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Inheritance of the *Bt* gene and *Striga gesnerioïdes* in transgenic line, 709A and line IT98K-205-8, resistant to *Striga gesnerioïdes in Burkina Faso*

Hamadou SIDIBE[*1], Jean Baptiste D.L.S. TIGNEGRE[2], Benoit T. J. BATIENO[1], Serge F.M. ZIDA[1], Léandre S. PODA[1], Romaric K. NANEMA[3], Jeremy T. OUEDRAOGO[4] et Mahamadou SAWADOGO[3].

[1]Institut de l'Environnement et de Recherches Agricoles du Burkina Faso (INERA)
[2]World Vegetable Centre (AVRDC), Bamako BP 320, Mali
[3]Université Joseph KI-ZERBO, École Doctorale Sciences et Technologie, Laboratoire Biosciences, Équipe Génétique et Amélioration des Plantes, 03 BP 7021, Ouagadougou 03, Burkina Faso
[4]African Union Development Agency (AUDA-NEPAD), Dakar, Senegal
[*]Auteur correspondent Email: hamasegua22@gmail.com , Tel: (00226) 71333129/ 00226 76431355

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ABSTRACT

Objective: Cowpea, *Vigna unguiculata* (L) Walp, a very important legume, is a source of protein for thousands of people worldwide. Cowpea is a crucial crop for achieving food security. However, several abiotic and biotic factors are constraints to its production. This study is a contribution to the development of lines resistant to the pod borer (*Maruca vitrata)* and *Striga gesnerioïdes*.

Methodology and results: one hundred (100) F_2 individuals' segregation were produced and evaluated for resistance to *Maruca vitrata* and *Striga gesnerioïdes*. The F_2 population derived from the cross between 709A (*Maruca* resistant) and IT98K-205-8 (Striga resistant) and their parents seeds were planted in pots of 10 (L) filled with topsoil which served as subtracts for plants. 2.5 g fertilizer (NPK) was applied before planting cowpea seeds. A single seed was planted per pot and watered daily. ELISA kits were used to determine the inheritance of the resistance induced by the *Bt* gene against the pod borers. The inheritance of the resistance *Striga gesnerioïdes* race 1 and Kp was determined through SSR1, a molecular marker linked to the gene for resistance to this weed. Inheritance of the genes (resistance to *Maruca vitrata* and *Striga gesnerioïdes*) and observed phenotypic ratios show that the expression of both genes of resistance is monogenic with dominance. 48% of the F_2 population has both resistance genes (to *Maruca vitrata* and *Striga* race 1 and KP).

Conclusion and application of results: Inheritance pattern showed that resistance to *Maruca vitrata*, race 1 and Kp of *Striga gesnerioïdes* were under the control of a single dominant gene. Understanding the mode of inheritance is a prerequisite for developing appropriate breeding strategies to develop cowpea lines with both stable resistance to *Maruca vitrata* and *Striga gesnerioïdes* and interesting agronomic traits (yield, earliness). A direct application of this study will allow to apply backross process for the improve cowpea varieties against these pests (Striga

and pods borer). Another application of this work will be to use enzyme-linked immunosorbent marker to control the entry of genetically modified organisms into Burkina Faso border.
Keywords: cowpea, *Maruca vitrata*, *Striga gesnerioïdes*, Inheritance, *Bt* Gene, Burkina Faso

INTRODUCTION

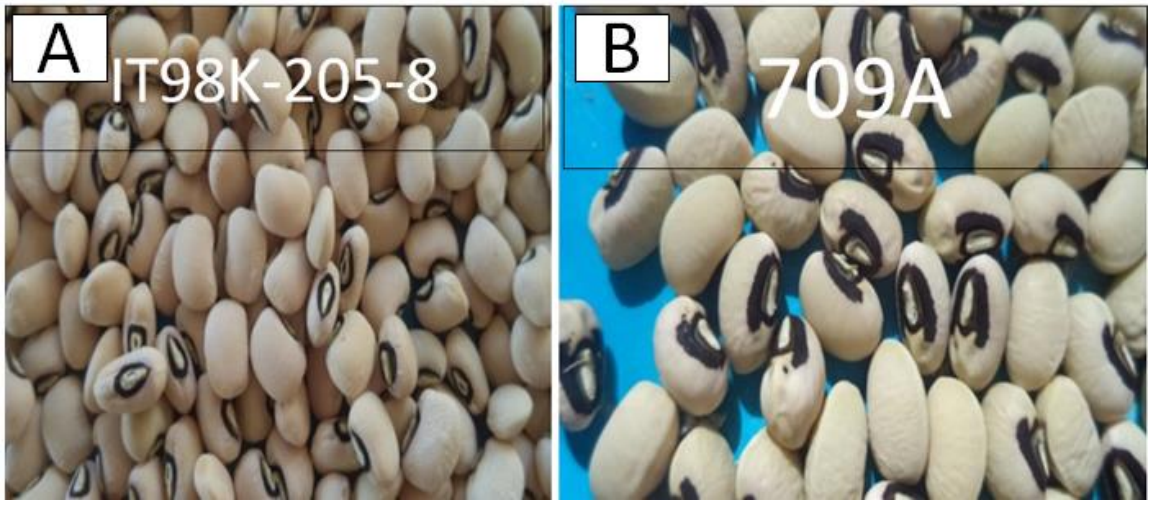
Cowpea is an important food in sub-Saharan Africa, particularly in the arid savannahs of West Africa (MURDOCK *et al.,* 2008). In Burkina Faso, its production was estimated at 630.960 tons in 2018, (Ilboudo *et al.,* 2014). Cowpea is a valuable source of vegetable protein, vitamins for humans and fodder for animals. Immature leaves and pods are consumed as vegetables (PASQUET *et al.,* 1998). It is referred as fertilizer crop in crop rotation system its good atmospheric nitrogen fixation ability. Cowpea can also provide substantial income to producers. However, cowpea production is hampered by biotic and abiotic constraints. Among the biotic constraints, insects are one of the major threats. Indeed, at all stages of vegetative growth, numerous insect pests attack cowpeas. The main insect pests of cowpeas in Africa are aphids; flower bud thrips, pod borers (*Maruca vitrata* FAB), pod-sucking bugs and stock browse (Dabire, 2001). Among them, *Maruca vitrata*, cowpea pod borer, causes crop damage resulting in very significant economic losses to growers. Indeed, this insect, which feeds on cowpea flowers and pods, can cause losses ranged from 20% to 80% (Atachi *et al.,* 2007). In response to this constraint, several control methods have been developed. The chemical method presents immediate results but involves several dangers for the environment, human and animal health. The chemicals used against *Maruca vitrata* are composed of two groups: pyrethroids and organophosphates. The pyrethroids are composed of deltamethrin, cypermethrin and lamda-cyalothrin, while the group of organophosphates contains fenitrothim and dimethoate (Guevremont *et al.,* 1989). The use of these products over time may induce resistance in insects. Using resistant varieties is a healthy, economic and environmental-friendly control method. However, this method has limitations because so far there is no cowpea variety with resistant to *Maruca vitrata* (Okeyo-Owuor *et al.,* 1991). Therefore, research has been undertaken under the *Bt* cowpea project funded by the African Agriculture and Technology Foundation (AATF) and resulted in the introgression of the *Bt* gene into improve varieties to increase yield. However, the inheritance of the *Bt* gene remains unknown. The present study aimed to determine the inheritance of the resistance induced by the *Bt* gene against *Maruca vitrata* as well as the inheritance of *Striga gesnerioïdes* resistance in cowpeas in Burkina Faso. The inheritance study of *Striga gesnerioïdes* resistance is explained by the fact that the recurrent parent used for the crosses is *Striga* Kp resistant.

MATERIALS AND METHODS

Experimental site: The study was conducted in pots under a sleeve cage at INERA/Kamboinsé during the rainy season 2013-2015.

Materiel vegetal: The genetic material used is composed of two (2) lines including one transgenic (709A) (photo 1) and one improved variety (IT98K-205-8) introduced from the International Institute of Tropical Agriculture (IITA). The transgenic line 709A, resistant to *Maruca vitrata* was obtained from genetic modification of line IT86D-1010 of Nigerian (IITA). It has white seeds, thin leaves and has an early cycle. Line IT98K-205-8 is an erect early maturing (60 days) variety with white seeds, it was developed in IITA.IT98K-205-8 is drought tolerant (250 to 550 mm) and resistant to four (4) of the seven races of *Striga*

gesnerioïdes identified in West and Central Africa (Muranaka *et al.*, 2008). SG1, SG2, SG3, SG5 occur respectively in Burkina Faso, Mali, Nigeria and Cameroon. Study of inheritance of the *Maruca vitrata* resistance gene, 709A line was crossed with the non-transgenic line, IT98k-205-8 susceptible to the pest *Maruca vitrata* but resistant to *Striga gesnerioïdes*. The F_1 seeds obtained from this crossing were advanced to F_2 by self-pollination. F_2 populations were generated from F_1 individuals from the cross (IT98K-205-8/709A). One hundred (100) F_2 seeds were used in the inheritance study.



(A): Conventional variety, IT98K-205-8, (B): transgenic variety, 709A
Photo 1: Seeds of different varieties of Cowpea

Molecular markers used: The molecular markers were composed of an enzyme immunoassay kit to detect the presence of the *Bt* gene and a microsatellite marker (SSR1) to detect resistance to *Striga gesnerioïdes*. This kit, called EnviroLogix QuickStix kit for Cry1Ab, is designed to detect the presence of the *Bt* protein. Kit consists primarily of a buffer solution and Cry1Ac and Cry1Ab protein detection kits (QuickStix). It's designed to be single-use test. The microsatellite marker or Simple Sequence Repeats (SSRs) has been used for the identification of *Striga gesnerioïdes* resistance gene. For this purpose, PCR reactions were run using a thermo cycler of the Eppendorf master cycler gradient 5332 version 2.30 marker. The marker has the sequence.

SSR1_
Forward: CAAGAAGGAGGCGAAGACTG
SSR1_ Revers: CCTAAGCTTTTCTCCAACTCC

Methodology: The study was conducted in pots under a sleeve cage at INERA/Kamboinsé during the rainy season 2013-2015. A segregating F_2 population was used to assess resistance to *Maruca vitrata* and *Striga gesnerioïdes*. The segregating population was planted in 10 (L) pots containing a mixture of sand and soil which served as a nutrient carrier for the plants. Mineral fertilizer (NPK) was applied at a dose of 2.5g before planting. In each pot, one seed was planted and watered daily. Seeds from each parent (IT98K-205-8 and 709A) served as a control. The F_1 and F_2 populations derived from the cross IT98K-205-8/709A, population seeds were used.

Production of segregated populations: Study of inheritance of the *Maruca vitrata* resistance gene, 709A line was crossed with the non-transgenic line, IT98k-205-8 susceptible to the pest *Maruca vitrata* but resistant to *Striga gesnerioïdes*. The F_1 seeds obtained from this crossing were advanced to F_2 by self-

pollination. One hundred (100) F_2 seeds were used in the inheritance study.

Identification of resistance to *Maruca vitrata* using the enzyme-linked immunosorbent marker: Leaves of the 100 F_2 individuals from the cross between IT98K-205-8/709A were individually tested with the QuickStix buffer solution to detect the presence of the Cry1Ac/Cry1Ab proteins as recommended by the manufacturer's (EnviroLogix Inc.) recommendations. Young cowpea leaves were collected and ground with a suitable pestle in a sterilized and labelled Eppendorf tube. Thereafter, 500µl of the previously diluted QuickStix buffer solution and stirred to homogeneity before dipping the test strip into the Eppendorf tube. Ten minutes later, le number of bands on the strip was recorded (Photo 2). A single band (B) which is the control line means that the solution does not contain the Bt protein. On the other hand, the presence of two bands infers the presence of the target protein (C).

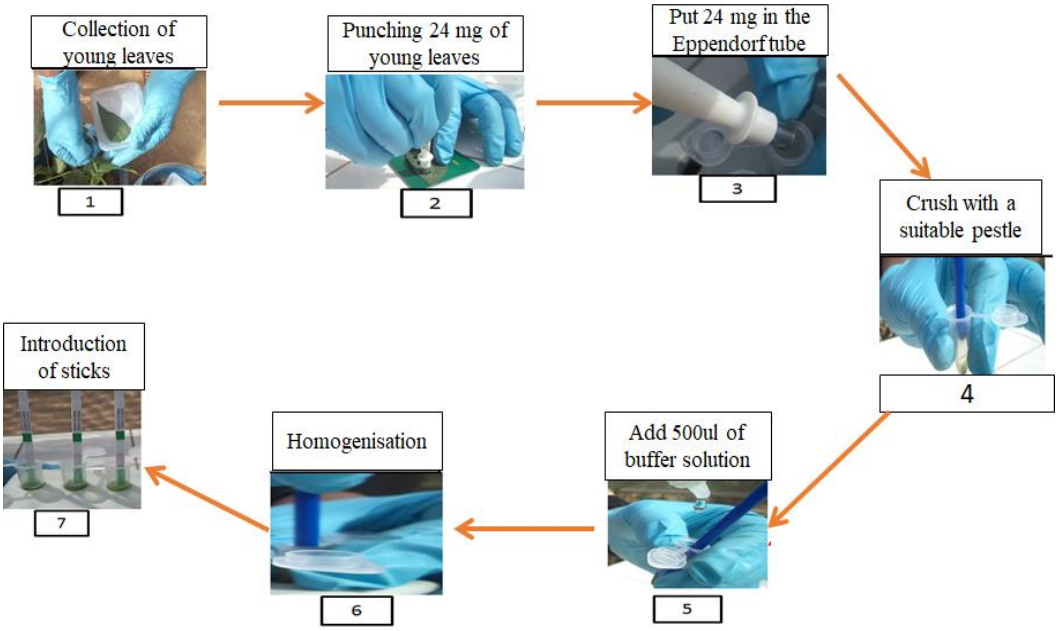


Photo 2: Procedure for checking the presence or the absence of the *Bt* protein

After the identification of the resistance to *Maruca vitrata*, *Striga gesnerioïdes* resistance of the second was done by using SSR1 molecular marker.

Identification of *Striga gesnerioïdes* resistance using the SSR1 marker: This step consisted of genomic DNA extraction from cowpea plants of the two parents and their 100 F_2 offspring, DNA amplification through, and electrophoresis with agarose gel.

i. **DNA extraction:** Fresh young leaves of cowpea were collected and ground on FTA card. The cards with DNA prints left dry at room temperature, a 1mm diameter disc is punched on these cards. This disc was washed twice with 200µl of FTA stamper and then rinsed twice with the same amount of TE stamper (Tris EDTA). It is then dried at room temperature and transferred to the PCR tube for amplification.

ii. **Amplification of microsatellite marker loci:** The amplification of the microsatellite loci was done by PCR, in a reaction volume of 25µl. Each PCR tube contained 2µl at (1µl of the forward and reverse, 10 µM); 5µl of PCR premix (the premix contains 1U of Taq polymerase, 250µM of Tris-HCL, 10mM KCl, 1.5 mM

MgCl2), 18µl of ultrapure water and finally the disc from the FTA card (2 ng of DNA) containing the genomic DNA to be amplified. The PCR program used consisted of: an initial denaturation for 2 min at 94°C, a denaturation of 45s at 94°C, an annealing of 45s at 51°C, 43 cycles comprising the denaturation steps of 45s at 94°C, annealing at 51°C for 45s and the elongation step at 72°C for 1mn 30s.

iii. Revelations of amplification products: Electrophoresis was run on 2% agarose gel in which Ethidium Bromide (BEt) was incorporated as fluorescent developer. 10 ul of amplified product was loaded in the gel and electrophoresis was run for 1 hour 10 ul of amplified product was loaded in the gel and electrophoresis was run for 2 hours in 0.5 × TBE (Tris Borate EDTA) buffer, at a voltage of 80 V and an intensity of 50 mA. At the end of the electrophoresis, the revelation of the amplified products was carried out under ultraviolet light. Images of the gels revealing the bands were taken with 30 minutes in 0.5 × TBE (Tris Borate EDTA) buffer, at a voltage of 80 V and an intensity of 50 mA. At the end of the electrophoresis, the revelation of the amplified products was carried out under ultraviolet light. Images of the gels revealing the bands were taken with a Canon Power Shot A620, 7.1 Megapixel camera.

Statistical analysis: The data collected for both *Bt* gene and *Striga gesnerioïdes*. resistance was subjected to chi-square test ($\chi 2$) with a degree of freedom equal to 1, using Xlstat Pro version 2013 software. The equation of the test uses is as follows:

$X^2 = \Sigma$ (observed numbers ─ expected numbers) 2/ expected numbers

This correction is applicable when the number of degrees of freedom is equal to one. The Chi-square test is used to calculate the probability that differences between observed and expected numbers are due to chance. The threshold for rejecting the null hypothesis was set at 5%. A segregation ratio was therefore declared unlikely when its Chi-square value corresponded to a probability of less than 5%. This test is a comparison of an observed frequency distribution with a theoretical frequency distribution. It is used in genetics where experimental results of cross-breeding for a given trait are compared with those resulting from Mendelian transmission for that trait. If the calculated Chi-square is lower than the control Chi-square, the sample under study has been extracted from a population with the same distribution of the trait under study. If the calculated chi-square is greater than the control chi-square, the study sample was drawn from a population with a different distribution of the trait of interest.

RESULTS

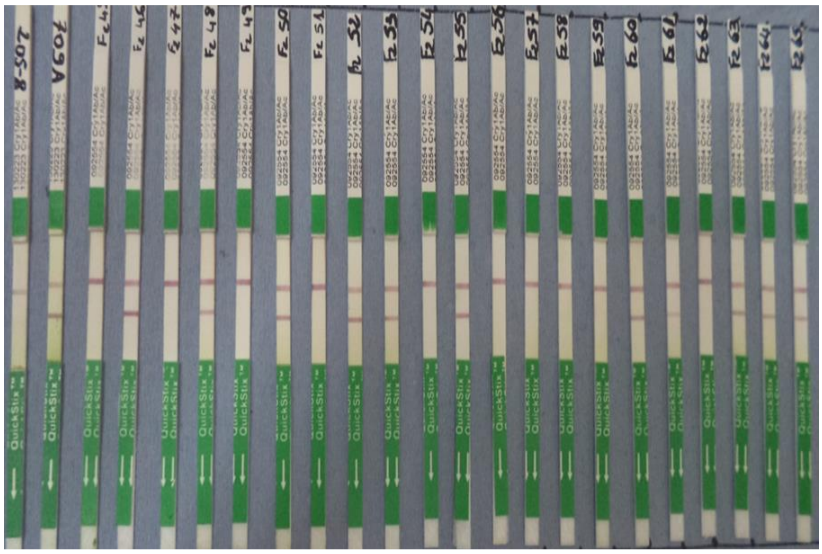
Genetic inheritance of *Maruca vitrata* resistance gene: The results of the test are recorded in Table 1 and reveal that out of the 100 F_2 individuals tested by ELISA, 67 individuals were resistant and 33 individuals are susceptible. The Chi-square test gives a value of 3.41 at the 5% threshold. The calculated Chi-square value (3.41) is lower than the control Chi-square value (3.84). This result rejects the null hypothesis and accepts the alternative hypothesis that the Bt gene inserted is dominantly expressed. The segregation of this F_2 population with a ratio of 3 resistants: 1 susceptible respects Mendel's law for the case of dominant monogenic gene. The expression of resistance to *Maruca vitrata* is therefore dominantly expressed.

Table 1: Results and Chi-square test of the segregated F_2 population for resistance to *Maruca vitrata*

Total	observed resistant	observed susceptible	Expected resistant	Expected susceptible	Ratio	X^2 (df=1)[a]	X^2 table	P (<5%)
100	67	33	75	25	3 :1	3,41	3,49	<0,01

(df=1) a: degree of freedom

Photograph 3 shows the results of a test for the presence or absence of the *Bt* protein in F_2 progeny from the cross IT98K-205-8/709A. The presence or absence and number of traits on the rods determine the status of each F_2 progeny. Resistance is indicated by the presence of two bands and susceptibility by a single band. This makes it possible to discriminate the 100 individuals of the F_2 progeny with respect to resistance or sensitivity to *Maruca vitrata.*



Two bands=presence of Bt protein; one band=absence of Bt protein
Photo 3: ELISA test of F_2 offspring derived from IT98K-205-8 /709A

Genetic inheritance of *Striga gesnerioïdes resistance* gene: Results showed 79 resistants for 21 susceptible out of 100 F_2 individuals. The calculated Chi-square value was 0.85 at the 5% threshold. This value (0.85) is lower than the theoretical Chi-square value (3.841) Table 2. This result rejects the null hypothesis and accepts the alternative one indicating that the gene for resistance to Striga races 1 and Kp is dominantly expressed. The segregation of this F_2 population with a ratio of 3 resistant: 1 sensitive respects Mendel's law of homogeneity. Resistance is dominant.

Table 2: Results and Chi-square test of the segregated F_2 population for resistance to Striga resistance

Total	Observed resistant	Observed susceptible	Expected resistant	Expected susceptible	Ratio	X^2 (df=1)[a]	X^2 table	P (<5%)
100	67	33	75	25	3 :1	3,41	3,49	<0,01

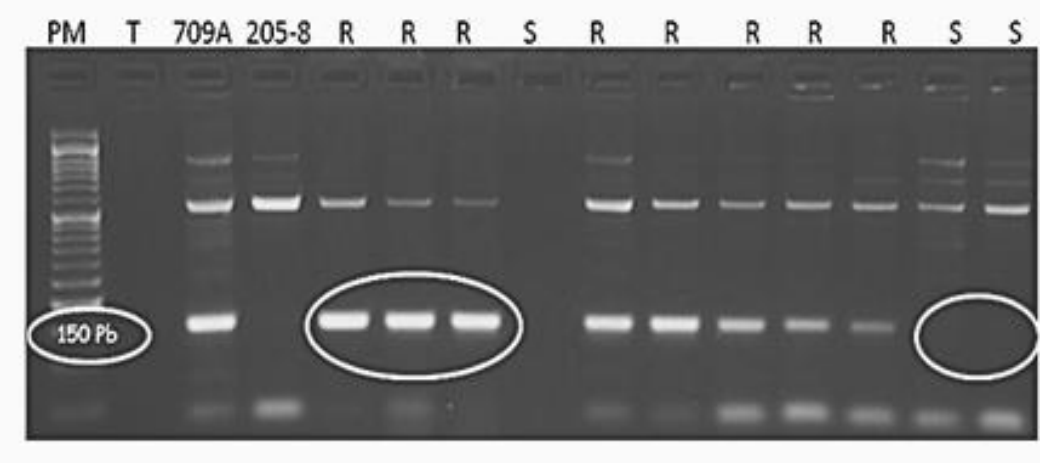


Photo 4: gel profiles showed the F_2 plants possessing the *Striga gesnerioïdes* resistance gene
PCR program: JB 52; Agarose gel: 2%; Revelation: BET: 5 µl; Migration time: 1h, voltage: 75 v, intensity: 50mA
R: resistant Striga gesnerioïdes and S: susceptible to Striga gesnerioïdes
709A: transgenic line resistant to *Maruca vitrata* and susceptible to *Striga gesnerioïdes*
IT98K-205-8: non-transgenic line, resistant to *Striga gesnerioïdes* and susceptible to *Maruca vitrata*

The gel profiles showed the F_2 plants possessing the *Striga gesnerioïdes* resistance gene. The band indicating resistance to *Striga* race 1 and Kp is located at 150 base pairs (Pb) from the bande. Resistant plants presented the band for resistance at 150 Pb which was absent for susceptible plants (Photo 4).

DISCUSSION

The stability of expression and transmission of transgenic genes has been extensively studied. Duan *et al* (1996) demonstrated by PCR analysis that the potato proteinase inhibitor II transgene inserted in rice had a stable inheritance pattern over the four generations studied. Müller et al (1992) also reported high meiotic transmission fidelity of the transgenes to their progeny. DNA and protein extraction from the different genetically modified rice lines confirmed the monogenic and dominant character of the Cry1Ab protein up to the sixth generation (R6) under field conditions (Datta *et al.*, 1990; Shimamoto *et al.*, 1989; Wu *et al.*, 2002). In this experiment, the Mendelian segregation pattern observed was 3/4 and 1/4. This segregation pattern of the cry1Ab gene (*Bt* gene) inserted in the rice genome showed that it is monogenic and dominantly inherited. The results of the present study are conforming to those of Datta *et al.*, (1990), WU *et al.*, (2002). The results of inheritance study of the *Striga resistance* gene of race SG1 and Kp revealed phenotypic ratio of 3: 1 and the probability at the 5%. Compared with Mendelian ratios, this ratio corresponds to that observed for a single gene. This result leads to the conclusion that the gene for resistance to *Striga gesnerioïdes* race 1 and Kp is dominantly expressed. It confirms the results

of Aggarwal and Ouedraogo (1989), Atokple *et al.,* (1985), Dube *et al.,* (2000) and Ouedraogo *et al.,* (2001) which state that the *Striga gesnerioïdes* race 1 resistance genes found in HTR and Wango-1 cultivars are not related to those found in B301 and IT82D-849 and that the Wango-1 resistance gene is the same in HTR unless these two genes are strongly related. Thus, the ratio obtained was consistent with the Mendelian ratio for a single gene. Indeed, Dube in (2000) found a phenotypic ratio of 3 resistant: 1 susceptible and concluded that the inheritance of the resistance gene to the *Striga gesnerioïdes* race SG1 in the two cowpea genotypes, HTR and Wango-1 is dominant and monogenic. AggarwaL and Ouedraogo (1989), and Ouedraogo *et al.,* (2001) asserted that these two varieties possess the Striga-resistant Rsg1 gene found the same results. This gene present in varieties IT93K-693-2 and B031 is monogenic and dominantly inherited. Investigation into the inheritance of the *Bt* gene and the inheritance of resistance gene to race 1 and Kp of *Striga gesnerioïdes* may have beneficial implications for cowpea improvement. These two genes, with monogenic and dominant inheritance, could be easily transferable into susceptible lines to both constraints. Since cowpeas are predominantly self-pollinated, pedigree and backcross selection may be the most appropriate methods to adopt for cowpea line breeding.

CONCLUSION AND APPLICATION OF RESULTS

The losses caused by *Maruca vitrata* and *Striga gesnerioïdes* in cowpea cultivation are considerable. These two constraints threaten the food security of several million people in West Africa. Due to the lack of natural sources of genetic resistance to *Maruca vitrata* in cowpeas, research has used modern plant breeding techniques through transgenesis to produce a genetically modified cowpea. In both research cases (resistance to *Maruca vitrata* and *Striga gesnerioïdes*), the results of the study of inheritance pattern showed that resistance to *Maruca vitrata* is under the control of a single dominant gene. The same results were observed for resistance to race 1 and Kp of *Striga gesnerioïdes* prevalent in Burkina Faso. Monogenic resistance to these dominant traits observed in both genes (*Bt* gene and resistance gene to race 1 and Kp of *Striga gesnerioïdes*) could be very easily transferred to lines sensitive to *Maruca vitrata* and *Striga gesnerioïdes* but with good agronomic qualities. Using both markers in cowpea plant breeding will help to reduce time for improvement varieties and may allow pyramiding of the two genes in cowpea varieties. Which will allow to fight effectively these two pests and raise the production of the small producers, protect the environment and their health.

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