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Authors: Sanou, Apolline, Traoré, Fousséni, Ba, Malick Niango, Dabiré-Binso, Clémentine L, Pittendrigh, Barry R, et al.

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Effects of Volatiles from *Clavigralla tomentosicollis* Stål. (Hemiptera: Coreidae) Adults on the Host Location Behavior of the Egg Parasitoid *Gryon fulviventre* (Crawford) (Hymenoptera: Scelionidae)

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Apolline Sanou^{1,2} , Fousséni Traoré¹, Malick Niango Ba^{1,3}, Clémentine L Dabiré-Binso¹, Barry R Pittendrigh⁴ and Antoine Sanon² 

¹Laboratoire Central d'Entomologie Agricole de Kamboinsé, Institut de l'Environnement et de Recherches Agricoles, Ouagadougou, Burkina Faso. ²Laboratoire d'Entomologie Fondamentale et Appliquée, UFR/SVT, Université Ouaga I Pr Joseph Ki-Zerbo, Ouagadougou, Burkina Faso. ³West and Central Africa Research Program. International Crops Research Institute for the Semi-Arid Tropics, Niamey, Niger. ⁴Department of Entomology, Michigan State University, East Lansing, MI, USA.

ABSTRACT: The egg parasitoid *Gryon fulviventre* is a potential biological control agent of *Clavigralla tomentosicollis*, a coreid pod-sucking pest of *Vigna unguiculata*. The host location behavior of naive parasitoid females was studied using a four-armed olfactometer. Two strains of *G. fulviventre* parasitoids from Burkina Faso and Benin were exposed to odors provided by healthy and infested pods as well as *C. tomentosicollis* females and males. The time spent in each odor zone was recorded to determine the preference of parasitoid females. Results show that odors from healthy pods, infested pods, and pest females did not attract the parasitoid. However, a significantly attractive response of both strains of *G. fulviventre* was recorded in the presence of volatiles from males of *C. tomentosicollis*. Moreover, experiments testing *G. fulviventre* females' behavior when simultaneously exposed to volatiles from cowpea pods (healthy and infested) and increasing numbers of *C. tomentosicollis* males revealed a significantly higher attraction of parasitoid females of both strains by volatiles from ten males of *C. tomentosicollis*. The results suggest that the males of the insect pest emit a pheromone used as kairomone by parasitoids to locate their host. The conditions determining this attractiveness at field level and its impact on host-searching efficiency are discussed.

KEYWORDS: *Gryon fulviventre*, odor source, parasitoid attraction, biological control, cowpea pod-sucking bug

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CORRESPONDING AUTHOR: Antoine Sanon, Laboratoire d'Entomologie Fondamentale et Appliquée, UFR/SVT, Université Ouaga I Pr Joseph Ki-Zerbo, Avenue Charles De Gaulle, Ouagadougou 7021, Burkina Faso. Email: sanonant@gmail.com

Introduction

Insect pests are an important constraint to cowpea (*Vigna unguiculata* (L.) Walp.) production in the tropics, including in Burkina Faso where the coreid pod-sucking bug, *Clavigralla tomentosicollis* Stål. (Hemiptera: Coreidae) is a key pest.¹ *Clavigralla tomentosicollis* nymphs and adults cause high economic losses on cowpea by feeding on the pods.² The damage is greater when the infestation occurs during flowering or pod-filling stage, and a treatment threshold is set at two to four larvae.³

Several control strategies, including cultural control methods and host plant resistance, have been explored with limited success.⁴ To date, the most effective control strategy relies on the spraying of synthetic insecticides. However, these pesticides are often not affordable to small-scale farmers and illiteracy can lead to their misuse which can generate many adverse effects.¹ To find healthy and ecologically sustainable control method, our research has focused on biological control. Biological control of *C. tomentosicollis* using parasitoids has been less explored. Natural parasitism by the egg parasitoid *Gryon fulviventre* (Hymenoptera: Scelionidae) has been recorded on over 52% of the target insects in Nigeria and Burkina Faso.^{5,6}

Natural parasitism by the egg parasitoid is low at the beginning of cowpea growing season but becomes high by the end of season leading to substantial reductions in the pest population.^{5,6} Although this observed high parasitism suggests a potential use of *G. fulviventre* to control *C. tomentosicollis*, to date, little attention has been given to this alternative control measure. Moreover, there is very little information on *G. fulviventre*'s bio-ecology and behavior.

The natural occurrence of parasitoids on a given pest does not necessarily make them effective biocontrol candidates. The selection of a parasitoid as a biological control agent relies on several requirements, including reproductive potential, effective mass rearing, and host-searching efficiency. Usually, parasitoid host searching is activated by a stimulus, such as complex volatiles from host insect and/or its host plant.^{7,8} In several parasitoid species, effective detection of this stimulus depends on the parasitoid's ability to discriminate between a blend of volatiles produced both by herbivore-damaged plants and host insects.^{9,10} High host-searching efficiency is also known to be a key factor determining the success of several parasitoid species used in large-scale biological control attempts.¹¹⁻¹³

Sources of volatile compounds that may influence the parasitoid's research behavior include the host plant (cowpea pods) and



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Figure 1. Four-arm olfactometer device used for the different tests. Picture: Apolline Sanou.

insect pest stages (particularly adults and eggs). Preliminary studies testing egg masses of *C. tomentosicollis* as volatiles source for females of *G. fulviventre* showed no significant host-searching behavior. Since then, in this study, we investigated the role of volatiles produced by cowpea pods and *C. tomentosicollis* adults on the host selection process of females from two strains of the parasitoid wasp *G. fulviventre*. The results could contribute to identify factors influencing host searching and to determine their importance in the implementation of a large-scale biological control strategy based on *G. fulviventre*.

Materials and Methods

Insect mass rearing

Strains of both *C. tomentosicollis* and *G. fulviventre* used in this study were derived from a laboratory mass rearing facility at the International Institute of Tropical Agriculture (IITA) in Cotonou, Benin. In the rearing room, the average temperature was $26.15^{\circ}\text{C} \pm 1.54^{\circ}\text{C}$ and the mean relative humidity was $65.9\% \pm 7.6\%$. *Clavigralla tomentosicollis* nymphs and adults were supplied with fresh pods of a continuous crop of the sensitive Benin landrace cowpea variety, *Kpodji-guè-guè*, and were reared in cylindrical boxes (1900 mL each). Two strains (one from Burkina Faso and one from Benin) of the parasitoid *G. fulviventre* were reared on eggs of *C. tomentosicollis*. For this purpose, one mated *G. fulviventre* female was introduced in a cylindrical box (360 mL) for 48 hours with fifty fresh eggs of *C. tomentosicollis*. Emerging bug larvae hatching from non-parasitized eggs were removed from the box. The parasitized eggs were then monitored, and the subsequent generation of parasitoids emerged 16 days after parasitism occurred. Both strains of parasitoids were used in rearing and experiments as well.

For the olfactory tests, naive emerging *G. fulviventre* females were kept by pairs with males for mating prior to testing them. Burkina Faso and Benin are quite different in their climatic conditions which can influence insect ecology and behavior. That is the reason why both parasitoid strains were used in the hypothesis of a behavioral difference.

Obtaining the odors for olfactory tests

Several sources were used to obtain the tested odors. First of all, cowpea pods were collected from field at the IITA station. The harvested pods were previously washed with tap water to clean any other odor before use. The pods were placed under a stream of tap water for a few minutes without scrubbing to remove any insect odors that may be initially present. This does not change the structure of the pod and its odors and allows infestation with a controlled number of insects.

Infested pods were obtained by placing freshly harvested and cleaned pods with *C. tomentosicollis* adults and nymphs for 24 hours (24-hour infested pods) and 48 hours (48-hour infested pods). A total of five pods of each type were used for the experiment. Pods infested in this way for 48 hours are damaged enough to change the volatile compounds they emit. The healthy pods were not exposed to the insect prior to the trial. Three-day-old males and females of *C. tomentosicollis* collected from the rearing boxes were also used as source of odor. The insects used to produce odors were not provided with food over the course of the test.

Olfactometer setup

The response of *G. fulviventre* to volatiles produced by cowpea pods and *C. tomentosicollis* adults was investigated using a four-armed olfactometer previously described.¹⁴ This device was expected to determine the discriminatory ability of insects to more than two sources of odor at the same time. The device allows testing three different odors and one control at the same time as in a previous study.¹⁵ The four-armed olfactometer is a complex device that includes a central unit called the orientation chamber in which the insect can move freely. The chamber has four openings 90° apart that allow the entry of air. A hole which ensures the exit of the air is in the middle of the room. The latter is finally covered with a transparent glass plate. From the end to the orientation chamber, the olfactometer consists of a glass tube containing charcoal for the purification of air, another container of distilled water for humidifying the air, and four flowmeters that calibrate the air inlet. Each flowmeter was connected to an odor source (Figure 1). Clean airflow (wind speed: 300 mL/min) was divided into four subflows, and each subflow passed through one of the four odor sources connected to the four arms of the olfactometer. The device thus made it possible to deliver air loaded with each of the four test odors into the exposure chamber. The air exit hole in the middle of the orientation chamber prevents an accumulation of odors inside.

Experimental procedure

Mated, three-day-old, naive *G. fulviventre* females (without any prior contact with cowpea pods or *C. tomentosicollis* adults) were introduced individually into the exposure chamber and their behavior was observed for a maximum of 5 minutes. Six sets of four-odor combinations (three odors and one control) were tested (Table 1). The choice of three,

Table 1. Details of experiments to determine the influence of volatiles produced by cowpea pods and *Clavigralla tomentosicollis* adults on the host location behavior of *Gryon fulviventre* females.

EXPERIMENTS	ODOR COMBINATIONS USED IN THE FOUR ARMS OF THE OLFACTOMETER
1	Healthy cowpea pods, 24-hour infested cowpea pods, 48-hour infested cowpea pods and clean air
2	3 <i>C. tomentosicollis</i> females, 5 <i>C. tomentosicollis</i> females, 10 <i>C. tomentosicollis</i> females and clean air
3	3 <i>C. tomentosicollis</i> males, 5 <i>C. tomentosicollis</i> males, 10 <i>C. tomentosicollis</i> males and clean air
4	Healthy pods, 48-hour infested pods, 5 <i>C. tomentosicollis</i> males and clean air
5	Healthy pods, 48-hour infested pods, 10 <i>C. tomentosicollis</i> males and clean air
6	Healthy pods, 48-hour infested pods, 15 <i>C. tomentosicollis</i> males and clean air

Table 2. Response of both strains of *Gryon fulviventre* females to clean air and odors from healthy pods, 24-hour and 48-hour infested cowpea pods.

	BURKINA FASO STRAIN					
	CLEAN AIR	24-H INFESTED PODS	48-H INFESTED PODS	HEALTHY PODS	N	P VALUE
Mean time spent, s	56 ± 10.3	83 ± 12.8	75 ± 11.4	79 ± 13.1	60	.495 ¹
Mean frequency of choice	0.192 ± 0.024	0.245 ± 0.03	0.290 ± 0.032	0.270 ± 0.035	60	.492 ²
	BENIN STRAIN					
	CLEAN AIR	24-H INFESTED PODS	48-H INFESTED PODS	HEALTHY PODS	N	P VALUE
Mean time spent, s	98 ± 17.8	90 ± 14.2	79 ± 12.5	67 ± 11.5	60	.558 ¹
Mean frequency of choice	0.197 ± 0.032	0.244 ± 0.034	0.305 ± 0.038	0.252 ± 0.032	60	.321 ²

¹Kruskal-Wallis test.²Friedman test; n=number of females tested; P value=probability with $\alpha=5\%$.

five, ten, and fifteen individuals of male and female for pheromone experiment was based on preliminary tests. The increasing number of individuals was expected to yield increasing amount of pheromone which affects parasitoid attraction.¹⁶ A test began once the female started moving, and the time spent in each odor field was recorded. Indirectly, the frequencies of entering in each odor field were recorded. Females remaining motionless for more than 2 minutes at the release point were discarded from the analysis. After testing five naive females, the positions of the odor sources were exchanged to correct for any potentially unforeseen asymmetry in the experimental setup. After testing ten naive female wasps, odor sources were renewed. In total, sixty naive female parasitoids from each strain were tested for each combination. Before each test, the arena was cleaned with laboratory alcohol. In the olfactometer room, the average temperature was 27.2°C and the mean relative humidity was 63.4%.

Data analysis

Data were analyzed to determine differences in the effect of the odor source on the behavior of female parasitoids. The Shapiro-Wilk and Levene tests in R (R Development Core

Team 2014) were used to analyze the times spent by the parasitoids in each odor source to check for normality and homogeneity of variances, respectively. Several series of transformations ($\log x$, $\log(x+1)$, \sqrt{x} , $\sqrt{(x+0.5)}$, $\ln x$, $\ln(x+1)$) were also used without normalizing the data. So, the Kruskal-Wallis test, as an alternative to analysis of variance, was used with $\alpha=.05$. Friedman tests^{17,18} with $\alpha=.05$ were used to analyze the frequency of choice of *G. fulviventre* females for each of the odors tested using XLSTAT752 software (Addinsoft; XLSTAT752).

Results

Response of parasitoid females to odors from healthy and infested cowpea pods (Experiment 1)

The time spent by the parasitoids in the arms receiving odors from cowpea pods and the clean air ranged from 56 to 98 seconds for both strains of parasitoid (Burkina Faso and Benin). Females of both *G. fulviventre* strains were not significantly attracted by volatiles from either healthy or damaged cowpea pods ($P=.495$ and $P=.558$, respectively; Table 2). Similarly, mean frequencies of parasitoid choice among the 4 odors tested did not significantly differ for the Burkina Faso ($P=.492$) and Benin ($P=.321$) strains.

Table 3. Response of *Gryon fulviventre* females to clean air and odors from increasing number of *Clavigralla tomentosicollis* females.

	BURKINA FASO STRAIN					N	P VALUE
	CLEAN AIR	3 FEMALES	5 FEMALES	10 FEMALES			
Mean time spent, s	77 ± 17.01	104 ± 16.6	112 ± 20.2	109 ± 20.5	60	.748 ¹	
Mean frequency of choice	0.211 ± 0.041	0.258 ± 0.043	0.308 ± 0.048	0.222 ± 0.040	60	.616 ²	
	BENIN STRAIN					N	P VALUE
	CLEAN AIR	3 FEMALES	5 FEMALES	10 FEMALES			
Mean time spent, s	108 ± 16	92 ± 14.6	114 ± 18.6	97 ± 17.8	60	.767 ¹	
Mean frequency of choice	0.333 ± 0.048	0.222 ± 0.040	0.208 ± 0.041	0.236 ± 0.041	60	.241 ²	

¹Kruskal-Wallis test.²Friedman test; n=number of females tested; P value=probability with $\alpha=5\%$.**Table 4.** Response of *Gryon fulviventre* females to clean air and odors from increasing numbers of *Clavigralla tomentosicollis* males.

	BURKINA FASO STRAIN					N	P VALUE
	CLEAN AIR	3 MALES	5 MALES	10 MALES			
Mean time in seconds	70 ± 12.3 ^{ab}	55 ± 11.2 ^b	110 ± 15.1 ^a	76 ± 11.8 ^{ab}	60	.037 ¹	
Mean frequency of choice	0.230 ± 0.029	0.219 ± 0.029	0.352 ± 0.042	0.197 ± 0.025	60	.226 ²	
	BENIN STRAIN					N	P VALUE
	CLEAN AIR	3 MALES	5 MALES	10 MALES			
Mean time in seconds	74 ± 12.4	74 ± 13.5	103 ± 13.3	90 ± 15.2	60	.330 ¹	
Mean frequency of choice	0.226 ± 0.031 ^a	0.179 ± 0.025 ^a	0.377 ± 0.038 ^b	0.174 ± 0.028 ^a	60	.001 ²	

¹Kruskal-Wallis test.²Friedman test: for each parameter and *G. fulviventre* strain, significant differences are indicated by different letters; n=number of females tested; P value=probability with $\alpha=5\%$.

Response of parasitoid females to odors from *Clavigralla tomentosicollis* females (Experiment 2)

The parasitoid females were not significantly attracted by odors emitted by females of *C. tomentosicollis* (Table 3). Differences in time spent in the four arms of the olfactometer, as well as frequencies of choice between odors, did not significantly differ for both strains (Table 3).

Response of parasitoid females to odors from *C. tomentosicollis* males (Experiment 3)

Gryon fulviventre females were attracted by odors from males of *C. tomentosicollis* depending on not only the strain tested but also concentration (Table 4). For the Burkina Faso strain, the time spent by the parasitoids in the arm diffusing volatiles from *C. tomentosicollis* males was significantly different ($P=.037$), whereas the frequency of choice among the four odor sources did not differ ($P>.05$). For the Benin strain, only the frequency of choice of *G. fulviventre* females for the odors from *C. tomentosicollis* males was significantly different ($P=.001$).

Discriminatory response of parasitoid females between combined odors from healthy pods, infested pods, and *C. tomentosicollis* males (Experiments 4–6)

As the compounds released by males of *C. tomentosicollis* attracted *G. fulviventre* females, we also tested their response to odors from an increasing number of males in comparison with volatiles from healthy and infested cowpea pods (Tables 5 to 7).

Females of both strains showed no significant difference for the time spent or the frequency of choice for the odor sources tested with five males (Table 5). However, females were attracted by the odors released by ten males of *C. tomentosicollis* as evidenced by the higher frequencies of choice in Burkina Faso strain ($P=.028$). Females in Benin strain showed a discriminatory capacity of the different odors ($P=.008$), but they did not discriminate odors between infested pods and ten males (Table 6). Again parasitoid females of both strains were not significantly attracted when the tested odors were provided by cowpea pods and 15 *C. tomentosicollis* males (Table 7).

Discussion

This study demonstrated that olfactory stimuli are used by *G. fulviventre* females to locate their host *C. tomentosicollis*.

Table 5. Discriminatory response of *Gryon fulviventre* females to volatiles from cowpea pods and five males of *Clavigralla tomentosicollis*.

	BURKINA FASO STRAIN					
	CLEAN AIR	HEALTHY PODS	48-H INFESTED PODS	5 MALES	N	P VALUE
Mean time spent, s	98 ± 17.4	114 ± 16.2	119 ± 17.3	116 ± 19.6	60	.952 ¹
Mean frequency of choice	0.194 ± 0.036	0.314 ± 0.043	0.319 ± 0.048	0.172 ± 0.034	60	.129 ²
	BENIN STRAIN					
	CLEAN AIR	HEALTHY PODS	48-H INFESTED PODS	5 MALES	N	P VALUE
Mean time spent, s	80 ± 10	96 ± 17.9	108 ± 18.3	130 ± 16.9	60	.331 ¹
Mean frequency of choice	0.180 ± 0.035	0.285 ± 0.048	0.266 ± 0.044	0.266 ± 0.045	60	.622 ²

¹Kruskal-Wallis test.²Friedman test; n=number of females tested; P value=probability with $\alpha=5\%$.**Table 6.** Discriminatory response of *Gryon fulviventre* females to volatiles from cowpea pods and ten males of *Clavigralla tomentosicollis*.

	BURKINA FASO STRAIN					
	CLEAN AIR	HEALTHY PODS	48-H INFESTED PODS	10 MALES	N	P VALUE
Mean time spent, s	98 ± 17.7	90 ± 23.4	124 ± 19.6	150 ± 19	60	.116 ¹
Mean frequency of choice	0.229 ± 0.03 ^{ab}	0.137 ± 0.031 ^a	0.234 ± 0.042 ^{ab}	0.398 ± 0.054 ^b	60	.028 ²
	BENIN STRAIN					
	CLEAN AIR	HEALTHY PODS	48-H INFESTED PODS	10 MALES	N	P VALUE
Mean time spent, s	88 ± 17.5	82 ± 15.8	58 ± 9.16	97 ± 14.4	60	.334 ¹
Mean frequency of choice	0.201 ± 0.035 ^{ab}	0.145 ± 0.026 ^a	0.293 ± 0.037 ^b	0.359 ± 0.044 ^b	60	.008 ²

¹Kruskal-Wallis test.²Friedman test: for each parameter and *G. fulviventre* strain, significant differences are indicated by different letters; n=number of females tested; P value=probability with $\alpha=5\%$.**Table 7.** Response of *Gryon fulviventre* females to discriminate volatiles from cowpea pods and fifteen males of *Clavigralla tomentosicollis*.

	BURKINA FASO STRAIN					
	CLEAN AIR	HEALTHY PODS	48-H INFESTED PODS	15 MALES	N	P VALUE
Mean time spent, s	103 ± 15.8	115 ± 16.6	101 ± 17.1	132 ± 17.1	60	.616 ¹
Mean frequency of choice	0.261 ± 0.042	0.216 ± 0.033	0.264 ± 0.043	0.258 ± 0.041	60	.997 ²
	BENIN STRAIN					
	CLEAN AIR	HEALTHY PODS	48-H INFESTED PODS	15 MALES	N	P VALUE
Mean time spent, s	70 ± 10.6	57 ± 10.2	84 ± 11.6	88 ± 14.4	60	.236 ¹
Mean frequency of choice	0.271 ± 0.036	0.199 ± 0.031	0.221 ± 0.034	0.305 ± 0.039	60	.412 ²

¹Kruskal-Wallis test.²Friedman test; n=number of females tested; P value=probability with $\alpha=5\%$.

However, the parasitoid females did not discriminate between volatiles produced by healthy and infested cowpea pods when they were exposed only to cowpea volatiles. For many parasitoid species, pest-damaged plants, including maize¹⁹ and soybean,^{20,21} release substances used as synomones by parasitoids to locate the pest-hosts. Our result suggests, in contrast to other

parasitoid species, that *G. fulviventre* females do not use volatiles from cowpea pods damaged by *C. tomentosicollis* to detect the presence of its host making this kind of indirect plant species defense against pests¹⁸ unavailable to *Vigna unguiculata*.

Previous studies demonstrated that some parasitoid species use their host's pheromones to locate host presence.²²⁻²⁶ We

hypothesize that *G. fulviventre* may be attracted either to a sexual pheromone used by *C. tomentosicollis* males to attract females or an aggregation pheromone released by the males. Such an assumption is based on previous observations indicating the possibility for parasitoids to use host-male insect's pheromones as kairomones to locate the hosts.²⁷⁻³¹

However, the results suggest that this odor attractiveness effect could depend on the number of *C. tomentosicollis* males used. At low and high densities (five and fifteen individuals, respectively), *G. fulviventre* females did not discriminate odors from the males. This finding suggests, on one hand, that a minimal amount of pheromone is first needed to trigger a response in parasitoids and that, on the other hand, the amount of pheromone released may in some way decrease once the pest densities have exceeded a certain threshold. Moreover, simply due to their regrouping in the olfactometer and regulating their density, the insects can inhibit the secretion of the congeners' attractive pheromone.³²⁻³⁴ Such inhibition involves the secretion of anti-aggregation substances,^{33,35} which would not be perceived by the parasitoid or which would not play an attractive role for *G. fulviventre* female. Otherwise, pheromone is a mixture of several compounds and some of the compounds would act as a repellent for parasitoids.^{36,37} An experimental olfactometer offers small and confined space distinct from real environmental conditions. Further experiments in actual environmental conditions could better establish what pest density, if any, triggers parasitoid infestation. Moreover, the precise identification of attractants for female parasitoids is an important step in considering the release of such substances to attract more parasitoids at the right time to prevent the growth of pest populations. In the wild, visual stimuli can also be involved, with or without olfactory signals, in triggering parasitoid activities.³⁸⁻⁴¹ Any role of this for host location behavior in *G. fulviventre* remains to be investigated. Provided that a precise attractive mechanism is characterized to ensure parasitism, then mass rearing and release of *G. fulviventre* at the beginning of the growing season when pest infestation initially occurs could potentially be used to control *C. tomentosicollis* populations.

Conclusions

The results obtained from this study are of great importance in the understanding of host-parasitoid interactions using a model that has been poorly studied, such as the egg parasitoid *G. fulviventre* and the major cowpea pod-sucking bug pest, *C. tomentosicollis*. The main findings indicated that the parasitoid females are attracted by olfactory cues provided by the males of their host species alone, cowpea pods (infested or not) being not attractive. Although precise determination of attractive volatile compounds emitted by *C. tomentosicollis* males remains for future research, the attractiveness of the involved compound depended on the density of males present and probably on the dose of volatiles produced. Subsequent studies could also characterize any role of other factors influencing the biological

potential of this control agent, a necessary step for developing a best practice strategy for an effective protection of cowpea fields in West Africa.

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Author Contributions

The research was conducted by ApS under the supervision of mentors listed as co-authors. All co-authors participated in defining the research methodology and writing/editing the paper.

ORCID iDs

Apolline Sanou  <https://orcid.org/0000-0003-2413-5535>

Antoine Sanon  <https://orcid.org/0000-0002-2046-8503>

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