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Persistence of Bioherbicidal Agents Formulated from the Multi-combination of the Wild and Mutant Strain of *Lasiodiplodia pseudotheobromae and Pseudomonas aeruginosa*

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Abstract: The success of applying bioherbicidal agents as biological control agents of weeds relies on the ability of the biological control agents to persist after its application and to remain viable after exposure to different environmental conditions. This research work was carried out to determine the persistence of bioherbicide formulated from multi-combination of the wild and mutant strain of *Lasiodiplodia pseudotheobromae* and *Pseudomonas aeruginosa* under field condition as well as soil carbon sequestration and soil functionality through the measurement of microbial biomass, total organic carbon and soil respiration. The viability of the multi formulated product was tested in two different trials after field at 3, 6 and 9 weeks after their application. The viability of the formulated bioherbicides was in the following orders: BH4>BH2>BH6>BH3>BH1>BH5>Control. BH4 showed the maximum number of viability with 4.0×10^5 CFU/g and 4.2×10^5CFU/g at the two field trial, for *Lasiodiplodia pseudotheobromae* and 18.3×10^2 CFU/g and 23.48×10^2CFU/g for *Pseudomonas aeruginosa,* respectively after 12 weeks of application. The results revealed that multi-combination of *Lasiodiplodia pseudotheobromae* and *Pseudomonas aeruginosa* into different "Pasta" formulation greatly enhanced the viability of the bioherbicidal agent used during this study as well as carbon sequestration and microbial biomass at the two trial fields.

Key words: Persistence · Bioherbicide · *Lasiodiplodia pseudotheobromae* · *Pseudomonas aeruginosa* · Multi-combination · Soil functionality

INTRODUCTION

Organic farming is a holistic production management system, which promotes and enhances agroecological systems (AES) health, including biological and nutrient cycles, biodiversity as well as soil biological activity [1-4]. It emphasizes the use of management practices in preference to the use of external and off-farm inputs, taking into consideration that regional conditions require locally adapted AESs [5], which is accomplished by using biological, agronomic and mechanical methods, instead of using synthetic inputs, to fulfil any specific function within the system [6].

Global food security has become a primary concern because of population growth and climate change [7], which has made the productivity of organic farming and its contribution in feeding the world increasingly important [8]. It has been observed that changes in climate which are reflected by higher temperatures and heat waves, changes in precipitation patterns, increased

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greenhouse gases and their interactions with other environmental stresses affect the sustainability of AESs and disrupt production [9]. It is suggested that changes in climate will affect both biotic and abiotic factors in cropping systems, threatening crops' sustainability and production [10]. Changes in climates could result in food insecurity to millions [11], especially in developing countries where different forms of resource limited, low-input and organic farming are being practiced [12-15].

Organic farming represents a broad set of practices that emphasize farming based on ecosystem management, integrated cropping and livestock systems, diversity of products and reliance on natural pest and disease management without the use of synthetic inputs. Jaradat and Boody [16] opined that ecosystem responses to changes in climate and variability may have implications for sustainability, biodiversity and ecosystem services available to society. Some changes in climate impact only biological systems (crops, farm animals and micro-organisms), while some others create further feedbacks to the climate through green house gas fluxes, eco-physiological changes and edaphic processes [17].

The ability of a soil-applied biocontrol agent to control its target pest depends on the survival of the microbial inocula in the soil. A microbial agent may be highly effective under laboratory conditions but be unpredictable in the field. This is because different environmental factors, such as soil temperature, soil moisture, as well as soil type, can all affect the establishment and survival of the microbial inocula in soil. By minimizing the decline of viable cells in the biocontrol product following application, the efficacy of the product can be greatly improved, but this is only possible through understanding the reasons for the decline [18]. There are a number of environmental factors that affect both the ability of the microbial agent to colonize the roots of target plants as well as its survival in the rhizosphere. These include temperature, moisture content, pH, soil composition and the activities of other microorganisms [19].

A formulated bioherbicide can be defined as a mixture of the active ingredient (the biological agent) within a carrier or solvent that delivers the active ingredient to the target weed and the adjuvants that improve the survival and effectiveness of the product in adverse environmental conditions [20-23]. Among the different possible formulations, the dry solid or powder formulations provide several advantages.

The pasta granular formulation is an extruded product composed of grain flour and the microbial agent. Pasta technology is adaptable to many different microorganisms and ingredients [24]. The formulation is usually a wheat gluten matrix derived from inexpensive wheat flour, kaolin, nutritional adjuvants and water, which house the microbial agent [25, 26]. Once these ingredients are combined, the dough is extruded through a small pasta-making machine into thin sheets. These sheets are air-dried and sieved to a specific size. The granules are usually between 0.6 mm and 1.4 mm in diameter [27]. High-speed extrusion machinery used to make foods such as spaghetti can be used to produce pasta in large-scale operations [24, 28]. In addition to the ability to produce pasta on a large scale, there are several other advantages to the pasta formulation. It is non-toxic, cost effective, convenient to store, simple to use and can be applied with agricultural machinery [29]. This research work was carried out to determine the viability of bioherbicide formulated from the multi-combination of the wild and mutant strain of *Lasiodiplodia pseudotheobromae* and *Pseudomonas aeruginosa* as well as soil functionality test under field condition with the aim of producing food and feed with minimal harm to ecosystems, animals or humans.

MATERIAL AND METHODS

Description of Site: A field experiment was carried out in the experimental plots of the Department of Agronomy, the University of Ilorin and the experimental plots of Nigerian Stored Product Research Institute. Ilorin is located on 8° 30' 0" North, 4° 33' 0" East. Mutant and wild strains of *Lasiodiplodia pseudotheobromae* and *Pseudomonas aeruginosa* were multi-combined with pasta granular formulations. The different formulations were applied at 500 g/ha applied to the soil on the different plots tested.

Source and Maintenance of Fungal Isolates: Fungal strains were isolated from small chlorotic and necrotic lesions on leaves of *Tridax procumbens* weeds collected from Ogbomoso and Ilorin in Nigeria. The weed leaves were surfaced sterilized for 2 minutes in 0.5% sodium hypochlorite, rinsed in sterile distilled water and placed on Difco Potatoes Dextrose Agar (PDA) plates at 24 °C with 12 hours light, for 3-7 days. Fungal identification was carried out according to the procedure described by Samson and Van Reenen-Hoekstra [30]. Cultural and microscopic morphology was used to confirm

Lasiodiplodia pseudotheobromae isolates. Koch's postulates were applied to establish the pathogenic status of pure isolates of *Lasiodiplodia pseudotheobromae* on weed from which they were isolated. Purified *Lasiodiplodia pseudotheobromae* isolates were then preserved by storing a hyphal fragment and spore suspension in a 1:1 skim milk (10% v/v) to glycerol (4 0% w/v) solution and then stored at 4 °C. Isolates were revived by thawing a vial containing the fungus to room temperature. The content was aseptically spread on the surface of 15-cm diameter Petri dishes containing Difco PDA. The plates were then incubated at room temperature with natural light for 1–2 weeks.

Isolation and Characterization of Pure Cultures from the Bacteria: One gram of soil was removed from the wheat rhizosphere with the help of sterilized spatula and the soil sample was placed inside an aluminum foil from the farm of Nigerian Stored Product Research Institute. It was then placed in a sterile test tube and dissolved in 10 mL of distilled water to make the stock. The suspension was filtered through sterile glass wool. Serial dilution was done to the necessary dilution factors and pour-plated. The plates were left to gel and then incubated. The bacteria plates were incubated at 37 °C for 48 hours on Kings agar. At the end of each incubation period, the colonies were counted and sub-cultured onto fresh media maintained on slants from Kings agar and preserved at 4 °C in the refrigerator according to Fawole and Oso [31]. Tentative identification of bacterial isolates was done using the Bucchanans and Gibbons [32].

Exposure of *Lasiodiplodia Pseudotheobromae* to Uv Light to Induce Mutation: This experiment was carried out in order to see whether by mutation we could improve the amount of phytotoxic metabolites in the medium. This was carried out by preparation of fresh PDA plate to grow the organisms. After the growth of the organisms, cork borer was used to obtain several mycelia plugs from the culture into a sterile PDA plate. The sterile plate containing several mycelia plugs were placed under UV lamp at 300 nm wavelength at a distance of 30 cm to the plates. At a different time interval (30, 60 and 90 minutes), 5 mycelia plugs were withdrawn and used as inoculants. The mycelia plugs from the wild type culture served as the control.

Exposure of *Pseudomonas aeruginosa* to UV light to induce Mutation: This experiment was also carried out in order to see whether by mutation we could improve the amount of phytotoxic metabolites in the medium. *Pseudomonas aeruginosa* was cultured for 3 days at 35 °C in a moist air atmosphere supplemented with 2.5% CO_2. Plates were then flooded with 10 mL of 3 mM potassium phosphate buffer, pH 7.0 and bacteria was scraped off the agar with a bent glass rod. Ten mL culture suspensions were adjusted to 1 x 10^7 colony forming units (CFU/mL) was placed in the bottom half of 100 mm diameter Petri dishes and exposed to U.V. The sterile plate containing the bacteria culture was placed under UV lamp at 300 nm wavelength at a distance of 30 cm to the plates. The suspensions were mechanically rocked during irradiation to ensure even dose distribution. Samples were removed at a different time interval (30, 60 and 90 minutes) and kept in the dark at 4 °C to prevent photo-reactivation effects as described by Carson and Peterson [33].

Preparation of Pasta Granules: The procedures developed by Connick *et al*. [25, 34] and optimized by Elzen et *a*l. [29] were used. Thirty-two g semolina, a coarse durum wheat flour, 6 g kaolin, 2 g sucrose, 20 mL of fungal and bacterial inoculum serving as an active ingredient and 3 mL of deionized water were thoroughly mixed in a dish. The dough was then rolled through a small, hand-operated pasta machine (Marcato Model Ampia 150, Padova, Italy) into sheets, which were folded and extruded 10–15 times at different roller gap settings until it became homogeneous. The dough sheets were then extruded, without refolding, at a narrow setting to yield a 1-mm thick sheet. The sheets were then placed on aluminum foil and air-dried at ambient laboratory conditions [28 ± 2 °C, 33 ± 2% relative humidity (RH)]. The dried sheets were ground in a grinder into granules and sieved to specific sizes (501– 2000 µm). Their initial viability was determined by serial dilution method by plating the different dilution on nutrient agar and potatoes dextrose agar. The end-product Pasta granules were packed into transparent polyethylene bags (120 × 200 mm, 0.025 mm).

The various pasta granules formulated into various bioherbicides were designated as followed:

- BH1= 32 grams of semolina + 6 g kaolin + 20 mL of glycerol + wild strain of *Lasiodiplodia pseudotheobromae* (Lp) + *Pseudomonas aeruginosa* (Pa 30 minutes) + glucose + sucrose + fructose + dextrose + lactose sugar + peptone
- BH2= 32 grams of semolina + 6 g kaolin + 20 mL of glycerol + mutant strain of *Lasiodiplodia pseudotheobromae* (Lp 1 hour 30 minutes) + mutant strain of *Pseudomonas aeruginosa* (Pa 1hour 30 minutes),

- BH3 = 32 grams of semolina + 6 g kaolin + 20 ml of glycerol + *Lasiodiplodia pseudotheobromae* (Lp 30 minutes) + glucose + sucrose + fructose + dextrose + lactose sugar + peptone + *Pseudomonas aeruginosa* (Pa 30 minutes),
- BH4 = 32 grams of semolina +6 g kaolin +20 mL of glycerol + mutant strain of *Lasiodiplodia pseudotheobromae* (Lp 1 hour 30 minutes) + glucose+ sucrose + fructose + dextrose + lactose sugar + peptone + mutant strain of *Pseudomonas aeruginosa* (Pa 1hour 30 minutes),
- BH5= 32 grams of semolina + 6 g kaolin +20 mL of glycerol + wild strain of *Lasiodiplodia pseudotheobromae* (Lp) + wild strain of *Pseudomonas aeruginosa* (Lp)
- BH6= 32 grams of semolina + 6 g kaolin +20 mL of glycerol + mutant of *Lasiodiplodia pseudotheobromae* (Lp 1 hour) + mutant strain of *Pseudomonas aeruginosa* (Pa 1 hour) + glucose+ sucrose + fructose + dextrose + lactose sugar + peptone.

Recovery and Enumeration of *Lasiodiplodia Pseudotheobromae* Agents from Soil: *Lasiodiplodia pseudotheobromae* populations in soil from the two field trials were enumerated by soil dilution plating onto a Potato dextrose agar PDA. After incubation for 6 days at 30°C, the identity of each colony was then noted. *Lasiodiplodia pseudotheobromae* populations in soil from the pot trial were enumerated with 0.1 mL aliquot from 10^{-5} dilution of the soil samples by spreading dilution plating onto PDA containing cycloheximide (BDH, UK; 125 mg/L), streptomycin sulphate (Sigma; 350 mg/L) and tetracycline (Sigma; 50 mg/L) after incubation for 5 days at 23–25°C. Fungal identification was carried out according to the procedure described by Samson and Van Reenen-Hoekstra [30]. Cultural and microscopic morphology was used to confirm *Lasiodiplodia pseudotheobromae* isolates.

Recovery and Enumeration of *Pseudomonas Aeruginosa* Agents from Soil: One gram of soil was removed from the two field trials with the help of sterilized spatula and the soil sample was placed inside an aluminum foil. It was then placed in a sterile test tube and dissolved in 10 mL of distilled water to make the stock. The suspension was filtered through sterile glass wool. Serial dilution was done to the necessary dilution factors and pour-plated. The plates were left to gel and then incubated. The bacteria plates were incubated at 37°C for 48 hours on Kings agar. At the end of each incubation period, the colonies were counted. Tentative identification of bacterial isolates was done using Bucchanans and Gibbons [32].

Soil Carbon Sequestration: The soil samples were taken from NSPRI site and UNILORIN at depths of (0–20 cm). It was collected from two points using an auger. From each field, soil samples were collected from a total of ten augering points and bulked together to give a composite sample. All soil samples taken from the field were air-dried on trays. After drying, the clods were broken using a porcelain mortar and the ground soil sieved through a 2 mm mesh sieve and dried at 38 °C until constant weight. The sieved samples were stored in plastic containers and taken to Nigerian Stored Product Research Institute, Chemistry Laboratory (NSPRI). This experiment was carried out before and after the application of the formulated pasta granules on the two trial sites. Three subsamples for each of the cleaned and sieved samples were used for the total carbon content (TOC) determination. The soil's TOC was determined in the dried sediments using a LECO CR-412 Carbon Analyzer (LECO Corp., St. Joseph, MI, USA). The microbial biomass carbon (MBC) was determined in the (0– 0.20) m depth soil samples with the method of the fumigation–extraction with chloroform [35]. The samples determined in triplicate were incubated in desiccators for 24 h at room temperature in the presence and absence of chloroform vapor. Moreover, the soluble C was extracted with 0.5 M K_2SO_4 and this was followed by digestion with $K_2Cr_2O_7$ and titration with $FeSO_4$.

The soil quality indexes such as: (i) physical indicators i.e., soil texture [36], (ii) soil pH, measured in deionized water was determined with a glass electrode pH meter at a soil/solution ratio of 1:1 (weight/volume) and (iii) organic nitrogen reserves (total N), measured using Kjeldahl's procedure by using the sieved and dried soil samples. Soil microbial activity was evaluated by using the metabolic quotient (the specific soil respiration of the microbial biomass, qCO_2), the C mineralization quotient (the fraction of total organic C mineralized throughout the incubation, qM) and the Microbial Biomass C/Total Organic C (MBC/TOC) ratio [37].

RESULTS AND DISCUSSION

Out of all the bioherbicides formulated into pasta granules BH4 showed the maximum number of viability with 4.0×10^5 CFU/g and 4.2×10^5CFU/g (Tables 1 and 2) at the field trial Unilorin and NSPRI for *Lasiodiplodia*

Table 1: Persistence of *Lasiodiplodia pseudotheobromae* in the soil after application of the pasta granules for the field studies at Unilorin

S/N	Days after application (per gram of soil)	Mean number of CFU/ml* Bioherbicides BH1	BH2	BH3	BH4	BH5	BH6	Control	Maximum	Minimum	Open air temperature Rainfall(mm)
1	3weeks	4.52	4.98	4.52	5.19	4.00	4.37	0.00	30.9	15.0	52.1
2	6weeks	3.93	4.32	3.95	4.51	3.61	4.17	0.00	34.0	16.9	23.2
3	9weeks	3.21	3.95	3.56	4.04	2.91	3.75	0.00	37.8	25.0	32.1

*No of CFU × 10^5 dilution

Means shown were calculated using data obtained from three replication.

Table 2: Persistence of *Lasiodiplodia pseudotheobromae* in the soil after application of the pasta granules for the field studies at NSPRI

S/N	Days after application (per gram of soil)	Mean number of CFU/ml* Bioherbicides BH1	BH2	BH3	BH4	BH5	BH6	Control	Maximum	Minimum	Open air temperature Rainfall(mm)
1	3weeks	4.52	5.00	4.52	5.32	3.61	4.73	0.00	32.9	16.0	54.1
2	6weeks	3.83	4.32	3.85	4.41	3.51	4.17	0.00	37.0	17.9	27.2
3	9weeks	3.11	3.65	3.46	4.02	2.85	3.85	0.00	39.8	26.0	38.1

* No of CFU × 10^5 dilution

Means shown were calculated using data obtained from three replication.

Plate 1: Map of Nigeria showing the location of the experiment site

pseudotheobromae and 18.3 × 10^2 CFU/g and 23.48 × 10^2 CFU/g for *Pseudomonas aeruginosa* respectively after 12 weeks of application (Tables 3 and 4). The initial viability of the various products for BH4 was 27.28 × 10^2CFU/g for *Pseudomonas aeruginosa* (Figure 2) and 6.00 × 10^5 CFU/g for *Lasiodiplodia pseudotheobromae* immediately after formulation (Fig. 1).

The minimum viability was obtained from BH5 with viability of 2.95 × 10^5 CFU/g and 2.85 × 10^5CFU/g (Tables 1 and 2) at the field trial in Unilorin and NSPRI, for *Lasiodiplodia pseudotheobromae* and 5.2 × 10^2 CFU/g and 6.68 × 10^2 CFU/g for *Pseudomonas aeruginosa*, respectively after 12 weeks of application (Tables 3 and 4). No significant differences were observed in clay across the treatments (Table 5). Significant ($P<0.05$) higher values were obtained for NSPRI for silt (26.16), sand (36.19), TOC (3.62), qCO_2 (0.94), qM (1.93, MBC/TOC (0.71), MBC (267.40) and Rb (22.63) than the values obtained at Unilorin after pasta granules application. Increased values were observed for the indicators after pasta granules application in the two locations.

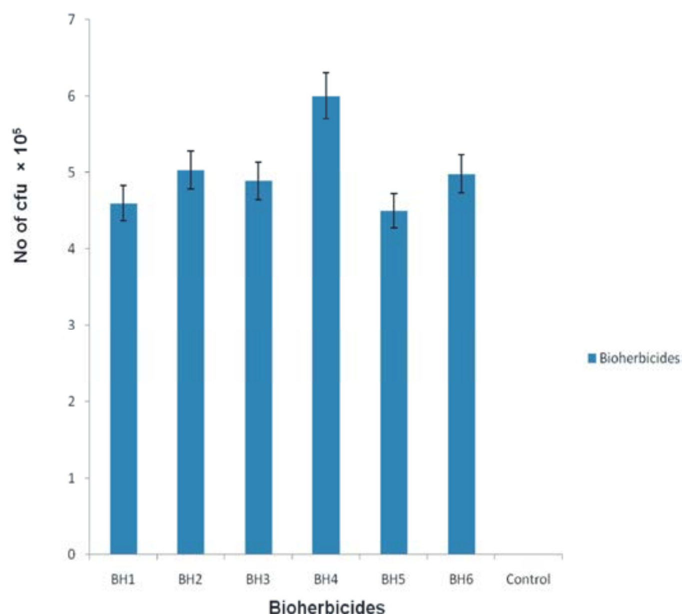

Fig. 1: Initial Viability of *Lasiodiplodia pseudotheobromae* formulated into different Pesta granular formulations Error bar=standard error of sbserved values

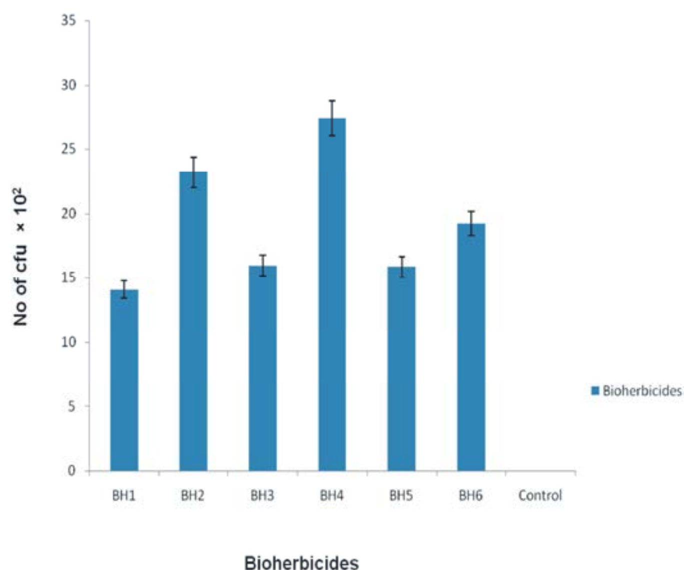

Fig. 2: Initial Viability of *Lasiodiplodia pseudotheobromae* formulated into different Pesta granular formulations Error bar=standard error of sbserved values

Table 3: Persistence of *Pseudomonas aeruginosa* in the soil after application of the pasta granules for the field studies at Unilorin

S/N	Days after application (per gram of soil)	Mean number of CFU/ml* Bioherbicides BH1	BH2	BH3	BH4	BH5	BH6	Control	Maximum	Minimum	Open air temperature Rainfall(mm)
1	3weeks	13.08	20.10	15.30	22.5	12.78	18.6	0.00	30.9	15.0	52.1
2	6weeks	11.32	18.70	13.80	20.20	9.23	15.8	0.00	34.0	16.9	23.2
3	9weeks	8.22	16.80	11.70	18.30	5.20	13.3	0.00	37.8	25.0	32.1

* No of CFU × 10^2 dilution

Means shown were calculated using data obtained from three replication.

Table 4: Persistence of *Pseudomonas aeruginosa* in the soil after application of the pasta granules for the field studies at NSPRI

S/N	Days after application (per gram of soil)	Mean number of CFU/ml* Bioherbicides BH1	BH2	BH3	BH4	BH5	BH6	Control	Maximum	Minimum	Open air temperature Rainfall(mm)
1	3weeks	13.78	22.10	15.78	25.68	13.90	18.90	0.00	32.9	16.0	54.1
2	6weeks	10.24	20.32	12.70	24.11	11.23	16.21	0.00	34.0	16.9	23.2
3	9weeks	9.32	18.52	8.32	23.48	6.68	14.70	0.00	39.8	26.0	38.1

* No of CFU × 10^2 dilution

Means shown were calculated using data obtained from three replication

Table 5: Soil functionality test before and after the application of pasta granules at the two locations

Parameters	Locations: Before NSPRI	Before UNILORIN	After NSPRI	After UNILORIN	SEM
Physical indicators					
Clay (%)	30.46	29.83	32.13	30.00	1.45
Silt (%)	25.38^b	23.16^c	26.10^a	23.29^c	0.69
Sand (%)	35.18^b	30.28^d	36.19^a	32.34^c	1.22
Chemical indicators					
pH	6.78^b	7.42^{ab}	6.92^b	7.83^a	0.28
N (gkg^{-1})	2.89^c	2.13^d	3.93^b	4.23^a	0.44
TOC (%)	2.52^b	1.78^c	3.62^a	1.84^c	0.39
Functional indicators					
qCo2 (10^{-2} h^{-1})	0.76^b	0.48^c	0.94^a	0.52^c	0.10
qM (%)	1.89^a	1.62^c	1.93^a	1.73^b	0.07
MBC/TOC	0.63^b	0.47^c	0.71^a	0.62^b	0.05
Microbial indicators					
MBC (μgCg dry $soil^{-1}$)	256.10^b	243.50^d	267.40^a	247.60^c	4.78
Rb (mg C-CO_2kg^{-1}soil)	20.18^b	15.63^d	22.63^a	16.32^c	1.49
Rcum (mg C-CO_2kg^{-1}soil)	495.60^b	378.40^d	499.30^b	389.50^c	29.72

Means with different superscripts within the same row are significantly (P<0.05) different. TOC = total organic carbon, MBC = microbial biomass carbon, Rb = basal respiration, Rcum = cumulative respiration, qCO_2 = metabolic quotient, qM = carbon mineralization quotient, MBC/TOC = microbial biomass TOC ratio

DISCUSSION

Weed biological control by specific micro-organisms is an accepted strategy for weed management [38, 39]. High levels of efficient and consistent recovery of formulated micro-organisms from soil are important for the accurate evaluation of establishment and field persistence of biocontrol agents. In the present study, the efficiency of formulated pasta granules containing wild and mutant strains of *Lasiodiplodia pseudotheobromae* and *Pseudomonas aeruginosa* as bioherbicidal agents.

The formulation of a bioherbicide is a tool with which the storability and ease of application of a biocontrol agent can be improved and to some extend the negative influence of environmental factors can be reduced as well. Understanding the needs of a biological control agent for survival during processing and storage as well as for proliferation and effectiveness in performing its biological mission are the basis for the development of an appropriate formulation [40].

After exposure to different environment conditions the bioherbicidal agents from Lasiodiplodia *pseudotheobromae* and *Pseudomonas aeruginosa* still maintain their viability on the field trial. BH4 had the highest number of viability at the two sites. Appropriate formulation might be responsible for the persistence of *Lasiodiplodia pseudotheobromae* and *Pseudomonas aeruginosa* on the field during the trial test. Also, the appropriate combination of the bioherbicidal agents, adjuvant and the pasta granules, allow the partial releases of nutrient and easy access to moisture. It has been claimed that suitable formulation technology may help in

solving some of the environmental constraints, particularly moisture requirements that often hinder the advancement of bioherbicide candidates beyond the discovery and evaluation phase. Propagules of foliar-applied fungal agents generally require free water to germinate and penetrate weeds. This leaf wetness requirement and its interaction with the ambient temperature often determine the outcome of a mycoherbicide application [41].

The persistence of a fungal biocontrol agent in the soil can be influenced not only by the type of formulation used, but also by the type and concentration of propagules incorporated, amendments added to the formulation and the initial dosage applied to the soil.

The deleterious rhizobacteria (DRB) belonging to the *Pseudomonas* group are known to be promising candidates for weed biological control [42, 43, 44]. A cell suspension of these DRB has been found to be an effective bioherbicide. However, the use of bacterial cell suspensions in large field scale is hard to manage due to difficulty in handling and storage. Therefore, development of robust formulations, ensuring simple handling, long shelf life and high cost-efficiency, can help to overcome such difficulties. The selection of the appropriate carrier, enabling the dispersion of the biological material to the target plant, is essential for the successful development of a formulation [45].

Also, it was found that the amendment of soy flour and oatmeal increased the soil persistence of *F. solani* f. sp. *cucurbita*, a biocontrol agent for Texas gourd, incorporated in an alginate formulation [46]. In addition, formulated macroconidia were more persistent in the soil than microconidia. Hebbar *et al.* [47] worked with *F. oxysporum* EN-4, a pathogen of *Erythroxylum coca* var. *coca*, concluded that the substrates amended to an alginate formulation not only determined the number of microconidia produced but also enhanced their conversion to chlamydospores which is critical for long-term persistence in the soil. For *Talaromyces flavus,* a biocontrol agent for plant pathogens formulated in alginate pellets, it was found that the propagule type, the initial inoculum level in the pellets, the type of filler material used to make the pellets and the initial concentration added to the soil each significantly affected fungal survival in and proliferation into soil from the pellets. Preparations incorporating conidia or ascospores using bran or Pyrex as bulky material resulted in a greater number of CFU in the soil compared to formulations prepared using fungal biomass [48]. The application of mycoherbicides to the soil in a granular formulation can be advantageous over a liquid application, not only because of greater stability but also because the bioagent is able to produce secondary spores on the granule surface [49].

Two major limiting factors that have an impact on mycoherbicides are temperature and moisture requirements [50, 51]. TeBeest *et al*. [52]considered temperature to be less important than moisture in most cases because many fungal pathogens will infect plants over a broad range of temperatures. However, there is often an interaction between temperature and moisture that has a greater effect than temperature alone. Free moisture and leaf wetness duration can significantly affect the ability of the fungal pathogen to germinate, produce penetration structures and ultimately cause plant infection. This requirement of leaf wetness duration often increases when temperatures are in suboptimal ranges.

Movement of bioherbicidal inoculum away from the point of inoculation will be an important factor in the success of a biocontrol agent applied to soil, especially when the target pest is not very mobile in soil. The role of percolating water is considered important in distributing micro-organisms through the soil [53]. Movement of bioherbicidal agents from *Lasiodiplodia pseudotheobromae* and *Pseudomonas aeruginosa* to the lower parts of soil profiles is indicative that they were released from the pasta granules.

The mutation might have enhanced the survival of these bioherbicidal agents. Mutation induction has become an established tool in strain improvement to supplement existing strains and to improve species in certain specific traits. Therefore, several approaches including chemical mutation, UV irradiation and genetic engineering to obtain high yield strains have been given a priority in the last decades [54].

Applications of chemical herbicides sometimes have a drastic effect on the soil composition and microbial availability in the soil, which consequently affect carbon sequestration and soil functionality. As a matter of fact, herbicides are able to kill a large portion of the soil's microbial population before their degradation takes place [55]. But in this study, application of organic fertilizer in the form of pasta granules was applied to two different sites. It was observed that there was an increase in total organic and microbial biomass after 9 weeks of application [56].

It had been observed that retention of soil C and N may be increased by using organic residues with low C:N ratio to maintain soil fertility, combined with large temporal diversity in crop sequences in organic farming

[57, 58]. High soil organic matter (SOM) in organic farming is usually associated with richer food webs and higher biological activity that drive soil ecological services [59]. The retention of high SOM is of important implications for regional and global C and N budgets, sustained crop production, enhanced environmental quality as well as enhancement of macroclimate modification [14, 17, 58-61].

CONCLUSION

This study has shown that pasta formulations can be used for effective delivery of from bioherbicidal agents containing *Lasiodiplodia pseudotheobromae* and *Pseudomonas aeruginosa* to the soil. Moreover, it was observed that the application of the pasta granules containing the bioherbicidal agents used at the two various field led to an increase in soil functionality indicators. BH4 formulation had the highest viability among all the tested bioherbicidal formulations during field's trial and at different environmental conditions. Therefore, application of bioherbicidal formulation from BH4 could lead to an increase in yields of agricultural products by contributing to food security and a sustainable agriculture system.

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