



Full Length Article

In vitro and *In vivo* Efficacy of some Fungicides against *Phytophthora nicotianae*

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Abstract

This study was conducted in order to determine the *in vitro* and *in vivo* efficacy of some chemicals against *Phytophthora nicotianae*, which is one among the *Phytophthora* spp. that are responsible for tomato root and crown rot disease. We tested various active substances against this disease pathogen. In the experiment, varied concentrations of “metalaxyl 35%”, “fludioxonil 25 g/L + metalaxyl-M 10 g/L”, “ametoctradin 300 g/L + dimethomorph 225 g/L”, “hymexazol 360 g/L” and “tolclofos methyl 30% + thiram 30%” active substances were used. In *in vitro* studies, EC₅₀/EC₉₀ values that inhibited sporangium formation and mycelium growth of *P. nicotianae* was calculated separately for each active substance. In pot experiments, application doses of active substances were applied to tomato seedlings and the active substance that prevents the disease best was determined. As a result of *in vitro* studies, “metalaxyl 35%” provided the lowest EC₅₀/EC₉₀ concentration that prevents mycelium growth by 50% and 90%. The lowest EC₅₀/EC₉₀ concentration values that decreased the sporangium formation by 50% and 90% were obtained from “ametoctradin 300 g/L + dimethomorph 225 g/L.” In pot the experiment, “Fludioxonil 25 g/L + metalaxyl-M 10 g/L” exhibited the highest efficacy against *P. nicotianae*. © 2018 Friends Science Publishers

Keywords: Fungicide; *In vitro*; *In vivo*; *Phytophthora nicotianae*; Tomato

Introduction

Phytophthora species in Oomycetes cause destructive damages on both agricultural production and natural ecosystems (Kroon *et al.*, 2012; Meng *et al.*, 2014). With the developments in biochemical analyses and molecular techniques, over a 100 species have been found in *Phytophthora* (Meng *et al.*, 2014; Miao *et al.*, 2016). One of the species of *Phytophthora* is *Phytophthora nicotianae* Breda de Haan (= *P. parasitica* Dastur) (Meng *et al.*, 2014). *P. nicotianae* is one of the destructive and prevalent soil-borne pathogen that causes diseases on plants. This pathogen causes disease in numerous plants such as tomato, pepper, and notably tobacco in many countries (Rodríguez *et al.*, 2012; Morales-Rodríguez *et al.*, 2014). The pathogen causes damping-off in tomato during nursery period and crown and root rot disease, and fruit rots in future periods. Seedlings that are struck by disease generally do not grow well and die. In grown plants, it causes spots varying from dark brown to black on the stem of the plant at soil level (Elena, 2000).

There are a few fungicides for the control of *Phytophthora* species that cause damping-off and crown and root rot disease in plants (Miao *et al.*, 2016).

Efficacy of active substances such as azoxystrobin, dimethomorph, fluazinam, fosetyl-l, ethazol, metalaxyl and oxathiapiprolin on different development periods of *P. nicotianae* were studied (Ioannou and Grogan, 1984; Matheron and Porchas, 2000; Bittner and Mila, 2014). It was found that ethazol is more efficient than metalaxyl against mycelium growth, sporangium formation, zoospore and chlamyospore germination (Ioannou and Grogan, 1984). It was reported that azoxystrobin, dimethomorph and fluazinam active substances could be used for the control of this disease during one or several periods of the life cycle of *P. nicotianae* (Matheron and Porchas, 2000).

Symptoms of crown and root rot disease originating from *P. nicotianae* are seen in tomato production fields, especially in greenhouses at early periods, in Turkey. Damages resulting from this pathogen are increasing consistently. There are licensed Plant Protection Products against soil-borne disease pathogens, which cause crown and root rot disease in vegetables in Turkey. However, it is generally indicated in their labels that they are efficient against pathogens such as *Rhizoctonia solani*, *Fusarium* spp., and *Phythium* spp. As far as we know, there is no Plant Protection Product that may have obtained license by being tested against *P. nicotianae*.

In this study, it is aimed to determine the *in vitro* and *in vivo* efficacies of some fungicides, which is licensed against damping-off and soil-borne diseases, which could possibly be effective against the soil-borne pathogen *P. nicotianae*.

Materials and Methods

Fungal Isolate

Experiments were carried out at the Faculty of Agriculture and Natural Sciences of Duzce University in 2015–2016. *P. nicotianae* isolate used in the experiment was obtained from Batı Akdeniz Agricultural Research Institute. The isolate was isolated during the previous studies in the Institute. Pathogen was developed on Carrot agar (CA) (carrot juice: 200 mL, distilled water: 800 mL, agar: 20 g).

Fungicides

Commercial products that contained active substances such as “metalaxyl 35%”, “fludioxonil 25 g/L + metalaxyl-M 10 g/L”, “ametoctradin 300 g/L + dimethomorph 225 g/L”, “hymexazol 360 g/L” and “tolclofos methyl 30% + thiram 30%” were used as fungicides in *in vitro* and *in vivo* studies.

Determination of Efficacy of Different Fungicide Concentrations against Mycelium Growth

Efficacy of different concentrations of some fungicides against mycelial growth of *P. nicotianae* were experimented in *in vitro* conditions. In the experiment, different concentrations of fungicides that contained “metalaxyl 35%”, “fludioxonil 25 g/L + metalaxyl-M 10 g/L”, “ametoctradin 300 g/L + dimethomorph 225 g/L”, “hymexazol 360 g/L” and “tolclofos methyl 30% + thiram 30%” active substances were used. In the study, metalaxyl 35%, fludioxonil 25 g/L + metalaxyl-M 10 g/L, ametoctradin 300 g/L + dimethomorph 225 g/L, hymexazol 360 g/L and tolclofos methyl 30% + thiram 30% active substances were used in concentrations of 0 (control), 0.1, 0.3, 1, 3, 10, 30, 100 a.i. µg/mL. In order to prepare these concentrations of fungicides, stock solutions via active substance doses were prepared. Required amount of fungicide solutions were mixed with sterilized under 1 atm. pressure at 120°C in autoclave and cooled up to 45–50°C the Carrot agar medium. This medium was poured into the plastic petri dishes of 90 mm. Five replicate plates were used for each concentration. Agar discs in 5 mm diameters were taken from the edges of one-week old culture of *P. nicotianae* with the help of cork-borer and they were placed in the center of these petri dishes. The petri dishes were incubated at 25±1°C for a week. One week later, their colony diameters were measured in two different directions and their averages were calculated. By utilizing from the colony diameters obtained, mycelial growth inhibition rate of different concentration of fungicides was found by using the formula:

$$\text{mycelial growth inhibition (\%)} = [(dc-dt)/dc] \times 100(\%)$$

dc: average diameter of colony in control, dt: average diameter of colony in treatment group (Nisa *et al.*, 2011; Parveen *et al.*, 2013).

Determining Efficacy of Different Concentrations of Fungicides on Formation of Sporangium

The experiment was carried out in sterile soil solution. Garden soil of 10 g was put into 1 liter distilled water and was mixed in magnetic stirrer for one hour. It was waited for 24 h for the soil particles in the solution to settle. At the end of this period, the solution was filtered through 4-fold cheesecloth and sterilized in autoclave at 120°C under pressure of 1 atm (Matheron and Porchas, 2000; Hu *et al.*, 2008). Twenty five mL of this solution was put in 9 cm petri dishes. To prepare different concentrations of fungicides, necessary amount of fungicide solutions were mixed with sterile soil solution in petri dishes. Five agar discs with diameters of 5 mm were put into sterile soil solution which contained different concentrations of fungicides. These agar discs were taken from the edge of one-week old culture of *P. nicotianae* that was developed in carrot agar medium. These petri dishes were incubated at 25±1°C for 48 h. At the end of this period, sporangia were counted under a microscope (three fields of view (10X) for each mycelial disc) and their average was taken (Miao *et al.*, 2016). Each treatment consisted of five replicate petri dishes. Efficacy of different concentrations of fungicides on formation of sporangium was calculated by using the formula:

$$\text{efficacy on formation of sporangium (\%)} = [(dc-dt)/dc] \times 100(\%)$$

dc: number of sporangium in control, dt: number of sporangium in the ones on which application was made." (Nisa *et al.*, 2011).

Determining Efficacies of Fungicides against Root and Crown Rot Disease in Tomato

Efficacy of fungicides against *P. nicotianae* pathogen, which causes root and crown rot disease in tomato, were determined by modifying the method of Elena (2000). Six-weeks-old Falcon variety tomato seedlings in pots were used in the pathogenicity test. Mixture of sterilized soil, sand and fertilizer (1:1:1) was put in pots of 10 cm and tomato seedlings were planted in pots. For inoculation, two holes were made in the pot soil next to the tomato seedlings. Discs of 10 mm were taken from the one-week-old *P. nicotianae* that was grown in carrot agar medium (Elena, 2000). Discs were placed in two holes (4 discs per pot). Fungicide application was performed after inoculation. Label doses of fungicides were prepared by mixing the fungicides in tap water and 50 mL was applied to each pot.

The pots were kept in climate chamber under white fluorescent light (16 h day/ 8 h night) at 25°C and 70% humidity. Ten days following the application, tomato seedlings were removed and evaluated based on 0–4 scale value (Table 1) (Neher and Duniway, 1992). The experiment was established using seven replicate according to randomized block design.

Statistical Analysis

By using SPSS 16.00 statistics software in computer environment, EC₅₀/EC₉₀ values of fungicides against *P. nicotianae* were determined (Nisa *et al.*, 2011). Based on scale values obtained from pot experiment, disease percentages were determined by using Tawnsend-Heuberger formula, and percentage efficacy of fungicides from disease percentages was determined by using Abbott formula. Statistical analysis was made with SPSS 16.0 statistics software.

Results

Determining the Efficacy of Different Concentrations of Fungicides on Mycelium Growth

A series of fungicide concentrations were used to determine mycelial growth inhibition of *P. nicotianae*. Metalaxyl at concentration of 10 µg/mL was found to be the most effective mycelial growth inhibition of *P. nicotianae* with 100% efficacy. Among the test fungicides, fludioxonil + metalaxyl-M and ametoctradin + dimethomorph showed the highest mycelial growth inhibition with 100% efficacy at 30 a.i. µg/mL concentration, and tolclofos methyl + thiram showed the highest inhibition with 100% efficacy at 100 µg/mL concentration. It was determined that Hymexazol fungicides did not have sufficient efficacy at tested concentrations (Table 2).

The fungicides that were used in the experiment were also evaluated in terms of their EC₅₀ and EC₉₀ value. EC₅₀ value (value where mycelium growth is inhibited by 50%), was 0.328, 0.393 and 0.393 µg/mL, respectively, for metalaxyl, ametoctradin + dimethomorph, fludioxonil + metalaxyl-M fungicides. EC₅₀ values of tolclofos methyl + thiram and hymexazol fungicides were determined as 19.601 µg/mL, 8210.542 µg/mL respectively. A similar relation can also be seen in EC₉₀ values, which correspond to values mycelium growth is inhibited 90%. metalaxyl, fludioxonil + metalaxyl-M, ametoctradin + dimethomorph fungicides obtained lower EC₉₀ values than tolclofos methyl + Thiram and hymexazol fungicides. The lowest EC₉₀ value of 3.234 µg/mL was demonstrated by metalaxyl (Table 2).

Determining the Efficacy of Different Concentrations of Fungicides on Formation of Sporangium

Fungicides that were used in the experiment affected the

sporangium formation of *P. nicotiana* in different concentrations. The lowest concentration that completely inhibited sporangium formation was demonstrated by ametoctradin + dimethomorph and fludioxonil + metalaxyl-M at 10 µg/mL. Toleclofos methyl + thiram fungicide completely inhibited sporangium formation at a concentration of 30 µg/mL (Table 3).

In terms of concentrations that inhibited sporangium formation at 50% and 90% rates, the lowest EC₅₀ concentration was 0.005 µg/mL, and lowest EC₉₀ concentration was 0.374 µg/mL by ametoctradin + dimethomorph fungicide. The highest EC₅₀ concentration was 2.885 µg/mL, and the highest EC₉₀ concentration was 32670 µg/mL by hymexazol (Table 3).

Determining the Efficacy of Some Fungicides on Root and Crown Rot Disease in Tomato Seedlings

According to obtained results, fludioxonil + metalaxyl-M had the highest efficacy with a rate of 98.21%. The lowest efficacy was shown by hymexazol with a rate of 64.71% (Table 4). The difference between the fungicides used in the experiment in terms of their efficacy percentage was significant as a result of the variance analysis. Three groups were created based on the results of Tukey's test, and the highest group included fludioxonil + metalaxyl-M, metalaxyl and ametoctradin + dimethomorph.

Discussion

Biofumigants, essential oils and biological preparations are used against soil-borne diseases as an alternative to chemical compounds (Poza *et al.*, 2002; Rodriguez *et al.*, 2012; Lu *et al.*, 2013). However, chemical compounds are more commonly used. In European Union, synthetic compounds such as 1,3-dichloropropene and chloropicrin are used against soil-borne diseases with special authorization (Rodriguez *et al.*, 2012). In addition, chemicals such as azoxystrobin, dimethomorph, fluazinam, fosetyl-Al, tolclofos-methyl, thiram, ethazol, metalaxyl, are also used (Ioannou and Grogan, 1984; Matheron and Porchas, 2000).

Since metalaxyl and its isomer metalaxyl-M are chemically highly stable compounds, they are very suitable for soil applications. Metalaxyl-M can be absorbed easily by plant root and transmitted to green part. It was conclusively proved by studies that metalaxyl and metalaxyl-M are effective fungicides against pathogens that are members of Oomycetes. In a study conducted in Arizona, the effects of azoxystrobin, dimethomorph, fluazinam, fosetyl-Al and metalaxyl active substances on mycelium growth and sporulation of *P. capsici*, *P. citrophthora*, and *P. parasitica* pathogens were researched. In that study, EC 50 and EC 90 value of metalaxyl active substances on mycelium growth of *P. parasitica* were, respectively, 0.38 µg/mL and 40.2 µg/mL. EC₅₀ and EC 90 values which inhibited

Table 1: Scale value

Scale no	Description
0	Healthy
1	1-25% of the root system is diseased
2	26-50% of the root system is diseased
3	51-75% of the root system is diseased
4	76-100% of the root system is diseased

Table 2: Effect of different fungicides on mycelial growth inhibition of *P. nicotianae*

Fungicide	Percent mycelial growth inhibition of <i>P. nicotianae</i> (%)							EC50 $\mu\text{g/mL}$	EC 90 $\mu\text{g/mL}$
	0.1 $\mu\text{g/mL}$	0.3 $\mu\text{g/mL}$	1 $\mu\text{g/mL}$	3 $\mu\text{g/mL}$	10 $\mu\text{g/mL}$	30 $\mu\text{g/mL}$	100 $\mu\text{g/mL}$		
Metalaxyl (35%)	30.59	46.81	66.22	87.50	100	100	100	0.328	3.234
Ametoctradin (300 g/L) + Dimethomorph (225 g/L)	12.66	69.13	72.03	72.82	77.57	100	100	0.393	9.939
Fludioxonil (25 g/L) + Metalaxyl-M (10 g/L)	34.05	49.06	58.71	72.65	86.60	100	100	0.393	10.170
Tolclofos methyl (30%) + Thiram (30%)	8.72	7.79	8.10	11.53	13.08	52.96	100	19.601	408.715
Hymexazol (360 g/L)	1.87	3.12	3.74	4.05	6.23	4.67	27.41	8210.542	4390000

Table 3: Effect of different fungicides on sporangium formation of *P. nicotianae*

Fungicide	Inhibition of sporangium formation of <i>P. Nicotianae</i> (%)							EC50 $\mu\text{g/mL}$	EC 90 $\mu\text{g/mL}$
	0.1 $\mu\text{g/mL}$	0.3 $\mu\text{g/mL}$	1 $\mu\text{g/mL}$	3 $\mu\text{g/mL}$	10 $\mu\text{g/mL}$	30 $\mu\text{g/mL}$	100 $\mu\text{g/mL}$		
Ametoctradin (300 g/L) + Dimethomorph (225 g/L)	87.86	84.98	88.27	98.56	100	100	100	0.005	0.374
Tolclofos methyl %30 + Thiram (30%)	40.66	73.03	75.93	80.50	93.36	100	100	0.139	4.245
Fludioxonil 25g/l + Metalaxyl-M (10 g/L)	37.68	56.37	94.62	97.17	100	100	100	0.178	1.010
Metalaxyl (35%)	29.89	37.43	69.55	84.64	87.43	91.9	94.97	0.396	15.102
Hymexazol (360 g/L)	31.06	46.38	52.77	42.55	51.49	27.66	42.55	2.885	32670

Table 4: Effects of some fungicides on root and crown rot disease in tomato seedlings

Fungicide	Disease incidence %	Efficacy %
Fludioxonil (25 g/L + Metalaxyl-M 10 g/L)	1.79	98.21a*
Metalaxyl (35%)	3.57	96.43a
Ametoctradin (300 g/L + Dimethomorph 225 g/L)	7.14	92.86a
Tolclofos methyl (30%) + Thiram (30%)	10.71	88.78ab
Hymexazol (360 g/L)	30.36	64.71b
Control	69.64	

*Means in the same column with different super script letters are significantly different; a-b - $P < 0.05$

Sporangia formation were lower than 1 $\mu\text{g/mL}$ for both of them (Matheron and Porchas, 2000). In our study, EC50 and EC90 values of metalaxyl on mycelium growth of *P. nicotianae* were, respectively, 0.328 $\mu\text{g/mL}$ and 3.234 $\mu\text{g/mL}$. EC 50 and EC 90 values that inhibited Sporangia formation were 0.396 $\mu\text{g/mL}$ and 15,102 $\mu\text{g/mL}$. While the results we obtained were similar to those of EC90 values showed some difference (Ioannou and Grogan, 1984; Matheron and Porchas, 2000).

In our study, EC50 and EC 90 value of fludioxonil + metalaxyl-M fungicide, which also includes the active substance of metalaxyl-M, on mycelium growth of *P. nicotianae* were, respectively, 0.393 $\mu\text{g/mL}$ and 10.170 $\mu\text{g/mL}$. EC 50 and EC 90 values that inhibited Sporangia formation were determined as 0.178 $\mu\text{g/mL}$ and 1.010 $\mu\text{g/mL}$. In another study conducted in Spain, EC50 and EC90 values of mefenoxam on mycelium growth supported the results we obtained (Morales-Rodríguez et al., 2014).

Both active substances of ametoctradin +

dimethomorph are effective against *Phytophthora* spp. Active substance of ametoctradin is a fungicide that has a high effect on zoospore formation, zoospore release, mobility of zoosporangiums and their germination directly (Gold et al., 2010; Merk et al., 2011). A study determined that the efficacy of dimethomorph active substance on the mycelium growth, zoospore germination and sporangium formation of *Phytophthora* was, in terms of EC50 values, 0.24, 0.10 and 0.46 $\mu\text{g/mL}$, respectively (Jackson et al., 2012). In our study, its EC50 and EC90 value on the mycelium growth of *P. nicotianae* was, respectively, 0.393 $\mu\text{g/mL}$ and 9.939 $\mu\text{g/mL}$. EC 50 and EC 90 value that inhibited sporangia formation was determined as 0.005 $\mu\text{g/mL}$ and 0.374 $\mu\text{g/mL}$. Our study showed that sporangia formation is affected more by this fungicide than mycelial growth.

Even though tolclofos methyl + thiram mixture is used against soil-borne diseases, it is well-known that it is more effective against *R. solani* (Smiley et al., 1990; Ahmed et al., 2002). Our study on tolclofos methyl +

thiram fungicide demonstrated that it had medium-level efficacy on mycelium growth of *P. nicotianae*. EC50 and EC90 value on mycelium growth of *P. nicotianae* was respectively, 19.6 µg/mL and 408.715 µg/mL. Sporangia formation was affected more by fungicide. EC 50 and EC 90 values that inhibited sporangia formation were, respectively, 0.139 µg/mL and 4.245 µg/mL.

Hymexazol fungicide is very effective against soil pathogens in general. However it is ineffective against members of *Oomycotina*, including many species of *Phytophthora*. It is used in optional environments and isolations made from soil, in order to obtain *Phytophthora* spp. isolates by inhibiting *Phythium* species (Delen, 2016). The results obtained from experiment also support this. EC50 and EC90 value of *P. nicotianae*'s mycelial growth was, respectively 8210.542 µg/mL and 4390000 µg/mL. EC 50 and EC 90 values that inhibited sporangia formation were determined as 2.885 µg/mL and 32670 µg/mL.

According to results of pot experiments, fludioxonil 25 g/L + metalaxyl-M 10 g/L fungicide showed the highest efficacy against *P. nicotianae*. Metalaxyl-M is considered to be the source of this efficacy. Hymexazol fungicide, which obtained the lowest efficacy as a result of *in vitro* studies, obtained above-average efficacy with a rate of 64.71% despite the fact that it obtained the lowest efficacy in inhibiting mycelium growth. In addition to its fungicide efficacy, hymexazol also has a characteristic of accelerating plant growth by increasing lateral root and capillary root development (Delen, 2016). It is thought that obtained efficacy comes from this.

Conclusion

It was determined through *in vitro* and *in vivo* studies that fungicides containing the active substances of metalaxyl, fludioxonil + metalaxyl-M, ametoctradin + dimethomorph were more effective against *P. nicotianae*. Fungicides containing active substances of tolclofos methyl + thiram and hymexazol did not demonstrate expected efficacy against *P. nicotianae*. It was generally determined that fungicides used in experiment were more effective against sporangium formation than mycelia development. Metalaxyl, fludioxonil + metalaxyl-M, ametoctradin + dimethomorph were the most effective against *P. nicotianae* on tomato seedling.

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