaction between DAI and the group, multiple different pairwise combinations are statistically significant (Table 4). It indicates that bacterial stalk rot infection in this study progressed at a different rate between the maize lines in group 1 compared to group 2, with some of the maize lines in group 2 already showing S and HS levels of responses against *D. zeae* at 5 DAI (Fig. 4 and Table 2). In general, maize lines in group 2 have a smaller number of lines with R to MS resistance level than group 1, and this pattern is consistent across different DAI.

Table 3. ANOVA results

	df	Sum Sq	Mean Sq	F value	Pr(>F)
line	623	178.93	0.2851	19.823	< 2.2e-16 ***
dai	2	32.55	16.2751	1123.289	< 2.2e-16 ***
dai:Group	2	1.825	0.9125	62.977	< 2.2e-16 ***
Residuals	5447	78.921	0.0145		

Table 4. Pairwise contrast of DAI vs group

Contrast	Estimate	SE	df	t.ratio	p.value
Group1 dai5 - Group2 dai5	-0.1262	0.00545	5447	-23.135	<.0001
Group1 dai5 - Group1 dai10	-0.057	0.00503	5447	-11.33	<.0001
Group1 dai5 - Group2 dai10	-0.2334	0.00545	5447	-42.796	<.0001
Group1 dai5 - Group1 dai15	-0.1419	0.00503	5447	-28.218	<.0001
Group1 dai5 - Group2 dai15	-0.3532	0.00545	5447	-64.783	<.0001
Group2 dai5 - Group1 dai10	0.0692	0.00545	5447	12.687	<.0001
Group2 dai5 - Group2 dai10	-0.1072	0.00574	5447	-18.671	<.0001
Group2 dai5 - Group1 dai15	-0.0157	0.00545	5447	-2.887	0.0451
Group2 dai5 - Group2 dai15	-0.2271	0.00574	5447	-39.552	<.0001
Group1 dai10 - Group2 dai10	-0.1764	0.00545	5447	-32.348	<.0001
Group1 dai10 - Group1 dai15	-0.0849	0.00503	5447	-16.888	<.0001
Group1 dai10 - Group2 dai15	-0.2963	0.00545	5447	-54.335	<.0001
Group2 dai10 - Group1 dai15	0.0915	0.00545	5447	16.774	<.0001
Group2 dai10 - Group2 dai15	-0.1199	0.00574	5447	-20.881	<.0001
Group1 dai15 - Group2 dai15	-0.2114	0.00545	5447	-38.761	<.0001

Given that at different DAI, the disease responses are mostly statistically significant even when considering the different groups (Fig. 5 and Table 4). It is suggested that in classifying disease resistance of maize lines against *D. zeae*, the scoring should rely more on disease response taken at the latter DAI when the disease progression has reached its maximum. These data can be used in breeding programs or commercial combinations for hybrid production to create new lines or development of hybrids with resistance against bacterial stalk rot.

CONCLUSION

In conclusion, lines in group 1 showed better resistance compared to group 2. In addition, the screening method of stabbing inoculation by means of the hypodermic needle can be feasibly used for routine large-scale screening experiments. The method used in this study can be used to generate data to support future studies in the field of phenomics and genomics.

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