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Impact of Antioxidants and Micronutrients as Fungicides Alternatives in Improvment the Antagonism of *Trichoderma* spp. Against *Sclerotinia sclerotiorum*

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Running title: Improvement of *Trichoderma* spp. Antagonism against *Sclerotinia sclerotiorum*
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ABSTRACT

S. sclerotiorum is a strong and resting soil pathogen with high resistance to synthetic fungicides. The biocontrol agent *Trichoderma* spp. alone provides moderate control of this fungus. In present study, Potassium tartrate, ascorbic acid, folic acid, thiamine and micronutrients mixture were used to improve the number of spores, fresh and dry weights, crude extracts, volatile compounds, and secondary metabolites produced by four isolates of *Trichoderma* against pathogen. A onetime amendment of thiamine (0.1%) or micronutrients mixture improves the antagonism efficacy of the most *Trichoderma* isolates showing complete prevention of pathogen growth and inhibition of sclerotia formation.
Keywords: sclerotia formation, thiamine, *Trichoderma* species, secondary metabolites

INTRODUCTION

S. sclerotiorum is capable of causing significant yield losses in numerous crops in Egypt and worldwide. It is capable of infecting flowers, leaves, fruits or stems (Attanayake *et al.,* 2013) with wide host range of over 400 plant species worldwide, many of them are economically important crops (Garg *et al.,* 2010). The fungus differentiates and propagates by forming sclerotia which are crucial in its survival for long periods of unfavorable conditions (Bolton *et al*., 2006). Chemical management is largely unreliable as fungicide-resistant strains of the pathogen may develop (Li *et al.,* 2008). All these reasons are driving the researchers from all over the world to develop alternative methods which are, by definition, safe in the environment and are rapidly biodegradable, one such strategy is use of biocontrol agents, but for example, biological control of soil-borne diseases of bean provides a moderate level of disease suppression in both greenhouse and field (Duffy *et al.,* 1997). For improving the efficiency of bioagents, several reports assessing the addition of some chemical compounds. In this connection, Alejandro and Blanka (2011) evaluated the effect of KHCO3 in the growth and development of *S. cepivorum* and on its interaction with *Trichoderma* strain R39. Charles *et al.* (1997) studied the biocontrol efficacy of *T. virens* in combination with fungicides against cotton seedling disease pathogens.

Humic, folic acids with furfural and *T. harzianum* as soil drench resulted in highly reduction in the incidence of root rot of cantaloupe grown under plastic house conditions comparing with fungicides treatment (El–Mougy *et al.,* 2014). Sodium bicarbonate and micronutrients mixture formula showed synergistic effect with *T. harzianum* against white mold disease (Yousef *et al.,* 2015). Addition of micronutrients particularly borax increased the biocontrol activity of *T. viride* against *F. oxysporum* f. sp *cubense* (Sanjeev 2008). In our previous work Yousef *et al.* (2013) noticed the direct effect of some micronutrients mixtures and antioxidants which showed good results in inhibiting the growth and sclerotia formation of *Rhizoctonia solani*. In this article, improving the growth and antagonicity of four *Trichoderma* spp. vs *S. sclerotiorum* growth and sclerotia formation by simple addition of antioxidants and micronutrients are discussed.

MATERIALS AND METHODS

Fungal isolates

Isolates of *T. harzianum* (T1, T4 &T8) and *T. koningii* (T6) were isolated from the rhizosphere of healthy bean plants at Ismailia governorate, Egypt. They were kindly identified by Mycological Research and Plant Disease Survey Department, Plant Pathology Research Institute, Agricultural Research Center, Giza, Cairo, Egypt according to Park *et al.* (2005). *Sclerotinia sclerotiorum* the causal organism of bean white mold was isolated from naturally infected bean plant collected from bean fields cultivated in Ismailia governorate, Egypt. The isolated fungus was identified based on cultural and microscopic morphological characters (Hanlin and Richard, 1998).

Effect of antioxidants and micronutrients on growth and sporulation of *Trichoderma* spp.

The mycelial growth of *Trichcoderma* isolates was studied in gliotoxin medium (GM, g/l) 2, ammonium tartrate; 2, dipotassium hydrogen orthophosphate; 25, glucose; 0.001, ferrous sulfate and 20, agar dissolved in one liter distilled water. Tested materials were potassium tartrate (A) 300 ppm, ascorbic acid (A.A) 100 ppm, folic acid (FA) 20 ppm, mixture of micronutrients (MN) 150 ppm which consists of (Zn sulfate 500 ppm, Mn sulfate 500 ppm, Cu sulfate 100 ppm, Boric acid 100 ppm and Selenium 1.0 ppm), thiamine (TH) 100 ppm and combination between (A+MN) was added to sterilized GM at the rate of 450 ppm. The prepared media were poured in Petri dishes. The mycelial disc of each *Trichoderma* isolates was placed in the center of the Petri plate and incubated at 23°C. The growth diameter for each *Trichoderma* spp. was measured. To determine the number of spores, for each isolate, the fungal growth on GM medium was filtered and the filtrate of each treatment was used to prepare serial dilutions up to 10-6 dilution, poured in

plate and incubated at 23°C. Growing colonies were counted daily and expressed as colony forming units (CFU). Three replicates for each treatment were used.

Effect of different antioxidants and micronutrients on the antagonistic potential of different *Trichoderma* spp. against *S. sclerotiorum*

The antagonistic activity was studied by using the dual culture technique (Cherif and Benhamou, 1990) in Petri dishes with PDA and placing equidistantly a disc (5mm in diameter) with mycelium of sclerotinia and on the other side of the Petri dish, a disk of the desired treated *Trichoderma* isolates was also placed. The control was a disk from the untreated *Trichoderma*. The plates were incubated at 23°C and the mean colony diameter of the pathogen was measured. The percent of inhibition growth was calculated. The degree of antagonism between each *Trichoderma* isolates and the pathogen was scored on scale of 1-5 as proposed by (Bell *et al.* 1982).

Effect of fungicides alternative on the activity of *Trichoderma* spp. crude extracts against *S. sclerotiorum*

S. sclerotiorum growth inhibition by substances secreted by *Trichoderma* spp. in liquid medium was determined as follow: In 250 ml Erlenmeyer flasks, 50 ml of GM was amended with tested compounds as mentioned before and each was inoculated with two discs of active mycelium of each *Trichoderma* isolates and incubated at 23°C for 14 days. The supernatant was filtered, centrifuged at 10,000 rpm for 20 min. and sterilized by millipore membrane (0.25 µm) filtration, 20 ml of the desired filtrate were mixed with 80 ml PDA media. The mixture was poured in Petri dishes and each was inoculated by disk (5mm) with active mycelium of *S. sclerotiorum* was placed at the center of each dish. Then incubated at 23°C for 10 days and mycelial growth was measured in all treatments and the results were expressed as the percentage of mycelial growth inhibition relative to the control (Carisse *et al*., 2001). The number and weight of sclerotia of the fungus were determined after 10 days.

Effect of fungicides alternative on the activity of *Trichoderma* spp. volatile compounds against *S. sclerotiorum*

The effect of volatile compounds produced by *Trichoderma* isolates, e.g. grown in liquid media supplemented by the antioxidants was demonstrated using the technique of Boubekeur *et al.* (2012). A mycelial disc of 5 mm diameter of the pathogen and another of treated *Trichoderma* isolates were put each in the center of each Petri-dish containing PDA medium. The lids were removed aseptically and the bottom of each dish containing the antagonist was placed inverted on that contains by the pathogen and was scaled tightly by three layers of parafilm to prevent the loss of volatile substances. Petri-dishes containing PDA without antagonist served as control. The average diameter of growth in the treatments was measured after 10 days of incubation at 23°C. The effect of volatile compounds was expressed in terms as mycelial growth inhibition, number and weight of sclerotia was also determined.

Effect of fungicides alternative on the activity of *Trichoderma* spp. secondary metabolites against *S. sclerotiorum*

Treated *Trichoderma* isolates were cultured on GM overlaid with a sterilized cellophane membrane (Dennis and Webster, 1971). A mycelial disc (5mm diameter) was cut from the margin of an actively growing culture tested compounds amended with and untreated (as the control) *Trichoderma* colony was placed on the center of cellophane overlaid on GM. After incubating the plates at 23°C in dark for 2 days, the cellophane membranes in all plates were removed and a disc (5mm diameter) of *S. sclerotiorum* culture was placed at the center of each plate. The plates were incubated at 23°C for 7 days and the diameter of the *S. sclerotiorum* colony in each plate was measured. The number and weight of formed sclerotia were also determined.

Statistical analysis:

Treatments were arranged in completely randomized blocks design. The means were compared using Duncan multiple range test at probability level (P) ≤ 0.05, using the statistical analysis software CoStat (version 6.4).

RESULTS AND DISCUSSION

Efficacy of antioxidants and micronutrients on improving growth and sporulation of *Trichoderma* spp.

The data in Table (1) shows the significant effect of some antioxidants and micronutrients on linear growth, spore number, fresh and dry weights of four *Trichoderma* spp. The mean colony diameter of *T. harazianum* T1 in the thiamine amended medium recorded 9 cm instead of 6 cm in control one (Fig 1) with 3.7 fold increase in dry weight. Potassium tartrate had positive effect on growth parameters of *T. harazianum* T4 and *T. koningii* T6. In addition folic acid and thiamine lead to 2.2 and 2.1 fold increase in dry weight of *T. harazianum* T4 respectively. On the contrary, *T. harazianum* T8 was slightly affected by the addition of antioxidants.

The positive effect of antioxidants and micronutrients was an expected result as most of them improved the metabolic activity and can be used by the fungus as a nutrients or coenzyme. Ivashechkin *et al.* (2015) mentioned that addition of an antioxidant increased the biomass yield of *Lentinus tigrinus*, as well as *Cunninghamella japonica*.

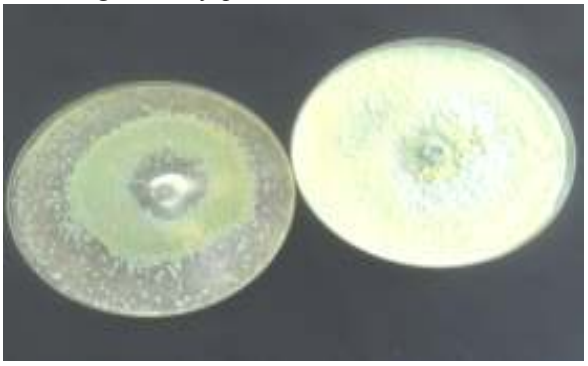

Fig. 1. Effect of thiamine in improving *T. harzianum* (T1) growth, the plate in left without thiamine and the right plate treated with thiamine

Table 1. Effect of some antioxidants and micronutrients on linear growth, spore number, fresh and dry weights of *Trichoderma* spp. after 10 days from incubation

Isolates	Treatments	LG*	Spore N. ($x10^{-6}$)	Fresh w. (gm)	Dry w. (gm)	Increase in dry w. (fold)
T. harzianum T1	Control	6.00 e	15.7 op	3.29 B	0.12 F	
	K tartrate	6.68 c	26.3 mn	7.19 x	0.53 s	4.4
	Micronutrients	6.43 d	21.0 nop	6.13 A	0.34 B	2.8
	Ascorbic acid	6.60 cd	13.3 p	6.44 z	0.41 x	3.4
	Folic acid	6.85 b	20.7 nop	6.99 y	0.49 v	4.1
	Thiamine	9.00 a	23.0 no	8.62 w	0.56 p	4.7
T. harzianum T4	Control	4.00 n	47.0 kl	12.00 s	0.35 A	
	K tartrate	9.00 a	68.3 abcd	19.64 c	0.63 l	1.8
	Micronutrients	4.17 mn	66.7 bcde	20.00 a	0.64 k	1.8
	Ascorbic acid	9.00 a	41.7 l	18.00 f	0.32 C	0.0
	Folic acid	9.00 a	71.7 abcd	13.47 mn	0.76 d	2.2
	Thiamine	4.50 jkl	70.7 abcde	19.30 d	0.72 e	2.1
T. koningii T6	Control	3.50 qr	56.3 ij	18.90 e	0.49 v	
	K tartrate	9.00 a	56.3 ij	13.17 o	0.66 i	1.3
	Micronutrients	4.33 lm	68.3 abcd	14.19 k	0.67 h	1.4
	Ascorbic acid	4.80 h	68.7 abcd	15.69 i	0.77 c	1.6
	Folic acid	5.43 f	59.7 ghi	20.03 a	0.51 t	1.1
	Thiamine	4.53 ijk	67.3 abcd	12.47 q	0.59 o	1.2
T. harzianum T8	Control	4.10 n	49.3 jk	13.05 m	0.30 D	
	K tartrate	4.50 jkl	49.7 jk	11.11 t	0.54 r	1.8
	Micronutrients	4.33 lm	65.3 bcd	11.20 t	0.65 j	2.2
	Ascorbic acid	4.37 kl	50.0 jk	9.80 v	0.23 E	0.0
	Folic acid	4.73 h	60.7 fghi	14.20 k	0.36 z	1.2
	Thiamine	4.67 hij	64.7 defghi	16.00 h	0.41 x	1.4

***linear growth**

Effect of antioxidants and micronutrients on improving antagonism degree of different *Trichoderma* spp. against *S. sclerotiorum*

Table (2) shows a great variation in the antagonistic effect of treated and untreated *Trichoderma* spp. against *S. sclerotiorum*. Where the antagonicity of *T. harzianum* T1 not affected by treatment, The untreated *T. harzianum* T4, *T. koningii* T6 and *T. harzianum* T8 revealed 59.2%, 41.9% and 44.4% of *S. sclerotiorum* inhibition respectively. Addition of potassium tartrate, micronutrients and ascorbic acid enhanced the antagonism leading to complete inhibition of *S. sclerotiorum* growth. Thiamine showed the same effect with *T. koningii* T6 and *T. harzianum* T8. Where folic acid showed it with *T. harzianum* T8 only. The mechanisms of action of *Trichoderma* against fungal pathogens include secretion of antibiotics, competition for space and nutrients and production of lytic enzymes (Harman *et al.*, 2004) and this can be improved the biocontrol potential of *T. viride* against *M. phaseolina* in micronutrients amended medium (Sundaravadana and Alice, 2006) as *Trichoderma* spp. prefer and grow well in acidic pH and in soil or media have high organic matter (Upadhyay and Rai, 1979).

Effect of fungicides alternative on improving crude extract, secondary metabolites and volatile compounds activity of *Trichoderma* spp. against *S. sclerotiorum*

According to Table (3) using Trichoderma crude extracts, the pathogen linear growth is completely suppressed with folic acid and thiamine addition in variants with *T. harzianum* T1. By *T. koningii* T6 the 100% efficiency is noted with all additives and by *T. harzianum* T8 as well, except potassium tartrate. The secondary metabolites of *T. harzianum* T4 greatly enhanced by K tartrate from no effect to complete linear growth inhibition of the pathogen. The secondary metabolites of *T. koningii* T6 inhanced by all additives to complete inhibition of the pathogen linear growth. Comparing to previous results, volatile compounds of Trichoderma isolates is slightly affected by the additives except *T. harzianum* T8 which showed complete linear growth inhibition of the pathogen with the addition of potassium tartrate, micronutrients and thiamine.

Table 2. Effect of antioxidants and micronutrients on antagonism degree of different tested *Trichoderma* spp. against S. *sclerotiorum* after 7 days from incubation

Isolates	Treatments	%inhibition of *S. sclerotiorum*	Bell scale
T. harzianum T1	Control	74.4	2 c
	K tartrate	57.4	3 b
	Micronutrients	75.6	2 c
	Ascorbic acid	81.5	2 c
	Folic acid	72.2	2 c
	Thiamine	72.2	2 c
T. harzianum T4	Control	59.2	3 b
	K tartrate	100.0	1 d
	Micronutrients	100.0	1 d
	Ascorbic acid	100.0	1 d
	Folic acid	83.3	2 c
	Thiamine	83.3	2 c
T. koningii T6	Control	41.9	3 b
	K tartrate	100.0	1 d
	Micronutrients	100.0	1 d
	Ascorbic acid	100.0	1 d
	Folic acid	83.3	2 c
	Thiamine	100.0	1 d
T. harzianum T8	Control	44.4	3 b
	K tartrate	100.0	1 d
	Micronutrients	100.0	1 d
	Ascorbic acid	100.0	1 d
	Folic acid	100.0	1 d
	Thiamine	100.0	1 d

All *Trichoderma* isolates with all antioxidants (Table 4) showed great effect in the new sclerotia formation by the pathogen. Crude extracts, secondary

metabolites and volatile compounds of *T. harzianum* T1 are very effective in inhibition of sclerotia formation of the pathogen. Addition of antioxidants has no remarked effect in its efficiency except thiamine. Addition of all antioxidants to *T. koningii* T8 caused complete inhibition of new sclerotia formation. The activity of secondary metabolites of *T. koningii* T6 is high and further increased to inhibit completely the new sclerotia formation with addition of potassium tartrate and micronutrients mixture where the fungus have no inhibition effect by its volatile compounds in untreated case, the addition of micronutrients steeply increase it to 100% efficiency

Table 3. Effect of antioxidants and micronutrients on the activity of antagonistic crude extract, secondary metabolites and volatile compounds of *Trichoderma* spp. against *S. sclerotiorum* after 10 days from inoculation

isolates		%inhibition of L.G.		
		C. E	S. M	V. C
T. harzianum T1	Control	70	77.8	51.5
	K tartrate	0.0	0	1.9
	Micronutrients	80	0	21.9
	Ascorbic acid	70	77.8	5.6
	Folic acid	100	100	0
	Thiamine	100	100	76.7
T. harzianum T4	Control	0.0	0	0
	K tartrate	100	100	0
	Micronutrients	0.0	0	13.9
	Ascorbic acid	0.0	0	70.4
	Folic acid	70	61.0	7.4
	Thiamine	0.0	0	0
T. koningii T6	Control	80	77.8	0
	K tartrate	100	100	0
	Micronutrients	100	100	27.8
	Ascorbic acid	100	100	0
	Folic acid	100	100	0
	Thiamine	100	100	0
T. harzianum T8	Control	80	77.8	6.5
	K tartrate	80	77.8	100
	Micronutrients	100	94.4	100
	Ascorbic acid	100	100	75.6
	Folic acid	100	100	77.0
	Thiamine	100	94.4	100

C.E=crude extract, S.M= secondary metabolites and V.C=volatile compounds

Table 4. Effect antioxidants and micronutrients on improving crude extract, secondary metabolites and volatile compounds activity of *Trichoderma* spp. against sclerotia production and weight of scelerotia of *S. sclerotiorum*

Isolates	Treatments	% inhibition of sclerotia production			Wt. of sclerotia (gm)		
		C.E	S.M	V. C	C. E	S. M	V. C
	S. sclerotiorum	**0.0**	**0.0**	**0.0**	**0.8 a**	**0.5 a**	**0.6 a**
T. harzianum T1	Control	78	100	87	0.1 b	0 i	0.1 mn
	K tartrate	63	64	72	0.1 b	0.1 f	0.21 e
	Micronutrients	68	61	72	0.1 b	0.1 d	0.13 hi
	Ascorbic acid	70	89	75	0.1 b	0.005 i	0.15 fg
	Folic acid	65	93	71	0.1 b	0.005 i	0.21 e
	Thiamine	67	100	100	0.09 b	0 i	0 o
T. harzianum T4	Control	34	29	62	0.28 a	0.23 b	0.17 f
	K tartrate	47	100	64	0.18 a	0 i	0.22 de
	Micronutrients	44	72	100	0.23 a	0.12 d	0.1 kl
	Ascorbic acid	49	57	100	0.14 a	0.12 d	0 o
	Folic acid	54	78	87	0.12 a	0.073 h	0.08 lm
	Thiamine	35	56	100	0.27 a	0.203 c	0 o
T. koningii T6	Control	51	92	0	0.13 a	0.003 i	0.2 e
	K tartrate	59	100	67	0.11 a	0 i	0.31 b
	Micronutrients	58	93	100	0.10 a	0.003 i	0 o
	Ascorbic acid	43	100	37	0.17 a	0 i	0.09 jk
	Folic acid	66	89	7	0.11 a	0.002 i	0.23 cd
	Thiamine	56	93	13	0.12 a	0.003 i	0.21 e
T. harzianum T8	Control	56	100	12.7	0.13 a	0 i	0.25 c
	K tartrate	62	96	100	0.10 a	0.001 i	0 o
	Micronutrients	51	93	100	0.15 a	0.001 i	0 o
	Ascorbic acid	25	100	100	0.32 a	0 i	0 o
	Folic acid	54	93	100	0.12 a	0.003 i	0 o
	Thiamine	58	100	100		0 i	0o

In discussion of these results there are several lines of evidence, Morid and Zafari (2013) found that the highest specific activity of the chitinase enzymes were found in isolates *T. brevicompactum* and *T.*

koningiopsis grown in medium containing manganese micronutrient. Peciulyte and Lugauskas (2000) reported that antifungal activity against 13 fungal species dependent on copper sulfate or copper oxychloride concentration in the medium. Metal ions may influence the growth rate, mass, sporulation and enzymatic activity of produced fungal mycelium (Gadd, 1993).

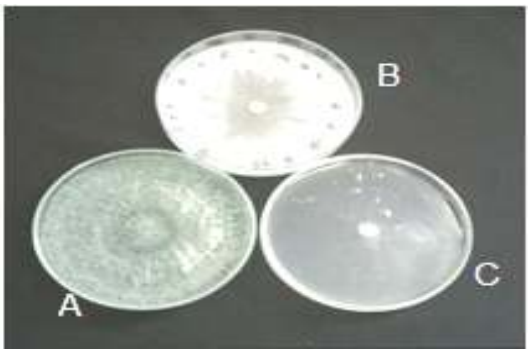

Fig. 2. PDA Plates shown the efficacy Efficiency of volatile compounds of compared with untreated one, A; Potassium tartrate treated *T. harzianum* T8; B, control *S. sclerotiorum*; C, complete inhibition of B by the action of volatile compounds of A

Sclerotia differentiation is triggered by high oxidative stress, Georgiou (1997) found that sclerotial biogenesis in *S. rolfsii* was accompanied by the accumulation of high levels of peroxidized lipids. This theory was supported by other studies showing that vitamin C and carotene as a hydroxyl radical scavengers inhibited sclerotial differentiation of *S. rolfsii*, *S. minor*, *S. sclerotiorum*, and *R. solani* (Georgiou *et al.,* 2006) and by relating the thiolredox state of these fungi to their sclerotial differentiation (Patsoukis and Georgiou, 2008). Cherednichenko *et al*. (2002) found that beta-carotene increased the viability of *T. reesei* 6/16 protoplasts to the greatest extent and the effect of ascorbic acid depended on the presence of Fe ions. Moreover, Addition of antioxidants at concentrations less than 5 mM can lead to fungal growth, increase resistance structures, and stimulate secondary metabolite gene expression and accumulation by *Aspergillus flavus* RCP08108 (Nesci *et al.,* 2003).

The advantage of *T. harzianum* isolates can be explained by its production of vast types of secondary metabolites as harzianic acid, dimethyl-harzianic acid, homoharzianic acid, 1-hydroxy-3-methyl-anthaquinone, 1,8-dihydroxy3-methyl-anthraquinone, harzianolide, trichoharzin, harziandione, cyclonerodiol, harzianopyridone, melanoxadin, trichodenones A, B and C, harziphilone, fleephilone, mevalonolactone, harzianolide, MR566B, tricho setin, trichorzianines A, B, trichokindins I- VII, harzianins HC, trichorozins I–IV, isonitrin A and D (Keswani *et al.,* 2014). In support of our results, Reverberi *et al*., (2005) documented a link between increased secondary metabolites production and oxidative stress based on chemical induction.

Our results put the studied antioxidants and micronutrients in enhancers list which play a positive role in production of primary and secondary metabolites of *Trichoderma* spp. involved in biocontrol of *S. sclerotiorum* growth and its sclerotia formation and differentiation.

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تـأثير مضـادات الأكسـدة والعناصـر الصـغرى كبـدائل للمبيـدات الفطريـة فـى تحسـين التضـاد للتريكودرمـا ضـد اسكليروتينيا اسكليروشيورم

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اسكليروتينيا اسكليروشيورم مسبب مرضى قوى يستقر فى التربة ومقاوم بدرجة عالية للمبيدات. يعطى العامل الحيوى التريكودرما بمفرده نتائج متوسطة لمقاومة هذا الفطر. فى الدراسة الحالية استخدم البوتاسيوم ترترات، حمض الاسكوربيك، حمض الفوليك ،ثيامين وخليط العناصر الصغرى لتحسين عدد جراثيم، الوزن الطازج والجاف، مستخلص التريكودرما والمركبات المتطايرة والمركبات الثانوية المنتجة من أربعة عزلات للتريكودرما ضد المسبب المرضى. مجرد اضافة الثيامين ٠.١% أو خليط العناصر الصغرى تحسن كفائة التضاد لمعظم عزلات التريكودرما موضحة منع نمو المسبب المرضى وتثبيط تكوين الاسكليروشيا.