

Article

Impact of Three Entomopathogenic Fungal Isolates on the Growth of Tomato Plants—Ectoapplication to Explore Their Effect on *Tetranychus urticae*

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Abstract: *Tetranychus urticae* is an important pest of tomato crops globally, affecting plant yield and growth. *Beauveria bassiana* and *Metarhizium robertsii* have the potential to control *T. urticae*. We investigated the influence of two *B. bassiana* (i.e., WG-12 and WG-19) isolates and one *M. robertsii* (WG-02) isolate when colonizing different plant organs (leaves, stems, and roots) and their influence on the growth of tomato plants, through foliar, root-dipping, and seed-soaking application techniques. We also examined the acaricidal activity of the three isolates against *T. urticae* (female adults), spraying tomato leaf discs with each isolate separately. After 28 days, WG-12 and WG-19 colonized 97 and 91% of the leaves after foliar inoculation, whereas WG-02 exhibited the lowest leaf colonization (76%). The height of the tomato plants, the root length, the number of leaves, and the weight of the biomass above and below the ground were enhanced significantly after inoculation with WG-02 vs. *B. bassiana* isolates and control. The complete mortality of *T. urticae* was caused by WG-12 and WG-02 after 10 days, whereas WG-19 killed 94% of the adults. For the effective management of *T. urticae*, we propose the application of the WG-02 isolate since it provides complete protection and promotes the growth of tomato plants.

Keywords: colonization; entomopathogenic fungi; biological control



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1. Introduction

Tomato, *Solanum lycopersicum* L. (Solanales: Solanaceae), is a species of high economic importance that is widely cultivated worldwide since it adapts to various agro-climatic environments [1–4]. The global tomato production is approximately 180,000,000 tons [5]. This fruit is also ranked second after the potato crop in terms of production and consumption [6]. However, tomato is attacked by several mites belonging to the Tarsonemidae, Eriophyidae, and Tetranychidae families, resulting in serious losses [7–10]. Among the tetranychids, *Tetranychus urticae* Koch (Acari) is a destructive pest of economic importance to tomato crops globally [8,9]. *Tetranychus urticae* damages a wide spectrum of plants, including ornamentals, vegetables, medicinals, and orchards [11–13]. This polyphagous pest has been recorded to feed on >1100 plant species from >140 plant families [14,15]. It causes an increase in water stress and a decrease in plant transpiration and photosynthesis by consuming leaf cell substances (e.g., chlorophyll) [16]. The feeding activity of *T. urticae* negatively affects plant yield and growth, causes leaf discoloration, also known as “bronzing”, or, more rarely, plant death [17–19]. *Tetranychus urticae* is short-lived and can rapidly

develop high population densities, requiring approximately 7 days from the egg to the adult stage under optimal conditions (i.e., 27 °C and 55–60% humidity) [15,20]. Females may oviposit up to 150 eggs during their lifetime [15,21]. In addition, *T. urticae* reproduces by arrhenotokous parthenogenesis, where females emerge from fertilized eggs, while males emerge from unfertilized eggs [15,22]. Consequently, through this type of reproduction, a single unmated female can establish a mixed population after oedipal copulation (a female mates with her male progeny), resulting in serious economic losses [23,24].

Chemical acaricides have been widely used globally for the management of *T. urticae* [19,25,26]. However, several studies over the years have revealed that this pest has developed resistance to organophosphates [27,28], carboxamides [29], carbazates [25], pyrazols [30–32], quinazolines [33], organosulfurs [34], and organometallics [32]. Totally, *T. urticae* is resistant to 96 active ingredients in 526 cases; therefore, it is considered one of the most resistant pests among the arthropods [35]. Several factors contribute to the rapid development of acaricide resistance in *T. urticae*, such as the ability to reproduce by arrhenotokous parthenogenesis, large number of offspring, and short life cycle [36,37].

Many predatory mites have been used in controlled growth environments, such as greenhouses, to effectively manage *T. urticae*, but this approach is not as common in field crops where pesticide applications are dominant [38–40]. Another biocontrol option is the use of entomopathogenic fungi (EPF) against *T. urticae*, which can contribute to the natural population regulation of this pest [41]. In a biological control strategy, EPFs could replace conventional acaricides [41] or act synergistically with other biocontrol agents, such as predatory mites [42–44]. In addition, EPFs are not detrimental to non-target organisms or the environment, and are safe for humans [37]. Fungi are usually more advantageous over other microbial biopesticides (e.g., viruses and nematodes) because of their broader host selectivity since one isolate can attack and control different arthropod pests simultaneously [45–48]. Upon contact, the fungal conidia are attached to the host and penetrate the cuticula by secreting cuticle-degrading enzymes [49,50]. Then, the hyphae colonize the host's body, leading to its death [51,52].

Several EPF species of Ascomycota, especially of the genera *Metarhizium* and *Beauveria*, have the potential to control *T. urticae* [53–55]. These fungi can also form symbiotic associations with plants (endophytes), living in plant tissues without symptoms through colonization [56,57]. Furthermore, EPFs have the potential to endophytically colonize a variety of plant species, improving plant growth and enhancing yields, while being harmful to pests [55,58–60]. There is evidence that EPFs make plants more resistant to stress caused by biotic or abiotic factors, enhance plant nutrient uptake, and excite hormone production, thereby contributing to plant growth [59,61,62].

Taking into account the limited knowledge related to the impact of isolates of entomopathogenic fungi on *T. urticae* and tomatoes in Pakistan [63], the current work aims to investigate for the first time the impact of three isolates of *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Cordycipitaceae) (WG-12 and WG-19) and *Metarhizium robertsii* J.F. Bisch., Rehner & Humber (formerly known as *Metarhizium anisopliae* (Metchnikoff) Sorokin) (Hypocreales: Clavicipitaceae) (WG-02) on the colonization and growth of tomato plants. Furthermore, the acaricidal efficacy of these isolates were tested against *T. urticae* after ectoapplication.

2. Materials and Methods

2.1. Mite Culture

Tetranychus urticae used in the bioassays was initially collected from a tomato field of the University of Agriculture, Faisalabad, Pakistan. This population had been nurtured for five years on the tomato cultivar (variety Moneymaker) susceptible to mites in an environment-controlled climate chamber set at a cycle of 16: 08 h light: dark, at 27 °C and 65% relative humidity (RH) [64]. Adult males are conical dorsally, while females are oval dorsally, and smaller than males, approximately 0.3–0.5 mm in length [65].

2.2. Tomato Plants

Tomato seeds (variety Moneymaker) were purchased from the local market and were sown in a seedling tray filled with moss and watered daily. This is the most promising cultivar and is extensively cultivated by Pakistani tomato growers [66,67]. For a period of three weeks post-sowing in the nursery, the seedlings were transferred to 3 L plastic pots filled with Sphagnum Peat Moss and were maintained in an environment-controlled chamber with a 16: 08 h light: dark cycle (27 °C, 65% RH). The plants were irrigated after a 3-day interval and fertilizer (macro- and micro-nutrients) was applied at 15-day intervals. The plants showed uniform growth characteristics and were used in further bioassays.

2.3. Suspensions of *Beauveria bassiana* and *Metarhizium robertsii* Isolates

In the current study, we used two entomopathogenic *B. bassiana* isolates (WG-12, WG-19) and one *M. robertsii* isolate (WG-02) [68]. Maintenance of these isolates was carried out on PDA (potato dextrose agar) (BD-Difco, Franklin Lakes, NJ, USA) stored in a refrigerator set at 4 °C, at the Microbial Control Laboratory, University of Agriculture, Faisalabad, Pakistan. Activation of the isolate was carried out in Petri dishes on SDA (sabouraud dextrose agar) (BD-Difco, Franklin Lakes, NJ, USA). Parafilm (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was used to seal the dishes and they were incubated for 10 days in 16: 08 h light: dark cycle at 25 °C (ICP-260, Memmert GmbH, Schwabach, Germany). These dishes produced an abundant amount of conidia two weeks post-inoculation. The layers of the conidia exiting from the media were scraped off with the help of a sterilized scalpel into a 10 mL tube. A falcon tube with a volume of 50 mL was used to suspend a portion of harvested conidia, containing a sterilized solution (30 mL) with 0.05% Tween 80 (Merck, Kenilworth, NJ, USA). For homogenized mixture, the conidia suspension was vortexed using a laboratory magnetic mixer (IRMECO GmbH, Lütjensee, Germany) with 8 glass beads. Measurement of the desired concentration was performed under the microscope with a Neubauer-Improved hemocytometer (Marienfeld, Germany). To measure conidia viability, two 60 mm Petri dishes with SDAY (sabouraud dextrose agar with 1% yeast) were inoculated with 0.1 mL (1×10^6 conidia/mL) of the conidial suspension, then sealed using parafilm and stored for 18 h for incubation at 25 °C with a photoperiod of 14: 10 h light: dark. Two SDAY dishes per *B. bassiana* and *M. robertsii* isolates were utilized and two counts including 200 conidia were recorded for each dish. Sterile cover slips were placed on the dishes after incubation. Conidia germination was considered successful when the germ tube length was $2 \times$ the conidia diameter [69,70]. This measurement was performed at magnification $400 \times$ using a microscope Euromex BB.1152-PLi microscope (Euromex Microscopen bv, Arnhem, The Netherlands). Before initiation of the experiments, conidia viability was $>92\%$.

2.4. Inoculation Methods of Tomatoes with Suspensions of *Beauveria bassiana* and *Metarhizium robertsii* Isolates

Surfaces of seeds of tomato were sterilized with ethanol (70%) for 60–120 s, and later with a sodium hypochlorite solution (1.5%) for 120–180 s. The seeds were washed thrice using sterile distilled H₂O to remove excess sodium hypochlorite, and then superfluid water was removed by blotting on sterile paper towels. The final rinsed H₂O was spread on an SDA dish to evaluate the efficacy of the surface sterilization process [71]. Before inoculation, the viability of each entomopathogenic isolate was determined through germination tests as in the previous section. Three different methods were used for each isolate for their endophytic establishment in tomato plants as follows [72]: (1) Seed inoculation method: The sterilized seeds were immersed in a suspension of 1×10^8 conidia/mL in a 100 mL glass bottle with the respective fungus for 16–24 h [59,73]. Seeds that were treated with 0.01% Triton X-100 acted as controls. Then, seeds were placed on a sterile kitchen towel to air dry for an interval of 20 min. The seeds treated with WG-12, WG-19, and WG-02, and control were sown separately in 0.5 L volume plastic pots (8 cm \times 10 cm height: diameter). Pots contained three-times-sterilized autoclaved plant substrates comprising

compost and vermiculite. The planting material was autoclaved at 121 °C for 30 min, and left for 20 h intervals within each sterilization [73,74]. Next, the material was allowed to cool for 72 h before being transferred to the pots. The latter were maintained in a climatic chamber with a 16: 08 h light: dark cycle (27 °C, 65% RH) using fluorescent bulbs (Philips, Karachi, Pakistan) [75]. The pots were irrigated on alternate days [55], and 3 mL Hoagland solution (Merck KGaA, Darmstadt, Germany) was applied to each pot per week. (2) Foliar application: Six- to eight-week-old plants, raised from the sterilized seeds sown in the pots as described previously, were sprayed with a suspension (1×10^8 conidia/mL, 0.5 mL per leaf) of each isolate [76] with the help of a hand-held plastic sprayer (Kissan Ghar, Sargodha, Pakistan). Plants that were treated with 0.01% Triton X-100 acted as controls. Plants were maintained in an incubator with 16: 08 h light: dark cycle (27 °C, 65% RH) [64]. (3) Root-dipping: To observe the colonization of EPFs by root-dipping method, sterilized seeds were sown in pots as described previously. Then, a 15-day-old plant was gently removed from the soil, and its roots were immersed in a 300 mL volume of suspension (1×10^8 conidia/mL), corresponding to WG-12, WG-19, or WG-02, for 1.5 h [77]. Plants that were treated with 0.01% Triton X-100 acted as controls. Afterward, the plant was put back in the pot and kept in an incubator with 16: 08 h light: dark cycle (27 °C, 65% RH) [64].

2.5. Evaluation of Endophytic Colonization of *Beauveria bassiana* and *Metarhizium robertsii* isolates in Tomato Plants

The plants were uprooted gently to avoid any breakage of roots and other tissues. Per plant, five pieces of randomly selected leaves of 10 mm² each, stems of 10 mm, and roots were cut with a sterilized blade [72]. They were sterilized with 1% NaClO for 300 s, then washed thrice with distilled H₂O, and dried on sterile tissue paper [78]. The final rinsed H₂O was spread on SDA media to check the efficiency of the disinfection process of each fungal isolate [79]. No contamination was observed in any of these dishes after sterilization of leaves, stems, and roots. After the sterilization process, with the help of a scalpel, the edges of the samples were trimmed and cut into pieces of 5 mm² for leaves, 5 mm for stems, and 5 mm for roots [72]. These inoculated plant samples were placed in dishes containing the respective *Beauveria* or *Metarhizium* selective media as described by Rivas-Franco et al. [80]. Different dishes were used per group of leaves, stems, and roots per plant. The tissues were pressed in the selective media carefully to develop contact with the media [72], and the dishes were sealed with parafilm. Dishes were incubated for two weeks in an incubator at 26 °C, 16: 08 h light: dark cycle, and 84% RH [72]. Fungal growth was observed after 7, 14, 21, and 28 days of inoculation at the interface of the plated tissues, which were re-isolated and identified relying on conidia/conidiophores structures through microscope (BB.1152-PLi, Euromex Microscopen bv, Arnhem, The Netherlands) and comparing them with the original culture of each fungal isolate [72]. Different dishes with leaves, stems, and roots were prepared per exposure to evaluate the fungal growth originating from the same plant. The experiment was repeated independently using nine plants per inoculation method, including a total of 81 plants (i.e., 9 plants \times 3 isolates \times 3 inoculation methods). Data were presented as % colonization: number of tissues of plants exhibiting fungal outgrowth per total number of plant tissues) \times 100 [72].

2.6. Influence of Endophytic *Beauveria bassiana* and *Metarhizium robertsii* Isolates on Plant Growth

The effect of the endophytic colonization of the isolates WG-12, WG-19, and WG-02 was evaluated on different plant growth parameters, by sowing new tomato seeds as described in the Section 2.4. The plants were inoculated with 1×10^8 conidia/mL of each fungal isolate separately by seed-soaking, foliar, and root-dipping methods. Per plant, the following growth physical parameters were evaluated 26 days post-inoculation: plant height (cm), i.e., distance between the tip of stem and base of the plant, using a steel ruler; stem diameter (mm), i.e., up to 30 mm from the ground, using Vernier caliper; number of leaves per plant; root length (cm), i.e., axial root, using a steel ruler; root dry weight (mg), using Shimadzu (Japan) balance; and above ground biomass (mg), i.e., leaves and

whole stem. Above- and below-ground parts of the tomato were first dried inside an oven at 70 °C, and then weighed separately [72]. The same parameters for control plants were also recorded. The experiment was repeated independently using nine plants per inoculation method, including a total of 108 plants, i.e., 9 plants × 4 (isolates and control) × 3 inoculation methods.

2.7. Direct Action of *Beauveria bassiana* and *Metarhizium robertsii* Isolates against *Tetranychus urticae* under Laboratory Conditions

The bioassay was conducted in three Petri dishes (90 mm diameter) (subreplications) containing, each, a 3 cm diameter tomato leaf disc [81]. Each disc was put with the adaxial surface on water-saturated cotton to keep the leaf moist/fresh [82]. Twenty 1-day-old female adults [64] were placed separately in each disc [81]. Then, discs with the mites were sprayed with 1 mL [81] of the suspension (1×10^8 conidia/mL) of each isolate, using an airbrush (Master Multi-purpose Airbrush, USA). Control leaves were sprayed with 1 mL of H₂O [37]. After spraying, leaves were left to air-dry for about 300 sec [82]. Afterward, dishes were covered with a PVC membrane (polyvinyl chloride), with fine holes for aeration [83], and stored in an incubator at 27 °C and 65% RH with a photoperiod of 16: 08 h light: dark. The mortality data were collected after 5, 7, and 10 days. Different leaves with mites were prepared per exposure. Observations were carried out under a stereomicroscope (Leica Wild M3B, Heerbrugg, Switzerland). Mites were considered dead if they could not move their appendages when touched with a camel hair brush [82]. The dead mites were placed on new plates containing a wet filter paper and stored in the same conditions for 3 days to observe any fungal growth to confirm their mortality caused by EPF [37,84]. The experiment was repeated four times using new plant leaves (i.e., 3 subreplications × 4 isolates and control × 4 replications).

2.8. Statistical Analysis

The data for the mortality of *T. urticae* were computed with Abbott's formula [85]. All the data were transformed using the formula $\log(x + 1)$ to normalize variance prior to statistical analysis [86,87]. Regarding the EPF colonization experiment, the main effects were fungal isolate, inoculation method, different parts of the plant, and interval. Colonization was the response variable. The colonization data underwent a four-way analysis of variance (ANOVA). Data of plant growth were separately analyzed for plant height, stem diameter, root length, root dry weight, above-ground biomass, and number of leaves. The response variable was the plant growth of each part of the plant. The main effects were EPF isolate and inoculation method. The data for different plant growth parameters (i.e., plant height, stem diameter, root length, root dry weight, above-ground biomass, and number of leaves) were analyzed using two-way ANOVA. Regarding mortality data, exposure interval and inoculation methods were the main effects. Mortality was the response variable. Mortality data were subjected to two-way ANOVA. In all cases, the main effects and their interactions are considered in the analyses. Mortalities observed in the controls were <5%. Means were distinguished using the Tukey test (HSD) [88] at a level of significance of 5%. All analyses were carried out with the Minitab statistical package [89].

3. Results

3.1. Colonization by Endophytic Entomopathogenic Fungi *Beauveria bassiana* and *Metarhizium robertsii* Isolates in Tomato Plants

All the main effects and their associated interactions were significant, apart from isolate × plant part × interval, and isolate × methods × plant part × interval (Table S1). Among the inoculation methods, significantly different colonization rates were recorded throughout the experiment for WG-12. The colonization rate of the WG-12 isolate in the different parts of the tomato remained low, regardless of the inoculation method, reaching 46.66% in the leaves by the foliar method (Table 1). After 14 days, colonization by the foliar method was moderate in leaves (68.14%), whereas colonization of stems (47.40%) and roots (37.77%) was lower. This trend was observed after 21 days, with a higher colonization in

leaves (83.70%) than stems (62.96%) and roots (49.63%) with the foliar method. Twenty-eight days later, leaf colonization reached 97.03% with the foliar inoculation method. However, stem and root colonization ranged between 47.40 and 76.29% with the three inoculation methods.

Table 1. Colonization (mean% \pm SE) of tomato plants with WG-12, WG-19 isolates of *Beauveria bassiana*, and WG-02 isolate of *Metarhizium robertsii* under three different inoculation methods (foliar, root-dipping, and seed-soaking). Per each plant part, within each column, means followed by the same lowercase letters are not significantly different (DF = 2, 26; Tukey HSD test at $p = 0.05$).

WG-12				
Intervals	Method	Leaf Colonization	Stem Colonization	Root Colonization
7 days	Foliar	46.66 \pm 1.92 a	34.81 \pm 1.85 a	26.67 \pm 1.92 a
	Root-dipping	25.18 \pm 1.48 b	21.48 \pm 1.48 b	32.59 \pm 1.73 a
	Seed-soaking	28.14 \pm 2.15 b	37.03 \pm 1.17 a	18.51 \pm 1.85 b
	<i>F</i>	38.5	30.4	14.8
	<i>p</i>	<0.01	<0.01	<0.01
14 days	Foliar	68.14 \pm 2.15 a	47.40 \pm 1.73 a	37.77 \pm 1.57 a
	Root-dipping	34.07 \pm 1.73 b	28.14 \pm 2.15 b	41.48 \pm 1.48 a
	Seed-soaking	39.25 \pm 2.06 b	45.92 \pm 2.06 a	23.70 \pm 1.95 b
	<i>F</i>	84.7	28.9	31.0
	<i>p</i>	<0.01	<0.01	<0.01
21 days	Foliar	83.70 \pm 1.95 a	62.96 \pm 1.17 a	49.63 \pm 2.25 a
	Root-dipping	44.44 \pm 1.57 b	35.55 \pm 1.92 c	46.66 \pm 2.72 a
	Seed-soaking	46.66 \pm 1.92 b	54.07 \pm 1.73 b	37.03 \pm 1.17 b
	<i>F</i>	146.0	72.5	9.4
	<i>p</i>	<0.01	<0.01	<0.01
28 days	Foliar	97.03 \pm 2.25 a	76.29 \pm 1.61 a	55.55 \pm 1.11 a
	Root-dipping	53.33 \pm 1.92 b	47.40 \pm 1.73 c	58.51 \pm 1.48 a
	Seed-soaking	59.25 \pm 2.06 b	66.66 \pm 1.57 b	50.37 \pm 1.17 b
	<i>F</i>	129.0	80.2	10.6
	<i>p</i>	<0.01	<0.01	<0.01
WG-19				
7 days	Foliar	40.74 \pm 1.33 a	31.58 \pm 1.48 a	22.96 \pm 1.17 a
	Root-dipping	19.25 \pm 1.33 c	17.03 \pm 1.17 b	28.14 \pm 3.09 a
	Seed-soaking	25.92 \pm 2.06 b	32.59 \pm 1.73 a	12.59 \pm 0.74 b
	<i>F</i>	46.4	35.1	16.3
	<i>p</i>	<0.01	<0.01	<0.01
14 days	Foliar	57.77 \pm 1.57 a	43.70 \pm 1.17 a	31.85 \pm 1.85 a
	Root-dipping	30.37 \pm 1.61 c	22.96 \pm 1.17 c	34.07 \pm 1.73 a
	Seed-soaking	36.29 \pm 1.17 b	39.25 \pm 1.33 b	17.77 \pm 1.57 b
	<i>F</i>	96.8	79.0	26.3
	<i>p</i>	<0.01	<0.01	<0.01
21 days	Foliar	74.81 \pm 1.48 a	56.29 \pm 1.17 a	42.96 \pm 1.95 a
	Root-dipping	38.51 \pm 1.48 b	29.63 \pm 1.17 c	41.48 \pm 1.48 a
	Seed-soaking	40.74 \pm 1.73 b	46.66 \pm 1.57 b	34.81 \pm 1.48 b
	<i>F</i>	168.0	105.0	6.9
	<i>p</i>	<0.01	<0.01	<0.01
28 days	Foliar	91.11 \pm 1.11 a	72.59 \pm 2.06 a	47.40 \pm 2.06 ab
	Root-dipping	48.14 \pm 1.48 b	40.74 \pm 1.73 c	52.59 \pm 1.73 a
	Seed-soaking	52.59 \pm 1.73 b	59.25 \pm 1.73 b	43.70 \pm 1.17 b
	<i>F</i>	260.0	74.6	6.9
	<i>p</i>	<0.01	<0.01	<0.01

Table 1. Cont.

WG-12				
Intervals	Method	Leaf Colonization	Stem Colonization	Root Colonization
WG-02				
7 days	Foliar	24.44 ± 1.57 a	18.51 ± 1.48 a	15.55 ± 1.11 a
	Root-dipping	10.37 ± 1.61 b	13.33 ± 1.11 b	19.25 ± 1.73 a
	Seed-soaking	7.40 ± 0.74 b	11.85 ± 1.48 b	5.18 ± 0.97 b
	<i>F</i>	44.2	6.5	30.6
	<i>p</i>	<0.01	<0.01	<0.01
14 days	Foliar	42.96 ± 2.51 a	26.66 ± 1.57 a	19.25 ± 1.73 b
	Root-dipping	14.81 ± 0.97 b	17.03 ± 1.17 b	25.18 ± 1.85 a
	Seed-soaking	12.59 ± 1.73 b	18.51 ± 1.48 b	9.62 ± 1.17 c
	<i>F</i>	83.6	13.4	23.6
	<i>p</i>	<0.01	<0.01	<0.01
21 days	Foliar	58.51 ± 1.85 a	49.63 ± 1.61 a	38.51 ± 1.48 a
	Root-dipping	27.40 ± 2.34 b	31.11 ± 1.57 b	33.33 ± 1.57 b
	Seed-soaking	21.48 ± 1.48 b	26.66 ± 1.92 b	16.29 ± 1.17 c
	<i>F</i>	107.0	50.7	67.2
	<i>p</i>	<0.01	<0.01	<0.01
28 days	Foliar	76.29 ± 1.17 a	67.40 ± 1.73 a	41.48 ± 2.15 a
	Root-dipping	30.37 ± 1.95 b	35.55 ± 1.57 b	39.25 ± 1.73 a
	Seed-soaking	28.14 ± 1.48 b	32.59 ± 1.73 b	22.22 ± 1.57 b
	<i>F</i>	299.0	131.0	32.8
	<i>p</i>	<0.01	<0.01	<0.01

During the experimental period, there were significant differences among inoculation methods in WG-19 colonization rates (Table 1). The percentage of WG-19 colonized leaves, stems, and roots of tomato plants was low, not exceeding 40.74% regardless of the inoculation method, after 7 days. The percentage of colonization further increased at 14 days post-inoculation, with the highest percentage recorded for leaves (57.77%) with foliar inoculation, while the lowest percentage was reported for roots (17.77%) by seed-soaking inoculation. Similar results were obtained after 21 days, where leaves after foliar inoculation (74.81%) showed the highest colonization percentage, while colonization on tomato stems by the same inoculation method was lower (56.29%). However, in the rest of the plant part/inoculation method, colonization remained below 46.66%. At 28 days, an increase in colonization was observed in all cases, but, again, the highest percentages were found in leaves (91.11%) and stems (72.59%) after inoculation by the foliar method.

Significant differences were recorded in the colonization percentages of WG-02 to the different tomato parts when comparing inoculation methods at all observed intervals (Table 1). The percentage of colonization of the WG-02 isolate in the leaves, stems, and roots of tomato plants was very low and ranged from 5.18 to 24.44%, after 7 days. Fourteen days later, there was a slight increase in the colonization rate for all cases, reaching 42.96%. Twenty-one days after inoculation, the WG-02 isolate exhibited 58.51% colonization on leaves by the foliar method. However, colonization ranged from 16.29 (root by seed-soaking method) to 49.63% (stem by foliar method). After 28 days post-inoculation, the highest colonization percentages were reported in leaves (76.29%) and stems (67.40%) using the foliar method, while the proportion of colonized WG-02 isolate in the remaining cases did not exceed 41.48%.

3.2. Effect of Inoculation with *Beauveria bassiana* and *Metarhizium robertsii* Isolates on Tomato Plant Growth

All main effects/interactions were significant for the growth of the plant parts (Table S2). Inoculation with isolate WG-02 significantly improved the height of tomato plants compared to inoculation with isolates WG-12 or WG-19, or non-inoculated plants (control)

(Table 2). In the case of isolate WG-02, significant differences were also observed among the three inoculation methods; i.e., the root-dipping method enhanced plant height more (27.02 cm) than seed-soaking (24.34 cm) and the foliation method (20.11 cm).

Table 2. Plant growth parameters (plant height, stem diameter, root length, root dry weight, above-ground biomass, and number of leaves/plant) (mean \pm SE) of tomato plants inoculated with three isolates of *Beauveria bassiana* (WG-12 and WG-19) and *Metarhizium robertsii* (WG-02) using three different methods (foliar, root-dipping, and seed-soaking). Within each row, means followed by the same lowercase letters are not significantly different (DF = 3, 35; Tukey HSD test at $p = 0.05$). Per response, within each column, means followed by the same uppercase letters are not significantly different (DF = 2, 26; Tukey HSD test at $p = 0.05$).

Plant Growth Parameter						
Plant Height (cm)						
Method	WG-12	WG-19	WG-02	Control	F	p
Foliar	16.02 \pm 0.26 Bb	15.84 \pm 0.24 Bb	20.11 \pm 0.37 Ca	14.81 \pm 0.52 Bb	40.1	<0.01
Root-dipping	22.10 \pm 0.27 Ab	19.78 \pm 0.37 Ac	27.02 \pm 0.23 Aa	16.45 \pm 0.21 Ad	245.0	<0.01
Seed-soaking	21.87 \pm 0.31 Ab	18.62 \pm 0.36 Ac	24.34 \pm 0.33 Ba	15.76 \pm 0.22 ABd	142.0	<0.01
F	146.0	36.6	118.0	5.5		
p	<0.01	<0.01	<0.01	0.01		
Stem diameter (mm)						
Foliar	3.61 \pm 0.19 Ba	3.17 \pm 0.18 Ba	3.75 \pm 0.13 Ba	3.68 \pm 0.16 Aa	2.4	0.08
Root-dipping	4.48 \pm 0.25 Aab	3.90 \pm 0.16 Ab	5.31 \pm 0.24 Aa	2.55 \pm 0.32 Bc	20.6	<0.01
Seed-soaking	3.86 \pm 0.15 ABab	3.58 \pm 0.18 ABab	4.26 \pm 0.27 Ba	3.38 \pm 0.20 ABb	3.3	0.03
F	4.7	4.2	12.1	6.0		
p	0.01	0.02	<0.01	<0.01		
Root length (cm)						
Foliar	13.72 \pm 0.52 Cb	12.88 \pm 0.42 Cb	16.12 \pm 0.26 Ca	10.43 \pm 0.31 ABc	35.2	<0.01
Root-dipping	18.27 \pm 0.28 Ab	16.51 \pm 0.24 Ac	19.37 \pm 0.30 Aa	9.45 \pm 0.24 Bd	267.0	<0.01
Seed-soaking	15.66 \pm 0.25 Bb	14.38 \pm 0.37 Bc	17.43 \pm 0.21 Ba	11.27 \pm 0.31 Ad	77.1	<0.01
F	37.2	25.7	38.3	38.3		
p	<0.01	<0.01	<0.01	<0.01		
Root dry weight (mg)						
Foliar	23.52 \pm 0.25 Cb	17.62 \pm 0.22 Cc	31.08 \pm 0.36 Ca	15.63 \pm 0.23 ABd	642.0	<0.01
Root-dipping	29.16 \pm 0.38 Ab	25.56 \pm 0.45 Ac	38.36 \pm 0.36 Aa	16.51 \pm 0.29 Ad	571.0	<0.01
Seed-soaking	26.28 \pm 0.26 Bb	21.42 \pm 0.34 Bc	34.62 \pm 0.30 Ba	15.23 \pm 0.30 Bd	718.0	<0.01
F	86.2	126.0	113.0	5.3		
p	<0.01	<0.01	<0.01	0.01		
Above-ground biomass (mg)						
Foliar	165.80 \pm 2.20 Cb	151.17 \pm 2.48 Cc	194.27 \pm 1.59 Ca	98.62 \pm 1.27 Ad	422.0	<0.01
Root-dipping	210.88 \pm 3.45 Ab	187.47 \pm 2.70 Ac	276.47 \pm 1.12 Aa	96.55 \pm 1.66 Ad	952.0	<0.01
Seed-soaking	198.23 \pm 2.03 Bb	176.96 \pm 2.17 Bc	223.39 \pm 1.65 Ba	97.51 \pm 1.56 Ad	843.0	<0.01
F	77.3	57.5	796.0	0.5		
p	<0.01	<0.01	<0.01	0.63		
Number of leaves/plant						
Foliar	21.17 \pm 0.49 Cb	19.72 \pm 0.40 Cc	24.26 \pm 0.29 Ca	18.92 \pm 0.61 Ac	25.5	<0.01
Root-dipping	32.11 \pm 0.47 Ab	26.65 \pm 0.36 Ac	35.27 \pm 0.35 Aa	20.21 \pm 0.43 Ad	261.0	<0.01
Seed-soaking	25.06 \pm 0.36 Bb	21.66 \pm 0.44 Bc	28.01 \pm 0.41 Ba	19.74 \pm 0.39 Ad	81.0	<0.01
F	153.0	77.8	241.0	1.8		
p	<0.01	<0.01	<0.01	0.18		

No significant differences in stem diameter were found by the foliar method between the fungal isolates and the control (Table 2). However, inoculation with WG-12 and WG-19 isolates with the root-dipping methods significantly increased stem diameter (i.e., 4.48 and

3.90 cm for WG-12 and WG-19, respectively) in comparison to inoculation by the foliar method (i.e., 3.61 and 3.17 cm for WG-12 and WG-19, respectively). Regarding the WG-02 isolate, inoculation with the root-dipping method resulted in a significantly wider stem (5.31 cm) than the other two methods (i.e., 4.26 and 3.75 cm for seed-soaking and foliar and methods, respectively).

The root was significantly longer with WG-02 isolate regardless of the inoculation method, when comparing to WG-12 and WG-19 isolates, and non-inoculated plants (Table 2). Inoculation with the three fungal isolates with the root-dipping method significantly enhanced root length (16.51, 18.27, and 19.37 cm for WG-19, WG-12, and WG-02 isolates, respectively) in contrast to the seed-soaking (14.38, 15.66, and 17.43 cm for WG-19, WG-12, and WG-02 isolates, respectively) and foliar method (12.88, 13.72, and 16.12 cm for WG-19, WG-12, and WG-02 isolates, respectively).

The root dry weight was significantly higher with the three inoculation methods using WG-02, in comparison to plants inoculated with WG-19 and WG-12, and non-inoculated plants (Table 2). Significant differences were noted among the inoculation methods for the three fungal isolates. The root dry weight was the highest with the root-dipping method (25.56, 29.16, and 38.36 mg for WG-19, WG-12, and WG-02 isolates, respectively), followed by the seed-soaking (21.42, 26.28, and 34.62 mg for WG-19, WG-12, and WG-02 isolates, respectively) and foliar method (17.62, 23.52, and 31.08 mg for WG-19, WG-12, and WG-02 isolates, respectively).

Inoculation with the three fungal isolates by the root-dipping method significantly increased the above-ground biomass weight (i.e., 187.47, 210.88, and 276.47 mg for WG-19, WG-12, and WG-02 isolates, respectively), compared to the seed-soaking (176.96, 198.23, and 223.39 mg for WG-19, WG-12, and WG-02 isolates, respectively) and foliar method (151.17, 165.80, and 194.27 mg for WG-19, WG-12, and WG-02, respectively) (Table 2). Regardless of the inoculation method, significant differences were noted among fungal isolates and controls in the above-ground biomass weight (i.e., WG-02 > WG-12 > WG-19 > control).

Concerning the number of leaves, the inoculated plants showed a significantly higher number of leaves than the control plants, regardless of the method used, except for the WG-19 isolate when inoculated by the foliar method, where the number of leaves did not vary significantly with the number of non-inoculated plants (i.e., 19.72 and 18.92 leaves/plant, for WG-19- and un-inoculated plants, respectively) (Table 2). The performance of WG-02 using the three inoculation methods resulted in a significantly higher number of leaves (24.26, 28.01, and 35.27 leaves/plant for the foliar, seed-soaking, and root-dipping methods, respectively), followed by WG-12 (21.17, 25.06, and 32.11 leaves/plant for the foliar, seed-soaking, and root-dipping methods, respectively), and WG-19 (19.72, 21.66, and 26.65 leaves/plant for the foliar, seed-soaking, and root-dipping methods, respectively). Inoculation by root-dipping method with the three isolates significantly enhanced leaf production in contrast to the seed-soaking and foliar methods.

3.3. Mortality Caused by *Beauveria bassiana* and *Metarhizium robertsii* Isolates on *Tetranychus urticae* in the Laboratory

All of the main effects/interactions (i.e., interval DF = 2, 107, $F = 2264.5$, $p < 0.01$, EPF isolate DF = 2, 107, $F = 252.2$, $p < 0.01$, and interval \times EPF isolate DF = 4, 107, $F = 50.6$, $p < 0.01$) were significant. Significant differences were observed in adult mortality caused by the three fungal isolates after 5 days, with WG-02 (57.91%) showing moderate adult mortality, whereas mortality caused by WG-12 (45.41%) and WG-19 (29.58%) were low (Table 3). Mortality increased significantly after 2 days in all cases, with high mortality caused by WG-02 (81.25%), followed by WG-12 (68.75%) and WG-19 (63.33%). Complete mortality was caused by WG-12 and WG-02, while, in WG-19, adult mortality reached 94.58%, after 10 days.

Table 3. Mortality (mean% \pm SE) of *Tetranychus urticae* female adults treated with *Beauveria bassiana* (WG-12 and WG-19) and *Metarhizium robertsii* (WG-02) isolates. Within each row, means followed by the same uppercase letter do not differ significantly. Within each column, means with the same lowercase letter do not differ significantly (DF = 2, 35 in all cases, Tukey HSD test at $p = 0.05$).

Isolate	Exposure			F	p
	5 Days	7 Days	10 Days		
WG-12	45.41 \pm 1.14 Cb	68.75 \pm 1.64 Bb	100.00 \pm 0.00 Aa	561.0	<0.01
WG-19	29.58 \pm 1.29 Cc	63.33 \pm 1.42 Bc	94.58 \pm 1.29 Ab	587.0	<0.01
WG-02	57.91 \pm 2.08 Ca	81.25 \pm 1.39 Ba	100.00 \pm 0.00 Aa	212.0	<0.01
F	82.4	38.0	17.4	-	-
p	<0.01	<0.01	<0.01	-	-

4. Discussion

Entomopathogenic fungi have been artificially inoculated and successfully established as endophytes in several crops of economic importance, including tomato plants [73,77,90,91]. The results of our study demonstrated that the isolates tested were capable of endophytically colonizing the leaves, stems, and roots of tomato plants using three different inoculation methods. However, the percentage colonization of tomato tissues depended on the inoculation method and fungal isolate. It has been previously documented that these factors can affect the successful establishment of EPFs as endophytes [57,92,93]. In the current investigation, the three fungal isolates exhibited the highest percentage of leaf colonization by foliar inoculation as opposed to the root-dipping and seed-soaking methods. Allegrucci et al. [94] reported that foliar spray inoculation had a superior effect on the colonization percentage of an Argentine *B. bassiana* strain (LPSC 1067) on tomato leaves, compared to seed immersion and root-dipping methods. A significantly higher colonization of *M. robertsii* (80.0%) and *B. bassiana* (72.5%) on maize leaves was achieved by foliar spray than the seed inoculation (50 and 60% for *B. bassiana* and *M. robertsii*, respectively) [95]. Different inoculation methods (i.e., root/soil drenching, leaf soaking, and stem injection) failed to establish the colonization of the three *B. bassiana* isolates LPP139, LEF140, and LEF141 on tomato plants, whereas only LPP139 was able to successfully colonize all parts of the plant when inoculating water-stressed seedlings [96].

Interestingly, in each fungal strain, we noticed significant differences in the colonization percent among the different parts of tomato plants, regardless of the inoculation method used. The highest abundance of fungi was found in leaves, followed by stems and roots. Isolates WG-12 and WG-19 showed higher foliar colonization when compared to the WG-02 isolate, especially by the foliar method. Russo et al. [97] reported variation in the colonization performances of five *B. bassiana* and two *Metarhizium* isolates on the organs of soybean plants using different inoculation techniques. The leaf aspersion method benefited the establishment of fungi on leaves rather than the stems and roots, while *B. bassiana* strains showed a greater colonization rate over *Metarhizium* strains. The factors that caused an increase in the fungal colonization of leaves compared to the other organs are not yet clear. This could be due to variations in physiological conditions among the plant parts [97], an issue that merits further investigation.

The endophytic presence of EPFs in host plants has been reported to be beneficial, not only by protecting the plant from its enemies (e.g., pathogens or arthropods), but also by promoting plant development and yield [97–100]. Our results confirmed the promotion of plant growth, since, in most combinations (inoculation method/isolate), the three fungal isolates significantly enhanced the growth of tomato plants above and below the ground, increased the number of leaves per plant, the root dry weight, and the weight of the biomass above the ground, in comparison to the control plants. Several studies have shown the positive effect of different strains of both fungal species on the development of a wide range of host plants, including tomatoes [73,78,97,101–103]. Even though the mechanisms of plant growth enhancement by EPFs have not been clarified yet, some hypotheses attempted

to clarify this issue. For instance, Jaber and Ownley [91] attributed the growth enhancement in colonized plants to the production of bioactive substances by *Beauveria* spp. González-Pérez et al. [102] correlated the high level of chlorophyll content found in inoculated plants with *M. robertsii* strains to an increase in their biomass, and the resulting plants were more vigorous than non-inoculated ones. Again, the inoculation method and fungal isolate affected plant growth. Our findings indicate that the three tested isolates have different effects on the growth enhancement of tomato plants. Furthermore, our results revealed that the inoculation method significantly affected plant growth, regardless of the isolate used here. Root-dipping inoculation proved more beneficial for the development of the plant organs and the biomass, followed by seed-soaking and foliar-spraying. In addition, root-dipping inoculation with WG-02 significantly enhanced plant growth in comparison to the plants inoculated with *B. bassiana* isolates or control plants.

Concerning the acaricidal activity against *T. urticae*, all three isolates killed 100% of the exposed adult females after 10 days, except WG-19 which resulted in 94.58% mortality. Interestingly, WG-02 exhibited higher mortality after 5 days compared to *B. bassiana* strains. The differences in the virulence of the three isolates against *T. urticae* may be related to differences in the protease activity of EPF [49,104]. Elhakim et al. [52] observed higher protease activity in the *M. robertsii* isolate compared to the *B. bassiana* isolate, which was correlated with the elevated virulence of the former strain against *T. urticae*. Furthermore, in a recent study, Khamis Al-Zahrani et al. [37] found that the activity of protease in *B. bassiana* was the lowest vs. other fungi belonging to the genera *Fusarium*, *Aspergillus*, *Scopulariopsis*, and *Penicillium* after a screening performed on skimmed milk agar medium. All EPFs were obtained from *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) with the exception of one *Fusarium* isolate obtained from *T. urticae*.

5. Conclusions

Our results revealed a multifactorial activity of the WG-12, WG-19, and WG-02 isolates that can be utilized to protect tomato plants from *T. urticae* and enhance plant growth at the same time. The WG-02 isolate was by far the most effective isolate that provided complete protection in tomatoes from the mite and promoted plant development in several ways. The application method of the EPFs should be taken into consideration since it played a significant role in fungal colonization, plant growth, and protection against *T. urticae*. Taking into account the results of the current study, we recommend the foliar spraying of WG-02, since, from a practical point of view, it is the easiest method that can be used in the field [93]. As a next step, further research should be conducted to demonstrate that the fungal isolates of *M. robertsii* and *B. bassiana* of the present study can act as endophytes protecting tomato plants against *T. urticae* or other mite species in the greenhouse and in the field.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy14040665/s1>, Table S1. ANOVA parameters for main effects and associated interactions for colonization of *Beauveria bassiana* and *Metarhizium robertsii* isolates with three inoculation methods in tomato plants (Total DF = 971). Table S2. ANOVA parameters for main effects and associated interaction for plant growth (Total DF = 109).

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