ANTIFUNGAL ACTIVITY OF ROOT EXTRACT OF *ASCLEPIAS SYRIACA* L. ON CAUSAL AGENTS OF APPLE BITTER ROT

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Abstract. *During storage, apple fruits are exposed to the potential infection by various phytopathogenic fungi. The damage that occurs in this period can be substantial due to the fact that the fruit is already formed and ready for market placement. Some of the most common fungi in apple storage are Penicillium spp., Alternaria spp., Colletotrichum spp., Monilinia spp., Botrytis cinerea. Control of these fungi mostly relies on chemical treatments before harvest. The negative impact on the environment, pesticide residues in soil and plant parts and resistance in target organisms, are just some of the consequences caused by frequent use of chemical pesticides, which is why research and application of biopesticides in plant protection is desirable. In previous years, the weed species Aclepias siryaca has been spreading intensively in the Balkans, including Serbia. Supposedly, it has arrived in Serbia from the neighboring country of Hungary, where it was mostly grown as a honey plant. Today, this plant causes great damage to the ruderal and arable agricultural lands. With its large habitus, it is a great competitor to other plants, both spontaneous and cultivated crops, especially in wheat, sunflower, soybean, barley, rapeseed crops, in orchards and vineyards. Chemical measures have so far proved to be the most successful form of control of this plant, applied in the early stages of development, but the best way is still a combination of mechanical and chemical methods. Although this plant can cause great damage on the one hand, on the other hand, there is the potential to find practical application in the control of some other weed species and phytopathogenic microorganisms. This trial examines antifungal effect of root extract of invasive plant species Asclepias syriaca against causal agents of apple bitter rot – Colletotrichum spp., with an aim to evaluate its potential as a biofungicidal treatment of apple fruits.*

Key words: *fruit storge, essential oils, biopesticides, Asclepias syriaca, Colletotrichum acutatum, Colletotrichum gloeosporoides*

INTRODUCTION

Apple is a fruit that occupies a very important place in human nutrition due to its multiple health benefits for adults and children. It is characterized by a wealth of vitamins and minerals, as well as water, carbohydrates, organic acids, necessary for the healthy functioning of the human body (KESEROVIC ET AL., 2016). Worldwide, apple production is undoubtedly important, both because of the exceptional nutritional values in the fresh state and because of the possibility of processing apple fruits in the form of jams, marmalades, juices etc (JELOCNIK ET AL., 2011). In the period between 2010 - 2020 we can see a steady increase in apple production in the world. Asia with over 50 million tons has the largest share in the mentioned period, followed by Europe, America, Africa and Oceania (FAO, 2020). Serbia is also an increasing producer of apples from year to year. According to the data of the Statistical office of the Republic of Serbia, the total area under apple orchards in 2017 on the territory of the Republic of Serbia was 25,281 ha. The largest producers in Serbia, according to KESEROVIC (2014), are the municipality of Subotica, followed by Grocka, Čačak and Arilje.

Today, when intensive farming system is represented, it is possible to market only healthy and high quality products. The apple fruit may be exposed to infection by phytopathogenic fungi not only in the growing season, but also during transport or storage, when the damage is much greater because the product is already formed and ready for placement. Infection by phytopathogenic fungi can greatly reduce the market and nutritional value of the fruit (PETRES ET AL., 2017).

The most common phytopathogenic fungi present on apple fruits are *Penicillium* spp., *Alternaria* spp., *Colletotrichum* spp., *Monilinia* spp., *Botrytis cinerea*. Their activity and harmfulness depend on a combination of several factors, starting with the sensitivity of the host, the presence of pathogens as well as environmental factors (GRAHOVAC ET AL., 2011). According to GRAHOVAC (2014), on the apple fruits, in Serbia, there are two species of the genus *Colletotrichum* – *C. acutatum* and *C. gloeosporoides.* Fungi of the genus *Colletotrichum* are cosmopolitan and aggressive species, which have a wide range of hosts, including pear, apple, cherry or tomato fruits (ZIVKOVIC ET AL., 2012). Today, in plant protection, especially apples, chemical control measures are dominant, which entail harmful effects on the environment in the form of pesticide residues in the soil or fruit, and thus on human health. That is why the integrated approach in production as well as the application of biological preparations is becoming more frequent (SOVLJANSKI ET AL., 2004).

Control of apple rot pathogens is necessary during production, before harvest, but it is also desirable after harvest, due to potential infection by various phytopathogenic fungi also in this period (GRAHOVAC ET AL., 2011). However, due to ecological issues, there are no currently available treatments for apple rot after harvest. The use of beneficial microorganisms or their metabolic products, as well as the use of essential oils and the application of plant extracts are possible ways of biological control application in plant protection (STEVIC, 2013), and present a great opportunity for apple fruits rot control after harvest.

Weed species *Asclepias syriaca* L. originating from the northern parts of central and northeastern United States and Canada (POPOV, 2016), today is widespread both in Europe and also in the Republic of Serbia (POPOV, 2013). It is a cosmopolitan species that can cause great damage to ruderal habitats where it is mostly present, however, recent literature states the potential of its antifungal effects (POPOV ET AL., 2020), and therefore in this study, extracts obtained from this plant species were tested for the activity against causal agents of apple bitter rot pathogens – *Colletotrichum*.

MATERIAL AND METHODS

PREPARATION OF *ASCLEPIAS SYRIACA* EXTRACT

Samples of *Asclepias syriaca* roots were collected in the vicinity of Novi Sad, on the sandy shores of the Danube River. The root was taken out during the fruiting period of the plant at a depth of 0-30 cm. The aqueous extract provided for this work was prepared by the method of Kalinova and the associates (2012). First, the cleaned silk root is cut into pieces 0.5 to 3 cm long, then it is dried to an absolutely dry mass, after which it is ground finely to obtain particles the size of one millimeter. 100 g of dry root mass was immersed in one liter of distilled water and shaken at room temperature for 24 hours in a shaker at a speed of 120 rpm. The extract thus obtained was filtered through filter

paper and centrifuged at 5000 rpm after which it is diluted to a concentration of 80 grams of dry mass of roots / 1 l of distilled water.

The methanol solution was obtained by mixing 80 g of dry root mass in 11 of 95% methanol at a temperature of 24 ° C in a shaker in the dark. After stirring, filtration was performed as with the aqueous extract. In the next step, methanol was evaporated in vacuo at 40 ° C to give a dry residue of 33.0332 g dry mass / l of distilled water.

ISOLATES OF PHYTOPATHOGENIC FUNGI

Two isolates of phytopathogenic fungi were used in the experiment. Both isolates belong to the genus *Colletotrichum*. One of the isolates used was *Colletotrichum gloeosporoides* (code ČA2) and the other species was *Colletotrichum acutatum* (code 166). Both isolates originate from apple fruit, and were identified to the species level using conventional, species-specific PCR. The isolates are part of the permanent collection of microbiological cultures of the Department of Plant and Environmental Protection, Faculty of Agriculture, University of Novi Sad. During the experimental setup, the isolates were cultured on PDA medium and incubated at 25 ° C for a period of five days. At the end of the incubation period, mycelial sections 3 mm in diameter along the colony circumference were formed and used for further trials to examine the effect of *A. syriaca* extract on the isolates in *in vivo* and *in vitro* assays.

IN VITRO TEST

The effect of aqueous and methanol extract of *A. syriaca* on the apple fruit rot pathogens from the genus *Colletotrichum* was investigated by the method of monitoring radial growth of isolates on potato dextrose agar (PDA) medium with incorporated extract. In the experiment, appropriate amounts of distilled sterile water and prepared extracts were added to the molten, cooled (50 ° C) PDA medium to obtain the desired concentrations of extract in the medium (0.04 and 0.08 ml of extract / ml of medium). Homogenization of the mixture was performed on a magnetic stirrer, and then the mixture was poured into Petri dishes. In the control variant, only sterile, distilled water was added to the PDA medium. After medium solidification, sections of mycelium with a diameter of 3 mm were placed in the center of the Petri dish. The experiment was set up in three replicates.

The effect of aqueous and methanol extract of *A. syriaca* was determined after three and six days of incubation of Petri dishes prepared in this way at 25 ° C in the dark, by measuring the diameters of the developed colonies in two perpendicular directions.

IN VIVO TEST

Due to the antagonistic effect recorded in *in vitro* experiments, the antifungal activity of *Asclepias syriaca* extract on *Colletotrichum gloeosporides* and *Colletotrichum acutatum* was also examined in *in vivo* assays. The extract that exhibited the most pronounced mycelial growth inhibition in *in vitro* assay was selected for further *in vivo* experiments.

Holes with a diameter of 4 mm and a depth of 3 mm were made on the sterilized surfaces of apple fruits of the Golden Delicious cultivar. Two openings were formed on each fruit, the experiment was set up in three repetitions, one fruit with two openings represented one repetition.

Undiluted *A. syriaca* water extract, 10% solution of *A. syriaca* extract or fungicide with active ingredient combination boscalid + pyraclostrobin at concentration of 1 g in 250 ml of water was introduced into the openings on the fruit. Immediately after the treatment, fragments of mycelium with a diameter of 3 mm were introduced into the openings so that the mycelium rests directly on the tissue of the apple.

Table 1

Tested treatments

Treatment	**Concentration /amount of application**
A. syriaca extract	100%
	10%
Synthetic fungicide *(boscalid +pyraclostrobin*)	1 g in 250 ml of water

Inoculated apple fruits treated with sterile, distilled water were used as a control. One half of treated, inoculated fruits was placed in humid chambers at room temperature, and the other half in cold storage at 2±0,5°C. The evaluation was performed after six and ten days of fruits incubation at room temperature, and for cold stored fruits evaluation was performed after 1-month storage and additional 7 days of incubation at room temperature. The evaluation was performed by measuring the diameter of the developed necrotic (rot) lesions.

STATISTICAL ANALYSIS

Data was processed by analysis of variance, and the significance of the differences was determined by Duncan's post hoc test in the *Statistica* 13.5 software package.

RESULTS AND DISCUSSION

IN VITRO TEST

According to the results recorded after 3 and 6 days of incubation, the analysis of variance showed that the fungal isolates, extracts and concentrations of extracts had a significant effect on the mycelial growth diameter of the tested isolates ($p < 0.01$).

As shown in Tables 2 and 3, the *A. syriaca* plant extract significantly inhibited mycelial growth of the tested isolates compared to the control. Higher concentration of aqueous extract of *A. syriaca* caused complete inhibition of mycelial growth of both isolates, regardless incubation duration.

After 3 days of incubation (Table 2), the aqueous extract showed significantly stronger inhibition of mycelial growth of the tested isolates compared to the methanol extract, regardless of the applied concentration. A higher concentration of methanol extract did not

cause significantly stronger inhibition compared to a lower concentration; while the lower concentration of the aqueous extract had a significantly weaker effect compared to the high concentration of the isolate *C. gloeosporioides*, while in the case of the isolate *C.acutatum* both concentrations of the aqueous extract completely inhibited the development of the isolate.

Table 2

Duncan's test of multiple intervals: the effect of A. syriaca extract on the mycelial diameter of the tested isolates after three days of incubation

Isolate	*A. syriaca* extract	Concentration (ml extract / ml medium)	Mycelium diameter (mm)
***C. gloeosporioides* (ČA-2)**	Aqueous extract	0.08	3.00[a]
		0.04	11.25[d]
	Methanol extract	0.08	14.75[e]
		0.04	14.5[e]
	control-distilled water		40.00[g]
***C. acutatum* (166)**	Aqueous extract	0.08	3.00[a]
		0.04	3.00[a]
	Methanol extract	0.08	8.50[b]
		0.04	8.50[b]
	Control - distilled water		23,5[f]

values followed by the same letter are at the same level of significance

The results obtained after 6 days of incubation (Table 3) also suggest that the aqueous extract caused significant inhibition of mycelial growth of the tested isolates compared to the methanol extract. Higher concentration of the aqueous extract led to complete inhibition of the growth of both isolates. However, in the case of *C. gloeosporiodes* isolates, the lower concentration of the aqueous extract showed a significantly weaker effect compared to the higher concentration and achieved growth inhibition at the same level as lower concentration of methanol extract. Compared to the untreated control, the methanol extract also showed significant inhibition of isolate growth.

Table 3

Duncan's test of multiple intervals: the effect of *A. syriaca* extract on the mycelial diameter of the tested isolates after six days of incubation

Isolate	*A. syriaca* extract	Concentration (ml extract / ml medium)	Mycelium diameter (mm)
***C. gloeosporioides* (ČA-2)**	Aqueous extract	0.08	3,00[a]
		0.04	27,00[fg]
	Methanol extract	0.08	30,25[hi]
		0.04	27,25[fg]
	control-distilled water		76,00[m]
***C. acutatum* (166)**	Aqueous extract	0,08	3,00[a]
		0,04	3,00[a]
	Methanol extract	0,08	8,50[b]
		0,04	8,50[b]
	control-distilled water		23.5[i]

values followed by the same letter are of the same level of significance

***IN VIVO* TEST**

Since in *in vitro* test the aqueous extract showed a significantly more pronounced inhibition of the development of the tested isolates, this extract was chosen to examine its inhibitory effect on the causes of bitter apple rot on the apple fruits themselves.

1. **Incubation at room temperature**

After 6 and ten days of incubation of fruits at room temperature, analysis of variance showed that the applied treatments significantly influenced the development of necrosis on artificially inoculated fruits.

As shown in Table 4, in the case of isolates of *C. gloeosporioides*, it was observed that the inhibitory effect of all applied treatments led to significant inhibition of necrosis development compared to control, and no significant differences were observed between treatments with synthetic fungicide (*boscalid + pyraclostrobin*) and different concentrations of *A. syriaca* root extract. In *C. acutatum* isolates, the synthetic fungicide showed the most significant inhibition of necrosis development, while the effect of *A. syriaca* root extract was completely absent regardless of the concentration of application - developed necrosis on apple fruits treated with the extract was at the same level of significance with untreated control.

Table 4

Duncan's test of multiple interverses: the influence of the examined extracts of *A. syriaca* on the development of necrosis on apple fruits artificially inoculated with *C. gloeosporioides* and *C. acutatum*, after six days of incubation.

Isolate	Treatment	Necrosis diameter (mm)
***C. gloeosporioides* (ČA-2)**	Synthetic fungicide (boscalid + pyraclostrobin)	3,00 ± 0.00[a]
	Undiluted water extract of *A. syriaca*	4,87 ± 2.53[a]
	10% *A. syriaca* water extract	6,50 ± 4.11[a]
	Untreated control	16,25 ± 4.77 [b]
***C. acutatum* (166)**	Synthetic fungicide (boscalid + pyraclostrobin)	3,75 ± 1.16[a]
	Undiluted water extract of *A. syriaca*	13,25 ± 1.28[c]
	10% *A. syriaca* water extract	11,00 ± 2.14[b]
	Untreated control	11,75 ± 1.04[bc]

values followed by the same letter are of the same level of significance

Table 5 shows the results of the development of necrosis on apple fruits after 10 days of incubation. In *C. gloeosporioides* isolates, all applied treatments maintained the effect of significant inhibition of necrosis development after 10 days compared to untreated control. However, treatment with the synthetic fungicide stood out and provided significantly lower development of necrosis on apple fruits and compared to apple fruits treated with different concentrations of *A. syriaca* root extract.

In the case of *C. acutatum* isolates, the applied treatments with *A. syriaca* extract did not provide a satisfactory effect, regardless of the concentration of application - the development of necrosis on the treated fruits was at the same level of significance with the untreated control. Synthetic fungicide significantly inhibited the development of necrosis compared to untreated control and treatments in which the extract was applied.

Table 5

Duncan's test of multiple intervals: the influence of the examined extracts of *A. syriaca* on the development of necrosis on apple fruits artificially inoculated with *C. gloeosporioides* and *C. acutatum*, after 10 days of incubation

Isolate	Treatment	Necrosis diameter (mm)
***C. gloeosporioides* (ČA-2)**	Synthetic fungicide (boscalid + pyraclostrobin)	3.00 ± 0.00[a]
	Undiluted water extract of *A. syriaca*	12.37 ± 6.76[b]
	10% *A. syriaca* water extract	10.62 ± 8.25[b]
	Untreated control	32.12 ± 7.38[c]
***C. acutatum* (166)**	Synthetic fungicide (boscalid + pyraclostrobin)	7.87 ± 3.23[a]
	Undiluted water extract of *A. syriaca*	23.87 ± 4.52[b]
	10% *A. syriaca* water extract	22.50 ± 3.12[b]
	Untreated control	23.75 ± 5.28[b]

values followed by the same letter are of the same level of significance

2. Incubation in cold storage

On fruits that were incubated in a cold storage for a month, no symptoms of bitter rot were observed on the day of storage termination.

After storage, the fruits were additionally incubated at room temperature for 7 days, and analysis of variance showed that the applied treatments significantly influenced the development of necrosis on the fruits.

As shown in Table 6, after additional incubation at room temperature, in the case of *C. gloeosporioides* isolates, a complete inhibitory effect on the development of necrosis was observed in all treatments applied compared to the untreated control.

However, in the case of *C. acutatum* isolates, the applied treatments did not show a satisfactory effect, regardless of the concentration of application. The development of necrosis on the treated fruits was at the same level of significance as the untreated control, in the case of each applied treatment.

Table 6

Duncan's test of multiple intervals: influence of tested *A. syriaca* extracts on the development of necrosis on apple fruits artificially inoculated with *C. gloeosporioides* and *C. acutatum*, after one month of cold storage and additional incubation for seven days at room temperature

Isolate	Treatment	Necrosis diameter (mm)
***C. gloeosporioides* (ČA-2)**	Synthetic fungicide (boscalid + pyraclostrobin)	3.00 ± 0.00^{a}
	Undiluted extract water of *A. syriaca*	3.00 ± 0.00^{a}
	10% *A. syriaca* water extract	3.87 ± 1.64^{a}
	Untreated control	16.00 ± 3.21^{b}
***C. acutatum* (166)**	Synthetic fungicide (boscalid + pyraclostrobin)	15.00 ± 4.78^{a}
	Undiluted water extract of *A. syriaca*	18.75 ± 3.06^{a}
	10% *A. syriaca* water extract	16.00 ± 1.85^{a}
	Untreated control	17.12 ± 6.42^{a}

values followed by the same letter are of the same level of significance

Based on the obtained results, it can be concluded that the aqueous extract of *A. syriaca* show more pronounced inhibition of *C. acutatum* mycelium growth on nutrient medium compared to *C. gloeosporoides*, while in *in vivo* test,on apple fruits, inhibitory effect of *A. syriaca* aqueous extract on necrosis development was observed only in the case of *C. gloeosporoides*. This result can be explained by the different temperature requirements of these two fungi. According to Grahovac (2014), the species *C. gloeosporoides* is thermophilic compared to the species *C. acutatum*, therefore it has been proven to have lower virulence on apples under low temperature, i.e. when storing fruits at low temperatures. This fact can be related to the fact that the aqueous extract on apple fruits significantly controlled species *C. gloeosporoides*, compared to *C. acutatum* in *in vivo* test, but only in the case of storing the fruit at a lower temperature. The absence of activity of the extract on *C. acutatum* in the *in vivo* test without storage of fruits at low temperature cannot be explained by differences in virulence of isolates, since in control, untreated variants the diameter of fruit necrosis

was higher in the case of *C. gloeosporioides*. It remains for additional research to establish the reason for the lack of effect on the species *C. acutatum* in the *in vivo* test.

There is no data in the available literature to date indicating the antimicrobial activity of *A. syrias* in the control of phytopathogenic microorganisms. However, members of the *Asclepiadaceae* family are listed as medicinal plants applicable in human medicine, exhibiting antimicrobial effects. The most studied species of *A. curassavicahas* shown antibacterial and antifungal activity in several studies (Hemavani and Thippeswamy, 2012). Accordingly, as this study has shown, *A. syriaca*, as a member of the family *Asclepiadaceae*, exhibits antimicrobial activity against phytopathogenic organisms of the *Colletotrichum* genus. However, for now, the proven antifungal effect on apple fruits only on the species *C. gloeosporoides*, does not justify further examination in this direction because the dominant cause of bitter fruit rot in our warehouses in the period 2009 - 2014. was the species *C. acutatum* (Grahovac, 2014), for which only the in vitro antifungal activity of the tested agent was proven.

CONCLUSIONS

Having in mind long - lasting consequences of intensive pesticide use in plant protection for the environment and humans, in order to protect plants, attention should be focused on non - chemical control measures. As this study showed, the extract of the root of the plant *A. syriaca*, exhibits antimicrobial activity against phytopathogenic organisms of the genus *Colletotrichum*. However, for now, the proven antifungal effect on apple fruits only on *C. gloeosporoides* does not justify further examination in this direction because *C. acutatum* is dominant causative agent of bitter fruit rot in our apple storages, for which only the *in vitro* antifungal action of the tested agent has been proven. Further research should focus on examining causes of the lack of effect of *A. syriaca* extract on *C. acutatum* on apple fruits, *in vivo*, and the possible improvements.

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