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Germination and Agronomic Traits of *Phaseolus vulgaris* L. Beans Sprayed with *Trichoderma* Strains and Attacked by *Acanthoscelides obtectus*

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Abstract: *Acanthoscelides obtectus*, one of the world's most important post-harvest pests, attacks wild and cultivated common beans (*Phaseolus vulgaris* L.). Four *Trichoderma* strains, *Trichoderma arundinaceum* IBT 40,837 (=Ta37), a wild-type strain producer of trichothecene harzianum A (HA); two transformed strains of Ta37, Ta37-17.139 (Δ*tri17*) and Ta37-23.74 (Δ*tri23*); and *T. brevicompactum* IBT 40,841 (=Tb41), a wild-type strain producer of the trichothecene trichodermin, were evaluated to determine the effect of these compounds on the virulence of *A. obtectus* and the effect of these strains on the seed's capacity of germination and on the agronomic traits of the plants grown from these seeds. Treatments of bean seeds with different *Trichoderma* strains provided varying survival rates in *A. obtectus* adults, so life survival of insects after Tb41 strain application was reduced to 15 days. Δ*tri17* and Tb41 strains sprayed on *P. vulgaris* beans resulted in low weight losses (1.21 and 1.55%, respectively). In spite of the low germination percentage of beans treated with Δ*tri23* strain (lower than the germination percentages of the rest of the fungal strains applied), this treatment encouraged a greater Wet Weight of Aerial Part of the plants grown from both damaged and undamaged beans. High germination rates of Ta37 and Δ*tri17* strains (higher than with the rest of treatments), did not turn into a greater Wet Weight Aerial Part and Wet Weight of Root System in the future plants developed. Linear regression between the number of exit holes and the wet weight aerial part on the one hand, and between the number of exit holes and the wet weight root system on the other, showed interaction, so Δ*tri23* and Tb41 strains behaved differently in comparison to their respective control treatments. The number of exit holes of beans treated with Δ*tri23* or Tb41 was negatively correlated with both the wet weight aerial part and the wet weight root system in *P. vulgaris* plants. Δ*tri23* sprayed on undamaged beans caused the greatest Wet Weight Aerial Part and wet weight root system in plants. Due to the good results obtained by Δ*tri23* and Tb41 strains in this work, more studies for *A. obtectus* control, *P. vulgaris* plant growth and trichothecenes production by these strains should be explored, in order to advance in the knowledge of how these fungi could be used in the field crop, together with the application of management strategies to mitigate risks for farmers and to minimize environmental contamination.

Keywords: bean weevil; post-harvest pest; biological control; weight loss; germination capacity; agronomic traits

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1. Introduction

Acanthoscelides obtectus (Say) (Coleoptera: Chrysomelidae: Bruchidae), the bean weevil, is one of the world's most important post-harvest pests in dry beans (*Phaseolus vulgaris* (L.) (Fabaceae)) [1,2]. It is mainly found in South America and Africa and in the Mediterranean area [3–5], where adults attack bean seeds while they are still in the field and continues to cause damage during storage. These attacks can lead to the total loss of stored bean seeds [6,7] as the larvae enter the seeds not only to feed on them but also to transform from larva to adult inside [8]. The population of this insect pest can grow exponentially and can completely destroy the stored crops in a few months [9]. Until now, the control of *A. obtectus* insects in storage is based on the application of synthetic insecticides [10], or is carried out through physical barriers, such as the use of hermetic packaging [11]. The continuous application of synthetic insecticides has led to concerns about pest resistance, risk to human health and environmental contamination [12]. All these reasons have motivated the search for more sustainable alternatives for pest control [13]. Recently, different sustainable alternatives to control this pest have been described, such as those through the use of essential oils [14], fungi [15–19] or bacteria [20]. The use of fungi to control invertebrate pests, weeds and plant diseases during the last years has grown due to the numerous commercial products that have been marketed or are still under development [21] to minimize side effects on the indigenous-beneficial organisms and on the environment.

Trichoderma (teleomorph Hypocrea, Ascomycota, Dikarya) is a well-studied fungal genus currently consisting of more than 200 molecularly defined species [22]. Trichoderma species are generally considered cosmopolitan and prevalent components of different ecosystems in a wide range of climatic zones [23]. However, some species are ubiquitous while others are limited to specific geographical areas [24]. Many *Trichoderma* spp. are non-pathogenic soil-borne fungi considered opportunistic, avirulent and plant symbionts which are able to colonize the roots of many plants [24]. Up to now, it is known that these fungi are highly beneficial for agriculture due to their ability to protect crops against diseases and to improve overall crop yields [25]. These *Trichoderma* strains can perform their biocontrol activity by several mechanisms, the most important being mycoparasitism, antibiosis and competition. Furthermore, the most efficient biocontrol strains display, simultaneously or sequentially, more than one of those biocontrol strategies [26].

Trichoderma strains hold a huge potential to produce a wide variety of secondary metabolites in their genomes [27,28], such as pyrones [6-pentyl-2H-pyran-2-one (6-PP) derivatives] [29,30], butenolides [31], peptaibols [32,33], terpenes (e.g., trichothecenes, triterpenes) [34–37] and gliotoxin, viridin, harzianopyridone and harziandione [28,38].

Terpene compounds have a variety of roles in mediating antagonistic and beneficial interactions between organisms [39]. These interactions can occur between arthropods and host plants infected with fungi, but there is a lack of information related to the impact of mycotoxins, as for example, volatile mycotoxins or intermediates of their biosynthesis, produced by saprofitic-beneficial fungi on visiting herbivores [40].

Trichothecenes, a group of non-volatile sesquiterpenoid mycotoxins, have a central core of fused cyclohexene/tetrahydropyran rings [41,42]. Most of these compounds are phytotoxic, have high antibiotic activity and high toxicity for human and animals, resulting in skin or intestinal mucosa irritation and effects in the immune and nervous system [43]. Up to now, the knowledge about trichothecenes interaction with plants is very limited, and their phytotoxic activity is widely known because they suppress the defense response in host plants [44]. However, the trichothecene harzianum A (HA), produced by *T. arundinaceum* (Zafari, Gräfenhan & Samuels), is not harmful for plants when assayed in vivo, and it also induces the expression of plant defense genes linked to the salicylic acid (SA) and jasmonic acid (JA) pathways [35].

The aims of this work were: (1) to determine the effect of compounds and intermediates produced by different wild-type and transformant *Trichoderma* strains sprayed over *P. vulgaris* beans against *A. obtectus* adults; (2) to analyse the effect of these strains on the germination capacity of beans and on the agronomic traits of the plants grown from beans

treated with the selected fungal strains, and that later were damaged or undamaged by *A. obtectus* larvae.

2. Materials and Methods

2.1. Fungal Strains Evaluated

Four *Trichoderma* strains were evaluated: *Trichoderma arundinaceum* IBT 40,837 (=Ta37) and *T. brevicompactum* IBT 40,841 (=Tb41), two wild-type strains producers of trichothecenes harzianum A (HA) and trichodermin, respectively [45], and Ta37-17.139 (Δ*tri17*) and Ta37-23.74 (Δ*tri23*), two transformants of Ta37, isolated in previous works, in which the genes *tri17* and *tri23*, respectively, both involved in the HA biosynthetic pathway, were deleted [37,46]. Δ*tri17* and Δ*tri23* mutants do not produce HA, but in both cases accumulate trichodermol, one of the intermediates in the synthesis of HA [37,46].

2.2. Insect Collection and Rearing

The original population of *A. obtectus* was collected during the years 2017, 2018 and 2019 from storages located in the Protected Geographical Indication (PGI) "Alubia de La Bañeza-León" (EC Reg. n.256/2010 published on 26 March 2010, OJEU L880/17). The common bean (*Phaseolus vulgaris* L.) "Canela" variety, was used to feed the different *A. obtectus* stages. To keep the insects under laboratory conditions before and after experiments the methodology described by Rodríguez-González et al. [16,19,20] was used.

2.3. Fungal Culture Conditions

PPG medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used for the growth of the fungal isolates according to the methodology described by Lindo et al. [47]. In order to obtain fungal spores and to calculate the final concentration of spores used in the experiments, the methodology described by Rodríguez-González et al. [16,20] was used.

2.4. Design of Experiments

2.4.1. Experiment 1: Effects of Beans Sprayed with *Trichoderma* Strains on *A. obtectus* Insects

With a manual loading Potter Tower (Burkard Scientific Limited, Po Box 55 Uxbridge, Middx UB8 2RT, UK) (Figure 1a), one ml of the spores' suspension (1×10^7 spores/mL) of each *Trichoderma* strain was directly applied on 40 *P. vulgaris* beans placed in a Petri dish (90 mm diameter) (Figure 1b) following the methodology described by Potter [48]. Distilled water was used as a control treatment and carrier in all the treatments with fungal isolates. Beans (treated with *Trichoderma* strains or theirs controls) were transferred to a structure made up of five circular plastic containers (Figure 1c). Four containers (A, B, C and D) (40 mm diameter and 70 mm high) with a central container (E) (120 mm diameter and 60 mm high) connected to the other four containers by plastic cylinders (70 mm long and 7 mm in diameter). Containers B and D were arranged diagonally and filled with the 40 beans treated with the *Trichoderma* strain. Containers A and C (controls) were filled with the 40 beans treated with the controls. In the central container, 20 *A. obtectus* adults (10 males and 10 females) were released. Four treatments (4 *Trichoderma* strains) with four replicates of 20 adults were used for each treatment. After 24 h once *A. obtectus* adults decided their location in containers A, B, C or D, beans (treated with *Trichoderma* strains or their controls) and insects were transferred back to Petri dishes where the treatments had been applied with the Potter tower. The insect mortality of *A. obtectus* adults in contact with the beans was recorded daily. After 15 days, the weight loss (2×40 beans) due to the insects' attacks on beans (treated with *Trichoderma* strains or their controls) in each Petri dish and treatment were recorded.

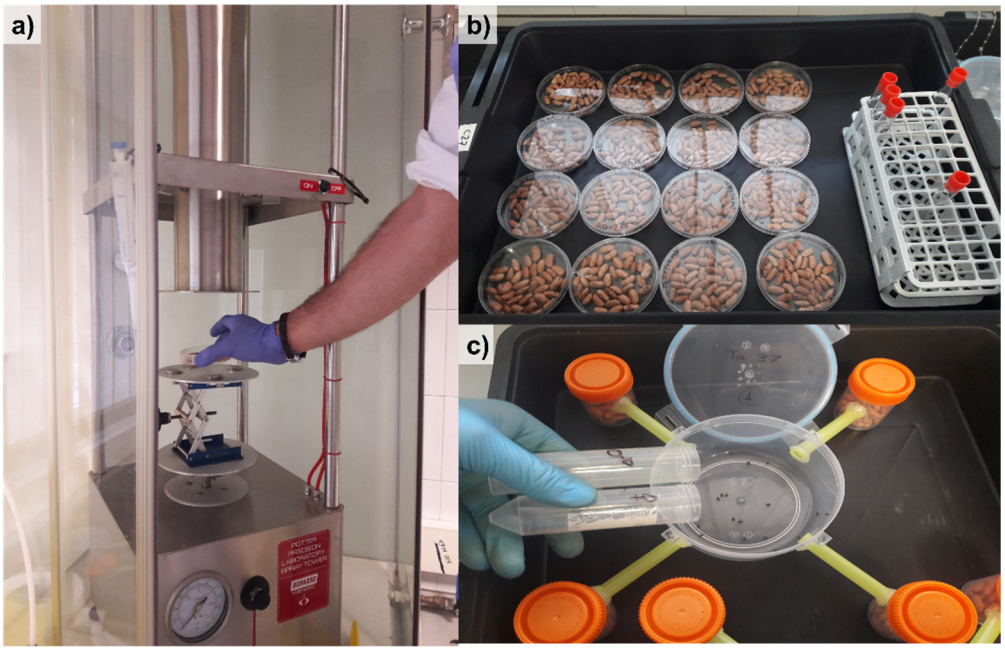

Figure 1. (**a**) Potter Spray Tower; (**b**) sprayed beans in Petri dishes; (**c**) structure of plastic containers used in the experiment.

2.4.2. Experiment 2: Evaluation of the Germination Capacity of Beans

One-litre polypropylene pots filled with peat (TYPical, Brill, Georgsdorf, Germany) were stored in climatic chambers under controlled conditions in order to test the germination capacity of bean seeds previously in contact with *Trichoderma* spp. (inoculated as described in Section 2.4.1) or control samples (sprayed with distilled water as described in Section 2.4.1) and *A. obtectus* insects. Each pot was filled with 250 mL of water prior to sowing. Five undamaged beans (1 seed without any hole from every Petri dish, described above; see Section 2.4.1, Experiment 1) were randomly selected and sown in five pots, each bean in an individual pot. Five damaged beans (beans with at least one hole per bean of every container caused by *A. obtectus*), were selected and sown in five pots, each bean in an individual pot. Controlled conditions were maintained for 45 days, which involved exposing seeds to a photoperiod of 16 h light, day/night temperatures of 25 °C/16 °C, 60% RH and brightness of 3500 lux. Irrigations were performed every 4 days with about 250 mL tap water per pot according to the methodology described by Mayo et al. [49,50]. A nutrient solution was added on the 2nd-4th week according to the methodology described by Rigaud and Puppo [51]. Plants were removed after 45 days and the parameters "wet weight" and "dry weight" (72 h in an oven, at 82 °C) of the aerial part and root system were calculated. Four replicates for each fungal isolate were used.

2.5. Statistical Analysis

Experiment 1. Mortality data of *A. obtectus* insects were submitted to survival analysis estimator using IBM SPSS Statistics for Windows, Version 26.0. (Armonk, NY, USA: IBM Corp.), and the functions obtained from each treatment were compared with the Wilcoxon-Gehan test ($p < 0.05$).

Experiment 2. A randomly completed experiment using the Generalized Linear Model (GLM) procedure, with four treatments and four replicates was subjected to ANOVA, using IBM SPSS Statistics for Windows, Version 26.0. (Armonk, NY, USA: IBM Corp.). Differences ($p < 0.05$) among weight losses of plants grown from damaged beans were examined by mean comparisons using Fisher least significant difference (LSD) tests.

Experiment 3. Germination capacity of beans was submitted to the Kaplan–Meier estimator and the functions obtained from each treatment were compared by the log-rank test (Mantel-Cox) ($p < 0.05$). Analysis of covariance (ANCOVA) was used to examine

the effect of the number of exit holes (NEH) of damaged beans (fixed factor) on the Wet Weight of the Aerial Part (WWAP) of *P. vulgaris* plants (obtained from beans previously sprayed with different treatments) as a covariate. The linear regression coefficients of the NEH x WWAP interaction were tested using an F-test, considering a significance level at $p \leq 0.05$. Analyses were conducted using IBM SPSS Statistics for Windows, version 26.0. (Armonk, NY, USA: IBM Corp.). A randomly completed experiment using the Generalized Linear Model (GLM) procedure, with four treatments and four replicates (damaged and undamaged beans) was subjected to ANOVA using IBM SPSS Statistics for Windows, Version 26.0. (Armonk, NY, USA: IBM Corp.). Differences ($p < 0.05$) among WWAP and WWRS were examined by mean comparisons using Fisher least significant difference (LSD) test.

3. Results

3.1. Effects of Beans Sprayed with Spores of Trichoderma Strains on the Survival Rate of A. obtectus Insects

The treatment influences the survival rate of insects (Wilcoxon-Gehan = 13.631; df = 7,141; $p = 0.050$) (Figure 2). Insects subjected to treatments with Tb41 and Ta37 had a significant lower survival rate after 15 days, 0 and 20% respectively, than those subjected to their respective controls.

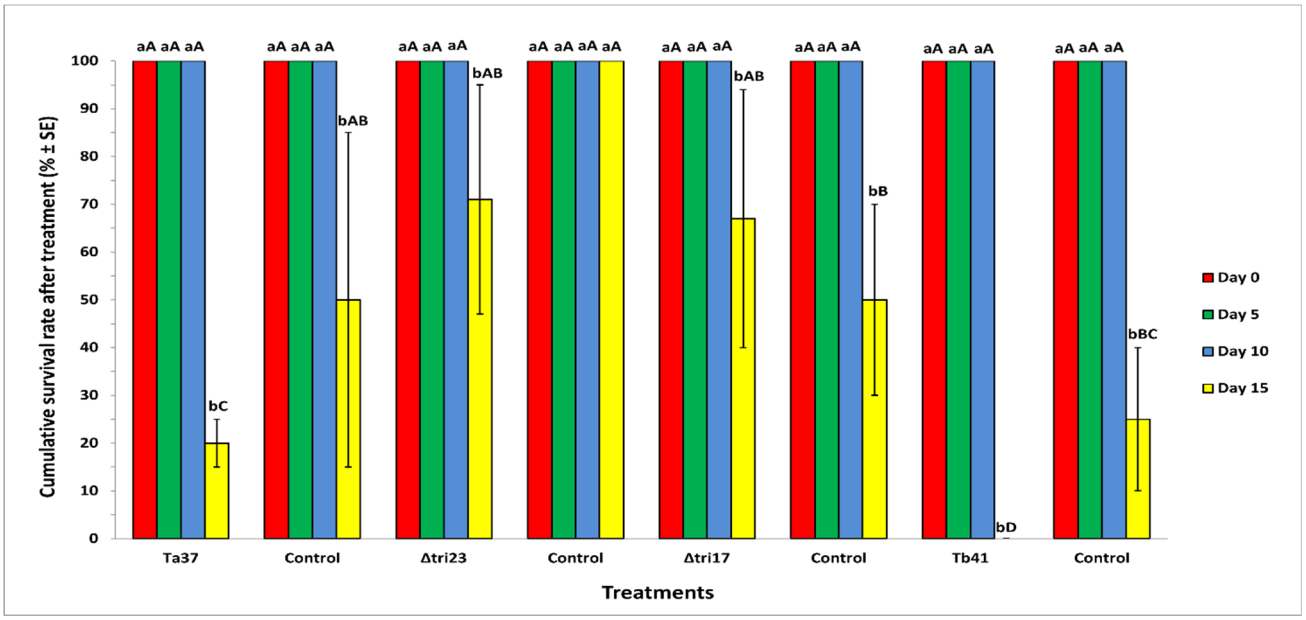

Figure 2. Mortality of *A. obtectus* exposed to beans sprayed with *Trichoderma* strains during the 15 days. Global comparisons of mortality rate for all treatments (Wilcoxon-Gehan = 13.631; df = 7,141; $p = 0.050$). Pairwise comparison among all treatments (Wilcoxon-Gehan). Different lowercase letters indicate significant differences among days within each *Trichoderma* strain or its control; ($p < 0.05$). Different capital letters indicate significant differences among treatments within the same day of evaluation; ($p < 0.05$).

Moreover, these strains, Tb41 and Ta37, provided a significant lower survival rate for the insect population than the rest of the fungal strains applied to the beans, Δ*tri17* and Δ*tri23*, whose insects had survival rates after 15 days of 67 and 71%, respectively (Figure 2).

3.2. Weight Losses of Beans Damaged by A. obtectus Larvae Treated with Trichoderma Strains and Controls

Weight losses in beans treated with *Trichoderma* strains ranged from 1.21% to 1.93%, whereas untreated beans (sprayed with distilled water) showed a weight loss between 1.58% and 3.99%. While beans treated with Δ*tri17* (F = 8.292; df = 1,9; $p = 0.037$) and Tb41 F = 7.082; df = 1,10; $p = 0.043$) strains showed a significantly lower weight loss than their

controls, differences were not significant between beans treated with Δ*tri23* strain and its control (Figure 3).

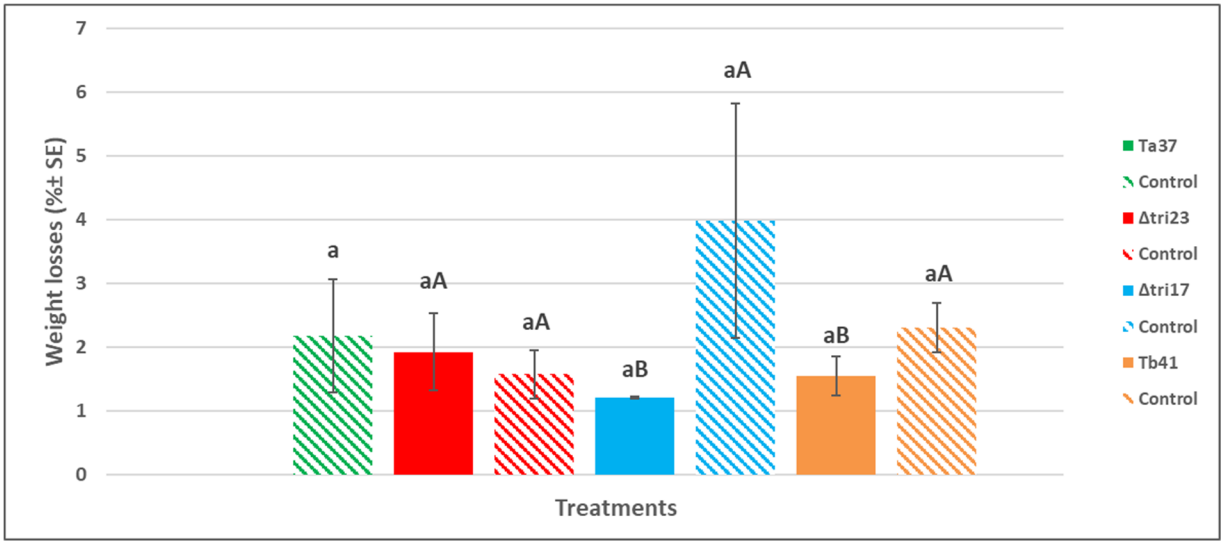

Figure 3. Weight losses (% ± SE) of treated beans (sprayed with *Trichoderma* strains) and their controls (sprayed with distilled water). Different lowercase letters indicate significant differences among treatments (*Trichoderma* strains on the one hand, and controls on the other hand) ($p \leq 0.05$), LSD test at 0.05. Different capital letters indicate significant differences between treated beans with *Trichoderma* strains and their controls ($p \leq 0.05$). Data obtained from the weight loss of 80 beans.

3.3. Effect of A. obtectus on the Germination of Beans and on Agronomic Traits of Future P. vulgaris Plants

3.3.1. Beans Sprayed with Spores of Trichoderma Strains and Damaged by *A. obtectus* Larvae

Table 1 shows that the type of *Trichoderma* strain used influences the germination capacity of damaged beans (log-rank $\chi^2 = 15.424$; df = 5,28; $p = 0.009$). Thus, damaged beans previously sprayed with Δ*tri17* had a higher germination percentage than damaged beans treated with Δ*tri23*. In addition, Δ*tri17* and Tb41 treatments allowed the beans to have a significantly lower number of exit holes per bean (1 and 1.33) and a lower weight loss (1.21% and 1.55%) than their respective controls.

Although damaged beans previously sprayed with Δ*tri23* strain had a lower germination percentage than both the control and the damaged beans treated with the rest of the fungal strains. Δ*tri23* treatment provided plants with a significantly higher wet weight aerial part (26.68 g) than Δ*tri17* treatment.

3.3.2. Beans Sprayed with Spores of Trichoderma Strains and Undamaged by *A. obtectus* Larvae

Treatments with *Trichoderma* strains used in the present work affected the germination capacity of undamaged beans (log-rank $\chi^2 = 40.663$; df = 7,152; $p < 0.001$), so beans sprayed with strain Δ*tri23* had a germination percentage higher than beans sprayed with Tb41 strain but lower than beans sprayed with Ta37 strain (Table 2).

The wet weight aerial part of plants grown from undamaged beans subjected to Δ*tri23* strain was significantly higher (35.03 g) than the control (25.38 g). Additionally, the wet weight aerial part of plants grown from beans treated with this strain was significantly higher than the wet weight aerial part of plants grown from beans treated with the other *Trichoderma* strains.

Regarding the root system, undamaged beans sprayed with Δ*tri23* strain provided plants with a higher wet weight root system (4.92 g) than those grown from beans treated with the rest of the *Trichoderma* strains, Ta37, Δ*tri23* and Tb41, with weights of 3.52, 3.19 and 2.15 g, respectively (Table 2).

Table 1. Germination capacity of damaged beans sprayed with different *Trichoderma* strains and agronomic traits of *P. vulgaris* plants after 45 days (and their controls).

Treatments	Beans				Number Exit Holes * (Mean ± SE)		Bean Weight Losses * (% ± SE)		Weight Wet Aerial Part * (Mean ± SE)		Weight Wet Root System * (Mean ± SE)	
	Total	Germinated	Not Germinated	Germination (%)								
Ta37	-	-	-	-	-	χ^2	-	χ^2	-	χ^2	-	χ^2
Control	-	-	-	-	-	df *P*	-	df *P*	-	df *P*	-	df *P*
Δ*tri23*	8	5	3	62.50	2.25 ± 0.69 aA [a,b]	0.594	2.21 ± 0.73 aA [a,b]	0.020	26.68 ± 2.04 aA [a,b]	0.917	1.96 ± 0.37 aA [a,b]	0.947
Control	3	3	0	100.00	1.33 ± 0.33 aB	(1,9) 0.441	1.58 ± 0.38 aB	(1,9) 0.887	24.83 ± 1.78 aA	(1,9) 0.338	1.87 ± 0.12 aA	(1,9) 0.331
Δ*tri17*	3	3	0	100.00	1.00 ± 0.00 bB	3.225	1.21 ± 0.02 bB	12.771	21.25 ± 2.45 aB	1.247	1.15 ± 0.36 aA	3.109
Control	8	7	1	87.50	4.25 ± 2.01 aA	(1,9) 0.050	4.71 ± 2.30 aA	(1,9) ≤0.001	21.24 ± 2.88 aA	(1,9) 0.264	1.79 ± 0.48 aA	(1,9) 0.078
Tb41	3	2	1	66.66	1.33 ± 0.27 bAB	3.400	1.55 ± 0.31 bAB	4.752	26.55 ± 0.95 aA	0.222	1.59 ± 0.18 bA	8.333
Control	9	6	3	66.66	2.66 ± 0.66 aAB	(1,10) 0.047	2.91 ± 0.57 aAB	(1,10) 0.043	27.00 ± 2.90 aA	(1,10) 0.637	2.65 ± 0.34 aA	(1,10) 0.004
					Trich. Ctrl.		*Trich.* Ctrl.		*Trich.* Ctrl.		*Trich.* Ctrl.	
				χ^2	5.971 4.230	χ^2	4.704 4.454	χ^2	9.290 1.901	χ^2	5.018 1.241	
				df	(2,11) (2,17)	df	(2,11) (2,17)	df	(2,11) (2,17)	df	(2,11) (2,17)	
				P	0.037 0.048	*P*	0.044 0.045	*P*	0.010 0.387	*P*	0.081 0.538	

* Test for equality of germination capacity of damaged beans between treatments and controls (Log Rank; Mantel-Cox). [a] Different lowercase letters indicate significant differences between treated and untreated bean (control) within each strain; ($p < 0.05$). [b] Different capital letters indicate significant differences among fungal strains (on one hand) and among their controls (on the other hand); ($p < 0.05$).

Table 2. Germination capacity of undamaged beans sprayed with different *Trichoderma* strains and agronomic traits of *P. vulgaris* plants after 45 days (and their controls).

Treatments	Beans				Weight Wet Aerial Part * (Mean ± SE)		Weight Wet Root System * (Mean ± SE)	
	Total	Germinated	Not Germinated	Germination (%)				
Ta37	20	16	4	80.00	24.73 ± 1.77 bB [a,b]	χ^2 = 3.573	3.52 ± 0.36 aB [a,b]	χ^2 = 0.029
Control	20	14	6	70.00	29.30 ± 1.67 aA	df(1,38) p = 0.05	3.62 ± 0.40 aA	df(1,38) p = 0.865
Δ*tri23*	20	15	5	75.00	35.03 ± 1.63 aA	χ^2 = 12.342	4.92 ± 0.53 aA	χ^2 = 2.262
Control	20	15	5	75.00	25.38 ± 2.11 bB	df(1,38) $p \leq 0.001$	3.70 ± 0.46 aA	df(1,38) p = 0.133
Δ*tri17*	20	16	4	80.00	23.38 ± 2.31 bB	χ^2 = 7.487	3.19 ± 0.84 aB	χ^2 = 1.081
Control	20	14	6	70.00	32.16 ± 2.66 aA	df(1,38) p = 0.006	3.62 ± 0.56 aA	df(1,38) p = 0.298
Tb41	20	9	11	45.00	26.98 ± 1.68 aB	χ^2 = 1.790	2.15 ± 0.24 aC	χ^2 = 1.011
Control	20	13	7	65.00	23.58 ± 1.22 aB	df(1,38) p = 0.181	2.50 ± 0.24 aB	df(1,38) p = 0.315

		Trich.	Ctrl.		*Trich.*	Ctrl.
	χ^2	22.173	16.672	χ^2	21.475	8.454
	df	(3,76)	(3,76)	df	(3,76)	(3,76)
	p	$p \leq 0.001$	$p \leq 0.001$	p	$p \leq 0.001$	p = 0.037

* Test for equality of germination capacity of undamaged beans between treatments and controls (Log Rank; Mantel-Cox). [a] Different lowercase letters indicate significant differences between treated and untreated beans (control) within each strain; ($p < 0.05$). [b] Different capital letters indicate significant differences among fungal strains (on one hand) and among their controls (on the other hand); ($p < 0.05$).

3.4. Agronomic Traits of Plants Grown from Damaged Beans

3.4.1. Wet Weight Aerial Part in Relation to the Number of Exit Holes in Beans

The interaction between the wet weight aerial part and the number of exit holes was not performed with the Ta37 strain due to lack of data (Figure 4a).

The wet weight aerial part of plants as function of the number of exit holes in damaged beans between beans sprayed with Δ*tri23* and its control was not significantly different (F = 1.251; df = 1,9; p = 0.296). The linear regression coefficients of the wet weight aerial part x number of exit holes interaction between Δ*tri23* and its control treatments were significantly different (F = 7.766; df = 1,9; p = 0.024). Beans treated with Δ*tri23* provided an increase in the wet weight aerial part of the plants when the number of exit holes was lower (Figure 4b).

The wet weight aerial part of plants as function of the number of exit holes in damaged between beans sprayed with Δ*tri17* and its control was not significantly different (F = 0.000; df = 1,9; p = 0.997). The linear regression coefficients of the wet weight aerial part x number of exit holes number of exit holes between Δ*tri17* and its control treatments were not significantly different (F = 12.916; df = 1,9; p = 0.409). The wet weight aerial part of plants and number of exit holes of beans were not correlated in beans treated with Δ*tri17* strain (Figure 4c).

The wet weight aerial part of plants as function of the number of exit holes in damaged beans between beans sprayed with Tb41 and its control was not significantly different (F = 0.941; df = 1,9; p = 0.357). The linear regression coefficients of the wet weight aerial part x number of exit holes interaction between Tb41 and its control treatments were significantly different (F = 3.2740; df = 1,9; p = 0.104). Beans treated with Tb41 provided plants with bigger wet weight aerial part whenever the number of exit holes declined (Figure 4d).

3.4.2. Wet Weight Root System in Relation to the Number of Exit Holes in Beans

The interaction between wet weight root system and number of exit holes was not performed with the Ta37 strain due to lack of data (Figure 5a).

The wet weight root system of plants as function of the number of exit holes in damaged beans between beans sprayed with Δ*tri23* and its control was not significantly different (F = 1.236; df = 1,9; p = 0.299). The linear regression coefficients of the wet weight root system x number of exit holes interaction between Δ*tri23* and its control were significantly different (F = 8.377; df = 1,9; p = 0.020). The number of exit holes of beans treated with Δ*tri23* was negatively correlated with the wet weight root system of the plants grown from seed (Figure 5b).

The wet weight root system of plants as function of the number of exit holes in damaged beans between beans sprayed with Δ*tri17* and its control was significantly higher on beans sprayed with the control than in beans treated with Δ*tri17* strain (F = 16.420; df = 1,9; p = 0.004). The linear regression coefficients of the wet weight root system x number of exit holes interaction between Δ*tri17* and its control treatments were not significantly different (F = 0.554; df = 1,9; p = 0.478). The wet weight root system was not correlated with the number of exit holes in beans treated with Δ*tri17* strain (Figure 5c).

The wet weight root system of plants as function of the number of exit holes in damaged beans between beans sprayed with Tb41 strain and its control was not significantly different (F = 0.165; df = 1,9; p = 0.694). The linear regression coefficients of the wet weight root system x number of exit holes interaction between Tb41 strain and its control were significantly different (F = 5.690; df = 1,9; p = 0.041). The number of exit holes in beans treated with Tb41 strain was negatively correlated with the wet weight root system of the plants grown from seed (Figure 5d).

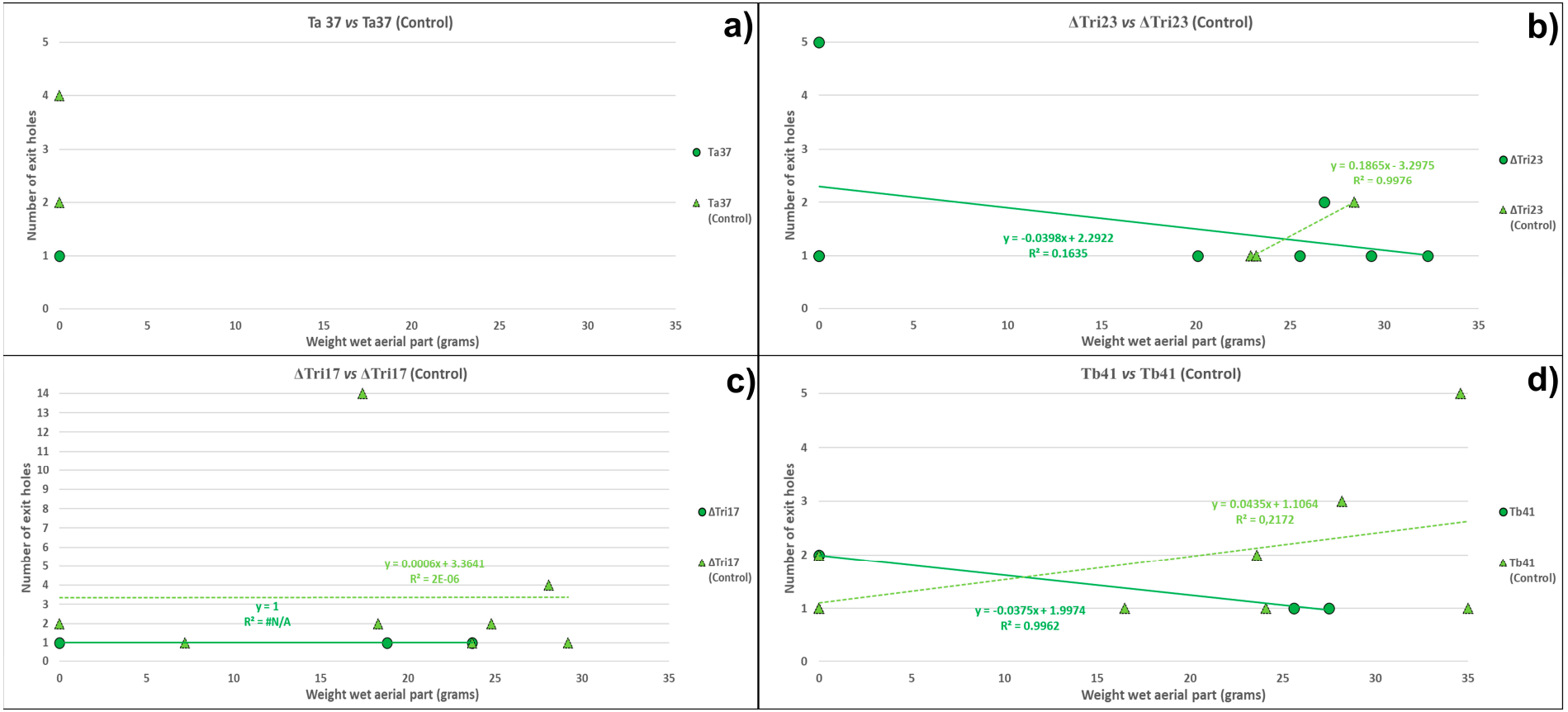

Figure 4. Linear regression of the wet weight aerial part (WWAP) of *P. vulgaris* plants (*x*-axis) versus the number of exit holes (NEH) of *P. vulgaris* beans (*y*-axis). "Round Intense Green Point" means bean sample sprayed with *Trichoderma* strains: Ta37, (**b**) Δ*tri23*, (**c**) Δ*tri17* and (**d**) Tb41. "Triangular Light Green Point" means the controls beans sample sprayed with distilled water: (**a**) Ta37 (control), (**b**) Δ*tri23* (control), (**c**) Δ*tri17* (control) and (**d**) Tb41 (control). Linear regression trendlines are coloured based on the treatment ("Continuous Intense Green Line" represents bean samples sprayed with *Trichoderma* strains; "Discontinuous Triangular Light Green Line" indicates bean samples sprayed with distilled water).

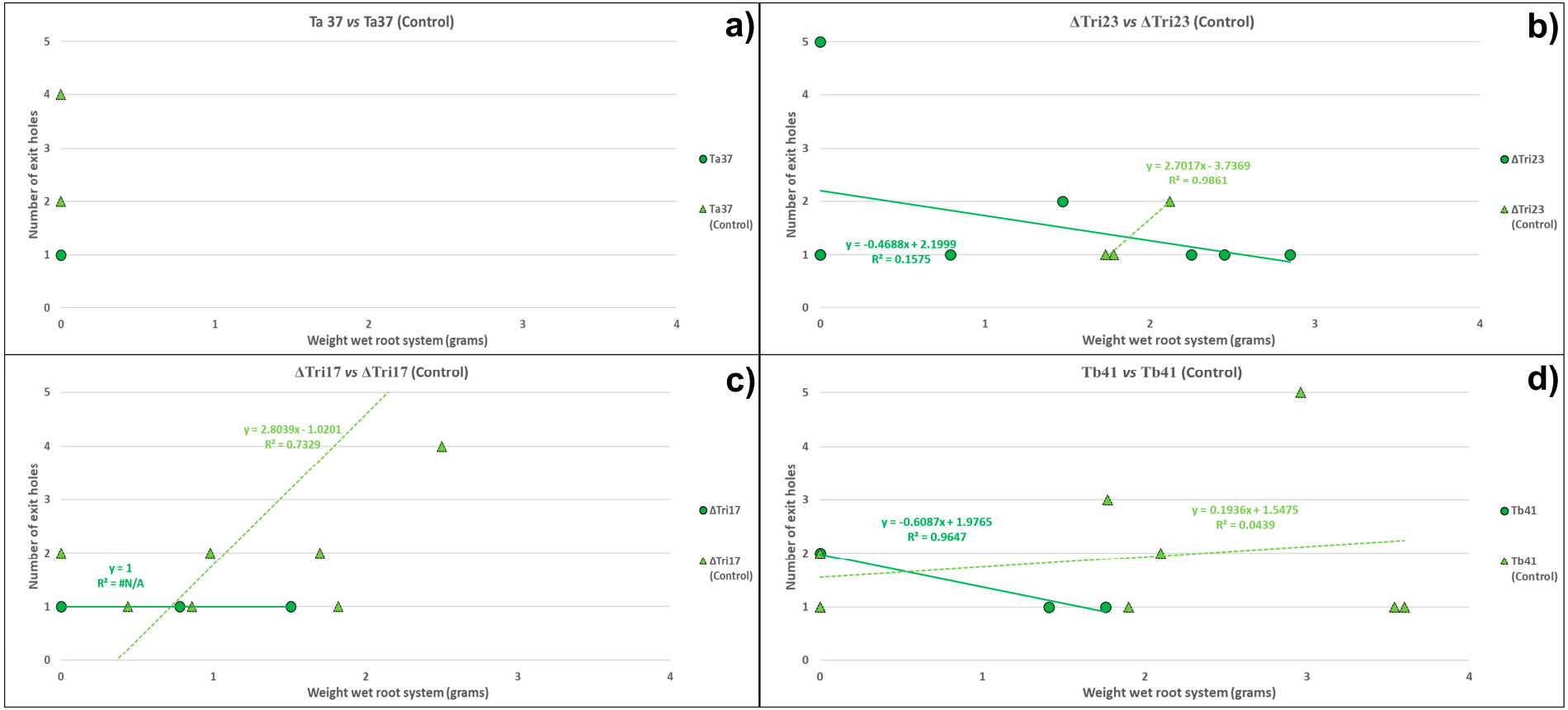

Figure 5. Linear regression of wet weight root system (WWRS) of *P. vulgaris* plants (*x*-axis) versus number of exit holes (NEH) of *P. vulgaris* beans (*y*-axis). "Round Intense Green Point" means bean samples sprayed with *Trichoderma* strains: Ta37, (**b**) Δ*tri23*, (**c**) Δ*tri17* and d) Tb41. "Triangular Light Green Point" means the controls beans samples sprayed with distilled water: (**a**) Ta37 (control), (**b**) Δ*tri23* (control), (**c**) Δ*tri17* (control) and (**d**) Tb41 (control). Linear regression trendlines are coloured based on the treatment ("Continuous Intense Green Line" represents bean samples sprayed with *Trichoderma* strains; "Discontinuous Triangular Light Green Line" indicates bean samples sprayed with distilled water).

3.4.3. Agronomic Traits in Relation to the Number of Exit Holes in Beans Based on the Strain Applied

The linear regression coefficients of the wet weight aerial part x number of exit holes interaction among the different *Trichoderma* strains applied to damaged beans were significantly different (F = 16.852; df = 2,11; p = 0.004). The greatest increase in the wet weight aerial part of plants in relation to the decrease in the number of exit holes of beans was observed in beans treated with the Δ*tri23* strain (Figure 6a).

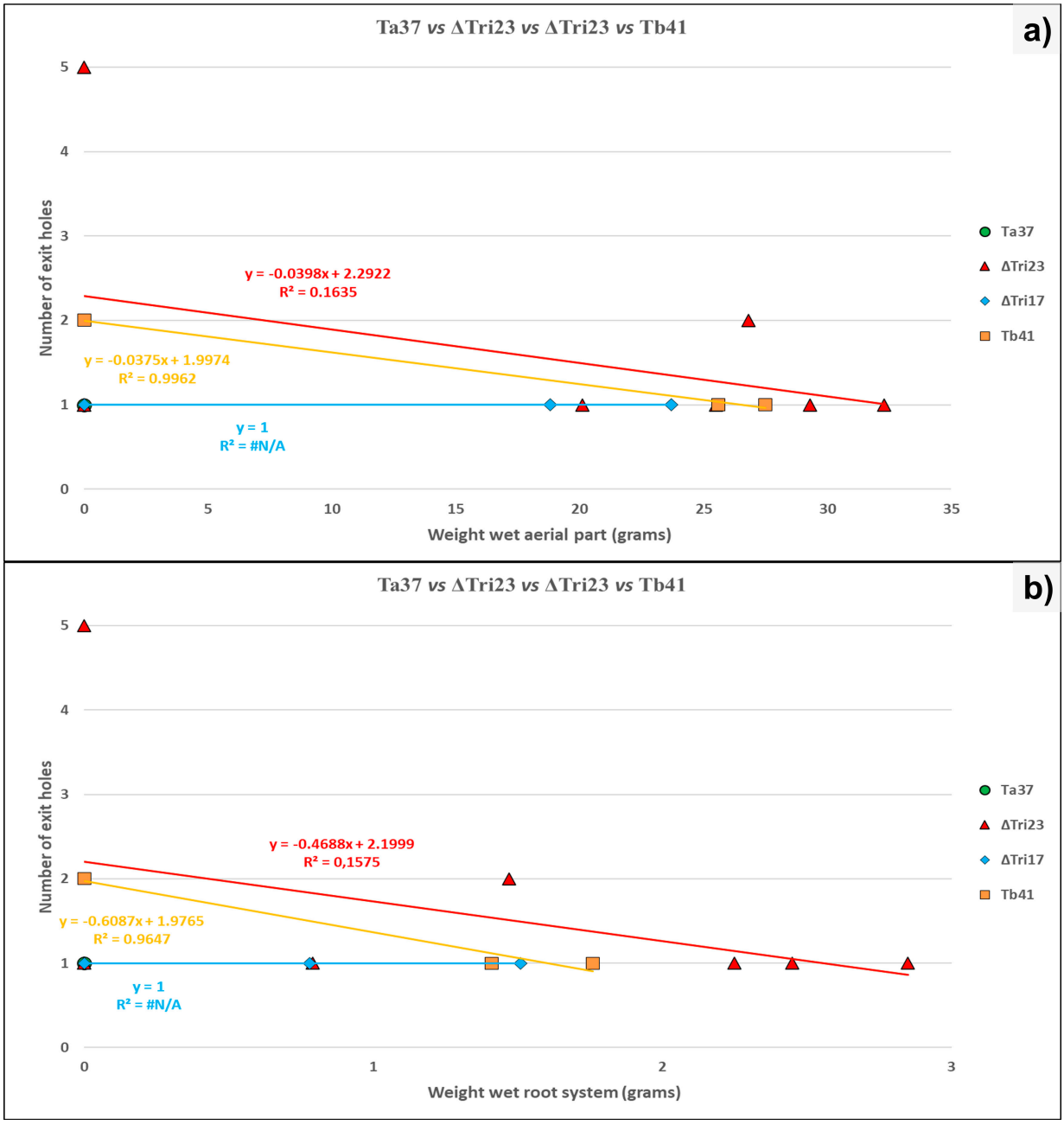

Figure 6. (**a**) Linear regression of wet weight aerial part (WWAP) of *P. vulgaris* plants (*x*-axis) versus Number of Exit Hole (NEH) of *P. vulgaris* beans (*y*-axis). (**b**) Linear regression of wet weight root system (WWRS) of *P. vulgaris* plants (*x*-axis) versus number of exit holes (NEH) of *P. vulgaris* beans (*y*-axis). "Round Green Point" means bean samples sprayed with Ta37. 'Triangular Red Point" means bean samples sprayed with Δ*tri23*. "Diamond-shaped Blue Point" means bean samples sprayed with Δ*tri17*. "Square Orange Point" means bean samples sprayed with Tb41. Linear regression trendlines are coloured based on the strain ("Green Line" represents Ta37 strain, 'Red Line' represents Δ*tri23* strain, 'Blue Line' represents Δ*tri17* strain, 'Orange Line' represents Tb41 strain).

The linear regression coefficients of the wet weight root system x number of exit holes interaction among the different *Trichoderma* strains treatments applied to damaged beans were significantly different (F = 15.556; df = 2,11; $p = 0.003$). The greatest increase in the wet weight root system of plants in relation to the decrease in the number of exit holes of beans was observed in beans treated with Δ*tri23* strain (Figure 6b).

3.5. Comparison of Agronomic Traits between Plants Obtained from Damaged or Undamaged Beans

Regarding the beans sprayed with the fungal strains used in the present work, results indicated that undamaged beans sprayed with Δ*tri23* strain provided plants with significantly greater wet weight aerial part and wet weight root system (35.03 and 4.92 g, respectively) than those grown from damaged beans sprayed with the same strain. Moreover, undamaged beans sprayed with Δ*tri23* strain provided plants with the significantly greatest wet weight aerial part and wet weight root system when compared with undamaged beans sprayed with the rest of the strains. Furthermore, plants grown from beans sprayed with Δ*tri17* and Tb41 strains reached significantly higher wet weight root system (3.19 and 2.15 g, respectively), than plants grown from damaged beans and sprayed with the same strains (Table 3, left side).

Regarding the beans sprayed with distilled water (controls), results indicated that undamaged beans sprayed with the control of Δ*tri17* provided plants with significantly greater wet weight aerial part (32.16 g) than those grown from beans sprayed with the control of Δ*tri17* but previously damaged by *A. obtectus* larvae. In addition, undamaged beans sprayed with the control of Δ*tri17* provided plants with the significantly greatest wet weight aerial part and wet weight root system. Furthermore, plants grown from beans in the controls of Δ*tri23* and Δ*tri17* treatments had significantly greater wet weight root system (3.70 and 3.62 g, respectively) than plants grown from damaged beans under the same control treatments. Besides, plants grown from undamaged beans in the control of Δ*tri23*, had a significantly greater Wet Weight Root System, than plants grown from undamaged beans in Tb41 control (Table 3, right part).

Table 3. Comparison of agronomic traits (grams; mean ± SE) of *P. vulgaris* plants grown for 45 days from damaged and undamaged beans sprayed with *Trichoderma* strains and their controls.

Treatments	Seed Condition	Agronomic Trait: Wet Weight Aerial Part		Agronomic Trait: Wet Weight Root System	
Ta37	U.*	24.73 ± 1.77 B	F df *P*	3.52 ± 0.36 B	F df *P*
Ta37	D.**	-		-	
Δ*tri23*	U.	35.03 ± 1.63 aA [a,b]	7.082 (1,18) 0.016	4.92 ± 0.53 aA [a,b]	9.775 (1,18) 0.006
Δ*tri23*	D.	26.68 ± 2.04 bA		1.96 ± 0.37 bA	
Δ*tri17*	U.	23.38 ± 2.31 aB	0.073 (1,17) 0.790	3.19 ± 0.84 aB	5.858 (1,17) 0.038
Δ*tri17*	D.	21.25 ± 2.45 aA		1.15 ± 0.36 bA	
Tb41	U.	26.98 ± 1.68 aB	0.098 (1,8) 0.762	2.15 ± 0.24 aB	4.921 (1,8) 0.043
Tb41	D.	26.55 ± 0.95 aA		1.59 ± 0.18 bA	
		Trich. (U.)	*Trich.* (D.)	*Trich.* (U.)	*Trich.* (D.)
	F	7.813	1.448	6.564	0.969
	df	(3,50)	(2,6)	(3,50)	(2,6)
	p	≤0.001	0.307	≤0.001	0.432

Treatments	Seed Condition	Agronomic Trait: Wet Weight Aerial Part		Agronomic Trait: Wet Weight Root System	
Control	U. *	29.30 ± 1.67 AB	F df *P*	3.62 ± 0.40 AB	F df *P*
Control	D. **	-		-	
Control	U.	25.38 ± 2.11 aBC [a,b]	0.012 (1,16) 0.912	3.70 ± 0.46 aA [a,b]	3.329 (1,16) 0.046
Control	D.	24.83 ± 1.78 aA		1.87 ± 0.12 bA	
Control	U.	32.16 ± 2.66 aA	6.705 (1,18) 0.019	3.62 ± 0.56 aAB	4.560 (1,18) 0.047
Control	D.	21.24 ± 2.88 bA		1.79 ± 0.48 bA	
Control	U.	23.58 ± 1.22 aC	1.213 (1,17) 0.286	2.50 ± 0.24 aB	0.027 (1,17) 0.872
Control	D.	27.00 ± 2.90 aA		2.65 ± 0.34 aA	
		Ctrl. (U.)	Ctrl. (D.)	Ctrl. (U.)	Ctrl. (D.)
	F	3.855	1.141	1.880	1.256
	df	(3,53)	(2,13)	(3,53)	(2,13)
	p	0.014	0.350	0.144	0.317

[a] Different lowercase letters indicate significant differences between damaged and undamaged beans (seed condition) within each treatment and agronomic trait (wet weight aerial part or Wet Weight Root System); LSD test at 0.05. [b] Different capital letters indicate significant differences among fungal strains (on one hand) or among control treatments (on the other hand) within each seed condition and agronomic trait (wet weight aerial part or wet weight root system); LSD test at 0.05. * Seed condition: Undamaged (U.); ** Seed condition: Damaged (D.).

4. Discussion

Application of *Trichoderma* strains and their controls (=with distilled water) on beans provided different survival rates in the insects. Tb41 significantly reduced the survival of the insects until 20 days after the treatment's application, and it was the only treatment to obtain a survival rate of the insects of 0% at that time. On the other hand, Δ*tri23* was the treatment that provided the greatest survival rate of insects, so the cumulative survival rate after 15 days of the treatment was 71%. This longer life span of the insects allowed them to lengthen the reproductive period, which led to a greater weight loss of beans due to cotyledon damage caused by this insect's larvae (see below for a detailed explanation). Previous works have addressed modification of insect development by treatment of their host (seeds in this case) with fungi. Coppola et al. [52] reported that treatments with the fungal biocontrol agent *T. atroviride* strain in tomato plants induce responses in insect pests with different feeding habits: as for example the noctuid moth *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) and the aphid *Macrosiphum euphorbiae* Thomas (Hemiptera: Aphididae), assuring that the tomato plant–*Trichoderma* interaction has a negative impact on the development of moth larvae and on aphid longevity. Coppola et al. [52] confirmed that these effects were attributed to a plant response induced by *Trichoderma* that was associated with transcriptional changes of a wide array of defense-related genes. Many studies have described the potential of *Trichoderma* spp. as a natural control agent against some targeted insects, as for instance, its effect against the Egyptian cotton leafworm *Spodoptera littoralis* (Lepidoptera: Noctuidae), achieving a 20% of larvae survival rate [53]. *T. harzianum* provided pathogenicity against pest such as the beetle larvae *Tenebrio molitor* (Coleoptera: Tenebrionidae) [54] or *Xylosandrus crassiusculus* (Coleoptera: Curculionidae) [55]. In this experiment, Tb41 proved to be able of controlling larvae of *A. obtectus* insect pest since it was able to decrease the survival rate of the insect to 0%. This, this fungus can be considered a highly effective tool for the control of this insect pest in the larval stage.

Application of treatments on *P. vulgaris* beans modified the behavior of *A. obtectus* adults and their attacks on exposed beans. Δ*tri17* and Tb41 strains conferred effective protection against the insect pest, since these strains caused the beans to have lower weight loss (1.21 and 1.55%, respectively) than their respective controls, due to the low number of larvae that ingested their cotyledons, a fact that can be confirmed by observing the lower NEH of the insects in beans treated with these strains. On the other hand, the greatest weight loss was observed in beans sprayed with Δ*tri23*, which favoured a higher concentration of *A. obtectus* adults, and later the attack of their larvae once they hatched from the eggs laid on the beans. There are other references showing modification of insect development and behavior by treatment of their host's seeds with fungi. The attraction of insects towards their plant hosts when they are infected with fungi has already been described in previous studies and can be produced by the Volatile Organic Compounds (VOC's) emitted by them. Most VOC's in plants are products or by products of primary metabolic pathways [56,57]. *Sithobion avenue* (Hemiptera: Aphididae) was attracted by the VOC's produced by their plant host when infected with *Fusarium* strains that produced the trichothecene derivative nivalenol (NIV) [40]. On the opposite side, aphids were repelled by VOC's produced by their hosts when infected with *Fusarium* strains producing deoxynivalenol (DON), another trichothecene derivative [40,58]. The application of *T. citrinoviride* and *T. harzianum* strains on beans "canela variety" reduced the attack of *A. obtectus* larvae, obtaining less damaged beans and lower number of holes on damaged beans than not treated beans [15,20]. The studies carried out by Akello and Siroka [59] reported that the inoculation of fungal isolates (one of them, *T. asperellum* M2RT4) in bean seeds reduced the population of *Acyrthosiphon pisum* Harris (Homoptera: Aphididae) compared to population growth observed in control seeds. Menjivar-Barahona [60] described the reduction of whitefly population in tomatoes inoculated with *T. atroviride*. According to Rodriguez-González et al. [17], the treatment of vine wood trunks with different *Trichoderma* strains and *Beauveria bassiana* (GHA strain) reduced the population of *Xylotrechus arvicola* (Coleoptera: Cerambycidae) larvae bored into *Vitis vinifera* grapevine wood. Other genera of entomopathogenic fungi, *Metarhizium*

spp., *Beauveria* spp., *Isaria* spp., have also been shown to be highly effective for the control of other pests of seeds or stored products by applying these fungi on their hosts. For example, *Metarhizium anisopliae* (TR 106 strain) and *Beauveria bassiana* (TR 217 strain), against the adults of the cowpea weevil, *Callosobruchus maculatus* F. (Coleoptera: Chrysomelidae: Bruchinae) in laboratory obtained a very successful biological control of this insect pest in laboratory through the two isolated described [61]. *B. bassiana* (GHA strain) proved to have a high inhibition capacity in *A. obtectus* eggs [15]. Or the brown planthopper (*Nilaparvata lugens*), that is an insect pest of rice (*Oryza sativa*), in which granular formulation of *Isaria javanica* was able to control *N. lugens* populations in rice fields [62].

Damaged beans previously sprayed with Δ*tri17* and Tb41 had a higher germination percentage (100 and 66%, respectively) than those subjected to Δ*tri23* (62.50%) and their respective controls. Although damaged beans previously sprayed with Δ*tri23* had a lower germination percentage than the control and the beans treated with the rest of the fungal treatments, Δ*tri23* provided plants with a significantly higher wet weight aerial part (26.82 g) than Δ*tri17*. With regards to undamaged beans, healthy beans previously sprayed with Δ*tri23* treatment had a high germination percentage (75%) and plants grown from seeds subjected to this treatment had a wet weight aerial part significantly larger than the control and also higher than those from other *Trichoderma* strains. In relation to the root system, undamaged beans sprayed with Δ*tri23* provided plants with a higher WWRS than plants from beans treated with the rest of the *Trichoderma* strains. Regardless of the seed condition, the application of Δ*tri23* provided plants with a great wet weight aerial part, in spite of its poor percentage of germination in comparison to the control or to the rest of the strains applied, whereas high germination rates of Ta37 and Δ*tri17* (greater than their controls or than the rest of the assessed strains) did not lead to great wet weight aerial part or wet weight root system values in the plants obtained. The use of biological control agents is one of the alternatives for seed treatment to reach greater sustainability in agriculture [63,64]. *Trichoderma* strains are widely used in seed treatment, but little is known about the possible interactions between *Trichoderma* spp. and seeds in the early stages of germination [65], or the dosage to be applied. Dalzotto et al. [64] obtained a high germination percentage in *P. vulgaris* seeds treated with *Trichoderma*. Another example is the inoculation of tomato seeds with *T. harzianum* (Rifai T-22 strain) through a conidia solution of 2×10^7 colony forming units per gram of seeds, which had a positive effect on seed germination and growth in in vitro culture [65]. The improvement in the germination of seeds treated by *Trichoderma* strains may be due to the production of hormones from *Trichoderma* [64]. *T. harzianum* produces harzianic acid and isoharzianic acid, which promote plant growth [66]. On the opposite, an excessive production of indole acetic acid (IAA), ethylene [67], auxins and cytokinins hormones [68] inhibit cell division and elongation, impairing both germination and the development of seedling [66].

Linear regression coefficients of both the number of exit holes x wet weight aerial part and the number of exit holes x wet weight root system interactions were significantly different between Δ*tri23* or Tb41 strains and their respective control treatments. The number of exit holes of beans treated with Δ*tri23* or Tb41 was negatively correlated with both the wet weight aerial part and the wet weight root system in *P. vulgaris* plants.

The application of *Trichoderma* strains on bean seeds provided different wet weights in the plants grown for 45 days. Undamaged beans sprayed with Δ*tri23* provided plants not only with significantly greater wet weight aerial part and wet weight root system (35.03 and 4.92 g, respectively) than those grown from damaged beans sprayed with the same strain, but also greater than the plants from their respective control and higher than the plants grown from the seeds treated with the rest of the strains.

The studies performed by Chang et al. [69], Hermosa et al. [70] and Studholme et al. [71] described the beneficial effects of *Trichoderma* in horticultural crops such as: cucumber, periwinkle, chrysanthemum and lettuce, based on an improvement of their seed germination, vegetative growth and flowering. Furthermore, works made by Björkman et al. [72], Yedidia et al. [73], Björkman [74], Harman [75], Vargas et al. [76], Azarmi et al. [77] and

Pereira et al. [78] in crops such as cucumber, maize, bean and tomato, emphasized correlations between previous inoculation with *Trichoderma* spp. and increases of root growth or shoot biomass production (increases in weight, shoot length and leaf area). Azarmi et al. [77], explained that *T. harzianum* (T-969 isolate) and *Trichoderma* spp. directly applied to tomato seeds yielded plants with greater shoot height and diameter, and larger shoot fresh and dry weights. Application of *Trichoderma* inoculum at an early stage of crop growth maximizes its benefits in terms of root development and nutrient uptake [66].

5. Conclusions

Treatments of bean seeds with different *Trichoderma* strains provided different survival rates in *A. obtectus* adults, so life survival of insects after Tb41 strain application was reduced to 15 days. Δ*tri17* and Tb41 strains decrease weight losses of *P. vulgaris* beans (1.21 and 1.55%, respectively). Regardless of the seed condition, the application of Δ*tri23* promoted plants with a great wet weight aerial part, in spite of their poor percentage of germination in comparison to their control or the rest of the strains applied, whereas high germination rates of Ta37 and Δ*tri17* strains (greater than their controls or than the rest of the assessed strains) did not lead to great wet weight aerial part or wet weight root system values in the plants obtained. Linear regression between number of exit holes and wet weight aerial part on one hand, and between number of exit holes and wet weight root system on the other showed interaction, so Δ*tri23* and Tb41 behaved differently in comparison to their respective control treatments. The number of exit holes of beans treated with Δ*tri23* or with Tb41 was negatively correlated with both the wet weight aerial part and the wet weight root system in *P. vulgaris* plants. Undamaged beans sprayed with Δ*tri23* strain provided plants with the greatest wet weight aerial part and Wet Weight Root System. Due to the good results obtained by Δ*tri23* and Tb41 in this work, more studies for *A. obtectus* control, *P. vulgaris* plant growth and trichothecenes production by these strains should be explored, in order to advance the knowledge on how these fungi could be used in the field crop, together with the application of management strategies to mitigate risks for the farmers and to minimize the environmental contamination.

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