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Alleviation of Nematode-Mediated Apple Replant Disease by Pre-Cultivation of *Tagetes*

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Abstract: Apple replant disease (ARD) is a severe problem in orchards and tree nurseries caused by yet unknown soil biota that accumulate over replanting cycles. This study tested the contribution of nematodes to ARD, and cultivation of *Tagetes* as a control option. In a pot experiment, *Tagetes patula* or *Tagetes tenuifolia* were grown in ARD soil, incorporated or removed. Nematodes extracted from untreated ARD soil and washed on 20-µm sieves induced ARD symptoms when inoculated to apple plantlets growing in a sterile substrate. In contrast, nematodes from *Tagetes* treated ARD soil did not reduce root growth compared to uninoculated plants, irrespective of *Tagetes* species and incorporation. In plots of five apple tree nurseries or orchards, either *Tagetes* or grass was grown on ARD soil. Nematodes extracted from the grass plots and inoculated to apple plantlets significantly reduced plant growth compared to nematodes from *Tagetes* plots for all five farms. Apple rootstocks showed overall a significantly higher increase in shoot base diameter when grown on *Tagetes*-treated plots compared to grass plots, while this effect differed among farms. Plant-parasitic nematodes were too low in abundance to explain plant damage. In conclusion, *Tagetes* alleviated ARD by changing the nematode community in soil.

Keywords: *Tagetes*; marigold; apple replant disease; nematodes; pest control; soil biome management; *Malus*

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1. Introduction

Apple replant disease (ARD) has been recognized throughout pome fruit production regions of the world and has been studied extensively [1–4]. When establishing a new orchard on replant sites, trees commonly exhibit poor growth that shows in the significantly reduced shoot growth, necrosis, and patchy blackening of root cells, impaired root hair development, and low cell vitality, which may lead to root death [5]. As a result, fruit yield and quality are significantly reduced [6]. Although consensus regarding the causality of replant disease has not been fully realized, mitigating measures such as soil pasteurization or fumigation significantly improved the growth of apple plants, which gives evidence that the disease is caused by biotic factors [7]. Originally, replant disease, also known as soil fatigue, was described as the phenomenon that soil gradually loses its capacity to support growth of a specific plant after replanting without any obvious reasons, especially if plant damage cannot be attributed to known pathogens or plant-parasitic nematodes [8,9].

However, many different pathogens of apple plants have been occasionally found in ARD soil or in apple roots growing in ARD soil, including fungi, bacteria, oomycetes, and nematodes [10–17], while none of them was consistently associated with disease severity. Apple roots show a non-systemic, localized reaction where they are in direct contact with ARD soil, resulting in local brownish discoloration and decreased ^{15}N uptake [18]. Accumulation of phenolic compounds or phytotoxins in disease-affected parts of the roots has been discussed to play a role in the disease [16,19]. Recently, a transcriptomic analysis of the molecular responses of apple plants to ARD soils showed peculiar defense reactions to biotic stress, especially up-regulation of genes for phytoalexin synthesis [20,21].

Nematodes are abundant in soils and play an essential role in ecosystem functions and services [22]. Some of the nematodes, mainly the endoparasitic phytonematodes [23,24], are a major limiting biotic factor of productivity [25,26]. These nematodes migrate through the soil in search of a host plant, invade roots, and feed on the cytoplasm of cells. Etiological relations between some nematodes and microbes in soil-borne disease complexes are known [27,28]. *Pratylenchus penetrans* was reported to exacerbate apple replant disease (ARD), in addition to unknown biotic factors [29,30]. The peculiar role of free-living, putatively non-parasitic nematodes in the development of ARD was demonstrated in pot experiments with apple plantlets growing in sterile substrate [31]. The microbial fraction extracted from ARD soil hardly induced phytoalexins in the root, while in roots exposed to the washed nematode fraction (including body-associated microbes), phytoalexins increased by 1.7 log units. The combination of nematodes and microbes further increased phytoalexins by 3.7 log units [31]. This gave evidence that a nematode-microbe complex induced ARD, while *P. penetrans* and other plant-parasitic nematodes were hardly detected.

Effective management of nematode-mediated diseases is still challenging. Apple growers consistently rely upon the use of pre-plant soil fumigation [32], which has revealed a large site-to-site variation in efficiency and often only a short-term growth response [33]. The application of synthetic nematicides targeting nematodes poses health and environmental risks because of animal and human toxicity [34,35]. There is emerging social pressure to develop non-fumigant strategies. Crop rotation is often not applicable in fruit and nursery production systems. Anaerobic soil disinfection or biofumigation with plants or seed meal of Brassicaceae could mitigate ARD [36,37]. These methods are limited by high time requirements, costs, logistical challenges, and often by the environmentally undesirable use of plastic. Biological control options may be costly and of unpredictable efficacy [38,39]. A few *Malus* genotypes with tolerance to ARD have been described but further breeding is necessary to get tolerant rootstocks with growth advantage in different ARD soils [40].

A potential alternative management option of nematodes in ARD is the pre-plant application of *Tagetes*, which has demonstrated the capacity to provide effective and long-term suppression of nematode pests in various other agricultural systems. *Tagetes* species (often referred to as marigold) are cultivated all over the world for ornamental purposes and for industrial use. They produce several potentially bioactive compounds among which alpha-terthienyl is acknowledged as one of the most toxic chemicals present in the marigold tissues and roots [41]. They exhibit insecticidal, nematicidal, fungicidal, antiviral, and cytotoxic activities [41,42], and were reported to be suppressive to several soil-borne plant pathogenic fungi such as *Rhizoctonia solani* and *Fusarium solani* [43,44]. Recently, pre-cropping with *Tagetes* revealed increased growth of apple in two ARD soils by 175% or 52%, respectively [37,45]. The effect of *Tagetes* on the nematodes related to ARD and its consequences on apple plant growth has not been explored.

The aim of this study was to elucidate the role of nematodes in ARD, and to investigate whether *Tagetes* pre-culture alleviates ARD by changing the nematode community in soil. The efficacy of *Tagetes* pre-culture to improve apple plant growth in ARD soils was evaluated in field trials at a diverse selection of tree nurseries and apple growing farms, and the effect on plant-parasitic and non-parasitic nematodes was investigated. In biotests, the effect of nematodes extracted from *Tagetes* treated and untreated ARD soils on growth

and ARD symptoms of susceptible apple plantlets was investigated. Furthermore, we tested the efficiency of different *Tagetes* species and soil incorporation on ARD in pot tests.

2. Materials and Methods

2.1. Pot Experiment on the Effect of Tagetes with or without Incorporation into Soil on Nematode-Mediated ARD

In a pot experiment, we investigated whether nematode communities recovered from ARD soil affect apple plant growth and whether pre-culture of *Tagetes* in ARD soil reduces the negative effect of the nematode community on apple plant growth. Since different *Tagetes* species produce different biocidal compounds, which may be exuded or not from the root, we tested whether *T. patula* and *T. tenuifolia* differ in their effect on nematode-mediated ARD, and whether incorporation of *Tagetes* into the soil is necessary for mitigation of nematode-mediated ARD. The soil was obtained from a field in the Pinneberg area near Heidgraben, Germany (53°41′57.1″ N 9°40′59.4″ E). Since 2009, the rootstock of the cultivar 'Bittenfelder Sämling' was planted repeatedly in two-years cycles [46]. The soil was sampled around the roots of plants at a depth of 0–30 cm and sieved through a 5-mm mesh. Samples were stored at 4 °C for 1 week before the pot experiment. Seeds of *T. patula* 'Single Gold' or *T. tenuifolia* (Saatzucht Bardowick, Bardowick, Germany) were nursed separately in sterile sand. Six two-week seedlings were transferred into each pot filled with 1 L of the ARD soil. Plants were grown for 8 weeks until flowering. To investigate the effect of plant incorporation into soil, *Tagetes* plants were removed from 20 pots, chopped into 0.5-cm pieces, and 50 g per pot was mixed with the soil. *Tagetes* in the rest of the 20 pots were removed and not incorporated into the soil. Control pots were not treated with *Tagetes*, and kept bare. Four weeks after incorporation, nematode communities (plant-parasitic and free-living) were extracted from each pot by centrifugal floatation using $MgSO_4$ at 1.18 specific density [47]. Briefly, 250 mL soil were suspended with a tablespoon of kaolin in 400 mL water in 1 L centrifuge tubes and pelleted at 1800 g for 4 min. The soil was re-suspended in 400 mL of the $MgSO_4$ solution, centrifuged at 1800 g for 4 min. Nematodes from the supernatant were collected on 20-µm sieves, washed with sterile water, and transferred to 10 mL sterile water. Nematodes were inoculated to in vitro propagated and acclimatized, 5-weeks old M26 apple plantlets growing in 500 mL sterile sand, by equally distributing the suspension into four 5-cm deep 1-cm wide holes in 2 cm distance around the shoot. Pots were placed in a randomized complete block design in the greenhouse. The fertilizers, Hakaphos NPK (+Mg) (15:10:15(+2)) (Compo, Münster, Germany) (0.5 g per pot) and 36% calcium carbonate (Vereinigte Kreidewerke, Söhlde, Germany) (2 g per pot) were applied weekly. Plants were watered every 2–3 days as required. The greenhouse conditions were 22 ± 2.5 °C, 60 ± 8.7% relative humidity, and a 16 h photoperiod. Pots were sampled eight weeks after inoculation to determine shoot length, shoot fresh mass, leaf fresh mass, and root fresh mass. Overall, the plant growth assay comprised 20 replicates of each treatment, which were: inoculation with nematodes from ARD soil that was either treated with *T. patula*, *T. tenuifolia*, *T. patula* with incorporation, *T. tenuifolia* with incorporation, or untreated bare ARD soil. Additional controls were not inoculated with nematodes. Those plants were grown either in sterile substrate, or directly in the untreated ARD soil.

2.2. Effect of Tagetes in Mitigating ARD in Apple Growing Farms and Tree Nurseries

Two apple-growing farms with either organic (farm M) or conventional (farm J) practice, and three tree nurseries either in Northrhein-Westfalia (farm L) or Schleswig-Holstein (farms S, C) were selected where apple plants were repeatedly replanted and problems with ARD were reported (Table 1). The soils differed in texture, pH as well as N and C contents (Table 2). In in-field trials, plots were either kept under grass to maintain the status of ARD, while avoiding soil erosion and weed growth, or cultured with *T. patula* 'Nemamix' (farms S, C, M, J) or *T. erecta* (farm L) for the vegetation period of 2019. Plants were chopped and incorporated by mulching into the soil before planting of apple (Table 1).

The effect of the *Tagetes* pre-culture on apple plant growth was determined by measuring the increase in shoot diameter during the vegetation period of 2020. In the tree nurseries, the diameter of 40 rootstock plants of the middle rows of each plot was taken at the base (above the soil) shortly after planting and in November before uprooting. In the apple orchards, trees (excluding border plants) were marked at 20 cm above the grafting, and diameter was measured twice (in orthogonal direction) each in May and November 2020.

Table 1. Experimental details of field trials in companies.

Farm	*Tagetes* Treatment (2019)			Apple Cultivation (2020)		
	Species	Sowing	Incorporation	Apple Plants	Measured Plants Per Treatment	Number of Blocks
L, tree nursery	*T. erecta*	Jun	Sep	Rootstock A2	160	4
S, tree nursery	*T. patula* 'Nemamix'	May	Apr (2020)	Rootstock M9	160	4
C, tree nursery	*T. patula* 'Nemamix'	May	Nov	Rootstock 'Bittenfelder'	235	6
M, organic orchard	*T. patula* 'Nemamix'	May	Nov	'Red Prince' on M9	28	1
J, orchard	*T. patula* 'Nemamix'	May	Nov	'Sweet Tango' on M9	24	2

Table 2. Location and soil properties of experimental sites.

Farm	Location	Soil Type	Clay [%]	Silt [%]	Sand [%]	pH ($CaCl_2$)	C [%]	N [%]
L	Northrhine-Westfalia 51.83254, 7.42113	Braunerde-Podzol	7.4	16.0	76.6	5.9	2.95	0.18
S	Schleswig Holstein 53.67823, 9.73118	Podzol-Parabraunerde	5.9	41.9	52.2	4.7	1.59	0.11
C	Schleswig Holstein 53.63304, 9.70630	Podzol	5.1	5.5	89.4	5.3	2.45	0.14
M	Lower Saxony 53.50748, 9.68593	Kleimarsch	30.5	64.0	5.5	5.9	3.37	0.32
J	Lower Saxony 53.47639, 9.59167	Pseudogley-Braunerde	7.2	32.6	60.2	5.5	2.00	0.16

To determine the contribution of nematodes to ARD, soil samples from all plots were collected in November 2020 at a depth of 0–20 cm with 6–8 sampling points between apple plants. The soil samples were stored at 4°C before nematode communities were extracted. To determine the number of nematodes of plant-parasitic genera and non-parasitic nematodes, 250 mL soil aliquots were extracted using an Oostenbrink elutriator [47]. Nematodes were collected on three mounted 45-µm sieves, washed into a beaker, and transferred onto an Oostenbrink dish to get a clean sample. After 48 h, the nematodes in the Oostenbrink dish were collected on a 20-µm sieve and transferred to a glass cylinder. After sedimentation for 2 h, the supernatant was syphoned off to leave 10 mL suspension, of which 1 mL was used for counting of nematodes on a counting slide under an Olympus SZX12 stereomicroscope at 40×–80× magnification (Olympus, Hamburg, Germany).

2.3. Biotest on ARD-Induction by Nematodes from Apple Plots with Preceding Tagetes or Grass Cultivation

For the biotest, nematode communities were extracted from 1 L soil samples from apple plots with preceding *Tagetes* cultivation or control plots with preceding grass cover. The extraction was done by centrifugal floatation in $MgSO_4$ at 1.18 specific density [47], as described above. Nematodes were collected on 20-µm sieves, washed with sterile water,

and transferred to glass cylinders. The nematodes were suspended in 10 mL sterile water and inoculated to apple plantlets grown in sterile sand and incubated in the greenhouse as described above. Shoot fresh mass, leaf fresh mass, root fresh mass, and shoot length were determined eight weeks after inoculation. Overall, the biotest comprised 10 replicates for both the *Tagetes* treated ARD soils and the grass treated ARD soils for each farm. Plants growing in uninoculated sterile sand served as control.

2.4. Statistical Analysis

Statistical analyses were done using the GLIMMIX procedure of the software package SAS 9.4 (SAS Institute Inc., Cary, NC, United States of America). Plant growth parameters were analyzed with the assumption of a normal distribution without data transformation, which was checked by Q–Q and residual plots. For multiple comparisons, the *p*-value was adjusted by the method of Tukey (ADJUST = TUKEY in the LSMEANS statement). To account for overdispersion, degrees of freedom were approximated by the method of Kenward-Roger (DDFM = KENWARDROGER). To analyze the effect of *Tagetes* (TAGETES = 1 or 0) on shoot base increase (SBI) in plots of five farms, the generalized linear mixed model SBI = TAGETES FARM was used, with PLOT as a random effect. The effect for each farm was estimated by using contrasts (LSMESTIMATE statement). The effect of preceding *Tagetes* cultivation on the proportion of plant-parasitic nematodes (PPN) was tested in a generalized linear mixed model PPN/TOTAL = TAGETES with FARM as random effect, binomial distribution and Logit transformation, using GLIMMIX. An effect was regarded as significant at the type III error $p \leq 0.05$.

3. Results

3.1. Effect of T. patula or T. tenuifolia, with or without Incorporation into Soil, on Nematode-Mediated ARD (Pot Experiment)

Washed nematode fractions from differently treated ARD soil (Heidgraben field), with or without *Tagetes* pre-culture and with or without soil incorporation of *Tagetes*, were tested for their effect on the growth of roots and shoots of apple plants. The plants that received this nematode inoculum from untreated ARD soil, or that were directly planted in ARD soil discolored roots. Overall, nematodes extracted from the different treatments significantly differed in their effect on the determined parameters of plant growth (MANOVA, $p < 0.0001$). Nematodes extracted from untreated ARD soil significantly reduced root fresh mass compared to nematodes from all *Tagetes* treatments of ARD soil (Table 3). Roots grown in ARD soil or grown in substrate with nematodes from ARD soil did not significantly differ in fresh mass, but were 44–68% smaller than in the control without nematodes. In contrast, apple roots inoculated with nematodes from the *Tagetes* treatments did not significantly differ from the control without nematodes. Incorporation of *Tagetes* was not a significant factor ($p = 0.84$), while root fresh mass had a 'trend' to be increased in treatments with *T. patula* compared to *T. tenuifolia* ($p = 0.0547$), as revealed by the respective contrasts in ANOVA.

Table 3. Effect of preceding *Tagetes patula* or *T. tenuifolia* cultivation in apple replant disease (ARD) soil, and incorporation into soil after growth, on nematode communities with respect to their inhibitory effect on the growth of apple M26 plantlets.

Source of Nematodes Inoculated to Apple Plantlets	Plant Growth Parameter [1]			
	Shoot Length (cm)	Shoot Fresh Mass (g)	Leaf Fresh Mass (g)	Root Fresh Mass (g)
Untreated ARD soil	13.7 ± 3.8c	3.7 ± 1.4c	2.3 ± 0.9c	1.9 ± 0.6b
T. patula treated ARD soil	18.4 ± 1.4ab	4.9 ± 1.2a	3.0 ± 0.9ab	3.7 ± 0.6a
T. patula treated ARD soil (with incorporation)	18.4 ± 3.5ab	4.9 ± 0.9ab	3.0 ± 0.6ab	3.8 ± 0.8a
T. tenuifolia treated ARD soil	11.9 ± 5.0c	3.5 ± 0.9c	2.0 ± 0.6c	3.4 ± 1.1a
T. tenuifolia treated ARD soil (with incorporation)	14.9 ± 3.3bc	3.9 ± 1.3bc	2.4 ± 0.8bc	3.4 ± 1.1a
Control without nematodes	21.2 ± 2.2a	5.3 ± 0.5a	3.3 ± 0.5a	3.4 ± 1.0a
M26 directly grown in ARD soil	5.8 ± 5.3d	1.6 ± 0.3d	0.9 ± 0.2d	1.1 ± 0.6b

[1] Different letters indicate significant differences (ANOVA, Tukey's adjustment, $n = 20$, $p \leq 0.05$).

Shoot parameters were significantly reduced by nematodes from ARD soil compared to the control without nematodes (Table 3). Treatments with *T. patula* alleviated this effect, whether incorporated into soil or not. However, nematodes from ARD soil that was treated with *T. tenuifolia* did not significantly differ in their effect on shoot parameters compared to nematodes from untreated ARD soil. Incorporation of *Tagetes* was not a significant factor, as revealed by the respective contrasts in ANOVA ($p > 0.08$). Only *T. tenuifolia*, which produced more shoot biomass than *T. patula*, showed a trend for increased shoot growth in the biotest when treatments with and without incorporation are compared. Shoot parameters were most affected by direct growth in ARD soil compared to growth in substrate that was inoculated with nematodes.

3.2. Effect of Preceding Tagetes Cultivation on Apple Shoot Growth in Apple Orchards and Tree Nurseries

In three apple orchards and two tree nurseries, the increase in apple trunk diameter over a season was compared among plots that have been pre-cultivated either with *Tagetes* or with grass before planting apple rootstocks. In general, apple plants cultivated in *Tagetes* pre-cultured ARD soils grew significantly better than those in the untreated grass plots (ANOVA, $p = 0.0004$; Figure 1). The trunk diameter at the base increased in grass plots by 2.3 mm and in *Tagetes* plots by 3.0 mm, on average. However, growth significantly differed among farms ($p = 0.0001$), and the effect of *Tagetes* was dependent on the farm (interaction effect FARM × TAGETES, $p = 0.0001$). The *Tagetes* effect was strongly evident in farms S and C, and less pronounced in farms J and L (Figure 1). Moreover, blocks of farm C where hollows cause temporary water logging showed a pronounced positive *Tagetes* effect on apple trunk growth. In farm M, conditions during this season did not allow for increase of the trunk diameter on average; thus, no effect of *Tagetes* was realized.

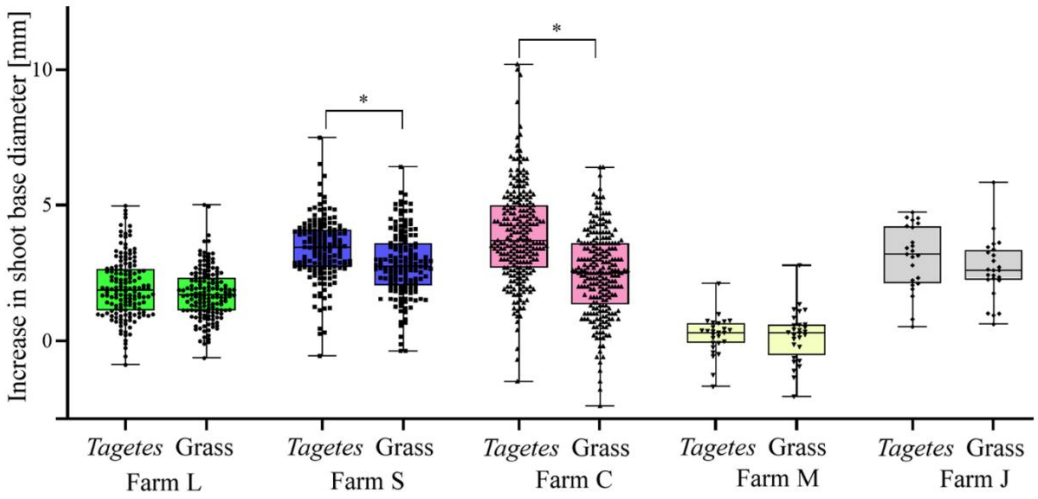

Figure 1. Increase in shoot base diameter of apple plants grown in ARD soils that were pre-cultured with *Tagetes* or grass. Crosses indicate means; boxes indicate medians and quartiles; stars indicate significant treatment effects as revealed by the respective contrasts in a generalized linear mixed model ($p < 0.05$).

3.3. Effect of Preceding Tagetes Cultivation on Plant-Parasitic Genera and Total Nematodes in Apple Orchards and Tree Nurseries

The numbers of plant-parasitic nematodes in the apple orchards and tree nurseries were generally low, with 77 individuals per 100 mL soil on average (Table 4). The genus *Pratylenchus* that was previously linked to ARD was only detected in low numbers in two tree nurseries, farms S and C. The genera *Rotylenchus*, *Meloidogyne*, or *Trichodorus* were each detected in two of the farms. *Paratylenchus* was the most abundant plant parasite and detected in three farms. None of the genera was detected in all ARD soils. In apple plots with preceding *Tagetes* cultivation, the proportion of plant-parasitic nematodes was

significantly lower than in the ARD plots ($p = 0.0002$). Farm L was characterized by the highest number of non-parasitic nematodes, while only four *Paratylenchus* were found. A 16 % increase of the non-parasitic nematodes was achieved under pre-cultivation with *Tagetes erecta*, while *Paratylenchus* became undetectable. In farm S, the plant-parasitic nematodes were reduced by 97% and non-parasitic nematodes were reduced by 11% under pre-cultivation with *T. patula*. In farm M, the non-parasitic nematodes achieved a 55% increase under pre-cultivation with *Tagetes* and *Meloidogyne* was reduced by 64%. In farm J, the pre-cultivation with *Tagetes* increased the abundance of non-parasitic nematodes by 43% and the genus *Paratylenchus* by 36%. In soil of farm C, the non-parasitic nematodes were increased by 331% under the pre-cultivation with *Tagetes* while *Rotylenchus* was reduced by 42%.

Table 4. Numbers of plant-parasitic and non-parasitic nematodes per 100 mL soil in tree nurseries or apple-growing farms in adjacent plots that were cultivated with *Tagetes* (*Tag*) or grass (G) before replanting of apple rootstocks.

	Tree Nursery L		**Tree Nursery S**		**Tree Nursery C**		**Apple Orchard M**		**Apple Orchard J**	
Genus/Type	**G**	***Tag***	**G**	***Tag***	**G**	***Tag***	**G**	***Tag***	**G**	***Tag***
Rotylenchus			4		112	80				
Meloidogyne			8				44	16		
Paratylenchus	4		80						128	200
Pratylenchus			8		20					
Trichodorus			56	4	4					
Non-parasitic	2744	3176	1656	1472	256	1104	384	594	1024	1464

3.4. Biotest with Inoculation of Nematodes from Apple Plots with Preceding Tagetes or Grass Cultivation

Nematodes from plots that were not treated by *Tagetes* before replanting of apple rootstocks caused browning and size reduction of roots of inoculated apple plantlets, which is typical for ARD (Figure 2). A two-factor ANOVA revealed significantly higher root fresh mass of plants that were inoculated with nematodes from *Tagetes*-treated ARD soils compared to grass-treated ARD soils ($p = 0.0001$). The factor FARM also had a significant effect on root fresh mass ($p = 0.0001$). The interaction of FARM and TREATMENT was not significant ($p = 0.14$). Contrasts revealed for each farm a significant *Tagetes* effect on root fresh mass in the biotest ($p < 0.007$), while the difference between treatments was most pronounced for farm L, followed by farms S and M (Figure 3).

Shoot fresh mass and shoot length were significantly larger when plants were inoculated with nematodes from the *Tagetes* treated ARD plots compared to inoculation of nematodes from the grass treated ARD plots ($p = 0.0001$, Table 5). The factor FARM had a significant effect on shoot fresh mass ($p = 0.0089$), but not on shoot length ($p = 0.33$). The respective interactions of FARM and TREATMENT were not significant. Contrasts revealed for each farm a significant *Tagetes* effect on shoot fresh mass ($p < 0.006$), while the difference between treatments was most pronounced for farm L, followed by farm S (Table 5).

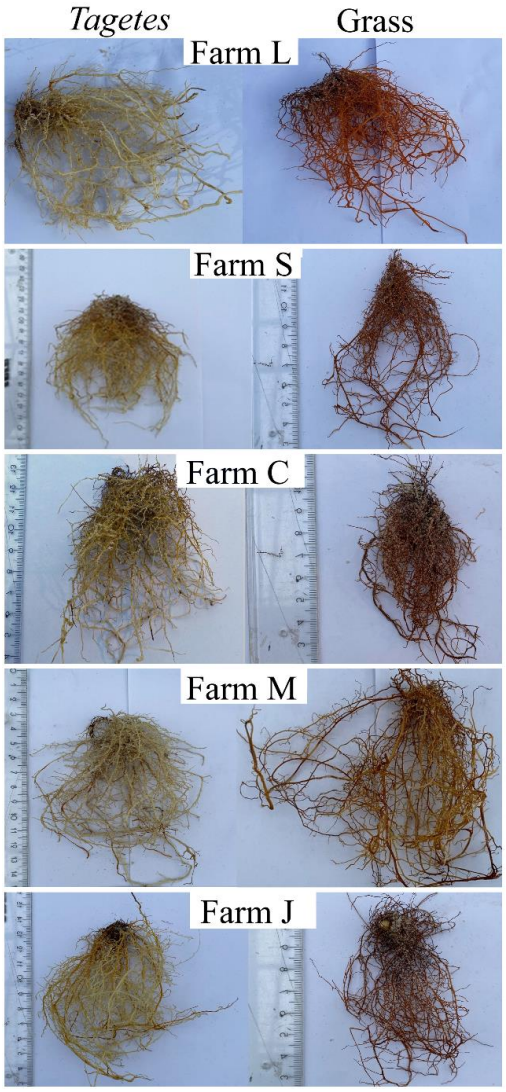

Figure 2. Discoloration and stunting of apple roots in the biotest of nematode fractions extracted from soils of plots in tree nurseries (farms L, S, C) and apple orchards (farms M and J), which were pre-cultured with either *Tagetes* or grass before planting of apple rootstocks.

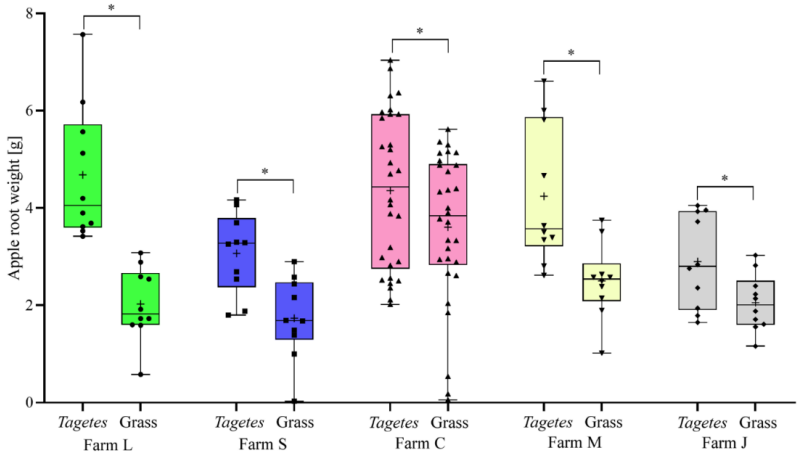

Figure 3. Root fresh mass of apple plantlets in response to inocula of nematodes recovered from ARD soils that were pre-cultured with *Tagetes* or grass before replanting apple. Crosses indicate means; boxes indicate medians and quartiles; stars indicate significant treatment effects as revealed by the respective contrasts in a generalized linear mixed model.

Table 5. Vegetative growth of apple M26 plants in pots inoculated with nematodes extracted from ARD soils pre-cultured with *Tagetes* or grass.

Farm	Treatment	Plant Growth Parameter [1]		
		Shoot Length (cm)	Shoot Fresh Mass (g)	Root Fresh Mass (g)
L	*Tagetes*	6.2 ± 1.1 a	3.5 ± 2.9 a	4.9 ± 1.4 a
	Grass	5.1 ± 0.8 b	1.8 ± 0.5 b	2.0 ± 0.8 b
S	*Tagetes*	7.1 ± 1.1 a	3.0 ± 0.6 a	3.1 ± 0.8 a
	Grass	4.7 ± 1.3 b	1.6 ± 0.8 b	1.7 ± 0.8 b
C	*Tagetes*	6.5 ± 1.4 a	2.3 ± 0.7 a	4.3 ± 1.5 a
	Grass	4.9 ± 1.1 b	1.5 ± 0.6 b	3.6 ± 1.5 b
M	*Tagetes*	7.3 ± 0.8 a	3.1 ± 0.3 a	4.2 ± 1.4 a
	Grass	5.3 ± 0.8 b	2.1 ± 0.3 b	2.5 ± 0.8 b
J	*Tagetes*	6.6 ± 0.7 a	2.3 ± 1.0 a	2.9 ± 1.0 a
	Grass	5.3 ± 0.9 b	1.3 ± 0.6 b	2.1 ± 0.6 b

[1] Mean ± SD (n = 10). Different letters indicate significant differences between *Tagetes* and grass-treated ARD plots revealed by ANOVA for each farm separately.

4. Discussion

In this study, the efficacy of *Tagetes* pre-culture to improve apple plant growth in ARD soils has been shown in field trials of a diverse selection of orchards and tree nurseries. All studied farms had major problems with ARD, especially the tree nurseries that typically replant apple rootstocks every year or every second year, depending on their specialization on rootstock production or grafting, respectively. The seasonal increase in shoot base diameter on *Tagetes* plots was on average 30% higher than on adjacent control plots where grass was grown instead (Figure 1). This effect was pronounced for the tree nurseries, while the apple-growing farm J showed the same trend. The other apple-growing farm experienced unfavorable conditions during the season, so that the stems did not show detectable increase in diameter. Therefore, an effect of preceding *Tagetes* cultivation on stem growth could not be detected there. However, when nematodes were extracted from these plots and inoculated to susceptible M26 apple plantlets, the effect of the preceding *Tagetes* cultivation on how the nematodes affected apple roots became very clear for all farms (Figure 3). In contrast to the nematode fraction from *Tagetes* plots, the nematodes from ARD plots with preceding grass cultivation caused the discolored and stunted roots that are typical for ARD (Figure 2). This confirms our previous finding that the nematode fraction contained one main driver of ARD [31]. As in this study, the nematode fraction was obtained from soil by floatation on a dense $MgSO_4$ solution, and collection and washing on a 20-µm sieve. The nematode fraction thus contained the microbes that were associated with the nematode bodies. These microbes were shown to synergistically enhance ARD symptoms together with the nematode fraction, but they had hardly any effect when inoculated to apple plantlets alone [31]. While floatation is a standard technique to retrieve nematodes from soil [48], and the fraction on the 20-µm sieve mainly contains nematodes when microscopically analyzed, it might contain other small organisms and microbes associated with small organic particles, which may play a role in ARD. This needs to be ruled out in future studies. Neither in the previous nor in the present study played plant-parasitic nematodes a significant role in ARD. They were only detected in low numbers in the ARD soils. None of the genera was detected in all farms and could be associated with ARD. The genus *Pratylenchus* was only detected in two tree nurseries. It has been frequently reported in association with ARD [14,29,49–51], with a damage thresholds of 50 *Pratylenchus* per 100 mL soil [52]. However, in other studies the reduction of *Pratylenchus* by nematicides did not improve tree growth in ARD affected orchards [32], or was hardly correlated with the gain in plant growth (R^2 = 0.186) [53], or ARD symptoms were observed despite reduction of *Pratylenchus* by soil treatment [30,52]. In an orchard in Washington, a heavy ARD infestation was treated by either fumigation or *Brassica* seed meal [54]. While *Pratylenchus* reached high densities in apple roots in the second year after fumigation but not in the Brassica seed meal treatment, this difference was not reflected by trunk

increase or apple yield. Plant-parasitic nematodes thus can eventually increase damage of ARD-affected roots but did not have a relevant contribution to ARD at least in our study. Nevertheless, the *Tagetes* treatment in our study further reduced the relative abundance of phytoparasites within the nematode community, which is an added benefit. The count data showed that *Tagetes* changed the structure of the nematode community, while the total number of nematodes in soil tended to increase (Table 4). This means, that Tagetes did not generally affect all nematode species in soil, but presumably those that are mostly living close to roots.

In the pot experiment, both *T. patula* and *T. tenuifolia* significantly reduced plant damage by the nematode fraction from the treated ARD soil that was inoculated to apple plantlets. For the *T. patula* treatment, nematode-mediated ARD was abolished, as shoot and root growth did not significantly differ from the uninoculated control. The *T. tenuifolia* treatments were not equally efficient. Shoot growth was less improved compared to the *T. patula* treatments, and roots also showed this trend. The farms applied mainly *T. patula* to reduce ARD, but farm L used *T. erecta* instead. Notably, farm L showed the most pronounced reduction of nematode-mediated ARD among the farms in the biotest (Figure 3). In contrast, the effect of *T. erecta* pre-culture on stem growth in the field was not significant for this tree nursery, while the other two nurseries significantly improved stem growth after *T. patula* pre-culture. This coincided with the overall lower plant growth in farm L compared to the other two tree nurseries. It should be considered that the rootstock A2 cultivated by farm L may be more susceptible to ARD than M9. In farm M, lack of trunk growth in the field completely prevented the detection of a *Tagetes* effect, while the biotest clearly revealed a significant treatment effect. The farms differed in the type of cultured rootstock, soil type, soil texture, and other parameters, so that the differential effect of the *Tagetes* species might be better reflected by the growth of apple plants in the more controlled biotest. *Tagetes patula* was reported to be attractive for root invasion by *P. penetrans* and other endoparasites, but the nematodes cannot multiply in the roots [55]. Cultivation of *Tagetes* as pre-culture reduced populations of root-knot and lesion nematodes and substantially increased yield in the subsequent crop (melon, tomato, or potato) [56,57]. This led to the view that *Tagetes* acts as a trap crop. However, metabolites released from the roots of mature *Tagetes* plants act against diverse herbivorous and non-herbivorous nematodes, especially thiophene compounds like α-terthienyl [58–60]. A study on the amount of nematicidal thiophenes in roots of *Tagetes* species revealed the highest concentration in *T. tenuifolia*, followed by *T. patula* and *T. erecta* [59]. Interestingly, when comparing the amount of thiophenes produced by the roots per area of cultivated *Tagetes*, then probably *T. minuta* has the highest effect on nematodes, followed by *T. patula*, *T. erecta*, and *T. tenuifolia* (320/39/14/12 mg m^{-2}, respectively). This coincides with the slightly better performance of *T. patula* compared to *T. tenuifolia* in our pot experiment.

At the farms, *Tagetes* was incorporated into soil either in autumn or in spring. It is unclear whether soil incorporation of *Tagetes* contributes to the effect on ARD. In the pot experiment, incorporation of *T. patula* had no effect on growth of apple plantlets in the subsequent biotest with the inoculated nematode fraction. The treatment with *T. tenuifolia* showed a trend for increased shoot growth when the soil was incubated with the chopped and incorporated plants compared to the treatment without incorporation. This might be explained by a green manure effect on nematodes, rather than an introduction of nematicidal compounds from the shoot, because actively growing roots of *Tagetes* act most effective on nematodes, while root extracts or other parts of the plant are less efficient [61,62].

Tagetes cultivation is already applied in tree nurseries to control *P. penetrans*. However, farmers are not yet aware that it is also a management option against ARD. *Tagetes* cultivation is less expensive compared to physical soil disinfection, and less damaging to the environment compared to chemical soil fumigation. However, it needs to be investigated for how long *Tagetes* cultivation suppresses the ARD causing biota, and to what extent production losses during *Tagetes* cultivation is outweighed by the gain in soil health.

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