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Reactions of Maruca Resistant Transgenic Cowpea to *Cowpea Aphid-Borne Mosaic Virus*

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Abstract

Cowpea (*Vigna unguiculata* L. [Walp.]) in one of the main grain legumes contributing to food security and poverty alleviation in Sub-Saharan Africa. To control the highly damaging legume pod borer *Maruca vitrata* F., transgenic cowpea lines expressing the insecticidal Cry1Ab Bt protein were developed. In this study, we evaluated the impact of Cry1Ab transgene expression on the susceptibility of four cowpea lines (named IT97K-T, IT98K-T, Gourgou-T and Nafi-T) and their respective non-transgenic near isogenic lines (IT97K, IT98K, Gourgou and Nafi) to *Cowpea aphid-borne mosaic virus* (CABMV) in greenhouse conditions. In a preliminary quality control test by enzyme-linked immunosorbent assay, the presence of Cry1Ab protein in transgenic seed lots ranged from 59% to 72%, with no significant differences among the lines (χ^2 = 3.26; p = 0.35). Upon virus inoculation, all cowpea lines exhibited mosaic symptoms with similar severity between 7- and 11-day post-inoculation. No significant differences were observed in symptom severity. Significant differences were found between cowpea lines for time of symptom onset, virus accumulation in plants and days to 50% flowering. However, while comparing pairs of transgenic lines and corresponding non-transgenic lines, virus accumulation showed not significant differences whatever the pair. Time of symptom onset and days to 50% flowering did not also differ significantly between pairs of cowpea lines except Nafi/Nafi-T in which transgenic Nafi-T showed earlier symptoms (7.4 ± 0.7 vs. 8.9 ± 0.8 days post-inoculation) and shorter

flowering time (37.3 ± 0.6 vs. 42 ± 1.7 days after sowing). Overall, these findings improve our understanding of the effects of Cry1Ab gene mediated genetic modification on cowpea infection by Cowpea aphid-borne mosaic virus, with potential implications for environmental safety assessment.

Keywords

Cowpea, Cry1Ab, Cowpea Aphid-Borne Mosaic Virus, Environmental Safety

1. Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is an important grain legume grown worldwide in tropical and sub-tropical areas. According to statistics from the Food and Agriculture Organization (FAO) for 2022, up to 97% of the cowpea production came from Africa [1]. The largest producers were Nigeria (4.13 million tons) Niger (2.87 million tons), and Burkina Faso (0.83 million tons), all three countries accounting for about 80% of the word production. Cowpea grain and leaves are particularly rich in proteins (25.4% - 27.6%) [2] with high contents in the essential amino-acids lysine and tryptophane [3]. Therefore, the crop plays an important role as food and feed. Dual purpose varieties producing appreciable amounts of both grain and fodder are being promoted in some areas [4] [5] [6]. Besides its nutritional use, cowpea is a valuable source of income through the selling of grain and fodder in local markets and exports [7]. In addition, being a leguminous plant, cowpea can fix atmospheric nitrogen which is valuable for improving soil quality. Therefore it is usually grown in association with cereals in farmers' fields [8] [9].

Cowpea production is affected by for many pests and diseases, especially in Sub-Saharan Africa. Over 140 cowpea-infecting viruses were reported worldwide with only 12 species occurring so far in Africa [10] [11]. Viruses are transmitted by insect vectors that also inflict direct damage as pests to vegetative parts of the cowpea plants. Several other groups of insects including *Lepidoptera*, *Coleoptera*, *Homoptera*, *Hemiptera*, *Thysanoptera*, and *Diptera* affect cowpea production and seed storage [12] [13]. Among these pests, the legume pod borer *Maruca vitrata* F. is one of the most important threats, especially for the major cowpea growing West African countries [8] [14]. This lepidopteran insect pest can cause yield losses ranging between 25% and 85% in cowpea [15] [16] and several control approaches involving chemical and biological control or host plant resistance were unsatisfactorily [16]. Therefore, the use of the insecticidal protein Cry1Ab from *Bacillus thuringiensis* (Bt) was envisaged, and genetically engineered (GE) pod borer resistant (PBR) cowpea were successfully developed [17]. However, these PBR cowpea events were not suitable for deployment in the field. Consequently, Cry1Ab Bt gene was introgressed from selected events into well adapted and farmers' preferred cowpea varieties in each country including Bur-

kina Faso, Ghana, and Nigeria [17].

The sole purpose of the genetic engineering of the Bt gene into cowpea was to induce resistance against the legume pod borer *M. vitrata*. One of the major concerns with genetic modification of plants is the possible occurrence of unintended effects inducing additional undesirable agronomic characteristics, such as increased disease susceptibility or impaired food nutrients production [18] [19]. Side-effect of genetic modifications in plants are related to human and environmental safety an involve altered nutritional composition, production of new allergens, gene flow from GE plants to related species, and antibiotic resistance in pathogenic bacteria, among others [20].

Cowpea mosaic disease caused by *Cowpea aphid-borne mosaic virus* (CABMV) is one of the most damageable virus disease of cowpea, particularly in West Africa [21]. Yield losses caused by the disease depend on cowpea cultivars and can reach 87% in susceptible ones [22]. CABMV is seed-transmitted and also propagated by aphids in a non-persistent manner [10]. It is also mechanically transmissible in laboratory and confined field experiments [23] [24]. In Burkina Faso, CABMV was found to be most prevalent (97.04%) of all cowpea-infecting viruses in the country [11]. Therefore, in this study, the susceptibility to CABMV of the Cry1Ab PBR cowpea being developed was assessed as part of their agronomic evaluation for possible unintended effects.

2. Materials and Methods

2.1. Plant Material

Four transgenic cowpea lines (named IT97K-T, IT98K-T, Gourgou-T and Nafi-T) and their respective non-transgenic lines, *i.e.* conventional lines (IT97K, IT98K, Gourgou and Nafi) were used. Each transgenic line shared similar genetic make-ups with its conventional counterpart except the presence of the cry1Ab gene in the transgenic line. All seeds were taken from the National biosafety laboratory seed collection.

Seeds from each cowpea line were soaked for about 15 hours and transferred on wet blotting paper in Petri dishes until germination started. Small portions of about 50 mg were removed from the cotyledon without disturbing the embryo for the detection of Cry1Ab protein. Three pre-germinated seeds with known status were sown per cowpea line in 10-liter buckets in a biosafety containment greenhouse. The buckets were arranged following a complete randomized design with three replications per treatment.

2.2. Serological Detection of Cry1Ab Protein

To ascertain the presence or absence of Cry1Ab protein in transgenic and conventional cowpea, respectively, seed stocks were analyzed by enzyme-linked immunosorbent assay (ELISA) using the QualiPlate kit for Cry1Ab/Cry1Ac (Envirologix Inc., USA). Seed portions collected as indicated above, were placed in 1.5 Eppendorf tubes, and homogenized in 300 µL of extraction buffer using disposa-

ble pipette tips. The antigen extraction buffer was composed of phosphate buffer saline, pH 7.4 containing 2% of polyvinylpyrrolidone (PVP-40). After centrifugation of the extracts at 10,000 xg for 10 min the supernatants were incubated in duplicates. Non-transgenic seeds (negative controls) were tested in triplicates and the threshold for Cry1Ab detection was determined as the average of their absorbance readings plus three times the standard deviation [25].

2.3. Virus Inoculation

Cowpea aphid-borne mosaic virus isolate BF72 of our collection was first propagated on susceptible cowpea cultivar "Gorom local" by mechanical inoculation. To prepare the inoculum, leaves showing symptoms were collected and 1g was ground in 5 ml of inoculation buffer (a mixture of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4, adjusted to pH 7.4). The leaf extract was filtered on muslin cloth and carborundum 600 mesh was added. The inoculum was rubbed with fingers onto the leaves of germinated cowpea seedlings 7 days after sowing (DAS). Inoculated plants were monitored daily for symptom appearance.

2.4. Assessment of Virus Multiplication in Plants

CABMV accumulation was assessed by double antibody sandwich ELISA [26]. Polyclonal rabbit antibodies raised against CABMV particles were used as coating antibodies. The same antibodies linked to alkaline phosphatase were used as conjugate. Last fully expanded leaves of inoculated plants were collected and pooled by cowpea line. Plant extracts were prepared by grinding 1g of leaf in 10 mL of antigen extraction buffer. Extracts were centrifuged as indicated above and tested in triplicates.

2.5. Assessment of Symptoms and Days to 50% Flowering

For each cowpea line, the appearance of CABMV-induced symptoms was monitored daily. Symptom severity was assessed three weeks post-inoculation using the 1 - 5 scale proposed by [27] where 1 represents no visible symptom and 5 indicates very severe symptoms (mosaic associated with leaf deformation and reduction of plant growth). The number of days to 50% flowering and virus multiplication in inoculated plants were also assessed.

2.6. Statistical Analysis

All data were collected using Microsoft Excel and then imported into R software [17] for statistical analysis. The data were expressed as mean ± standard deviation of three replicates. Assumption for normality was assessed through QQ-plots and the Shapiro-Wilk test, while homogeneity of variances was assessed using the Levene test. Analysis of variance (ANOVA) was used to compare data measurements across cowpea lines. Post-hoc tests for the separation of the means were conducted using Fisher's LSD with the significance level set at $p < 0.05$.

3. Results

3.1. Presence of Cry1Ab Protein in Cowpea Seed Lots

The results of the analysis of the presence of the Cry1Ab protein in the seeds of transgenic and conventional cowpea lines are summarized in Table 1. The presence of the Cry1Ab protein was confirmed in all transgenic varieties at varying levels between 59% and 72%. Statistical analysis of the results using the chi-square test did not reveal significant differences (χ^2 = 3.26; p = 0.35). The presence of the Cry1Ab protein at rates less than 100% indicates that parts of the seeds (27.9% - 41%) of all the transgenic seed lots do not contain the Bt toxin. No transgenic seed was found in conventional cowpea seed lots.

Table 1. Proportion of Cry1Ab protein containing seeds in cowpea lines.

Cowpea line[a]	Seeds		
	Total	Positive	Negative
IT97K-T	61	44 (72.13%)	17 (27.87%)
IT98K-T	57	41 (71.93%)	16 (28.07%)
Gourgou-T	61	36 (59.02%)	25 (40.98%)
Nafi-T	32	20 (62.5%)	12 (37.5%)
IT97K	15	0 (0%)	15 (0%)
IT98K	15	0 (0%)	15 (0%)
Gourgou	15	0 (0%)	15 (0%)
Nafi	15	0 (0%)	15 (0%)

[a]The names of transgenic cowpea lines end with "-T" to distinguish them from their respective conventional counterparts.

3.2. Symptom Development and Severity

In response to CABMV inoculation, the plants showed various mosaic symptoms (Figure 1). In the yellow mosaic type was characterized the leaves were predominantly yellow indicating severe chlorosis. The vein-banding pattern was also associated with chlorosis leaving areas with chlorophyll along the veins. A green mosaic type also occurred with less obvious chlorosis but associated with leaf deformation. The different types of symptoms were observed indistinctively on the various cowpea cultivars.

Table 2 summarizes three parameters of the development of symptoms. All plants inoculated with the virus extract developed clear symptoms during the first- and second-week post inoculation. The time of the onset of the symptoms ranged between 7- and 11-day post-inoculation (dpi). Symptoms appeared earlier in cowpea lines Nafi-T with disease incidence of 66% at 7 dpi. Most inoculated plants showed clear symptoms up to 10 dpi. Symptoms appeared at 11 dpi

were observed on 1 out of 9 plants only, in cowpea lines Gourgou and IT97K-T. One-way ANOVA indicated that not all cowpea lines developed symptoms in the same time frame ($F_{7,64} = 3.09$; $p = 0.007$).

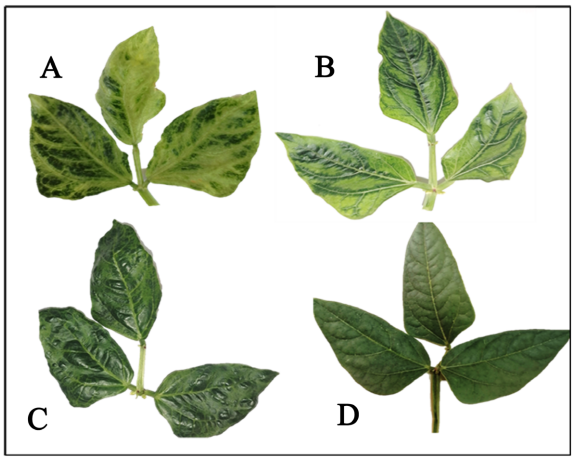

Figure 1. Main leaf symptoms exhibited by the cowpea lines following CABMV inoculation: severe yellow mosaic (A), mosaic with vein-banding (B), green mosaic with leaf deformation (C), healthy leaf (D).

Table 2. Symptoms time of onset and severity rating.

Cowpea line[a]	Symptom onset time (dpi)[b]	AUDPC[c]	Symptom severity rating
IT97K	8.6 ± 1.0 ab	288.9	3.3 ± 0.5
IT97K-T	8.9 ± 1.1 ab	261.1	3.6 ± 0.5
IT98K	8.1 ± 1.1 bc	322.2	3.6 ± 0.5
IT98K-T	8.9 ± 0.8 ab	266.7	3.6 ± 0.5
Gourgou	9.1 ± 1.3 a	233.3	3.3 ± 0.5
Gourgou-T	8.9 ± 0.8 ab	261.1	3.6 ± 0.5
Nafi	8.9 ± 0.8 ab	250.0	3.3 ± 0.5
Nafi-T	7.4 ± 0.7 c	372.2	3.7 ± 0.5

[a]The names of transgenic cowpea lines end with "-T" to distinguish them from their respective conventional near-isogenic lines. [b]dpi: days post inoculation. Means followed by the same letter(s) within the symptom onset time column are not significantly different. Cowpea lines did not differ significantly in symptom severity. [c]AUDPC: area under disease progress curve.

In comparing transgenic cowpea lines with their non-transgenic counterparts, the onset of symptoms was significantly different ($p = 0.002$) in the pair Nafi/Nafi-T only. All other pairs showed similar durations for the appearance of symptoms. The earliness of the development of symptoms in Nafi-T was also associated with a higher area under disease progress curve (AUDPC).

The symptom severity scores were comprised in a very narrow range between

3.3 and 3.7. No statistical difference was found among mean severity scores ($F_{7,64}$ = 0.594; p = 0.76).

3.3. Virus Accumulation in Plants

The average absorbance readings reflecting the levels of viral accumulation in plants varied between 1.213 ± 0.015 in cowpea line Gourgou-T and 1.597 ± 0.019 in Nafi-T.

There was a significant cowpea line effect ($F_{7,16}$ = 34.62; p < 0.001) (Figure 2). Interestingly, on the one hand, no significant differences were observed between pairs of transgenic/non-transgenic line IT97K/IT97K-T (p = 0.556), IT98K/IT98K-T (p = 0.238), Gourgou/Gourgou-T (p = 0.065), and Nafi/Nafi-T (p = 0.162). On the other hand, marked differences were found between most of the other combinations of cowpea line. Notably, each of the two lines Gourgou and Gourgou-T significantly differed from any other cowpea line (p < 0.001) by their lower absorbance readings.

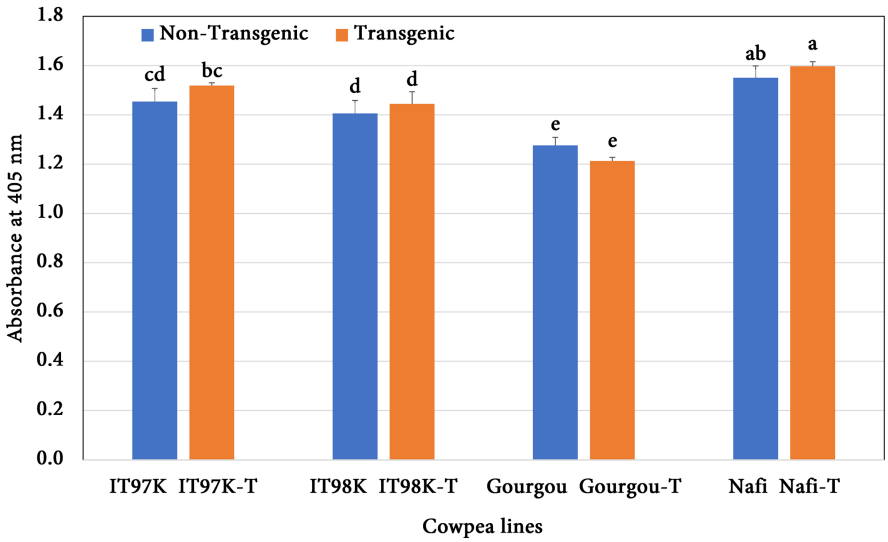

Figure 2. Virus accumulation in transgenic and corresponding near isogenic non-transgenic cowpea lines. Identical letters on the top of the bars indicate groups with non-significant difference in virus accumulation.

3.4. Days to 50% Flowering

The results of the assessment of days to 50% flowering (DF50) of cowpea lines are shown in Figure 3. Taken together, the DF50 varied on average between 34.3 ± 1.5 days and 42.0 ± 1.7 days. As indicated by one-way ANOVA, differences in DF50 across the cowpea lines were significant ($F_{7,16}$ = 8.17; p = 0.0003).

Comparisons between the transgenic cowpea lines and their corresponding non-transgenic parents showed a significant difference between Nafi and Nafi-T only (p = 0.0037). No significant differences were found between cowpea lines in pairs IT97K/IT97K-T (p = 0.243), IT98K/IT98K-T (p=0.634) and Gourgou/Gourgou-T (p = 0.243).

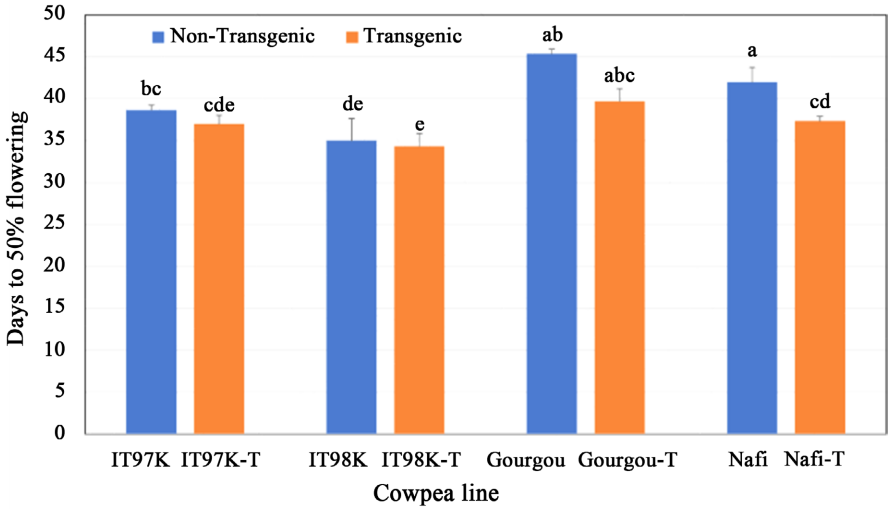

Figure 3. Days to 50% flowering in transgenic and non-transgenic cowpea lines. Identical letters on the top of the bars indicate groups with non-significant difference in days to 50% flowering.

4. Discussion

Whether seeds are from genetically modified (GM) crops or not, their quality is of utmost importance at any step of crop development and release. Particularly, the authenticity and accurate labelling of transgenic seed lots are essential for any downstream use [28]. In this study, authentic transgenic and non-transgenic seeds were needed for assessing the susceptibility of cowpea lines to CABMV. Therefore, it was worth testing the seed lots for the presence or absence of Cry1Ab Bt toxin. The results showed that significant amounts (27.9% - 41%) of non-transgenic seeds were found in transgenic seed lots. This undesirable presence of conventional seeds in transgenic seed lots is likely due to the fact that the seeds were produced from non-homozygous parents [29]. Mislabeling of the seed lots was unlikely since all conventional seed lots were found authentic.

Upon mechanical inoculation of CABMV, all cowpea lines developed mosaic symptoms within 7 to 11 days post-inoculation (dpi). Similar results were reported earlier while inoculating CABMV to cowpea [22] and passionfruit [30]. The overall earliness and narrow range of symptom onset in cowpea lines was consistent with high efficiency attributed to the mechanical inoculation of the virus [31]. Therefore, it is expected that the cowpea lines show lower levels of CABMV infection parameters in field conditions under aphid transmission of the virus. In fact, plant-vectors interactions which may be unfavorable to virus transmission due to plant resistance to aphids should be taken into account [31].

The levels of virus accumulation, as reflected by absorbance readings, varied significantly among cowpea lines. Notably, Gourgou and Gourgou-T lines exhibited significantly lower absorbance readings compared to other lines, indicating reduced viral accumulation. This reduced accumulation in the two lines could imply potential genotype characteristics conferring some resistance to virus

multiplication. No significant differences were found between any pair of transgenic/non-transgenic cowpea lines, suggesting that the genetic modification does not account for variations in viral accumulation.

The assessment of day to 50% flowering (DF50) revealed a range between 34.3 ± 1.5 days and 42.0 ± 1.7 days with significant differences between cowpea lines. DF50 depends on the cowpea genotype [32] which accounted for the differences observed. The pairs of transgenic and non-transgenic cowpea lines did not display any significant difference excepted the Nafi-T/Nafi pair.

5. Conclusion

This study investigated the susceptibility of genetically modified pod borer resistant (PBR) cowpea lines expressing Cry1Ab protein to Cowpea aphid-borne mosaic virus (CABMV). The inoculation of the virus resulted in development of mosaic symptoms in all cowpea lines within 7 to 11 days post-inoculation, with some variation in symptom onset and severity among the different lines. Viral accumulation and days to 50% flowering varied significantly among cowpea lines. However, transgenic cowpea lines and their corresponding near isogenic lines showed similar reactions for all the major disease parameters. This indicates that the PBR cowpea lines do not require additional CABMV control measures beyond what is applicable to non-transgenic lines.

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Conflicts of Interest

The authors declare no conflict of interest regarding the publication of this paper.

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