

***In vitro* assessment of fusarium wilt disease expression in banana population: a fast-track approach for resistance screening**

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ABSTRACT

Fusarium wilt (TR4) threatens global banana production, creating an urgent need for efficient resistance screening methods. This study aimed at standardising an efficient *in vitro* bioassay for the early selection of Foc TR4-resistant banana populations. An *in vitro* bioassay using a conidial concentration of 10^6 conidia mL⁻¹ for 100 F₁ progenies from a cross between a susceptible wild *Musa acuminata* (WB3) and a tolerant local cultivar, Pisang Jari Buaya (PJB). The disease symptoms were calculated using the Disease Severity Index (DSI) and progenies were statistically categorised into resistance categories according to previously developed criteria. The standardised *in vitro* bioassay was highly reproducible and effectively induced the full range of disease symptoms. Screening the F₁ population showed a continuous distribution of DSI values, with a mean of 3.58 and a range of 1.7 to 6.0 which indicates quantitative inheritance. Notably, the population showed transgressive segregation, with 23% of the population classified as Highly Resistant (HR) or Resistant (R). However, analysis of variance revealed significant environmental variation ($P < 0.001$) and a non-significant genotypic effect, resulting in a low heritability estimate. This dominance of environmental noise indicates that phenotypic selection in *in vitro* conditions requires high replication to improve accuracy. Despite this challenge, the identification of transgressive segregants highlights the genetic potential of this germplasm. These findings validate the bioassay as a high-throughput screening tool but suggest that future breeding efforts must integrate marker-assisted selection (MAS) to overcome environmental variance and precisely capture resistance alleles.

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1. INTRODUCTION

Banana (*Musa spp.*) is an important staple food crop for millions of people in tropical and subtropical regions (Gonçalves et al., 2021; Khiabani, 2022). Its production is estimated at 139 million tonnes (Mt) and the producers of this crop are mainly found in three continents: Asia, Africa and Latin America. India is the largest producer with 37.6 million tonnes (FAOSTAT, 2024).

Global production is largely dominated by the Cavendish subgroup which accounts for about half of the total global production and supports global trade (FAO, 2019a). This production is severely threatened by Fusarium wilt caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4). This pathogen overcomes the resistance of Cavendish cultivars and many local varieties, jeopardising a significant portion of the global banana supply (Cheng et al., 2019). Foc TR4 is a highly virulent, clonal lineage whose pathogenicity is facilitated by a

complex repertoire of virulence factors including accessory chromosomes and effector genes that enable host colonisation and defence evasion (Czislowski et al., 2018; Yang et al., 2020). A key aspect of its management challenge is the pathogen ability to produce long-lived chlamydospores that persist in soil for decades, making infected fields unfavourable for banana cultivation (Dita et al., 2010).

Current control measures including chemical, biological and cultural practices are largely ineffective and unsustainable for managing Foc TR4 in the long term (Dita et al., 2018). The development and introduction of genetically resistant varieties is considered the only viable long-term solution. However, conventional banana breeding faces a significant hurdle due to the crop's long generation time, polyploidy and high sterility especially among elite edible cultivars (Wu and Yi, 2022). These biological constraints make the introgression of resistance traits from wild relatives a slow and difficult process.

One of the major bottlenecks in resistance breeding is the phenotyping of breeding populations (Fiorani and Schurr, 2013; Chawade et al., 2019). Conventional field-based screening for Foc TR4 resistance, while ideal, is time consuming, resource-intensive and confounded by environmental variability, creating a significant constraint for breeding programmes (Noordin et al., 2022).

To mitigate the constraint of field screening, several early bioassays have been developed, including pot-based and hydroponic systems under controlled conditions (Khiabani, 2022; Wu and Yi, 2022). *In vitro* bioassays are considered a promising alternative, providing better advantages in terms of speed, space efficiency and control. Studies by Wu et al. (2010) and subsequent modifications (Wu and Yi, 2022) have shown the utility of *in vitro* systems for phenotyping established banana genotypes. However, their application has primarily focused on evaluating existing cultivars rather than enabling genetic studies in breeding populations.

Furthermore, while modern breeding strategies including Marker-Assisted Selection (MAS) hold great promise, their implementation is hampered by the limited availability of reliable and fast high-throughput phenotyping platforms such as standardised *in vitro* bioassay that are required to validate molecular markers linked to Foc TR4 resistance (García-Bastidas et al., 2019).

This study addresses the critical gap between the need for efficient genetic improvement and the lack of suitable high-throughput phenotyping tools for genetic analysis. We introduce a novel application of a standardised *in vitro* bioassay, not merely for routine genotype screening but specifically as a high-throughput phenotyping platform for a structured pre-breeding programme. While the assay methodology builds upon established principles (Wu and Yi, 2022), its innovative application lies in the large-scale screening of a segregating mapping population. This approach enables for the first time in this specific genetic context the quantitative analysis of inheritance patterns and the characterisation of Foc TR4 resistance thereby directly bridging phenotyping and genetic mapping.

The specific objectives of this study were: 1. To establish a reproducible *in vitro* screening technique for the early detection of Foc TR4 resistance in banana; 2. To assess the phenotypic disease response and categorise the resistance levels of F_1 progenies derived from a cross between a susceptible wild *Musa acuminata* and a tolerant cultivar, Pisang Jari Buaya; 3. To evaluate the population genetic variation and estimate the heritability of Foc TR4 resistance.

2. MATERIALS AND METHODS

2.1. Establishment of F_1 population for Foc TR4 resistance

The susceptible wild diploid banana relative *Musa acuminata* spp. *malaccensis* (WB3) and tolerant local cultivar (PJB) were crossed to generate an F_1 mapping population of 100 progenies for the study of resistance to *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4). Four replicates of Pisang Berangan were included as controls in the experimental block. Controlled hand pollinations were conducted as described by Ortiz and Vuylsteke (1994). To prevent non-target pollination, the female inflorescence of WB3 was covered with a cotton bag one day prior to bract opening to avoid contamination with pollen from unknown sources. The male buds from PJB were also bagged to ensure that a bract with fresh pollen was available for pollination the following day. Pollinations were performed early in the morning between 7:00 and 7:30 h using freshly dehiscent male flowers (pollen) of PJB and immediately applied onto the receptive stigmas of the female flowers. The pollinated inflorescence was re-bagged and maintained until fruit set to prevent any external pollen contamination.

Mature fruits were harvested 100–120 days after pollination and stored at room temperature (25–30°C). Seeds were separated from the pulp by washing in tap water and subsequently soaked in a beaker of water for 30 minutes. Since the majority of floating seeds typically lacked an embryo or endosperm, only sunken seeds were utilized.

The population was developed through embryo culture followed by micropropagation for multiplication of each seed progeny (Indrayanti et al., 2019). Seeds were surface sterilised by quick rinse with 70% ethanol for 60 seconds. Embryos were excised using a scalpel blade and cultured on basal Murashige and Skoog (MS) media. The cultures were maintained in the dark to promote germination prior to being exposed to 16 hours of light to induce shoot formation. To promote the development of multiple shoot formation, root and leaf tissues, the explants were pruned and transferred to fresh basal MS media supplemented with coconut water and 2mg l^{-1} 6-Benzylaminopurine (BAP). The explants were subcultured into rooting media composed of basal MS media and activated charcoal. Through this process, a mapping population appropriate for subsequent *in vitro* screening tests was developed.

To mitigate contamination, all procedures were performed under aseptic conditions in a laminar flow hood. Cultures were monitored weekly, and any contaminated plantlets were recorded and excluded from the final dataset. Data from these contaminated units were treated as missing values in the statistical analysis.

2.2. Plant material preparation

For *in vitro* inoculation, rooted plantlets with a height of 3.5 - 4.0 cm were selected and their roots were trimmed to approximately 0.5 cm (Fig. 1a). The plantlets were then transferred into glass jars containing half-strength MS media excluding sucrose, with one plantlet per jar (Fig. 1b). The cultures were maintained at $25 \pm 2^\circ\text{C}$ under a photoperiod of 12 to 16 hours, provided by cool white fluorescent lamps at an intensity of $50\text{--}65 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Yorgancilar et al., 2015; Ndayihanzamaso et al., 2022). After a period of 1 to 2 weeks of growth, plantlets that had reached a pseudostem height of about 4.5 to 5.0 cm, developed 2-3 fully expanded leaves and produced at least 3 - 4 white roots were selected for *in vitro* inoculation (Fig. 2) (Wu et al., 2010).



Figure 1a: Suitable plantlets for disease assessment *in vitro*



Figure 1b: Healthy plantlets in half MS media ready for inoculation against Foc TR4



Figure 2: One to two month plantlets with 4.5-5cm height, fully expanded leaves and three to four healthy roots

2.3. Preparation of Foc TR4 inoculum for *in vitro* screening

The Foc TR4 isolate used in this study was obtained from the Institute of Biological Sciences Laboratory, Universiti Malaya, Malaysia. Inoculum preparation was conducted in a biosafety cabinet, and all equipment was autoclaved to ensure sterility. Prior to use, cheesecloth was cut into 8 cm and placed in a glass Petri dish.

Under aseptic conditions, a piece of Foc TR4 VCG (0121) inoculum was excised from the active growing margin of a 4-5-day old colony on potato dextrose agar (PDA) and transferred into a glass jar containing Armstrong FOC sporulation media, which was then sealed with plastic wrap. This culture was incubated at 25°C with continuous shaking at 150 rpm for 7 days to promote fungal growth and sporulation (Table 1).

Immediately after the incubation, the sporulation medium was carefully filtered through four layers of cheesecloth to separate the fungal spores from the mycelia. The filtrate was collected in a sterile beaker and the spore concentration was quantified using a haemocytometer. The resulting spore suspension was at 4°C until needed for inoculation. Based on biosafety regulations, the beaker, funnel, and cheesecloth were autoclaved immediately after use.

Table 1: Armstrong sporulation media composition

Media Composition	Quantity (g/l)
Sucrose	20
MgSO ₄	0.4
KCl	1.6
KH ₂ PO ₄	1.1
Ca(NO ₃) ₂	5.9
FeCl ₂	0.01
MnSO ₄	0.01
ZnSO ₄	0.01

2.4. Foc TR4 disease challenge assessment via *In vitro* inoculation

During the inoculation in the biosafety cabinet, the concentration of conidia was adjusted to 10^6 conidia ml^{-1} using sterile distilled water. This concentration was selected based on established protocols (Wu et al. 2010; Wu and Yi, 2022). Sterile filter paper discs measuring 5 mm were prepared to serve a dual purpose: providing an essential carbon source (since the MIS medium lacks sucrose) and acting as a physical substrate to standardise spore-to-root contact.

The filter paper discs were soaked in the spore suspension (10^6 conidia ml^{-1}) for 10-20 minutes. Inoculation was conducted on each plantlet by placing one filter paper disc onto the surface of the half-strength MIS medium. The plant cultures were incubated at 25°C for 24 days under a photoperiod providing $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 12 to 16 hours

(Figure 3). At 24 days, disease severity was assessed using the 6-point scale described by Wu et al. (2022): 1 - no discolouration of the pseudostem, 2 - discolouration in $\leq 1/2$ the height of the pseudostem, 3 - discolouration in $\geq 1/2$ the height of the pseudostem and/or the leaf stalk, 4 - $\leq 50\%$ of the leaves wilting or yellowing, 5 - $> 50\%$ of the leaves wilting or yellowing, and 6 - the entire plantlet wilting.

Disease severity index score was graded *in vitro* (Table 2). The Disease Severity Index (DSI) for each progeny was calculated as the arithmetic mean of scores across replicates:

$$DSI = \frac{\sum Score_i}{n}$$

where $Score_i$ is the rating for an individual replicate and n is the number of replicates.

The resistance level of each F_1 progeny was classified according to the statistical probability criteria established by Wu and Yi (2022). Unlike conventional methods based solely on mean DSI, this classification was based on the frequency distribution of severity scores across replicates. For each progeny, the probability $P(y)$ of observing a specific disease severity score y (1 - 6) was calculated as:

$$P(y) = \frac{n_y}{N}$$

where n_y is the number of replicates assigned the score and N is the total number of replicates per progeny ($N = 4$). This calculation generated a probability distribution for each progeny ($P(y=1)$, $P(y=2)$, ..., $P(y=6)$). This probability distribution was evaluated against the logical conditions outlined in Table 2 to assign the progenies into one of five resistance categories: Highly Resistant (HR), Resistant (R), Moderately Resistant (MR), Susceptible (S), or Highly Susceptible (HS).



Figure 3: Visual disease symptoms of banana plantlets screened against Foc TR4 in the *in vitro*

Table 2: Standards for classifying resistance against *Fusarium oxysporum* f. sp. *cubense* (Wu and Yi, 2022)

Symptom rating probability distribution	Level of resistance to <i>Fusarium</i> wilt
$P(y = 2) \geq 50\%$, $P(y = 1) \leq 50\%$	HR
$P(y = 2) \geq 50\%$, $P(y = 3) \leq 50\%$	R
$P(y = 3) \geq 50\%$, $P(y = 2) < 50\%$ or $P(y = 4) \leq 50\%$	MR
$P(y = 4) \geq 50\%$, $P(y = 3) < 50\%$ or $P(y = 5) \leq 50\%$	S
$P(y = 5) \geq 50\%$, $P(y = 4) < 50\%$ or $P(y = 6) \leq 50\%$	HS

*HR = Highly resistance, R = Resistance, MR = Moderately resistance, S = Susceptible, HS = Highly susceptible

2.5. Experimental design and data analysis

The experiment was subjected to a randomized complete block design (RBCD) with four replicates per progeny (100 progenies x 4 replicates) including Pisang Berangan as the control. Due to the large population size, the blocking factor was defined as the temporal experimental run, where each replication represented a distinct execution batch performed sequentially. This design was employed to control for systematic variation inherent in laboratory experimental screening such as fluctuations in inoculum preparation, media batches and technical handling across days.

Data were analysed using a two-way Analysis of Variance (ANOVA) in R (v4.5.1). The assumptions of ANOVA were checked by visual inspection of residual plots. Broad-sense heritability (H^2) was estimated based on the ratio of genotypic to phenotypic variance: $H^2 = \sigma_g^2 / \sigma_p^2$ (Falconer and Mackay, 1996). Variance components were computed using the expected mean squares method (Keber et al., 2006) as follows:

$$\sigma_g^2 = \frac{MS_g - MS_e}{r}$$

$$\sigma_p^2 = \sigma_g^2 + \sigma_e^2$$

$$\sigma_e^2 = MS_e$$

Where σ_g^2 , σ_p^2 and σ_e^2 are the genotypic, phenotypic, and error variances, respectively; MS_g genotypic mean square, MS_e = environmental variance (error mean square) and r = the number of replications.

Since the mapping population consists of clonally propagated F_1 progenies, broad-sense heritability (H^2) was estimated to capture the total genetic variance (additive, dominance, and epistatic variance) transferable to the clones. The genetic variance (σ_g^2) was derived by partitioning the Mean Squares from the ANOVA, where the variation among clonal replicates within genotypes provided the estimate of environmental error (σ_e^2) (Bernardo, 2020).

3. RESULT AND DISCUSSION

3.1 *In vitro* bioassay standardisation

A standard inoculum concentration of 10^6 conidia mL^{-1} previously established by (Wu et al., 2010), was used to challenge the plantlets. This high infection pressure successfully produced the full range of disease reactions including both susceptible and resistant phenotypes, enabling clear differentiation between the progenies. The resistant and susceptible phenotypes were statistically categorised as described previously by Wu and Yi, (2022), validating the functionality of the bioassay. The parental lines exhibited moderate variation, with a DSI mean of 4.5 and Coefficient of Variation (CV) of 11.1% for the susceptible parent WB3 (*M. acuminata* ssp. *malaccensis*) and DSI mean of 2.67, CV of 17.7% for the tolerant parent PJB across four replicates (Table 3). This confirms that the protocol demonstrates consistent symptom induction and provides quantitative measures of precision for the early identification of Foc TR4 resistance.

A critical factor of this standardisation was the specific control of the infection interface. To ensure a controlled infection process, the bioassay was conducted on half-strength MIS medium without sucrose. As described by Couteaudier and Alabouvette (1990), carbon is a limiting substrate for Foc growth. Therefore, the sterile filter paper disc served a dual function: it acted as a substrate and as a restricted carbon source to support initial fungal colonisation. This carbon-limiting environment prevented excessive overgrowth of the pathogen in the medium and directed the mycelial colonisation toward the plantlet roots. By creating a localised point of nutrient availability, the protocol ensured that disease symptoms resulted from active root infection rather than environmental suffocation, thereby standardising the infection pressure.

This standardised protocol represents a significant methodological advancement. It offers a rapid, cost-effective, and space-efficient alternative to time-consuming, labour-intensive, and unpredictable field trials due to environmental variation, making it suitable for the high-throughput screening required in modern breeding programmes. Our results present an effective method to phenotype segregating breeding populations, which corroborates with the study of Wu et al., (2010). One of the advantages is that the method produces disease-free, genetically uniform plantlets (Justine et al., 2022), overcoming the limitation associated with conventional sucker multiplication, which can facilitate the spread of soil-borne diseases such as Foc TR4 (Chukwu et al., 2025). Our bioassay offers a controlled environment, reducing the environmental variation that shows bias in field-based screening, ensuring the observed phenotypic variation is more accurate reflection of the plant response among progenies.

Table 3: Descriptive statistics of DSI for the F_1 population and parental lines

Genotype	Mean (DSI $\pm$ SE)	Standard Deviation (SD)	Range (Min -Max)	Coefficient of Variation (%)
F_1 population	3.58 $\pm$ 0.08	0.96	1.7-6	23.20%
WB3 (P)	4.5 $\pm$ 0.27			11.1%
PJB (P)	2.67 $\pm$ 0.27			17.7%

*SE: Standard error; F_1 : First Filial generation, WB: Wild Banana, PJB: Pisang Jari Buaya, P: Parent, DSI: Disease Severity Index

3.2 Phenotypic variation in the F_1 mapping population

The F_1 progenies derived from the cross between the susceptible parent (WB3) and the tolerant parent (PJB) showed significant phenotypic variation in their disease expression response to Foc TR4. The population mean Disease Severity Index (DSI) was 3.58, intermediate between the parental means (WB3: 4.5 ± 0.35 ; PJB: 2.67 ± 0.28), with a continuous distribution ranging from 1.7 to 6.0 (Table 3; Fig. 4). This continuous variation is a hallmark of quantitative inheritance, suggesting that resistance to Foc TR4 in this population is controlled by multiple genes. This provides critical insight for breeding programmes, indicating that stacking several resistance alleles will be necessary to develop durable resistance (Rocha et al., 2021).

Furthermore, the population exhibited transgressive segregation, with progenies exhibiting phenotypes more extreme than either the susceptible or tolerant parent which corroborates with studies by Buregyeya et al. (2021) and Nirubana et al. (2021). The significant coefficient of variation (CV = 23.20%, SD = 0.96) reflects phenotypic dispersion within the population, consistent with expectations for traits governed by multiple minor effect alleles (Adedugba et al., 2023; Begna and Teressa 2024).

This suggests that favourable resistance alleles from PJB and WB3 have been recombined in novel combinations in the offspring. The identification of transgressive segregants, specifically those surpassing the resistance donor parent validates the genetic potential within this germplasm. This phenomenon highlights the genetic potential that is visible in the parental lines and indicates that sexual recombination plays a significant role in the discovery of potential novel combinations of genes, which could form the basis for mapping studies (Epstein et al., 2023).

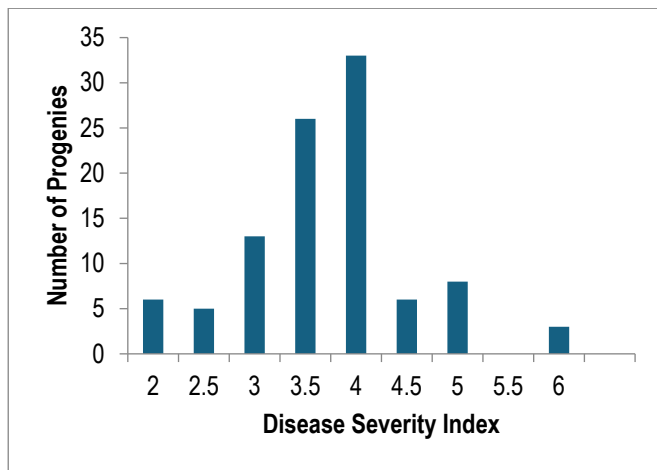


Figure 4: Frequency distribution of Disease Severity Index (DSI) in the F_1 population

3.3 F_1 population disease resistance classification for identification of promising breeding candidates

The disease response of the F_1 progenies to Foc TR4 infection was categorised based on disease severity scores, revealing a distinct resistance spectrum. The mean Disease Severity Index (DSI) showed a significant decreasing trend from the Highly susceptible (HS) to the Highly resistant (HR) category. The reliability of this classification was statistically confirmed by Tukey's HSD test, which showed significant differences ($P < 0.05$) in mean DSI among all five resistance categories (Fig. 5). Based on the statistical probability criteria used in this study, the population categorisation was: 7.6% Highly Resistant (HR), 15.2% Resistant (R), 60.9% Moderately Resistant (MR), 13.0% Susceptible (S), and 3.3% Highly Susceptible (Fig. 6). The majority of the population (83.7%) exhibited some level of resistance (HR, R and MR). The disease expression of progenies in the Highly Resistant (HR) and Resistant (R) classes provides evidence of the successful transfer of resistance alleles from the parental materials to the breeding population.

The segregation of the 100 progenies across five grading classes provides further evidence for the quantitative inheritance of Foc TR4 resistance, suggesting that the trait is influenced by multiple genes. This finding aligns with genetics studies on Fusarium resistance in bananas and other crops. For instance, resistance of Foc Race 1 resistance in bananas has been reported to be controlled by a minimum of two dominant genes with epistatic interaction (Arinaitwe et al., 2019). However, the continuous variation observed in our study implies a polygenic control, where the cumulative effect of several genes each with a minimal contribution determines the final phenotypic expression. This difference may be attributed to the specific virulence of Foc TR4 or the distinct genetic background of the wild diploid parent used in our study. Similarly, there is a study by Xu et al., (2023) on wheat

resistance to Fusarium head blight specified multiple quantitative trait loci (QTL) controlling the complex trait. This is crucial, as it suggests that breeding for durable resistance will require the combination of multiple favourable alleles.

The identification of 22.8% of the population in the HR and R categories highlights these progenies as the most valuable candidates for downstream breeding and genetic analysis. These progenies are of critical importance for two main reasons, i). They represent candidates for accelerated advancement to field trials to assess their tolerance under natural infection pressure and performance over time, ii). The distinct phenotypic segregation among HR, R, S and HS progenies makes this population ideal for genetic studies. These populations can be employed in linkage or QTL mapping to identify molecular markers associated with resistance genes (Arinaitwe et al, 2019; Ma et al.,2022).

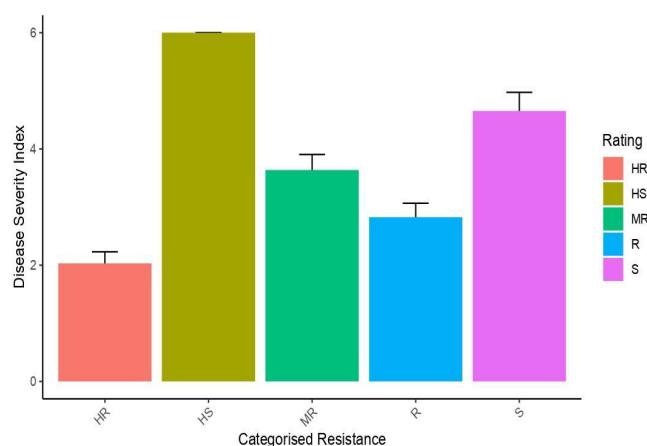


Figure 5: Bar chart showing the distribution of progenies across resistance classes

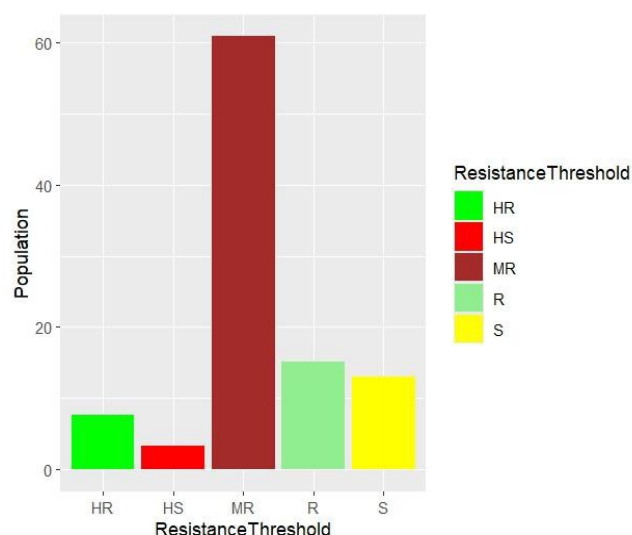


Figure 6: Statistical classification identifies promising breeding candidates

3.4 Analysis of variance and heritability of Foc TR4 resistance

The disease response of banana progenies to Foc TR4 infection was analysed via Analysis of Variance (ANOVA)

to partition the observed phenotypic variation into its components. The ANOVA showed a highly significant effect of replication, indicating large environmental variation across the experiment (Table 4). This finding highlights the importance of the blocked experimental design in controlling for micro-environmental fluctuations inherent in high-throughput *in vitro* systems which aligns with Festing, (2014) study.

In contrast, the genotypic effect (progenies) was not statistically significant, suggesting that the genetic variance was small compared to the environmental variance under these experimental assays. Consequently, the estimate of broad-sense heritability (H^2) was low indicating that the phenotype is heavily influenced by non-genetic factors. This predominance of environmental variation highlights the sensitivity of the *in vitro* bioassay to experimental conditions, a challenge that is consistent with the findings of Thangavelu et al. (2025), who reported that resistance ratings were highly unstable when plants are subjected to variable screening environments.

Despite the low heritability estimate, the wide phenotypic distribution (Section 3.2) suggests that genetic potential exists but is difficult to quantify at the individual progeny level due to the high residual error. This observation has a critical methodological implication consistent with the low heritability findings by Arinaitwe et al. (2019) which is that phenotypic selection for this trait requires high replication to overcome environmental noise to achieve better accuracy. Collectively, these results highlight the complexity of phenotyping quantitative traits under *in vitro* conditions and suggest that molecular markers (MAS) which are unaffected by environmental variance will be essential for more precise selection in this population.

Table 4: Analysis of variance for the Disease Severity Index (DSI) of banana progenies screened for Foc TR4 resistance

Source of variation	Degree of freedom	Mean Squares
Replication	3	23.39***
Progenies	100	1.48 ^{ns}
Residual	241	1.39

*ns = non-significant at 0.05 probability level, *** = Significant at 0.001 probability level

4. CONCLUSION

This study successfully standardised an *in vitro* bioassay using a 10^6 conidia mL^{-1} inoculum for high-throughput phenotyping of Foc TR4 resistance in banana. The protocol proved reproducible and successfully identified 22.8% of the screened population as Resistant and Highly Resistant. By applying this assay to a population from controlled parental lines, we bridge a key methodological gap by enabling rapid, controlled phenotyping of breeding populations for genetic studies. The population displayed a continuous range of disease severity with significant

transgressive segregation, confirming that Foc TR4 resistance is a quantitative trait governed by multiple genes. Methodologically, the analysis of variance revealed significant block effects, demonstrating that high replication is essential to overcome environmental noise in *in vitro* phenotyping.

While this bioassay serves as a powerful initial screen, further steps are required to validate these findings. Although the observed transgressive segregation strongly supports the successful hybridization of the parental lines, molecular markers should be employed to confirm the genetic fidelity of the individual segregants. Additionally, the resistant candidates identified in this study should be advanced to field trials to assess the durability of their resistance under natural infection conditions.

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