UDK 631.466:632.954
Scientific paper

Received: 11 October 2025
Revised version accepted: 20 October 2025
Doi number: 10.5937/34ah-61426

Isolation, characterization, and herbicidal potential of soil actinobacteria

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SUMMARY

The effect of 22 actinobacteria isolates from arable soil on seed germination and the growth of maize, *Setaria glauca*, and *Sorghum halepense* was examined under laboratory conditions. Suspensions of eight isolates showed varying levels of inhibitory effects on seed germination and seedling growth. Based on the screening results, five actinobacterial isolates exhibited strong inhibition of one or both weed species, with minimal negative impact on maize. The SI I isolate was identified as the best candidate for further testing, as inhibition for maize was less than 18%, while it exceeded 81% for the weeds.

Keywords: arable soil actinobacteria, seed bioassay, maize, weeds, bioherbicide.

INTRODUCTION

The increasing demand for food has driven the development of agriculture, primarily through the use of chemical agents and mechanization. A major challenge in modern farming is the presence of weed species competing with cultivated plants. Weeds can cause significant economic losses, estimated between $100 million and $26 billion worldwide, and can reduce crop yields by up to 34% (Oerke, 2006; Ahmad et al., 2023). Weed science has made considerable progress in managing weeds effectively, affordably, and safely across various crops. However, the

overuse, misuse, and unprofessional application of synthetic herbicides create environmental and health risks, degrade food quality and safety, and highlight the need for alternative approaches (Gaines et al., 2020). One promising alternative involves natural compounds known as allelochemicals. Allelopathy has been studied as a means to reduce dependence on synthetic herbicides, as allelochemicals possess phytotoxic properties that can suppress the growth of weeds. These natural substances could help address environmental pollution, herbicide resistance, and organic weed management. They are environmentally safe, rapidly degradable, highly selective, and effective. Many natural products have been isolated from lower organisms such as algae, bacteria, and fungi, and are used commercially in plant protection. However, unlike other natural products used as fungicides, bactericides, insecticides, and acaricides, very few have herbicidal activity (Copping, 2001). Since 2014, 47 preparations based on microorganisms (biofungicides and bioinsecticides) have been registered in the European Union, but not a single bioherbicide. The challenges in developing bioherbicides are believed to arise from factors such as application methodology, dosage, timing, the specificity of microorganisms to host plants, and metabolite production (Hajnal Jafari et al., 2020).

Actinobacteria (formerly Actinomycetes) are among the most widespread groups of microorganisms in nature. They are present in large numbers in both cultivated and uncultivated soils and waters across various regions worldwide. Actinobacteria are Gram-positive bacteria that produce numerous secondary metabolites, with the genus *Streptomyces* from the Streptomycetaceae family being particularly notable for this capability (Mallik, 2001; Li et al., 2002). Secondary metabolites can be classified into several groups, such as amino acids, peptides, nucleosides, lactones, amides, and macrolides, some of which have been successfully developed as commercial herbicides (Shi et al., 2020). The first commercial herbicide based on actinobacteria or *Streptomyces* strains, specifically *Streptomyces toyocaensis*, is Anisomycin, which demonstrated good effectiveness against *Echinochloa crus-galli* and *Digitaria sanguinalis*, without recorded phytotoxic effects on sugar beet, tomato, and other vegetable crops (Yamada et al., 1972). Bilanafos (bialafos), obtained through fermentation from the soil actinobacteria *Streptomyces hygroscopicus*, was introduced in Japan in 1984 as the Meiji Herbiac herbicide to control various annual and perennial weed species (Tachibana and Kaneko, 1986). The efficacy of the product Opportune, formulated as a combination of cells of *Streptomyces acidiscabies* and its fermentation metabolites, was evaluated by Bailey (2014) for controlling many weed species. Consequently, this study aimed to examine, under laboratory conditions, the effects of different arable soil actinobacteria isolates on seed germination and seedling growth of maize, and *Setaria glauca* and *Sorghum halepense*, which are among the most common weeds in this crop.

MATERIAL AND METHODS

Seed source and isolation of actinobacteria. Seeds of *Sorghum halepense* L. and *Setaria glauca* L. were collected from the fields in the suburbs of Belgrade (Nova Galenika and

Batajnica, respectively) in October 2022. Seeds of the maize hybrid NS3022 (Institute of Fields and Vegetables, Novi Sad) were used in the trial.

Samples were collected from arable fields for further isolation. For preparation, 10 g of each sample was mixed with 90 mL of sterile distilled water, and multiple serial dilutions were spread onto synthetic agar containing sucrose, starch, ammonia agar, starch casein agar (SCA), and potato dextrose agar (PDA). These plates were incubated at 27°C for seven days. From the resulting colonies, 22 single colonies were transferred onto SCA and PDA media. The SCA was purified using the streak plate technique (Jarak and Đurić, 2006).

Morphological characteristics of actinobacterial isolates. Five actinobacterial isolates were inoculated on SCA, and colony color, shape, diameter, surface, as well as spores and conidiospores, were examined. Spores were observed under a microscope (CX41RF Olympus, Düsseldorf, Germany), while the Gram staining procedure for actinobacteria was performed using crystal violet and Lugol's solution (Knežević-Vukčević and Simić, 2015). The actinobacterial isolates were also 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 and 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). 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, with actinobacterial isolates incubated for 5 days at 28°C. After incubation, the plates were flooded with Lugol's solution and examined 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 (as a change in the color around the colony from green to 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 a low 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 formation 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 color change from green to blue indicated a positive reaction. For melanin production, actinobacterial isolates were inoculated into the ISP6 and ISP7 media. The isolates were considered to produce melanin when dark-brown or black diffusible pigments formed in the medium.

Screening test of the herbicidal potential of primary actinobacterial extracts. In a screening test, a primary inoculum of 22 actinobacterial isolates was prepared in Erlenmeyer flasks containing 150 mL of starch casein broth (SCB) and incubated for 7 days in an orbital shaker incubator at 140 rpm and 28°C. Culture filtrates were aseptically obtained by filtration through Whatman No. 2 filter paper. The surface of the tested seeds was sterilized with a 5% sodium hypochlorite solution (NaOCl) for 2 minutes, followed by three rinses with sterilized distilled water to prevent inhibition of germination by fungal or bacterial toxins. Ten disinfected seeds were placed in each sterilized Petri dish, with filter paper, and moistened with 5 mL of the primary actinobacterial extract from each isolate. The controls were prepared in the same way, but uninoculated SCB was used instead of the primary filtrate. All dishes were sealed with parafilm to prevent evaporation and placed in an incubator (VELP, Incubator FOC) at 28±1°C in the dark. After 7 days, the final germination percentage was calculated, and the lengths of the radicle and shoot of the seedlings were measured.

Seed test of the herbicidal potential of actinobacterial supernatant. In a seed test, 120 mL of SCB was inoculated with 6 mL of the primary inoculum of five isolates and incubated for five days in an orbital shaker incubator at 140 rpm and 28°C. Supernatant filtrates were aseptically obtained by filtration through Whatman No. 2 filter paper and centrifuged for 20 min at 10,000 rpm. The resultant supernatant was collected, and 5 mL of each supernatant was transferred to a Petri dish containing sterile filter paper and seeds prepared as in the screening test. Controls were prepared in the same way as the uninoculated SCB that was used instead of the supernatant filtrate. All dishes were sealed with parafilm to prevent evaporation and placed in an incubator (VELP, Incubator FOC) at 28±1°C in the dark. After 7 days, germination percentage was calculated, and seedling growth (radicle and shoot length) was measured. The experimental design was a randomized complete block with four replications (10 seeds per Petri dish), repeated twice, and the data were then combined for analysis.

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 Tukey's Honest Significant Difference (HSD) test.

RESULTS AND DISCUSSION

Screening test of the herbicidal activity of primary actinobacterial extracts. In a preliminary test of herbicidal potential on weed seeds (*S. glauca* and *S. halepense*) and crops (maize), of the 22 actinobacterial isolates tested, only 8 showed varying levels of inhibitory effect on seed germination and seedling length (Table 1). The remaining isolates did not exhibit significant herbicidal activity (data not shown).

The measured parameters of maize seedlings (radicle and shoot length) showed statistically significant differences only for isolate SI X, while inhibition of these two parameters by the other 7 isolates was ≤ 24%. Additionally, isolates SA VIII and SI I had stimulatory effects, as the seedling lengths were greater than those in the control. In contrast, the seeds and seedlings of

S. glauca and *S. halepense* were more sensitive, as all measured parameters were significantly inhibited (Table 1). Inhibitions of shoot growth in *S. glauca* seedlings exceeded 78% for all isolates except SC VIII, where inhibition was 44.5%, while radicle length was inhibited by more than 71% for all 8 actinobacteria isolates. A similar trend was observed in *S. halepense* seedlings, where inhibition of shoot growth was in the range of 70-100%, and inhibition of radicle length ranged from 92-100% (Table 1). In both weed species, seed germination was the least sensitive parameter.

Table 1. Effects of actinobacterial isolates on seed germination, radicle, and shoot length of the tested plants.

Isolate	Maize			*Setaria glauca*			*Sorghum halepense*		
	*Final germination (%)	Shoot length (cm)	Radicle length (cm)	*Final germination (%)	Shoot length (cm)	Radicle length (cm)	*Final germination (%)	Shoot length (cm)	Radicle length (cm)
Control	97.5 a	3.44 a	4.28 ab	90.0 a	5.90 a	1.72 a	67.5 a	11.17 a	3.84 a
CH III	90.0 ab	3.38 a	3.27 ab	25.0 cd	1.28 bc	0.34 b	5.0 c	0.95 b	0.08 b
CH IV	85.0 ab	3.22 ab	4.08 ab	32.5 bcd	1.06 c	0.42 b	12.5 bc	2.34 b	0.30 b
CH IX	70.0 b	3.06 ab	3.35 ab	32.5 bcd	0.96 c	0.14 b	0.0 c	0.00 b	0.00 b
SA VIII	80.0 ab	3.98 a	4.48 a	7.5 d	0.81 c	0.21 b	22.5 b	2.74 b	0.31 b
SC V	62.5 c	3.04 ab	3.94 ab	32.5 bcd	1.00 c	0.28 b	7.5 c	0.53 b	0.13 b
SC XII	65.0 c	3.05 ab	3.87 ab	57.5 b	3.28 b	0.50 b	12.5 bc	3.40 b	0.38 b
SI I	72.5 b	4.13 a	4.89 a	22.5 d	0.65 c	0.16 b	15.0 bc	0.84 b	0.21 b
SI X	80.0 ab	2.36 b	1.71 b	52.5 bc	1.29 bc	0.38 b	0.0 c	0.00 b	0.00 b

*Differences were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's HSD (Honestly Significant Difference) test. Means in the same column referring to the same parameter are marked with different letters (a, b, c, d) only when the differences are significant ($p < 0.05$).

Similar to our findings, research by other authors has shown that actinobacteria, as sources of herbicidal compounds with significant allelopathic potential, can be widely used to protect plants from weed species. Singh et al. (2018), in experiments with endophytic actinomycetes (*Nocardiodes* sp.1, *Nocardiodes* sp.2, and *Actinomandura* sp.), achieved 60% inhibition of seed germination in the weed species *Ageratum conyzoides*. Their research also indicated that the reduction in germination depended on the type of nutrient medium used to obtain the culture filtrates. For the weed species *Parthenium hysterophorus*, the greatest reduction in seed germination (80%) was observed with the filtrate of the isolate *Saccharopolyspora* sp. grown in SCA. Priyadharsini and Dhanasekaran (2015) screened the allelopathic potential of 45 actinobacteria isolates on *Cyperus rotundus* seeds. Among the tested isolates, six showed maximum inhibitory potential, while 17 caused minimal inhibition of germination in this weed species.

Seed test of the herbicidal activity of actinobacterial supernatant. Based on the evaluation of the screening test results, five actinobacterial isolates (CH III, CH IV, CH IX, SA VIII, SI I) showed a strong inhibitory effect on one or both weed species, with minimal negative

impacts on maize (Table 2). Among these five isolates, only SI I exhibited the highest level of herbicidal activity.

Table 2. Effects of actinobacterial secondary metabolites on seed germination, radicle, and shoot length of the tested plants.

Isolate	Maize			*Setaria glauca*			*Sorghum halepense*		
	*Final germination (%)	Shoot length (cm)	Radicle length (cm)	*Final germination (%)	Shoot length (cm)	Radicle length (cm)	*Final germination (%)	Shoot length (cm)	Radicle length (cm)
Control	97.5 a	5.19 a	3.95 ab	77.5 a	7.26 a	1.69 a	65.0 a	9.11 a	3.14 a
CH III	90.0 ab	1.82 e	2.94 cd	17.5 bc	0.69 bc	0.43 bc	7.5 bc	1.55 bc	0.30 b
CH IV	85.0 bc	3.76 bc	3.41 bc	27.5 bc	1.38 bc	0.13 c	10.0 bc	0.73 bc	0.03 b
CH IX	92.5 ab	3.12 cd	3.85 ab	22.5 bc	1.16 bc	0.29 c	12.5 b	0.76 b	0.14 b
SA VIII	72.5 d	3.44 bc	2.80 cd	25.0 bc	1.80 b	0.23 c	5.0 bc	0.53 bc	0.00 b
SI I	90.0 ab	4.27 ab	4.43 ab	15.0 c	0.94 bc	0.03 c	5.0 bc	0.54 bc	0.00 b

*Differences were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's HSD (Honestly Significant Difference) test. Means in the same column referring to the same parameter are marked with different letters (a, b, c, d) only when the differences are significant ($p < 0.05$).

Maize seed germination was inhibited by up to 26%, while seedling growth parameters were more sensitive. Statistically significant differences in maize shoot length were observed for four of the five tested actinobacteria isolates, with inhibition levels ranging from 18-65%. Slightly lower sensitivity was observed for radicle length, with significant differences noted for two isolates. Inhibition of all measured maize parameters below 18% occurred only with isolate SI I. In contrast, significant differences in seed germination and seedling growth of *S. glauca* and *S. halepense* were found for all five tested actinobacteria isolates. Seed germination was inhibited by more than 65% in *S. glauca* and over 81% in *S. halepense* (Table 2). The radicle and shoot lengths of *S. glauca* were inhibited by at least 75%. *S. halepense* seedlings showed even greater sensitivity, with inhibition levels ranging from 83-100%.

Based on the results of the secondary test, isolate SI I can be identified as a potential source of herbicidal metabolites and included in further testing. Research by other authors has shown that secondary metabolites isolated from different streptomycetes can be compounds with herbicidal activity. Priyadharsini et al. (2013) investigated the herbicidal activity of isolates of *Streptomyces* sp. KA1-3 against the weed species *Cassia occidentalis* and *Cyperus rotundus*. These studies demonstrated that under *in vitro* conditions, the bioactive compound N-phenylpropanamide has an inhibitory effect of up to 80% on the germination of these weed species. Among the initially selected 102 bacterial strains, Bo et al. (2019) found that only three isolates showed a considerable inhibitory effect, but a clear herbicidal effect was achieved with the *Streptomyces anulatus* strain-329. A positive correlation was observed between the phytotoxic activity and the concentrations of this actinobacterial extract at both pre- and post-emergence applications in the tested weed species (*Digitaria sanguinalis, Sorghum bicolor, Echinochloa crus-galli, Agropyron smithii, Solanum nigrum, Aeschynomene indica, Xanthium*

strumarium, Calystegia japonica). Additionally, the results confirmed that the *Streptomyces* strain-329 contains a natural active compound with contact and selective activity.

Characteristics of actinobacterial isolates. Morphological, physiological, and biochemical characteristics of five selected isolates (CH III, CH IV, CH IX, SA VIII, SI I) are presented in Tables 3-6.

Table 3. Morphological characteristics of actinobacterial isolates.

Isolate	Colony shape	Colony color	Colony diameter	Colony surface	Conidiospores	Gram reaction	Spore shape
CH III	circular	green	ø 6 mm	powdery	cylindrical	+	round
CH IV	circular	grey	ø 5 mm	dusty	cylindrical	+	round
CH IX	circular	pink	ø 4 mm	dusty	cylindrical	+	round
SA VIII	irregular	green	ø 3 mm	rough	cylindrical	+	round
SI I	circular	white	ø 4 mm	powdery	cylindrical	+	round

Table 4. Growth, pigmentation of aerial and substrate mycelium, and diffusible pigment production of actinobacterial colonies on ISP medium.

Medium		CH III	CH IV	CH IX	SA VIII	SI I
ISP 2	growth	good	medium	medium	good	good
	aerial mycelium	white	white	white	grey	grey
	substrate mycelium	green	orange	burgundy	light brown	dark brown
	soluble pigment	/	orange	light brown	light brown	/
ISP 3	growth	good	low	good	good	good
	aerial mycelium	beige	white	pink	grey	grey
	substrate mycelium	dark brown	brown	burgundy	dark brown	light brown
	soluble pigment	dark brown	brown	/	/	/
ISP 4	growth	good	low	medium	good	good
	aerial mycelium	beige	grey	dark grey	grey	grey
	substrate mycelium	green	light brown	dark brown	light brown	light brown
	soluble pigment	/	yellow	grey	/	/
ISP 5	growth	medium	medium	low	good	low
	aerial mycelium	beige	grey	pink	grey	beige
	substrate mycelium	light brown	light brown	pink	brown	beige
	soluble pigment	light brown	light brown	/	/	/
ISP 6	growth	good	medium	low	low	low
	aerial mycelium	pink	white	beige	white	white
	substrate mycelium	beige	brown	beige	dark brown	black
	soluble pigment	/	brown	/	dark brown	black
ISP 7	growth	good	low	low	good	medium
	aerial mycelium	beige	beige	dark brown	beige	grey
	substrate mycelium	green	brown	dark brown	dark brown	dark brown
	soluble pigment	brown	brown	light brown	dark brown	dark brown

Table 5. Physiological characteristics of actinobacterial isolates.

Isolate	pH medium				NaCl [%]			Temperature					
	3	6	7	9	3	6	9	5	16	22	28	35	40
CH III	-	++	++	++	++	++	++	-	++	++	++	+	-
CH IV	-	++	++	+	+	+	+	-	+	+	++	+	-
CH IX	-	++	++	+	++	+	+	-	+	++	++	++	++
SA VIII	-	++	++	++	++	++	++	-	+	++	++	++	-
SI I	-	++	++	++	++	++	++	-	+	++	++	++	+

(-) low growth; (+) medium growth; (++) good growth

Table 6. Biochemical characteristics of actinobacterial isolates.

Isolate	Gelatin hydrolysis	Starch hydrolysis	Catalase	Citrate	Melanin production	Carbon source		
						saccharose	dextrose	glucose
CH III	-	+	+	-	-	-	+	+
CH IV	+	+	+	-	-	-	+	+
CH IX	-	+	+	-	-	-	+	+
SA VIII	+	-	+	-	+	-	-	+
SI I	+	+	-	-	+	-	-	-

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

CONCLUSION

Synthetic herbicides have played a crucial role in the history of weed management. Despite their effectiveness, prolonged use has led to the development of resistance in some weeds to certain active ingredients. Allelopathy has been explored as a means to reduce dependence on synthetic herbicides, as natural compounds (allelochemicals) possess phytotoxic properties that can suppress the growth of weeds. Many natural products have been isolated from microorganisms and are used commercially in plant protection. Preliminary results from this research indicate that suspensions of certain actinobacterial isolates investigated exhibit herbicidal properties. Isolate SI I proved to be the most promising candidate for further studies, as it is necessary to identify and isolate the most effective bioactive compounds from this source and to validate the current results under field conditions, including different weeds and crops.

ACKNOWLEDGEMENT

This research was funded by the Ministry of Science, Technological Development, and Innovation of the Republic of Serbia (Contracts No. 451-03-136/2025-03/200214).

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Izolacija, karakterizacija i herbicidni potencijal zemljišnih aktinobakterija

REZIME

U laboratorijskim uslovima testiran je uticaj primarnog inokuluma 22 izolata aktinobakterija izolovanih iz obradivog zemljišta, na klijavost semena i dužinu korenka i stabaoca kukuruza, *Setaria glauca* i *Sorghum halepense*. Suspenzije osam izolata aktinobakterija pokazale su različite nivoe inhibitornog delovanja na klijavost i porast klijanaca testiranih biljaka. Na osnovu rezultata dobijenih u preliminarnom testu, odabrano je pet izolata aktinobakterija koje su ispoljile značajan

inhibitorni efekat na jednu ili obe korovske vrste, a najmanji negativni uticaj na kukuruz. Izolat SI I izdvojio se kao najbolji kandidat za dalja ispitivanja jer je inhibicija merenih parametara kukuruza bila manja od 18%, a kod korovskih vrsta veća od 81%.

Ključne reči: aktinobakterije, biotest, kukuruz, korovi, bioherbicid.