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Original Paper

Germination Performance of Cowpea (*Vigna Unguiculata*) Seeds Treated with Entomopathogenic Isolates

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Abstract*— The use of bio-control measures as alternative to chemical pesticides is increasingly encouraged on the basis of food safety as chemical pesticides are dangerous to human health. However, limited attention is paid to the impact of these bio-control measures on the germination performance of treated seeds. In this study, the germination performance of stored cowpea seeds treated with isolates of entomopathogens (Beauveria bassiana, Metarhizium anisopliae, Verticillium lecanii and Bacillus thuringensis) usually used as bio-pesticides was investigated. The pure isolates were subjected to liquid fermentation technology using sucrose water as the substrate and the liquid medium. The isolates obtained through liquid fermentation were also inoculated into talc powder as solid medium. The germination viability of the cowpea seeds treated with the liquid and solid mediums of the isolates and stored for ten (10) weeks was determined using the standard germination test adopting the seed germination vigour (SGV) and the seed germination ability (SGA) as parameters. The study revealed that the cowpea seed treatment with the isolates in their liquid and solid mediums showed no significant impact on SGV and SGA when compared with control at the end of 1st and 5th week storage post-treatment. However a significant effect was seen at the end of 10th week storage post-treatment with the liquid medium of Verticillium lecanii showing better germination performance (SGV=58.33±1.67 and SGA=71.67±3.33) among other liquid medium isolates when compared with the control (SGV=68.33±1.67 and SGA=80.00±2.89), and the solid medium of Bacillus thuringensis revealing better germination performance (SGV=58.33±3.33 and SGA=71.67±1.67) among other solid isolates when compared with the control (SGV=68.33±1.67 and SGA=80.00±2.89). This study concludes that isolates of Beauveria bassiana, Metarhizium anisopliae, Verticillium lecanii and Bacillus thuringensis do not reduce germination performance of cowpea seeds when used as bio-control against cowpea storage pest. However, at longer storage post-treatment of cowpea seeds with the isolates, the germination performance may be reduced.

Keywords*— *Germination viability, Entomopathogens, Beauveria bassiana, Metarhizium anisopliae, Verticillium lecanii, Bacillus thuringensis.

I. INTRODUCTION

Cowpea (Vigna unguiculata) which is popularly known as "Beans" is an important staple crop in sub-saharan Africa and food legume in the world, serving human consumption needs as well as being a good source of quality fodder for livestock [1]. It provides a significant amount of dietary calories and serves as a good source of vitamins, minerals and substantial amount of dietary protein [2]. Cowpea seeds and whole grains are rich in carotenoids, precursors of vitamin A, a very good source of vitamin C and contains appreciable amounts of vitamin B complex like thiamin, riboflavin, niacin [3]. It is also rich in minerals like calcium, magnesium, potassium and phosphorous. It also has appreciable amount of iron, zinc and sodium [4].

As at 2017, more than 7.4 million tons of dried cowpeas were produced worldwide, with Africa producing nearly 7.1 million [5]. However, under specific pedoclimatic conditions, abiotic and biotic stresses could cause huge economic and productive loses to cowpea cultivation and storage. Of particular concern is the food safety as regards the toxicity caused by frequent use of chemical pesticides during storage for preservation of the cowpea seed and grain against the cowpea weevils (Callosobruchus maculatus). Callosobruchus maculatus causes damage by reducing the grain weight (dry matter reduction), makes them unsuitable for human and animal consumption, contaminated with live or dead insects, dejection and fragments, and depreciation of the nutritional and commercial values of the infested cowpeas and cause poor germination ability [6].

Cowpea weevil is usually controlled with the use of chemical pesticides, however its use has resulted in serious health implications to man and his environment [7]. These chemical contaminants. as a result of their use in control of stored seed and grain pests can lead to acute poisoning or long-term diseases, such as cancer [8]. An estimated 600 million (almost 1 in 10 people) in the world fall ill after eating contaminated food inclusive of chemical contaminants and 420,000 die every year, resulting in the loss of 33 million healthy life years [9]. In order to provide alternatives to the use of these synthetic pesticides, the use of biological pesticides are

now advocated as they are best used when incorporated into a well-designed integrated pest management (IPM) program. Biological pesticides otherwise known as bio-pesticides are certain types of pesticides derived from natural materials such as microorganisms, plants, animals and certain minerals [10]. They do not have any residue problem, which is a matter of substantial concern for consumers. Microbial bio-pesticides constitute the largest group of broad-spectrum bio-pesticides, which do not target non-pest species and are environmentally non-threatening [11].

Several studies have shown the biopesticidal activity of fungal isolates such as Beauveria bassiana, Verticillium lecanii and Metarhizium anisopliae and that of bacteria isolates especially Bacillus thuringensis against different orders of insect pest in agriculture, especially Lepidoptera, Diptera, Coleoptera, Hymenoptera, and Hemiptera, as well as against other organisms such as mites and nematodes. These microbes are entomopathogenic in nature and specialize in killing insects by penetrating their cuticles into the haemocoel especially the entomopathogenic fungi [12] or sometimes by ingestion before infection is initiated in several cases of Bacillus thuringensis [13]. These organisms have been well studied over the years as biological pest control agents with broad-spectrum activity producing unique insecticidal compounds [14, 15].

Protection of seeds against pathogens and pests using pesticides should not come at the expense of seed quality especially the seed germination viability [16]. Pesticide treatments may have the potential to affect seed germination and overall viability depending on the type of pesticide used, the concentration applied, and the specific characteristics of the seeds themselves [17]. The distribution and persistence use of synthetic chemicals for field and stored pest negatively affects the growth and physiological parameters of the crop including the germination performance of the seed [18]. Cypermethrin, a synthetic pyrethroid insecticide applied to cowpea seed to protect it against attack by C. maculatus was found to inhibit early germination [19].

Owing to the problem of toxicity impacted on treated cowpea seed by different synthetic insecticides applied to protect it against cowpea weevil attack, many studies have explored biological options as potential alternatives to the deleterious synthetic insecticides. However, limited studies have shown the impact of these biological insecticides on the germination viability of the cowpea seed. Beauveria bassiana, Metarhizium anisopliae, Verticillium lecanii and Bacillus thuringensis have been shown in several studies to be eco-friendly and effective against several agricultural field and stored pests and therefore, are considered suitable candidates in research efforts towards developing microbial-based options for protecting cowpea seeds against C. maculatus attack.

This study therefore aimed to investigate the stored cowpea seeds germination performance when treated with Beauveria bassiana, Metarhizium anisopliae, Verticillium lecanii, and Bacillus thuringensis as bio-pesticides.

II. Materials And Methods

A. Source and Maintenance of the Entomopathogenic Isolates

Fungal isolates- *Beauveria bassiana, Metarhizium anisopliae Verticillium lecanii*, and bacterial isolate- *Bacillus thuringensis* were obtained from the Reference Laboratory section of Bio-crops Biotechnology Limited, Abuja, Nigeria. The microbial isolates were subjected to morphological and biochemical test for confirmation. Microbial cell culture was done using Potato Dextrose Agar for fungi and Nutrient Agar for bacteria. Fungal and bacterial culture plates were incubated at room temperature ($28^0C \pm 2^0C$) for 96 and 72 hours respectively. The emerging colonies after incubation period were discretely isolated and sub-cultured repeatedly on freshly prepared Potato Dextrose Agar for fungi and Nutrient Agar for bacteria to obtain pure isolates. The pure isolates were maintained on agar slants bottles and stored at 4^0C for further use. The pure isolates were later subjected to liquid fermentation to maximize the microbial load using sucrose water as substrate and serving as the liquid medium.

B. Source of Cowpea Seeds

Cowpea seeds (Black-eye pea) were collected directly from the study cowpea farm at the National Biotechnology Research and Development Agency, Abuja Nigeria to ensure that no storage pesticide have been used on the seeds. The seeds were packed in a tightly sealed sterile bag and placed inside a tightly covered container and kept at the freezer (0^0F) for further use.

C. Innoculation of the Entomopathogenic Isolates into Talc Powder as Solid Medium

The talc stones were collected from Ejiba town in Yagba West Local Government Area of Kogi State, Nigeria and were processed into fine powder using the mechanical grinding machines. The talc powder was packed into tightly sealed water-proof nylons and then sterilized appropriately using autoclave with a holding time of 15 minutes at 1210C. The sterilized talc powder was stored at room temperature.

The fermented isolates of Beauveria bassiana, Metarhizium anisopliae, Verticillium lecanii, and Bacillus thuringensis in their liquid medium with microbial load of 2.56 x 104CFU/ml, 4.4 x 106CFU/ml, 2.16 x 105CFU/ml and 3.66 x 106 CFU/ml respectively were at 20ml inoculated each into 50g of sterilized talc powder, sealed appropriately and stored at room temperature (280C + 20C). The talc powder-entomopathogenic isolate mixtures at their concentrations were used for cowpea seed treatment after one month of storage.

D. Assessment of the Germination Performance of Cowpea Seeds treated with the Entomopathogenic Isolates

The germination performance of cowpea seeds treated with entomopathogenic isolates was assessed as described by [20]. Cowpea seed lots treated with the entomopathogenic isolates were subjected to germination viability test as follows:

1) Cowpea Seed Treatment with Entomopathogenic Isolates

Twenty (20 ml) of each of the microbial isolates in its liquid medium with concentrations of 2.56×10^4CFU/ml for *Beauveria bassiana*, 2.16×10^5CFU/ml for *Metarhizium*

anisopliae, 4.4 x 10^6CFU/ml for *Verticillium lecanii*, , and 3.66 x 10^6CFU/ml for *Bacillus thuringensis* were each sprayed to 200g of the cowpea seed in sealed sterilized transparent containers. Also 20g of the microbial isolates in its solid medium with concentration of 6.24 x 10^5CFU/g for *Beauveria bassiana*, 6.16 x 10^5CFU/g for *Metarhizium anisopliae*, 5.9 x 10^5CFU/g for *Verticillium lecanii*, and 8.99 x 10^5CFU/ml for *Bacillus thuringensis* were each applied to 200g of the cowpea seed in a sealed sterilized transparent containers and mixed properly. Both carriers of the microbial isolate treatments were used for germination viability study at the end of 1st, 5th and 10th weeks of storage at room temperature (28 $\pm$ 2^0C). The untreated cowpea seeds stored at same room temperature for same time period was used as control.

2) Germination Viability Study

Germination viability of cowpea seeds treated with entomopathogenic isolates were determined by standard germination test using the Top of Paper Method as described by [20]. The Top of Paper method for determining germination viability is to provide seeds with a substrate (water-moistened paper) that makes water available at proper amount for the seeds to take up into themselves and to germinate.

With a sterilized hand, paper-towel (double-folded) was snugged into sterilized petri-dishes. Appropriate amount of distilled water was added to completely moisten the paper without soaking it. The paper towel in the petri-dishes was kept moist as found necessary. Twenty (20) cowpea seeds treated with the entomopathogenic isolates and the untreated cowpea seeds serving as control were each spread uniformly on the distilled-water moistened paper towel in three replicates, ensuring that none of the seeds touch each other. The petri-dish lids were closed and labeled appropriately and placed in warm area where some light is present. The germination viability study was conducted each for the 1st, 5th and 10th week post-treatment storage.

3) Germination Count

During each of the germination viability study for 1st, 5th and 10th week post-treatment storage, germination counts were taken at 24 h-interval for 6 days after sowing [20]. Seeds were considered germinated when the tip of the radicle had grown free from the seed coat, otherwise known as radical emergence [21]. Germinated seeds were removed after each day counting. The study parameters considered were seed germination vigour (SGV) and seed germination ability (SGA), both representing germination viability [22]. The SGV was calculated based on the germination percentage on the half of the complete days of the experiment (on the 3rd day of the testing), according to the formula by [22]:

$$G\% = \frac{Total\ Number\ of\ seeds\ Germinated}{otal\ Number\ of\ seeds\ Tested}\ X\ 100 \quad \text{.....(1)}$$

The SGA was also calculated using same (1) based on the percentage of seed germination on the final day of the testing (6th day of the testing).

E. Statistical Analysis

Data generated in this study were expressed as mean value $\pm$ standard error of mean (S.E.M). Comparisons between different groups were carried out by Analysis of Variance (ANOVA). Significant differences between the control and experimental groups were determined by Duncan Multiple Range Test (DMRT) using the statistical package for social sciences (SPSS) version 26.

III. Results And Discussion

A. Results

TABLE I. Germination Viability Of Cowpea Seeds Treated With The Liquid Medium Of The Entomopathogenic Isolates

Treatment Samples	Week 1		Week 5		Week 10	
	SGV (%)	SGA (%)	SGV (%)	SGA (%)	SGV (%)	SGA (%)
Control	78.33±6.01^a	93.33±1.67^a	78.33±1.67^a	86.67±1.67^a	68.33±1.67^d	80.00±2.89^d
Beauveria Bassiana	75.00±2.89^a	93.33±1.67^a	75.00±0.01^a	88.33±1.67^a	46.67±1.67^{ab}	58.33±1.67^b
Metarhizium anisopliae	76.67±3.33^a	86.67±3.33^a	75.00±0.01^a	81.67±1.67^a	41.67±1.67^a	50.00±0.01^a
Verticillium Lecanii	68.33±6.01^a	91.67±1.67^a	78.33±1.67^a	86.67±1.67^a	58.33±1.67^c	71.67±3.33^c
Bacillus Thuringensis	76.67±4.41^a	90.00±2.89^a	76.67±3.33^a	86.67±4.41^a	48.33±1.67^b	60.00±0.01^b

Values are in mean ± S.E. Values between experimental treatments on each column bearing the same superscript are not significantly different at 5% level (P>0.05).S.E = Standard error of Mean. SGV= Seed Germination Vigour, SGA= Seed Germination Ability.

TABLE II. Germination Viability Of Cowpea Seeds Treated With The Liquid Medium Of The Entomopathogenic Isolates

Treatment Samples	Week 1		Week 5		Week 10	
	SGV (%)	SGA (%)	SGV (%)	SGA (%)	SGV (%)	SGA (%)
Control	78.33±6.01^a	93.33±1.67^a	78.33±1.67^a	86.67±1.67^a	68.33±1.67^d	80.00±2.89^d
Beauveria Bassiana	75.00±2.89^a	93.33±1.67^a	75.00±0.01^a	88.33±1.67^a	46.67±1.67^{ab}	58.33±1.67^b
Metarhizium anisopliae	76.67±3.33^a	86.67±3.33^a	75.00±0.01^a	81.67±1.67^a	41.67±1.67^a	50.00±0.01^a
Verticillium Lecanii	68.33±6.01^a	91.67±1.67^a	78.33±1.67^a	86.67±1.67^a	58.33±1.67^c	71.67±3.33^c
Bacillus Thuringensis	76.67±4.41^a	90.00±2.89^a	76.67±3.33^a	86.67±4.41^a	48.33±1.67^b	60.00±0.01^b

Values are in mean ± S.E. Values between experimental treatments on each column bearing the same superscript are not significantly different at 5% level (P>0.05).S.E = Standard error of Mean. SGV= Seed Germination Vigour, SGA= Seed Germination Ability.

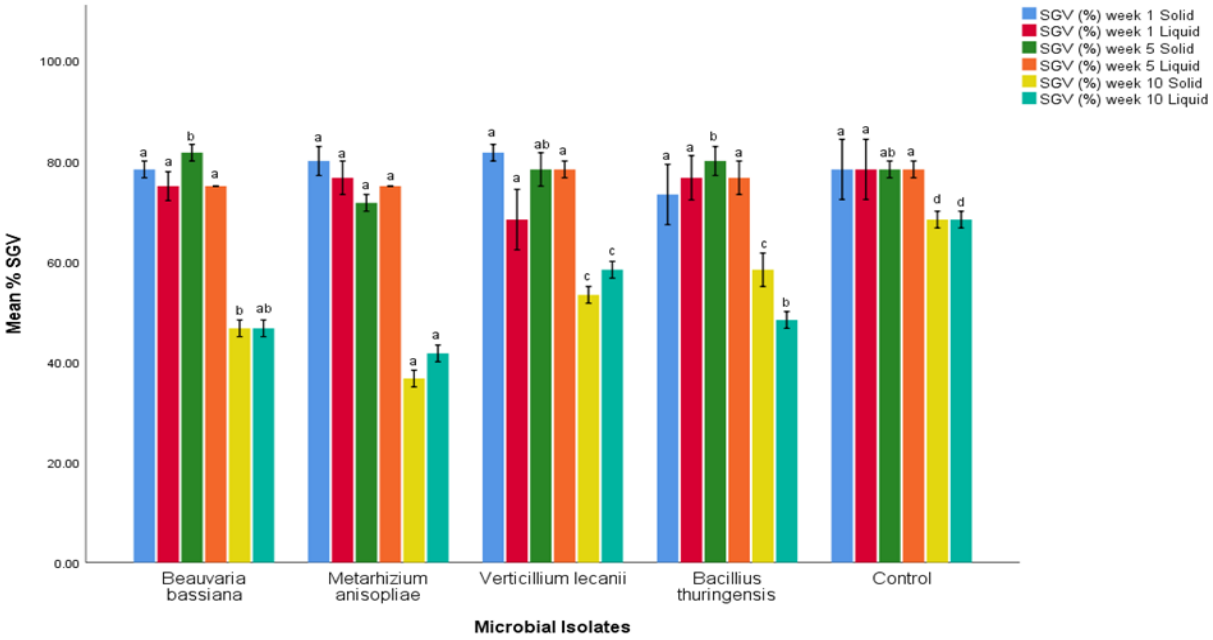

Fig. 1. Comparative Analysis of the Seed Germination Vigour (SGV) of Cowpea seeds Treated with both Solid and Liquid Mediums of the Entomopathogenic Isolates

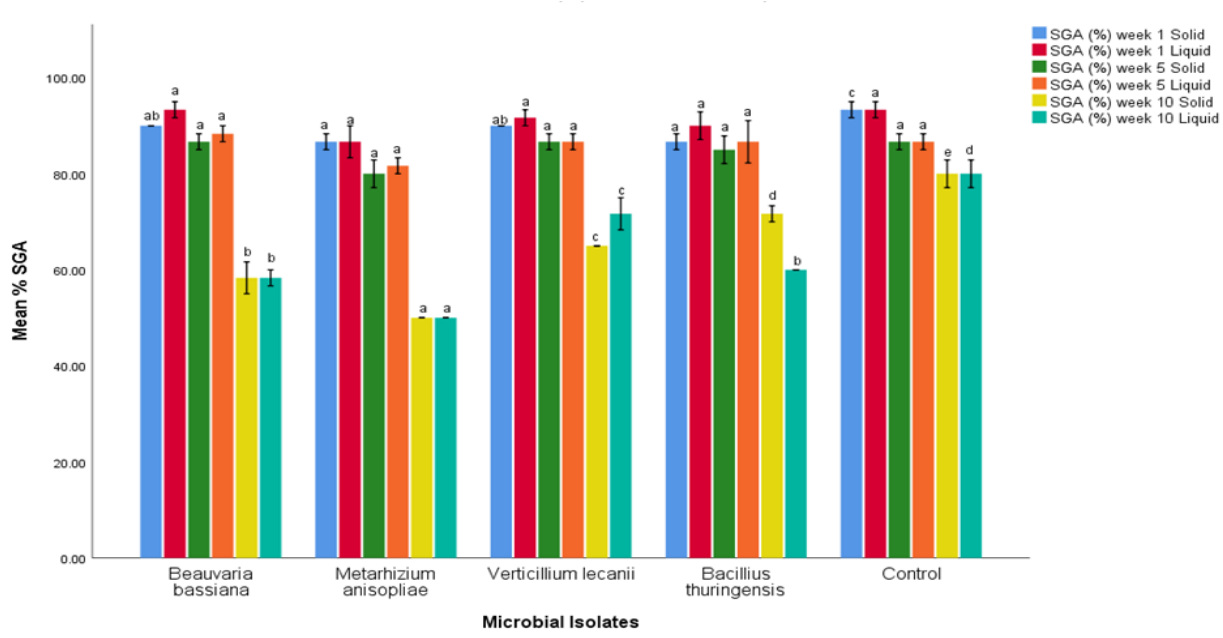

Fig. 2. Comparative Analysis of the Seed Germination Ability (SGA) of Cowpea seeds Treated with both Solid and Liquid Mediums of the Entomopathogenic Isolates

B. Discussion

In this study, the entomopathogenic isolates used to treat the cowpea seeds as biological control measure did not reduce the standard germination performance of the treated cowpea seeds under laboratory condition after 5 weeks of post-treatment storage at room temperature when compared with the control. However, the study as shown in tables 1 and 2 revealed a significant reduction difference in standard germination performance after the 10th week post-treatment storage at room temperature. The treatment of the cowpea seeds with both the liquid and solid mediums showed the same germination performance especially after the 1st and 5th week's post-treatment storage, thereby indicating that the medium or carrier of the entomopathogenic isolates used in this study did not influence the standard germination performance after 5 weeks post treatment storage.

The germination vigour and ability of the liquid and solid mediums of the entomopathogenic isolates showed different

significant reduction in germination performance after 10 weeks post-treatment storage. The liquid medium of the Verticillium lecanii isolate showed better germination performance than other liquid medium of the entomopathogenic isolates with 58% and 71% germination vigour and germination ability respectively when compared with the control that had 68% and 80% germination vigour and germination ability respectively. However, the solid medium of Bacillus thuringensis showed better germination performance more than other solid medium of the isolates with 58% and 71% germination vigour and germination ability respectively when compared with the control that had 68% and 80% germination vigour and germination ability respectively. This reduced viability in both the treatments and the untreated (control) after the 10th week storage when compared with 1st and 5th week storage is in line with the study of [23] which revealed that long-term storage reduced viability of cowpea seeds by between 4 and 12% regardless of the temperature and relative humidity of the storage environment. The significant difference recorded after longer storage period (10 weeks) when compared with the short (1 week) and medium (5 weeks) storage period in both the control and the treatments is an indication that the longer the cowpea seeds are stored, the higher the cowpea seed ageing effect on seed germination indices. However, treatment with entomopathogenic isolates as microbial based-pesticides may increase the cowpea seed ageing effect on seed germination vigour and germination ability.

Microbial biocontrol inoculants for seed treatments when subjected to prolonged periods of storage negatively impact seed viability and may ultimately limit efficacy in the field [24]. It is therefore suggested that cowpea seed lots treated with the entomopathogenic isolates used in this study, are not allowed for a longer storage period specifically when the seeds are stored for planting, so as to ensure good germination performance.

The impressive seed germination vigour and ability in the short-term (1 week) and medium term (5 weeks) storage period showed by the entomopathogenic treated seeds in this study was also reported by [25], where bean seeds treated with fungal isolates, Beauveria bassiana and Metarhizium robertsii showed impressive germination potentials and plant growth in the field more than the control during the first 21 days of planting with germination indices taken at the interval of 7, 14 and 21 days. Similarly, entomopathogens such as Beauvaria bassiana was reported in the study of [26] to improve chili seed germination vigour especially when soaked with the fungal suspension before planting. The inability of the entomopathogens used in this study to negatively affect cowpea seed germination over short storage period may be as a result of their endophytic properties to plants.

Modulation of seedling development by endophytes such as fungal and bacterial entomopathogens is likely as a result of the continuous symbiosis of these microbes in colonizing seed tissues and thus, reliably participating in the development process such as the widespread capacity of fungal and bacterial entomopathogens to produce plant signal molecules (such as nitric oxide) growth regulators (such as auxins and ethylene) which could be the reflection of co - evolutionary association of microbes and plants [27]. An increase in the germination of seeds after treatment with fungal entomopathogens can also be caused by the fungus ability in producing phyto-hormones that stimulate seed germination [26].

IV. Conclusion

This study concludes that isolates of Beauveria bassiana, Metarhizium anisopliae, Verticillium lecanii and Bacillus thuringensis do not reduce germination performance of cowpea seeds when used as microbial pesticide against cowpea storage pest. However, at longer storage post-treatment of cowpea seeds with these entomopathogenic isolates, the germination performance may be reduced

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