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ORIGINAL PAPER

ANTIFUNGAL ACTIVITY OF LENTINULA EDODES EXTRACTS AGAINST PHYTOPHTHORA INFESTANS PHYTOPATHOGENIC FUNGI

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Abstract: **Phytophthora infestans** is an oomycete that is responsible for the late blight disease of potatoes and tomatoes and othe**r**several plant species. Late blight affects foliage of both potato and tomato as well as potato tubers and tomato fruit. Disease management during the production of vegetable crops has become a major concern in all over the world. In last years the biological control of tomato and potato late blight has attracted much attention. The objectives of this study wereto present preliminary experimental researches consisting in testing of the treatements based on Lentinula edodes (Shiitake) extractsapplied to Lycopersicon esculentum Mill. in order to disturb the activity of Phytophthora infestans fungi.The treatements have been made using aqueous extractof Lentinula edodes (Shiitake). The vegetal biological material consisted of Lycopersicon esculentum Mill. plantlets, cultivated in vitro or ex vitro, inoculated with Phytophthora infestans. After the infection has become active, the treatements based on Lentinula edodes (Shiitake), in different concentrations (2 %, 4 % or 6 %), have been applied to the planlets, to test their influence on the activity of Phytophthora infestans fungi.The best experimental results have been noticed for the experimental variant which used the treatement based on aqueous extract of Lentinula edodes (Shiitake), 6 % concentration, used on Lycopersicon esculentum Mill. plantlets of Elisabeta variety, obtained and infected with Phytophthora infestans in ex vitro conditions.

Key words: Shiitake, Lycopersicon esculentum Mill., aqueous extract, in vitro, ex vitro.

Introduction

Late blight caused by *Phytophthora infestans* is one of the most serious disease to tomato production (K. LAMSAL& al., 2013[1]). Control of plant disease is mostly based on cultural practices, chemical treatments or genetic resistance in host plants (R. C. SHATTOCK, 2002[2], R. N. STRANGE, 1993 [3],H. TRAN& al., 2007 [4]). Biocontrol of late blight using several antagonistic microorganisms, or plant growth-promoting rhizobacteria (PGPR) represents an attractive alternative for disease management (K. LAMSAL& al., 2013[1]).

The antifungal activity of the lentin protein from the *Lentinula edodes*, mushrooms has been studied by P. H. K. NGAI and T. B.NG. (2003) [5], on *Botrytis cinerea* fungi.

In addition to the first experiment presented by the two Chinese scientists, this paper presents the results of testing the action of the *Lentinula edodes*, extracts on *Lycopersicon esculentum* Mill. plantletspreviously infected with phytopathogenic fungi, *Phytophthora infestans*.The plantlets have been infected in a controlled way through mechanical wound at the leaf level and through dropping 2 ml of liquid culture of *Phytophthora infestans* mycelium on the affected area (Y. COHEN&al., 1994 [6],M. ALVES & al.,2013 [7]).

Lentinula edodes, spores have been provided from an ordinary mushroom, while the stem has been isolated in the laboratory to obtain the mycelium. The fungus has been obtained from the mycelium and the mushroom has been dried to be preserved in optimum conditions. After that, the mushroom has been minced, homogenized in water and it has been kept at the 4ºC for 7 days to facilitate the transfer of the interest substances from the fungi into the liquid (N. BĂBEANU& al., 2008 [8]), and thus the extract has resulted.

The goal of our work was to investigate the antifungal activity of *Lentinula edodes*(Shiitake), aqueous extracts against *Phytophthora infestans*phytopathogenic fungi.For this proposestreatmentswith*Lentinula edodes*(Shiitake)aqueous extracts were applied on *Lycopersicon esculentum* Mill. infected plantlets, obtained *in vitro* or *ex vitro* conditions.

Materials and Methods

Biological materials

The biological materials used for the experiments were: *Elisabeta* and *Rio Grande*seeds of *Lycopersicon esculentum*, *Lentinula edodes* (Shiitake) mushrooms both provided from the commercial market, and a pure *Phytophthora infestans* cultureprovided fromthe Microbiology Laboratory - Faculty of Biotechnology, University of Agronomical Sciences and Veterinary Medicine Bucharest.

Methods

Germination of tomato seeds. In order to obtain*in vitro* tomato plantlets, seeds were surface sterilized for a brief rinse with 70 % ethylic alcohol and for 10 min. in 2 % sodium hypochlorite, followed by a 3x10 min. rinse with sterile distilled water(N. BĂBEANU & al., 2008 [8]).Seeds were than cultivated on PDA (potato extract - dextrose - agar) nutritive medium and incubated *in vitro* conditions.

Ex vitro tomato plantlets were obtained from the seeds immersed in

water at 50ºC, for 30 min. Seeds were than sown in a plastic pot (5 cm diameter each) filled with commercial soil containing 10 % perlite. Seedlings were grown in a greenhouse at 23-25ºC.

***In vitro* culture of*L. edodes*.** Fruit bodies of *Lentinula edodes*, were surface sterilizing and cutting out a piece of trama using a sterile scalpel. The pieces were placed in Petri dishes on 2 % ME (maltextract-agar) or PDA (potato extract - dextrose-agar) media and incubated at 25˚C for a week. After the mycelium growing on the medium surface, mycelia agar discs (5 mm diameter), obtained from the active growth areas were placed on an aseptic medium with hydrated cereals. After the complete colonization of the cereals substrate with the mushroom mycelium, this was used as inoculums for cultivation of a nutritive medium with wood and incubated in a dark at 20˚C, for 5 weeks (P. STAMETS, 1993 [9], E. GEAMĂN SANDULESCU & I. GEAMĂN, 2001 [10], A. O. ANTOCE & L. D. DINU, 2002 [11]) – according to *Training Manual on Mushroom Cultivation Technology* [12].

Extracts preparation. *Lentinula edodes* carpophores were dried at room temperatures and grounded for extracts preparation. The powder resulted was separated into three samples, of 10 g each and used for preparation of 3 concentrations (2 %, 4 % or 6 %) of aqueous extracts. The aqueous solutions were then centrifuged at 5.000 rpm for 10 minutes. The supernatant was kept at 4˚C and used to determine the antifungal activity of *L. edodes* extracts (G. FIDLER & al., 2013 [13]).

Tomato seedling infection. The 30 day old plantlets obtained from *in vitro* and *ex vitro* conditions, have been transferred on fresh artificial nutritive medium / substrate. At the moment of the transfer every plantlet was inoculated in two ways: by piercing of a real leaf and by superficial harming of the stem with a syringe needle, dropping 1 ml of *Phytophthora infestans*liquid culture over every lesion. To avoid a potential contamination, the whole mechanical harming process and the inoculation assay were made over a collector container in the hood with air laminar flux. In order to facilitate the infection, in the new container it was ensured a humidity of over 90 % (U. ZŁOTEK&W. WÓJCIK, 2014 [14]).

***In vitro* evaluation of antifungal activity of *L. edodes* extracts.** The antifungal effect of *Lentinula edodes*(Shiitake), aqueous extracts was performing according P. H. K. NGAI and T. B.NG method (2003) [5]. To determine the antifungal activities of tested extracts, sterile filter paper discs (5 mm diameter), soaked in aqueous extracts were placed at 1.0 cm distance from the *Phytophthora infestans* colony obtained on PDA (potato extract - dextrose - agar) medium after 24 hours of incubation at 25˚C. After 96 hours of incubation at 25˚C occurrence of fungal growing inhibition was observed at the interaction area between extracts and fungal mycelium.

Experimental variants

Tomato seedlings treatments were consisted by using various concentrations of *L. edodes* extracts. The efficacy of extracts on infected tomato plants was compared with inhibitory effects of a copper sulphate solution (1.0 %) and stamycin solution (0.1 %) against *P. infestans* (Table 1). Each experimental variant was made in 3 repetitions.

Table 3. Experimental variants for evaluation treatments for antifungal activity against *P. infestans*

Experimental variants	***Lycopersicon esculentum* Mill. plantletsRio Grande and Elisabeta varieties**		
	Elisabeta (*in vitro*) treatment	**Elisabeta (*ex vitro*) treatment**	**Rio Grande (*ex vitro*) treatment**
1	*Lentinula edodes*(Shiitake) 2 % aqueous extract	*Lentinula edodes*(Shiitake) 2 % aqueous extract	*Lentinula edodes*(Shiitake) 2 % aqueous extract
2	*Lentinula edodes* (Shiitake) 4 % aqueous extract	*Lentinula edodes* (Shiitake) 4 % aqueous extract	*Lentinula edodes* (Shiitake) 4 % aqueous extract
3	*Lentinula edodes* (Shiitake) 6 % aqueous extract	*Lentinula edodes* (Shiitake) 6 % aqueous extract	*Lentinula edodes* (Shiitake) 6 % aqueous extract
4	1.0 % Copper sulphate solution	1.0 % Copper sulphatesolution	1.0 % Copper sulphatesolution
5	0.1 % Stamycin solution	0.1 % Stamycin solution	0.1 % Stamycin solution
6	Control first (infected and not treated)	Control first (infected and not treated)	Control first (infected and not treated)
7	Control second (not infected and not treated)	Control second (not infected and not treated)	Control second (not infected and not treated)

Results and Disccussion

Effects of ***L. edodes*extracts** on *P. infestans* infected **Elisabeta** tomato plants performed ***in vitro*** conditions shows the following results: treatments consisting of 2 % *L. edodes* extract, the signs of the infection seven days after were visible on almost entire surface of the plantlet. All leaves were yellow and curled, with small signs of necrosis. But the leaves were not faded. Big damages could not be seen, but the uniformity of the infection still represented a danger.Seven days after from application of 4 % *L. edodes* aqueous extract could not stop the development of disease symptoms. Practically, all the samples showed partially faded leaves, with yellow margins and necrosis and curled tops

with necrosis spots.

The plantlets treated with 6 % *L. edodes* extract, after seven days, presented some infection symptom signs, such as: turning yellow of the leaves ends and small necrosis spots where has been the pathogen inoculation. The leaves were not dried, but the signs of the infection are strongly visible at over 60 % of the area of the plantlets.

Extracts applied on infected **Elisabeta**or**Rio Grande** tomato plants obtained in *ex vitro* conditions hadthe follow effects:

The Elisabeta plants treated with 2 % *L. edodes* extract, seven days after treatment, showed 15 % small green leaves with small yellow spots. The rest of the plant was yellow, with clear signs of infection on the margins of the leaves, and the damaged areas due to pathogen inoculation, were necrosed. Some of the plantlets have lost their cotyledon us leaves, but the general aspect of the plantlet is positive, the infection being less visible than in the case of the plantlets treated with 0.1 % stamycin solution or 1.0 % copper sulphate solution.

Unlike the experimental variants corresponding to *ex vitro* treatments, tomato plants treated with 4 % *L. edodes* extract showed, seven days after, weaker signs of the infection. Cotyledonuos leaves and first row of the real leaves were faded at over 80 % of the plantlets. The leaves from the upper half side showed signs of yellowing with necrosis on top of some leaves, and in the area of the lesions caused by pathogen inoculation. On the other hand, leaves treated with 6 % *L. edodes* extract showed small yellow spots of necrosis. In this case, the number of faded leaves was very small and the plantlets shape was close to a normal one.

The Rio Grande tomato plants treated with 2 % *L. edodes* extract, showed weak leaves and signs of turning yellow. The leaves exhibits necrosed areas and curled edges.

Follow the treatments with 4 % *L. edodes* extract, the plantlets showed strong signs of turning yellow and necrosis on the margins and on top of the leaves. The cotyledon us leaves dried and fell, and the real leaves were a little faded. It was not noticed an important difference comparing to the Rio Grande variants treated with 2 % *L. edodes* extract. The plantlets degrade slowly than the control ones, but they do not show any sign of recovery.

The plantlets treated with 6 % *L. edodes* extract, showed yellow leaves, with curled and necrosed margins. An important part of them are dried and the general aspect is slightly faded, the signs of infection being strongly visible.

In Figure no. 1 it was shown the influence of the treatements with 2 % *L. edodes* applied to Elisabeta or Rio Grandetomato varieties previously infected with *P. infestans*, upon the average of the period of viability (days). The data recorded reflect a better resistance of Elisabeta tomato variety comparing to Rio Grande variety. Elisabeta plantlets obtained in *ex vitro* conditions had an average

value of the viability period of 9.7 (± 0.57) days, unlike the same type cultivated *in vitro* conditions, which had an average viability period of only 8.9 (± 0.72) days. Rio Grande type cultivated in *ex vitro* conditions allowed recording similar results, with an average period of viability of 8.7 (± 0.66) days.

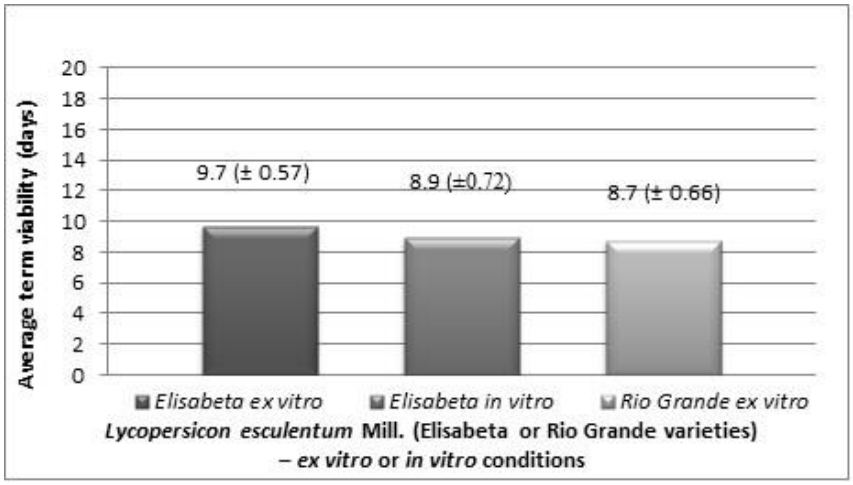

Fig. 1. Effects of 2 % *L. edodes* extract treatments on infected tomato plants, upon average of the viability period (days)

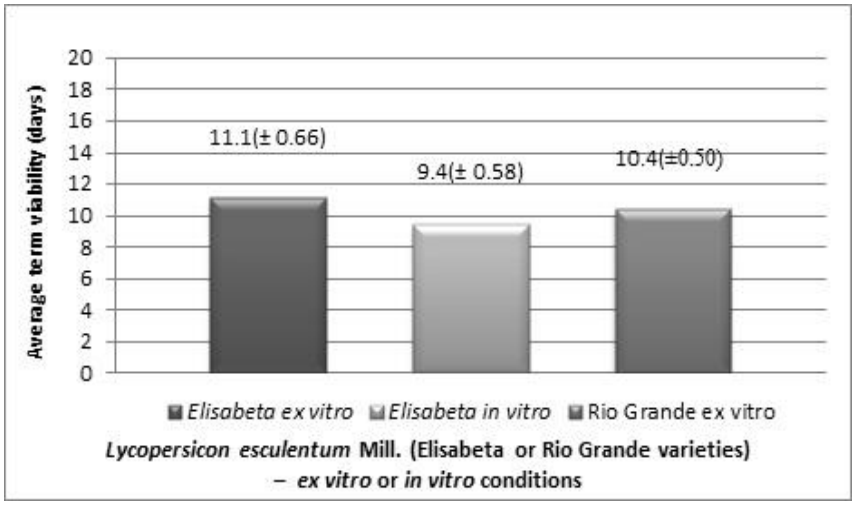

Fig. 2. Effects of 4 % *L. edodes* extract treatments on infected tomato plants

The experimental results for the average of the viability period recorded as a result of applying treatement with 4 % *L. edodes* extract on infected Elisabeta

or Rio Grande tomato varieties are shown in Fig. 2. Unlike the treatement with 2 % aqueous extract, Elisabeta variety resisted on average 11.1 (± 0.66) days in *ex vitro* conditions and in *in vitro* conditions, Elisabeta plantlets had a survival rate of 9.4 (± 0.58) days. Rio Grande variety after this treatement reached an average of the viability period of 10.4 (± 0.50) days, one day more than the previous treatment.

In the case of treatements with 6 % *L. edodes* extract applied to infected Elisabeta or Rio Grande tomato varieties,have been recorded the best results, with a rise of the viability period of over 50 % (Fig.3).

Elisabeta tomato plants cultivated in *ex vitro* conditions is the most longevity type, 19.1 (± 0.75) days, the average viability period being almost double comparing to the treatments with 2 % or 4 % *L. edodes* extracts. The same type obtained *in vitro* had an average viability period of 14.8 (± 0.53) days, being more longevive comparing to the treatement with 2 % or 4 % *L. edodes* extracts.

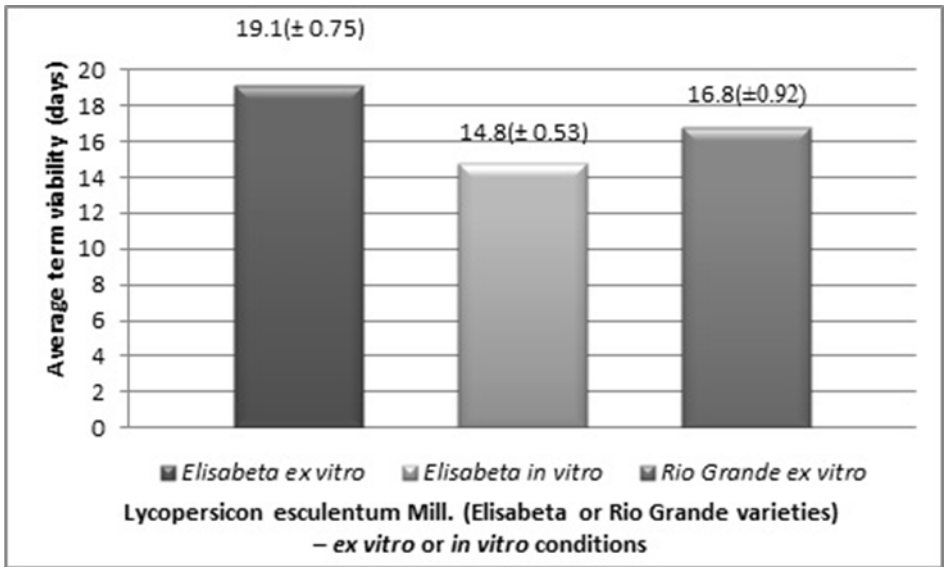

Fig. 3. Effects of 6 % *L. edodes* extract treatments on infected tomato plants

Rio Grande tomato variety cultivated in *ex vitro* conditions reached to an average of viability period of 16.8 (± 0.92) days comparing to 8.7 (± 0.66) days, as it was recorded after the first treatement.

Also, it can be seen from the recorded experimental results that for Elisabeta variety, the average values of the viability period (days), are bigger in *ex vitro* conditions comparing to the ones *in vitro*.

These results support the idea that identifying the optimum concentration levels of L. edodes aqueous extracts and correlating them with the

right tomato variety. Treatments can be realized in order to disturb the activity of *Phytophthora infestan* fungi, at least in *ex vitro* conditions.

Results recorded seven days after on i*n vitro* treatment with 1.0 % copper sulphate solution, applied to artificially infected Elisabeta tomato plants with *P. infestans* did not show any sign of recovery. The degradation of the plantlets treated with copper sulphate solution was almost as fast as the ones untreated. The young leaves showed necrosis as a sign of an advanced infection. Also, *ex vitro* treatments with copper sulphate1.0 % solution applied on infected Elisabeta tomato plants, they showed a rapid degradation which was faster than the untreated ones. The plantlets were faded over 75 %, and in less than 14 days, they were all died. Explanation may be that the treatments with 1.0 % copper sulphate solution were too strong for the plantlets.

The infected Rio Grande tomato plants treated with copper sulphate1.0 % solution show visible signs of decline determined by the pathogen and the toxicity of the substance. The leaves of the plantlets are weak, yellow, they have their tops curled down and they show small signs of necrosis. The results are similar to those observed at Elisabeta tomato variety, both *in vitro* and *ex vitro* conditions.

Treatments with **0.1 % stamycin**, applied to ***in vitro*** infected **Elisabeta** plants showed clear signs of infection, but the general aspect is better than the ones treated with copper sulphate 1.0 % solution. In addition, new leaves had appeared which did not show signs of infection. In ex vitro conditions most of the **Elisabeta** plants treated with antibiotic did not have their cotyledon us leaves anymore, and the real leaves were partially faded and yellow, after seven days from the application of the treatment based on 0.1 % stamycin solution. The signs of necrosis were visible at over 60 % of the leaves, especially on the curled margins. After seven days from the antibiotic treatments application on Rio Grande infected plants most of the plantlets were yellow completely and they were showing spots of necrosis. The leaves were faded and the new ones were already showing the signs of disease.

Conclusions

The main conclusions from the current study were:

- all the infected tomato plants treated with different concentrations of *L. edodes* aqueous extracts had a slower evolution of the disease comparing with those not treated;
- between the various concentrations of *L. edodes* extracts used in experiments against *P. infestans*, 6 % *L. edodes* extracts applied on infected tomato plants significantly reduced the severity of disease in plants caused by artificial inoculation with *P. infestans.*

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