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Glavni urednik/Editor-in-chief
Matevž Likar, Slovenija / Slovenia, matevz.likar@bf.uni-lj.si

Odgovorna urednica/Managing editor
Anita Jemec Kokalj, Slovenija / Slovenia, anita.jemec@bf.uni-lj.si

Oblikovanje/Design
Ajda Fortuna

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Original Research

Effect of prepubertal caffeine consumption and recovery on adult erectile tissue functions in Wistar rats

Shakiru Ademola Salami [1,*], Bashua Omotoyosi Kassim [1], Michael Olabode Allen [1], Hussein Mofomosara Salahdeen [1], Babatunde Adekunle Murtala [1]

Abstract

The study examines the impact of caffeine consumption during prepubertal development and recovery on the contractile function of the adult corpus cavernosum in Wistar rats. Prepubertal male rats consumed distilled water (vehicle) and caffeine (5 mg/kg). A third group consumed caffeine and was allowed a period of recovery. Cavernosa tissues were excised at adulthood, and their contractile functions in the presence of Ca^{2+}, K^{+}, indomethacin, glibenclamide, methylene blue, L-N-nitro-arginine-methyl-ester (L-NAME), and sodium nitroprusside (SNP) were assessed. K^{+}-induced increase in contraction in the caffeine-treated group was similar to that in the recovery group. Ca^{2+} ions, however, increased contraction significantly in the caffeine group compared to the recovery group. Acetylcholine-mediated relaxation (%) was significantly higher in the recovery as compared to the caffeine treated after incubation with indomethacin and methyl blue. Acetylcholine-mediated relaxation, however, was higher in caffeine as compared to the recovery group after incubation with glibenclamide and L-NAME. Relaxation in the presence of SNP was significantly reduced in recovery than in the caffeine-treated group. Prepubertal caffeine intake had an erectogenic effect on the cavernous tissues in the presence of glibenclamide, nifedipine, and L-NAME. Inhibitors of prostacyclin (indomethacin) and guanylyl cyclase (methylene blue) militate caffeine-induced relaxation. These effects were reversed after recovery.

Keywords

Prepubertal caffeine consumption, erectile tissue function, recovery, methyl blue, indomethacin, L-NAME, glibenclamide

1 Department of Physiology, Lagos State University College of Medicine, Ikeja Lagos State Nigeria

*** Corresponding author:**
E-mail address: shakiru.salami@lasu.edu.ng

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Učinek predpubertetnega uživanja kofeina in okrevanja na funkcije odraslih erektilnih tkiv pri podganah Wistar

Izvleček

Študija preučuje vpliv uživanja kofeina med predpubertetnim razvojem in okrevanjem na kontraktilno funkcijo odraslega kavernoznega telesa pri podganah Wistar. Podganji samci v predpuberteti so uživali destilirano vodo (nosilec) in kofein (5 mg/kg). Tretja skupina je uživala kofein in ji je bilo dovoljeno obdobje za okrevanje. Tkiva kavernoze so bila izrezana v odrasli dobi in ocenjena je bila njihova kontraktilna funkcija v prisotnosti Ca^{2+}, K^{+}, indometacina, glibenklamida, metil modrega, L-NAME in natrijevega nitroprusida. S K^{+} povzročeno povečanje krčenja v skupini, zdravljeni s kofeinom, je bilo podobno kot v skupini, ki je okrevala. Ioni Ca^{2+} pa so znatno povečali kontrakcijo v skupini s kofeinom v primerjavi s skupino, ki je ozdravela. Relaksacija (%), posredovana z acetilholinom, je bila bistveno višja pri okrevanju v primerjavi s kofeinom, zdravljenim po inkubaciji z indometacinom in metil modrim. Relaksacija, posredovana z acetilholinom, pa je bila večja pri kofeinu v primerjavi s skupino za okrevanje po inkubaciji z glibenklamidom in L-NAME. Sprostitev v prisotnosti natrijevega nitroprusida je bila znatno zmanjšana pri okrevanju kot v skupini, zdravljeni s kofeinom. Predpubertetni vnos kofeina je imel erektogeni učinek na kavernozna tkiva v prisotnosti glibenklamida, nifedipina in L-NAME. Zaviralci prostaciklina (indometacin) in cGMP (metilno modro) spodbujajo sprostitev, ki jo povzroča kofein. Po okrevanju so bili učinki obrnjeni.

Ključne besede

Uživanje kofeina v predpuberteti, funkcija erektilnega tkiva, okrevanje, metilno modro, indometacin, L-NAME, glibenklamid

Introduction

Scientists have been fascinated by research in caffeine for decades. Caffeine ranks as one of the most frequently ingested active substances (Nawrot et al., 2003; Scorza et al., 2022). Apart from coffee, which serves as the commonest source, caffeine is usually present in prescription drugs, soft or energy drinks, gums, tea leaves, and stimulants (Mahoney et al., 2019). Absorption of caffeine in humans is almost optimal, with 100% bioavailability after oral intake (Sepkowitz, 2013).

A study reported a reduced likelihood of having erectile dysfunction when young adults consume around three saucers of coffee (about 170-375 mg/kg) daily (Lopez et al., 2015). A prospective cohort investigation of 40–75-year-old men found that prolonged coffee intake is not related to the development of erectile dysfunction (Lopez et al., 2018). Yang et al. (2008) also reported that caffeine intake (10 and 20 mg/kg) enhanced the erectile functions of diabetic rats through upscaling cavernous cyclic guanosine monophosphate (cGMP) activity.

On the contrary, the maternal exposure of rats to caffeine was reported to adversely affect the birth weight, cytoarchitecture of the testes, and serum testosterone level of the male offspring in adulthood (Ogunwole et al., 2015). Caffeine treatment for four weeks in adult male rats also impaired the body, reproductive organ weight, sperm characteristics, and testicular cytoarchitecture (Oluwole et al., 2016). The effects were, however, reported to be reversed in recovery groups.

The foregoing reports, along with others in the literature on caffeine consumption and its direct effect on reproductive functions, have not only been contradictory but also inconclusive in both human and animal studies. Furthermore, fewer studies have identified or targeted the significance of prepubertal caffeine exposure on erectile functions in adulthood. The significance of recovery or weaning from caffeine on erectile functions after exposure is also not fully determined.

This study investigates caffeine consumption during prepubertal development and recovery and the contractile activity and function of the adult corpus cavernosum in Wistar rats.

Materials and Method

Caffeine preparation and Wistar rats

The caffeine was purchased from Aesar Johnson Matthew Company, 26 Park Ridge Road, MA, U.S.A. Before daily treatment, the caffeine was freshly prepared using distilled water. The prepubertal rats (2 weeks old) were sourced from the Central Animal House, College of Medicine, Ibadan. They were acclimatized for one week and fed a pelletized rat diet and water. The ethics approval (Ref. No: AREC/2022/050) for this study was granted by the Lagos State University College of Medicine Animal Ethics Committee.

Experimental design and treatment

Eighteen prepubertal male rats were randomly divided into three equal groups. Group 1 (the control) was given distilled water (a vehicle for caffeine). Group 2 was given caffeine orally at a dose of 5 mg/kg (Metro et al., 2017) for the entirety of the study. Group 3 rats were treated with caffeine orally (5 mg/kg) for six weeks but were allowed six weeks of recovery without caffeine administration.

Serum collection for testosterone assay

The serum was carefully aspirated using a Pasteur pipette into an Eppendorf tube and stored at -4°C. Serum testosterone was assayed using a testosterone ELISA kit (Monobind Inc, Lake Forest, CA, U.S.A.)

The excision of the cavernosa tissues and cavernosa contractile functions studies

The cavernosa tissues were excised after the 12th week of the treatment. The procedures for the excision of the cavernosa tissue, composition of the P.S.S., mounting in the organ chamber, and recording of the isometric contraction using a data capsule system were as previously described (Salami et al., 2017). Using a force isometric transducer that is connected to the Ugo Basile (model 17400, Italy) data acquisition system, the contractile responses of the cavernosa tissues to increasing doses of phenylephrine (10^{-9}-10^{-5} M) and acetylcholine (10^{-9}-10^{-5} M) were recorded. In addition, cavernosa contractile responses to increasing doses of Ca^{2+} (10–60 mM) and K^{+} (10–60 mM) in calcium and potassium-free tissue chambers were recorded. Finally, the acetylcholine-mediated relaxation of the cavernosa tissues following incubation with indomethacin, glibenclamide, methylene blue, nifedipine, L-NAME, and sodium nitroprusside (10^{-4} M) was determined and recorded to investigate how prepubertal caffeine ingestion and recovery have influenced contractile mechanisms guided by the individual agents. Care was taken to wash the tissue three times before another drug was introduced.

Statistical Analysis

All the data were expressed as mean ± standard error of the mean (S.E.M.). One-way and two-way analysis of variance (ANOVA) were carried out with Tukey's multiple comparisons using GraphPad Prism 8.0 software. Statistical significance was taken at $p < 0.05$

Results

Table 1 showed that the testosterone level was significantly reduced in the recovery group

Table 1. Effect of caffeine treatment and recovery on testosterone concentration in male Wistar rats.

Tabela 1. Učinek zdravljenja s kofeinom in okrevanja na koncentracijo testosterona pri samcih podgan pasme Wistar.

	Control	Caffeine	Recovery
Testosterone (ng/ml)	4.16 ± 0.35	4.60 ± 0.28	1.63 ± 0.42*

N= 6, * p<0.05, Value expressed as mean S.E.M.

Effect of prepubertal caffeine ingestion and recovery on cavernosa tissue contraction to phenylephrine (10^{-9}- 10^{-5} M), potassium chloride and calcium chloride.

Phenylephrine-mediated contraction of the cavernosa tissue was significantly greater in the caffeine group than in the recovery group. The contraction was, however, significantly reduced in the control group when compared to the caffeine and recovery groups (Figure 1). Cumulative doses of extracellular potassium chloride influx significantly increased the contraction of the cavernosa tissue

in the caffeine and recovery groups when compared to the control (Figure 2). The extracellular influx of cumulative doses of calcium chloride significantly increased the cavernous tissue contraction in the caffeine group as compared to the recovery group (Figure 3).

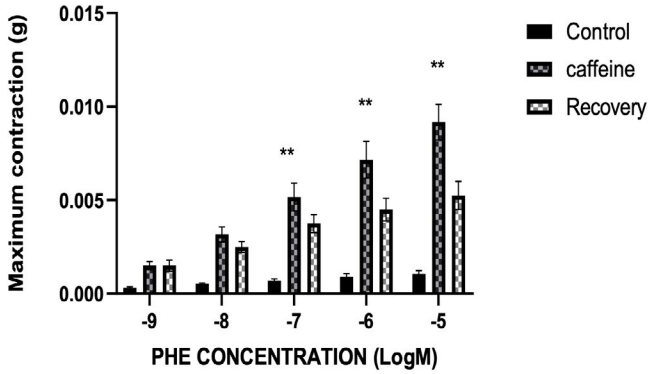

Figure 1. Maximum contraction of cavernosa tissue to cumulative doses of phenylephrine (10^{-9} – 10^{-5} M). N=6, ** = p < 0.01.

Slika 1. Največja kontrakcija kavernoznega tkiva na kumulativne odmerke fenilefrina (10^{-9} – 10^{-5} M).
N=6, ** = p < 0,01.

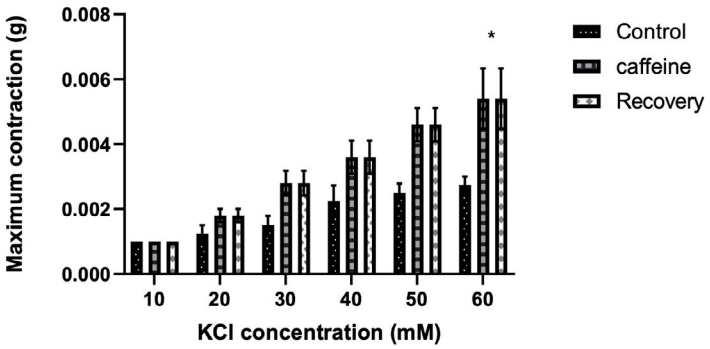

Figure 2. Maximum contraction of cavernosa tissue to influx of potassium chloride (10 – 60 mM), N = 6, *...p < 0.05

Slika 2. Največja kontrakcija kavernoznega tkiva na dotok kalijevega klorida (10 – 60 mM), N = 6, *...p < 0,05

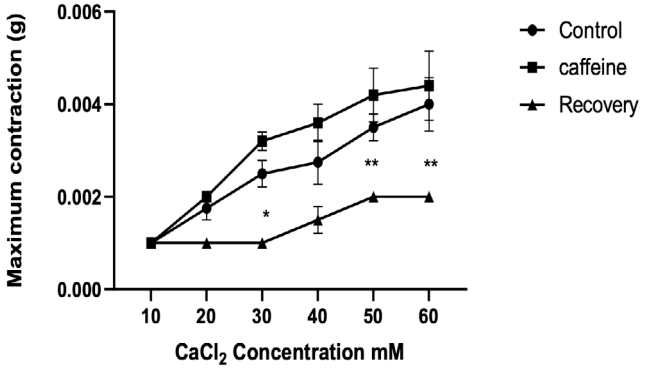

Figure 3. Maximum contraction of cavernosa tissue to influx of calcium chloride (10 – 60 mM), N = 6, *...p < 0.05, **...p < 0.01.

Slika 3. Največja kontrakcija kavernoznega tkiva na dotok kalcijevega klorida (10 – 60 mM), N = 6, *...p < 0,05, **...p < 0,01.

Effect of caffeine ingestion and recovery on acetylcholine-mediated relaxation response following incubation with indomethacin, glibenclamide, and methylene blue

As shown in Figure 4, acetylcholine-mediated relaxation (%) was significantly increased in the recovery group following incubation of the cavernous tissue with indomethacin (10^{-4} M). However, acetylcholine-mediated relaxation (%) was significantly reduced in the recovery group (Figure 5) following the incubation of the cavernous tissue with glibenclamide (10^{-4} M). The relaxation (%) to the cumulative dose of acetylcholine following the incubation of the cavernosa tissue in methyl blue was significantly increased in the recovery group (Figure 6).

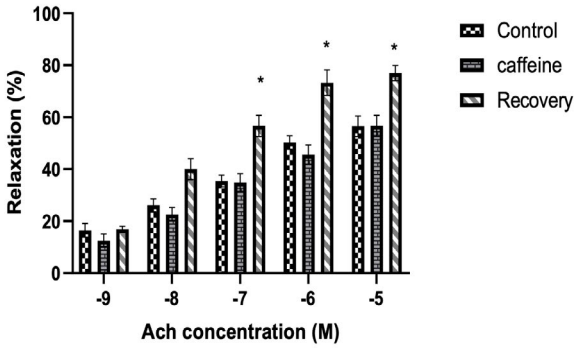

Figure 4. The relaxation (%) of the cavernosa tissue to cumulative doses of acetylcholine (10^{-9} – 10^{-5} M) after the incubation of the cavernosa tissue in 10^{-4} M indomethacin. N = 6, *...p < 0.05.

Slika 4. Relaksacija (%) kavernoznega tkiva na kumulativne odmerke acetilholina (10^{-9} – 10^{-5} M) po inkubaciji kavernoznega tkiva v 10^{-4} M indometacinu. N = 6, *...p < 0,05.

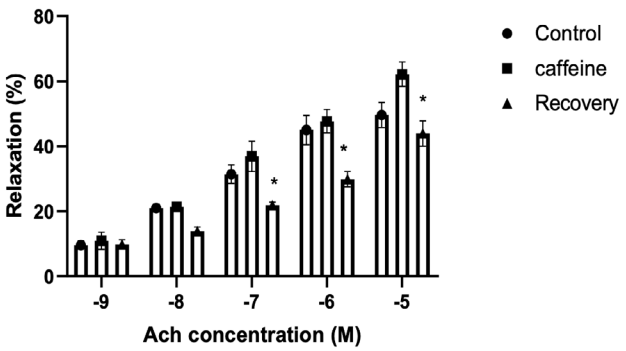

Figure 5. The relaxation (%) of the cavernosa tissue to cumulative doses of acetylcholine (10^{-9} – 10^{-5} M) after the incubation of the cavernosa tissue in glibenclamide (10^{-4} M). N = 6, *...p <0.05.

Slika 5. Relaksacija (%) kavernoznega tkiva na kumulativne odmerke acetilholina (10^{-9} – 10^{-5} M) po inkubaciji kavernoznega tkiva v glibenklamidu (10^{-4} M). N = 6, *...p <0,05.

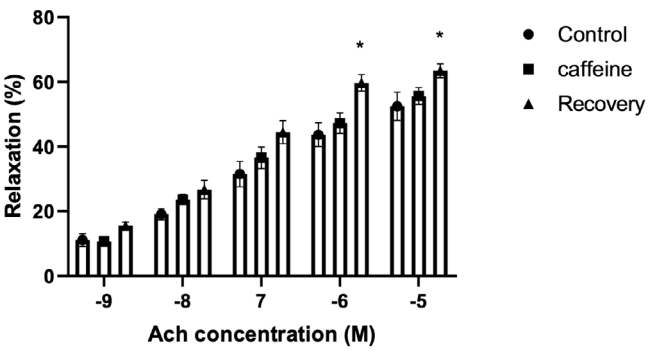

Figure 6. The relaxation (%) of the cavernosa tissue to cumulative doses of acetylcholine (10^{-9} – 10^{-5} M) after the incubation of the cavernosa tissue in methylene blue (30 µM). N = 6, *...p < 0,05.

Slika 6. Sprostitev (%) kavernoznega tkiva na kumulativne odmerke acetilholina (10^{-9} – 10^{-5} M) po inkubaciji kavernoznega tkiva v metilensko modrem (30 µM). N = 6, *...p < 0,05.

Effect of caffeine ingestion and recovery on acetylcholine-mediated relaxation response after incubation with nifedipine, L-NAME, and sodium nitroprusside (SNP)

Acetylcholine-mediated relaxation (%) showed no significant changes in the caffeine and recovery groups when compared with the control group following incubation of the cavernous tissue with nifedipine (Figure 7). Furthermore, acetylcholine-mediated relaxation (%) was higher in the caffeine compared to recovery and control following the incubation of the cavernous tissue in L-NAME (Figure 8). The relaxation (%) response of cavernosa tissue to increasing doses of SNP after the precontraction of the cavernosa tissue with phenylephrine was significantly reduced in the recovery group (Figure 9).

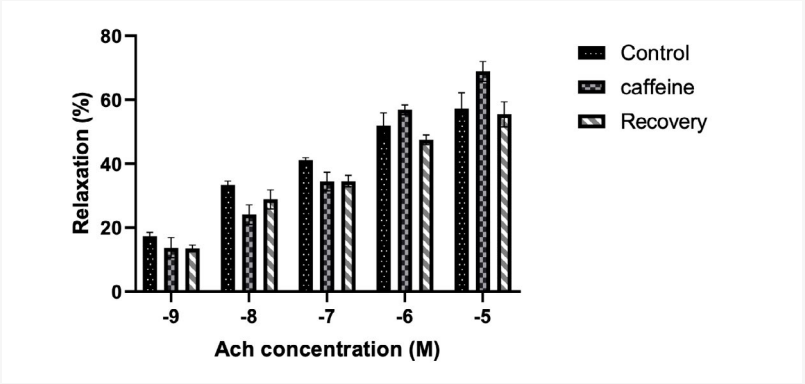

Figure 7. The relaxation (%) of the cavernosa tissue to cumulative doses of acetylcholine after the incubation of the cavernosa tissue in nifedipine (10^{-4} M)

Slika 7. Sprostitev (%) kavernoznega tkiva na kumulativne odmerke acetilholina po inkubaciji kavernoznega tkiva v nifedipinu (10^{-4} M)

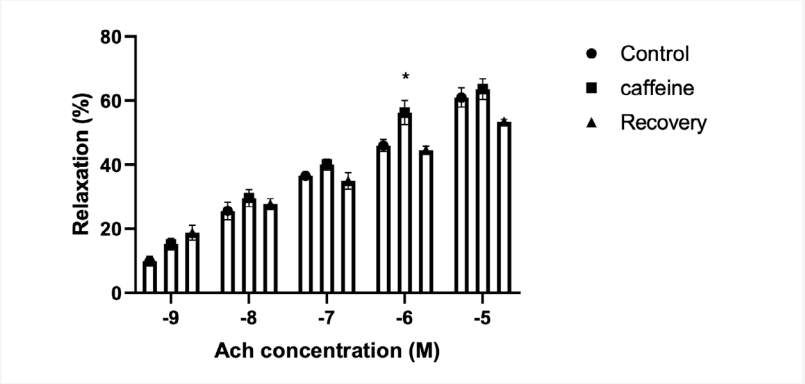

Figure 8. The relaxation (%) of the cavernosa tissue to cumulative doses of acetylcholine after the incubation of the cavernosa tissue in L-NAME (10^{-4} M). N = 6, *...p < 0,05.

Slika 8. Sprostitev (%) kavernoznega tkiva na kumulativne odmerke acetilholina po inkubaciji kavernoznega tkiva v L-NAME (10^{-4} M). N = 6, *...p < 0,05.

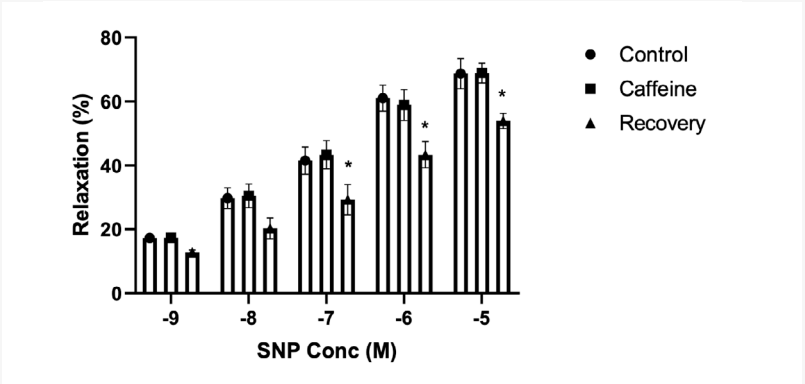

Figure 9. The relaxation (%) of the cavernosa tissue to cumulative doses of sodium nitroprusside (SNP) after the precontraction of the cavernosa tissue in phenylephrine. N = 6, *...p < 0,05.

Slika 9. Sprostitev (%) kavernoznega tkiva na kumulativne odmerke natrijevega nitroprusida (SNP) po predkontrakciji kavernoznega tkiva v fenilfrinu. N = 6, *...p < 0,05.

Discussion

The testosterone level of the recovery group in this study was significantly lower than the caffeine and control groups (table 1). Oluwole et al. (2016) earlier reported an increase in testosterone levels in caffeine and an insignificant decrease in the recovery group, albeit during adult exposure to caffeine. Furthermore, the dosage of caffeine administered in the study was far higher (20 mg/kg and 40 mg/kg) than that reported in the current study (5 mg/kg). The juxtaposition of our current study and that by Oluwole et al. (2016) emphasizes the importance of caffeine dosage and prepubertal caffeine exposure on testosterone levels. It is possible that caffeine's inhibition of aromatase activity caused the increase in serum testosterone levels in this study. Aromatase is the major enzyme involved in the conversion of androgens into estrogens in adult Leydig cells (Carreau, 2007). Additional studies have linked coffee to high levels of testosterone, sex hormone-binding globulin, and low levels of estrogen (Svartberg et al., 2003; Ramlau-Hansen et al., 2008). The absence of the inhibitory impact of caffeine during the recovery period may also account for the significantly lower testosterone levels in the recovery group compared to the control and caffeine groups.

This study found that phenylephrine-mediated contractions increased significantly in the caffeine group as compared to the control and recovery groups (Figure 1). The increase in contraction of the cavernosa tissue of the caffeine group may be due to the caffeine-induced presence of catecholamines, adenyl cyclase, and cyclic adenosine monophosphate (cAMP). Although these factors were not estimated in the current study, caffeine consumption has been reported to increase circulating levels of catecholamine, adenyl cyclase, and cAMP (Nabofa and Alada, 2020). The elevation of these factors during the period of caffeine exposure and their gradual decline in recovery helped shape the contractile responses of the cavernosa tissues to phenylephrine in this study.

The contraction of the cavernosa tissue after adding doses of potassium chloride caused significantly higher contractions in the caffeine and recovery groups (Figure 2). This suggests that a caffeine-induced increase in the activity of the voltage-operated Ca^{2+} channels (stimulated by the K^{+} influx) persisted in the cavernosa tissue during the recovery period. However, cumulative doses of calcium chloride resulted in significantly reduced contraction in the recovery group when compared to the caffeine and control groups (Figure 3). Caffeine-containing coffee was reported to inhibit K^{+}-induced contractions in aortic and ileal smooth muscle. Caffeine also inhibited Ca^{2+}-induced contraction in vascular and intestinal smooth muscle (Watanabe et al., 1992). Our results show that caffeine-induced contraction varies in the cavernosa tissue as compared to the vascular and intestinal smooth muscle.

The relaxation of the cavernosa tissue was diminished (Figure 4) in the presence of a prostacyclin inhibitor (indomethacin) in the caffeine-treated group. It could be suggested that caffeine promotes the output of prostacyclin in cavernosa tissue, similar to what was reported by Eccheverri et al. (2010). The prostacyclin-releasing effect of caffeine was not observed in the recovery group (Figure 4).

There is no difference in the relaxation response of cavernosa tissue following incubation with methylene blue (a guanylyl cyclase inhibitor) in the caffeine-treated group or the control. Relaxation (%) of the cavernosa tissue was, however, higher in the recovery group (Figure 6). This shows that caffeine exposure may impair the activity of the soluble guanylyl cyclase-nitric oxide (NO)-cGMP pathway during recovery from caffeine. The incubation of the cavernosa tissue with an adenosine triphosphate-sensitive K^{+}-channel inhibitor (glibenclamide) significantly reduces the acetylcholine-mediated cavernosa relaxation in the recovery group as compared to the caffeine group (Figure 5). This suggests that caffeine consumption mitigates the detrimental effect of glibenclamide on cavernosa relaxation.

The acetylcholine-mediated relaxations (%) in the cavernosa tissues were higher in the caffeine group than in the recovery group after incubation with nifedipine (Figure 7). Nifedipine stimulates relaxation by inhibiting voltage-gated large-conductance calcium channel activity (Elliott and Ram, 2011). According to Williams et al. (2018), nifedipine and diltiazem appear to increase sexual function. Nifedipine had no negative impact on sexual function, according to Doumas and Douma (2006). The current study demonstrates that caffeine treatment potentiates nifedipine-induced cavernosa tissue relaxation.

Neurons, sinusoidal endothelium, and corporeal smooth muscle cells are potential nitric oxide producers in the penis (Cartledge et al., 2001). Nitric oxide is thought to interact with certain molecular targets to elicit a variety of functional effects. Numerous regulatory variables affect both its production and activity in the penis (Priviero et al., 2007). Through increased NO bioavailability, caffeine improves endothelial function (Higashi, 2019). In the current study,

after the incubation of cavernosa tissues with a nitric oxide synthase inhibitor (L-NAME), the caffeine-treated group showed a considerably higher relaxation response to acetylcholine than the recovery and control groups (Figure 8). The observed increase in the caffeine group could be the result of alternative nitric oxide synthase-independent ways of caffeine-induced relaxation. Caffeine has been found to boost prostacyclin output from the perfused rat mesenteric vascular bed (Echevarri et al., 2010). According to Joshi et al. (2012), the ryanodine receptor found in cavernosa tissue is crucial for relaxation. Ryanodine also enhanced prostaglandin production, indicating that caffeine may be stimulating a ryanodine receptor (Kong et al., 2008) to cause the relaxation seen in the caffeine-treated group and aid in the release of nitric oxide, as reported by Umemura et al. (2007). This was observably absent in the recovery group.

Lastly, the maximum (%) relaxation response of the cavernosa tissue to SNP, which was comparable to the control in the caffeine-treated group, may indicate that caffeine has no adverse effects on erectile function and that its withdrawal can decrease the release of nitric oxide, as was seen in the recovery group (Figure 9).

Conclusion

Prepubertal caffeine intake improved testosterone levels. Caffeine-induced cavernosa tissue relaxation was not impaired with glibenclamide, nifedipine, or L-NAME. Caffeine-induced cavernosa tissue relaxation was impaired with indomethacin and methylene blue. These effects were reversed during the recovery.

Author Contributions

Conceptualization: S.A.S., Methodology: S.A.S. & H.M.S., Software: S.A.S., B.A.M., Validation: S.A.S., Formal Analysis: S.A.S., H.M.S. & B..AM., Investigation: B..OK., B.A.M., Resources: S.A.S., H.M.S., B.O.K., Data Curation: S.A.S., H.M.S., B.O.K., M.O.A., Writing – Original Draft: S.A.S., Writing – Review & Editing: S.A.S., H.M.S., B.A.M., M.O.A., Supervision: S.A.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

Ethical Disclosures

The study was also certified by the Lagos State University College of Medicine Animal Ethics Committee with reference number AREC/2022/054.

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Original Research

Environmental alterations on potency of eugenol content of basil: an antihypertensive herb

Palshikar Gautam [1,*], Gadhave Sandeep [1], Mahadik Shubhashree [1], Mali Prashant [1], Patil Rajesh [1]

Abstract

Medicinal plants such as Tulsi (Basil) are used to treat patients with cardiovascular diseases, which may occur due to ailments of the heart and blood vessels and comprise heart attacks, cerebrovascular diseases, hypertension, and heart failure. The plant has a long traditional medicinal history. In India, Tulsi is worshipped by Hindus and is abundantly found in all regions. Each part of a plant, like stems, roots, seeds, leaves, flowers, and fruits, has its own curative properties and functions. Research has found various herbal therapies that successfully reduce high blood pressure with diet, exercise, stress management, and supplements. Herbal therapy demand increases worldwide for treating various diseases due to better acceptability with the human body, lesser side effects and low cost. Environmental changes have an impact on the availability of herbal constituents along with their therapeutic efficacy. If harvesting is done correctly and in accurate conditions, it yields more potency. Plant samples were used in different seasons, times and places, and identification and analysis were performed. Morphological, microscopical and extractive values appear to change. Maximum levels of ethanol extract with higher concentrations of eugenol were obtained in the rainy season, at high altitudes, and in the morning.

Keywords

Alkaloids, Environment, Fluctuations, Herbal therapy, Identification

1 Sinhgad College of Pharmacy, Wadgaon Budruk, Pune- 411041, Maharashtra, India

*** Corresponding author:**
E-mail address:
gautampalshikar@rediffmail.com

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Okoljske spremembe vsebnosti evgenola v baziliki: antihipertenzivna rastlina

Izvleček

Zdravilne rastline, kot je tulsi (bazilika), se uporabljajo za zdravljenje bolnikov s srčno-žilnimi boleznimi, ki se lahko pojavijo zaradi obolenj srca in ožilja in vključujejo srčni infarkt, cerebrovaskularne bolezni, hipertenzijo in srčno popuščanje. Rastlina ima dolgo tradicionalno zdravilno zgodovino. V Indiji hindujci častijo Tulsi, ki ga je mogoče v izobilju najti v vseh regijah. Vsak del rastline, kot so stebla, korenine, semena, listi, cvetovi in plodovi, ima svoje zdravilne lastnosti in funkcije. Raziskave so odkrile različne zeliščne terapije, ki uspešno znižujejo visok krvni tlak z dieto, telesno vadbo, obvladovanjem stresa in dodatki. Povpraševanje po zeliščni terapiji za zdravljenje različnih bolezni po vsem svetu narašča zaradi boljše sprejemljivosti the zdravil, manjših stranskih učinkov in nizkih stroškov. Okoljske spremembe vplivajo na razpoložljivost zeliščnih sestavin skupaj z njihovo terapevtsko učinkovitostjo. Če je nabiranje opravljeno pravilno in v natančno določenih pogojih, lahko optimiziramo vsebnost učinkovin v zeli. Uporabljeni so bili vzorci rastlin v različnih letnih časih in nadmorski višini. Pri analiziranih vzorcih smo opazili morfološke, mikroskopske in ekstraktivne vrednosti. Najvišje ravni etanolnega ekstrakta z višjimi koncentracijami evgenola so bile dosežene v deževnem obdobju, na najvišji nadmorski višini.

Ključne besede

Alkaloidi, okolje, nihanja, zeliščna terapija, identifikacija

Introduction

Basil (*Ocimum basilicum*) is a well-known Ayurvedic medicinal plant consisting of an antioxidant compound, eugenol, that helps to lower blood pressure by acting as a calcium channel blocker (Peixoto-Neves et al., Cohen, 2014). Calcium channel blockers prevent the movement of calcium into the heart and arterial cells, allowing the blood vessels to relax (Tabassum and Ahmad, 2011). Basil extracts helped relax blood vessels and thin the blood, which in turn helped reduce blood pressure (Umar et al., 2010). It plays a major role in the traditional "Ayurveda" and "Unani" systems as a tonic for the mind and body to cure illnesses in humans (Kumaret et al., 2010). Herbs well known to treat cardiac diseases are *Daucus carota*, *Nerium oleander*, *Amaranthus Viridis*, *Ginkgo biloba*, *Terminalia arjuna*, *Picrorhiza kurroa*, *Salvia miltiorrhiza*, *Tinospora cordifolia*, *Mucuna pruriens*, *Hydrocotyle asiatica*, *Bombax ceiba*, and *Andrographis paniculata*. The active phytochemicals found in these plants are flavonoids, polyphenols, plant sterols, plant sulphur compounds, and terpenoids (Bachheti et al., 2022). *Astragalus membranaceous* (Synonym *Astragalus propinquus* Schischkin. in the Missouri Botanical Garden plant list), another Chinese herb, contains Astragaloside IV, which is the plant's primary bioactive compound widely used as an antioxidant and for protection against ischemic-associated CVDs (Zhang et al., 2006). For the treatment of cardiac diseases, many mediators are used, such as diuretics, sympatholytic agents, renin inhibitors, angiotensin-converting enzyme (ACE) inhibitors, calcium channel blockers, β-adrenergic and α1/β-adrenergic antagonists, vasodilators (Hashemi et al., 2017). These drugs have various side effects, including muscle cramps, abnormal heart rate, blurred vision, skin rash, vomiting, kidney failure, extreme tiredness, headache, and oedema (Sinha and Agarwal 2019). Current growth in accepting alternative medicines and natural products has drawn attention to traditional medicines for treating cardiac diseases (Singh et al., 2015). Approximately 75% to 80% of the world's population, predominantly in developing countries, uses herbal medicines for primary healthcare because of their better compatibility with the human body, lower costs than novel pharmaceuticals, and fewer side effects (Rastogi et al., 2016). Various active constituents are present in basil; some show a high effectiveness rate and fast relief, while others take a certain amount of time for recovery and healing from diseases (Bhooshitha et al., 2020). Plant-derived natural medicine is a vital health resource with various applications, including

heart disorder prevention and management (Mounika et al., 2021). Plants are the primary sources of the continuous need for new drugs with lesser side effects and lower costs (Silva et al., 2021). Hence, there is an urgent interest in safe, effective, green, and more economical drug candidates

Hypertension acts as an increasing disorder in the universe and disturbs the economic condition of each nation (Karachaliou, 2020). As per the report of the World Health Association, in 2010, there were more than 375 million patients with cardiac disabilities, and approximately 4.5 million patients died yearly (Cho et al., 2018.). Approximately 900 medicinal herbs have been shown as remedies for the treatment of hypertension. Several herbal active components have demonstrated their importance in treating increased blood cholesterol levels in humans (Tran et al., 2020). The present study involves detecting changes in the eugenol content in basil leaves through the HPTLC chromatographic pattern of the components (Thomas et al., 2020).

Materials and Methods

Collection and Identification of Plant material

The plant material (leaves) was collected in every season of the year, i.e. Rainy (June –September), Winter (October-January), and Summer (February-May) from places of different altitudes, i.e. Low (100 m a.s.l.), Medium (500 m a.s.l.), and High (1000 m a.s.l.) at 6.00 am by manually. A voucher specimen was deposited in the Herbarium of Botanical Survey of India, Pune.

Assessment of quality of plant materials

The plant materials were assessed as per WHO guidelines for macroscopy, i.e. colour, odour, taste, size and shape, microscopy and leaf constants, i.e., stomatal number, index, vein islet number, termination number, palisade ratio, proximate analysis and Phytochemical screening, i.e. total ash, extractive value, solubility. Microscope equipped with lenses providing a wide range of magnification and a substage condenser, a graduated mechanical stage, objectives with a magnification of 10x and 40x was used.

Method of extraction

1 kg plant material was dried under shade, coarsely powdered and extracted with ethanol using the soxhlet apparatus of Borocil for six cycles of continuous hot extraction process. Extracts are stored in a refrigerator at a cool temperature.

Establishment of qualitative photo profile of successive solvent extracts

The extracts obtained from successive solvent extraction were then subjected to various qualitative chemical tests to determine the presence of various phytoconstituents like alkaloids, glycosides, carbohydrates, phenolics and tannins, proteins and amino acids, saponins and phytosterols (Elvino Nortjie et al., 2022). HPTLC study was performed for eugenol at 2650 standard area under the curve using mobile phase as Chloroform:Benzene (6:4) at 250nm wavelength (Table 1).

Results and Discussion

The plant leaf colour is dark green, with a strong odour and taste, and its shape is oblong, simple, petiolate, and exstipulated. The entire margin has a tapering base and acuminate apex, a leathery touch, and a smooth and shining texture. The outermost cell wall comprises single-layered epidermal cells followed by compactly arranged barrel-shaped parenchymatous cells. The vascular bundle is arc-shaped, conjoint, and collateral closed and enclosed by a parenchymatous bundle sheath. Vessels with pitted

Table 1. HPTLC parameters

Tabela 1. Pogoji HPTLC analize.

Plant Name	Phyto Constituent	Std. Area (Under Curve)	Mobile Phase	Wavelength (nm)
Basil	Eugenol	2650	Chloroform: Benzene (6:4)	250

thickening, anomocytic or anisocytic Stomata, glandular, multicellular uniseriate (40 to 110 µm) trichomes, Prismatic calcium oxalate crystals and starch grains are present.

Phytochemical analysis shows the presence of volatile oils such as eugenol. The eugenol content shows varying proportions with varying seasonal conditions and at different times and places of altitude (Table 2).

Qualitative chemical examination of extracts shows the presence of volatile oils such as eugenol. HPTLC chromatogram of Eugenol % Yield mg/g in basil leaf extracts show variations at different seasons, times and places. In the months from June to September (rainy season), in the morning at high altitudes, there is more yield. The values are expressed as mean SEM; P<0.05 (Two-way ANOVA followed by Tukey's multiple comparison test) (Table 3). Calibration curves show variations in the concentration of eugenol with the area under the curve. The presence of eugenol in ethanolic extract was confirmed by HPTLC fingerprinting. The yield was 3.23 mg/ gm at Rf value 0.51, which is more than summer 1.68 mg/g and winter 2.55 mg/g at altitude of 615 and 200 m a.s.l. at evening time (Table 4; Figures 1, 2). Current research work can be useful for the selection of month, place and time of harvesting crude drugs. According to Stefanakis et al. (2022), the highest content obtained for all five species when leaves and/or inflorescences were analyzed was during the summer months and especially July, except for S. rosmarinus, where the highest yield was obtained during June, i.e. rainy season. According to Gautam et al. (2024), crude drug activity variation is related to the season, time, and place of collection of the plant, and it is proportional to the changes in the active components of the herbal drug.

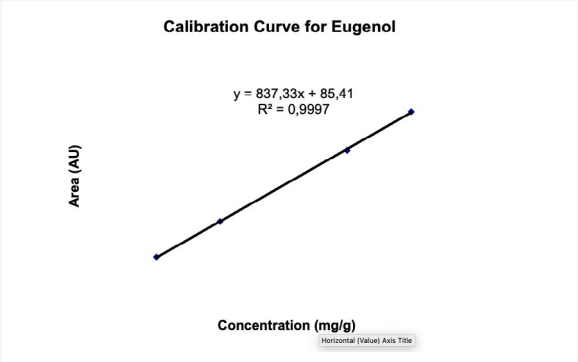

Figure 1. Calibration curve of eugenol.

Slika 1. Kalibracijska krivulja za evgenol.

Table 2. % of eugenol in basil leaf extract (n = 3).

Tabela 2. % evgenola v ekstraktu iz listov bazilike (n = 3).

Altitude	Month											
	1	2	3	4	5	6	7	8	9	10	11	12
Low	6.43* ±0.37	6.10 ±1.30	6.10 ±1.00	6.10 ±1.00	6.10 ±1.00	6.10* ±1.00	6.43* ±1.52	6.33 ±1.52	6.43 ±1.15	6.00 ±1.00	5.77 ±1.15	5.77 ±1.15
	6.33* ±0.57	6.00 ±1.00	6.00* ±1.00	6.00 ±1.00	6.00 ±1.00	7.00* ±1.00	7.43* ±1.52	7.33 ±1.52	7.33* ±1.15	7.00 ±1.00	6.67 ±1.15	6.67 ±1.15
Middle	6.67* ±0.47	6.33 ±0.77	6.33* ±0.57*	6.33 ±0.57	6.33 ±0.57	6.33* ±0.57	6.67* ±1.15	6.67* ±1.15	6.67* ±0.57	6.33 ±0.57	6.00* ±1.00	6.00* ±1.00
	6.43* ±0.37	6.10 ±1.30	6.10 ±1.00	6.10 ±1.00	6.10 ±1.00	6.10* ±1.00	6.43* ±1.52	6.33 ±1.52	6.43 ±1.15	6.00 ±1.00	5.77 ±1.15	5.77 ±1.15
	6.33* ±0.57	6.00 ±1.00	6.00* ±1.00	6.00 ±1.00	6.00 ±1.00	7.00* ±1.00	7.43* ±1.52	7.33 ±1.52	7.33* ±1.15	7.00 ±1.00	6.67 ±1.15	6.67 ±1.15
High	6.67* ±0.47	6.33 ±0.77	6.33* ±0.57*	6.33 ±0.57	6.33 ±0.57	6.33* ±0.57	6.67* ±1.15	6.67* ±1.15	6.67* ±0.57	6.33 ±0.57	6.00* ±1.00	6.00* ±1.00

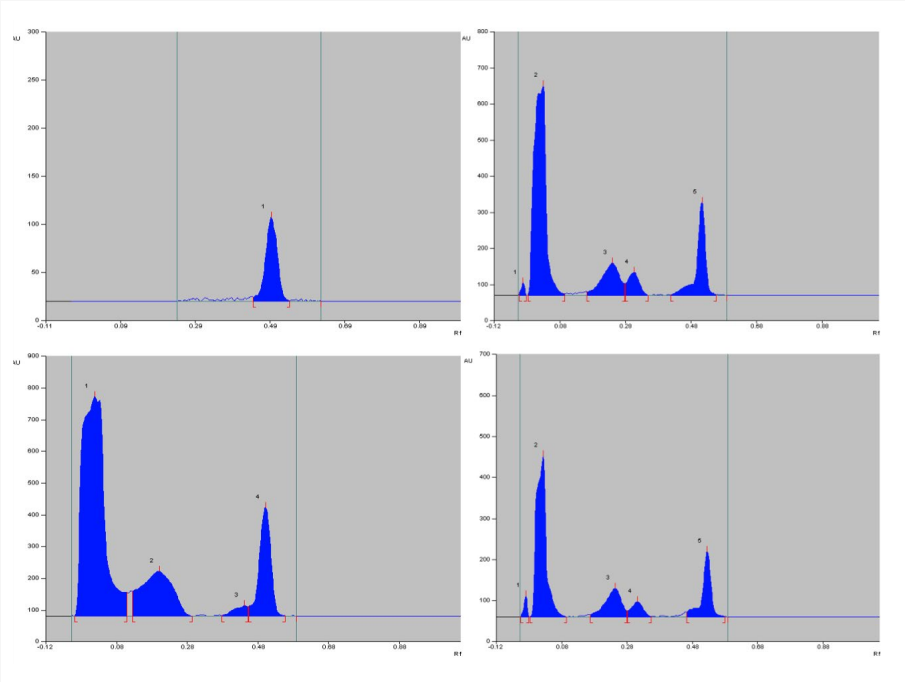

Figure 2. Profiles of eugenol standard (upper left), ethanolic extract in April (upper right), ethanolic extract in July (lower left), and ethanolic extract in November (lower right).

Slika 2. Profili evgenol standarda (zg. levo), etanolnega ekstrakta v aprilu (zg. desno), v juliju (sp. levo) in v novembru (sp. desno).

Table 3. HPTLC Yield of eugenol in basil leaf extract (mg/g FW, n = 3).

Tabela 3. HPTLC koncentracija evgenola (mg/g) v ekstraktih iz listov bazilike (mg/g sveže mase, n = 3).

Altitude	Month											
	1	2	3	4	5	6	7	8	9	10	11	12
Low	2.97 ±0.51	2.83 ±0.72	2.8 ±0.50	2.93 ±0.15	2.73 ±0.50	2.87 ±0.49	2.93 ±0.28	3.2 ±0.60	3.27 ±0.46	3.03 ±0.49	2.9 ±0.51	2.87 ±0.55
Middle	2.3 ±0.17	2.17 ±0.15	2.13 ±0.28	2.23 ±0.15	1.87 ±0.30	2.43 ±0.32	2.5 ±0.36	2.53 ±0.50	2.6 ±0.20	2.37 ±0.58	2.23 ±0.55	2.2 ±0.68
High	2.43 ±0.05	2.33 ±0.15	2.27 ±0.05	2.5 ±0.10	2.23 ±0.05	2. 80 ±0.10	2.77 ±0.15	2.87 ±0.05	2.93 ±0.11	2.7 ±0.10	2.57 ±0.05	2.53 ±0.05

Table 4. HPTLC analysis of eugenol

Tabela 4. HPTLC analiza evgenola.

Season	Area (AU)	Yield (mg/g)
Summer	3302.7	1.68
Rainy	8819.5	3.23
Winter	5292.8	2.55

Qualitative chemical examination of extracts shows the presence of volatile oils such as eugenol. HPTLC chromatogram of Eugenol % Yield mg/g in basil leaf extracts show variations at different seasons, times and places. In the months from June to September (rainy season), in the morning at high altitudes, there is more yield. The values are expressed as mean SEM; $P<0.05$ (Two-way ANOVA followed by Tukey's multiple comparison test) (Table 3). Calibration curves show variations in the concentration of eugenol with the area under the curve. The presence of eugenol in ethanolic extract was confirmed by HPTLC fingerprinting. The yield was 3.23 mg/ gm at Rf value 0.51, which is more than summer 1.68 mg/g and winter 2.55 mg/g at altitude of 615 and 200 m a.s.l. at evening time (Table 4; Figures 1, 2). Current research work can be useful for the selection of month, place and time of harvesting crude drugs. According to Stefanakis et al. (2022), the highest content obtained for all five species when leaves and/or inflorescences were analyzed was during the summer months and especially July, except for *S. rosmarinus*, where the highest yield was obtained during June, i.e. rainy season. According to Gautam et al. (2024), crude drug activity variation is related to the season, time, and place of collection of the plant, and it is proportional to the changes in the active components of the herbal drug.

Conclusions

The study concluded that the environmental changes related to the season, time, and place of collection of the plant are proportional to the changes in the active components of the herbal plant. In July, eugenol content was higher at 6.00 am and 1000 meters high altitude.

Author Contributions

Conceptualization, G.P., R.P.; Methodology, G.P., S.G.; formal analysis, G.P.; investigation, G.P.; resources, S.M.; data curation, G.P.; writing—original draft preparation, G.P., R.P.; writing—review and editing, G.P., R.P.; visualization, S.M.; supervision, G.P., R.P. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interes

The authors declare no conflict of interest.

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Original Research

Rhizobacteria-*Pseudomonas guguanensis* Isolated from Mines Area Assists Green-Remediation of Cadmium by *Brassica juncea*: a Promising Environment Sustainable approach

Sarita Sharma [1,2*], Meenu Saraf [2]

Abstract

This study investigated how Cd-tolerant rhizobacteria isolated from the mine area and landfill site influence the phytoremediation efficacy of *Brassica juncea* plants in Cd-contaminated soils. Out of four cadmium-tolerant rhizobacteria, isolate SMHMZ4 showed the promising phytoextraction efficacy of *B. juncea*. Isolate SMHMZ4 was identified as *Pseudomonas guguanensis* and submitted to NCBI GenBank under accession number MZ145097. These rhizobia were reported for the first time to support Cd-phytoremediation using *B. juncea*. Compared with the non-inoculated control, SHMMZ4 treatment significantly improved the germination of *B. juncea* seeds and increased soluble Cd in soil by 7.78 times. Growth and health parameters, pigment and Cd accumulation in roots and shoots of isolate SHMMZ4 inoculated *B. juncea* grown in individual soil contaminated with 94.95 µg g^{-1} $CdCl_2$ were significantly increased. Pot experiments showed that SHMMZ4 could transfer Cd from soil to roots, from roots to shoots. The translocation, bioconcentration, and bioaccumulation coefficient values were 1.28, 1.22, and 1.72 times higher, respectively, than in the non-inoculated control. The present study demonstrates that the rhizobacteria amendments to *B. juncea* are believed to be a promising method for green remediation of cadmium-polluted areas.

Keywords

Rhizobacteria, metal(s), phytoremediation, atomic absorption chromatography, bioconcentration factor, translocation factor

1 Department of Microbiology, Institute of Sciences, Humanities, and liberal studies (IISHLS), Indus University, Ahmedabad - 382115, Gujarat (India)

2 Department of Microbiology and Biotechnology, University of School of Sciences, Gujarat University, Ahmedabad - 380009, Gujarat (India)

*** Corresponding author:**
E-mail address: saritasharma.ishls@indusuni.ac.in
sarita191087@gmail.com

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Rhizobacteria *Pseudomonas guguanensis*, izolirana iz območja rudnikov, pomaga pri zeleni sanaciji kadmija z *Brassica juncea*: obetaven okoljski trajnostni pristop

Izvleček

Naša študija je preučevala, kako rizobakterije, tolerantne na kadmij, izolirane iz območja rudnika in odlagališča, vplivajo na učinkovitost fitoremediacije rastlin *Brassica juncea* v tleh, onesnaženih s kadmijem. Izmed štirih rizobakterij, tolerantnih na kadmij, je izolat SMHMZ4 pokazal obetavno učinkovitost fitoekstrakcije *B. juncea*. Izolat SMHMZ4 je bil identificiran kot *Pseudomonas guguanensis* in predložen NCBI GenBank pod pristopno številko MZ145097. Prvič so poročali, da te rizobije podpirajo Cd-fitoremediacijo z *B. juncea*. V primerjavi z neinokulirano kontrolo je tretma s SHMMZ4 znatno izboljšal kalitev semen *B. juncea* in povečalo topnost Cd v tleh za 7,78-krat. Rastni parametri, fiziološko stanje, kopičenje pigmenta in kadmija v koreninah in poganjkih *B. juncea* inokulirane z izolatom SHMMZ4, gojene v posameznih tleh, onesnaženih s 94,95 µg g^{-1} $CdCl_2$, so se znatno povečali. Poskusi v loncih so pokazali, da lahko SHMMZ4 vplivana na privzem kadmija iz tal v korenine in iz korenin v poganjke. Vrednosti translokacijskega koeficienta, biokoncentracijskega koeficienta in bioakumulacijskega koeficienta so bile 1,28, 1,22 oziroma 1,72-krat večje, kar je bilo bistveno višje od neinokulirane kontrole. Ta študija dokazuje, da so kombinacija *B. juncea* z rizobakterijami zelo obetavna metoda za zeleno sanacijo s kadmijem onesnaženih področij.

Ključne besede

Rizobakterije, kovine, fitoremediacija, atomska absorpcijska kromatografija, biokoncentracijski faktor, translokacijski faktor

Introduction

In recent decades, metal(s) contamination of soil has become a serious concern due to anthropogenic activities such as growing industrialization, mining activity, heavy input of various fertilizer applications, weedicides, pesticides, and land irrigation with polluted water sources (Sharma et al., 2021a; Sharma and Saraf, 2023b). Given that metal(s) are quickly incorporated into the food chain through fruits, vegetables, and other edible plant components, metal(s) contamination in agricultural soils is particularly significant (Kaur et al., 2018; Prajapati et al., 2022). Elevated concentrations of potentially hazardous metal(s) in soils are recognized worldwide as a major contributor to environmental deterioration, endangering human health and the ecosystem (Ekoa Bessa et al., 2021). For 25 to 30 years, cadmium (Cd), a dangerous non-essential transition metal, can accumulate in plants and animals. Reactive oxygen species (ROS) were created, photosynthesis and respiration were inhibited, enzyme activities were altered, and plants' capacity to absorb nutrients was diminished when exposed to cadmium (Wang et al., 2022). Sensitive remediation techniques for metal(s)-contaminated soils are desperately needed to protect the environment and reduce the detrimental effects of metal(s) bioaccumulation and biomagnification in biological systems. Many physical, chemical, and/or biological solutions are now available to clean up contaminated environments (Sharma I., 2020). On the other hand, the high cost and labour intensity of physical and chemical procedures are their limitations. Additionally, secondary pollutants such as sludge and piles are produced by chemical operations and will be harsh to the environment (Zainab et al., 2020).

According to Jeyasundar et al. (2021), "green remediation" refers to a gentle in situ remediation technique that considers the environmental impact of remediation measures at every stage of the process to maximize the overall environmental value of a clean-up. Because they are effective at creating biomass, plants like *Brassica juncea* are perfect for producing bioenergy (Ying et al., 2021; Sharma and Saraf, 2023a: 2023c; 2023d). According to Amin et al. (2018), plants are believed to be a low-cost way to remove metal(s) with relatively modest environmental management issues. Additionally, rhizobacteria-assisted phytoremediation of metal(s)-contaminated soils, which promotes plant development, is being investigated as an affordable, environmentally friendly option for soil management. Therefore,

to achieve the objectives of "green remediation," plant growth-promoting rhizobacteria (PGPR) from the Zawar mines in Udaipur, Rajasthan, India, and the Pirana garbage dumpsite in Ahmedabad, Gujarat, India, along with the bio-energy crop *B. juncea*, were integrated into this study. The efficacy of phytoremediation may be limited by factors such as plant production, pollutant bioavailability, and plant resistance to stress caused by metal(s) (Antoniadis et al., 2021). The use of various types of organic and inorganic substances as amendments, plant growth promoters, beneficial microorganisms, and genetically engineered microorganisms has been reviewed and suggested by numerous researchers as assisted phytoremediation techniques (Rathore et al., 2019; Saraf et al., 2017; Rostami and Azhdarpoor, 2019). These techniques are thought to help plant stabilization, promoting phytoextraction performance. The remediation potential of these processes is primarily achieved by increasing i) plant biomass, root surface area, and health (Shaikh et al., 2022); ii) metal bioavailability and microbial community composition; and iii) metal translocation within plants via microbe-metal-plant interactions (Sharma and Saraf, 2023a:2023b:2023d). Of these processes, microbial-assisted phytoremediation has recently attracted increased attention due to multiple efficient mechanisms. According to Sullivan and Gadd (2019), microorganisms can interact with metals, food, and toxins. Their exceptional adaptability and metabolic activities also show that they are particularly useful for remediation (Sharma I., 2020; Dabhi et al., 2021).

Determine whether toxic Cd-tolerant rhizobacteria can increase cadmium availability and translocation in *B. juncea* roots and shoots to enhance phytoremediation and make it a viable choice for improving Cd-contaminated locations. This was the main goal of the current investigation. Recent studies (Din et al., 2020; Zhang et al., 2020; Sharma and Saraf, 2023a) focused on single strain-single host plant interactions; nevertheless, microbial populations in the field are always complex mixtures.

Materials and Methods

Metal-tolerant rhizobacteria isolation and selection

Earlier, we recovered 91 multi-metal-tolerant rhizobacteria: 40 from the Pirana dumpsite in Ahmedabad, Gujarat, India, and 51 from the Zawar mines in Udaipur, Rajasthan, India. Because of their strong resistance to Cd, four rhizobacteria were selected for additional study (Sharma et al., 2020; 2021; 2022a: 2023c). We explored how these rhizobacteria affected *B. juncea's* cadmium accumulation and plant growth.

Identification of Cd-tolerant rhizobacteria using 16S rRNA gene sequence

Isolates with greater resistance to cadmium, plant growth-promoting traits, and elevated cadmium accumulation in plants were selected for identification. The pure culture's DNA was taken out. Using Agarose Gel Electrophoresis, a single band of high molecular weight DNA was discovered, indicating the sample's purity. Using 16S rRNA primers, a portion of the gene was amplified by PCR. One unique PCR amplicon band was visible when the sample was resolved on an Agarose Gel. SLS's PCR Purification kit (column-based purification) was used to purify the PCR amplicon to eliminate contaminants. Using the BDT v3.1 Cycle sequencing kit and 16S rRNA Primers, the DNA sequencing reaction of the PCR amplicon was carried out using an ABI 3730xl Genetic Analyzer at SLS Research Pvt. Ltd., Surat, Gujarat, India (Saitou and Nei, 1987). BLAST was run using the gene sequence against the NCBI Genbank database. Various alignment software programs were used to align the first ten sequences, which were selected based on their maximum identity score. Here's a summary of the primers (Sharma and Saraf, 2023c).

Primer name	Sequence (5′–3′)
27F	AGA GTT TGA TCM TGG CTC AG
1492R	CGG TTA CCT TGT TAC GAC TT

Soil sampling and metal spiking

Surface soil (0–15 cm) samples were collected at five separate locations from an agricultural tract near Jagatpur village near Gota, Ahmedabad, Gujarat. The soil samples were bulked up to form a composite sample after being air-dried, crushed, and sieved to a particle size of 2 mm. $CdCl_2$ was used as a Cd source to spike the soil sample. The soils were spiked, meaning that 1000 g of air-dried parent soil (at a 10:1 solid:liquid ratio) received 100 mL of 1000 mg L^{-1} metal stock, and the mixture was incubated for four weeks to stabilize the metals in the soil. According to Sharma

and Saraf (2023c), the previously indicated spiking was intended to produce a target concentration of about 100 µg g^{-1}. The atomic absorption spectrophotometer was used to assess the metal concentrations in the contaminated soil after applying the acid digestion method. Following the protocols mentioned above, each spike of soil produced had Cd values of 94.95 µg g^{-1} after four weeks. These spike soils were then used in pot studies.

Rhizobacterial inoculum preparation

The creation of rhizobacterial inoculum is achieved by growing rhizobacterial isolates in 250 mL Erlenmeyer flasks containing 100 mL of sterilized nutrient broth modified with 100 µg mL^{-1} of Cd. Flasks were in a shaking incubator set at 37°C ± 2°C and 120 rpm for 48 hours. After centrifuging the bacteria for 15 minutes at 10,000 rpm, they were twice cleaned with sterile distilled water to extract the cells. Cell pellets were resuspended in phosphate buffer (pH 7.0) and adjusted to an absorbance of 1.5 at 600 nm (about = 5.6 × 10^8 cfu mL^{-1}) using a spectrophotometer (Systronics 166).

Pot experiment preparation

The soil was first oven-dried for 72 hours at 70°C. It was then sieved using a 2 mm sieve and autoclaved for 15 minutes at 121°C. After being sterilised, 1000 g of soil were placed into 6-by-7-inch plastic pots and individually spiked with 100 µg g^{-1} Cd using six different treatments: rhizobacterial isolates (SMHMZ2, SMHMZ4, SMHMP4, and SMHMP23, Control (H2O with no external metal or inoculums added), and Control (M, spiked with Cd). It employs *B. juncea* as a plant to screen rhizobacterial isolates for Cd phytoextraction. Before inoculation, all spike pots were kept for four weeks to attain metal-in-soil equilibrium, and the bags were arranged in a randomized factorial pattern. In total, six treatments with four duplicates produced twenty-four black poly bags.

Rhizobacteria's influence on soil metal mobility

The rhizobacterial strains were cultivated in 100 mL conical flasks with 50 mL of Nutrient broth and incubated at 37°C ± 2°C and 200 rpm on a Remi, India shaker. After being centrifuged at 8000 rpm for 10 minutes, the bacterial cells were removed from the broth after 24 hours. They were then twice cleaned with phosphate buffer (pH 7.0) and resuspended in 5 mL of sterile double-distilled water. Each bacterial cell suspension's optical density was adjusted to 1.5 using a UV spectrophotometer. (Systronics 166). 100 g of the soil contaminated with 100 µg g^{-1} $CdCl_2$ was placed into 50 ml falcon tubes, and a 1 mL aliquot of SMHMZ2, SMHMZ4, SMHMP4, and SMHMP23 rhizobacterial cell suspension was added (Treatment 2). The tubes were shaken at 200 rpm at room temperature. Treatment 1: (control - Cd spike soil + no rhizobacterial inoculation) received a 1 mL application of sterile distilled water. After seven days, 10 mL of sterile double distilled water was added to each set of falcon tubes to remove water-soluble cadmium. All falcon tubes were centrifuged for 10 minutes at 8000 rpm to get rid of any dirt particles. The resulting solution was then filtered through Whatman filter paper No. 40. The amounts of Cd in the filtrate were measured using an atomic absorption spectrophotometer (ELICO SL 194) (Rajkumar and Freitas, 2008).

Seed germination test

To investigate the impact of bacterial inoculation on seed germination in the presence of metal contamination, seed germination tests were conducted using an adapted version of He et al. (2013) methodology. Two folded pieces of filter paper were placed on the bottom of each clean, sterile glass petri plate for this use. 100 µg mL^{-1} Cd was added to either 10 mL of bacterial solution or sterile tap water (Control). The procedure described by Ndeddy Aka et al. (2016) was used to sterilize *B. juncea* seeds. After being incubated at room temperature for two hours in 10 mL of rhizobacterial consortium suspension on a shaker at 150 revolutions per minute, sterile seeds were placed in each petri dish (30 seeds per plate). Every therapy was administered three times. After seven days, the number of seeds germinating in each petri dish was counted. To measure the seedlings' growth characteristics, three randomly selected seedlings (shoot length and root length) were taken from each plate. The germination percentage and vigour index were computed using the following formula (Rathod et al., 2021).

$$\text{Germination rate [\%]} = \frac{n \times 100}{N}$$

Where n is the number of germinated seeds after seven days, and N is the number of total seeds.

Vigour Index = % germination × Total length of seedling (Shoot length + Root length)

Cadmium and Cd-tolerant rhizobacteria's effect on plant growth

The *B. juncea* seeds were sterilised for the pot tests using the procedure described by Ndeddy Aka et al. (2016). After being sown for two hours in either sterile water (control) or rhizobacterial cultures, sterilized *B. juncea* seeds were allowed to dry at room temperature. Ten infected and non-inoculated seeds were planted at a depth of 5 cm in each container (6 by 7 inches) containing 1000 g of soil. Eight days after germination, the pots were pruned, each containing seven seedlings. After ten days, bacterial suspensions (10 mL per pot) were added to the soil surrounding the root. When the pots were watered again, the leachate was collected in a plastic tray underneath the treatment pot and reintroduced to the pots. Every day, deionized water was used to irrigate the seedlings. The experiment's overall plan called for a maximum of six treatments, each with four replicates:

- *B. juncea* + non-contaminated soil (control H2O)
- *B. juncea* + metal-contaminated soil (spiked with-Cd)
- *B. juncea* + metal-contaminated soil (spiked with-Cd) + rhizobacterial isolates (SMHMZ2, SMHMZ4, SMHMP4, and SMHMP23)

After 30 days, each plant was carefully removed from its pot and given a thorough rinse with distilled water to remove any remaining dirt. After measuring each plant's height and fresh weight, the plants were divided into roots and shoots. Plant tissues, including roots and shoots, were placed inside separate polyethylene bags and dried at 80°C for 72 hours. Plant tissues that had been baked were ground into a powder using a mortar and pestle, and then a Wensar PGB 200 scale was used to weigh each plant individually. Plant samples were appropriately tagged and kept in polyethylene bags for further examination.

Chlorophyll pigment estimation (chlorophyll a, b and carotenoids)

A 0.5 g leaf was homogenized with acetone at an 80% (v/v) concentration and filtered through filter paper to estimate the amount of chlorophyll pigment. A spectrophotometer was used to test the absorbance of the filter at wavelengths of 663, 645, and 480 nm for carotenoids, chlorophyll a, and b, respectively (Lichtenthaler & Wellburn, 1983).

Proline content

Proline levels were ascertained using the Bates et al. (1973) protocol. We crushed a 500 mg plant sample in 10 mL of 3% sulfosalicylic acid. This was centrifuged at 13,000 rpm for 15 minutes. A 2 mL supernatant sample was heated to 100°C and mixed with 2 mL of ninhydrin and glacial acetic acid. Immediately, the test tubes were submerged in an ice bath to halt the reaction. A 4.0 mL toluene sample was added to the reaction mixture and vortexed for one minute. After removing the aqueous layer, the red toluene layer's absorbance was measured at 520 nm. The standard curve was made using the L-proline.

Total phenols

The creation of rhizobacterial inoculum is achieved by growing rhizobacterial isolates in 250 mL Erlenmeyer flasks containing 100 mL of sterilized nutrient broth modified with 100 µg/mL of Cd. Flasks were in a shaking incubator set at 37°C ± 2°C and 120 rpm for 48 hours. After centrifuging the bacteria for 15 minutes at 10,000 rpm, they were twice cleaned with sterile distilled water to extract the cells. Cell pellets were resuspended in phosphate buffer (pH 7.0) and adjusted to an absorbance of 1.5 at 600 nm (about = 5.6 × 10^8 cfu mL-1) using a spectrophotometer (Systronics 166).

Flavonoid content

According to Zhishen et al. (1999), the amounts of flavonoids were tested. The dried sample of 100 mg was crushed in 3 mL of 100% alcohol. Four mL of double-distilled water, 3 mL of 5% $NaNO_2$, and 3 mL of 10% $AlCl_3$ were mixed with 1 ml of the extract sample. For 10 minutes, the mixture was incubated with 2 mL of NaOH and 2 mL of distilled water. The absorbance was measured at 510 nm. The standard for determining flavonoid content was quercetin.

Plant metal(s) measurement

Each dried plant sample (roots and shoots) was carefully weighed using a balance before being added to a 100 mL Erlenmeyer flask with 9 mL of HCL, 3 mL of concentrate HNO_3 (69 %), and 1 mL of H_2O_2 for the Cd analysis. The mixture was heated on a hot plate at 100°C in a fume hood until white fumes were released. After digestion, the mixture was allowed to cool before being diluted with

distilled deionized water to a volume of 50 mL. Before the Cd concentrations in the samples were measured using an Atomic Absorption Spectrophotometer (ELICO SL 194), the resultant solution was twice filtered using filter paper (Ndeddy Aka et al., 2016; Edulamudi et al., 2019).

Evaluating the efficacy of phytoremediation

Phytoextraction indices are a helpful technique for determining the phytoextraction potential of *Brassica juncea* in conjunction with PGPR for removing Cd from polluted soil. The following are the most commonly used phytoextraction indices (Lindsay and Norvell, 1978; Amin et al., 2018):

Bioconcentration factor (BCF)

The bioconcentration factor (BCF) was calculated as the metal concentration ratio in plant roots to soil (Lindsay and Norvell, 1978; Amin et al., 2018):

$$\text{Bioconcentration factor [BCF]} = \frac{[Metal]root}{[Metal]soil}$$

Bioaccumulation coefficient (BAC)

The bioaccumulation coefficient (BAC) was calculated as a ratio of Cd in shoots to Cd in soil (Lindsay and Norvell, 1978; Amin et al., 2018):

$$\text{Bioconcentration factor [BCF]} = \frac{[Metal]shoot}{[Metal]soil}$$

Translocation factor (TF)

The translocation factor (TF) was calculated as a ratio of Cd in plant shoots to Cd in plant roots (Lindsay and Norvell, 1978; Amin et al., 2018):

$$\text{Bioconcentration factor [BCF]} = \frac{[Metal]shoot}{[Metal]root}$$

Statistical Analysis

In this experiment, a randomized block design was utilized. Every experiment was run in triplicate. Each treatment's outcomes were given as an arithmetic mean plus standard error. One-way analysis of variance (ANOVA) was performed on the data using IBM SPSS Statistics version 22 (SPSS Inc. Chicago, USA). The homogeneity of variance was assessed using the Levene test, and differences between the averages of all treatments were tested at $p \leq 0.05$ significance using Duncan's Multiple Range Test (DMRT).

Results

Identification using 16s rRNA sequence

Molecular identification of isolates of rhizobacteria SMHMZ4 was conducted based on the outcomes of phytoextraction efficiency of Cd by *B. juncea*. The partially amplified and sequenced 16S mRNA gene was searched for sequence homology using BLAST. It was discovered that the partial nucleotide sequences of the bacterial strains transcribed by SMHMZ4 and Pseudomonas guguanensis strain CC-G9A were reasonably similar. They were recognized by phylogenetic analysis as gram-negative bacteria (Fig. 1). Partial sequences of rhizobacterial strains tagged with SMHMZ4 were given the following accession codes, which were then added to the NCBI GenBank database under the accession number MZ145097.

Rhizobacteria's influence on soil metal mobility

In this work, the levels of water-soluble Cd in soil were examined to assess the effectiveness of individual rhizobacteria in promoting soil Cd solubilization. When compared to the control treatment, soil inoculation with SMHMZ4 increased the quantity of soluble Cd in the soil by a factor of 7.78 (Fig. 2).

Cadmium-tolerant rhizobacteria's effect on germination of *B. Juncea* seedlings under Cd stress environment

Table 1 shows that rhizobacterial strain inoculation of seeds significantly increased plant height, root length, seed germination, and vigour index ($p<0.05$) compared to control. For seedlings treated with SMHMZ4 and SMHMP23, maximum germination% of 80% and 76.66%, respectively, were registered. With Cd cultivation, uninoculated seedlings exhibited the lowest germination rate (43.33 %), considerably ($p<0.05$). The results showed that seeds treated with SMHMZ4 and SMHMP23 had considerably ($p<0.05$) higher seedling vigour indexes, 768.0 and 691.77, respectively. The seeds treated with Cd yielded the lowest value (326.4).

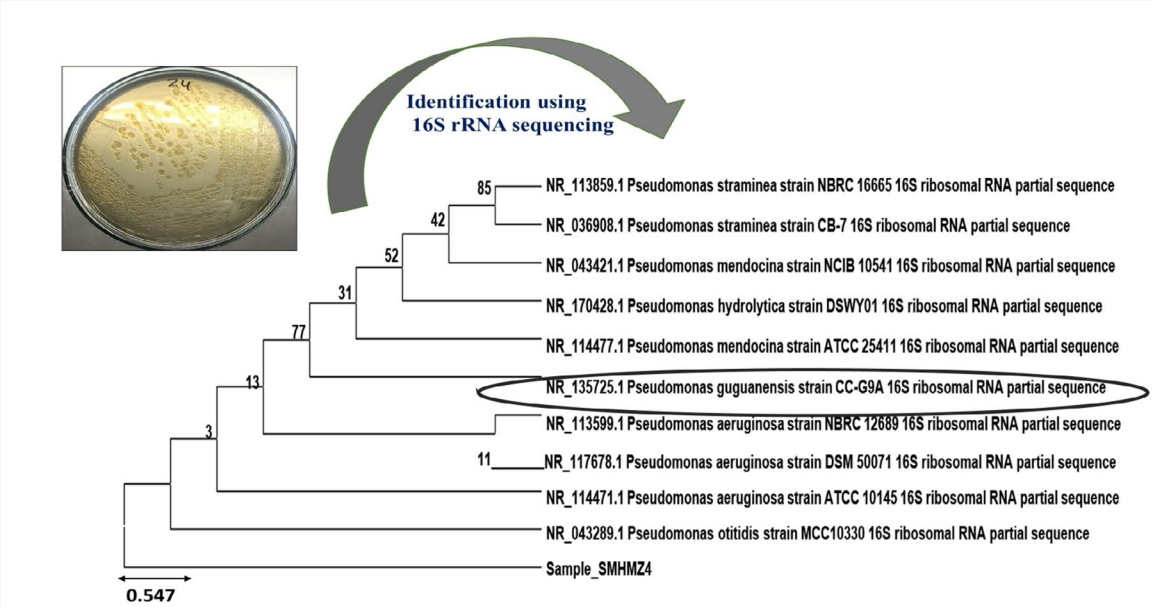

Figure 1. The Phylogenetic relationship of rhizobacterial strain coded with SMHMZ4 closely related sequences based on partial 16S rRNA gene sequence.

Slika 1. Filogenetsko razmerje seva rizobakterij, kodiranega s tesno povezanimi sekvencami SMHMZ4 na podlagi delnega zaporedja gena 16S rRNA.

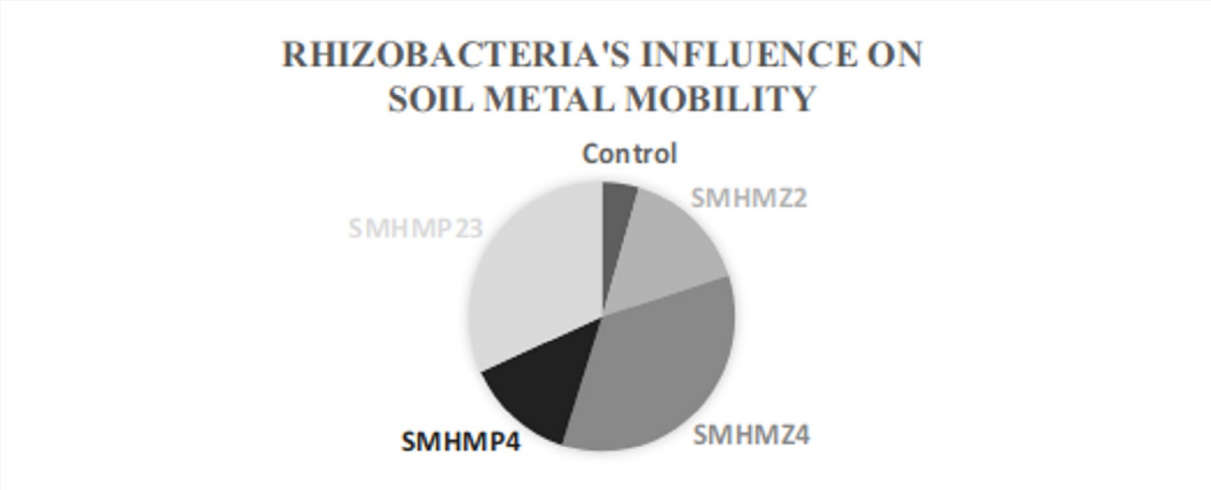

Figure 2. Effect of Cd-tolerant rhizobacterial isolates on the concentration of water-soluble Cd in soil. Treatment 1 Control (metal contaminated soils (100 µg g^{-1} $CdCl_2$) with no bacteria inoculation); Treatment 2 (metal-contaminated soils + individual rhizobacteria). According to Duncan's Multiple Range Test ($p<0.05$), similar letters in the same column are statistically non-significant. Data are means (n = 3±SD), with ain superscript indicating considerably higher values, and later alphabets indicating significantly lower values.

Slika 2. Vpliv izolatov rizobakterij, tolerantnih na Cd, na koncentracijo vodotopnega kadmija v tleh. Obdelava 1 Kontrola (s kovinami onesnažena tla (100 µg g^{-1} $CdCl_2$) brez inokulacije bakterij); Obdelava 2 (s kovinami onesnažena tla + posamezne rizobakterije). Po Duncanovem Multiple Range Testu ($p<0,05$) so podobne črke v istem stolpcu statistično neznačilne. Podatki so povprečja (n = 3±SD), z nadnapisom, ki označuje znatno višje vrednosti, kasnejše črke pa označujejo bistveno nižje vrednosti.

Table 1. Effect of Cd-tolerant rhizobacteria and Cd on shoot length, root length, vigour index, and germination of *B. juncea* seedlings.

Tabela 1. Vpliv Cd in rizobakterij tolerantnih na Cd na dolžino poganjkov, dolžino korenin, indeks vitalnosti in kalitev kalic *B. juncea*.

Treatments	Shoot length (cm)	Root length (cm)	Vigor index	Germination (%)
Control (H_2O)	3.80±0.06^{a}	4.40±0.06^{c}	519.33±6.3^{e}	63.33
Control (M)	3.76±0.09^{a}	3.77±0.09^{d}	326.44±7.2^{f}	43.33
SMHMZ2 (M)	3.70±0.09^{a}	4.50±0.06^{c}	555.55±2.2^{d}	66.67
SMHMZ4 (M)	3.83±0.07^{a}	5.90±0.06^{a}	768.00±9.2^{a}	80
SMHMP4 (M)	3.43±0.12^{b}	5.37±0.03^{b}	586.66±10.2^{c}	66.67
SMHMP23 (M)	3.60±0.06^{ab}	5.87±0.09^{a}	691.77±6.47^{b}	76.66

Note: According to Duncan's Multiple Range Test (p<0.05), similar letters in the same column are statistically non-significant.
Data are means (n = 4±SD), with a superscript indicating considerably higher values and later alphabets indicating significantly lower values.

Effect of Cd-tolerant rhizobacteria on *B. juncea* growth under Cd stress condition

In comparison to the same level of Cd stress without inoculation, the results showed that rhizobacteria SMHMZ4 significantly (p≤0.05) improved the root length (1.33-fold)), shoot length (1.94-fold), fresh weight of shoot and root (1.15-fold and 1.80-fold), and dry weight of shoot and root (1.46-fold and 2.22-fold) of *B. juncea* at 94.95 µg g^{-1} of Cd contaminated soil (Fig.3 and Table 2).

Table 2. Effect of Cd-tolerant rhizobacteria and Cd stress on the growth of *B. juncea*.

Tabela 2. Vpliv Cd in rizobakterij tolerantnih na Cd na rast *B. juncea*.

Cd stress	Shoot length (cm)	Root length (cm)	Fresh weight of Shoot (g Pot^{-1})	Dry weight of Shoot (g Pot^{-1})	Fresh weight of Root (g Pot^{-1})	Dry weight of Root (g Pot^{-1})
Control (H_2O)	13.25±0.06^{e}	6.15±0.09^{d}	35.36±0.16^{e}	2.19±0.03^{d}	1.84±0.12^{c}	0.168±0.0^{c}
Control	10.63±0.09^{f}	4.68±0.18^{e}	30.71±0.05^{f}	1.48±0.01^{e}	1.47±0.03^{d}	0.139±0.0^{d}
SMHMZ2	14.28±0.26^{d}	6.75±0.06^{c}	36.46±0.04^{d}	2.27±0.01^{c}	2.00±0.04^{bc}	0.188±0.0^{bc}
SMHMZ4	25.75±0.06^{a}	8.16±0.01^{a}	40.88±0.12^{a}	3.21±0.03^{a}	3.33±0.04^{a}	0.373±0.02^{a}
SMHMP4	15.45±0.06^{c}	7.55±0.06^{b}	37.11±0.03^{c}	2.30±0.01^{c}	2.15±0.01^{b}	0.205±0.0^{b}
SMHMP23	24.38±0.13^{b}	7.96±0.05^{a}	40.05±0.08^{b}	3.08±0.03^{b}	3.2±0.01^{a}	0.353±0.0^{a}

Note: According to Duncan's Multiple Range Test (p<0.05), similar letters in the same column are statistically non-significant.
Data are means (n = 4±SD), with asuperscript indicating considerably higher values and later alphabets indicating significantly lower values.

Figure 3. Pot experiment rhizobacteria assist phytoremediation of Cd using *B. juncea* as a plant.

Slika 3. Rizobakterije v loncu pomagajo pri fitoremediaciji Cd z uporabo *B. juncea* kot rastline.

Effect of Cd and Cd-tolerant rhizobacteria on pigments, total phenolic content, flavonoid content, and proline of *Brassica juncea*

Rhizobacteria SMHMZ4 considerably raised the carotenoid and chlorophyll a and b content of the plant as compared to the non-inoculated control plant (Fig. 4). *B. juncea* treated with hazardous metal (Cd) showed higher amounts of total phenols, flavonoids, and proline; these levels increased even more following inoculation with Cd-tolerant rhizobacteria. After a month of exposure to Cd toxicity, the total phenols, flavonoids, and proline content level in a *B. juncea* plant (187.67, 61.32, and 7.86%, respectively). But under Cd-stressed conditions, supplementation with SMHMZ4 further raised total phenols, flavonoids, and proline levels by 124.46, 73.09, and 66.11%, respectively (Table 3).

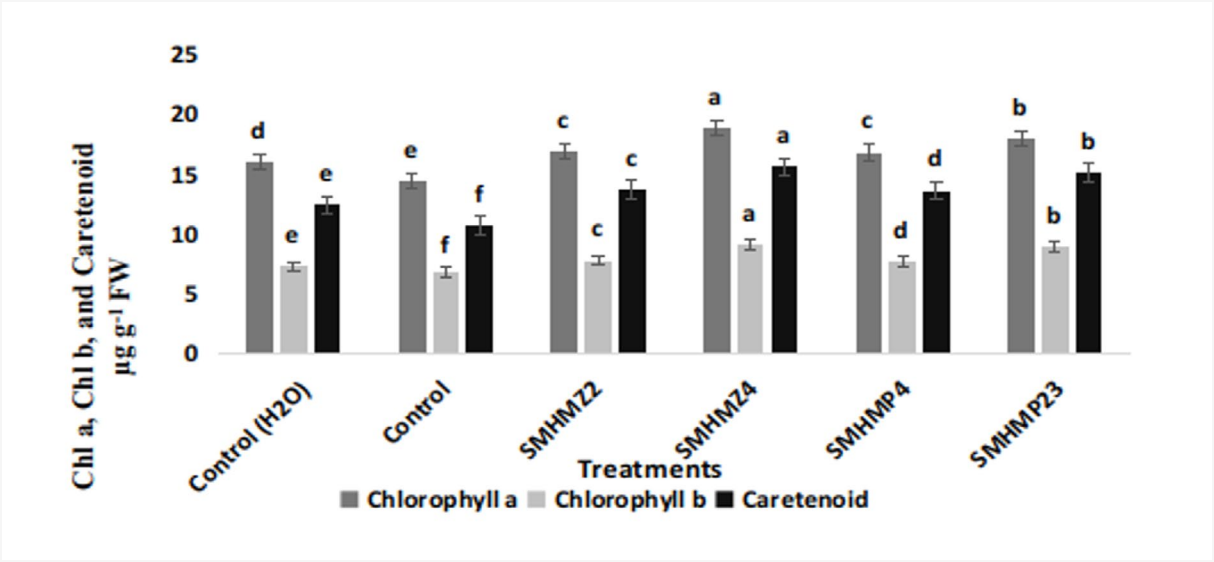

Figure 4. Effect of Cd and Cd-tolerant rhizobacteria on pigments (Chlorophyll a and b, carotenoid content) of *B. juncea* under pot experiments. Note: According to Duncan's Multiple Range Test ($p<0.05$), similar letters in the same column are statistically non-significant. Data are means (n = 4±SD), with ain superscript indicating considerably higher values, and later alphabets indicating significantly lower values.

Slika 4. Vpliv Cd in rizobakterij tolerantnih na Cd, na fotosintezne pigmente (klorofil a in b, vsebnost karotenoidov) *B. juncea* v lončnem poskusu. Opomba: Po Duncanovem testu večkratnega razpona ($p<0,05$) so podobne črke v istem stolpcu statistično nepomembne. Podatki so povprečja (n = 4±SD), z nadnapisom, ki označuje bistveno višje vrednosti, kasnejše črke pa označujejo bistveno nižje vrednosti.

Table 3. Effect of Cd-tolerant rhizobacteria and Cd stress on the level of total phenols, flavonoids, and proline content in *B. juncea*.

Tabela 3. Vpliv Cd in rizobakterij tolerantnih na Cd na raven skupnih fenolov, flavonoidov in vsebnosti prolina v *B. juncea*.

Cd stress	Total phenol content mg g^{-1} DW	Flavonoid content mg g^{-1} DW	Proline µmole g^{-1} DW
Control (H_2O)	5.03±0.15e	1.06±0.03f	3.94±0.0e
Control	14.47±0.09d	1.71±0.03e	4.25±0.0d
SMHMZ2	17.21±0.10c	2.44±0.05c	4.49±0.09c
SMHMZ4	32.48±0.14a	2.96±0.03a	7.06±0.10a
SMHMP4	15.20±0.10d	2.26±0.03d	4.33±0.0cd
SMHMP23	31.23±0.06b	2.80±0.03b	6.60±0.0b

Note: According to Duncan's Multiple Range Test ($p<0.05$), similar letters in the same column are statistically non-significant.
Data are means (n = 4±SD), with ain superscript indicating considerably higher values and later alphabets indicating significantly lower values.

Effect of Cd-tolerant rhizobacteria and Cd on the growth tolerance indices (TIs) of *B. Juncea*

Our study examined B. juncea's tolerance indices (TIs) in the presence of Cd and Cd-tolerant rhizobacteria. The TIs for root and shoot lengths, fresh and dried weights of the roots and shoots, and weights during Cd treatment are displayed in Table 4. Other treatments include metal stress without bacterial inoculation and treatment with bacterial inoculation plus individual metal.

Table 4. Effect of Cd-tolerant rhizobacteria and Cd stress on the growth of *B. juncea*.

Tabela 4. Vpliv Cd in rizobakterij tolerantnih na Cd na rast *B. juncea*.

Cd stress	Tolerance Index (TI_{shoot_L})	Tolerance Index % (TI_{root_L})	Tolerance Index (Ti_{shoot_FW})	Tolerance Index (Ti_{shoot_DW})	Tolerance Index % (Ti_{root_FW})	Tolerance Index % (Ti_{root_DW})
Control	80.19±0.71^{e}	76.02±2.85^{d}	86.84±0.16^{e}	67.16±0.34^{d}	79.62±1.62^{e}	82.35±0.0^{c}
SMHMZ2	107.74±2.0^{d}	109.76±1.05^{c}	103.10±0.12^{d}	103.07±0.22^{c}	108.97±2.27^{d}	111.76±2.4^{b}
SMHMZ4	194.34±0.49^{a}	132.64±0.24^{a}	115.61±0.33^{a}	146.36±1.16^{a}	181.11±2.35^{a}	220.59±12.82^{a}
SMHMP4	116.60±0.49^{c}	122.76±1.05^{b}	104.95±0.09^{c}	104.55±0.41^{c}	116.71±0.26^{c}	122.06±1.47^{b}
SMHMP23	183.96±0.94^{b}	129.43±0.84^{a}	113.24±0.24^{b}	140.34±1.36^{b}	173.91±0.22^{b}	210.30±4.41^{a}

Note: According to Duncan's Multiple Range Test (p<0.05), similar letters in the same column are statistically non-significant.
Data are means (n = 4±SD), with ain superscript indicating considerably higher values and later alphabets indicating significantly lower values.

Cadmium accumulation in *B. juncea* plant

When compared to non-inoculated controls in this study, there was a significant (p ≤0.05) increase in Cd accumulation in the root and shoot tissues of *B. juncea* due to the increase in plant biomass and metal availability brought about by inoculation with toxic Cd-tolerant rhizobacteria treatments (Table 5). For example, the rhizobacteria SMHMZ4 significantly increased the Cd level in *B. juncea* roots and shoots tissues by 24.03 and 71.71%, respectively (p≤0.05).

Table 5. Effect of Cd and Cd-tolerant rhizobacteria on accumulation of Cd in root and shoot of *B. juncea*.

Tabela 5. Vpliv Cd in rizobakterij tolerantnih na Cd na kopičenje Cd v korenini in poganjku *B. juncea*.

	Root Concentration (µg g^{-1} DW)	Shoot Concentration (µg g^{-1} DW)
Control (H2O)	ND	ND
Control	95.32±0.05d	95.05±0.05e
SMHMZ2	114.25±0.03c	134.12±0.01d
SMHMZ4	118.23±0.14a	163.21±0.13a
SMHMP4	114.03±0.01c	138.11±0.04c
SMHMP23	117.22±0.14b	156.29±0.10b

Note: According to Duncan's Multiple Range Test (p<0.05), similar letters in the same column are statistically non-significant.
Data are means (n = 4±SD), with ain superscript indicating considerably higher values and later alphabets indicating significantly lower values.

Phytoremediation potential of *Brassica juncea*

Inoculation with Cd-tolerant rhizobacteria led to a considerable increase in Cd accumulation by the roots and shoots of *B. juncea* compared to the non-inoculated control. The *B. juncea* root and shoot exhibit a significant ($p \leq 0.05$) increase in Cd content due to the presence of SMHMZ4. The Cd-SMHMZ4 inoculated treatment's TF, BCF, and BAC values were 1.28, 1.22, and 1.72, respectively, considerably ($p \leq 0.05$) higher than the non-inoculated control. In the non-immunized control, the Cd TF, BCF, and BAC levels were 1.01, 0.99, and 1.0, respectively.

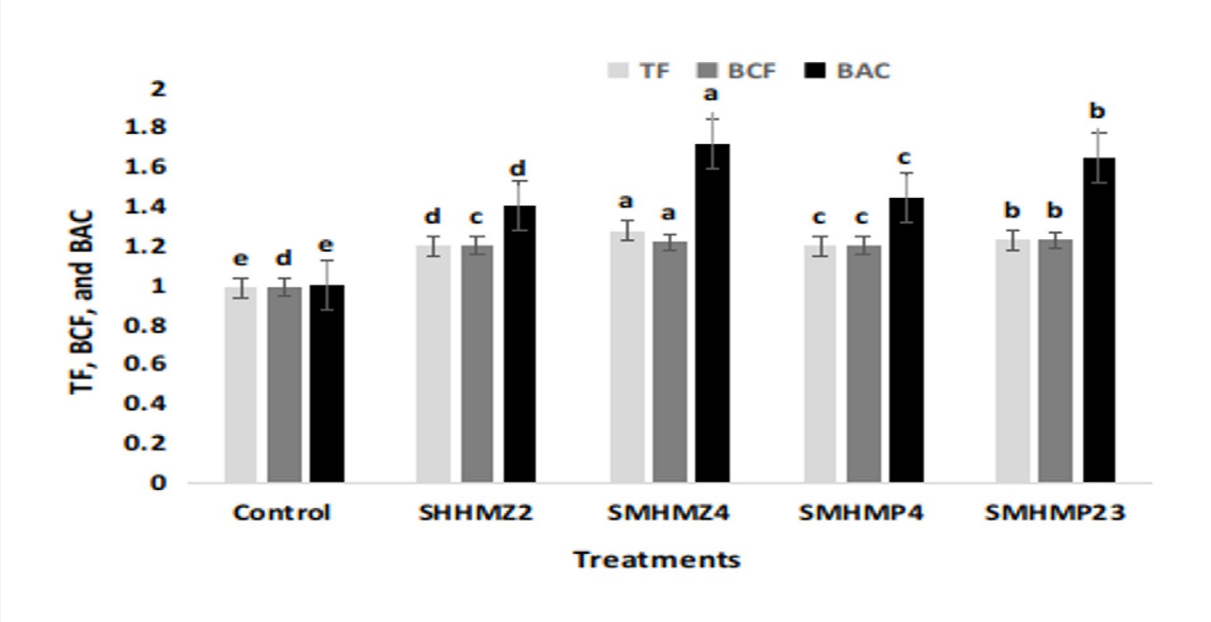

Figure 5. Effect of Cd and Cd-tolerant rhizobacteria on pigments (Chlorophyll a and b, carotenoid content) of *B. juncea* under pot experiments. Note: According to Duncan's Multiple Range Test ($p<0.05$), similar letters in the same column are statistically non-significant. Data are means (n = 4±SD), with ain superscript indicating considerably higher values, and later alphabets indicating significantly lower values.

Slika 5. Vpliv Cd in rizobakterij tolerantnih na Cd, na fotosintezne pigmente (klorofil a in b, vsebnost karotenoidov) *B. juncea* v lončnem poskusu. Opomba: Po Duncanovem testu večkratnega razpona ($p<0,05$) so podobne črke v istem stolpcu statistično nepomembne. Podatki so povprečja (n = 4±SD), z nadnapisom, ki označuje bistveno višje vrednosti, kasnejše črke pa označujejo bistveno nižje vrednosti.

Discussion

Based on the outcomes of phytoextraction efficiency of *B. Juncea*, molecular identification of rhizobacteria SMHMZ4 was carried out and identified as *P. guguanensis* SMHMZ4 and submitted to NCBI Genebank with accession number MZ145097 (Fig. 1).

The low availability of metal(s) in soils is another factor restricting phytoextraction, in addition to the constrained biomass of plants. Rhizobacteria can transform dangerous metal(s) into less toxic forms that plants can absorb, hence increasing the availability of metal(s) in soils (He et al., 2013). Water-soluble Cd levels demonstrated low levels of Cd availability in soils lacking a bacterial inoculum (control set). All of the rhizobacteria utilized in this investigation are capable of both insoluble Cd solubilization and Cd tolerance (Fig. 2). The synthesis of organic acids like indole-3-acetic acid, phosphate solubilization, and oxidation-reduction reactions may be connected to the increase in water-soluble metal concentrations brought on by rhizobacteria (Sharma and Saraf, 2023a).

Germination parameters were investigated to learn more about plant tolerance to cadmium, the quality of seeds, and the ability of rhizobacteria to promote plant growth. The health of the seedlings produced, including their capacity to withstand a range of stressful conditions,

is assessed by the Vigor Index. As compared to the non-inoculated control, the results displayed in Table 1 demonstrate that inoculating *B. juncea* seeds with SMHMZ4 raised the shoot length, root length, seed germination, and vigour index by 1.02-fold, 1.56-fold, 1.85-fold, and 2.35-fold, respectively. Increased synthesis of many metabolites, including GA, cytokinins, alpha-amylase, and IAA, was linked to the better health of seedlings (Sharma et al., 2023a: 2023c).

Compared to uninoculated plants growing in non-contaminated soil, cadmium poisoning considerably shortened the length of the roots and shoots (Table 2). Also, metal(s) can prevent plants from absorbing nutrients, leading to stunting, chlorosis, and even plant death (Sadiq et al., 2017). Rhizobacteria provide essential nutrients to their host plants, eliminate toxins that hinder their growth, and support one another through biochemical and physical processes. This enhances the intake of minerals and nutrients and promotes the growth of plant roots and shoots. Plants inoculated with SMHMZ4 exhibited more chlorophyll content than non-inoculated control plants, with SMHMP23 following closely behind (Fig. 4). Similar results in Fe-contaminated soil were reported by Jinal et al. (2019).

The tolerance index values of plants inoculated with PGPR were higher than those of the non-inoculated controls (Table 4). Compared to the control, SMHMZ4 dramatically increased the Cd concentration in the root and shoot in the current investigation (Table 5). A similar observation was made by Din et al. (2020), who reported that the synthesis of plant growth substances like IAA, siderophore production, and the solubilization of minerals like phosphorus caused the improved mineral and nutrient uptake resulting from the bacterial inoculation. These processes promote plant root elongation and shoot growth. Ammonia and HCN generation are crucial factors that have been proven to significantly affect biocontrol and may also indirectly impact root and plant growth (Rochlani et al., 2022). The capacity of a metal(s) to restrict root and/or shoot growth in a medium is used to determine a plant's tolerance to heavy metal stress. If the TI value is less than one, it indicates that metal contamination caused stress to the plant, which led to a net reduction in biomass. On the other hand, plants that have evolved tolerance and a net increase in biomass (hyperaccumulator) are indicated by TI values greater than one (or > 100%). In contrast to the control treatments, the plant is not impacted by metal pollution if the TI value is 1 (Amin et al., 2018).

Compared to non-inoculated controls, the results demonstrated that treatments containing rhizobacteria under Cd stress had tolerance index values of more than one, indicating that plants developed tolerance significantly and with a notable net increase in biomass. Plants that were injected with PGP rhizobacteria experienced a reduction in metal stress. Additionally, the microbial inoculations resulted in a significant ($p \leq 0.05$) increase in root-to-shoot translocation. These findings align with previous research (Mendoza-Hernandez et al., 2019; Jeyasunda et al., 2021) that found that *Brassica* plants inoculated with different microbial strains had higher Cd concentrations than the corresponding controls.

According to Wang and colleagues (2022), inoculants derived from Plant growth promoting bacteria (PGPB) consortia, both rhizospheric and endophytic, enhanced plant growth (6.9%–22.1%), facilitated *B. juncea's* Cd uptake (230.0%− 350.0%), increased Cd phytoextraction efficiency (343.0%− 441.0%), and improved soil Cd removal rates (92.0%− 144.0%). According to Ali et al. (2021), the utilization of toxic metal-resistant polymer-grafting resin (PGPR) for metal(s) alleviation is not only cost-effective and environmentally friendly, but it also fosters plant growth by reducing the stress caused by metal(s) and generating compounds that stimulate plant growth. A comparable study on the synergistic effects of *Pseudomonas aeruginosa*, *Burkholderia gladioli*, and plant growth-promoting rhizobacteria on several physiological and biochemical activities of 10-day-old *Solanum lycopersicum* seedlings under Cd stress was published by Khanna et al. in 2019. In seedlings treated with Cd toxicity, total phenols, flavonoids, and proline levels increased by 30.2, 92.72, and 59.51%, respectively (Table 3). In contrast, *P. aeruginosa* (M1) supplementation raised proline, flavonoids, and total phenols levels in Cd-stressed seedlings by 51.3%, 28.33, and 60.11 per cent, respectively. Total phenols, flavonoids, and proline levels in Cd-treated seedlings increased by 111.7, 13.04, and 83.6%, respectively, upon *B. gladioli* (M2) supplementation (Khanna et al., 2019).

By characterizing metal accumulation and translocation behaviours in plants, the bioconcentration factor (BCF), bioaccumulation coefficient (BAC), and translocation factor (TF) values (Fig. 6) help determine plant suitability for phytoremediation. According to Amin et al. (2018), plants that have BAC, TF, and BCF values greater than one are considered potential phytoextractors and acceptable for phytoextraction, while those that have TF and BCF

values less than one are not suitable for phytoextraction or phytostabilization. In our investigation, *B. juncea's* capacity to absorb additional Cd from the soil with a translocation factor value >1 was enhanced by the rhizobacteria SMHMZ4 (Fig. 5).

Conclusions

The current study concluded that Cd negatively affects *Brassica juncea* plant development and health indices (total phenol, flavonoid, and proline). Nevertheless, inoculating plants with *Pseudomonas guguanensis* SMHMZ4 multi-metal tolerant rhizobacterial strains not only shields them from Cd-induced growth inhibition but also promotes plant growth, biomass production, and metal bioavailability in the soil, all while simultaneously increasing Cd accumulation in the aerial part of the plant caused by *B. juncea*. *P. guguanensis* SMHMZ4 rhizobacteria are essential for boosting the values of the translocation factor, bioconcentration factor, and bioaccumulation coefficient; these factors improve the effectiveness of phytoremediation and reduce the amount of Cd in polluted soil. The findings showed that rhizobacteria-rich *B. juncea* amendments were more successful at cadmium green remediation. Additionally, because *B. juncea* plant species may supply valuable biomass that can be used to create income and remediate places contaminated with metals, they have ecological and economic significance. After harvesting, the biomass could be burned and disposed of, or the metals could be extracted and used to make biofuels again.

Author Contributions

Conceptualization: S.S., Methodology: S.S. & M.S., Software: S.S., Validation: S.S., Formal Analysis: S.S., Investigation: B.OK., S.S., Resources: S.S., Data Curation: S.S., Writing – Original Draft: S.A.S., Writing – Review & Editing: S.S., M.S., Supervision: S.S. All authors have read and agreed to the published version of the manuscript."

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Conflicts of Interest

The authors declare that they have no known conflicts of interest associated with this study.

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Original Research

Allelopathic effect of aqueous extract from selected invasive plants on germination and growth of Tartary buckwheat

Aurora Maria Anžlovar [1], Sabina Anžlovar [2*]

Abstract

Allelopathic compounds released by invasive plants can directly affect neighbouring plants by interfering with their germination and/or suppressing their growth. In this study, we investigated the allelopathic effect of aqueous extract from three invasive plants: Japanese knotweed (*Fallopia japonica*), Canadian goldenrod (*Solidago canadensis*) and stinkwort (*Dittrichia graveolens*) on the germination and early growth of Tartary buckwheat (*Fagopyrum tataricum*). All three aqueous extracts had almost no effect on grain germination but significantly reduced the growth of buckwheat seedlings. In addition, aqueous extracts obtained from a 2-fold serial dilution of a 10% extract of *D. graveolens* inhibited the growth of buckwheat seedlings in a dose-dependent manner. The results showed that root length was significantly more reduced than shoot length, while grain germination remained largely unaffected. The roots were more severely damaged than the shoots and were not only shorter but also thicker and darker in colour. The effect was dose-dependent.

Keywords

allelopathy, *Fagopyrum tataricum*, invasive plant extract, *Fallopia japonica*, *Solidago canadensis*, *Dittrichia graveolens*

1 Bežigrad Grammar School, Peričeva 4, SI-1000 Ljubljana, Slovenia

2 Biotechnical Faculty, Department of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia

* **Corresponding author:**
E-mail address: sabina.anzlovar@bf.uni-lj.si

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Alelopatski učinek nekaterih invazivnih rastlin na kalivost in rast tatarske ajde

Izvleček

Alelopatske spojine invazivnih rastlin lahko neposredno vplivajo na sosednje rastline tako, da motijo njihovo kalitev in/ali zavirajo njihovo rast. V tej študiji smo raziskovali alelopatsko delovanje vodnih izvlečkov treh invazivnih rastlin: japonskega dresnika (*Fallopia japonica*), kanadske zlate rozge (*Solidago canadensis*) in smrdljivke (*Dittrichia graveolens*) na kalitev in zgodnjo rast tatarske ajde (*Fagopyrum tataricum*). Vsi trije vodni izvlečki skoraj niso vplivali na kalitev semen, poleg tega pa so vodni izvlečki, pridobljeni iz 2-kratne serijske redčitve 10-odstotnega ekstrakta *D. graveolens,* zavirali kalitev ajde v odvisnosti od koncentracije. Pri tretiranih rastlinah je bila rast korenin močneje prizadeta kot rast poganjka, pri čemer smo opazili tudi zadebelitev in rjavenje korenin. Učinek je bil odvisen od koncentracije.

Ključne besede

alelopatija, *Fagopyrum tataricum*, izvlečki invazivnih rastlin, *Fallopia japonica*, *Solidago canadensis*, *Dittrichia graveolens*

Introduction

Invasive plants are an important environmental management problem exacerbated by climate change and socio-economic globalization. They contribute to biodiversity loss and ecosystem degradation, and their occurrence is increasing (Pyšek and Richardson, 2010).

Allelopathy is defined as a phenomenon that encompasses both the positive and negative effects of plants on other organisms through chemical substances, described as allelochemicals (secondary metabolites), that plants produce to obtain a competitive advantage over other plants, animals and microbes (Schandry and Becker, 2020). Allelopathy is a common invasion mechanism across the plant phylogeny. Anti-plant allelopathic compounds can directly affect neighbouring plant tissues, disrupting germination and/or the growth of seedlings or mature plants (Kalisz et al., 2021). Since the invasive nature of plants is likely related to their ability to produce specialized allelopathic compounds that can inhibit the growth of neighbouring native plants (Kalisz et al.2021), their large biomass could be used as a source of substances that inhibit or slow the growth of plants, so they could also be used to minimize the growth and spread of other invasive plants.

We tested three invasive plants: Japanese knotweed (*Fallopia japonica*), Canadian goldenrod (*Solidago canadensis*) and stinkwort (*Dittrichia graveolens*). These species are aggressive colonizers in new environments and form dense monospecific stands. The allelopathic character of *S. canadensis* and *F. japonica* has been well documented (Kato-Noguchi, 2021; Kato-Noguchi and Kato, 2022). The root exudates, extracts, essential oil, and rhizosphere soil of *S. canadensis* suppressed the germination, growth and arbuscular mycorrhizal colonization of several plants (Kato-Noguchi and Kato, 2022). Allelochemicals such as flavanols, stilbenes and quinones were also detected in the knotweed methanol root extract. Some of these allelochemicals may be released into the rhizosphere soil through the decomposition process of their parts and the exudates of their rhizomes and roots (Kato-Noguchi, 2021). *D. graveolens* is a plant native to the Mediterranean region that is spreading northwards in Europe. Due to its chemical composition and biological activities, especially its terpene and phenolic content, it has attracted increasing research interest. Moreover, the invasive nature of *D. graveolens* seems to be related to its ability to produce specialized metabolites that can inhibit the growth of other species, leading to the elimination of competing vegetation and the spread of climate change. (Ponticelli et al., 2022)

The leaf extract of *S. canadensis*, the rhizome extract of *F. japonica* and the shoot extract of *D. graveolens* were used for the experiments. Sun et al. (2022) demonstrated that the aqueous extract (50 g/L) from the aerial parts of *S. canadensis* significantly inhibited the germination rate

of *Zoysia* grass, while root and litter extracts showed no significant effect on germination. In *Fallopia*, most of the secondary metabolites are stored in the underground rhizomes (Chen et al., 2013; Frantík et al., 2013). The extract from the shoots of *D. graveolens* was selected because its allelopathic effect on the germination and growth of some weed species is already known (Almhened et al., 2021).

Tartary buckwheat is a very resistant plant that accumulates abundant bioactive compounds that help it survive in harsh environments, such as cooler climates and higher altitudes. In many mountainous regions where other crops cannot survive, Tartary buckwheat is grown as a staple crop because of its short growing season, high ecological adaptability, and tolerance to nutrient-poor conditions (Zou et al., 2023). Tartary buckwheat is more bitter and contains more rutin than common buckwheat (*Fagopyrum esculentum*). It also contains a wide range of bioactive compounds such as flavonoids, phenolic acids, triterpenoids, phenylpropanoid glycosides, bioactive polysaccharides, bioactive proteins and peptides, as well as D-chiro-inositol and its derivatives (Zou et al., 2023). In addition, Tartary buckwheat has been reported to have an allelopathic activity against weeds (Vieites-Álvarez et al., 2023). The results obtained by Vieites-Álvarez et al. (2024) indicate that Tartary buckwheat can sustainably control weeds through plant interference, such as competition or allelopathy and is effective against both monocot and dicot weeds. Therefore, Tartary buckwheat can be considered a very suitable candidate for testing the strength of the allelopathic effect of invasive plants.

Materials and Methods

Plant material

Shoots of *S. canadensis* were collected during the flowering period in Ljubljana, Slovenia (N 46° 4' 19.86", E 14° 25' 52.25'’). Fresh leaves were separated from the shoot and air dried at room temperature in the dark and ground in the mill (IKA, IKA-Werke M20, IKA, Germany).

Fresh shoots of *D. graveolens* were collected during the flowering period in September (det. S. Strgulc Krajšek) in Ljubljana, Slovenia (N 46°6'17.17", E 14°28'54.36"). The shoots were air-dried at room temperature in the dark and ground in the mill.

Rhizomes of *F. japonica* were collected in November in a dense *F. japonica* stands next to stream Mali Graben, Ljubljana, Slovenia (N 46°02'33.9"; E 14°27'00.9"). Fresh rhizomes were washed in tap water, dried, cut in a 1-cm thick reel, frozen in liquid nitrogen, lyophilized (5 days, 0.002bar) (Christ Alpha 1-4LSC, Christ, Germany), and ground in a cutting mill (SM 200; Retsch, Germany).

The grain of Tartary buckwheat was obtained from Rangus Mill (Šentjernej, Slovenia, https://www.mlinrangus.si/en/).

Preparation of aqueous extracts

Ground plant material (5 g) was suspended in 100 ml distilled water and placed on a shaker (Laboshake 500, Gerhardt, Germany), where it was shaken at 130 rpm for approximately 24 hours. After aqueous extraction, the suspension was filtered under pressure through filter paper (Whatman filter paper 520A, pore size 15-18 µm, Ge Healthcare Life Sciences) to remove plant particles and obtain approximately 70 ml of 5% (w/v) aqueous extracts. The extracts were prepared fresh before the experiment and used immediately.

The yield of extract (extractable component) expressed on dry weight basis of pulp was calculated according to the following equation:

$$\text{Yield (\%)} = (W1 \times 100) / W2$$

where W1 is the weight of the extract residue obtained after solvent removal, and W2 is the weight of the dry plant material before the extraction.

For the dilution preparation of aqueous extract of *D. graveolens*, a 10% (w/v) aqueous extract was prepared and serially diluted with distilled water to produce 5%, 2.5%, 1.25% and 0.625% aqueous extracts.

Germination and early growth test

For the germination test, sterile covered crystallizing dishes (9 cm diameter, 3 cm height) with two layers of autoclaved filter paper were used, which were moistened with 8 ml test solution (5% aqueous extract of the rhizome of *F. japonica*, the leaf extract of *S. canadensis*, and shoot extract of *D. graveolens*). In addition, for the concentration-dependent experiment, sterile covered crystallizing dishes (9 cm diameter, 3 cm height) with two layers of autoclaved filter paper were used, which were moistened with 8 ml test solution (10%, 5%, 2.5%, 1.25% and 0.625%

aqueous extracts of *D. graveolens*). Distilled water was used as a control treatment in both experiments. For each test solution, three replicates with 10 grains in a 2x2 cm arrangement in covered crystallizing dishes were used. The germination test took place in a growth chamber at 22 °C, 60% humidity and a photoperiod of 16 hours. The germination experiment lasted four to five days, with the growth of the seedlings being terminated on the seventh day. The germinated grains were counted and examined every day at intervals of about 24 hours. A grain was considered germinated when its radical had emerged. On the seventh day of the experiment, the root and shoot length were measured, and the number of lateral roots was counted as an indicator of seedling development and growth. The roots were separated from the shoots, and the fresh mass was weighed.

Statistical analysis

For the statistical analyses, the mean values and standard errors were calculated for all treatments. Means were compared between treatments using One-way ANOVA and the Bonferroni-Holm post-hoc test (MS Excel, Daniel's XL Toolbox). The level of significance was set at a P value < 0.05.

Results and Discussion

The germination of Tartary buckwheat grain was not significantly affected by the 5% aqueous extracts of the three invasive plants (Table 1). Similarly, a 5% extract of *F. japonica* had no effect on radish seed germination on days 5 and 7 (Šoln et al., 2021), while a 10% root extract of *F. japonica* significantly reduced radish seed germination (Šoln et al., 2023). Both extracts delayed the germination of radish (Šoln et al., 2021), which was confirmed by our experiment in Tartary buckwheat grain (Table 1). The variability of seed germination depends on the plant tested; crop seeds are more sensitive to *F. japonica* extracts than weed seeds (Kato-Noguchi, 2022). Since Tartary buckwheat has high ecological adaptability and tolerance to nutrient-poor and other unfavourable conditions (Sofi et al., 2023; Zou et al., 2023), it is not surprising that its germination was not affected by the extract of *F. japonica*. On the other hand, the leaf extract of *F. japonica* significantly impaired the germination of common wheat (*Triticum aestivum*), especially at the highest concentration (0.05 g/mL), at which only one-third of the seeds germinated (Levačić et al., 2023).

The aqueous extract of *D. graveolens* had no effect on the germination of Tartary buckwheat grain (Table 1). On the other hand, a 5 % aqueous extract of *D. graveolens* proved to be effective against wheat (*Triticum aestivum*) and common ragweed (*Ambrosia artemisiifolia* L.) germination, as it delayed germination and significantly reduced the germination rate (Grašič et al., 2016).

The germination of Tartary buckwheat was not affected by the extract of *S. canadensis*, as the germination rate on the first day was 63 % (Table 1). This inhibition is comparable to that reported for the aboveground aqueous extract of *S. canadensis* on *Zoysia japonica* seeds, which significantly reduced the germination rate to 60% at a concentration of 50 g L-1 (Sun et al., 2021). The 2.5% extracts of *S. canadensis* leaves also significantly inhibited radish seed germination only at the beginning, while the difference was no longer significant on the fourth day compared to the control, reaching 76% (Anžlovar and Anžlovar, 2019). Tartary buckwheat grain appears to be more resistant to *S. canadensis* extract compared to the other seeds tested: *Raphanus sativus*, *Lactuca sativa*, *Triticum aestivum*, *Setaria viridi* (see Kato-Noguchi and Kato 2022), as the germination rate was not significantly reduced across all days (Table 1).

Overall, the invasive plant extracts had only a moderate, non-significant effect on the germination of buckwheat grain, but the results showed that these extracts significantly inhibited the growth of 7-day-old Tartary buckwheat seedlings compared to the control (Figure 1). The early growth of the treated seedlings was slower than that of the control group. Figure 1 shows that all extracts significantly inhibited root and shoot growth compared to the control, with the extract of *F. japonica* having a significantly lower effect on the growth of Tartary buckwheat than *S. canadensis* and *D. graveolens*. Nevertheless, the root extract of *F. japonica* significantly reduced the length of shoots and roots of Tartay buckwheat seedlings compared to the control (Figure 1). These results are consistent with those of other studies that have shown that the aqueous root extract of *F. japonica* reduces root length significantly more than shoot length or seed germination (Šoln et al., 2021, 2022, 2023; Kato-Noguchi 2022). Both treatments with *S. canadensis* and *D. graveolens* resulted in significantly shorter root and shoot lengths compared to *F. japonica* extracts, while there was no difference between *S. canadensis* and *D. graveo-*

Table 1. Germination of Tartary buckwheat grain treated with 5% aqueous extracts of *F. japonica*, *S. canadensis*, and *D. graveolens* for four days. Data are means ± standard error (N = 30). Different letters indicate statistically significant differences within rows (p <0.05).

Tabela 1. Delež kalivosti zrn tatarske ajde tretiranih z vodnimi izvlečki *F. japonica*, *S. canadensis* in *D. graveolens* tekom 4 dni. Podatki so povprečja ± standardna napaka (N = 30). Različne črke prikazujejo statistično značilne razlike med tretmaji (p <0,05).

	Germination rate (%)			
	Control	***F. japonica***	***S. canadensis***	***D. graveolens***
Day 1	73 ± 9[a]	77 ± 3[a]	63 ± 9[a]	77 ± 3[a]
Day 2	87 ± 9[a]	77 ± 3[a]	80 ± 10[a]	77 ± 3[a]
Day 3	87 ± 9[a]	80 ± 6[a]	80 ± 10[a]	83 ± 3[a]
Day 4	90 ± 6[a]	90 ± 0[a]	80 ± 10[a]	83 ± 3[a]

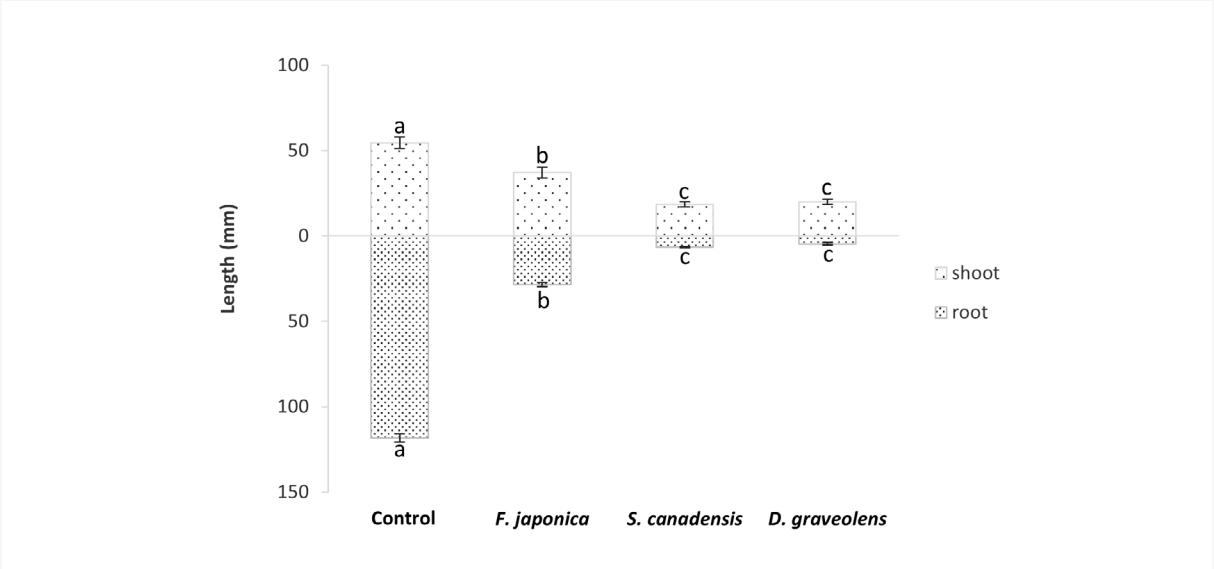

Figure 1. Shoot and root length of Tartary buckwheat seedlings after 7 days of growth and treatment with 5% aqueous extracts of *F. japonica*, *S. canadensis* and *D. graveolens*. Shown are means ± standard error is shown (N=30). Different letters indicate statistically significant difference between treatments for each seelding part separately (One-way ANOVA ..... p <0.05).

Slika 1. Dolžina poganjka in korenine kalic tatarske ajde po 7 dneh rasti in tretmaju z vodnimi izvlečki *F. japonica*, *S. canadensis* in *D. graveolens* Prikazana so povprečja ± standardne napake (N = 30). Različne črke prikazujejo statistično značilne razlike (p <0,05).

lens treatments (Figure 1). The roots of seedlings treated with *S. canadensis* and *D. graveolens* extracts were 94% and 96 % shorter, respectively, compared to the control. Similar growth inhibition was observed in *Raphanus sativus* and *Lactuca sativa* treated with *S. canadensis* (Butcko and Jensen 2002) and *D. graveolens* (Omezzine et al., 2011), while seed germination was not affected (Butcko and Jensen 2002), comparable to our results.

Plant roots show morphological plasticity and play an important role in tolerance to various stress factors (Karlova et al., 2021). The reduction of lateral roots in buckwheat seedlings was significantly affected by both extracts, *S. canadensis* and *D. graveolens*, compared to the control, as 90% of the treated seedlings did not develop lateral roots (Figure 2). The *F. japonica* extract also significantly affected the formation of lateral roots, as the number of roots per seedling decreased by almost 50 % compared to the control. Šoln et al. (2021) reported that the reduction of lateral roots in radish seedlings was differentially affected by the concentration of *F. japonica* extract; lower

concentrations (0.5%) stimulated lateral root formation with up to 25% higher root number per seedling than in control, while higher concentrations (5%, 10%) reduced lateral root formation by up to 54%, which is similar to the result in buckwheat seedlings (Figure 2). Zhang et al. (2023) investigated the effects of salt stress on the growth of Tartary buckwheat and found that low salt stress treatment promoted root growth and improved seedling root activity, while medium and high salt stress treatment reduced root growth compared to the control. In several crops, such as rice, wheat and *Arabidopsis*, the root elongation rate decreased under salt stress, while increased elongation of lateral roots was observed in *Silene vulgaris* and *Brassica napus* (Arif et al., 2019). Root elongation is the result of cell division and cell expansion in the root apical meristem, and salt stress can alter root elongation by both promoting and reducing cell division and expansion (West et al., 2004).

Figures (1 and 2) show that the extract of *F. japonica* was significantly less effective than that of *S. canadensis* and *D. graveolens*, which is also a consequence of the low extraction yield (Table 2). The extract of *F. japonica* contained less dry matter and had the lowest yield compared to the extracts of *S. canadensis* and *D. graveolens* (Table 2). Despite the highest yield of the *S. canadensis* extract, the effects on buckwheat morphology were greater when treated with the *D. graveolens* extract, so we decided to conduct a concentration-dependent experiment with *D. graveolens*.

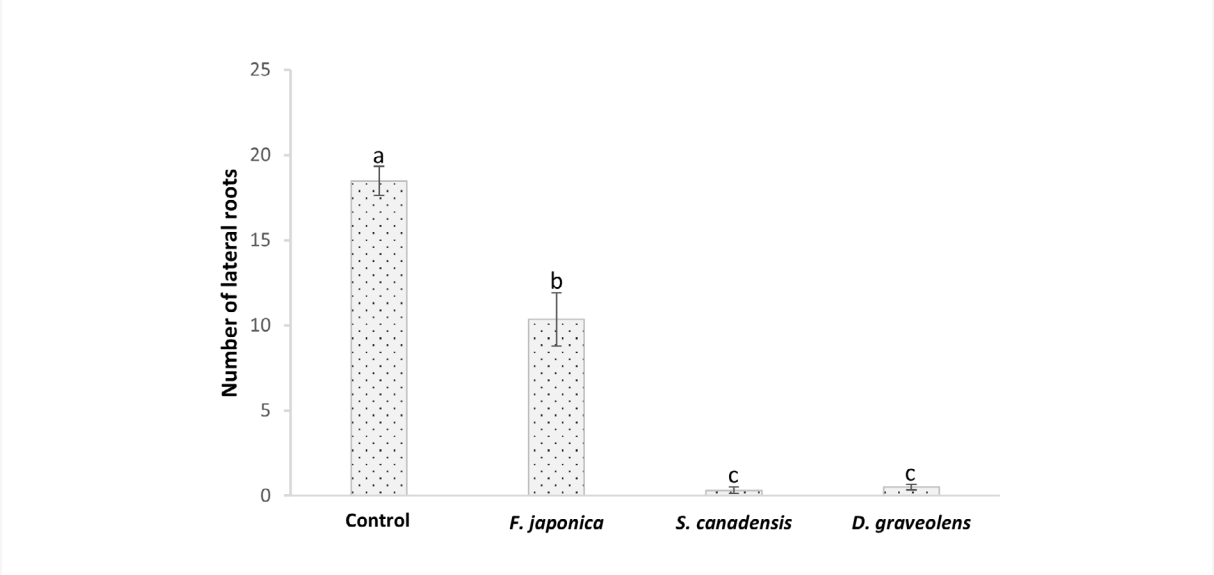

Figure 2. Number of lateral root of Tartary buckwheat seedlings after 7 days of growth and treatment with aqueous extracts of *F. japonica*, *S. canadensis* and *D. graveolens*. Mean value ± SE is shown (N=30). Different letters indicate statistically significant difference among treatments (p <0.05).

Slika 2. Število stranskih korenin kalic tatarske ajde po 7 dneh rasti in tretmaju z vodnimi izvlečki *F. japonica*, *S. canadensis* in *D. graveolens* Prikazana so povprečja ± standardne napake (N = 30). Različne črke prikazujejo statistično značilne razlike (p <0,05).

Table 2. Yield of aqueous extracts. The yield was calculated as the percentage of extract dry mass according to the starting material. Data are means (N = 3).

Tabela 2. Izkoristek vodnih izvlečkov. Izkoristek je delež suhe snovi, glede na maso začetnega materiala, izražen v odstotkih. Podatki so povprečja (N = 3).

Extract	Yield (%)
F. japonica	13.27±0.61
S. canadensis	38.00±0.20
D. graveolens	25.87±0.76

Aqueous extracts obtained from a 2-fold serial dilution of a 10% extract of *D. graveolens* moderately affected the germination of Tartary buckwheat grain (Table 3). After 24 hours, 73% of the control grain had germinated, and a significant difference in germination rate was observed between the control and the treatment at 1.25% to 10%, while the lowest treatment was comparable to the control. After 48 hours, the germination rate was comparable to the control in all treatments (Table 3).

Despite the small effect on the germination rate of *Dittrichia*-treated grain, further growth of Tartary buckwheat seedlings was significantly reduced (Figure 3). The only exception was the 0.625% extract, which actually promoted the growth (Figure 3). Almhemed et al. (2021) reported that the aqueous extract of *D. graveolens* at concentrations of 2%, 6%, and 10% inhibited the germination and growth of some weed species. The germination rate and growth varied depending on the weed species, with greater effects on germination than on growth. Delayed germination and significantly reduced germination rate were also observed in wheat (*Triticum aestivum*) and common ragweed (*Ambrosia artemisiifolia* L.) treated with 5% aqueous extracts of *D. graveolens* (Grašič et al., 2016). In contrast, the results of Abu Irmaileh et al. (2015) showed that the ethanolic extract of *D. graveolens* at 200 ppm reduced root length significantly more than shoot length or seed germination, which is similar to our results (Figure 3, Table 3).

The shoots were less affected by the aqueous extracts of *D. graveolens* than the roots (Figure 3). Here, the different concentrations appear to inhibit shoot growth equally, except for the weakest concentration, which appears to be at the same level as the control and has no significant effect on shoot growth. The inhibition of root growth is dose-dependent (Figure 3). Similarly, volatile monoterpenoids of *Salvia leucophylla* inhibited root growth of *B. campestris* in a dose-dependent manner, while hypocotyl growth remained largely unaffected (Nishida et al., 2005). The morphological and biochemical analyses showed that the size of mature cells was not altered in either hypocotyls or roots but that the monoterpenoids specifically decreased the mitotic index in the root apical meristem, while they did not reduce the mitotic index in the shoot apical region (Nishida et al., 2005). These results suggest that the allelochemicals may affect the growth of other plants in their vicinity by inhibiting cell proliferation in the root apical meristem. This is consistent with the finding that the sensitivity of root growth is the best indicator of the phytotoxicity of allelochemicals, as the root is highly permeable to chemicals (Ponticelli et al., 2022).

The same reduction pattern was observed in the fresh mass of the shoots and roots of the buckwheat seedlings (Figure 4). After seven days, the inhibitory effect of the 10% *D. graveolens* extract was significant and reduced the root mass by 95%. The 5% and 2.5% extracts showed similar effects: Root mass was significantly smaller, while shoot mass did not decrease significantly. The shoot and root mass of the 0.625% treatment corresponded to the control level.

The morphological observations showed that the allelopathic effects of the aqueous extracts of *D. graveolens* on Tartary buckwheat were mainly observed on the roots of the seedlings (Figure 5). They were more severely damaged than the shoots. The roots of Tartary buckwheat seedlings treated with the 10% *D. graveolens* extracts were

Table 3. The germination rate of Tartary buckwheat grain was treated with different concentrations of *D. graveolens* aqueous extracts over five days. Data are means ±standard error (N = 30). Different letters indicate statistically significant differences within rows (p <0.05).

Tabela 3. Delež kalivosti zrn tatarske ajde tretiranih z različnimi koncentracijami vodnega izvlečka *D. graveolens* tekom 5 dni. Podatki so povprečja ± standardna napaka (N = 30). Različne črke prikazujejo statistično značilne razlike med tretmaji (p <0,05).

	Germination rate (%)					
	Control	**0.625%**	**1.25%**	**2.5%**	**5.0%**	**10.0%**
Day 1	73 ± 7a	60 ± 6a	37 ± 7ab	43 ± 9ab	33 ± 7ab	7 ± 3b
Day 2	90 ± 6a	90 ± 6a	80 ± 6a	90 ± 10a	77 ± 9a	87 ± 3a
Day 3	90 ± 6a	90 ± 6a	80 ± 6a	90 ± 10a	80 ± 6a	87 ± 3a
Day 4	97 ± 3a	97 ± 3a	83 ± 9a	90 ± 10a	90 ± 0a	87 ± 3a
Day 5	97 ± 3ab	97 ± 3ab	100 ± 0a	97 ± 3ab	90 ± 0b	87 ± 3ab

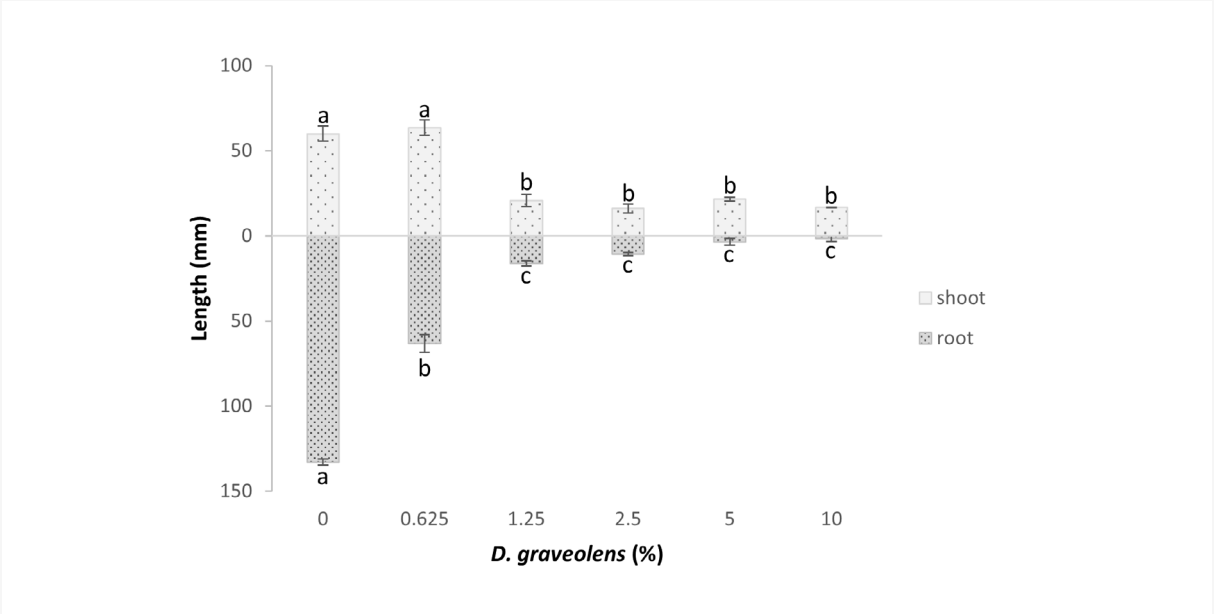

Figure 3. Shoot and root length of Tartary buckwheat seedlings after 7 days of growth treated with different concentrations of *D. graveolens* aqueous extracts. Mean value ± SE is shown (N=30). Different letters indicate statistically significant difference among treatments ($p < 0.05$).

Slika 3. Dolžina poganjka in korenine kalic tatarske ajde tretiranih z različnimi koncentracijami vodnega izvlečka *D. graveolens* po 7 dneh rasti. Prikazana so povprečja ± standardne napake (N = 30). Različne črke prikazujejo statistično značilne razlike ($p < 0,05$).

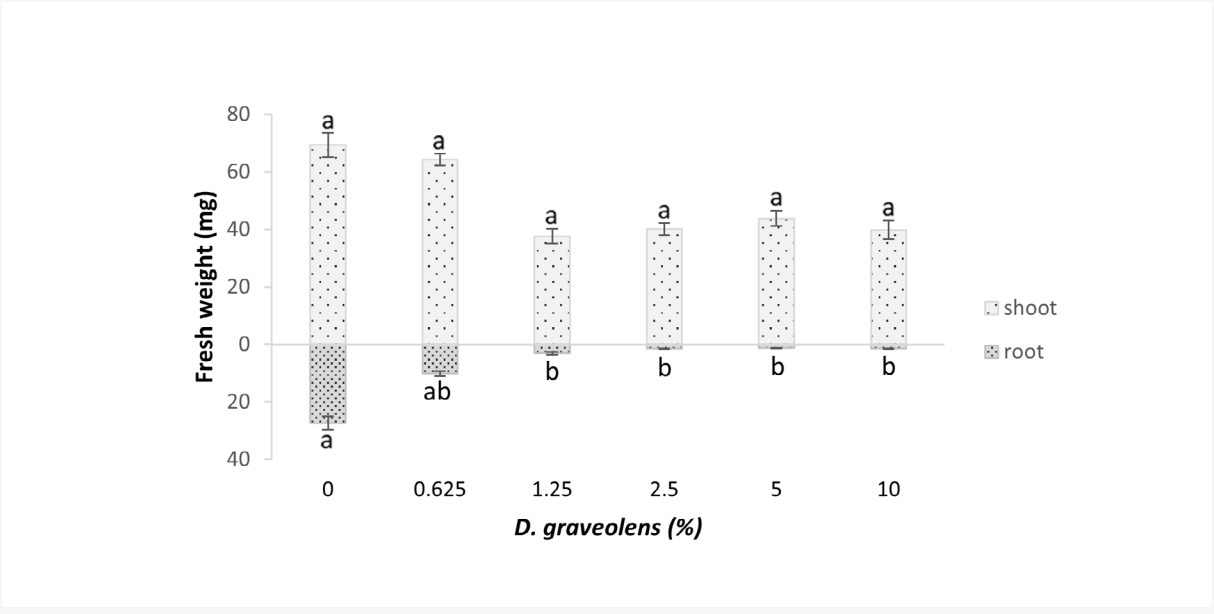

Figure 4. Fresh mass of Tartary buckwheat seedlings after 7 days of growth and treatment with different concentration of *D. graveolens*. Mean value ± SE is shown (N=30). Different letters indicate statistically significant difference among treatments ($p < 0.05$).

Slika 4. Sveža masa kalic tatarske ajde tretiranih z različnimi koncentracijami vodnega izvlečka *D. graveolens* po 7 dneh rasti. Prikazana so povprečja ± standardne napake (N = 30). Različne črke prikazujejo statistično značilne razlike ($p < 0,05$).

not only shorter but also thicker and darker in colour. The same observation was made in radish roots treated with rhizome extracts of *F. japonica* (Šoln et al., 2021). The roots of radish seedlings treated with a 10% extract were shorter and thicker due to the shorter and wider shape of their cortex cells (Šoln et al., 2022) and the reduced cell length in the root cap (Šoln et al., 2023). In addition, these cells exhibited various ultrastructural and biochemical changes, which could be the reason for the more than 60% shorter root length of the treated radish seedlings compared to the control (Šoln et al., 2023). The morphological adaptation, in which the roots become shorter and thicker, was also observed in plants that respond to water stress (Yetgin 2024).

The invasive nature of *D. graveolens* appears to be related to its ability to produce allelochemicals, which significantly inhibit the growth of Tartary buckwheat seedlings in a dose-dependent manner. The results showed that the root length of Tartary buckwheat seedlings was reduced significantly more than shoot length, while germination remained largely unaffected.

Author Contributions

Conceptualization, A.M.A.; methodology, A.M.A.; software, A.M.A.; validation, A.M.A.; formal analysis, A.M.A.; investigation, A.M.A.; data curation, A.M.A.; writing—original draft preparation, A.M.A.; writing—review and editing, S.A. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Review article

Microbiome manipulation – the future of inflammatory skin disease treatment?

Ema Rezar [1], Maša Vodovnik [1,*]

Abstract

The manipulation of the human microbiome presents a transformative frontier in addressing prevalent dermatological conditions, like acne and atopic dermatitis. Strategies for skin and gut microbiome modification, such as microbiome transplantation and oral or topical application of probiotics, prebiotics, and postbiotics, offer promising solutions for different skin disorders. Bacteriophages, viruses that target bacteria, also provide an alternative microbiome manipulation platform. However, despite the promising initial results, further investigation is essential to unravel the underlying mechanisms, assess efficacy, and ensure safety across diverse populations, as the interplay between microbial communities and skin health is very complex. In the transformative era of microbiome manipulation techniques it is important to ensure that these are applied beyond the realms of scientific exploration and benefit the global advancement of skin health. The aim of this review is to capture the increasing volume of research in this field that reflects a growing interest and dedication to advancing our understanding of microbiome manipulation techniques with the potential applications in dermatology. It represents an overview of the possibilities of treating the skin diseases via microbiome modulation are discussed, focusing on two of the most common inflammatory skin diseases of today: acne and atopic dermatitis.

Keywords

microbiome; skin diseases; biotechnology; dermatology; acne; atopic dermatitis

1 University of Ljubljana, Biotechnical Faculty, Dept. of Microbiology, Večna pot 111, 1000 Ljubljana, Slovenia

*** Corresponding author:**
E-mail address: masa.vodovnik@bf.uni-lj.si

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Manipulacija mikrobioma – prihodnost zdravljenja kožnih bolezni?

Izvleček

Manipulacija človeškega mikrobioma predstavlja izjemen potencial za zdravljenje pogostih dermatoloških stanj, kot so akne in atopijski dermatitis. Strategije manipuliranja kožnega in črevesnega mikrobioma vključujejo transplantacijo mikrobioma, peroralno ali topično uporabo probiotikov, prebiotikov in postbiotikov ter manipulacijo kožnega ali prebavnega mikrobioma z uporabo bakteriofagov. Začetne raziskave, v katerih so omenjene strategije uporabili za zdravljenje nekaterih pogostejših kožnih bolezni so pokazale obetavne rezultate. Naraščajoč obseg raziskav na tem področju pa odraža željo po napredku razumevanja in uporabe tehnik manipulacije mikrobioma v dermatologiji. Ker so interakcije med mikrobioto in kožo zelo kompleksne, je za čim uspešnejši prenos novih znanj v prakso nujno dobro poznavanje tako osnovnih mehanizmov v ozadju teh interakcij, kot ocene učinkovitosti zdravljenja ter zagotavljanje varnosti tovrstnih posegov pri različnih populacijah. Ključno je, da najnovejše informacije in odkritja s tega področja preidejo okvirje znanstvenega raziskovanja in s prenosom spoznanj v prakso lahko pripomorejo h globalnemu izboljšanju zdravja kože. V objavi povzemamo najnovejše raziskave na področju povezav med človeškim mikrobiomom in kože, s poudarkom na potencialih novih pristopih zdravljenja atopijskega dermatitisa in aken z manipulacijo mikrobioma kože in prebavil.

Ključne besede

mikrobiom; kožne bolezni; biotehnologija; dermatologija; akne; atopijski dermatitis

Introduction

Skin is the largest organ of the human body. It consists of three main layers – the epidermis, dermis, and subcutaneous tissue, with all three layers being prone to various skin conditions. It serves several important functions, the most important being its defensive role as it acts as a physical barrier between the external environment and the interior of the human body, protecting against the intrusion of microorganisms, ultraviolet (UV) radiation, mechanical, and chemical injuries, among others (Boer et al., 2016; Marks & Miller, 2019).

According to recent estimates, the human body is inhabited by approximately 3.8×10^{13} microorganisms (Sender et al., 2016), which are importantly contributing to the maintenance of our health and homeostasis. The concept of the human microbiome encompasses the entire population of microorganisms residing on or in the human body. They are found in various areas of the body, with the highest concentrations present in the digestive tract, nose, genitals and on the skin. Disruption of the microbial population balance, resulting from genetic or environmental factors, can lead to various health conditions, including skin-related issues (Ellis et al., 2019). This implies that reestablishment of the microbiome homeostasis via modulation strategies could potentially contribute to the treatment of various skin diseases (Yang et al., 2022).

There are more than 3,000 known skin diseases, varying in symptoms and the severity of the condition. They rank fourth on the list of the most common human diseases. At least one-third of the global population is estimated to be affected by at least one skin condition (Karimkhani et al., 2017). Tizek et al. (2019) conducted a study at the Bavarian Central Agricultural Festival to explore the prevalence of skin diseases among individuals outside medical facilities, who usually never seek medical attention for certain conditions. Random passers-by were examined for potential skin disorders. Out of a total of 2,701 individuals, at least one skin condition was detected in 64.5% of people. Two-thirds of the participants were unaware of the identified skin abnormalities. The results indicate that the prevalence of skin diseases may be much higher than commonly perceived. Although a smaller percentage of skin diseases leads to fatal outcomes, they still represent a significant health and economic burden. This includes physical, psychological, and socio-economic

consequences that affect both the patient and their family, ultimately impacting the healthcare system as well (Ahmed et al., 2016; Karimkhani et al., 2017; Seth et al., 2017). Most illnesses cause physical discomfort and deteriorate the quality of life for patients, subsequently affecting the psychological and social aspects of their lives. Patients often develop negative emotions such as shame and embarrassment, impacting both personal and professional relationships. The combination of these factors can lead to depression and even suicide (Ahmed et al., 2016; Karimkhani et al., 2017; Seth et al., 2017). In a study including 1510 participants, Yew et al. (2020) concluded that individuals with skin diseases more frequently experienced symptoms of depression, social isolation, loneliness – all accumulating to a lower quality of life. The socio-economic consequences include lost opportunities in professional life (indirect costs) and costs to the healthcare system (direct costs). For the year 2013 alone, in the United States, direct costs associated with skin diseases were estimated at $75 billion (including office visits and procedures, medications, vaccines, and other specific treatment-related procedures), and indirect costs at $11 billion (Lim et al., 2017). In addition to the widespread occurrence of skin diseases, the high costs of treatment are also attributed to the fact that these conditions often manifest as chronic and prolonged illnesses. Besides the prolonged and sometime unsuccessful treatments of common inflammatory skin conditions that may also result in unfavourable reactions to the traditional medications, the emergence of bacterial resistance to antibiotic therapies also represents a significant future challenge justifying the urge to search for novel treatment approaches.

Human microbiome-skin connection

The skin represents a habitat of millions of bacteria, fungi, and viruses that make up its microbiota. Cutaneous microorganisms play a crucial role in protecting the body against the invasion of pathogens and shaping our immune system. As the largest organ in the human body, healthy skin is colonized by trillions of microorganisms and serves as a physical barrier to prevent the entry of pathogens. When skin barrier is compromised or when the balance between commensals (harmless microorganisms) and pathogens is disrupted, the development of skin (or even systemic) diseases may occur (Grice & Segre, 2011; Zeeuwen et al., 2013; Oh et al., 2016). The skin microbiome is influenced by individual factors such as genotype, gender, age, lifestyle, as well as potential use of antibiotics and various cosmetic products. Furthermore, the diversity and location of microorganisms on the skin are influenced by environmental factors such as pH, moisture, sebum content, and the salinity of specific skin areas. Each of the skin areas with the specific microenvironment is populated by its own microbial community. *Propionibacterium*, *Corynebacterium* and *Staphylococcus* represent the three most dominant microbe genera in the skin microbiome, each importantly contributing to human health (Yang et al., 2022).

In sebum-rich areas of the skin, such as the face, back, etc., typically lipolytic species like *Cutibacterium acnes* (formerly *Propionibacterium acnes*) prevail (Zeeuwen et al., 2013; Lee et al., 2019). These species thrive in such environments due to their ability to degrade sebum produced by the sebaceous glands, which is facilitated by the extracellular lipolytic enzymes. The released fatty acids as a substrate for fermentation not only to the producing species but also for some other surrounding bacteria (Mayslich et al., 2021). In the areas rich with sebaceous glands, lipophilic commensal representatives of fungi are also found, such as *Malassezia restricta*, *M. globosa*, and *M. sympodialis*. These fungal species are present in the areas of the skin with different moisture contents, covering the entire surface. The greatest diversity of fungal species has been described on the feet surface (Zeeuwen et al., 2013; Jo et al., 2016; Byrd et al., 2018). Besides *Malassezia* species, also *Cryptococcus*, *Rhodotorula* and *Candida* species have been identified as skin commensals (Boxberger et al., 2021).

In addition to bacteria, *Archaea* belonging to *Thaumarchaeota* and *Euryarchaeota* were also shown to be a part of human skin microbiome. Analysis of *Thaumarchaeota* detected on human skin, placed them close to ammonia-oxidizing archaea from the soil. Although it remains to be proven, the role of these archaea could be explained by chemolithotroph ammonia turnover, which may influence the pH regulation of the human skin, natural protective barrier of the body (Moissl-Eichinger et al. 2017; Boxberger et al., 2021)

In addition to bacterial, archaeal and fungal communities; viruses, predominantly bacteriophages, also inhabit

skin surfaces. The latter are believed to regulate bacterial populations through their lytic activity, contributing to the maintenance of skin homeostasis (Boxberger et al., 2021). Metagenomic analysis has shown that the prevalent skin phages inhabit genera *Cutibacterium* and *Staphylococcus* (Liu et al., 2015). Other viruses were also identified (*Densovirus*, *Alphapapillomavirus*, *Human papillomavirus*, *Merkel cell polyomavirus*, *Molluscum contagiosum virus* etc.) and some of them were already linked to certain skin conditions (Boxberger et al., 2021).

Furthermore, increasing number of studies reveal strong connection between the skin conditions and gut microbiome. Significant differences in the composition of stool microbiota between individuals with acne and healthy controls were identified (Deng et al., 2018). In contrast to the healthy control group, acne patients exhibit reduced diversity in gut microbiota and an elevated ratio of *Bacteroidetes* to *Firmicutes*, which is associated with the western diet and other inflammatory diseases. This also implies the influence of the western diet on the onset of acne vulgaris, highlighting the potential for dietary adjustments and probiotic-based interventions in both preventing and managing this skin condition (Deng et al., 2018).

Both, the skin as well as gut microbiomes control the colonization of potentially pathogenic microorganisms, regulate the immune response, and are essential for the optimal functioning of the immune system. This suggests that maintaining balance within these communities if crucial for our health (Grice & Segre, 2011; Zeeuwen et al., 2013).

Skin microbiome manipulation by microbiome transplantation, probiotics, prebiotics and postbiotics

Manipulation of the skin microbiome opens new possibilities for the therapy of skin diseases and can be achieved in various ways (Arora et al., 2023). One of the potential approaches is the transplantation of the skin microbiome, which involves the application of skin microbiome from a healthy individual to the affected skin pre-treated by antiseptic agent. Although this method has the advantage of obtaining microorganisms from the natural environment, it has not been proven entirely reliable. Furthermore, only a limited number of bacteria can be obtained from the skin and the method is not suitable for serial use (requires suitable donors, can only be performed in an outpatient setting). Additionally, there is a risk of transferring pathogenic microorganisms to an already weakened microbiome, potentially causing more harm than benefit (Callewaert et al., 2021).

The skin microbiome can also be altered by applying probiotics, prebiotics, or postbiotics to restore the microbial balance on affected skin (Callewaert et al., 2021). Probiotics are live microorganisms that, in appropriate concentrations, provide beneficial effects to the host (e.g., traditionally bacteria belonging to the *Bifidobacterium* and *Lactobacillus* species). On the other hand, postbiotics are formulations of non-viable microorganisms or their structures or metabolic by-products that also contribute to maintaining host homeostasis (e.g., peptides, enzymes, vitamins) (Vallianou et al., 2020; Salminen et al., 2021). Prebiotics are substrates that stimulate the growth of specific health-promoting microorganisms (e.g., inulin and galacto-oligosaccharides). In contrast to skin microbiome transplantation, the production and use of such preparations is easier and more widely applicable (Arora et al., 2023). Another advantage of this approach lies in the possibility of using concentrated preparations, theoretically enhancing the effectiveness of therapy (Callewaert et al., 2021). Topically applied probiotics act through competition for binding sites, thereby preventing the colonization of potential pathogens (Lopes et al., 2017). However, the use of probiotics and postbiotics also has its limitations. The environment rich in sebum may be unfavourable for some probiotic bacteria which may not properly adapt to it. Moreover, the use of high concentrations of bacteria, their components, or products can induce an immune reaction and skin irritation (Callewaert et al., 2021).

The advantage of skin prebiotics is that they do not contain living microorganisms or their components, reducing the likelihood of skin immune reactions. Additionally, they are typically well-defined compounds with well predicted potential side effects. However, as an indirect method of microbiome modifications, this method may result in less obvious effects compared to therapies with probiotics or postbiotics. In addition, prebiotics may also stimulate non-target bacteria, leading to unpredictable effects on the skin microbiome, physiology, and immune response in different individuals (Callewaert et al., 2021).

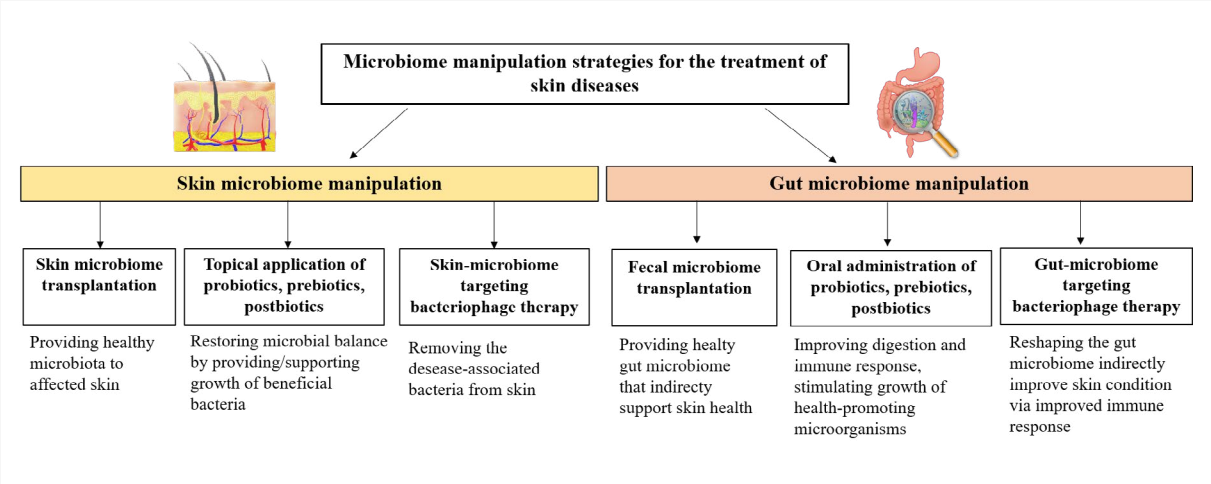

Figure 1. Overview of the microbiome manipulation strategies potentially applicable in inflammatory skin disease treatment.

Slika 1. Pregled strategij manipulacij človeškega mikrobioma s potencialom za zdravljenje vnetnih kožnih bolezni.

Skin microbiome manipulation in the treatment of acne

Acne represent the most common inflammatory skin disorder in the Western world, affecting approximately 85 % of the population, primarily adolescents (Yang et al., 2022). It is a multifactorial disease of the pilosebaceous unit (a unit composed of the hair follicle and sebaceous gland), influenced by both genetic and environmental factors (microbiome composition, hormonal and immune status of the individual, sebum production, diet, genetics, etc.) (De Pessemier et al., 2021). The condition is typically presented by open and closed comedones, red pustules, and yellowish papules, as well as inflamed nodules below or above the skin surface. Severe cases may lead to chronic scarring. Post-inflammatory hyperpigmentation can also be observed on the skin (Ayer & Burrows, 2006).

It is presumed that the skin commensal bacterium *Cutibacterium acnes* plays a significant role in the disease, with certain strains mediating the inflammatory response and leading to the formation of acne lesions (Gollnick et al., 2003). *C. acnes* is a Gram-positive, aerotolerant bacterium that is part of the skin microbiome. It thrives in lipid-rich environments, and it is often found in areas of the skin with the highest density of pilosebaceous units (face, neck, back, chest) (Spittaels et al., 2020). Discerning between strains that confer benefits to human health by inhibiting pathogen growth and those that pose a threat presents a substantial challenge, but also opens avenues for novel treatment strategies (O'Neill & Gallo, 2018). Further exploration of the intricate interplay between *C. acnes* strains and their impact on the skin microbiome holds promise for advancing our understanding of acne etiology and developing targeted therapeutic interventions.

The main therapeutic approach for treating acne currently relies on antibiotics, which may have side effects, and their excessive use raises concerns about the alarming spread of bacterial resistance. Moreover, the therapy is often unsuccessful, or the condition recurs after the end of the treatment. There is a need for the development of new therapeutics that are both safer and more effective (Newman et al., 2011).

In the study of Paetzold et al. (2019) microbiome samples from two healthy individuals were transplanted to individuals with acne. After three consecutive days of applications (once daily), the recipient's microbiome became more similar to that of the donor. It was revealed that the result of the transplantation reflected the composition of individuals' microbiomes (both recipients and donors) as well as the quantity and concentration of the bacteria used. This study demonstrated the potential use of live bacteria to regulate the composition of the skin microbiome (Paetzold et al., 2019).

Karoglan et al. (2019) tested the hypothesis that the application of *C. acnes* strains that are not associated with acne could positively impact the skin microbiome and

thereby contribute to a reduction in the extent of acne. They initially treated the skin with benzoyl peroxide for 7 days to significantly reduce the skin microbiota which was followed by application of a bacterial mixture of two (type C3 and K8, 50% each) or four live strains (C3, K8, A5, and F4, each contributing in varying proportions) with a total combined concentration of 10^6 colony-forming units per gram of *C. acnes* twice per day for 5 consecutive weeks. No adverse effects, visible irritation, or inflammation were observed. The number of comedones decreased. However, they did not detect any difference in the use of mixtures containing two or four strains (Karoglan et al., 2019). These findings inspired a Belgian company S-Biomedic to further develop products based on these probiotic mixtures, leading to the launch of their first product, Sencyr—a probiotic cream for acne treatment (S-Biomedic, n.d.).

A study performed by Lebeer et al. (2022) demonstrated the use of topical probiotics for acne treatment with lactobacilli. Selected strains (*Lacticaseibacillus rhamnosus* GG, *L. plantarum* WCFS1 and *Lactiplantibacillus pentosus* KCA1) were applied as a cream twice daily on the skin of volunteers with mild or moderate acne for eight consecutive weeks with a minimal dose of 10^6 colony forming units per application. The therapy successfully reduced inflammatory lesions on the participants' skin. A change in the composition of the skin microbiome with a decrease in the relative abundance of staphylococci was also observed. Even after individuals stopped using the cream, the reduction in acne persisted for several weeks, indicating that lactobacilli partially act by modulating the immune system (Lebeer et al., 2022).

In a recent study, the the efficacy of a fermentation lysate of *Lactiplantibacillus plantarum* VHProbi® V22 in ameliorating acne was tested by aplying the anti-acne skincare cream containing fermentation culture lysate o subjects with mild-to-moderate acne vulgaris for 4 weeks. Significant improvements in the acne lesion proportion ($P < 0.01$), transepidermal water loss ($P < 0.001$), and sebum secretion ($P < 0.05$) were observed in comparison to the baseline in the subjects, suggesting the treatment as a complementary option to the treatment of the above-mentioned conditions (Cui et al., 2022). Furthermore, a post-biotic containing heat-treated *Pediococcus acidilactici* LM1013 previously isolated from the Korean traditional fermented alcoholic beverage-makgeolli, has recently been demonstrated as effective *C. acnes* inhibitor (Bae et al., 2023).

Skin microbiome manipulation in atopic dermatitis

Atopic dermatitis (AD) is increasingly common inflammatory skin disease affecting around 34% of the world population. It occurs in all age groups, with the highest prevalence among younger children (Hadi et al., 2021). The disease is characterized by dysfunction of the skin barrier, chronic inflammation, and microbial imbalance on the skin. The development of the condition is significantly influenced by an individual's genetics and the environment (Leung & Guttman-Yassky, 2014). It is often associated with food allergies and asthma as the compromised skin barrier in AD patients allows the absorption of allergens from the environment through the skin, promoting systemic hypersensitivity to allergens, predisposing individuals to the development of food allergies and asthma (Brough et al., 2015).

The damaged skin of AD patients is typically associated with low bacterial diversity. An increased proportion of *Staphylococcus aureus* and *Staphylococcus epidermidis* has been found, while the proportion of other common skin commensals (such as *Cutibacterium*, *Corynebacterium*, *Streptococcus*, *Acinetobacter*, *Prevotella* and *Malassezia*) was found to be reduced (Kong et al., 2012). In healthy individuals, *S. aureus* is rarely detected on the skin (Guzik et al., 2005), while in AD patients, the density of skin colonization with *S. aureus* is strongly associated with the severity of the condition. The distribution over the body surface has also been shown to be linked to the distribution of dermatitis (e.g., on the face and limbs) (Tauber et al., 2016; Kennedy et al., 2017; Iwamoto et al., 2019).

Currently, the treatment of atopic dermatitis (AD) is based on the use of immune response inhibitors—corticosteroids and systemic immunosuppressants, which can cause severe side effects (Newsom et al., 2020). Prolonged use may lead to skin atrophy and disruption of the skin barrier function, resulting in increased water loss, reduced hydration levels, and increased skin transparency. The severity of these side effects depends on the strength, duration, and dosage of the treatment, as well as the morphological characteristics of the skin in different anatomical areas (Atherton, 2003).

Myles et al. (2016) investigated the impact of exposing the skin to various strains of Gram-negative bacteria belonging to *Roseomonas mucosa* and P*seudomonas aeruginosa* species, to improve the condition of atopic dermatitis (AD).

Strains of both species, isolated from the skin of healthy individuals demonstrated the ability to inhibit the growth of *S. aureus* in *in vitro* cell cultures. The effectiveness was also tested *in vivo* using mouse models of AD. They induced dermatitis similar to AD on the ears by applying a vitamin D analogue MC903, and then applied selected isolates of *R. mucosa* and *P. aeruginosa* to the skin once per day for three consecutive days. In mice treated with *R. mucosa* isolates, visible reduction in redness occurred and no observed side effects. On the other hand, applying *P. aeruginosa* isolates did not lead to improvement in the skin condition (Myles et al. 2016).

These findings led to a smaller clinical study testing the therapeutic capabilities of *R. mucosa* isolates. The study involved 10 adults with atopic dermatitis (AD) who applied the formulation twice a week for six consecutive weeks to any area of the body. No adverse effects or complications were recorded during the treatment, while visibly reduced redness was observed. Participants also reported reduced itching and a decreased need for corticosteroid use. Because the therapy proved to be safe, the study included five younger patients, aged nine to fourteen, who applied the formulation twice a week for 16 consecutive weeks. Similar results were reported, including visibly reduced redness, decreased itching, and a reduced need for corticosteroids. The results suggest that *R. mucosa* alleviates AD symptoms and could potentially represent a form of therapy in the future (Myles et al., 2018).

Keratinocytes contribute to defence against pathogens by secreting antimicrobial peptides. It is presumed that their deficiency is associated with a loss of protection against the spread of *S. aureus* on the skin (Howell et al., 2006). Nakatsuji et al. (2017) demonstrated that commensal bacteria of the skin microbiome, *Staphylococcus hominis*, provide selective protection against S. aureus by secreting lantibiotics, a type of antimicrobial peptides. Isolated strains were multiplied and applied to patients' skin. While they did not measure clinical improvement in symptoms, they detected a reduced level of *S. aureus* colonization, demonstrating the role of these commensal bacteria in providing protection against pathogens and preventing the dysbiosis of the skin microbiome than can lead to the development of a diseased condition (Nakatsuji et al, 2017). These findings led to the establishment of MatriSys Bioscience, with the goal of obtaining a single strain to be sold as a probiotic formulation for alleviating dermatitis symptoms (MatriSys Bioscience, n.d.).

Bacteriophage-assisted skin microbiome manipulation

Bacteriophages are viruses that infect bacteria, taking over their host and using it for reproduction. A bacteriophage can recognize, infect, and kill a specific type or even a particular strain of bacteria. Consequently, they play a crucial role in regulating bacterial populations (Palaniappan & Dayanithi, 2021). They can only multiply within host cells, making them active only at the site of infection where pathogenic bacteria are present (Abedon et al., 2011).

The excessive use of antibiotics in treating various diseases and in intensive livestock farming has led to the emergence of antibiotic resistance. The problem is further exacerbated by the lack of newly discovered antibiotic agents (Ventola, 2015). This is particularly significant in the treatment of skin diseases where antibiotics are frequently prescribed. The use of bacteriophages and phage cocktails in treating various diseases appears as a promising alternative to antibiotic treatment or, at the very least, a supportive therapy to existing treatment methods (Palaniappan & Dayanithi, 2021).

Bacteriophages exhibit the following advantageous characteristics: 1) they attack both Gram-positive as well Gram-negative bacteria, 2) they are highly specific to individual species and even strains of bacteria; 3) due to different mechanisms of action compared to antibiotics, they also act on antibiotic-resistant bacteria, 4) after infection, they replicate only locally and do not affect the rest of the microbial population, 5) their properties can be enhanced via genetic engineering; 6) identification, isolation, and production of bacteriophages for therapeutic purposes is cheaper than developing new antibiotic agents, 7) they have the ability to mutate (adapt) to the altered host characteristics, 8) resistance of bacteria to individual bacteriophages can be avoided by using bacteriophage cocktails, and 9) they are considered safe and do not induce unwanted side effects (Palaniappan & Dayanithi, 2021). Currently, bacteriophage therapy is only applied when all other forms of treatment have been exhausted (Palaniappan & Dayanithi, 2021). The limitations of bacteriophage treatment include an incomplete understanding of the phage life cycle and the potential for transduction of pathogenic genes. Optimal dosages, methods, and frequency of applications, as well as the duration of treatment and short-/ long-term effects for each therapy, need to be determined. There is currently

a lack of standardized guidelines for bacteriophage preparations manufacturing (Castillo et al., 2018).

The prolonged and excessive use of both topical and oral antibiotics in the treatment of acne has led to a significant resistance of *C. acnes* strains to antibiotics (Walsh et al., 2016). As a result, treatment is becoming less effective, and resistance to available antibiotics is one of the main reasons for treatment failure. Alternative approaches that reduce the presence of pathogens while not harming commensals are therefore necessary (Golembo et al., 2022). Bacteriophage therapy could potentially replace or complement current approaches to acne treatment. Golembo et al. (2022) identified and characterized 21 *C. acnes* bacteriophages. Three of them were used to prepare a phage cocktail. The cocktail was first tested on an *ex vivo* skin model of the epidermis to assess its infectivity upon topical application and the safety of the preparation. The product proved to be safe at all concentrations (100x, 10x and 1x fold concentrations of the maximal intended dose for human exposure and compared to tissues exposed to negative control; specific information on concentrations was not revealed) as no inflammation was detected. The results from the skin model experiment were sufficient for the next step, a clinical study, and animal model studies were deemed unnecessary.

Furthermore, the clinical study involved 75 participants with mild to moderate acne, divided into three groups. Each group applied the preparation once a day for four weeks. The first group applied the higher concentration, the second the lower concentration (a 2 log10 lower dose than the high concentration), and the third applied the same formulation but without bacteriophages (negative control). Cheek skin swabs underwent processing for bacterial DNA extraction and were subject to analysis through specific quantitative PCR (qPCR) targeting *Cutibacterium* spp. This aimed to assess the absolute quantity of this bacterium and its alteration from the baseline, relative to the vehicle, following the application of BX001. Only the first group, compared to the third, showed a significant reduction in the presence of the *C. acnes* bacterium one week after the last application (up to 24 %), indicating that a higher concentration is needed to achieve the results. Despite a month of daily application of the preparation, no development of bacterial resistance was observed. Additionally, there were no severe side effects, and any reported effects were similar to those in the control group, confirming the safety of the preparation (Golembo et al., 2022).

Shimamori et al. (2021) proposed and investigated the possibility of bacteriophage therapy as a potential strategy for treatment of atopic dermatitis without affecting the rest of the skin microbiome. An atopic mouse model was used to examine whether the *S. aureus* SaGU1 bacteriophage could be used as a tool to prevent disease exacerbation. Application of SaGU1 to the mouse's back skin reduced the concentration of *S. aureus* and improved the disease condition. The results suggest that treatment using the bacteriophage SaGU1 could be a promising clinical approach for atopic dermatitis (Shimamori et al., 2021).

Gut microbiome manipulation and inflammatory skin conditions

The gastrointestinal tract microbiome is a dynamic ecosystem influenced by various factors (including diet, genetics, and medical interventions), originating from the host or the host's environment. A healthy microbiome contributes to host health and colonization resistance by training the host immune system, nutrient sequestration, antimicrobial compound production and competition for binding sites with pathogens. Not only microorganisms but also different microbial compounds (vitamins) and metabolic products play an important role in the interaction of the microbiome with the immune system. Any sort of changes in the gut microbiome composition can potentially lead to some sort of inflammation, which manifest on the skin as well (McCuaig & Goto, 2023). Several therapeutic strategies therefore strive to improve the well-being of the gut microbiome and fortify its ability to remain in balance, indirectly contributing to skin health as well.

Gut microbiome may be manipulated via oral administration of probiotics, which are essentially microorganism formulations proven as efficient and harmless to humans (Rusu et al., 2019). In the context of both atopic dermatitis and acne, several studies have been conducted, mostly demonstrating positive effects of adjunctive therapy with probiotics in improving digestion, immune response, and other beneficial effects on the gastrointestinal tract and skin (Roessler et al., 2008; Yoshida et al., 2010; Drago et al., 2011; Thompson et al., 2020). Adjunctive probiotic therapy has proved even more important in the treatment of acne by antibiotics. Although the antimicrobial properties of anti-

biotics provide significant health benefits, their non-specificity strongly influences the composition and functioning of the microbiome, especially the gut (Mahmud et al, 2022). Disturbances in the balance and reduced diversity of the gut microbiota composition can subsequently lead to various health conditions, including skin-related issues (Forssten et al., 2014).

Results of an open-label study, aimed to investigate the efficacy of probiotics in mitigating the side effects associated with systemic antibiotics and their synergistic impact in treating inflammatory acne, showed some promising results. Forty-five females aged between 18 and 35 years were randomly assigned to three groups. Group A received probiotic supplementation, group B received the antibiotic alone (minocycline), and group C received both probiotics and the antibiotic. The probiotic product used in this study contained a combination of *Lactobacillus acidophilus* (5 billion CFU/capsule), *Lactobacillus delbrueckii* subspecies *bulgaricus* (5 billion CFU/capsule), and *Bifidobacterium bifidum* (20 billion CFU/capsule), encapsulated within an oil matrix in a two-piece hard gel capsule. Those in the probiotic group took capsules in the morning and evening, while the minocycline group took the antibiotic once after dinner. All three groups also used standard topical acne medication and a facial cleanser, following the same regimen throughout the 12-week study, with additional acne treatments prohibited. Over the 12-week study period, all groups showed a significant improvement in total lesion count, with group C exhibiting a notably greater reduction at the 8- and 12-week follow-up visits compared to groups A and B. The findings suggest that probiotics, when used in conjunction with systemic antibiotics, may offer a promising therapeutic approach for acne vulgaris by providing a synergistic anti-inflammatory effect while potentially minimizing adverse events associated with prolonged antibiotic use (Jung et al., 2013).

The effect of oral probiotic supplementation was also tested on children aged 4–17 with atopic dermatitis. Patients were given a daily pill containing a probiotic formulation (consisting of 10^9 colony-forming units of *Bifidobacterium animalis* subsp. lactis, *Bifidobacterium longum*, and *Lacticaseibacillus casei* in a 1:1:1 ratio, with maltodextrin as a carrier) or a placebo (containing only maltodextrin) for a duration of 12 weeks. The probiotic group showed improved AD severity scores and reduced use of topical steroids. While no significant increase in specific probiotics was observed, microbiome analysis revealed decreased *Faecalibacterium* and increased *Bacteroides* levels, suggesting a potential modulation of the gut microbiome. Results indicate the need for further research with larger cohorts and exploration of microbiome changes in different age groups. Safety and efficacy considerations for probiotic consumption were also noted (Climent et al., 2021).

Recently, B. Lee et al. (2023) showed potential for anti-aging effects of the probiotic strain *Limosilactobacillus fermentum* USM 4189 (LF 4189), using a D-gal-induced rat model. They examined various factors associated with skin aging, such as antioxidant capacity, skin elasticity, histological alterations, telomere length, and gene expression linked to apoptosis, senescence and oxidative stress. The experimental groups included 6 young rats receiving daily subcutaneous injections of 0.9% saline (Young group), 6 old rats receiving subcutaneous injections of 600 mg/kg D-gal to induce aging (Old group), a group consisting of aged rats treated with *L. fermentum* 4189 (1×1010 CFU/d) via oral administration (Old+4189), and a group with aged rats treated with metformin (300 mg/kg/d) via oral administration (Old+metformin). Results revealed that administering *L. fermentum* 4189 to aging rats significantly enhanced antioxidant capabilities, diminished lipid peroxidation, and improved skin elasticity compared to untreated aging rats. Histological analysis indicated that the administration of *L. fermentum* 4189 prevented the deterioration of skin structure, increased collagen fibers, and overall improved skin health. Additionally, LF 4189 mitigated telomere shortening, a marker of cellular aging, and influenced gene expression related to apoptosis, senescence, and oxidative stress. These findings suggest that oral administration of *L. fermentum* 4189 may provide antioxidative and anti-aging effects, positioning probiotics as a promising avenue for interventions to support skin health during the aging process (Lee et al., 2023).

Apart from probiotic supplementation, prebiotic oral supplements also offer an alternative option to modulate immune status via gut microbiota. The most common prebiotics are considered indigestible fibers, which remain undigested by the host and can only be fermented by commensal bacteria in the lower gastrointestinal tract. The benefits of these prebiotics are typically associated with the stimulation of short chain fatty acids (SCFAs) producing bacteria (Costa et al., 2021), but they may also improve immune response through the production of immunomodulating compounds and secondary bile acids and training the immune system by providing microbe-associated molecular

patterns (MAMPs) (McCuaig & Goto, 2023). The pectins, for example, have been found to promote SCFA production, particularly acetate. The conversion of acetate to propionate and butyrate varied depending on the resident microbiome community (Pascale et al., 2022). Costa et al. (2021) discuss the effects of another type of prebiotics, namely fructooligosaccharides, on inflammation and gut immune response. A study testing the potential benefits of fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) on adult women with acne revealed these probiotics may indirectly improve the condition. Twelve women with mild to moderate acne that participated in the study, receiving a daily food supplement containing FOS (100 mg) and GOS (500 mg) for three months. The results revealed significant reductions in fasting blood glucose levels and total cholesterol, suggesting a positive impact on metabolic health. While further investigation is needed, these findings imply a potential avenue for prebiotic supplementation in managing metabolic parameters in individuals with adult acne. In addition, the use of oral nutritional supplementation containing the abovementioned prebiotics has been shown to elevate stool colony counts of *Bifidobacteria* and *Lactobacilli*. This contributes to the maintenance of an efficient intestinal mucosal barrier, which could potentially result in the improvement of skin's health (Dall'Oglio et al., 2018).

Fecal microbiota transplantation (FMT) has also been tested for the restoration of gut microbiota in mice with atopic dermatitis (Kim et al., 2019; Kim et al. 2021; Mashiah et al., 2021; Jiang et al., 2023). Apart from the skin condition, the studies measured other parameters, including cytokine levels, blood parameters, histological parameters, and short-chain fatty acid (SCFA) levels. The results revealed a significant restoration of gut microbiota and associated parameters following FMT treatment and indicated promising therapeutic potential of FMT in atopic dermatitis, suggesting a novel approach for addressing the condition by modulating the immune via gut microbiota (Kim et al., 2019).

Conclusions

Microbiome manipulation strategies emerge as a promising frontier in the therapeutic landscape, offering innovative approaches for addressing skin conditions such as acne and atopic dermatitis. The skin microbiome, a complex ecosystem of microorganisms, can be modulated through various approaches, ranging from the transplantation to topical applications of probiotics, prebiotics, and postbiotics. These interventions aim to restore microbial balance, particularly in the face of dysbiosis associated with inflammatory skin diseases. While microbiome transplantation poses some challenges and potential risks, the scalable nature of probiotics and postbiotics provides more accessible and controlled means of manipulating the skin microbiome. Increasing number of studies showcase the potential of live bacteria and topical formulations in regulating the skin microbiome, offering hope for effective and scalable therapies.

Furthermore, the gut microbiome also presents promising avenue for intervention in skin diseases, as different studies imply the interconnectedness of the gut-skin axis and the role of the microbiome in influencing skin health. Probiotics administered orally have demonstrated positive effects in improving digestion, immune response, and overall gastrointestinal health, with implications for skin conditions like acne. Additionally, the emergence of bacteriophage therapy, targeting specific harmful bacteria offer a promising alternative to traditional antibiotic approaches. The specificity, adaptability, and potential to mitigate resistance make bacteriophages a valuable tool in possible skin disease treatments.

In conclusion, despite their prevalence acne and atopic dermatitis and approaches to their treatment covered in this manuscript represent only a small part of the challenges in dermatology. Several other acute or chronic inflammatory conditions (i.e. psoriasis) are also becoming increasingly prevalent in the modern world and should be addressed in future research. In recent years, increasing attention and resources have been dedicated to the development of biotechnological solutions for alleviating or treating skin diseases, which is expected to lead to several innovations in the upcoming years. However, these represent only the beginning of a long journey, which concludes with the improvement of the patient's condition, ideally leading to complete recovery. Investments in research as well as in preclinical and clinical studies are essential for the development of safe products with appropriate dosage and application methods. Additionally, it is important to establish scalable and cost-effective production of therapeutics with good manufacturing practices. The final cost of therapy is a result of multiple factors that must be considered. If the price is too high, significant progress in dermatology may be hindered, as only a handful of affected individuals would be able to afford the treatment.

In the future, we aspire to have not only effective but also accessible methods of treatment. To achieve this goal, it is crucial to change the perception of skin conditions, to accept them, and to end the stigmatization of those affected. Since chronic skin diseases are often not (directly) life-threatening, attention and resources are frequently redirected to other areas. However, the psychological and socio-economic impact of inflammatory skin diseases is often comparable to, if not greater than, that of other chronic health conditions. It should also be kept in mind that skin diseases are usually not only disorders of the skin but also indicative of larger, systemic illnesses and is therefore essential to approach patients in a systemic manner and to identify and treat the primary cause of the conditions.

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Conflicts of Interest

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ABS

Field Notes

Significant records of plants, algae, fungi, and animals in SE Europe and adjacent regions, 1

Simona Strgulc Krajšek [1*], Mihael J. Kocjan [2], Filip Küzmič [3], Žan Lobnik Cimerman [1], Lara Marc [1], Katja Potočnik [1], Ela Šenk [1]

Abstract

In this paper, we present four significant records of plants in Slovenia: a vascular plant Calepina irregularis, and three moss species, Ptychostomum torquescens, Sphagnum papillosum; Tortella fasciculata.

Keywords

Calepina irregularis; Ptychostomum torquescens; Sphagnum papillosum; Tortella fasciculata; vascular plants; bryophytes; mosses, flora; Slovenia

1 University of Ljubljana, Biotechnical Faculty, Department of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia

2 Društvo za raziskovanje mokrišč Slovenije (DRMS), Celovška cesta 30, SI-1000 Ljubljana, Slovenia

3 ZRC SAZU, Jovan Hadži Institute of Biology, Novi trg 2, 1000 Ljubljana, Slovenia

*** Editor**
For correspondence, see the individual chapters.

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Calepina irregularis (Asso) Thell., fam. Brassicaceae (vascular plant)

Contributor(s)	Filip Küzmič
Corresponding author	Filip Küzmič (filip.kuzmic@zrc-sazu.si)
Leg.	Filip Küzmič
Country	Slovenia
Statement of significance	A very rare occurrence of the species outside of (sub)Mediterranean phytogeographic zone.
Locality description	Dolenjska, Novo mesto, between the streets Na Loko and Defranceschijeva, 166 m a.s.l.
Habitat	lawn border by the trail covered with small stones, partly trampled
Date of observation	2024-04-30
GPS	N 45.803431°, E 15.164150°
Voucher	Herbarium LJS 13123

The species is readily recognisable by its white flowers, small, almost globose fruits, and non-pubescent aboveground organs. In the described locality, all specimens (more than 10) exhibited prostrate growth habit, slightly ascending at the tips of the inflorescences due to trampling.

The species is native to the area from the Mediterranean to Iran. In Slovenia, it was predominantly recorded in the westernmost parts of the submediterranean phytogeographic region, mostly in vineyard weed plant communities and ruderal vegetation (Balant, 2011; Glasnović & Jogan, 2009; Lešnik, 2001; Poldini, 1980, 2006; Poldini, Oriolo, & Mazzolini, 1998; Seljak, 1989; Šilc and Čarni, 2007; Trčak, 2014). The only known occurrence in another region is from the vicinity of the Vihre village in Eastern Slovenia (Trčak et al. 2008).

In the currently reported locality, several (more than 10) specimens were found aligning the non-shadowed trail. Species in the plant stand with their Braun-Blanquet abundance values taken in a plot of 10mx0.2m were: *Achillea millefolium* agg. (+), *Allium scorodoprasum* subsp. *scorodoprasum* (+), *Bellis perennis* L. (+), *Calepina irregularis* (Asso) Thell. (2), *Capsella bursa-pastoris* (L.) Med. (1), *Cardamine hirsuta* L. (+), *Carex hirta* L. (2), *Cerastium glomeratum* Thuill. (+), *Convolvulus arvensis* L. (+), *Cynodon dactylon* (L.) Pers. (+), *Geranium pusillum* Burm. fil. (+), *Lysimachia nummularia* L. (+), *Malva sylvestris* L. (+), *Myosotis arvensis* (L.) Hill (+), *Picris hieracioides* agg. (+), *Plantago major* L., s. str. (+), *Poa annua* L. (1), *Poa trivialis* L. (1), Poaceae (seedlings) (+), *Polygonum aviculare* agg. (+), *Potentilla reptans* L. (1), *Ranunculus repens* L. (1), *Taraxacum* sect. *Taraxacum* F. H. Wigg. (3), *Trifolium pratense* L. (+), *Trifolium repens* L. (2), *Veronica arvensis* L. (1), *Veronica persica* Poir. (1).

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Ptychostomum torquescens (Bruch & Schimp.) Ros & Mazimpaka, fam. Bryceae (moss)

Contributor(s)	Ela Šenk, Lara Marc, Simona Strgulc Krajšek
Corresponding author (email)	Simona Strgulc Krajšek (simona.strgulc@bf.uni-lj.si)
Leg.	Ela Šenk, Lara Marc
Country	Slovenia
Statement of significance	The first recent record of a rare species.
Locality description	Ljubljana, southwest of town Ig, Iški vintgar, Nature Reserve (RS, no. 80/2005), 350 m a.s.l.,
Habitat	soil on calcareous bedrock
Date of observation	2024-05-24
GPS	N 45.9114409°, E 14.4991750° (approximate coordinates)
Voucher	Herbarium LJU

Ptychostomum torquescens (Bruch & Schimp.) Ros & Mazimpaka (synonym, *Bryum torquescens* Bruch & Schimp.) is a rarely encountered moss species that is frequently confused with the much more widespread *P. capillare* (Hedw.) Holyoak & N. Pedersen. The species is found in most European countries and is considered least concern (LC) in the European Red List (Hodgetts et al., 2019). It grows in all neighbouring countries of Slovenia except Austria (Hodgetts & Lochhart, 2020). It is regionally extinct (RC) in Sweden, endangered (EN) in the Netherlands, vulnerable (VU) in Great Britain, Ireland, Bulgaria, and Hungary, and near threatened (NT) in Romania (Hodgetts & Lochhart, 2020). In Slovenia, it is listed as data deficient (DD) (Martinčič, 2024). We report the first data of its occurrence in Slovenia since 1960, the first one in central Slovenia. Two specimens collected by Martinčič are stored in Herbarium LJU (from Bela Krajina, 1955, and Trnovski gozd, 1960). There are also two older published records from the vicinity of the town Nova Gorica (Höhnel, 1893; Loitlesberger, 1909).

We sampled a few specimens on soil on calcareous bedrock in Iški Vintgar nature reserve. For species identification, we used identification keys from Holoyak (2021) and Frey et al. (2006). The species is morphologically similar to *P. capillare*. The only reliable differences between them are that *P. torquescens* is sinoicous and has pointed apical cells of paraphyses, and *P. capillare* is dioicous and has rounded apical cells of paraphyses (Holoyak, 2012). Both species also have similar habitat preferences. They grow in patches on calcareous rock or soil, usually in bright to moderately shaded places. In a dry state, both species are recognised by leaves that are spirally twisted like a screw. *B. capillare* also grows on bark, moderately acidic rock, and is common in urban areas, too (Holoyak, 2012). Identification of the species in the field is not possible.
We presume that *P. torquescens* might be overlooked in Slovenia due to its similarity to *P. capillare*.

Funding

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Sphagnum papillosum Lindb., fam. Sphagnaceae (moss)

Contributor(s)	Žan Lobnik Cimerman, J. Mihael Kocjan
Corresponding author	Žan Lobnik Cimerman (zan.lobnikcimerman@bf.uni-lj.si)
Leg.	J. Mihael Kocjan
Country	Slovenia
Statement of significance	Rarely reported taxon, listed in the near threatened (NT) category of the Slovenian Red List.
Locality description	Gorenjska, near Komenda, NE of the village Mlaka pri Komendi, left side of stream, 350 m a.s.l.
Habitat	alder forest, transitional mire
Date of observation	2023-01-01
GPS	N 46.216944°, E 14.566389°
Voucher	Herbarium LJU

Sphagnum papillosum (sect. *Sphagnum*) was recently found growing in a transitional mire in the Gorenjska region of Slovenia. The species has a known distribution in the Alpine (Julian Alps and Pohorje) and the pre-Alpine regions (Martinčič, 2024). It is listed as a near threatened (NT) taxon on the national Red List (Martinčič, 2016, 2024). The species is also present in Croatia, Italy, and Austria, in the latter being considered vulnerable (category 3, which roughly translates into VU using the IUCN system) (Hodgetts and Lockhart, 2020). In the year 1987, the dynamics of net photosynthesis of *Sphagnum papillosum* were investigated on a relic of the raised bog Goričica near Ljubljana, Slovenia (Gaberščik and Martinčič, 1987). Whether that population is still present up to the present day remains a question.

According to Martinčič (2024) the species is an exclusive element of raised bogs in Slovenia. Generally, it is known in Europe as a species of open acidic peatlands growing in distinct hummocks or more extensive carpets. It grows beside streams or in flushes in upland areas and transitional mires with a deep peat layer in the lowlands (Daniels and Eddy, 1990).

The unique cell morphology of *Sphagnum papillosum* gives rise to its name; hence, the surface of the chlorocysts on a cross-section of the branch leaves appears to be rough with minute projections. This is due to cell wall protrusions that form papillae (Anderson and Ammann, 1991). The fascicles consist of 4 branches, with usually two being pendent and two divergent. The whole plant is green, yellowish, or ochre (Daniels and Eddy, 1990).

Similar but with longer outgrowths on the surface of chlorocysts, commonly referred to as transverse lamellae or comb-fibrils, are also present in two other species, those being S. austinii and S. affine. Still, they have a wider distribution in northern European countries (Smith, 2004).

Tortella fasciculata (Culm.) Culm., fam. Pottiaceae (moss)

Contributor(s)	K. Potočnik, S. Strgulc Krajšek
Corresponding author	S. Strgulc Krajšek (simona.strgulc@bf.uni-lj.si)
Leg.	K. Potočnik
Country	Slovenia
Statement of significance	The first recent report for Slovenia.
Locality description	Gorenjska, Kranj, Nemilje, in the front yard of Nemilje 1504 m a. s. l.
Habitat	at the top of the stone wall
Date of observation	2024-05-28
GPS	N 46.2648756°, E 14.2297790°
Voucher	Herbarium LJU

Tortella fasciculata (Culm.) Culm. is a species from *T. bambergeri* agg. In the Red List of Bryophytes of Slovenia (Martinčič, 2016), *T. bambergeri* (Schimp.) Broth. s. lat. is listed as a data deficient species. It was found in the Alpine (Glowacki, 1908, 1910; Breidler 1891), Dinaric (Glowacki, 1913), and Pre-Alpine phytogeographical regions (Glowacki, 1910), but all records are more than 100 years old. As the recent molecular research by Köckinger and Hedenäs (2017) showed, *T. bambergeri* agg. consists of two species, namely *T. fasciculata* and *T. pseudofragilis* (Thér.) Köckinger & Hedenäs. *T. pseudofragilis* was already collected in Slovenia (Plemenice under Triglav) by I. Dakskobler (Martinčič, 2024) and Martinčič (2024) comments that the specimens reported by Glowacki (1910) could also refer to this species. The presence of *T. fasciculata* was not confirmed in Slovenia until our findings. However, Martinčič (2024) comments that the specimens reported by Glowacki (1910) from Kanal at Soča Valley might refer to *T. fasciculata*.

We sampled the species on the top of the rock wall near the stream in the front yard of Nemilje 1, where humidity is high for most of the year. According to the literature, the ecology is not typical, as the species is believed to be thermophyllous (Köckinger and Hedenäs, 2017). We used Köckinger and Hedenäs (2017) and Ottley and Blockeel (2019) for identification. The central strand was well-developed and composed of several cells. The leaves in the dry state were incurved up to the apex. Marginal cells were very papillose and mostly isodiametric. The plants were without sporophytes.

According to Köckinger and Hedenäs (2017), the species has a suboceanic-submediterranean distribution. Up to now, it has been recorded in several European countries, but in several European regions, its status is still unclear (Hodgetts & Lockhart, 2020). This species is considered the least concerned (LC) in the European Red List (Hodgetts & al. 2019).

The species is expected to be more widespread in Slovenia. Until we collect more distribution data, we suggest listing the species as data deficient with recent records (DD-n) in the Slovene Red List.

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Short Review

Fruits of japanese quince are a valuable commodity for the food and pharmaceutical industry

Samatova Sh. A. [1], Berdiyev M. F. [1], Ergasheva N. G. [1,*]

Abstract

The article presents analytical data on the medicinal content of the fruit of the Japanese quince (Chaenomeles japonica) introduced to Shakhrisabz. Based on the study of its growth and development under the conditions of introduction, it was determined that it is promising for Shahrisabz and will produce many goods in the new conditions. It was concluded that it is possible to spread it to other regions and enrich the food and pharmacopoeia industry with valuable raw materials by developing a scientifically based technology for increasing fertility in new conditions.

Keywords

introduction, shrub, fruit, pectin, enzyme, flavonoid

1 Department of Botany of Karshi State University, Uzbekistan

*** Corresponding author:**
E-mail address: mirtemir01554.gmail.com

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Plodovi japonske kutine so dragoceno blago za živilsko in farmacevtsko industrijo

Izvleček

V članku so predstavljeni analitični podatki o zdravilni vsebnosti plodov japonske kutine (Chaenomeles japonica), prinesene v Shakhrisabz. Na podlagi študije njegove rasti in razvoja v pogojih uvedbe je bilo ugotovljeno, da je obetavna in ekonomsko zanimiva rastlinska vrsta za dotično regijo. Ugotovljeno je bilo, da ga je možno razširiti tudi v druge regije in obogatiti živilsko in farmakopejsko industrijo z dragocenimi surovinami z razvojem znanstveno utemeljene tehnologije za povečanje rodnosti v novih razmerah.

Ključne besede

vnos, grm, plod, pektin, encim, flavonoid

Japanese quince is a new fruit-ornamental plant that is expanding its cultural area more and more. The advantages of this cultivar are its high economic efficiency and potential for expanding the area, easy reproduction, and resistance to diseases and pests, which allow it to grow without toxic chemicals. These properties increase the biological and ecological value of Japanese quince fruits and serve to save money (Fedulova et al. 2016).

Japanese quince (Chaenomeles japonica) is a small ornamental shrub belonging to the Rosaceae family and grows naturally in Japan. It grows up to 0.5-1.0 m tall in natural conditions and produces 2 cm thorns. Branches grow. The compact root system is located in the soil's upper layer up to 10 cm. The leaves are side-lobed, shiny, dark-green, broadly ovate, 3-5 cm long. The flowers are golden-red, with a large diameter of up to 4 cm, and hundreds of flowers are formed on one bush. The fruit is green to golden yellow, sour, and fragrant, and contains a large amount of organic acids (4-5%), pectin (06-2.6%) and aromatic substances. Jam, wine, juice and other products are made from its fruits (Osipov et al. 2013). It is a drought-resistant, light-loving decorative, fruit-bearing, honey-producing plant (Kaminskiy et al. 2013).

Currently, Japanese quince is being researched for preparing juices, aromatization and fibre extraction, determination of healing properties, use in functional nutrition and other purposes. The interest in growing it is increasing day by day due to the beneficial properties of its fruit. Regarding vitamin C content (100-230 mg/%), its fruit is not inferior to black currant. It contains five times more vitamin C than lemon. The amount of vitamin R (910 mg/%) is ten times more than that of apples (Osipov et al., 2013). The freshly picked fruits of the plant are hard and very sour, and they are difficult to eat. Still, the biologically active components, aroma and high fibre content are very suitable for industrial processing of the fruit (Baranowska-Bosiacka et al. 2017). As a result of research on the use of fruits in the food industry, it was found that the fruit of Japanese quince has high antioxidant properties and contains a large amount of phenols. It has been revealed that wine and liquor products made from fruits have high commercial potential (Tarko et al. 2014) and can be used as valuable products in functional nutrition (Prichko et al. 2014). Due to the high antioxidant properties of plant fruits, it is recommended that microcapsules be prepared from them. Antioxidant properties of microcapsules, the amount of phenolic compounds contained in them, as well as the effect of α-glucosidase, pancreatic lipase, acetylcholinesterase and 15-lipoxygenase enzymes in vitro, and it was found that they retain their healing properties even under such conditions (Turkiewicz et al. 2020).

Analyzes conducted to evaluate the activity of phenolic compounds obtained from the fruit of the plant confirmed that five compounds isolated from it: isoquercitrin, rutin, (+)-catechin, (-)-epicatechin and chlorogenic acid have antimicrobial properties against gram-positive and gram-negative bacteria. In particular, Enterococcus faecalis bacteria showed the highest sensitivity to these compounds. The analysis showed that Japanese quince fruit can be used as a promising antioxidant and antimicrobial agent to enrich the diet and replace chemical preservatives in the food and cosmetics industry (Urbanaviciute et al., 2020). The flavanols in Japanese quince fruit have made normal cells more resistant to apoptosis and cancer cells more

sensitive. Studies have shown that fruit polyphenols have a strong antiproliferative effect on prostate and breast cancer cells, inhibiting their growth but not reducing the number of healthy cells. It has also been found to inhibit the invasiveness of cancer cells and reduce the expression level of several genes involved in apoptosis, angiogenesis and metastasis (Lewandowska et al., 2013).

Japanese quince fruits contain large amounts of macro- and micronutrients, ascorbic acid, phenolic compounds, fibre, and low amounts of oxalates. The average amount of iron in freshly picked fruits is 0.516 mg/g, copper 0.146 mg/g, zinc 0.546 mg/g, magnesium 16.729 mg/g, and calcium 22.920 mg/g. In experimental conditions, it was observed that the concentration of lipid peroxides decreased in hepatocytes incubated with Japanese quince extract. Still, it did not affect the concentration of the active form of oxygen in mitochondria. It has been found that signs of apoptosis and necrosis are not observed in hepatocytes under the influence of any concentration of Japanese quince fruit extract. Japanese quince was considered to have a hepatoprotective effect due to the antioxidant and antiapoptotic effect of the aqueous extract of the fruits on hepatocytes (Baranowska-Bosiacka et al. 2017).

It was found that cookies enriched with freeze-dried (lyophilized) fruits of Japanese quince had a 2-3.5 times higher radical scavenging activity than the control option and fewer secondary products formed from lipid oxidation. Enriched cookies contained more volatile hexanal, heptanal, octanal, and 2-heptenal (E) than the control. Because of acetic acid predominance (7.05-23.37%) in the volatile properties of the cookies, they were rated as having a higher intensity of sourness and citrus odour. Biscuits stored for 16 weeks were found to have an increased amount of carbohydrates compared to freshly made ones, and carbohydrates that were not present in freshly made biscuits were formed. Consumers preferred cookies with 1.0 and 1.5% freeze-dried fruit over cookies with 6.0 and 9.0% freeze-dried fruit (Antoniewska et al. 2019).

The effect of procyanidin extract from Japanese quince fruits on the activity of metalloproteinases secreted by human peripheral blood mononuclear cells and human leukaemia cells was studied. It has been proven that the extract effectively inhibits enzyme activity. The efficiency of scavenging radical cations explains their antioxidant activity. It is concluded that Japanese quince polyphenols can be used for cancer prevention, and their biological activity mechanisms should be studied (Strek et al. 2007). Phytochemical analysis of fat and protein fractions in the residue released during winemaking from Japanese quince was carried out. It was determined that the oil extracted from residual products is rich in unsaturated fatty acids, tocopherols and phytosterols, and the protein contains all non-exchangeable amino acids. Fat and protein fractions extracted from waste products have been recommended for use in the food and cosmetic industries (Ben-Othman et al., 2023).

This plant was introduced to Uzbekistan as an ornamental plant not long ago. At the beginning of our century, following the relevant decisions of the Cabinet of Ministers, large construction works were carried out in the cities of Karshi and Shahrisabz, and new decorative plants were brought to the region and planted. In this regard, Japanese quince bushes introduced to Shahrisabz City are growing well and producing abundant crops. Since the plant is resistant to cold, it starts its vegetation in Shahrisabz conditions in early February. From the second half of February, the first buds begin to appear from the flower buds on the plant's one-year-old branches, and branches begin to appear from the vegetative shoots. Flowering begins in the first days of March and lasts until the middle of April. The first flowers turn into fruits in the first days of April. At the end of flowering, that is, from the second ten days of April, the growth of branches accelerates significantly. From May, the growth slows down, the fruits begin to ripen, and the growth of branches continues until July. In July and the first half of August, the plant's growth stops completely. From the second half of August, repeated growth at the expense of side branches begins and continues until late autumn. This state of plant growth and development has been observed in most of the subtropical and tropical, as well as Chinese-Japanese flora introduced to South Uzbekistan (Yoziev et al. 2001).

Author Contributions

For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, S.Sh, B. M., E. N.; writing—original draft preparation, S.Sh, B. M., E. N.; writing—review and editing, S.Sh, B. M., E. N. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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