INNSPUB

Journal of Biodiversity and Environmental Sciences | JBES
ISSN: 2220-6663 (Print); 2222-3045 (Online)
Website: https://www.innspub.net
E-mail contact: info@innspub.net
Vol. 28, Issue: 6, p. 141-149, 2026

RESEARCH PAPER OPEN ACCESS

In vitro assessment of Bambara groundnut M3 mutant genotypes for resistance to *Macrophomina phaseolina* (Tassi) Goid. in the seedling stage in Burkina Faso

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Key words: Bambara groundnut, Mutants, *M. phaseolina*, Resistance, Burkina Faso

Received: 26 May, 2026 **Accepted:** 10 June, 2026 **Published:** 15 June, 2026

DOI: https://dx.doi.org/10.12692/jbes/28.6.141-149

ABSTRACT

Bambara groundnut is one of the food legumes with significant nutritional potential. However, this crop is susceptible to numerous fungal diseases, particularly seedling blight caused by *Macrophomina phaseolina*. The objective of this study was to identify resistant genotypes to *M. phaseolina*. Thus, nineteen Bambara groundnut genotypes, including fifteen M3 mutants, were evaluated *in vitro* for their resistance to IS-246, an isolate of *M. phaseolina* that proved virulent in pathogenicity tests. Each genotype was sown into 10 glass dishes (500 mL) containing PDA culture medium, of which five dishes were inoculated with *M. phaseolina,* and five were left uninoculated as controls. The results showed that the germination rate ranged from 0% to 80%, with an average of 23.5% among the inoculated genotypes. In contrast, the germination rate of uninoculated genotypes ranged from 50% to 100%, with an average of 73.5%. The severity index ranged from 1.33 to 5 based on a scale of 0 to 5. The means separation test classified the genotypes into three groups: moderately resistant genotypes with severity indices between 1 and 2, the susceptible genotypes with severity scores between 3 and 4, and the highly susceptible genotypes with severity indices between 4 and 5. The genotypes KVS115_M3S12, KVS115_M3S6, KVS115_M3S5, and KVS259_M3S8, which showed moderate resistance, constitute a potential source of improvement for a Bambara groundnut breeding program in Burkina Faso.

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INTRODUCTION

Bambara groundnut is a high-calorie legume rich in plant protein that helps to fight hunger in sub-Saharan Africa. Its seeds offer a remarkable nutritional value, being rich in protein (18–24%), minerals, fats, fiber, carbohydrates, and energy (Mubaiwa *et al.,* 2017). In Burkina Faso, its production involved women (Ouoba *et al.,* 2016). From an agronomic perspective, Bambara groundnut improves soil fertility through symbiotic fixation of atmospheric nitrogen with rhizobia. However, biotic constraints such as *Macrophomina phaseolina* are factors limiting its productivity. It is a cosmopolitan pathogenic fungus that infects approximately 500 plant species from over 100 families (Sarr *et al.,* 2014). Among the fungi associated with Bambara groundnut leaf diseases in Burkina Faso, *Macrophomina phaseolina* is the most widespread pathogenic (Ouoba, 2017). It spreads both through the soil and via seeds. *M. phaseolina* causes seed rot, damping-off, and rot of the stems, collars, and roots, leading to the death of the host plant (Ouoba *et al.*, 2019). In case of an epidemic, this fungus causes yield losses that can reach 100% (Iqbal *et al.*, 2010). Due to its virulence and highly efficient mode of transmission, control strategies based on cultural, chemical, and biological methods are not sufficient to effectively manage it (Oladimeji *et al.*, 2012). The use of resistant varieties to reduce the yield losses caused by this pathogen has been identified as the most promising and cost-effective method (Iqbal *et al.*, 2010; Ouoba, 2017). Disease resistance/tolerance is one of the goals of mutation breeding in many crops. Diseases result from the interaction between the host plant and the pathogen. Induced mutations can alter this interaction between the host and the pathogen and block some stages of the infection mechanism (FAO/IAEA, 2020). It has been reported that many mutants derived from induced mutations exhibit improved resistance to various diseases, particularly fungal ones (Lebeda and Svabova, 2010). Furthermore, the work of Ouoba *et al.* (2019) identified five *M. phaseolina*-resistant Bambara groundnut genotypes. However, pathogens that evade resistance, especially those involving a single gene, emerge rapidly. This situation also occurs when a new, more aggressive strain of the pathogen emerges (FAO/IAEA, 2020). It is therefore crucial for a sustainable breeding program to combine several important resistance genes with new genotypes or varieties to achieve broad-spectrum resistance. This study aimed to identify potential sources of resistance to *M. phaseolina* among M3 mutants of Bambara groundnut. The resistant genotypes could be used in Bambara groundnut breeding programs for resistance to *M. phaseolina*.

MATERIALS AND METHODS

Study site

The study was carried out during the rainy seasons of 2024 at the Phytopathology Laboratory of the Center for Environmental, Agricultural, and Training Research (CREAF), located in Kamboinsé at coordinates 12°28'N and 1°32'W. It is one of INERA's leading phytopathology laboratories.

In vitro pathogenicity test

Plant material

Two varieties of Bambara groundnut, KVS235 and KVS115, were used for the pathogenicity test. The KVS235 variety is known for its high susceptibility to leaf spot diseases in the field (Ouoba, 2017), and KVS115 was chosen for its high seed germination rate (Fig. 1).

Fungal material

The fungal material consisted of three isolates of *M. phaseolina* (IS-246, IS-53-4, and IS-235) isolated from Bambara groundnut seed samples collected from farming communities in major production areas and research institutions (INERA) in Burkina Faso. The fungus *M. phaseolina* was identified using the fungus identification manual of Mathur and Kongsdal (2003), and the strains were isolated using the method described by Baikoro *et al.* (2023).

Preparation of inocula and inoculation of seeds

Each isolate was propagated in Petri dishes to serve as an inoculum. After three days of growth, single spores from each isolate were aseptically transferred

to five Petri dishes containing PDA medium and then incubated at 25 °C with 12-hour light/dark cycles for 3 days. For each isolate, each variety was sown on the Petri dishes, with 10 seeds per dish, each dish representing a replicate. The same varieties were sown in five uninoculated Petri dishes each to serve as controls. All dishes were incubated in the Incubation chamber.

Data collection

The parameters measured were the number of germinated seeds, pathogenicity, and disease index. Germinated seeds were counted 7 and 14 days after incubation. Pathogenicity was recorded 7 days after incubation using the severity rating scale developed by Rayatpanah *et al.* (2012), with scores ranging from 0 = healthy seed to 5 = infected seed that did not germinate. Severity indices were estimated using the scores according to the formula used by Williams and Singh (1981), and the result was expressed as a percentage.

Laboratory mutant screening

Plant material

The plant material used included 19 Bambara groundnut genotypes, including 15 third-generation mutant (M3) families, three parental varieties, and a control variety susceptible to *M. phaseolina* infection. The mutant families were derived from gamma-ray irradiation (60Co radiation source) of the three parental varieties at doses of 200 Gray (for varieties KVS234 and KVS259) and 250 Gray (for variety KVS115). These families were selected for their performance characteristics, notably high yield potential compared to the parental varieties for some, high 100-seed weights, and an early maturity cycle for others (Tingueri *et al.,* 2024). The list of plant material used is presented in Table 1.

Table 1. List of plant material

Plant material	List	Radiation dose	Number
Mutant families	KVS259_M3S5, KVS259_M3S2, KVS259_M3S6, KVS259_M3S3, KVS259_M3S8, KVS234_M3S5, KVS234_M3S7, KVS234_M3S16	200 Gray	8
	KVS115_M3S5, KVS115_M3S6, KVS115_M3S8, KVS115_M3S11, KVS115_M3S12, KVS115_M3S13, KVS115_M3S14,	250 Gray	7
Parent varieties	KVS115, KVS234, KVS259	0	3
Susceptible control	KVS235	0	1
Total			19

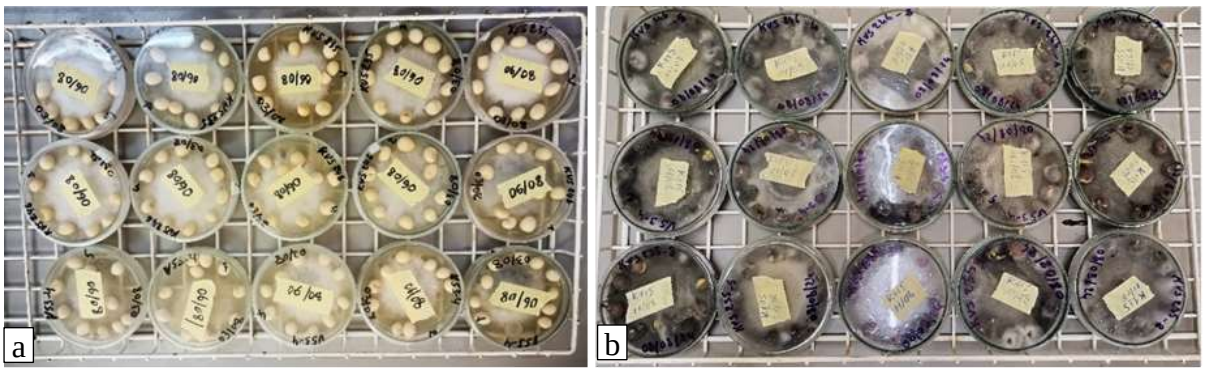

Fig. 1. Pathogenicity test setup: (a) uninoculated control plates, (b) inoculated dishes

Preparation of inocula and seed inoculation

One isolate was used for mutant screening: IS-246, which proved to be pathogenic and exhibited faster growth following the pathogenicity test. The seeds were sown in 500-ml glass jars (10 cm diameter) containing PDA medium, which had been previously sterilized in an autoclave at 120 °C for 20 minutes. All procedures were conducted aseptically in a laminar flow hood. For each genotype, ten jars were seeded, five of which were inoculated with the three-day-old isolate and five of which were uninoculated (controls), each

containing ten seeds. All jars were incubated at 25 °C, with 12-hour light/dark cycles.

Data collection

The following traits were recorded: the number of germinated seeds at 14 days after incubation, and the severity of infestation at 14 days after incubation, which was assessed using the 0–5 severity rating scale proposed by Rayatpanah *et al.* (2012).

Statistical data analysis

The germination and severity data were subjected to a normality test and analyzed using the R software via the RStudio interface. An analysis of variance (ANOVA) was performed using a linear fixed-effects model to evaluate the effect of genotype on the variables studied.

The model used is as follows: $Y_{ij}=\mu+G_i+\varepsilon_{ij}$, where Y_{ij} represents the observed value for genotype i at replicate j, μ is the overall mean, G_i is the fixed effect of genotype i, and $\varepsilon_{(ij)}$ is the associated random error, assumed to be normally distributed $N(0,\sigma^2)$.

For the comparison between inoculated and non-inoculated conditions, the following model was applied: $Y_{ijk} = \mu + G_i + T_j + (G \times T)ij + \varepsilon_{ijk}$, where T_j represents the treatment effect (inoculated vs. control), and $(G \times T)ij$ represents the genotype × treatment interaction.

When the effect of the factor under study was significant ($p < 0.05$), the means were compared using the Newman-Keuls multiple comparison test, with a significance threshold of 5%.

RESULTS

Pathogenicity test

Effects of M. phaseolina isolates on germination

All *M. phaseolina* isolates caused a significant decrease ($p<0.01$) in the germination rate at 14 days after incubation of the two Bambara groundnut varieties (KVS235 and KVS115) compared to the control, as revealed by the results across all isolates. The three isolates completely inhibited germination of the KVS235 variety compared to the uninoculated control, which recorded a germination rate of 60% (Fig. 2). Decreases in germination rates ranging from 38% to 71% were recorded for the KVS115 variety compared to the control.

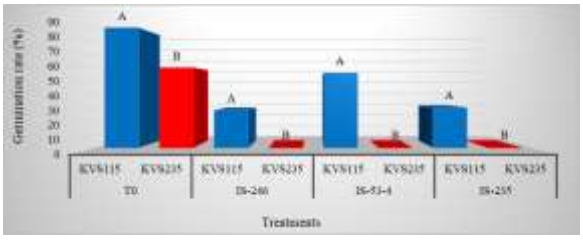

Fig. 2. Effect of *M. phaseolina* isolates on the germination of the KVS115 and KVS235 varieties

The *M. phaseolina* isolate IS-246 had the most negative impact on the germination of the KVS115 variety, with 71% decrease, while isolate IS-53-4 had the least impact on its germination, with 38% decrease compared to the control. Isolate IS-235 caused 67% reduction in the germination of KVS115 compared to the control.

Severity of M. phaseolina isolates

Symptoms induced by the isolates included seed rot, seeds completely invaded by mycelium that have failed to germinate (as in the case of the KVS235 variety), and discolored seedlings (Fig. 3–4). The results of the analysis of variance for the severity of attacks by the isolates revealed non-significant differences ($p>0.05$) between isolates for each variety. However, significant differences ($p<0.001$) between the two varieties were observed for each isolate.

Fig. 3. Symptoms of isolates on the KVS235 variety: (a) IS-235 effect, (b) IS-246 effect, (c) IS-53-4 effect (100% severity for all three isolates on KVS235)

Fig. 4. Symptoms of isolates on the KVS115 variety: (a) IS-235 effect, (b) IS-246 effect, (c) IS-53-4 effect (partially infested seeds)

A severity of 100% was recorded for the KVS235 variety for all *M. phaseolina* isolates. Severity values ranged from 80% (isolate IS-53-4) to 84% (isolates IS-246 and IS-235) for the KVS115 variety (Fig. 5).

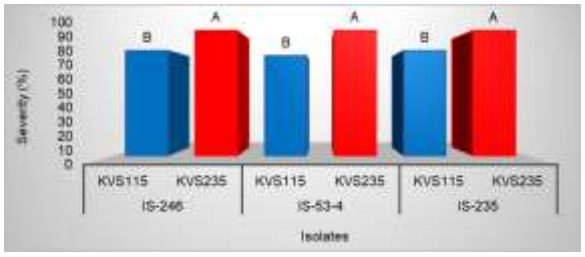

Fig. 5. Severity of isolates' attacks on the KVS235 and KVS115 varieties

Evaluation of the screening test

Effect of strain of M. phaseolina on Bambara groundnut germination rate

The results of the analysis of variance of the effect of *M. phaseolina* isolate IS-246 on the germination of the 19 genotypes revealed significant differences between the germination of the uninoculated controls and that of the mutants, parental varieties, and controls inoculated with the fungus (Table 2). On average, the germination rate of the controls was 73.5% while 23.5% for the inoculated samples.

Among the uninoculated control genotypes, the germination rate ranged from 50% for the KVS115_M3S11 mutant to 100% for the parental variety KVS115 (Fig. 6). Among the same inoculated genotypes, the germination rate ranged from 80% for the mutant KVS115_M3S12 to 0% for the mutants KVS259_M3S2, KVS234_M3S5, KVS234_M3S16, KVS115_M3S14, KVS115_M3S13, KVS259_M3S6, and the KVS235 variety. In most genotypes, inoculation with isolate IS-246 significantly reduced germination. These decreases in germination ranged from 5% to 100% relative to the control, depending on the genotype.

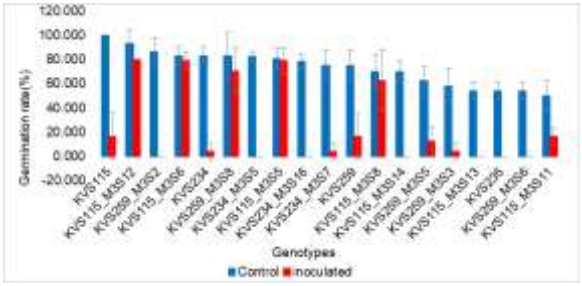

Fig. 6. Effect of isolate IS-246 on genotype germination rates

The Pearson correlation analysis (Table 3) between the germination rate under non-inoculated conditions (GRNI) and *M. phaseolina*-inoculated conditions (GRIN) showed a moderate, positive, and significant correlation coefficient ($r = 0.322$, $p < 0.05$). This result indicates a modest association between intrinsic germination vigor and performance against the pathogen. The low coefficient of determination ($r^2 = 0.104$) shows that more than 89% of the variation of the germination rate under inoculation was not explained by the control germination, suggesting that the resistance observed in certain mutants is largely specific to *M. phaseolina*.

Effect of the isolate IS-246 on disease severity

The strain IS-246 of *M. phaseolina* colonized and infected most of the seeds of the genotypes tested. The disease symptoms observed included seed rot, damping-off, and necrosis of the roots and leaves (Fig. 7).

Fig. 7. Severity of the *M. phaseolina* isolate on several mutants

Legend: (a) KVS115 control, (b) seed infection and necrosis of the taproot, (c) seedling emergence despite seed infection.

Table 2. Average performance of germination of control and *M. phaseolina*-inoculated samples

Parameter	Control	Inoculated	Mean	CV	F	Pr > F	Significant
GR (%)	73.50 ± 16.65 a	23.51± 5.70 b	48.5	72.6	38.5	<0.0001	Yes

CV: coefficient of variation; Pr: probability; means denoted by different letters are statistically different at the 5% significance level (Newman-Keuls test), GR: germination rate

Table 3. Pearson's correlation between the germination rate under inoculated conditions and under non-inoculated conditions

	GRIN	GRNI
GRIN	1.000***	
GRNI	0.322*	1.000***

GRIN: germination rate under inoculated conditions; GRNI: germination rate under non-inoculated conditions. *$p < 0.05$: significant, ***$p < 0.001$: highly significant

Table 4. Severity scores and reaction of genotypes

Genotypes	Severity score	Reaction
KVS115	4.33 ± 0.58 ab	HS
KVS259	4.33 ± 0.58 ab	HS
KVS259_M3S5	4.33 ± 0.58 ab	HS
KVS234	4.67 ± 0.58 ab	HS
KVS259_M3S3	4.67 ± 0.58 ab	HS
KVS115_M3S13	5.00 ± 0.00 a	HS
KVS115_M3S14	5.00 ± 0.00 a	HS
KVS234_M3S16	5.00 ± 0.00 a	HS
KVS234_M3S5	5.00 ± 0.00 a	HS
KVS235	5.00 ± 0.00 a	HS
KVS259_M3S2	5.00 ± 0.00 a	HS
KVS259_M3S6	5.00 ± 0.00 a	HS
KVS234_M3S7	3.67 ± 0.58 b	S
KVS115_M3S8	4.00 ± 0.00 ab	S
KVS115_M3S11	4.00 ± 0.00 ab	S
KVS115_M3S5	1.33 ± 0.58 c	MR
KVS259_M3S8	1.67 ± 0.58 c	MR
KVS115_M3S6	1.67 ± 0.58 c	MR
KVS115_M3S12	2.00 ± 0.00 c	MR
F	32.012	
Pr > F	<0.0001	

HS: Highly susceptible, S: susceptible, MR: Moderately resistant, CV: Coefficient of variation, F: Fisher's value, Pr: Probability. In the same column, values assigned the same letter do not differ significantly at the 5% level (SNK test).

The results showed highly significant differences ($p < 0.0001$) among genotypes for disease severity (Table 4). The mean disease severity scores ranged from 1.33 for the least susceptible genotype (KVS115_M3S5) to 5.00 for the most susceptible genotypes (KVS115_M3S13, KVS115_M3S14, KVS234_M3S16, KVS234_M3S5, KVS235, KVS259_M3S2, KVS259_M3S6). In addition to KVS115_M3S5, three other mutant genotypes (KVS259_M3S8, KVS115_M3S6, and KVS115_M3S12) were found to be moderately resistant to *M. phaseolina* with severity rates ranging from 1 to 2. The majority of the genotypes tested (60 %) were highly susceptible to *M. phaseolina* attacks, with severity rate from 4 to 5, while three (15%) genotypes were moderately susceptible, with severity scores ranged from 3 to 4. All parental varieties were highly susceptible to the *M. phaseolina* isolate and recorded a maximum severity score of 5, similar to that of the susceptible control KVS235.

The mean comparison test identified three groups of mutants based on their susceptibility to the *M. phaseolina* strain IS-246, including:

1. The group of highly susceptible mutants (HS) with severity scores between 4 and 5 (4<NS≤5). This

group consisted of eight mutant families, the three parental varieties, and the susceptible control variety.

2. The group of susceptible mutants (S) with severity scores ranging from 3 to 4 (3<NS≤4). This group includes only three families of mutants;
3. The group of moderately resistant mutants (MR), characterized by genotypes with severity scores (NS) ranging from 1 to 2 (1<NS≤2). This group consisted of four mutant families, three of which resulted from the mutation of the KVS115 variety (KVS115_M3S5, KVS115_M3S6, and KVS115_M3S12) and one from the mutation of the KVS259 variety (KVS259_M3S8). The moderately resistant mutant families originate from genetic groups 3 (KVS115_M3S5 and KVS115_M3S6) and 5 (KVS259_M3S8 and KVS115_M3S12).

DISCUSSION

In the present study, three isolates of *M. phaseolina* were evaluated for pathogenicity. They all led to the infestation of the seeds of the two tested varieties, with very high disease severity levels exceeding 80%. The ability of these three isolates to cause disease with very severe symptoms reflects their pathogenicity and virulence. Indeed, according to the scale described by Manici *et al.* (1992), an *M. phaseolina* isolate is pathogenic when it causes a disease with a severity level exceeding 60%. Ouoba *et al.* (2019) also reported that the fungus *M. phaseolina* proved to be highly pathogenic in Bambara groundnut in a pathogenicity test involving several fungal species. Langarica *et al.* (2008) and Rayatpanah and Dalili (2012) also observed high pathogenicity of *M. phaseolina* isolates in soybeans and sunflowers. Furthermore, the observed symptoms are similar to those described by Ouoba *et al.* (2019) in cowpeas, by Abawi and Pastor-Corrales (1990) in common beans (*Phaseolus vulgaris* L.), by Oladimeji *et al.* (2012) and Baikoro *et al.* (2023) in cowpeas (*Vigna unguiculata* L. Walp.). Bambara groundnut mutants exhibited differential responses (resistance or susceptibility) to the IS-246 isolate of the fungus *M. phaseolina* under *in vitro* culture conditions. Symptoms of seed rot, poor germination, dark necrotic lesions on roots and leaves, and damping-off were also observed. These symptoms (rot, damping-off, and necrosis) are typical of *M. phaseolina* infection and result from the secretion of hydrolytic enzymes that degrade cell walls, as reported by Ramos *et al.* (2016) and Marquez *et al.* (2021). Despite the high virulence of the *M. phaseolina* isolate under favorable laboratory conditions to its proliferation, the tested mutants exhibited different reactions to the fungus.

Four mutant genotypes were moderately resistant to infection. The positive but moderate correlation (r = 0.322) between germination rates under control and inoculated conditions indicates that a small fraction of the variation in response to *M. phaseolina* can be attributed to the general vigor of the seeds. However, most of the resistance expressed by the moderately resistant genotypes (KVS115_M3S5, KVS115_M3S6, KVS115_M3S12, KVS259_M3S8) is not predicted by their performance in the absence of the fungus, confirming the existence of active and specific resistance. These mutant genotypes, grown in greenhouses or under field conditions, may prove to be more resistant due to conditions that are less favorable to the fungus and more favorable to Bambara groundnut. These moderately resistant mutants may have acquired this resistance to infection caused by *M. phaseolina* through gamma mutagenesis, since the parental varieties from which they originated were all highly susceptible to the fungus. The differences observed in the response of the tested genotypes to *M. phaseolina* infection were also reported by Ouoba *et al.* (2019) in Bambara groundnut morphotypes collected from all agro-climatic zones of Burkina Faso. Similarly, Atiq *et al.* (2014) observed variability in the response of mung bean (*Vigna radiata* L.) genotypes to infection caused by *M. phaseolina*. Studies have identified host defense-related genes and proteins expressed during infection by *M. phaseolina*. Moderately resistant mutants may have acquired resistance genes. Indeed, studies on *Arabidopsis thaliana* have demonstrated that increased expression of defense-related genes could contribute to the development of resistance

against *M. phaseolina* (Saima and Wu, 2019). In addition, in sorghum, the studies of Sharma *et al.* (2014) revealed that the interaction between the plant and *M. phaseolina* led to the expression of antifungal genes, particularly during the first few hours following infection. Furthermore, according to Marquez *et al.* (2021), the host plant's susceptibility to *M. phaseolina* could be partially attributed to the pathogen's suppression of the auxin response.

CONCLUSION

This study revealed significant variability in the response of Bambara groundnut mutants to infection by *M. phaseolina* and identified two groups of genotypes: moderately resistant genotypes and moderately susceptible and highly susceptible genotypes. The four moderately resistant mutant genotypes (KVS115_M3S12, KVS115_M3S6, KVS115_M3S5, KVS259_M3S8) constitute valuable genetic resources for introgression into a program for pyramiding *M. phaseolina* resistance genes in Bambara groundnut. Given that the disease caused by *M. phaseolina* is strongly correlated with environmental conditions, it is essential to conduct screening trials of these genotypes in greenhouses and in the field to validate these resistances under real-world conditions. This would facilitate the selection of the best-adapted genotypes for different climatic conditions.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the "Projet d'Appui à l'Enseignement Supérieur (PAES)" for financial support for this work. They would also like to thank the Insectarium of Bobo-Dioulasso (IBD-CETT) and the FAO/IAEA Division for seed irradiation.

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