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Resistance to *Callosobruchus maculatus* Developed Via Gamma Radiation in Cowpea

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Abstract: Among the biotic stresses that affect cowpea (*Vigna unguiculata L*) productivity, infestation by cowpea bruchid beetles (*Callosobruchus maculatus*) is a major problem, causing yield losses of up to 100 %. To alleviate this problem, use of resistant varieties is a feasible approach for small-scale farmers. In Zambia, there are no reported sources of resistance to *C. maculatus*. The objectives of this study were: i) to evaluate certain cowpea mutants, generated at the University of Zambia, for resistance to *C. maculatus*; ii) to cluster the tested genotypes based on height, number of pods per plant, 100-seed weight, yield ha-1, number of eggs laid and adult emergence; and iii) to evaluate the candidate mutants for protein content. Experiments were conducted at three locations in 2014/15. The mutants, LT 11-5-2-2, BB 7-9-7-5 and BB-14-16-22, were found to be resistant to *C. maculatus* across locations and they out-performed their respective parents ($p< 0.05$) in the degree of resistance to *C. maculatus*. Cluster analysis, using selected agronomic parameters, showed that the selected resistant mutants (cluster B) were more similar to each other (95 %) than to Namuseba and Msandile (included as susceptible genotypes), which clustered at a similarity level of 78 % (cluster A). The mutants, LT 11-5-2-2, BB 7-9-7-5 and BB-14-16-22, showed resistance to *C. maculatus*, but their protein content was similar to their parents, indicating that this crucial trait had been maintained in the mutants.

Keywords: Bruchid; cluster analysis; mutations; protein; *Vigna unguiculata*.

1. Introduction

Cowpea (*Vigna unguiculata* (L.) Walp) is an important grain legume cultivated in sub-Saharan Africa. It is an important high-quality source of protein, especially for resource-poor farmers, who cannot afford animal protein. Its leaves and flowers can also be consumed and processed into hay and silage, resulting in nutritious livestock feed. The cowpea grain contains about 20 – 30 % protein, 1-2 % fat and 55 – 60 % carbohydrates on a dry-weight basis [1]. Its high biomass and good ground cover reduce soil erosion and improve soil fertility. Although it is drought tolerant and well adapted to sandy and poor soils, best yields are obtained in well-drained sandy loam to clay loam soils at pH between 6 and 7 [2].

Cowpea has good ability of associating with different species of *Rhizobia* (bacteria) in the soil to fix atmospheric nitrogen. It can fix about 70– 240 N kg ha^{-1} of atmospheric nitrogen per year [3] and a residue of fixed N deposit of 60-70 Kg N ha^{-1} is left in the soil for the next crop. Cowpeas are grown in rotation with or mixed with many cereals and tuber crops. Worldwide production of dry cowpeas stands at more than 5.4 million tons, with Africa producing nearly 5.2 million tons. Nigeria is the largest producer of cowpeas in Africa, accounting for 61 % of African and 58 % of global production. Niger is the second largest producer, followed by Burkina Faso, Myanmar, Cameroon, and Mali [4].

Cowpea yields have been characteristically low as a result of both abiotic and biotic stresses. Among the biotic stresses, infestation by cowpea bruchid beetles, also known as "cowpea weevil'' (*Callosobruchus maculatus*), is a major problem, which can cause in-storage yield losses of up to 100 %. Losses are caused by bruchid larvae that perforate the cowpea grain and feed on the contents, create holes and ultimately contaminate the grain [5]. Chemicals can be used to manage *C. maculatus*, but disastrous effects resulting from cowpea poisoning and environmental contamination have led to the limited use of chemical applications [6]. In addition, the high costs of chemicals make it difficult for small-scale farmers to afford the chemical approach. The development and use of

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resistant varieties is, therefore, a feasible and cost-effective solution. In Zambia, there are no reported sources of resistance to *C. maculatus*. It was for this reason that certain mutants that were generated by the University of Zambia, School of Agriculture Sciences, were evaluated for resistance to this pest. Mutations can generate new alleles and produce variants that are different from the parent, and which when advanced beyond M_5 generation yield non-segregating, distinct plants [7]. The identified resistant mutants may be evaluated and directly released as a variety or used as parents in breeding programs. Because protein content is a key nutrient in cowpea, it is essential to maintain it in the mutants alongside other desirable traits [8]. Crosses between distinct parental mutants or genotypes have been associated with hybrid vigor [9] and cluster analysis based on phenotypic or agronomic traits has been used to categorize genotypes into distinct groups [10, 11], which provides breeders with information on appropriate parental genotypes to consider for crossing.

Utilization of known resistant genotypes to *C. maculatus* is a challenge, as their performance is environment-specific, and in addition, resistance genes introduced into a new genetic background can easily break down [12-14]. We conducted this study to assess the performance of cowpea mutants for resistance to *C. maculatus.* The specific objectives were to i) evaluate certain cowpea mutants for resistance to *Callosobruchus maculatus*, ii) cluster tested genotypes based on agronomic traits and iii) evaluate the candidate mutants for protein content.

2. Materials and Methods

2.1. Study Sites

The experiments were conducted at three sites in Zambia: The University of Zambia (UNZA) field station, Lusaka (15°23' S, 28°19' E; altitude 1262 m), Chibombo (14°39' S, 28°05 E; altitude 1172 m), and Msekera (13°38' S, 32°38' E; altitude 1137 m). Chibombo and Msekera are approximately 100 km north and 593 km east of the capital city, Lusaka, respectively. The chosen sites were representative of the cowpea production areas. The rainfall received at the three sites is unimodal, with the rainy season stretching from November to March. These experiments were conducted in the 2014/15 cropping season.

2.2. Germplasm and Experimental Protocol

Eight genotypes (mutants and their parents) and two susceptible checks (Msandile and Namuseba) were used (see Table 1). The parents used were Lutembwe (LT PRT) and Bubebe (BB PRT). All the mutants used in this study were generated at the University of Zambia (UNZA) in collaboration with the National Institute for Science and Industrial Research (NISIR) in Zambia. The parental lines were subjected to 150 gray of gamma rays and resulting mutants were advanced to a stable mutation generation 8 (M_8). Approximately 3000 seeds per parental line were irradiated.

The experiment was laid out in a randomized complete-block design (RCBD) with three replications during the 2014/15 cropping season. Plants were established in six- row plots of 5 m length, with inter- and intra-row spacing of 0.6 m and 0.15 m, respectively, accommodating 33 plants per row. Two seeds were initially planted per hill but two weeks after germination, seedlings were thinned to one plant per hill. Standard cultural practices, such as weeding and fertilizer application, were followed at all experimental sites. Traits measured at each site were number of days to 50% flowering, plant height, number of pods per plant, number of seeds per pod, 100-seed weight, and yield ha^{-1}. After carefully harvesting (in April 2015) and isolating the pods for all the evaluated genotypes, threshing was subsequently done. The seeds were then taken to the laboratory at University of Zambia for in-storage evaluation of traits such as number of eggs laid and bruchid adult emergence. Details on how the bruchids were reared and how harvested test genotypes were infested are highlighted below.

2.3. Rearing and Inoculation of Experimental Bruchids

Rearing of *C. maculatus* beetles followed the modified procedure described by Swella and Mushobozy [5]. Adult (male and female) beetles were originally collected from infested cowpea seed in storage at the UNZA field station. The beetles were reared on a known susceptible genotype (Msandile). To initiate the culture, 10 adult individuals (1:1, male: female) were placed in ventilated glass containers (12 x 12 x 6 cm) containing susceptible cowpea genotype seed (~ 200 g). The insects were allowed to mate and oviposit on the cowpea under controlled laboratory conditions (27 °C and 60% RH). Two days later, the insects were removed from the containers. Containers were then left in the laboratory under the same conditions until new adult insects had emerged. Newly emerged adult insects were sexed, placed in Petri dishes (1:1, male: female) and allowed to mate for 12 hrs before they were used in the experiments or to start another culture. Only 2-3 day old, mated female adults were used for the experiments.

Evaluation of the test cowpea genotypes was done using the modified no-choice procedure, as described by Ponnusamy, *et al.* [15]. A completely randomized design (CRD) with three replications was used in the experiment. Cowpea genotypes were refrigerated for five days at 4-5°C to ensure that they were free from *C. maculatus* eggs and other postharvest insect infestations. Before initiating the experiment, seeds of test genotypes were removed from the refrigerator and kept at room temperature for 24 hrs. For each genotype, 20 seeds were placed in Petri dishes [8.7 x 1.2 cm (diameter x height)]. Four (2-3-day old) adult insects (1:1, male: female) were then placed in each Petri dish containing a test genotype and kept in the laboratory under controlled conditions (~ 27 °C and 60% RH). The insects were allowed 48 hrs to mate and oviposit on the cowpea, after which they were removed. Thereafter, the number of eggs laid on each test genotype was recorded using a magnifying glass. The materials were then maintained in the

laboratory under the same conditions as above until the adult insects had emerged. The number of adults emerged was recorded for each genotype in each replication. Furthermore, the mean number of days to adult emergence was also recorded.

2.4. Protein Evaluation

Candidate mutants (which were more resistant than the parental genotypes), together with their parents, were evaluated for protein content using the Kjeldahl method, as described by AOAC [16]. The cowpea genotypes were ground in an electric grinder. Protein was digested and distilled. The percentage nitrogen content was determined from the distillate by titration and it was then multiplied by a factor of 6.25 to obtain the corresponding protein content (%).

2.5. Data Analyses

The genotypic responses for number of bruchids emerged, days to emergence and protein content were evaluated using analyses of variance (ANOVA). The treatment means were separated using the Fisher's protected least significant difference (LSD) test at 5 % probability level. For the number of adults emerged, data were transformed by using the formula: $(X + 1)^{½}$. By transforming data, the assumption of normality needed for ANOVA was fulfilled. The relationship between number of eggs laid and adult emergence was determined using correlation analysis on all 90 paired data values across locations. Cluster analysis was performed using a multivariate analysis approach and single link cluster option. The parameters used in the cluster analysis were mean values, across locations, of pods per plant, plant height, number of seeds per pod, 100-seed weight, number of days to 50% flowering, yield ha^{-1}, no. of eggs laid and adult emergence. Data analyses were performed using GenStat [17] and we assumed a mixed-effects model, with locations being random and genotypes fixed effects.

3. Results

Significant differences among genotypes were observed for adult bruchid emergence at two individual locations [at UNZA ($P < 0.05$) and Chibombo ($P< 0.05$)] and across locations ($P< 0.05$) (Table 2). No significant differences among genotypes were detected at Msekera. The interaction between genotype (G) and location (L) [G × L] was also significant. The correlation (r) between number of eggs laid and adult emergence was computed to be 0.90 ($P < 0.001$). The transformed mean values for number of adults emerged (Table 3) showed that the mutant LT 11-5-2-2 had a lower mean value at each individual location and across locations as compared to the parent LT PRT and susceptible checks (Msandile and Namuseba). Similarly, the mutants BB 14-6-2-2- and BB 7-9-7-5 had significantly mean lower values for number of adults emerged as compared to their parental genotypes and susceptible checks across locations. The mean number of days to adult bruchid emergence at each location across genotypes was significantly different ($P= 0.013$; LSD= 2.42) (Table 2). Mean adult days to emergence values of 31.87, 33.22 and 29.03 were obtained in Chibombo, UNZA and Msekera, respectively. The G × L interaction was also significant.

Cluster analysis (Fig. 1) showed that the identified three mutants formed a cluster (B) at 95 % similarity as compared to Namuseba and Msandile (Cluster A), which showed 78% similarity to the rest of the genotypes. No significant differences were detected for crude protein content among tested genotypes ($P = 0.56$). Genotypes tested were resistant mutants (LT 5-2-2, BB 14-6-2-2 and BB 7-9-7-5) and their respective parents (LT PRT and BB PRT). The mean crude protein percentages among genotypes ranged from 24.2 to 26.0 % (Table 4).

4. Discussion

Breeding for resistant genotypes to combat cowpea yield losses in storage is the most logical and cost-effective approach to help small-scale farmers. In mutation breeding, a mutant is considered a candidate genotype for release or a parent in a breeding program if it is better in one or more traits as compared to the parental genotype. In this study, three mutants (LT 11- 5-2-2, BB 7-9-7-5 and BB 14-16-2-2) had a lower transformed mean number of *C. maculatus* adult emergence when compared to their parents and susceptible checks across locations. Genotype (G) × location (L) interaction (Table 2) was significant, implying that the genotypic performance with regards to number of adult bruchids emerged differed at each location. In this study, the genotypes were not significantly differentiated with regard to number of adult bruchids emerged at $P \leq 0.05$ in Msekera; implying that environmental factors had an influence on genotypic seed resistance to *C. maculatus*. Cruz et al. [18] found that factors, such as temperature, humidity and soil nutrients, had an effect on genotypic seed composition, which in turn had an effect on resistance to bruchid pest. Significant G × L interaction was detected for the mean number of days to emergence, indicating an influence of environmental effect on genotypes. Previous work has shown that adult emergence time is a function of seed texture, size and endogenous seed chemical composition, which are influenced by the environment [18]. In general, the average life cycle of the *C. maculatus* beetle ranges from 21 to 25 days on a susceptible genotype [19]. However, in this study, emergence time ranged from 26 to 35 days. Similar to what Amusa, *et al.* [20] obtained for the cowpea genotypes infested with *C. maculatus*. The emergence time differences observed might be attributed to the type of germplasm under study. Previous studies have demonstrated that, evaluation for number of adult bruchid emergence among cowpea genotypes is an inverse function of cowpea resistance to *C. maculatus* [21, 22]. In this regard, LT 5-2-2, BB 14-6-2-2 and BB 7-9-7-5 were found to be resistant across locations. High significant correlation ($r= 0.90$; $P< 0.001$) between number of eggs laid and number of adults emerged might imply that the

number of eggs laid could be used as an indirect selection criterion for resistance to *C. maculatus* in cowpea. However, the revelation that adult emergence is related to seed texture and thickness [18] challenges the utilization of number of eggs laid as an indirect selection criterion for resistance to *C. maculatus*.

Cluster analysis revealed that there was similarity among mutants that clustered in one group (Group B) at 95 % similarity. On the other hand, susceptible check genotypes clustered together (Group A) at a 78 % similarity with other genotypes. Group B included LT 5-2-2, BB 14-6-2-2 and BB 7-9-7-5, which were judged as resistant to *C. maculatus*. An earlier study revealed that resistance to *C. maculatus* in cowpea was determined by one to two major recessive genes [13]. However, the reports that these genes break down across time because of the coexistence of *C. maculatus* with cowpea may discourage breeders to introgress these genes into the adapted Zambian germplasm [12-14]. The mutants, LT 5-2-2, BB 14-6-2-2 and BB 7-9-7-5, were identified as resistant to *C. maculatus*. This meant that the mutation agent (gamma ray) created an allele or alleles that codes/code for resistance to *C. maculatus*. Further research should be carried out to establish the stability of this resistance. The fact that there was a gain-in-function with regard to resistance to *C. maculatus* trait in mutants implied that the created resistance allele(s) was/were likely dominant [23]. As a breeding strategy, a resistant mutant can be crossed with a susceptible genotype to obtain resistant hybrids. Another approach will be to advance a cross, followed by selection to maintain a desirable trait (resistance) along with other desirable agronomic traits. Alternatively, the identified mutants can be evaluated for possible release and marketing [24]. The revelation that there was no significant difference in protein content between the selected mutants and their parents implied that these mutants were as good a source of protein as their parents. Protein is an important trait to maintain in a cowpea breeding program. In this study, tested mutants and their respective parents were found to contain an adequate, medium level of protein content. Afiukwa, *et al.* [8] classified cowpea genotypes for protein content as low (< 20%); medium (20 – 30%) and high (> 30 %). In our study, protein content was between 20 to 30%, which was within the range for most cowpea genotypes [1].

5. Conclusion

We have found LT 11-5-2-2, BB 7-9-7-5 and BB-14-16-22 mutants to be resistant to *Callosobruchus maculatus.* Cluster analysis showed that the resistant genotypes clustered together at a similarity level of 95 %, whereas susceptible ones clustered at 78 % similarity level. As the mutants exhibited a gain-in-function phenotype when compared to their parental genotypes, the created resistance allele or alleles is/are likely dominant. This implies that resistant hybrids can be created by crossing resistant mutants (cluster B) with other genotypes, especially susceptible but otherwise desirable genotypes (cluster A). Identified mutants can also be evaluated and possibly released as varieties. Alternatively, in a breeding program, the mutants can be used as parents to introduce resistance to *Callosobruchus maculatus* in other germplasm and the resulting cross can be advanced through selection until resulting progeny are stable in regard to resistance to *C. maculatus*. The fact that no significant protein content differences between mutants and their respective parents were detected, implied that an important nutritional-security trait (protein content) remained unchanged in the mutants.

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Tables and Figure

Figure-1. A dendrogram depicting similarity relationships among cowpea genotypes. A and B show clusters with susceptible and resistant genotypes to *C. maculatus* respectively based on evaluated agronomic traits. BB PRT (Bubebe) and LT PRT (Lutembwe)- Parental genotypes; BB 14-16-2-2 and BB 7-9-7-5 are mutants created from BB PRT; LT 11-5-2-2, LT 4-2-4-1, LT 3-8-4-6 and LT 3-8-4-1 are mutants created from LT PRT

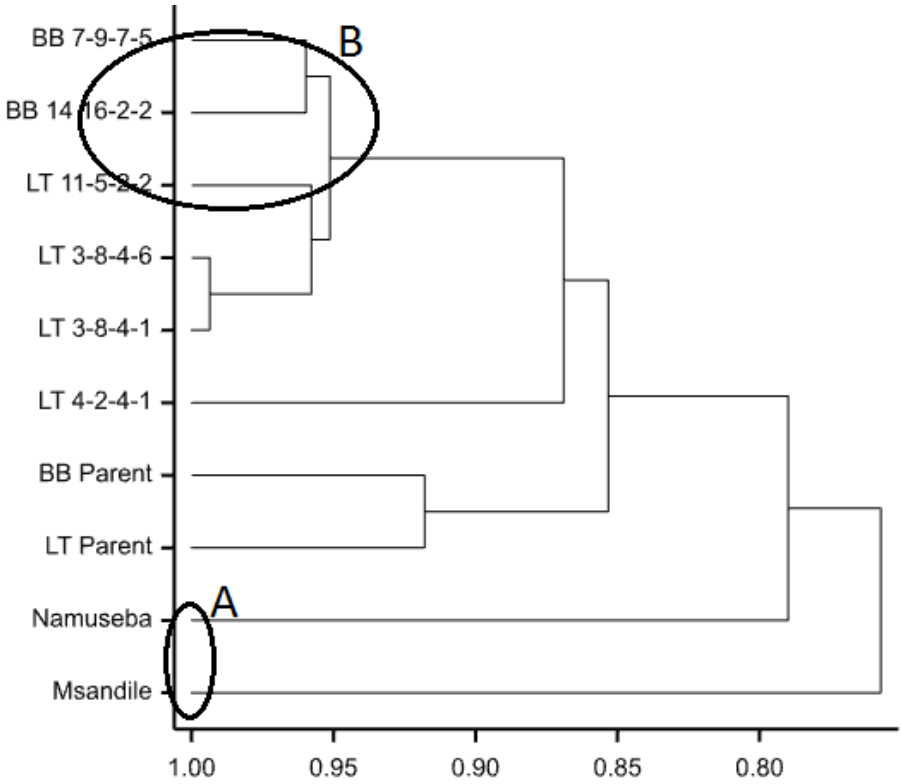

Table-1. Germplasm identity and characteristics used in the bruchid resistance study for field and lab evaluation
†BB PRT - Bubebe parental genotype; BB 14-16-2-2 and BB 7-9-7-5 are mutants created from BB PRT.
‡LT PRT - Lutembwe parental genotype; LT 11-5-2-2, LT 4-2-4-1, LT 3-8-4-6 and LT 3-8-4-1 are mutants created from LT PRT.

GENOTYPE	TYPE	CHARACTERISTICS
LT 11-5-2-2	Mutant	Early maturity, attractive seed coat color, pod formation above half plant height.
BB 14-16-2-2	Mutant	Medium maturity, tolerant to aphids, high above ground biomass.
LT 4-2-4-1	Mutant	Medium maturity, tolerant to aphids, attractive seed coat color.
LT 3-8-4-6	Mutant	Early maturity, high yielding, attractive seed coat color, high above ground biomass.
LT 3-8-4-1	Mutant	Indeterminate, attractive seed coat color, medium maturity, high yield.
BB 7-9-7-5	Mutant	Tolerance to *Colletotricum lindemuthianum*, determinate, medium maturity.
‡LT PRT	Parent	Attractive seed coat color, high yield but susceptible to aphids.
Msandile	Popular Genotype	Attractive seed coat color, big seeds, susceptible to bruchids.
Namuseba	Popular Genotype	Attractive seed coat color, susceptible to bruchids attack.
†BB PRT	Parent	High yields, susceptible to leaf and stem rust, susceptible to bruchids and aphids.

Table-2. Mean squares for number of adult bruchids emerged and days to adult bruchid emergence for individual locations and across locations

Source of variation	Multi-locations			Individual locations						
	df	Across locations		df	†UNZA		Chibombo		Msekera	
		‡No.	§Days		‡No.	§Days	‡No.	§Days	‡No.	§Days
Locations (L)	2	2.19	137.6*							
Rep/locations	6	0.57	14.7	2	0.14	8.7	0.95	18.5	0.9	16.9
Genotypes (G)	9	2.18*	36	9	1.71*	46.4*	1.97*	47.5**	1.66	11.8
G × L	18	1.69*	34.9**							
Error	54	0.78	13.2	18	0.68	17.1	0.62	11.3	1.04	11

*, ** - Significant at P< 0.05 and P< 0.01 respectively.
†UNZA- University of Zambia.
‡- Mean squares for transformed number of adult emerged.
§- Mean squares for number of days to emergence.

Table-3. Mean number of adult bruchid emergence and days to emergence for individual locations and across locations

Genotypes	Across Locations	†UNZA		Chibombo	
	‡Number	‡Number	§Days	‡Number	§Days
BB 14-16-2-2	3.34	2.16	30.33	4.43	34.33
BB 7-9-7-5	3.12	3.82	28.51	2.7	35.33
BB PRT	4.24	3.11	31.1	5.31	21.33
LT 4-2-4-1	4.18	3.41	26	4.74	33.00
LT 11-5-2-2	3.25	3.02	32	3.16	34.33
LT 3-8-4-1	3.72	3.35	35	4.12	32.00
LT 3-8-4-6	3.64	3.49	29	4.85	32.67
LT PRT	4.34	4.07	20.67	4.14	32.67
Msandile	4.39	4.76	31	4.17	33.00
Namuseba	4.26	4.47	26.67	3.4	30.00
LSD (α = 0.05)	**0.84**	**1.41**	**7.16**	**1.36**	**5.76**

†UNZA -University of Zambia
‡-Transformed number of adult emerged
§-Number of days to emergence

Table- 4. Protein content (%) measured on candidate mutants and their respective parents

Genotype	Type		Mean ± SE§
BB 14-16-2-2	Mutant		25.9 ± 1.1
BB 7-9-7-5	Mutant		26.0 ± 0.7
†BB PRT	Parent		24.2 ± 0.9
LT 11-5-2-2	Mutant		25.8 ± 0.5
‡LT PRT	Parent		24.7 ± 0.8

†BB PRT- Bubebe parental genotype; BB 14-16-2-2 and BB 7-9-7-5 are mutants created from BB PRT.
‡LT PRT- Lutembwe parental genotype; LT 11-5-2-2 is a mutant created from LT PRT.
§SE- Standard error of the mean computed from genotypic values across locations.