Proceeding Paper

Effect of Cacao Black Pod Rot Screening Method on Disease Reaction Determination †

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Abstract: Black pod rot, caused by several species of *Phytophthora*, is responsible for greater losses than any other disease affecting cacao. Breeders use various approaches to screen material for resistance to *Phytophthora* spp., however, the method used to assess disease reaction can influence outcomes. To determine how screening methods affect results, disease reactions of four cacao clones (BE 10, HY 271419, RIM 15 [MEX], and EET 236 [ECU]) were compared using incidence under field conditions, and lesion area following artificial inoculation. Disease incidence differed significantly among clones ($p < 0.0007$), ranging from 6.1% for BE 10 to 24.0% for HY 271419. Differences among clones were also detected based on lesion area ($p < 0.032$), however, their relative ranking differed: BE 10 (53.9 cm^2), HY 271419 (80.64 cm^2), RIM 15 [MEX] (95.7 cm^2), and EET 236 [ECU] (102.4 cm^2). These apparent differences observed in disease reaction among clones when comparing methods may be due to interactions with environmental conditions or differences in the pathogen species/isolates present. The improved understanding of the how screening methods used can affect the disease reaction determination and breeding outcomes in cacao germplasm will benefit breeders and farmers.

Keywords: *Theobroma cacao*; black pod; disease resistance; susceptibility; phenotyping

1. Introduction

Phytophthora species infect all tissues of *Theobroma cacao* (L.) plants and cause black pod rot (BPR), a disease responsible for the loss of up to 30% of global production [1]. BPR is responsible the greatest production losses of cacao around the world and resistance to this disease is a priority for breeding programs. BPR resistance can be quantified through disease incidence in the field or by measuring lesion size following artificial inoculation of pods [2,3], twigs, or leaves [4].

However, screening conditions impact the outcome in research trying to identify disease resistance. For example, the wounding of pods prior to inoculation affects results as this screens for post-penetration resistance only [5]. Nyadanu et al. [6] found field incidence to be strongly correlated with results from artificial inoculation on unwounded pods (r = 0.62), but only moderately correlated when pods were wounded prior to inoculation (r = 0.41). A similar analysis on germplasm from the CEPEC/CEPLAC (Centro de Pesquisas do Cacau/Comissão Executiva do Plano da Lavoura Cacaueira) Germplasm Bank in Bahia, Brazil also found a moderate correlation (r = 0.38) between field incidence and lesion area following artificial inoculation [7].

Although digital image analyses have been adopted in many host-pathogen research systems to quantify disease reaction, features such as dark red coloration of many pods,

sporulation in lesion centers, and ubiquitous cosmetic damage present on pods means time-intensive image processing step is required for implementing this technology in cacao [3]. Lesions are generally measured by hand; however, both manual and digital measurements of lesion area are very strongly correlated (r = 0.98) [3].

The collection of accurate disease reaction data is essential for the development of disease-resistant crops. The objective of this research was to determine if different methods used to screen cacao germplasm for resistance to BPR produced similar conclusions. The comparison was conducted by examining the relative disease resistance of four clones obtained using incidence under field conditions and lesion area following artificial inoculation.

2. Materials and Methods

2.1. Plant Material

Four clonal accessions (BE 10, HY 271419, RIM 15 [MEX], and EET 236 [ECU]) held at the USDA-ARS National Plant Germplasm System (NPGS) cacao germplasm collection at the Tropical Agriculture Research Station in Mayaguez, PR were evaluated using data collected on disease incidence in the field and disease severity following artificial inoculation. Each clone was represented by six trees planted in a randomized complete block design and were chosen for these experiments based on pod availability.

2.2. Field Incidence

Disease incidence was determined by recording the number of healthy and infected pods during monthly harvests from 2007–2011. To minimize the effect of productivity differences on disease incidence values, only months in which at least one tree of each clone had pods present were included in the analysis. Disease incidence per tree was calculated by dividing the number of diseased pods by the number of total pods:

$$\text{Disease Incidence} = \text{\# diseased pods}/(\text{\# healthy + diseased pods}) \qquad (1)$$

Disease incidence was compared among clones using a non-parametric analysis of variance (ANOVA) in SAS 6.2. Means were separated using post hoc nonparametric *t*-tests with a Bonferroni correction. Each evaluation date counted as a replication, and incidences for each tree were averaged to produce a single value per clone. Trees without pods were excluded from the analyses.

2.3. Detached Pod Inoculations

Unwounded pod inoculation assays were carried out on unripe, detached pods (four to five months old) using 1700 zoospores of *P. palmivora* from 10-day old cultures, with release induced using cold shock, as described in Ali et al. [8]. Inoculated pods were placed in Ziplock bags to prevent desiccation, and kept at 25 °C in the dark (Figure 1a).

All inoculations were carried out with a *P. palmivora* isolate H33, which was obtained from a stem canker on cacao in Hawaii [9], and was shown to be an aggressive isolate in preliminary experiments (Puig, unpublished).

After seven days, two lesion diameters were measured per pod and converted to radii by dividing by two. Lesion area was calculated as:

$$\text{Lesion Area} = \pi \times (r_1) \times (r_2) \qquad (2)$$

where r_1 and r_2 are the largest and smallest radii of the lesion, respectively (Figure 1b). Lesion area was compared among clones using an ANOVA and means were separated using Tukey–Kramer Comparison lines for the least squares means. The assay was carried out as a completely randomized design with 11 to 13 replications (pods) per clone.

(**a**)   (**b**)

Figure 1. Detached pod inoculations. (**a**) Necrotic lesion on pod of HY 271419 seven days after inoculation with zoospores of *Phytophthora palmivora*. (**b**) Lesion area was calculated using the formula: $\pi \times r_1 \times r_2$.

3. Results

Disease reaction, measured as incidence of black pod rot under field conditions, differed significantly among clones ($p < 0.0007$; DF = 3; X2 = 16.9), ranging from 6.1% for BE 10 to 24.0% for HY 271419 (Table 1). RIM 15 [MEX] and EET 236 [ECU] were moderately susceptible at 10.3% and 21.9% incidence, respectively. Although nearly a quarter of pods produced by HY 271419 became diseased (24%), this was not statistically different from RIM 15 [MEX] and EET 236 [ECU].

Table 1. Disease incidence of black pod rot (*Phytophthora* spp.) in four cacao (*Theobroma cacao* L.) germplasm clones under natural field conditions in Puerto Rico.

Clone	N	Mean [1,2]	Std Error	
BE 10	43	0.061	0.017	A
RIM 15 [MEX]	43	0.103	0.022	B
EET 236 [ECU]	43	0.219	0.041	C
HY 271419	43	0.240	0.047	BC

[1] Values multiplied by 100 represent the average percentage of infected pods present at each evaluation date.
[2] Clones sharing the same letters in the last column are not significantly different ($p \leq 0.05$).

Differences in disease reaction among clones were also detected based on lesion area ($p < 0.032$; numDF = 3; denDF = 25.1; F = 3.43). However, their relative ranking differed from results for incidence because HY 271419 was not as susceptible (Table 2). Lesion area ranged from 53.9 cm^2 for BE 10 to 102.4 cm^2 for EET 236 [ECU], with the only statistically significant difference in means detected between these two. All pods of EET 236 [ECU] developed lesions after inoculation. In contrast, only 13 of 15 (86.7%) of the RIM 15 [MEX], 10 of 13 (76.9%) of the HY 271419, and 8 of 11 (72.7%) of the BE 10 pods developed lesions.

Table 2. Lesion area (cm^2) on cacao (*Theobroma cacao* L.) pods 7 days post-inoculation with zoospores of *Phytophthora palmivora*.

Clone	N	Mean [1]	Std Error	
BE 10	11	52.9	42.9	A
HY 271419	13	80.6	55.2	AB
RIM 15 [MEX]	15	95.7	49.4	AB
EET 236 [ECU]	14	102.4	35.2	B

[1] Clones sharing the same letters in the last column are not significantly different ($p \leq 0.05$).

4. Discussion

High-quality disease reaction data are needed by breeding programs to develop durable, resistant cultivars. This study examined relative disease resistance of four clones using two assessment approaches: field incidence and quantification of lesion area following artificial inoculation. The artificial pod inoculation protocol described here was designed to incorporate pre- and post-penetration resistance by using a much lower number of zoospores than is used by other researchers [8,10]. Inoculum concentrations that result in 100% infection are less likely to reflect pre-penetration resistance.

The change in disease resistance ranking of clones when assessed using artificial inoculation instead of field evaluations was caused by the shift of HY 271419 from most susceptible (based on field incidence) to second most resistant clones when using data from artificial inoculation. This could be caused by HY 271419 having resistance to colonization, which is not reflected in field incidence data, as it does not take lesion size into account. Similar results were observed by Lawrence [11], were disease reactions of clones obtained in detached pod assays were not consistent with the reactions of the same clones to natural infection in the field.

Alternatively, the differences observed here may be due to pathogen-specific responses. Although *P. palmivora* is the only species found causing BPR in Puerto Rico so far [12,13], the isolates present in the field may differ from the Hawaiian isolate used in this study. Some host resistance is not durable when challenged with diverse isolates of the same species.

In addition, several fungi in the *Diaporthe*, *Lasiodiplodia*, and *Colletotrichum* genera have been found to cause necrotic lesions indistinguishable from those caused by *Phytophthora* spp. [13,14]. Disease incidence data collected in the field likely includes pods affected by various pathogens that cause similar symptoms. Although this information is important for farmers, the presence of multiple, unrelated pathogen may complicate genomic studies and make it difficult to accurately identify pathogen-specific resistance markers in germplasm.

5. Conclusions

This study shows that the assay used to screen for disease reaction can affect which clones are classified as resistant or susceptible. For best results, initial screening using less labor-intensive methods, such as evaluating disease incidence in the field, or artificial inoculation of leaf discs, should be followed by artificial pod inoculations of clones of interest. Thorough characterization of cacao germplasm will enable the identification of new sources of disease resistance and more effective, long-term disease control.

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