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Original scientific paper

Allelopathic effects of actinobacterial isolates on seed germination and early seedling growth of velvetleaf (*Abutilon theophrasti* Medik.)

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SUMMARY

This study aimed to examine *in vitro* allelopathic effects of actinobacterial isolates on seed germination and early seedling growth of velvetleaf (*Abutilon theophrasti*). Thirty-five actinobacterial isolates were obtained from soil and compost in different phases of composting. Also, an experiment variant involving the herbicide mesotrione was set in the recommended amount of application, as a reference standard.

The experimental results indicate statistically significant differences ($p<0.05$) between controls and all treatments with actinobacterial isolates regarding seed germination, and radical and shoot length. The highest inhibition (100%) was observed in seed germination and radical length in treatments with the isolates A10 and NOV2, compared to uninoculated starch casein broth (SCB) as control. Shoot length was shown to be the most sensitive parameter, where 100% inhibition was observed in the following treatments with actinobacteria: A010, A017, NOV2, NOV3, NOV4 and NOV5. Actinobacterial isolates showed a higher inhibitory effect on seed germination than treatment with the reference herbicide mesotrione.

Keywords: actinobacteria, velvetleaf, seed germination, allelopathy

INTRODUCTION

Synthetic herbicides have had a central role in the history of weed management. Although they are efficient in controlling weeds, their continual use has resulted in weeds developing resistance to some herbicides (Santos, 2009). Furthermore, reacting to health and environmental concerns over their indiscriminate usage, numerous countries have banned many of those agrochemicals (Eisler, 2000). Although it is hard to leave off herbicides, it is possible to reduce their use by applying integrated weed management approaches in field crop production (Anderson, 2007). As a result, there is a rapidly growing trend at present towards discovering effective and safer alternatives to synthetic herbicides. Strategies that are complementary to herbicides are gaining increasing importance and aim to reduce dependence on chemical control, and to

alleviate negative impact that these compounds impose on the environment.

Allelopathy is a biological process representing the activity of various biochemical agents of one organism on the germination, growth or reproduction of another organism, and the process can be utilized as a method of weed control (Marcías et al., 2007). This method is considered to be an efficacious, cost-effective and environmentally friendly approach to weed control. Allelopathic control of weeds can be applied either as a unique strategy in certain systems, for example in organic production, or in combination with other methods of integrated plant protection (Saxena & Pandey, 2001; Jabran et al., 2015). Another aspect of allelopathy is the applicability of different groups of soil microorganisms and their metabolites for weed control, which offers an important alternative to the use of chemical products (Inderjit, 2005).

Actinobacteria (class *Actinobacteria*) represent one of the most widely distributed groups of microorganisms in nature. They have an outstanding role as agents of biodegradation in soil and formation of humus. They are Gram-positive bacteria whose DNA has a high content of GC pairs (55%) and they are the source of numerous secondary metabolites (Wang et al., 1999). Actinobacteria own a significant antagonistic potential against a variety of pathogens of cultivated plants and human pathogens (Ouhdouch et al., 2001; Šantrić et al., 2018). They are also important for the production of antibiotics (Agarwal & Mathur, 2016) and enzymes (Mukhtar et al., 2017). The most frequent genus of actinobacteria is *Streptomyces*. Many of their secondary metabolites show fungicidal, insecticidal and herbicidal properties (Shi et. al., 2020).

Weed interference in agricultural fields reduces the quality and quantity of agricultural products, causing huge economic losses to farmers. Velvetleaf (*Abutilon theophrasti* Medik.) is a major weed in maize and other summer row crops in many European countries, including Serbia (Sattin et al., 1992; Travlos et al., 2012; Vrbničanin et al., 2017). This species is generally highly competitive for nutrients, light and moisture, and it is able to drastically reduce yields and harvesting efficiency (Nurse & Di Tommaso, 2005). To our best knowledge, there are no available reports on the allelopathic potential of different actinobacterial isolates against this weed species. The present study therefore aimed to evaluate *in vitro* the allelopathic potential of actinobacterial isolates and their effects on seed germination and early seedling growth (radical and shoot length) of *A. theophrasti*.

MATERIALS AND METHODS

Isolation of actinobacteria

Isolation was performed from samples collected from arable fields and casing soils. Samples weighing 10 g were mixed with 90 ml of sterile distilled water and several serial dilutions were spread onto synthetic agar with sucrose, starch ammonia agar and starch casein agar (SCA) and incubated at 27°C for seven days. Thirty-five subcultured single colonies were transferred onto potato dextrose agar (PDA), and SCA was purified by streak plate technique (Jarak & Đurić, 2006).

Seed material

Seeds of the weed species *A. theophrasti* were collected in a field located in Belgrade's Jakovo suburb in October 2019. All seeds were cleaned and stored in paper bags in the laboratory at the temperature of 20-22°C.

Preliminary testing of allelopathic potential of actinobacterial isolates

In a preliminary test, primary inoculums of thirty-five isolates were prepared in Erlenmeyer flasks containing 25 ml of starch casein broth (SCB) and incubated for 7 days in an orbital shaker incubator at 120 rpm and 28°C. Culture filtrates were aseptically obtained by filtration through Whatman No 2 filter paper. Seed surface of *A. theophrasti* was sterilized with 5% sodium hypochlorite solution (NaOCl) for 2 minutes and then rinsed three times with sterilized distilled water to avoid possible inhibition of germination due to fungal or bacterial toxins. Ten disinfected seeds were placed into each sterilized Petri dish with filter papers and moistened with 2 ml of the primary filtrate of each actinobacterial isolate. Two controls were prepared in the same way in which uninoculated SCB broth and sterilized distilled water were used separately instead of culture filtrate. All dishes were sealed with parafilm to avoid evaporation and placed in an incubator (VELP, Incubator FOC) at 28±1°C in the dark. After 5 days, nine isolates with allelopathic potential were observed based on inhibition assessment of seed germination and early seedling growth (radical and shoot length).

Secondary test of allelopathic potential of actinobacterial isolates

In a secondary test, 60 ml of SCB was inoculated with 3 ml of primary inoculums of nine isolates and incubated for 7 days in an orbital shaker incubator at

120 rpm and 28°C. Culture filtrates were aseptically obtained by filtration through Whatman No 2 filter paper. Two controls were prepared in the same way as the uninoculated SCB broth and sterilized distilled water that were used separately instead of culture filtrate. All dishes were sealed with parafilm to avoid evaporation and placed in an incubator (VELP, Incubator FOC) at 28±1°C in the dark. After 5 days, germination percentage was calculated and seedling growth (radical and shoot length) was measured (Bataineh et al., 2008). Also, treatment with the herbicide mesotrione (commercial product CHIEF, mesotrione 100 g/l, Adama, Israel) was carried out at the recommended field rate of 120 g a.i./ha, i.e. 1.2 l/ha, as a reference standard. Inhibition percentage, based on the changed germination and seedling growth, was calculated using the formula:

$$\% \text{ inhibition} = [(Xc-Xt)/Xc]\times 100$$

where Xc = % of germination or seedling growth in control; and Xt = % of germination or seedling growth in treatments with actinobacterial isolates or the herbicide.

The experiment design was a randomized complete block with four replications (10 seeds per Petri dish), repeated twice, and data were combined for analysis.

Morphological and cultural characteristics of actinobacteria isolates

Nine actinobacterial isolates were inoculated on SCA and colony color was checked. Spores were examined under a microscope (CX41RF Olympus, Düsseldorf, Germany), while the Gram staining procedure for actinobacteria was performed using crystal violet and Lugol solution (Knežević-Vukčević & Simić, 2015).

The actinobacterial isolates were inoculated onto several different ISP (International Streptomycetes Project) media: yeast malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salt starch agar (ISP4), glycerol asparagine agar (ISP5), peptone yeast extract iron agar (ISP6) and tyrosine agar (ISP7) (Himedia Laboratories, Mumbai, India). After 7 days, growth, pigmentation of aerial and substrate mycelium, and diffusible pigment production were observed (Shirling & Gottlieb, 1966; Wink, 2014).

Physiological and biochemical characteristics of actinobacterial isolates

Physiological characteristics were determined on SCA medium at different temperatures (5°C, 16°C, 26°C, 35°C) and pH levels (4, 7, 9), and the isolates were incubated at 27°C under different salt concentrations (3%, 5%, 7%). Their growth was measured after 7 days of incubation.

The starch hydrolysis test was performed on starch agar. Actinobacterial isolates were incubated for 5 days at 28°C. After incubation, the plates were flooded with Lugol's solution and inspected for the presence or absence of bright zones around colonies.

For utilization of carbon sources, the Hugh-Leifson medium was prepared and sterile carbon sources (1%) were added, including dextrose, sucrose and glucose. After incubation at 28°C for 7 days, a positive reaction was detected (green color around colony was turning yellow).

Gelatin hydrolysis was run on nutrient gelatin agar. Tubes were inoculated using the stub method and incubated at 27°C for 7 days along with uninoculated control tubes. After incubation, the tubes were kept at cold temperature. Hydrolyzed gelatin was detected in tubes with liquid media.

For catalase production, sterile yeast dextrose agar was inoculated with actinobacterial isolates and incubated at 28°C for 7 days. After incubation, a few drops of 3% hydrogen peroxide were added to each isolate, and the development of air bubbles indicated a positive reaction.

The use of citrate followed, and the SCA medium was inoculated with actinobacterial isolates and incubated at 30°C for 48 h. A change in color from green to blue indicated a positive reaction.

For melanin production, actinobacterial isolates were inoculated into the ISP6 and ISP7 media. The isolates were assumed to produce melanin when dark-brown or black diffusible pigments formed in the medium.

Statistical analysis

Data were analyzed by a one-way analysis of variance (ANOVA) using STATISTICA 8.0. software package. When F-values were statistically significant ($p<0.05$) treatments were compared using Fisher's Least Significant Difference (LSD) test.

RESULTS AND DISCUSSION

Characteristics of actinobacterial isolates

Thirty-five isolates of actinobacteria from field soil and casing soil for button mushroom were collected. After a preliminary test with *A. theophrasti* seeds, 9 isolates (A01, A02, A07, A010, A017, NOV2, NOV3, NOV4 and NOV5) showed inhibitory effect (of 50% and more) on seed germination and early growth. Another test was set up with those 9 isolates to examine their allelopathic activity on *A. theophrasti* seeds. Morphological and biochemical characteristics of the selected isolates are described in Tables 1 and 2.

Table 1. Morphological and cultural characteristics of actinobacterial isolates

Characteristics	A01	A02	A07	A09	A010	A017	A018	NOV2	NOV3	NOV4	NOV5
Colony color	grey	pink	cream	grey	green	grey	grey	green	white	pink	cream
Gram reaction	+	+	+	+	+	+	+	+	+	+	+
Spore form	round	round	round	round	round	round	round	round	round	round	round
ISP2 gowth	++	++	++	++	++	++	++	++	++	++	++
a. mycelium	grey	pink	grey	grey	green	grey	white	white	white	white	grey
s.mycelium	green	purpl	brown	yellow	brown	brown	brown	green	orange	pink	brown
soluble pigment	brown	purpl	-	-	brown	brown	brown	-	orange	brown	-
ISP3 gowth	++	++	++	+	++	++	++	++	+	++	-
a. mycelium	grey	pink	cream	cream	grey	grey	white	cream	white	pink	-
s.mycelium	green	purpl	green	yellow	brown	brown	cream	brown	brown	pink	-
soluble pigment	-	purpl	-	-	yellow	brown	-	brown	brown	-	-
ISP4 gowth	++	+	++	++	+	++	++	++	+	++	++
a. mycelium	grey	pink	cream	cream	green	green	grey	cream	grey	grey	cream
s.mycelium	green	pink	green	green	yellow	brown	cream	green	cream	brown	cream
soluble pigment	-	-	green	green	-	green	-	-	yellow	grey	-
ISP5 gowth	++	++	++	++	++	++	+	++	++	+	++
a. mycelium	green	pink	cream	cream	cream	grey	white	cream	grey	pink	cream
s.mycelium	orange	pink	cream	cream	cream	brown	white	brown	brown	pink	cream
soluble pigment	yellow	pink	yellow	-	-	brown	-	brown	brown	-	-
ISP6 gowth	++	++	++	++	++	+	+	++	++	+	+
a. mycelium	grey	grey	grey	pink	grey	cream	white	pink	white	cream	cream
s.mycelium	green	black	brown	brown	brown	brown	black	cream	brown	cream	cream
soluble pigment	brown	brown	brown	-	brown	brown	black	-	brown	-	-
ISP7 gowth	++	++	++	++	++	++	++	++	+	+	+
a. mycelium	grey	grey	cream	cream	green	brown	white	cream	cream	brown	cream
s.mycelium	grey	black	green	brown	brown	brown	black	green	brown	brown	cream
soluble pigment	green	brown	-	-	brown	brown	black	brown	brown	brown	-

(-) no growth; (+) minimal growth; (++) optimal growth

Based on their morphological and biochemical properties, the isolates were identified as members of the genus *Streptomyces,* family *Streptomycetaceae* (Shirling & Gottlieb, 1966; Wink, 2014).

Bioassay with actinobacterial isolates and weed seeds

The germination results for *A. theophrasti* seeds obtained with different actinobacterial isolates are presented in Table 3 and Figure 1. Our data showed that the highest final germination of *A. theophrasti* seeds was in both control treatments (sterilized distilled water – 85.0% and uninoculated SCB broth – 68.0%). The data also showed that the highest inhibition (100%) of *A. theophrasti* seed germination was achieved by treatments A010 and NOV2, compared to the SCB broth control. Conversely, inhibition was the lowest when the herbicide treatment was used (50%), compared to the sterilized distilled water control. In other treatments, the inhibition of seed germination ranged from 59% (NOV5) to 72% (A07) (Figure 1a). The obtained data revealed a highly significant effect ($p<0.05$) between the controls and all treatments with actinobacterial isolates and the herbicide mesotrione (Table 3).

Table 2. Physiological and biochemical characteristics of actinobacterial isolates

Characteristics	A01	A02	A07	A09	A010	A017	A018	NOV2	NOV3	NOV4	NOV5
pH medium:											
4	-	+	-	-	-	-	-	-	-	-	-
7	++	++	++	+	++	++	++	++	++	++	++
9	++	++	++	+	++	++	+	++	+	+	++
N_aCl (%):											
3	++	++	++	++	++	++	++	++	+	++	+
5	++	-	++	++	+	++	++	++	+	+	++
7	++	-	++	++	-	+	+	++	+	+	++
Temperature: (C°)											
5	-	-	-	-	-	-	-	-	-	-	-
16	++	+	+	-	+	+	+	++	+	+	++
26	++	++	++	++	++	++	++	++	++	++	++
35	-	++	-	+	++	++	++	+	++	++	++
Hydrolysis of gelatin	+	-	+	+	-	+	+	-	+	-	-
Catalase	+	+	+	+	+	+	-	+	+	+	+
Starch hydrolysis	+	-	+	+	-	-	-	-	+	+	+
Citrate	+	-	-	-	-	+	-	+	-	-	-
Melanin production	-	+	-	-	+	+	+	-	-	-	-
Saccharose	-	+	-	-	+	+	-	-	-	-	-
Dextrose	-	+	-	-	+	+	+	+	+	+	+
Glucose	-	+	-	-	+	+	-	+	+	+	+

(-) no growth; (+) minimal growth; (++) optimal growth

Table 3. Effects of different actinobacterial isolates and a herbicide on *Abutilon theophrasti* seed germination and early seedling growth (radical and shoot length)

Treatments	Final germination (%)	Radical length (cm)	Shoot length (cm)
Control (SDW)	85.00±3.26 **a**	2.04±0.50 **a**	2.56±0.33 **a**
Mesotrione	42.50±1.31 **c**	0.24±0.00 **bcd**	0.00±0.00 **c**
Control (SCB)	68.00±2.79 **b**	1.87±0.26 **a**	1.98±0.41 **a**
A01	23.75±1.18 **d**	0.86±0.03 **bc**	0.28±0.01 **bc**
A02	22.50±1.18 **d**	0.92±0.10 **b**	0.80±0.02 **b**
A07	18.75±0.88 **d**	0.62±0.02 **bc**	0.25±0.01 **bc**
A010	0.00±0.00 **e**	0.00±0.00 **d**	0.00±0.00 **c**
A017	25.00±1.00 **d**	0.48±0.00 **bcd**	0.00±0.00 **c**
NOV2	0.00±0.00 **e**	0.00±0.00 **d**	0.00±0.00 **c**
NOV3	20.00±1.26 **d**	0.45±0.01 **bcd**	0.00±0.00 **c**
NOV4	20.00±1.09 **d**	0.37±0.00 **cd**	0.00±0.00 **c**
NOV5	27.50±1.09 **d**	0.45±0.00 **bcd**	0.00±0.00 **c**

SDW - Sterilized distilled water; SCB - Starch casein broth; Data are reported as the mean ± standard deviation. Differences in final germination, shoot and radical length of *Abutilon theophrasti* were evaluated by one-way analysis of variance (ANOVA), completed by Fisher's Least Significant Difference (LSD) test. Means in the same column referring to the same parameter are marked with different letters (a, b, c, d) only when they differ significantly ($p < 0.05$).

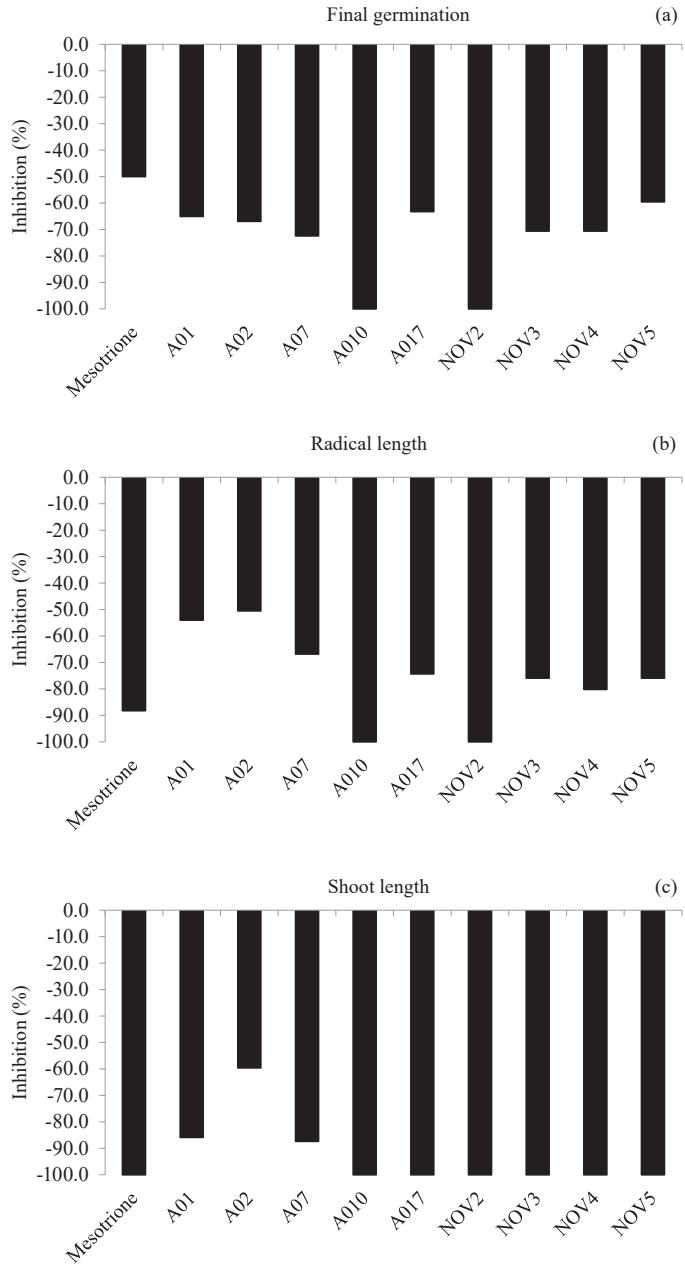

Figure 1. Inhibition (%) of final seed germination (a), radical length (b) and shoot length (c) under impact of different actinobacterial isolates and mesotrione herbicide

Literature data indicate a great allelopathic potential of actinobacteria, i.e. their secondary metabolites, on weed species. Priyadharsini et al. (2013) tested *in vitro* the herbicidal activity of a *Streptomyces* sp. isolate (KA1-3) on the weed species *Cassia occidentalis* L. and *Cyperus rotundus* L. The research showed that the bioactive compound N-phenylpropanamide had an inhibitory effect of up to 80% on seed germination of those weed species. Working with a *Streptomyces* sp. 8E-12 strain, Lee et al. (2003) isolated a herbicidal metabolite marked MHM, which stimulated leaf bleaching of the monocotyledonous weed species *Digitaria sanguinalis* and *Echinochloa crus-galli* under *in vivo* conditions.

The present study suggests that the tested actinobacterial isolates had adverse effects on the radical and shoot length of *A. theophrasti* (Table 3). The results of the seed germination test showed that two isolates, A010 and NOV2, achieved the highest inhibition (100%) of *A. theophrasti* radical length. Inhibition by other treatments with actinobacteria ranged from 50% to 80%, while mesotrione treatment caused 88% inhibition. On the other hand, shoot length was shown to be more sensitive as a parameter because 100% inhibition was observed in several treatments with actinobacteria (A010, A017, NOV2, NOV3, NOV4, and NOV5) and mesotrione (Figure 1b and 1c). Statistical analysis revealed signi ficant differences ($p<0.05$) between the controls and all treatments regarding both parameters (Table 3). Similarly, Singh et al. (2018) found several isolates with herbicidal action during a screening test that included over 300 endophytic actinobacteria. Applying secondary metabolites of those isolates on the weed species *Parthenium hysterophorus, Ageratum conyzoides,* and *Bidens biternata* before and after emergence, they observed a significant reduction in the length of their shoots and roots.

CONCLUSION

Of the 35 isolates of actinobacteria isolated from field soil and mushroom casing soil, 9 isolates (A01, A02, A07, A010, A017, NOV2, NOV3, NOV4 and NOV5) demonstrated allelopathic effects on seed germination and early shoot growth of the weed species *Abutilon theophrasti*. Based on morphological and biochemical characteristics, the isolates were identified as belonging to the genus *Streptomyces* of the family *Streptomycetaceae*. The isolates A010 and NOV2 demonstrated the best inhibitory potential (100%) for all measured parameters. In other treatments, inhibition of seed germination ranged from 59% to 72%, while shoot growth (i.e. radical and shoot length) decreased from 50% to 87%. The potential of actinobacteria for producing bioherbicide metabolites makes them a good candidate for biological weed control.

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Alelopatski efekti aktinobakterija na klijanje semena i rani porast klijanaca Teofrastove lipice (*Abutilon theophrasti* Medik.)

REZIME

Cilj ovog rada je bio da se ispitaju alelopatski efekti aktinobakterijskih izolata u *in vitro* uslovima na klijavost semena i rani porast klijanaca semena *Abutilon theophrasti*. Iz zemlje i komposta u različitim fazama kompostiranja dobijeno je 35 izolata aktinobakterija. Takođe, u ogled je uključen i tretman herbicidom (mezotrion) u preporučenoj količini primene, kao referentni standard. Na osnovu dobijenih rezultata može se utvrditi da postoji statistički značajna razlika ($p<0,05$) između kontrole (neinokulisana tečna skrobno-kazeinska podloga) i svih tretmana sa aktinobakterijskim izolatima na klijavost semena, dužinu korenka i stabaoceta. Najveća inhibicija (100%) klijanja semena i dužine korenka zabeležena je u tretmanima sa izolatima A10 i NOV2. Pokazalo se da je dužina stabaoceta osetljiviji parametar, jer je zabeležena inhibicija od 100% u većini tretmana A010, NOV2, NOV3, NOV4 i NOV5. Izolati aktinobakterija su pokazali veći inhibitorni efekat na klijanje semena u odnosu na tretman herbicidom mezotrionom.

Ključne reči: aktinobakterije, Teofrastova lipica, klijanje semena, alelopatija