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# Biological Control Activity Of Wild And Mutant Strains Of *Glomus versiforme* Against *Erysiphe flexuosa* Causing Powdery Mildew Disease In Cowpea

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## ABSTRACT

Biological control is the most current approach in sustainable agricultural system due to its potential of improving plant tolerance to pathogen and unfavorable environmental stresses. This research evaluates the biocontrol potentials of wild and mutant strains of *Glomus versiforme* (Gv), against *Erysiphe flexuosa* (E.f), the powdery mildew fungus of cowpea. Wild strain of *Glomus versiforme* was subjected to random mutagenesis using UV irradiation for a regime of 4, 8 and 12 minutes, after which they were evaluated for their antagonistic and biocontrol activity (wild and mutant) against *Erysiphe flexuosa* in a dual culture assay and green house respectively. Disease severity of various *Glomus versiforme* treatments was assessed 7 weeks after plantation. The isolates were able to inhibit radial mycelia growth of the pathogen. The antagonistic activity follows the order Gv8>Gv4>Gvw>Gv12. Green house observation after 7 weeks shows that Gv8 has the best agronomic parameters taking note of the plant height, number of leaves, fresh and dry weight of roots and shoots as compared to treatments with other mutant versions. The agronomic features with respect to *Glomus versiforme* treatments follows the order Gv8>Gv4>Gvw>Gv12>control>E.f. Gv8 shows the highest healthiness and lowest disease incidence. The disease incidence follows the order E.f>GV12>GVw>GV4>GV8.

**Keywords:** *Glomus versiforme*, cowpea, *Erysiphe flexuosa*, powdery mildew, mutagenesis, radial mycelia, biocontrol, Arbuscular mycorrhizal fungi.

## INTRODUCTION

For many decades and still counting, chemical pesticides has been used for pathogen control in agricultural system worldwide (Yoon et al.; 2013). In recent years, large number of synthetic chemical fungicides has been banned in the western world because of their undesirable attributes such as high and acute toxicity. Most synthetic fungicides have residual effects on crops which amount to acute diseases on consumers and are even said to cause environmental imbalance (Oyewole et al.; 2017). More so, the applications of fungicides for the control of seedling diseases are very expensive as farmers in the developing countries cannot afford. Furthermore, many pathogenic fungi have developed resistance against these chemical fungicides Hahn, M (2014). Thus, the increasing cost of chemical fungicides, coupled with concerns about the food and environmental safety of applying fungicides to food and the

development of disease resistance rendered this conventional approach unsuitable and underscored the need for alternative disease control options in crops (Kravchenko et al.; 2017).

Cowpea is one of the most important food product globally and a major contributor to global source of protein. It is a major food and cash crop in Nigeria and many developing countries (Langyintuo et al.; 2003). Though produced in other parts of the world, Nigeria remains the largest producer and consumer of cowpea (Oyewole et al.; 2017). It is very important for the livelihood of the poor people in developing countries and can serve a wide range of purpose as food crop, cash crop, and animal feed (Kravchenko et al.; 2017 and Alsanius et al.; 2019). Perhaps the coinage “*naman talaka*” (the poor man’s meat) by the Hausas of the west and central Africa, points to the perception about the nutritional attributes of cowpea grain. However, it is susceptible to powdery mildew caused by *Erysiphe flexuosa* during wet and humid conditions (Omomowo et al.; 2018). Powdery mildew is caused by *Oidium* spp. and *Erysiphe polygoni* and it can be controlled biologically using resistant varieties and chemically by the application of fungicides such as triadimefon (Fondevilla & Rubiales 2012). The symptoms are white, powdery growth consisting of oidia appearing on the upper surface of the leaf (Fondevilla & Rubiales 2012). Chlorotic and then brown patches also appear on the upper surface of the leaf, which finally result in the defoliation of the plant. Omomowo et al.; (2018) reported that *Erysiphe flexuosa* growing on or in stored cowpea causes a variety of losses, this include decrease in germinability, discoloration of parts (usually the germ or embryo) or all of the seed, heating and mustiness, various biochemical changes such as increase in fatty acids, reducing sugar and respiration, production of mycotoxins which if consumed may be harmful to man and animals. Elewa et al.; (2011). Reported that the pathogen decreases the quality of the cowpea through discoloration or change in taste (bad flavor or smell) and also decreases the nutritive value. Spontaneous heating and associated increase in respiration of the grain in storage has been reported to be due to microorganisms and are known to raise the temperature of the grain up to 70-75°C. The effect on seed germinability has been linked to the invasion of the embryo by these pathogenic storage fungi. Mannaa, & Kim (2017) reported the invasion of germ of seeds by species of *Erysiphe* and *Aspergillus* spp., which led to the rapid death of the germ. However, researchers in the recent years have used the wild strains of *Glomus versiforme* in the control of cowpea pathogen but its effectiveness is somewhat limited (Abdel-lateif, 2017).

*Glomus versiforme*, a potential Arbuscular mycorrhizal fungi has proven its biocontrol potentials in recent years such as in the control of *Fusarium oxysporum* of carrot as reported by Zhang et al.; (2014) but very little is known on its ability to control fungal pathogens of cowpea. Much effort has been paid to obtaining suitable formulations of Arbuscular mycorrhizal fungal inocula and appropriate ways for their application to the field (Sasvári, et al.; 2012). Ojha, & Chatterjee (2012) reported various versions of these biocontrol organisms which include *Trichoderma* spp., *Bacillus* spp., *Pseudomonas* spp., *Agrobacterium* spp, *Streptomyces* spp etc. However, many of the widely used microbes in the synthesis of bioactive compounds that aid pathogen control have certain limitations (Gemechu et al.; 2020). Changing their genomic constitution with the view to enhancing their potentials for the synthesis of bioactive compounds through mutagenesis followed by screening and selection to detect the new variants that might arise with different biosynthetic pathway responsible for antibiosis have in the past led to again of function (Sasvári, et al.; 2012). It should however be noted that not all mutations are harmful, there are numerous documented cases where beneficial mutations with survival advantages have risen in the population. Changes in genomic constitution can boost the activities of these biocontrol fungi (positive effect) in the synthesis of bioactive compounds to increase its effectiveness on these pathogenic life forms (Ojha & Chatterjee, 2012). The inability of the wild type strain to surpass the chemical treatments led to strain improvement of *Trichoderma harzianum* by UV mutagenesis for enhancing its secretion of bioactive compound against aflatoxigenic *Aspergillus* species (Özer & Arın 2014). This research is expected to bring about a multiple-fold reduction in the use of chemical pesticides, by exploring the use of mutants strains of *Glomus versiforme* in the control powdery mildew fungus of cowpea (*Erysiphe flexuosa*). The global consensus to reduce inputs to chemical fungicides which are perceived as being

hazardous by some consumer has provided opportunities for the development of this novel sustainable crop protection strategies. (Van-Lenteren, 2012).

## MATERIALS AND METHODS

The research was carried out at Microbiology/Biotechnology laboratory of Nigerian Stored Product Research Institute, km 3, Ilorin Kwara State, Nigeria A pure culture of bioagent and pathogen used for this study was obtained from the institute's culture bank and was maintained on Potato Dextrose Agar (PDA) slants at 26±2°C for further sub-culturing. Koch's postulate was used for pathogenicity assay of the pathogen.

### Exposure of *Glomus versiforme* to UV light to induce Mutation.

The UV mutant isolates were obtained according to methods described by Saini & Kshirsagar (2015) with slight modifications. Organism from stocked pure culture was seeded centrally on PDA plate for 5 days to have a pure culture. Spore suspension were prepared in normal saline (0.9%) of 10<sup>6</sup> spore/ml concentration. 10 ml spore suspension of wild *Glomus versiforme* was transferred to sterile petri dish and exposed to UV light ( $\lambda = 260$  nm) using UV trans-illuminator (Bio DOC-It™ System U.S.A Upland for different time intervals of 4 min, 8min and 12 min. The distance between spore suspension and UV source was adjusted to 15 cm. Then 20µl of sample from treated spore suspension was diluted with 980µl distilled water and these UV irradiated samples of each time interval were spread on petri plates containing Potato dextrose agar (PDA). The plates were incubated at 25° C for 5 days. Possible mutants were selected based on morphology, color, mycelia growth, period of sporulation, color of reverse plate as compared to the wild type. Slants were prepared and stored in the refrigerator at 4°C for further use. The mycelia plugs from the domesticated type culture serve as the control. Therefore, this research step has four treatments.

1. G.V WILD: Real strain of *Glomus versiforme*.
2. G.V 4: *Glomus versiforme* exposed for 4 minutes.
3. G.V 8: *Glomus versiforme* exposed for 8 minutes.
4. G.V 12: *Glomus versiforme* exposed for 12 minutes.

Antagonistic assay of *Glomus versiforme* (wild and mutant) against *Erysiphe flexuosa*

Using the method employed by Omomowo et al.; (2018) with little modification, *Glomus versiforme* wild isolates were evaluated *in-vitro* for its antagonistic and inhibition potential against *Erysiphe flexuosa* pathogens, using dual culture techniques. The *Erysiphe flexuosa* was surrounded at equidistance by three plugs of *Glomus versiforme*, and incubated at 25°C for 7 days. Isolates were then scored for extents of radial mycelia growth after seven days according to the method employed by Ararsa and Thangavel, (2013)

$$I = \frac{C-T}{C} \times 100$$

Where I = Mycelia inhibition in percentage, C= Mycelia diameter in control plate, T= Mycelia diameter in Antagonistic plate. The procedure was repeated for the mutants and their respective percentage mycelia inhibitions were estimated.

### Preparation of Artificially made *Erysiphe flexuosa*-infested pot and sowing of cowpea.

As described by (Omomowo et al., 2018) with little modification. Efficacies of different *Glomus versiforme* strains (wild and mutants) were tested in comparison with the control in an artificially made *Erysiphe flexuosa*-infested soil. The earthen pots were filled with autoclaved soil (soil, sand and organic manure in ratio of 3:1:1), *Erysiphe flexuosa* pathogen broth was used to infect the soil and was left at ambient temperature for 7 days for the pathogen to acclimatize.

Cowpea seeds were washed with sterilized water, soaked in 0.5% NaCl for 1 minute and dried. *Glomus versiforme* broth was then applied to the soil at 4cm depth, followed by the sowing of the seeds at 3cm depth. The biocontrol agent was finally applied above the seed and covered with soil. Artificially made *Erysiphe flexuosa*-infested pots were equally set without application of bioagent, Control pots do not receive any bioagent and pathogen inoculum (Puthur *et al.*, 2019). The set up was observed under greenhouse conditions for 7 weeks after which the agronomic parameters were measured according to the method employed by (Omomowo *et al.*, 2018). This was carried out in triplicates following the design described below:

#### TREATMENTS

1. Pot 1 Cowpea seed + *Erysiphe flexuosa* + G.vWild
2. Pot 2 Cowpea seed + *Erysiphe flexuosa* + G.v4
3. Pot 3 Cowpea seed + *Erysiphe flexuosa* + G.v8
4. Pot 4 Cowpea seed + *Erysiphe flexuosa* + G.v12
5. Pot 5 Cowpea seed + *Erysiphe flexuosa* only.
6. Pot 6 Cowpea seed only (CONTROL)

At the end of the 7th week, agronomic parameters were measured (plant height and number of leaves), the plant was uprooted, shoots and roots were separated, weighed before and after drying at 70°C.

#### **Determination of Percentage Disease Incidence and disease severity due to Root Colonization by *Glomus Versiforme***

According to the method of Elewa *et al.*; (2011), percentage disease incidence was estimated 3 weeks after plantation for pre-emergence of powdery mildew, 45 days for post-emergence, percentage powdery mildew (disease severity), and percentage healthy plant were also estimated. Percentage disease incidence were then calculated:

$$\text{Percentage Disease incidence} = \frac{\text{No. of infected plant}}{\text{Total No. of observed plant}} \times 100$$

The disease severity was scored using the method of Huang *et al.*; (2014). 5 degrees was scored using 0-5 scales.

- Degree 1 = 1-25% of leaves were diseased
- Degree 2 = 26-50% of leaves were diseased
- Degree 3 = 51-75% of leaves were diseased
- Degree 4 = 76-99% of leaves were diseased
- Degree 5 = plant dead.

The disease severity can then be estimated by the formula

$$\text{Disease severity} = \frac{\text{No. of infected plant} \times \text{their infected degree}}{\text{Total examine plant} \times \text{upper infected degree}} \times 100$$

#### **Statistical analysis using ANOVA.**

The data obtained in this study were subjected to analysis by one way ANOVA. P-values of 0.05 or less were considered significant and the means were compared by L.S.D. at 0.05 level of Significance using SPSS Software version 20.0. Three replicates were used throughout the experiment.

**RESULTS**

**In-Vitro Antagonistic Assay of Biocontrol Agents Against Cowpea Pathogen**

Antagonistic effect of wild and mutant strains of *Glomus versiforme* was tested against *Erysiphe flexuosa* *in vitro* as shown in table 1 below. Percentage mycelia inhibition was best in G.v8 with 73.0% followed by G.v4 and G.vW with 64.7% 59.7% respectively and lowest in G.v12 with 53.0% as shown in table 1 and figure 1 below. The antagonistic activity follows the order **G.v8 > G.v4 > G.vW > G.v12**.

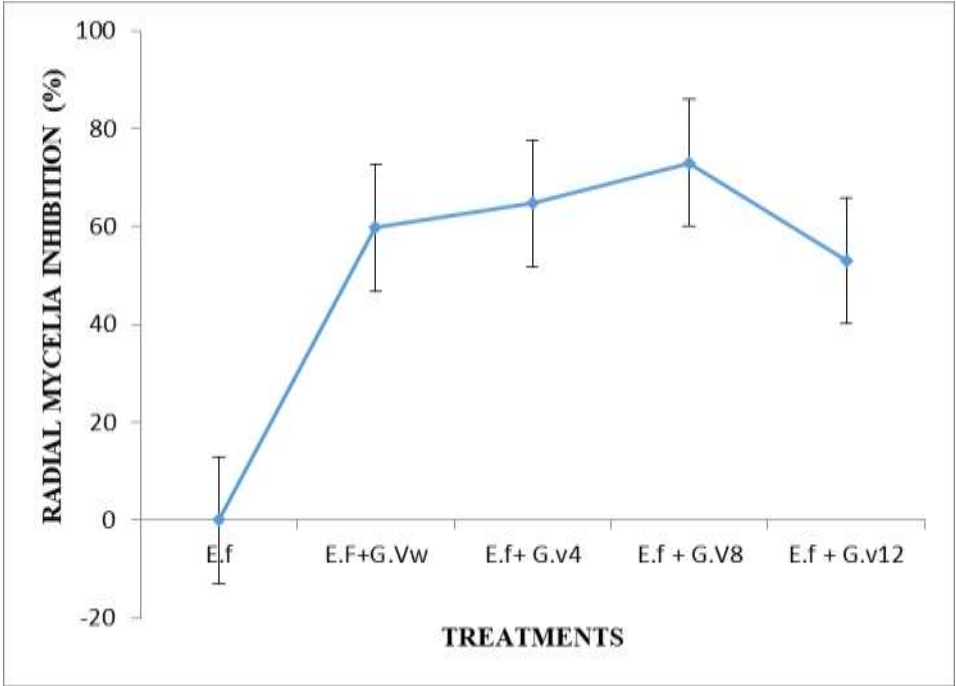
**TABLE 1**

**Antagonistic Assay of *Glomus versiforme* (G.v) Against *Erysiphe flexuosa* (E.f) Causing Powdery Mildew in Cowpea.**

STRAINS	DAY 5		DAY 8	
	MYCELIA GROWTH(cm)	MYCELIA INHIBITION (%)	MYCELIA GROWTH(cm)	MYCELIA INHIBITION (%)
<i>Erysiphe flexuosa</i>				
<b>Control (E.f)</b>	5.6	0.0	6.0	0.0
<b>G.VW</b>	2.3	58.8	2.4	59.7
<b>G.V4</b>	2.0	63.9	2.1	64.7
<b>G.V8</b>	1.5	72.9	1.6	73.0
<b>G.V12</b>	2.7	51.4	2.8	53.0
<b>LSD (0.05)</b>	0.049	0.429	0.049	0.516
<b>P LEVEL(0.05)</b>	***	***	***	***

All Values Are Means Of Three Replicates, \*\*\*: Mean Square Values Are Significant At P<0.001

E.f= *Erysiphe flexuosa* (control), G.vw=*Glomus versiforme* wild , G.v4=*Glomus versiforme* exposed for 4minutes, G.v8=*Glomus versiforme* exposed for 8minutes, G.v12= *Glomus versiforme* exposed for 12 minutes



**Fig 1: In-vitro antagonistic assay of *Glomus versiforme* (wild and mutant) against *Erysiphe flexuosa***

E.f= *Erysiphe flexuosa* (control), G.vw=*Glomus versiforme* wild, G.v4=*Glomus versiforme* exposed for 4minutes,G.v8=*Glomus versiforme* exposed for 8minutes, G.v12= *Glomus versiforme* exposed for 12 minutes

**Pot Assay of Biocontrol Agent Against Cowpea Pathogen**

Plant growth parameters was measured 7 weeks after plantation (49 days).G.v8 gave the best growth parameter in the green house followed by G.v4, G.vW,G.v 12 and control respectively as shown in table 2 and figure 2 below.The pathogen infested soil treatment negatively affects the growth parameters.*Glomus versiforme* treatments does not only prevent the proliferation of the pathogen but also improve growth parameters as compared to the control. The order of activity is as follows **Gv8+E.f>Gv4+E.f>GvW+E.f>Gv12+E.f>CONTROL>E.f**

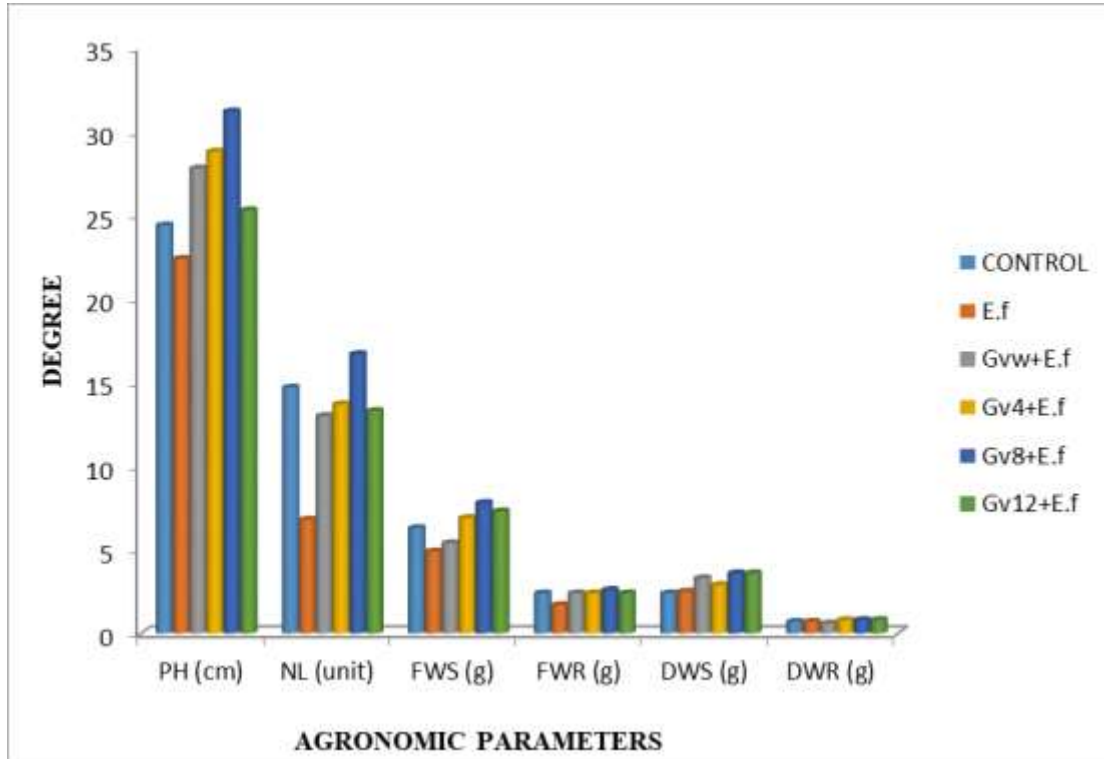
TABLE 2

Biocontrol Effects of *Glomus versiforme* (wild and mutant) on the growth parameters of cowpea seedlings in an *Erysiphe flexuosa*-infested soil

TREATMENTS	DRY WEIGHT (g)		FRESH WEIGHT (g)			
	PLANT HEIGHT (cm)		NO OF LEAVES (units)			
	SHOOT	ROOT	SHOOT	ROOT		
CONTROL	24.4	14.7	6.3	2.4	2.4	0.7
E.f	22.2	6.8	4.9	1.7		2.5
	0.7					
G.Vw+Ef	27.8	13.0	5.4	2.4		3.3
	0.6					
G.v4+Ef		28.8	13.7			6.9
	2.4		2.9			
	0.8					
G.v8+Ef		31.2		16.7		7.8
	2.6	3.6	0.8			
G.v12+Ef	25.3	13.3	7.3	2.4		3.6
						0.8
LSD (0.05)	1.103	2.385	0.254	0.127	0.110	
	0.103					
P LEVEL (0.05)	***	***	***	***	***	***
						**

All Values Are Means of Three Replicates. \*\*\* Mean Square Values Significant At P<0.001, \*\*: Mean Square Values Significant At P<0.01

**E.f**= *Erysiphe flexuosa* , **G.vw**=*Glomus versiforme* wild , **G.v4**= *Glomus versiforme* exposed for 4minutes, **G.v8**= *Glomus versiforme* exposed for 8minutes, **G.v12**= *Glomus versiforme* exposed for 12 minutes. **Control** = only cowpea seedling without *Glomus versiforme* and *Erysiphe flexuosa* treatment



**Fig 2: The effect of *Glomus versiforme* (wild and mutant) on the growth parameters of cowpea seedlings in an *Erysiphe flexuosa*-infested soil.**

**PH:** plant height, **NL:** number of leaves, **FWS:** fresh weight of shoot, **FWR:** fresh weight of root, **DWS:** dry weight of shoot, **DWR:** dry weight of root

**Effect of inoculating pathogen-infested soil with *Glomus versiforme* on the disease incidence in cowpea plant**

Disease incidence was very low in G.v8 seedlings with 3.7% and having 81.7% healthiness followed by G.v4 with 4.4% disease incidence and 79.5% healthiness as shown in table 3 and figure 3 below. Both the Gvw and G.v12 directly imitate each other in percentage healthiness but slightly differs in disease incidence with 7.6% and 8.5% respectively, while the worst result was obtained with *Erysiphe flexuosa*-infested soil with a very high disease incidence 27.5% and very low healthiness of 19.4%.

**Table 3**

**Effect of inoculating pathogen-infested soil with *Glomus versiforme* (wild and mutant) on the disease incidence in cowpea plant**

SOIL TREATMENT	% disease incidence			
	At seedling stage (3 weeks)		At maturity (7 weeks)	
	% pre-emergence	% post-emergence	% powdery mildew	%
healthy plant				
E.f	26.3	27.5	27.5	19.4
G.v.W	14.1	15.2	7.6	62.5
G.v4	7.6		8.3	4.4
79.5				
G.v8	6.7		7.6	3.7
81.7				
G.v12	12.4		16.5	8.5
62.6				
LSD (0.05)	0.392	0.402	0.392	0.395
P LEVEL (0.05)	***	***	***	***

All Values are means of three replicates  
SIGNIFICANT AT P<0.001

\*\*\*: MEAN SQUARE VALUES ARE

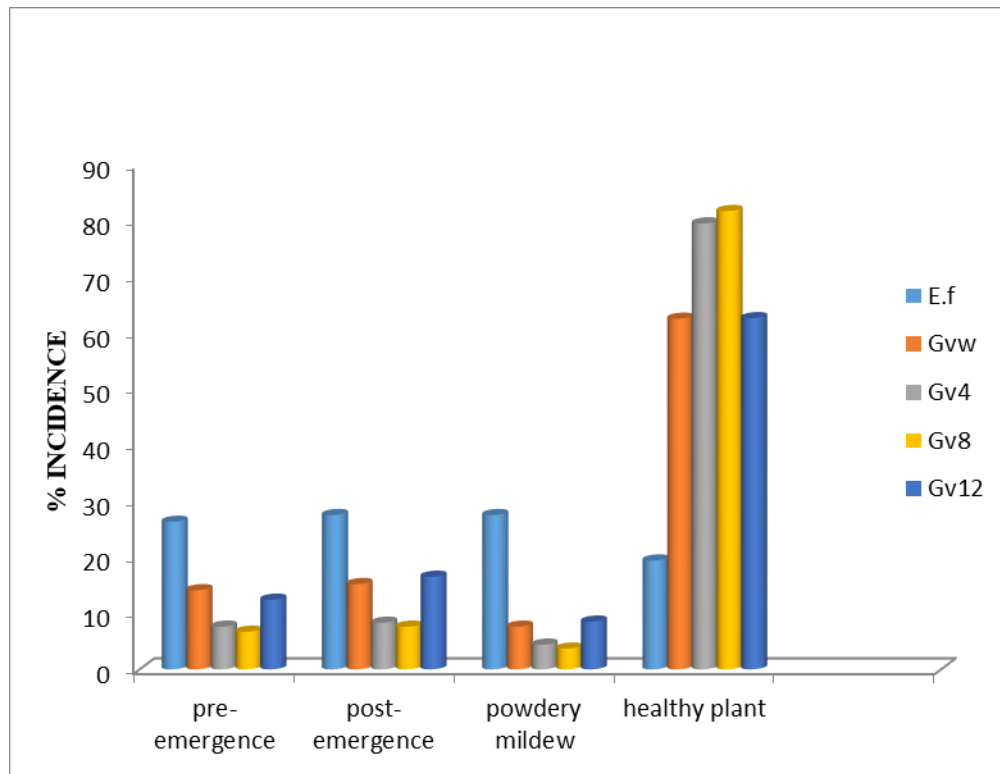


Fig 3 Effect of inoculating pathogen-infested soil with *Glomus versiforme* on the disease incidence in cowpea plant

E.f= *Erysiphe flexuosa* , G.vw=*Glomus versiforme* wild , G.v4= *Glomus versiforme* exposed for 4minutes, G.v8= *Glomus versiforme* exposed for 8minutes, G.v12= *Glomus versiforme* exposed for 12 minutes.

## DISCUSSION

The use of *Glomus versiforme* as a means of biological control agent is the major objective of this research, by developing a simple biotechnological method that can be adopted and applied to enhance effective and efficient control of powdery mildew pathogen that causes spoilage of cowpea thereby affecting food availability and great economic loss. Effective approaches to control plant diseases is largely affected by several biotic and abiotic stresses. Unfortunately, chemical pesticides do have attendant risks such as persistence in the environment, contamination of water table and even adverse health effects on consumers due to bioaccumulation of this complex chemicals on agricultural produce. One of the dramatic effect of colonization by Arbuscular mycorrhizal fungi on the host plant is the increase in the growth and development of cowpea plant and its tolerance to pathogen as reported by Akhtar & Siddiqui, (2008). Fortunately, this study demonstrate the use *Glomus versiforme* (mutants) as a biocontrol agent to suppresses the growth of *Erysiphe flexuosa* pathogen that causes powdery mildew in cowpea, it was very efficient and successful in inhibition of the pathogen by acting as a root covering which ultimately reduced the space of root region, resulting in the increase of biomass of plant. The yield of plants that were treated with biocontrol agent was significantly higher when compared to uninoculated plants

The characterized mutant isolates in table 1 above showed a high radial mycelia inhibition by preventing the proliferation of the pathogen which is considered as antibiosis. The antifungal metabolites secreted by the wild and mutant 8isolates, penetrates the cell wall of the pathogen and causes mycelia

malformation. Gv8 showed the highest antagonistic activity followed by the Gv4 when compared to the wild type strain, this is in agreement with the work of Anita and Ashwin, (2012) where *Trichoderma harzianum*, mutated by UV antagonizes Aflatoxigenic *Aspergillus* species. This result also goes in line with that obtained by Akhtar & Siddiqui, (2008) who observed that two species of *Glomus mosseae* were able to control wilt disease caused by *Fusarium oxysporum*.

The wild and mutant isolate of *Glomus versiforme* inhibited the mycelia growth of the pathogen in all cases without mycelia contact on dual culture assay due to the secreted inhibitory substances, with Gv8 giving the best result, followed by Gv4. This agrees with the work of St-Arnaud, et al., (2008) where mycelia proliferation of *Fusarium oxysporum* .sp .*chrysanthemi* in an *in vitro* dual culture system with the vesicular Arbuscular mycorrhizal fungus *Glomus intraradices* which prevented mycelia contact. Inhibitory activity of the biocontrol agent follows the order **Gv8> Gv4> Gvw> Gv12**.

UV mutagenesis is a random mutation with very high possibility of point mutation. It is possible that from millions of potential mutants generated, the exact strain with the highest biocontrol activity might have not been isolated. However, mutants isolated from 4 to 8 minutes UV irradiation gave the best result in antagonistic assay and pot assay against the pathogen when compared to the wild type strain, suggesting a mutation that might be a gain of function with increased metabolite secretion. This result is in agreement with the studies conducted by Singh et al., (2010) Gv12 showed lesser activity in antagonistic and pot assay as compared to the wild type, this may be due to UV mutagenesis causing dimerization, or amino acid substitution which might knock down the protein function (null mutation) responsible for antibiosis. This agrees with the work of Lopes et al.; (2012) where a non-synonymous mutation negatively affect the protein function.

Green house results in table 2 above showed that Gv8 gave the best agronomic parameters followed by Gv4, but *Erysiphe flexuosa* treatment showed the lowest activity. In fact, results of seedling germination and growth promotion seems to be consistent with the antagonistic effect produced on plate, suggesting that in-vitro proliferating conditions of the organism as antagonist against the cowpea pathogen is not affected while the organism is tested in pot. This result agrees with that obtained by Akhtar & Siddiqui, (2008) who reported that Arbuscular mycorrhizal plays antagonistic interaction with the soil-borne pathogen without any side effect to other beneficial organisms. This research also noted a decreased disease incidence which follows the order **Gv8<Gv4<Gvw< Gv12<E.f** while the degree of healthiness of the cowpea plants follows the order **Gv8>Gv4 > Gv12> Gvw> E.f**. On this basis, bioagent of fungi can be exploited for plant disease management program. It should however be noted that simple biotechnological techniques using UV irradiation gave the best results all through.

## CONCLUSION

This study has established a very high efficacy and great biocontrol potency of mutant strains of *Glomus versiforme* in the control of *Erysiphe flexuosa* both on plate and in pots. It is ecologically friendly and could facilitate more efficient use of this type of method on a commercial scale as an alternative to synthetic pesticides which has acute side effects on human health. It should however be noted that inputs of chemical pesticides in agricultural pest managements should be reduced and the need to augment pest control with microbial strains of *Glomus versiforme* or other reported versions which are mutational selection. This work is hoped to accelerate agricultural productivity and serve as alternative to chemical pesticides because of its safety in the environment. Mutant isolates when investigated further at the molecular level, may provide additional information as to genetic arrangement, regulation and type of mutation created by UV in the wild type strain of *Glomus versiforme* genes. On a final note, mutant strains of *Glomus versiforme* can be a useful source of biofungicide for the control of *Erysiphe flexuosa* causing powdery mildew in cowpea.

## Authors Contribution

Luka Yelwa Barde (Under Graduate Student) conducted field experiments and recorded field observations. Issa Funsho Habeeb (Biotechnologist) conceived the idea and supported the experiment and

wrote the concept and discussion. (Biotechnologist) Tabita Sule Gaba (Biologist) wrote the draft authenticate the manuscript, carried out the validation and reliability of the research instrument and result, TSG worked on the data analysis and references. Abba Alhaji Mohammed (Microbiologist) advised about the laboratory technique and conducted manuscript proofreading before submission. All authors read and approved the final version of the manuscript.

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