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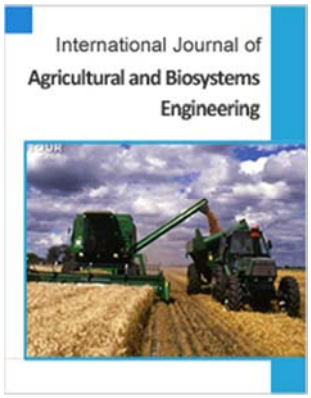


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# Environmentally Sustainable Production of *Metrhizium anisopliae* and *Beauveria bassiana* for Control of *Tuta absoluta*

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## Abstract

Extensive insecticide use can cause resistance in many insect-pests and also undesired side-effects on human and environment. Leaf miner, *Tuta absoluta*, is a new generation of devastating pests that rapidly spreading throughout the world causing up to 100% of tomato yield losses. In this view, it is a necessary consequence to apply environmentally-friendly strategies in order to control this pest effectively. As a result, research studies were undertaken in hope to find some waste materials that could be successfully used for sustainable mass-production of Lebanese isolates and their utilisation against *T. absoluta*. Eight different solid substrates (mostly waste materials) were tested for suitability for mass production of *M. anisopliae* and *B. bassiana*. High conidial yields of more than $10^{10}$ conidia $ml^{-1}$ for *M. anisopliae* and around 4.0 x $10^9$ conidia $ml^{-1}$ for *B. bassiana* were harvested from spoiled cereals. 2.0 - 4.0 x $10^9$ conidia $ml^{-1}$ for both spp. was collected from potato and carrot peel substrates. However, olive pomace (jifett), sawdust and orange peel substrates were found unsuitable for mass production. Carbon and nitrogen content of resultant conidia were affected by the substrate. The lowest C:N value of 4 was estimated for conidia of *B. bassiana* cultured on rice and the highest of 7.9 for *M. anisopliae* cultured on SDA. Conidial germination was exceeding 95% for all cultures. Total control of *T. absoluta* eggs was achieved under laboratory conditions using conidia from rice and burghul. Conidia of *M. anisopliae* from the same substrates caused 80% of egg mortalities under the same conditions. Good control of eggs and larvae in a greenhouse were observed after 3rd application of *B. bassiana* cultured on potato peels suggesting that this substrate is a potential candidate for sustainable mass production of EPF and their use in IPM strategies in the greenhouses.

## 1. Introduction

The development of resistance by many important insect pests, the continuous increase of chemical prices used for the pest control, along with the concern about the environment protection has encouraged studies and use of biological control (BC) [1, 2]. Entomopathogenic fungi are attracting attention as biocontrol agents for insect pests and are being considered as supplements or alternatives to synthetic insecticides [3].

*Beauveria bassiana* (Bals.) Vuill. (Hypocreales: Clavicipitaceae) and *Metarhizium anisopliae* (Metschn.) Sorokin (Hypocreales: Clavicipitaceae) are the common entomopathogens used in pest management [4], and are the natural enemy with great potential for crop protection worldwide [5]. Both entomopathogenic fungi (EPF) have been produced on a commercial scale in several countries, and, for example in Brazil solely, in considerable volumes of this pathogen are sold annually for the control of many pests [6, 7]. In addition, *B. bassiana* and *M. anisoplie* were isolated from Lebanese soils, mass-produced on solid substrates such as rice, wheat and burghul and their effectiveness was shown against varieties of aphids, a whitefly and spider mites under laboratory and greenhouse conditions [8]. Even though the studies showed that mass produced inoculum results in good control of targeted pests, it is still expensive to produce.

*Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) has become a key pest of tomato in several world regions and its geographic distribution is rapidly expanding [9]. The newly introduced pest from South America [10] is finding the shores of the Mediterranean [11, 12] where it can breed between 10-12 generations a year. The infestation of *T. absoluta* also reported on potato, aubergine, sweet peppers and common beans. It also occurs on weeds of Solanaceae family (*Solanum nigrum*, *Datura* spp., etc.). *T. absoluta* can cause 50-100% yield reduction on tomato crops and its presence may also limit the export. The larvae of *T. absoluta* mine the leaves producing large galleries and burrow into the fruit, causing a substantial loss of tomato production in protected and open filed cultivations. This pest damage occurs throughout the entire growing cycle of tomatoes. In tomato infestation found on apical buds, leaves, and stems, flowers and fruits, on which the black frass is visible. On potato, mainly aerial parts are attacked. However, damage on potato tubers has also been recently reported in Sudan and Ethiopia [13].

Effectiveness of chemical control is limited due to insect's nature of damage as well as its rapid capability of development of insecticide resistant strains [14, 15, 16, 17, 18]. Although several studies have shown fungal potential for the BC of eggs, larvae and pupae of *T. absoluta* [19, 20, 21, 22], the use of biological factors against *T. absoluta* are still largely under development and not ready to combat this pest effectively in a cost effective way.

In view of the above mentioned perspectives, the present research was undertaken to work, as far as possible, towards a low cost production of EPF by means of phasing out the use of expensive capital items and any imported ingredients. The development of production methods and formulations by substituting cheaper ingredients that come as waste products such as vegetable peels, sawdust, ground coffee, recycled oils, etc. would facilitate the cost-effective application of *B. bassiana* and *M. anisopliae* conidia for the control of tomato leaf miner *T. absoluta* under laboratory and greenhouse conditions.

# 2. Materials and Methods

## 2.1. Mass Production

Lebanese isolates of *B. bassiana* (LIB1) and *M. anisopliae* (LIM1) [8] were used in this study. Long term storage of the isolates was achieved by freezing conidia in 10% w/w Glycerol at -20°C.

When required conidia was re-hydrated by suspending in a small volume of sterile 0.03% Tween 80 solution, placed on SDA and incubated at 23 ± 2°C in the dark for 14 days. Following incubation conidia were scraped from sporulating colonies and suspended in 0.03% Tween 80 solution. In order to remove any hyphal fragments, the resultant conidial suspension then was filtered and used for further experimentation.

Materials that regarded as waste materials and came from food or other industries were tested in this study. Fresh potato and carrot peels were obtained from an orphanage (Al Moassassa Al Ijtimaiya, Tripoli, North Lebanon), ground coffee waste (Damour coffee shop, Central Lebanon) and orange peel (Fresh Juice, Damour, Central Lebanon) were collected and stored at 4°C until used. Saw dust from a local furniture making shop (Damour, Central Lebanon) and dry olive pomace (private household, Kffar Hammam, South Lebanon) were soaked in distilled water ($dH_2O$, 100g/200ml) for 24 hr prior to use. Whole grains of long parboiled rice and coarse burghul that have been infested with pest insects (private household, Damour, Central Lebanon) with added $dH_2O$ just before autoclaving (100g/80ml) were also used.

The preparation of the substrates was done by placing 100g of each substrate into roasting bags (36 cm x 15 cm, ZEC, China) and plastic pots that were previously used as yogurt containers (1 litre, Tamia, Lebanon). A strip of autoclavable tape (5cm) was fixed onto each bag and pot to reduce contamination during inoculation. Bags and pots containing substrates were plugged with cotton wool and sterilized by autoclaving at 121°C, 15psi for 35 min.

After cooling 2 cm of the tape was peeled off, sterile syringe needle was inserted and 2 ml of the conidial suspension ($10^7$ conidia $ml^{-1}$) was injected in each bag or pot. Upon needle withdrawal the puncture was immediately resealed. Inoculated bags were thoroughly massaged, whereas inoculated pots were vigorously shaken to mix inoculum with the substrate and then placed into plastic trays, and incubated at 25°C. When the substrates were fully colonized by fungal hyphae, the cotton plugs were removed and the content of each bag was kneaded and each pot was shaken for substrate aeration and maximum sporulation.

## 2.2. Yield Determination

The conidia were harvested 7 days and 14 days post inoculation in order to estimate the optimal harvesting period. One gram of each substrate was placed in 10 ml of 0.03% Tween 80 solution and shaken on a rotary shaker for

30 min at 380 rpm. Spore counting was done using Fusch Rosenthal hymocytometer. Note that yield from coffee substrate was harvested 7, 14 and 21 days post inoculation since the growth and sporulation on this substrate appeared one week later compared to the other substrates.

When the inoculated substrates reached the maximal production, each bag and pot were removed from the incubator and sides of each bag were split-opened from the bottom to the middle, whereas lids of the pots were taken off and kept at 25°C to allow rapid dehydration of the substrates. When the substrates were fully dehydrated, the aerial conidia were dislodged from the substrate by gentle agitation of the substrate through three sieves (500µm, 250µm and 45µm) stuck on top of each other. The sieves were covered and agitation of substrate was done manually for approximately 45 min to collect maximum quantity of fungal spore powder from each substrate. Resultant powder was collected from the tray and placed into sterile vials, weighed and stored in the refrigerator at 5°C until needed [23].

## 2.3. Quality Control

C:N ratios (carbon and nitrogen composition) as quality control parameters from conidia produced on SDA and selected substrates (rice, burghul, potato and carrot peels) were determined using modified methods of Springer-Klee for organic carbon and Kjeldahl method for total nitrogen [23].

Total carbon was determined using 20 ml $K_2Cr_2O_7$ (0.33M) and 26 ml conc. $H_2SO_4$ that were added to 0.5g of desiccated conidia in a glass test tube and left to cool (40-50°C). Tubes were placed in a block digest and heated at 160°C for 10 min. After cooling on icy water bath, the content of each tube was transferred into a 200 ml volumetric flask and the volume was made up to 200 mL with $dH_2O$. The flasks were shaken to homogenise the solution and then left to settle for 12 h. Titration was performed as follows: 20 ml of the solution was mixed with 2 ml of $H_3PO_4$ (85%) and $H_2SO_4$ (96%) mixture and 8 drops of indicator in 100 ml of $dH_2O$ was titrated against $F_2SO_4$ until colour shifted from violet to pale green. Two controls without any sample solution were heated or without heating in the block digest were also performed.

For total nitrogen determination, each sample of desiccated conidia (0.5 g) together with 3.35 g of catalyst ($K_2SO_4$/$CuSO_4.5H_2O$/Se at 100:10:1 ratio) and 10 ml of conc. $H_2SO_4$ was digested for 30 min at 200°C and then for 1.5 h at 350°C in a fume cabinet. After digestion for each batch of samples at least one blank (no conidia) and one standard ($(NH_4)_2SO_4$) were prepared. Recovery was determined between 95-105%. Distillation was carried out in the distillation unit.

Protein concentration in each sample was determined using method of Lowry [24]. Each dosage sample together with 1 ml of Lowry solution were added to 250 ml of distilled water and let to stand for 10 min. One hundred ml of Folin solution was then added, vortexed and incubated in the dark for 30 min. The optical density has been measured at 691 nm (BSA) using a spectrophotometer and standard curve was constructed using an equation: y=0.1908x+0.0845. Amounts of proteins in each sample were determined using the standard curve.

Spore germination test was performed for all conidia cultured on different substrates according to Ibrahim [25]. Briefly, 10 µl of conidial suspension in 0.03% Tween 80 ($10^5$ conidia $ml^{-1}$) was dispensed onto the centre of five replicate Petri dishes containing 15 ml of SDA. All plates were sealed with parafilm and incubated at 25±2°C in darkness. After 24 h incubation the incidence of germinated conidia was recorded for five hundred conidia (100 conidia per replicate). Conidia was considered to have germinated when the germ-tube was as long as conidial width.

Viability of conidia harvested from potato substrate and dried to 5% moisture content was tested after 24 hours of conidial inoculation of SDA media. Two conidial formulations were tested as follows: from two Petri dishes each cultured with *B. bassiana* or *M. anisopliae*, two loops were taken and then added to two separate Erlenmeyer flasks each containing 10 ml of autoclaved 0.03% Tween solution or two flasks each containing 50 ml of autoclaved reused vegetable oil. The four flasks then were sealed with parafilm and shaken on a rotary shaker for 20 min. There were 3 replicate dishes for each isolate and each formulation. Five little circles were drawn on the bottom of each Petri dish. Conidial suspensions were applied using a micropipette when 20 µm of each formulation were disposed into each marked circle. The inoculated Petri dishes were then sealed with parafilm and incubated for 24 hours. Conidial germination for both fungi was determined by staining the conidial germlings with Cotton Blue.

## 2.4. Pathogenicity Tests

Conidia harvested from SDA, rice, burghul, potato and carrot peals were compared for their virulence against *T. absoluta* eggs under laboratory conditions.

Adults of *T. absoluta* were collected from tomato greenhouses located in Jeita Keserwan (Mount Lebanon) in June, 2016. Healthy and free from any insect tomato plants (4 to 5 weeks old) were placed in cages (60cm high, 50cm wide and 50 cm long). About 80 adults of *T. absoluta* were released in each cage for 48 hr at room temperature (25±3°C) and natural light/dark photoperiod. After 2 days the plants were checked for eggs availability and these eggs were used for bioassay.

Conidial suspension of $10^7$ conidia $ml^{-1}$ was used for all treatments. Dry conidia from potato and carrot peels, rice, burgul and SDA were suspended in 0.03% Tween 80 solution. Leaves with *T. absoluta* infested eggs were used for laboratory assays. Each treatment consisted of 3 replicates with 18, 20 and 30 eggs per leaf, respectively. Each replicate was sprayed with 0.3 ml of conidial suspension using hand sprayer. Leaves were sprayed from both adaxial and abaxial sides and placed in sterile Petri dishes lined with moist filter paper. Control treatment consisted of same number of replicates and were sprayed with 0.03% Tween 80 solution

only. All treatments were randomly placed in clean trays and incubated at 23±1°C. After 7 days the number of dead eggs was recorded. Development of mycosis was observed 14 days after treatment.

The virulence of formulated conidia was also tested against *T. absoluta* in a greenhouse (Besri, South Lebanon) using procedures described below.

Seeds of tomato variety "Malakeh" were planted into trays containing seedling compost at the beginning of April, 2016 and grown for a month (till May) in a greenhouse. The resulting seedlings at 2-3 leaf-stage were transferred to the greenhouse (60 m x 8 m) located at 400 m above sea level. The experimental design was Complete Randomized Block Design with 6 tomato plants in each block with 5 blocks for each treatment. There were 6 treatments: Control 1 (C1) – 0.03% Tween 80 solution, Control 2 (C2) - 20% oil emulsion, Treatment 1 (T1) - *B. bassiana* in 0.03% Tween, Treatment 2 (T2) - *B. bassiana* + 20% oil emulsion, Treatment 3 (T3) - *M. anisoliae* in Tween and Treatment 4 (T4) - *M. anisopliae* + 20% oil emulsion. Spacing between each plant was 40 cm and each block was spaced 1 m apart from each other. The greenhouse doors were draped with nets in order to keep the tomato plants free from any pest invasion before the release of *T. absoluta* and also to protect the other tomato plants in neighbouring greenhouses from getting invaded by *T. absoluta* adults after their release, and to ensure the accuracy of the experiment. About 50 tomato leafminer adults were released onto the healthy and insect free tomato plants in the greenhouse. After 5 days the plants were checked for eggs and larval availability in order to start the treatments.

Conidial suspension of $10^8$ conidia $ml^{-1}$ of *M. anisopliae* and $10^7$ conidia $ml^{-1}$ of *B. bassiana* were used. Each replicate was sprayed with 200 ml of conidial suspension using two 1 L hand sprayers designed for spraying Tween suspension and oil formulations, respectively. Leaves were sprayed from both adaxial and abaxial sides. Adults of *T. absoluta* were released 5 days prior to the first spray on 6th of June. The dates of spraying were: 11th, 18th, and 25th of June. Two days after each treatment, six labelled leaves from each treatment were examined and number of eggs and larvae on each leaf recorded using glass magnifier. Leaf samples were also collected and brought to the laboratory in order to record presence of the fungi on the leaf surface and inside leaf mesophyll. Leaves were washed and shaken for 30 min in order to collect conidia from leaf surface. A drop from resulted suspension and a drop of Cotton Blue then were applied on a microscope slide and examined under the light microscope. In order to see if the conidia became endophytic by penetrating the leaf cuticle and continue to grow inside mesophyll, the leaves were surface-sterilised using 70% alcohol for 2 min and 4% bleach solution for 1 min while shaken in order to remove the remaining conidia found on the leaf surface. The sterilised leaves were then rinsed in sterile distilled water three times. The washed leaf cuts were then cultured on SDA for 7 days to see if the leaves became endophytic showing fungal growth. These procedures were repeated for each isolate and each treatment.

Temperature, solar radiation, relative humidity, dew point and leaf wetness data were taken from Lebanese Agricultural Research Institute (LARI, Lebaa, East of Saida, South Lebanon).

### 2.5. Statistical Analysis

A completely randomized design was used during mass production tests, conidial germination tests and pathogenicity bioassays for determination of egg mortality of *T. absoluta*. The data obtained on mean% germination, mean% egg mortality or mycosis were angularly transformed and then analysed statistically using analysis of variance (Two-way ANOVA). Significant differences (at $P \leq 0.05$) between the treatments over the time of weekly assessment after inoculation were determined by F-tests and mean separation was done using Tukey's test (SPSS, Version 16.0, 2007). Complete Randomized Block Design was used during greenhouse experiment and data analysed using analysis of variance (Three-way ANOVA).

## 3. Results and Discussion

### 3.1. Effect of Substrate on Fungal Colonization, Development and Conidial Production

Eight different solid substrates (mostly waste materials) were tested for suitability for mass production of *M. anisopliae* and *B. bassiana* by means of growth, development and sporulation. The results showed that not all of the substrates tested were suitable for fungal colonisation and further development. For example, extensive mycelial growth on burghul, rice, potato and carrot peels were noted within two days of inoculation with sporulation appearing a day or two later. Although fungal growth and sporulation of *B. bassiana* were observed on coffee substrate, the process of colonisation was somewhat slower in comparison to other substrates.

The number of spores on four solid substrates was determined after 7 and 14 days post inoculation for *M. anisopliae* and 7, 14 and 21 days for *B. bassiana*. Since similar yields of spore production in both, recycled plastic pots and roasting bags, were observed, the mean values for each substrate in Tables 1 and 2 are presented for yields determined for pots only. The yield of dry conidia is also shown [Fig. 1].

***Table 1.*** *Mean number of conidia $ml^{-1}$ produced by M. anisopliae on different solid substrates over period of 7 and 14 days in recycled plastic pots.*

| Substrate | 7 days [$10^9$ conidia $ml^{-1}$ ±SE*] | 14 days [$10^9$ conidia $ml^{-1}$ ±SE] |
|---|---|---|
| Carrot | 1.17± 0.47a | 2.99 ± 0.40a |
| Potato | 1.70± 0.10a | 2.63± 0.67a |
| Burghul | 1.10± 0.26a | 19.61± 0.26b |
| Rice | 0.91 ±0.08a | 10.03±0.12b |

*-standard error (SE); a,b - means of 5 replicates followed by the same small letter in a column are not significantly different according to Tukey's test ($P \leq 0.05$)

***Table 2.*** *Mean number of conidia ml$^{-1}$produced by B. bassiana on different solid substrates over period of 7, 14 and 21 days in recycled plastic pots.*

| Substrate | 7 days [$10^9$ conidia ml$^{-1}$ ±SE*] | 14 days [$10^9$ conidia ml$^{-1}$ ±SE] | 21 days [$10^9$ conidia ml$^{-1}$ ±SE] |
|---|---|---|---|
| Carrot | 0.65±0.08a | 3.35±0.05b | 2.65±0.61b |
| Potato | 1.04±0.03b | 4.31±0.03b | 4.08±0.45c |
| Coffee | - | 0.11±0.01a | 0.51±0.15a |
| Burghul | 1.38±0.02 b | 4.40±0.10 b | 1.86±0.36b |
| Rice | 1.44±0.07 b | 3.94±0.27 b | 1.72±0.11b |

*-standard error (SE); a,b,c - means of 5 replicates followed by the same small letter in a column are not significantly different according to Tukey's test (P≤0.05)

Results (Table 1) indicated that there was no significant difference observed in the yields of *M. anisopliae* cultured on 4 different substrates after 7 days post inoculation. However, 14 days post inoculation significantly higher number of conidia was harvested from burghul and rice compared to the both vegetable peels resulting in almost $2.0x10^{10}$ and $1.0x10^{10}$ conidia ml$^{-1}$, respectively. Spore counts for *B. bassiana* (Table 2) indicated that the different substrates significantly affected the spore production (F=930.92; P≤0.001). Seven days post-inoculation the highest yield was 1.44 $x10^9$ conidia ml$^{-1}$ on rice, 1.38 $x10^9$ conidia ml$^{-1}$ on burghul and 1.04 $x10^9$ conidia ml$^{-1}$ on potato peels. The lowest yield of $0.65x10^9$ conidia ml$^{-1}$ was observed on carrot peels. However, two weeks later similar high number of spores were collected from rice, burghul and two vegetable peels. Spore counts of $1.1x10^8$ conidia ml$^{-1}$ were determined on coffee. Three weeks post-inoculation number of spores has declined on almost all substrates used except for coffee (Table 2).

Results have also shown [Fig. 1] that regardless of substrate used the maximal conidial yield for *M. anisopliae* was achieved two weeks post inoculation. Total weights of harvested dry spore powders from 500g of each substrate resulted in 2g of pure conidia from carrot peels, 8g from potato peels, 27g from burghul and 25g from rice [Fig. 1]. Each value was significantly different from each other (F=207.136; P<0.0001) reflecting the data obtained using hemocytometer count method. For *B. bassiana* significant variations in total conidial yield (F=361.750; P<0.0001) was observed and depended on substrate it was harvested from. For example, the highest yield of 4.7g of pure conidia was collected from burghul substrate followed by rice, potato and carrot peels with weights of 4.6g, 2.1g and 1.5g, respectively.

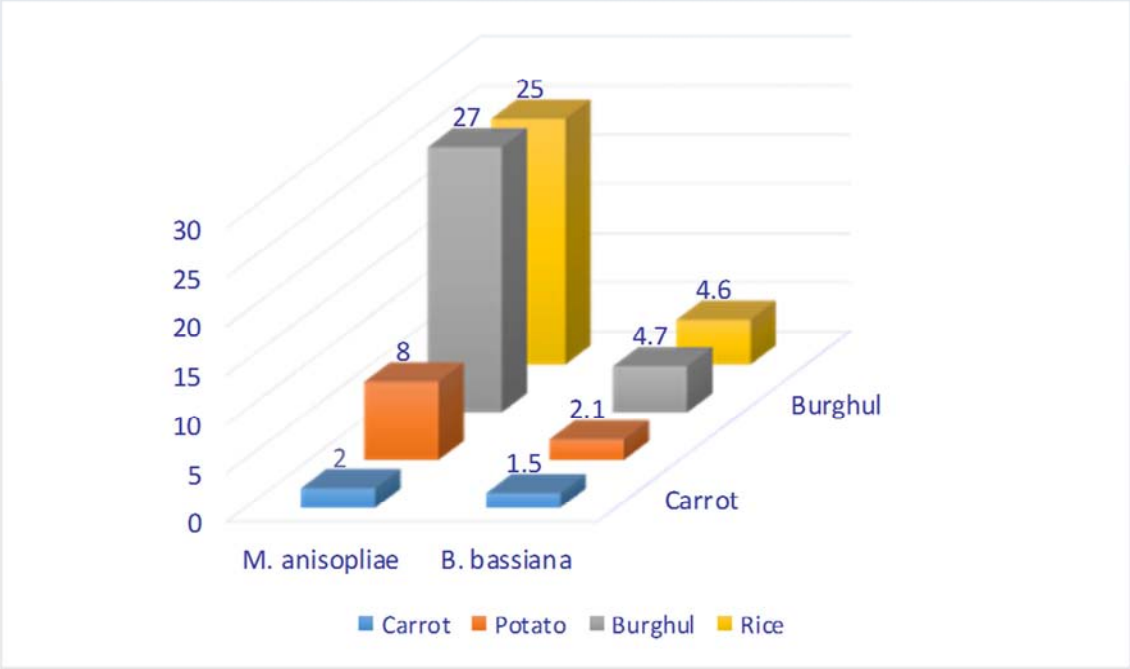


***Figure 1.*** *Total dry conidial weights (g/500g) harvested from different substrates in recycled plastic pots.*

Four out of eight substrates used in this study supported growth of entomogenous *M. anisopliae* and *B. bassiana* fungi confirming earlier work of Feng [4], Somasekhar [26] and Sergio [27] who found that fungi can be cost-effectively mass-produced on different solid substrates. Nutritional values of solid substrate are known to influence the yield of conidia [23, 28, 29]. High yields of conidia from burghul could be explained by high carbohydrates content, fibre, proteins, high levels of most vitamins and minerals [30]. Grains are cheap, easily available and act as best nutritive media for the mass multiplication of many micro and macro organisms. According to Ibrahim and Low [31] and Sharma [32], rice was found to be the most suitable media for the mass culture of *B. bassiana*. Although significantly lower

amounts of fungal propagules were harvested from potato and carrot peels which mainly consist of phenolic compounds (e.g. phenolic acids), high molecular cell wall components (e.g. cellulose, hemicelluloses, lignin, pectin, gums), glycoalkaloids (e.g. α-solanine and α-chaconine) and low amounts of starch, proteins and vitamins, these substrates supported colonisation, establishment and sporulation of isolates used in this study. Gopalakrishnan [33] and Tincilley [34] reported that carrot was found to be the cheapest and best suitable media for the large-scale production of deuteromycete fungi.

Coffee waste is known to contain large amount of organic compounds, i.e. fatty acids, lignin, cellulose, hemicelluloses and polysaccharides such as glucose and mannose and is commonly used in mushroom industry [35]. Why *M. anisopliae* fungus failed to colonize and develop on this substrate in present studies deserves further investigations.

Nevertheless, citrus peel contains essential oils (90% D-Limonene) which are known to be antimicrobial agents [36, 37] that may upset or fail the fungal digesters. Dried olive pomace (jifet), although contains soluble sugars, organic acids, fibre with values above 70%, balanced amino acid (75–80%) composition, its isoelectric point, however, is about pH 2.0 [38], a parameter that could be responsible for the fungus failing to establish itself on this particular substrate. Sawdust from unknown wood tree in the study also did not support growth and development of *M. anisopliae* and *B. bassiana* isolates. This is may be because most wood contain mainly cellulose and lignin [39] and also essential oils [40] that could also possess fungicidal properties. In addition, utilisation of solid substrates by microorganisms is affected by many other factors such as particle size, shape, surface-to-volume ratio, crystallinity, and porosity of the substrate, all factors that can influence the accessible surface area to both organism and enzymes [41]. Indeed, Yypsilos [42] reported that burghul wheat supported a higher yield of better quality conidia compared to millet grains because burghul provided a more accessible substrate. Present study also suggests that the nutritional content of burghul wheat is more favourable for conidial production of higher quality compared to other substrates and that the physical properties of burghul wheat grains allowed the fungus to access more nutrients.

## 3.2. Quality Control of Mass Produced Conidia

With the intention of elucidating pathogenicity mechanism and effects of culture substrates on virulence of produced inocula it was necessary to perform the quality control. Shah [43] has recommended endogenous C:N ratio (<5.2:1) of conidia in combination of other parameter as a virulence indicator in quality control. Conidia of *M. anisopliae* from carrot, potatoes, burghul and rice were tested and results were expressed in%. Results in Table 3 have revealed that substrates used in our study did affect the carbon content of resultant conidia (F=27.763; P<0.01). Amount of nitrogen accumulated in harvested conidia, was also significantly affected by a substrate it was cultured on (F=29.230; P<0.011). The lowest amount 33.56% of carbon was observed in conidia from potato peels, followed by carrot with 34.42%, burghul with 35.68%, rice with 43.62% and finally in SDA with the highest percentage of almost 48. Highest amount of nitrogen, however, was accumulated in conidia harvested from burghul (7.04%).

***Table 3.*** *Carbon and Nitrogen (%) and C: N ratios of M. anisopliae conidia cultured on 5 different media.*

| Substrate | Carbon%±SE* | Nitrogen%±SE | C/N ratio ±SE |
|---|---|---|---|
| Carrot | 34.42±4.42[a] | 5.70±0.30 [a] | 6.01±0.46 [a] |
| Potatoes | 33.56±3.44 [a] | 4.80±0.20 [a] | 7.03±1.00 [a] |
| Burghul | 35.68±3.56 [a] | 7.04±0.02[b] | 5.06±0.49 [a] |
| Rice | 43.62±2.66 [ab] | 6.34±0.12[b] | 6.72±0.39[a] |
| SDA | 47.90±1.98 [b] | 6.08±0.11[b] | 7.88±0.12[b] |

*-standard error (SE); a,b - means of 3 replicates followed by the same small letter in a column are not significantly different according to Tukey's test (P≤0.05)

Similar nitrogen content was observed in conidia from rice and SDA with the lowest quantity of 4.8% present in conidia from potato peels. The results in Table 4 illustrate insignificant differences in carbon content of *B. bassiana* conidia (F=2.505; P=0.198). The percentage values have been ranging between 35 and 40. Nitrogen content, on the other hand, was different (F=160.529; P=0.001) from carbon and observed to be highest in conidia grown on rice with value of 8.12 and lowest in conidia from SDA with value of 5.14. Calculated C:N ratio values also differed depending on media (F=27.012; P=0.004). For instance, conidia from rice had the lowest ratio of 4.31 followed by potato and carrot peels with the values of 5.86 and 5.96, respectively. The highest of 6.88 and 7.20 C:N ratio values were determined for conidia from burghul and SDA, respectively (Table 4).

***Table 4.*** *Carbon and Nitrogen (%) and C:N ratios of B. bassiana conidia cultured on 5 different media.*

| Substrate | Carbon%±SE* | Nitrogen%±SE | C/N ratio ±SE |
|---|---|---|---|
| Carrot | 40.4±0.09a | 6.78±0.17b | 5.96±0.03b |
| Potato | 39.9±0.08a | 6.81±0.20b | 5.86±0.02b |
| Burghul | 37.5±0.24a | 5.45±0.02a | 6.88±0.04c |
| Rice | 35.0±0.02a | 8.12±0.02c | 4.31±0.00a |
| SDA | 37.3±0.00a | 5.14±0.05a | 7.20±0.00c |

*-standard error (SE); a,b,c - means of 3 replicates followed by the same small letter in a column are not significantly different according to Tukey's test (P≤0.05)

The effect of each substrate on protein concentration in resultant conidia is shown in Table 5. Average protein concentration differed significantly between substrates (F= 4.39, *P*<0.05) with rice showing the highest protein concentration for both fungi (2.86 µg $ml^{-1}$ for *M. anisopliae* and 2.75 µg $ml^{-1}$ for *B. bassiana*). The lowest concentration of 0.88 µg $ml^{-1}$ for *B. bassiana* and 1.05 µg $ml^{-1}$ for *M. anisopliae* was determined for spores grown on SDA.

***Table 5.*** *Mean protein concentration for B. bassiana and M. anisopliae grown on different substrates.*

| Substrate | *B. bassiana* (µg/ml± SE*) | *M. anisopliae* (µg/ml ± SE) |
|---|---|---|
| Burgul | 1.99± 0.68ab | 2.72± 0.77b |
| SDA | 0.88± 0.66a | 1.05± 0.94a |
| Potato | 2.07± 0.67b | 2.64± 0.41b |
| Rice | 2.75± 0.73b | 2.86± 0.33b |

*-standard error (SE); a,b,c - means of 3 replicates followed by the same small letter in a column are not significantly different according to Tukey's test (P≤0.05)

High amounts of endogenous proteins in conidia of EPF may provide the amino acid pool necessary for protein synthesis and thus facilitate rapid germination and frequent appressorium formation in *Metarhizium* and *Beauveria* isolates. Endogenous supplies of amino acids for *de novo* protein synthesis were shown to be essential for initial events of germination and differentiation in *Colletotrichum lagenarium*, *M. anisopliae*, *Glomerella magna* and *C. truncatum*, while subsequent events, such as germ tube elongation, often used exogenous sources of carbon and nitrogen [44, 45, 46, 47, 48, 49]. In addition, proteolytic compounds (enzyme proteins) in entomopathogens have been always linked to their virulence [45]. Tomson [46] showed that *B. bassiana* strains having higher protein content caused significantly higher insecticidal activity and altered digestive enzymes level/ activity in insect than strains having lower protein content. According to the results presented here, conidia from burghul and rice contained the highest protein concentration in both spp. suggesting of these inocula being highly effective against insect pests. Indeed, these same isolates cultured on the same substrates were shown to be highly pathogenic against *T. absoluta* eggs treated with *B. bassiana* causing 100% mortality and up to 80% death in eggs treated with *M. anisopliae* [Fig. 2].

The germination test was conducted at 24 h post inoculation on SDA media. The test was done for both isolates straight after the harvesting. Overall, the germination for all treatments at 24 h was over 94.5% (Table 6). The substrates tested had no adverse effect on conidial viability and thus considered suitable for mass production.

***Table 6.*** *Germination (%) of M. anisopliae and B. bassiana after 24 h post inoculation on SDA media.*

| Substrate | *M. anisopliae* (%±SE*) | *B. bassiana* (%±SE) |
|---|---|---|
| Carrot | 98.7±0.12[a] | 98.3±0.02[a] |
| Potatoes | 98.8±0.09[a] | 98.5±0.02[a] |
| Burghul | 99.1±0.10[a] | 99.3±0.03[a] |
| Rice | 98.9±0.12[a] | 99.7±0.01[a] |
| SDA | 94.9±0.19[a] | 98.2±0.07[a] |

*standard error (SE); means of 3 replicates followed by the same small letter in a column are not significantly different according to Tukey's test (P≤0.05)

To obtain uniform conidial suspension and even application on the plant surfaces, fungal conidia often formulated in oils [50]. Conidial viability harvested from potato substrate and formulated in recycled vegetable oil were also determined in this study (Table 7). Vegetable oil used seemed to affect the germination of *M. anisopliae.* Ten percent reduction in viability could be due to changed chemical composition of recycled oil since preceding heating could have caused the oil to undergo a series of chemical reactions like oxidation, hydrolysis and polymerization [51] and thus became contributing factor to the decline of conidial viability.

***Table 7.*** *Mean germination (%± Standard Error) of B. bassiana and M. anisopliae in oil and 0.03% Tween solution.*

| Formulation | *B. bassiana* (%) | *M. anisopliae* (%) |
|---|---|---|
| 0.03% Tween | 97.40 ±0.60a | 93.35±0.35a |
| Recycled veg oil | 97.85±1.15a | 85±0.35b |

Means of 3 replicates followed by the same small letter in a column are not significantly different according to Tukey's test (P≤0.05)

Conidial quality could significantly affect conidial germination since the endogenous reserves of such inoculum are initially the sole source of nutrients available to conidia during the process of germination. In the study of Ibrahim [23] it was evident that spores containing lower amounts of C but higher quantities of N had also a greater germination rate. The results of current studies confirm the previously reported results [23]. Studies on bioherbicides have also suggested that carbon and nitrogen concentrations of nutritional conditions are responsible for rapid germination and enhanced pathogenicity of resultant inocula [47, 48].

## 3.3. Effect of Mass Production on Conidial Virulence

Eggs of *T. absoluta* were exposed to conidia of *M. anisopliae* and *B. bassiana* cultured on different solid substrates [Fig. 2]. The results obtained show that *M. anisopliae* inocula caused similar mortalities 7 days post exposure across all treatments ranging from 58% to 80%. Effectiveness of *B. bassiana* on leaf miner eggs, in contrast, was considerably affected by the substrate used (F=59.107; P≤0.001). Total control of *T. absoluta* was achieved spraying eggs with conidia from burghul and rice. Around 60% of eggs died from conidia cultured on carrot and potato peels. The lowest mortality in egg population was caused by conidia from SDA media resulting in around 37%. In general, *B. bassiana* isolate was more effective against eggs than *M. anisopliae*. Tween (control) treatment did not cause any death of *T. absoluta*.

Considerable number of treated eggs and larvae have died from fungal infection exhibiting mycelial growth and subsequent sporulation [Fig. 3]. Furthermore, all substrates have markedly affected the level of mycosis instigated by both, *M. anisopliae* and *B. bassiana,* (F=10.52 and F=47.317, respectively; P≤0.001) isolates. Interestingly that cadavers of dead eggs and larvae previously treated with *Metarhizium* have developed less mycosis than those treated with *Beauveria* [Fig. 3].

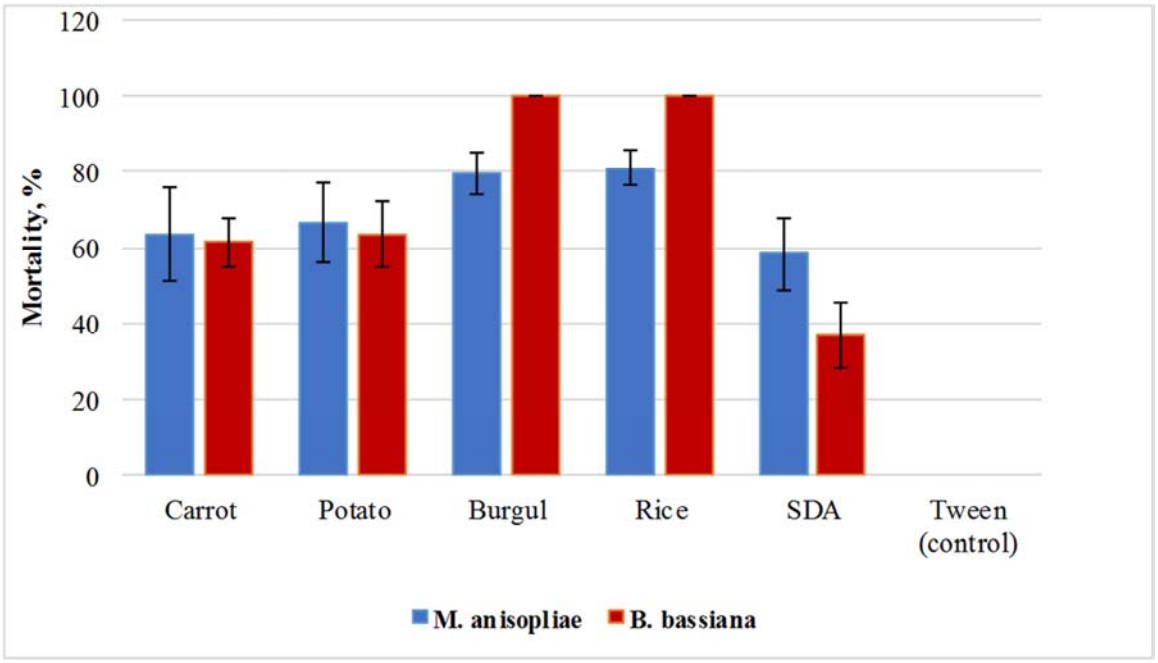


***Figure 2.*** *Percentage mortalities of Tuta absoluta eggs 7 days post inoculation with M. anisopliae and B. bassiana. Bars represent average values ± SE.*

Isolate (LIM1) in previous studies was noticeably more aggressive towards all target insects if grown on burghul or rice [23]. Results of this study show similar findings for *B. bassiana* where it was more aggressive against *T. absoluta* if grown on rice or burghul. Also, *B. bassiana* cultured on rice or other synthetic substrates was shown to be more effective than that produced on insect cadavers [49]. However, effectiveness of *M. anisopliae* against *Tuta* eggs was not affected by nutritional substrates [Fig. 2]. That is also in contrary to the previous reports of *M. anisopliae*'s virulence to be influenced by the culture medium [25, 43].

In addition, all dead eggs treated with *B. bassiana* conidia from rice have demonstrated 100% mycosis. On all eggs that did not hatch external fungal sporulation was observed in contrast to control treatments confirming the death due to fungal infection [Fig. 4]. Also, eggs treated with conidia from vegetable peels have been proven to die from fungal infection. In contrast to *M. anisopliae, B. bassiana* treated eggs have developed the lowest level of mycosis if conidia were grown on SDA.

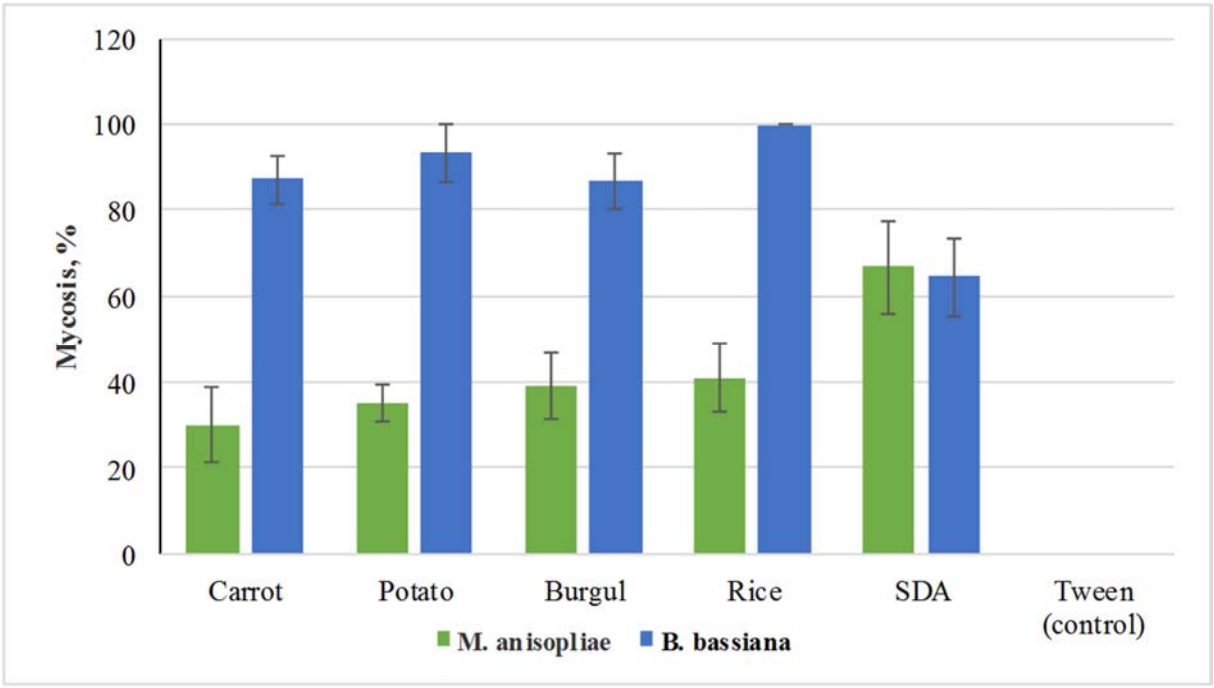


***Figure 3.*** *Percentage of mycosed Tuta absoluta eggs 14 days after inoculation with M. anisopliae and B. bassiana. Bars represent average values ± SE.*

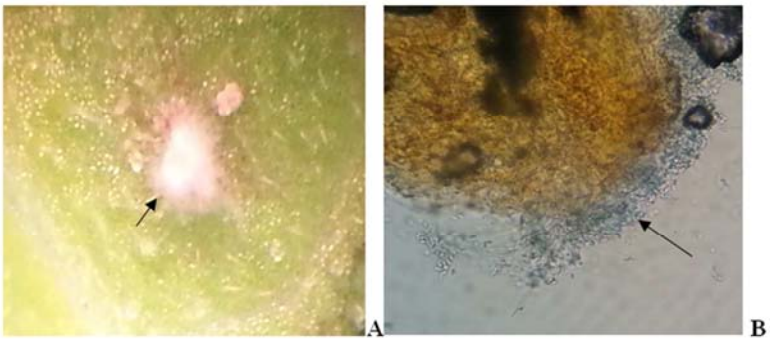


***Figure 4.*** *Dead eggs of T. absoluta. A. Mycosed egg at advanced stage of mycelial growth of B. bassiana; B. Light microscopy (x60) showing an egg with external fungal sporulation; Note: Black arrows indicate M. anisopliae spores on eggs' surface.*

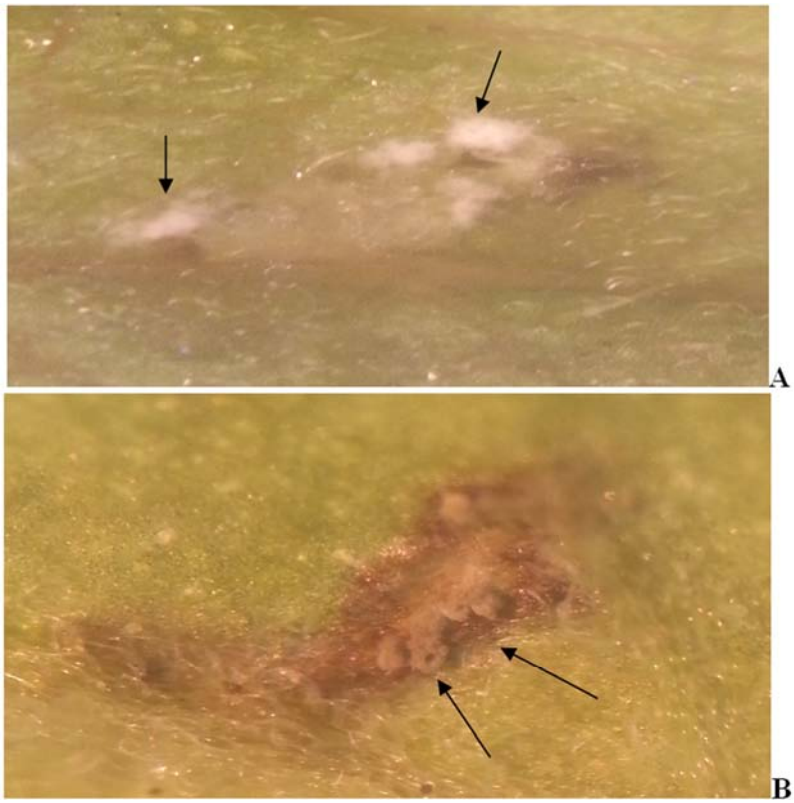


***Figure 5.*** *Dead larvae inside the leaf mesophyll. A. Showing fungal mycelial growth through leaf cuticle (arrows). B. Showing sporulation on the leaf surface out of the larvae that is still in the leaf mesophyll. Note: black arrows indicating clusters of fungal conidia of M. anisopliae.*

Isolates of *B. bassiana* was shown to induce high mortality to tomato leaf miner eggs and larvae [19]. The eggs were more susceptible than the first instars larvae [20]. Besides the ovicidal effect, mortality also occurred in the different instar larvae that hatched from treated eggs in present study. The mortality of larvae from treated eggs could have been initiated by the penetration of the fungus by the chorion of the egg before hatching or by contact with spores on the corium as observed by Rodriguez-Rueda and Fargues [52].

### 3.4. Efficacy of Fungal Formulations Under Greenhouse Conditions

Results in Table 8 show that after the first spray, there was significant difference in the number of eggs (F= 2.47; P=0.034) observed, where the highest mean number reached 1.6 eggs per leaf treated with *M. anisopliae* in oil emulsion and the lowest mean reached 0.86 eggs on plants treated with *B. bassiana* in oil emulsion. In addition, the results presented here (Table 8) show that the number of emerged larvae were very similar (F= 1.16; P≥ 0.05) across treatments. It also shows that second application of fungal pathogens had no effect neither on number of eggs nor on hatched larvae (F= 2.19; P≥0.05 and F= 0.80; P≥0.05, respectively). Interestingly that total number of eggs have consistently increased on tomato plants irrespective to treatments with the exception of 2$^{nd}$ control treatment where the lowest mean was 0.83 eggs per leaf observed suggesting that previously laid eggs have been hatched with no additional deposition of eggs. Statistical analysis of data presented in the Table 8 show that only after the third spray significant effect of *B.*

*bassiana* pathogen on number of eggs per leaf (F= 10.32; P≤0.05) was achieved. Also, significantly higher number of larvae was observed in positive control than in other treatments (F= 3.90; P=0.002) suggesting of cumulative effects of both pathogens. Control of *T. absoluta* was also reported using *M. anisopliae* and *B. bassiana* isolates under greenhouse conditions when $10^6$ conidia $ml^{-1}$ required to achieve 50% of pest mortality [53].

The maximum day temperatures during greenhouse experiment ranged between 23°C during 1st treatment and 29°C during the last 3rd application, whereas the lowest night temperatures were recorded 14.8°C and 22°C, respectively.

Efficiency of fungal application was confirmed by presence of numerous conidia in suspensions obtained from washing treated leaves (data not presented). The endophytic status of the fungi inside the leaf mesophyll of the treated leaves was not observed despite the fact that larvae were dying in the mines as a results of fungal infection [Fig. 5]. A study currently is conducted to determine the endophytic activities of the studied isolates against *T. absoluta*.

***Table 8.*** *Mean number of eggs and larvae (±SE) of T. absoluta per leaf after 1st, 2nd and 3rd applications of M. anisopliae and B. bassiana entomopathogens.*

| Treatments | 1st application | | 2nd application | | 3rd application | |
|---|---|---|---|---|---|---|
| | Eggs±SE | Larvae±SE | Eggs±SE | Larvae±SE | Eggs±SE | Larvae±SE |
| C1 | 0.93 ± 0.15 [a] | 1.93 ± 0.22 [a] | 1.06 ± 0.24 [a] | 1.93 ± 0.24 [a] | 3.00 ± 0.29 [a] | 2.56 ± 0.24 [b] |
| C2 | 0.93 ± 0.15 [a] | 1.43 ± 0.19 [a] | 0.83 ± 0.15 [b] | 1.76 ± 0.22 [a] | 2.60 ± 0.21 [a] | 2.06 ± 0.21 [a] |
| T1 | 0.90 ± 0.13 [a] | 1.56 ± 0.21 [a] | 1.73 ± 0.22 [a] | 1.76 ± 0.21 [a] | 1.36 ± 0.18 [b] | 1.63 ± 0.21 [a] |
| T2 | 0.86 ± 0.15 [a] | 1.56 ± 0.20 [a] | 1.33 ± 0.21 [a] | 2.06 ± 0.20 [a] | 0.96 ± 0.15 [b] | 1.30 ± 0.18 [a] |
| T3 | 1.33 ± 0.24 [b] | 1.70 ± 0.24 [a] | 1.06 ± 0.19 [a] | 2.00 ± 0.21 [a] | 2.66 ± 0.40 [a] | 1.86 ± 0.21 [a] |
| T4 | 1.60 ± 0.26 [b] | 1.26 ± 0.17 [a] | 1.26 ± 0.19 [a] | 1.53 ± 0.19 [a] | 3.16 ± 0.34 [a] | 1.86 ± 0.20 [a] |

[a],[b],[c] means of 5 block replicates followed by the same letter in a column are significantly different according to Tukey's test (P≤0.05).

## 4. Conclusions

This study shows that: 1) recycled materials such as potato and carrot peels and spoiled cereal grains could be used successfully for sustainable mass production of high quality inoculum; 2) recycled oils could also be considered as potential material for conidial formulations; 3) isolates of *M. anisopliae* and *B. bassiana* are good candidates for Integrated Pest Control strategies in greenhouses against *Tuta absoluta.*

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