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Viability of Entomopathogenic Fungi *Metarhizium anisopliae* (Metsch) Sorokin in Some Alternative Media and Different Shelf-Life

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ABSTRACT

Pest control by using chemical pesticides continuously can harm the environment. Therefore it is necessary to look for environmentally friendly technologies, one of which is the use of entomopathogenic fungi such as Metarhizium anisopliae. The alternative media should be easy to obtain and has a good shelf life so the growth of M. anisopliae should be optimum. This research aimed to obtain alternative media that provide the best growth and development at each different shelf-life. The research method used was an experimental method using a Completely Randomized Design (CRD) Factorial and it was repeated 4 times. The media factors were from PDA, bran, green beans, and maize. The shelf-life factors were at 21 and 42 days old (d-old). The diameter on maize media gave the highest diameter value of 8.57 cm at 42 d-old. Green bean media gave the best spore density results of 2.08×107. Whereas the germination capacity of the media ranged from 63.94% to 94.23%. Germination showed no significant difference at 21 and 42 d-old. Therefore, green beans and maize media were effectively used for propagation of M. anisopliae as a substitute for synthetic media.

Keywords:
Alternative media; conidial spore; germination; M. anisopliae; shelf-life

1. Introduction

Pest control by using chemical pesticides continuously can harm the environment. In addition, it can cause pest resistance, natural enemies killed, secondary pest outbreaks, pest resurgence etc. Therefore it is necessary to look for environmentally friendly pest management. Technology that is safe for the environment and humans is one of the pest management goals, one of them is by utilizing entomopathogenic fungi such as *Metarhizium anisopliae*. *M. anisopliae* is an entomopathogenic fungus that can be used in controlling pests. *M. anisopliae* has a broad spectrum of hosts and can infect more than 100 species of pests (Dirjen Tanaman Pangan, 2008). The fungus *M. anisopliae* was

proven to be able to infect several types of insects from the order Lepidoptera, Coleoptera, Hemiptera, and Isoptera (Novianti & Dewi, 2017).

The fungus *M. anisopliae* can be produced commercially as a bioinsecticide (Sambiran et.al., 2007). Propagation of *M. anisopliae* fungi generally uses synthetic media such as Potato Dextrose Agar (PDA), Maize Meal Agar (CMA), Oatmeal Agar (OMA), and selective media The Dodine Oatmeal (DOA) (Liu., et al, 2012). In fact, the multiplication of *M. anisopliae* fungus experienced obstacles because the synthetic media used were expensive and difficult to obtain. So that the alternative growth media are needed in the field of mass propagation of entomopathogenic fungi that has the same capabilities as synthetic media, affordable prices, and easy to obtain.

Media that can be used are media containing organic substances as a source of C, a source of N, inorganic ions in an amount sufficient for fungal growth. In addition, *M. anisopliae* fungi need carbohydrates for vegetative growth and protein for the formation of fungal conidia (Nurwibawanto, 2016). The media contains macro and micro nutrients for the growth of *M. anisopliae* (Sadad et.al, 2014). Bran, maize, and green beans can be used as an alternative media. The advantages of alternative media besides being easy to obtain, they also have affordable prices so that they can reduce farmers' production costs.

The use of *M. anisopliae* fungus in controlling pests in the field is still very limited. This occurs due to the difficulty in preparing the inoculum for application (Nurwibawanto, 2016). *M. anisopliae* fungi can generally be applied after the age of 21 days after inoculation, this is different from chemical pesticides which can be applied independent of time. This research aimed to find the best alternative growth media of *M. anisopliae* so that it is expected to control pests in the field. The purpose of this study was to obtain the shelf life with the highest viability and spore density on each alternative media.

2. Materials and Methods

The research method used was an experimental method using a Completely Randomized Design (CRD) Factorial. The media factors were from PDA, bran, green beans, and maize. The shelf-life factors were at 21 and 42 days old (d-old). Each treatment was repeated 4 times.

Preparation of alternative media

Maize and green bean washed and steamed for 25 min, then dried. Dried maize and green bean were put in plastic bags of 30 g each/petridish and then sterilized in autoclave. Then the Inoculum of *M. anisopliae* inoculated to sterilized bran, maize, and green bean. Inoculation was carried out in laminar air flow (LAF) to avoid contamination. The culture will be incubated in an incubator for 21 and 42 days at 20-23 ^{o}C. As check control, PDA medium was used.

Colony diameter of M. anisopliae

Colony diameter measurements were carried out every day until the media were 21-d-old and 42 –d-old. The diameter was measured using a ruler by taking 4 points followed by calculating the average diameter.

Radial diameter = (Ø A+ Ø B+ Ø C+ Ø D)/4

Ø A = Axis Diameter A

Ø B = Axis Diameter B

Ø C = Axis Diameter C

Ø D = Axis Diameter D

Calculation of conidia density and germination rate

After the fungus were 21 and 42-d-old, conidia density and germination were observed using a haemocytometer. A conidia suspension was taken as much as 0.2 mL using a type pipette at a dilution of 10-1, a conidia suspension was slowly dropped on the haemocytometer canal. Conidia density contained in the haemocytometer was calculated after 10 hr-incubation, with a magnification of 400x. The measurement was taken twice in each observed field followed by calculating the percentage of germination.

Data analysis

Data was analyzed using F test at the level of 5%. If the results of the F test are significantly different, a further test with Duncan Multiple Range Test / DMRT was carried out at the level of 5% to determine the highest growth.

3. Results and Discussion

3.1 Incubation Period of M. anisopliae

The average incubation period of *M. anisopliae* was 2 days on PDA, green beans, and maize media (Table 1). On the second day of observations *M. anisopliae* began to appear white mycelium which will turn green. Whereas in the bran media incubation period was 3 days. This is in line with research Novianti & Dewi (2017) showed that the average incubation period in the treatment of bran media was 3 days after incubation. Another case in the research of Gusnawaty *et.al,* (2013) state that the average incubation period of the fungus *Gliocladium* sp. were 2 days after incubation. Differences in genus or even fungal species can cause differences in the incubation period, nutritional requirements, optimal temperatures for growth and conidial formation (Kleespies and Zimmermann, 1992; Soetopo and Indrayani, 2007)

Table 1 Average incubation period of *M. anisopliae* in different media and shelf-life

Treatment	Shelf life (d-old)	Average Incubation Period (days)
PDA	21	2
	42	2
Bran	21	3
	42	3
Green beans	21	2
	42	2
Maize	21	2
	42	2

3.2 Colony Diameter of M. anisopliae in Different Media and Shelf-life

The *M. anisopliae* regression equation at the age of 21 days after inoculation in maize media was y= 1.65x+0.133 and R2= 0.98, it means the diameter growth in maize media will increase by 1.65% on each day. The increasing of diameter on PDA media were (1.44%), green beans (1.12%) and bran (0.30%) (Figure 1a). At the age of 42 days on maize media showed a quadratic model which has the highest diameter compared to other models with the following models y= -0.35x2 + 3.74x – 1.42 and R= 0.98 (Figure 1b).

Alternative media have more complex nutrients so that in the beginning the growth of fungi is not as optimal as PDA media (Aini and Rahayu, 2015). This is in line with Gandjar (2006) which states that the complex content in alternative media causes the fungus takes more time to break down into simple components so that the fungus can be used for its growth. In contrast to PDA media, nutrition is easily absorbed by fungi so that the initial growth/initiation runs faster. Afifah (2011) states that hyphae on PDA media grow faster than maize and rice media due to differences in nutrient content in each media. According to Saha., *et.al,* (2008); Aini and Rahayu (2015) explained that PDA media contain simple formulations so that nutrients are easily absorbed by fungi.

Maize media contains macronutrients carbon (C), hydrogen (H) and oxygen (O) and micro nutrients namely iron (Fe) and calcium (Ca). These nutrients are needed by *M. anisopliae* in the process of hyphal and mycelium growth. This causes the diameter growth on the 21st day to 42nd day run faster than other alternatives media. This is in line with the statement of Sadad., *et.al* (2014), media which contain macro and micro nutrients as needed can support the growth of fungi. Maize media does provide the highest average diameter compared to other alternative media, but the high average diameter growth does not mean providing high average spore density. Aryo*, et.al* (2017) showed that the highest average diameter of *M. anisopliae* fungi colony growth was obtained in isolates from Gadingrejo at 5 (d-old) with an average diameter reaching 7.80 cm and at 7 (d-old) reached 8.63. The highest spore density was found in isolates from UGM with a density of 2.25 × 109 spores/ml and that was greater than isolates from Gadingrejo which had a conidial density of 1.68×109 spores / ml. stated that maize also produced the greatest diameter colony than other alternatives media. Previous research reported that cereals such as maize can be used as substrates because they contain nutrients needed by the fungus for their growth so that they can colonize the substrate easily (Afifah & Saputro, 2020)

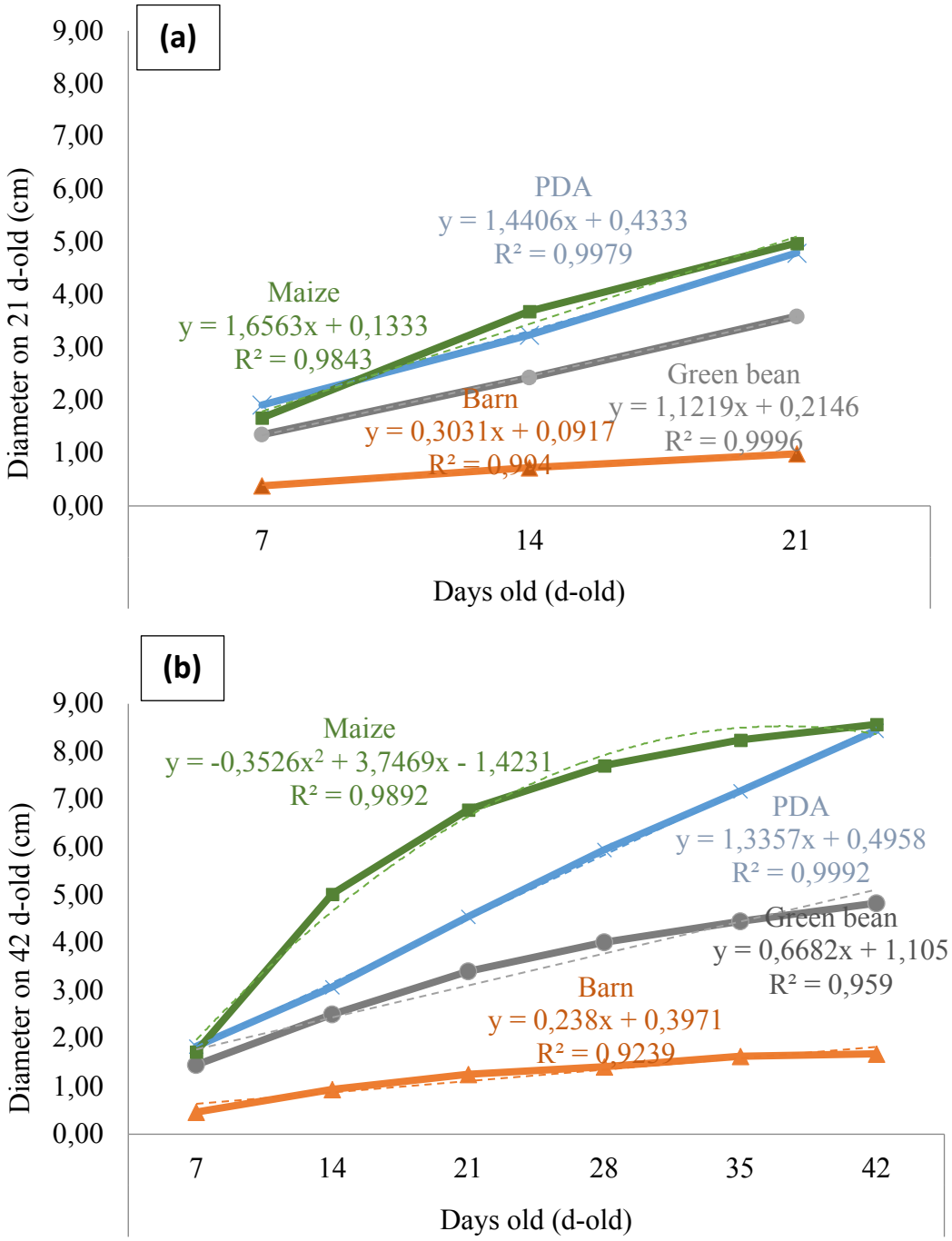

Figure 1. Diameter of *M. anisopliae* at shelf life (a) 21 d-old and (b) 42 d-old on maize, PDA, green beans and bran media

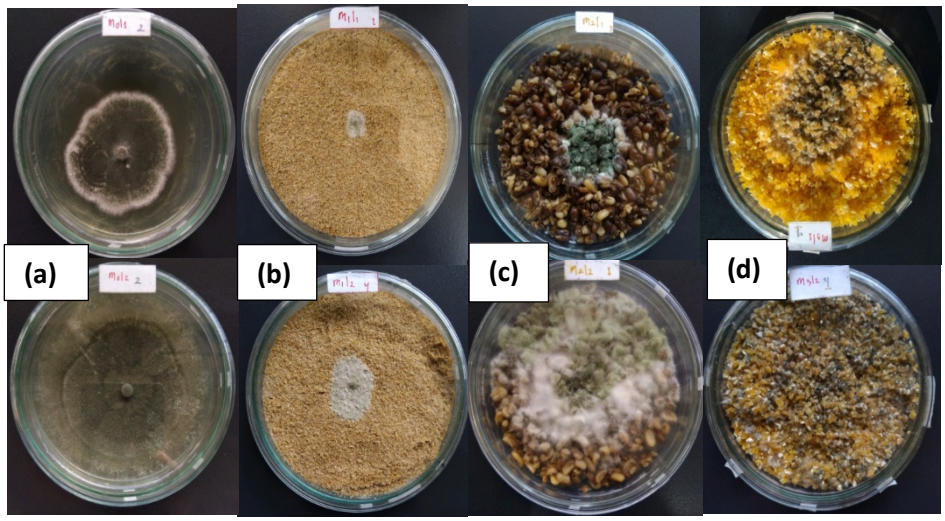

Figure 2. Colony appearance on PDA, bran, green beans, and maize media on 21 d-old (upper) and 42 d-old (bottom)

3.3 The density of M. anisopliae Konidia in Different Media and Shelf-life

The results of DMRT follow-up tests at 5% level showed that green bean media (spore density of 2.08×107 spores/ml) was significantly different from PDA, bran, and maize media (Table 4). This happens because the green bean media has a high protein content that is equal to 24% different from the protein content in other media which only contains 8.7% in bran media and 10% in maize media. Media with high protein content can accelerate the growth of *M. anisopliae* fungi. Nurwibawanto (2016) states that the protein content contained in the media plays a role in the formation of the conidia of the fungus *M. anisopliae*.

Protein contains nitrogen (N), nitrogen plays a role in supporting the vegetative process, namely the process of hyphal and mycelium growth. If vegetative growth goes well so generative growth (sporulation) goes well too. Proteins are needed for hypha formation and enzyme synthesis Gerraway and Evans, (1984); Sadad, (2014). In addition protein, carbohydrates and glucose are also needed for the growth and development of fungi. Novianti (2017) stated that entomopathogenic fungi require media with high glucose and protein content. *M. anisopliae* can grow and develop well on propagation media that contain high concentrations of carbohydrates.

Table 2. The average of density of *M. anisopliae* spores in several alternative media and different shelf-life.

Treatment	Spore Dencity/ml
Media	
PDA	$1{,}06 \times 10^{7}$ b
Bran	$4{,}34 \times 10^{6}$ c
Green Bean	$2{,}08 \times 10^{7}$ a
Maize	$1{,}04 \times 10^{7}$ b
Shelf-life (d-old)	
21	$7{,}44 \times 10^{6}$ b
42	$5{,}05 \times 10^{7}$ a

Note: The average value followed by the same letter shows no significant difference according to further DMRT tests at the 5% level.

In this experiment, the spore density in each media is still relatively low. This is due to the humidity during the experiment ranging from 47% - 59% and the daily temperature ranging from 25.9° - 28.4° celcius so that the growth and development of *M. anisopliae* are less than optimal. Factor that support the development of fungi was air humidity. High humidity is needed for the formation of sprout tubes of fungi. According to Prayogo & Santoso (2013) stated that the optimal humidity for the development of fungus is above 90%.

The spore density at the age of 42 days was significantly different from the age of 21 days culture, ie with a spore density of 5.05×10^7 spores/ml (Table 2). This happens because the longer the fungus is incubated, the more spores will be produced. Nuryati and Sujono, (2017) stated that the longer the incubation period, the growth of fungi will increase. According to Wahyunendo, (2002); Afifah, (2011) the increase in conidia is proportional to the longer incubation time until it shows the stationary point of growth.

3.4 M. anisopliae Germination Rate in Different Media and Shelf Life

The bran media gave the highest germination rate (94.23%) showed significantly different from the media of green beans, but not significantly different from the PDA and maize media (Table 3). The bran media contains 19.9% more fat than the green bean media which only contains 1.20% fat. These fats can trigger conidia germination (Kim., et.al, 2007; Prayogo, 2011). This is following Prayogo's research (2011) which states that both glycerol and fatty acids are a good source of energy for the growth and development of fungi. According to Prayogo & Santoso (2013) that the glycerol contained in vegetable fat is able to protect the structure of conidia from environmental factors that are less supportive so that the conidia can still germinate well.

Age of 21 days cultures gave the highest value to the germination capacity of *M. anisopliae* that is equal to 81.69%, but not significantly different from the age of 42 days. It is suspected that the longer shelf life of the fungus, the spore's germination capacity will decreases. This is in line with the research of Afifah (2011) the influence of the different type of media and the shelf life can also affect the germination. The germination capacity at 21 days in maize media was greater, which was 82.08%, although not significantly different at 42 days old.

Tabel 3 The average germination capacity of *M. anisopliae* in different media and shelf-life

Treatments	Germination Rate (%)
Media	
PDA	80,97 ab
Bran	94,23 a
Green Bean	63,94 b
Maize	75,33 ab
Shelf-life (d-old)	
21	81,69 a
42	75,54 a

Note: The average value followed by the same letter shows no significant difference according to further DMRT tests at the 5% level.

M. anisopliae spores are cylindrical and green in colour (Figure 3a). Spores will germinate after incubating for 24 hours. The germination power will be optimal if the sprout tube has grown beyond the initial spore length (Figure 3b). In the conidiophores the sporulation will occur to produce new spores (Figure 3c) after the spores are ripe then the spores will be separated from the conidiophores (Figure 3d).

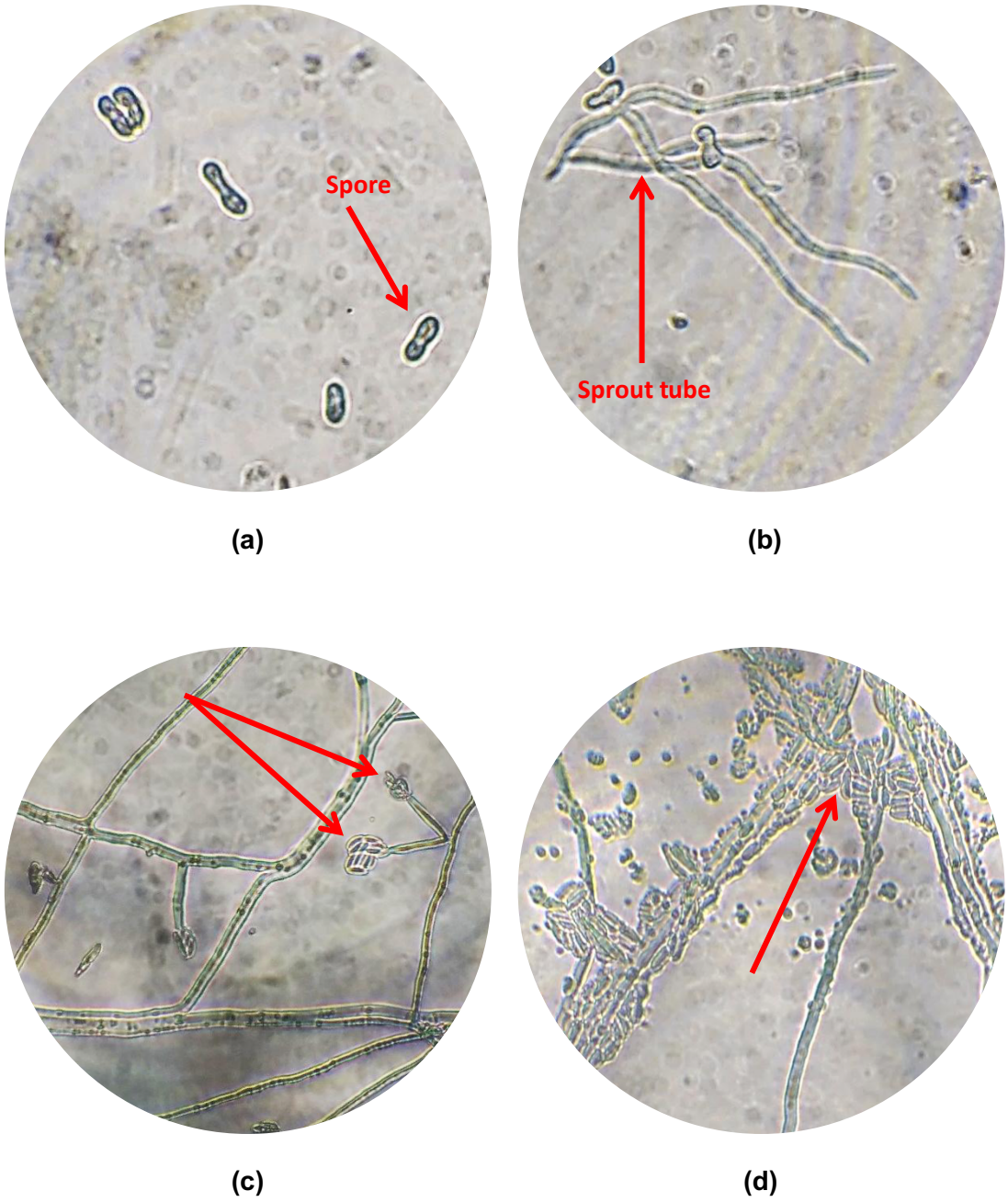

Figure 3 *M. anisopliae* spores which have not germinated (a), *M. anisopliae* spores which have emerged sprout tubes (b), Spores that have just emerged from conidiophores (c) Spores that have been released from conidiophores (d) (400 times magnification).

3.5 The difference in Weight of M. anisopliae Growing Media at 21 and 42 d-old

In the media of green beans with 42 d-old, the highest media weight difference was 2.64 g and was significantly different from other media (Table 3). This happens because the highest spore density is found in the green bean media. Existing nutrients in the green bean media are absorbed more by *M. anisopliae*, so that the weight of the media is reduced more. The statement is in accordance with research by Gusnawaty (2013) that the nutrients available to the media will be overhauled and absorbed by fungi for growth. Besides, fungus activity also causes a reduction in water content.

Table 4 Average differences in media weights before inoculation and after inoculation on several alternative media with different shelf lives.

Media	**Difference in Media Weight/g**	
	21 d-old	**42 d-old**
PDA	0,95 b	2,22 c
	B	A
Barn	0,27 c	0,81 d
	B	A
Green Bean	0,89 b	2,64 a
	B	A
Maize	1,05 a	2,43 b
	B	A

Note: The average value followed by the same letter in each column with a lowercase letter and each row with an uppercase letter shows no significant difference according to the DMRT at the 5% level. Lowercase letters indicate the type of media factor. Uppercase letters indicate the shelf life factor.

4. Conclusion

The diameter of the maize media at 42 days gives the highest average value of 8.57 cm. Green bean media gave the highest spore density results of 2.08 × 107. While the best germination was obtained in bran media with a value of 94.23% at the shelf life of 21 d-old. The highest difference in media weight occurred in the media of green beans with a shelf life of 42 d-old, 2.64 g. The starch-rich media such as green beans and maize could be alternative media for growing entomopathogenic fungi of *M. anisopliae* .

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