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Solid–liquid extraction of bioactive compounds as a green alternative for developing novel biostimulant from *Linum usitatissimum* L.

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Abstract

Background The interest expressed by the agriculture in the category of innovative biostimulants is due to the intensive search for novel preparations based on natural substances i.a. allelochemicals. Scientific research into the design and testing of next-generation biostimulants marks advances for sustainable agricultural production.

Results Our research represents an innovative and comprehensive approach to the use of *Linum usitatissimum* L. seed extracts in soybean cultivation using foliar treatments. A multi-tiered approach was adopted, involving both the assessment of soybean responses at the agronomic, morphological and biochemical levels. It was hypothesized that the extract would improve the growth and yield of soybean through mechanisms based on improved physiological and biochemical processes. The study showed that the extract was rich in micro- and macro-nutrients (in particular potassium and zinc), amino acids (with the largest proportion of glutamic acid), fatty acids (predominant oleic + elaidic acids) and carbohydrates (sucrose and glucose). Soybean responded positively by increased in plant height, number of pods and seed yield. The results confirmed that the tested biostimulant is not only an environmentally friendly product, but also is capable of increasing the growth and productivity of soybeans, thus increasing farmers' profit. The obtained yield was characterized by a lower total proteins pool (average decrease approx. 2%). The extract caused changes in the amino acid profile (especially in the case of proline and lysine) and fatty acid composition (significantly higher content of acids: heptadecanoic, octadecanoic, eicosanoic, eicosapentaenoic, docosanoic, erucic, tricosanoic).

Conclusions All the observations made confirm that the extract positively affected the soybean crop. Based on the study, it was concluded that solid–liquid extraction could be considered as a green alternative for prototyping a novel and ecological biostimulant.

Keywords Flaxseed, Biostimulant, Prototyping, Soybean, Water extraction, Infusion, Partial budget

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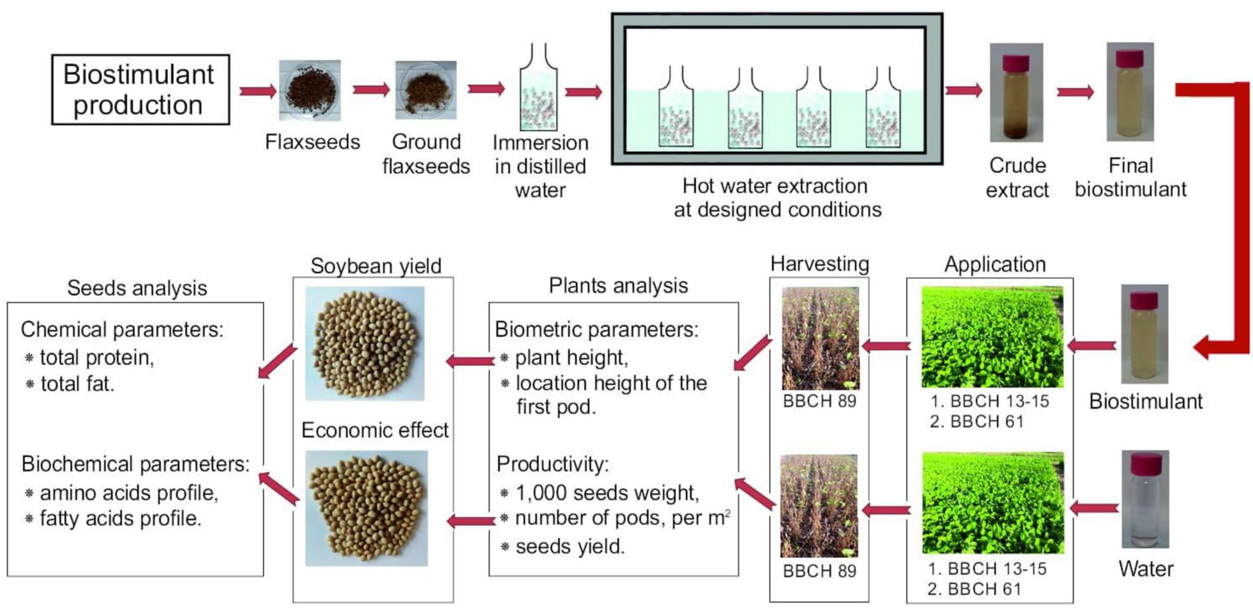
Graphical Abstract



Background

In recent years, the development of environmental awareness has taken place in scientific and social circles. The necessity of introducing pro-environmental changes in all fields of human activity has been noticed, including agriculture, which has become one of the economic sectors with a huge impact on the natural environment. The significant increase in demand for food and feed recorded on a global scale seems to put additional and constantly expanding pressure on this branch of the economy. Currently, the greatest challenge facing humanity seems to be developing the productivity of crops to feed the growing population while conserving natural resources [11]. To overcome these and achieve pro-environmental goals, scientists are focusing their efforts on developing solutions to reduce and/or rationalize the use of chemical fertilizers and pesticides. This will help prepare agriculture for the new coming green revolution, which is largely defined by cost-effective and ecologically friendly approaches to sustaining the agricultural sector [43]. The European farm-to-table strategy (EC COM/2020/381 [25]) is to reduce: (1) the use of chemical pesticides by 50%, (2) nutrient losses by at least 50%, and (3) fertilizer use by at least 20% by 2030. In addition, a plan to bring 25% of farming land under organic cultivation is fundamental. On this road to agricultural change, it seems that genetic improvements and better varieties will be insufficient, and the key to achieving these goals will be biostimulants. These products are natural preparations that have gained a lot of interest in recent years, mainly due to their role in stimulating plant growth and yield and reducing the impact of abiotic and biotic stresses [43]. The current Regulation (EU) 2019/1009 [56] defines biostimulants as "products that stimulate plant nutritional processes regardless of the nutrient content of the product, whose sole purpose is to improve one or more of the following characteristics of the plant or its rhizosphere: nutrient use efficiency, abiotic stress tolerance, quality characteristics, availability of limited nutrients in the soil or rhizosphere". Thus, given the definition provided, biostimulants do not directly affect pathogens and biotic stresses, but are mainly used to improve crop yields under both adverse and optimal environmental conditions [14]. Higher yields in accordance even with organic practices are the main advantages of these bioproducts [32]. Depending on the type of crop, biostimulants can be applied as: seed priming [50], soil drenches, foliar spray, and additives to hydroponic solutions [32]. It should also be emphasized that these products can be applied according to different schemes, individually adapted to crop plants and environmental conditions, i.e., regularly at the vegetative stage or preventively before or during stress [30, 32]. Biostimulants can be produced from a whole range of raw materials, which can include various natural compounds such as beneficial microorganisms, protein hydrolysates, humic acids, seaweed extracts, and amino acids [64, 65], plant extracts. Therefore, usually, biostimulants are classified by origin rather than by the chemical structure of the compounds included in their composition [13, 24, 47]. The most important aspect, however, is the fact that these agricultural bioproducts are produced from raw materials rich in bioactive compounds that can exhibit action in many ways, including independent, additive, and/or synergistic. However, the exact mechanisms of action behind the biostimulant effect are still unidentified [30]. Currently, the mode of action of agronomical bioproducts is identified indirectly through plant responses at the biochemical, physiological, and molecular levels [36]. The highly complex nature of biostimulants and the broad heterogeneity of their chemical composition are responsible for the wide range of biochemical, cellular, and physiological responses they induce in crop plants. These responses include improved seed germination and seedling development [31, 67], changes in secondary metabolism [22], and improved yield and yield quality [45, 52].

Studies are available in the literature describing the use of plant extracts as effective natural biostimulants to support crop plants [1, 12, 26, 39, 53, 54, 57, 69, 75, 76]. Du Jardin [24] coined the term "botanicals" for plant-derived biostimulants. The results of few studies show that botanical extracts can be produced from different morphological parts, including seeds, leaves, flowers, or roots, of higher plants belonging to the families Amaryllidaceae, Brassicaceae, Ericaceae, Fabaceae, Fagaceae, Moringaceae, Plantaginaceae, Poaceae, Rosaceae, Solanaceae, Theaceae and Vitaceae [4, 5, 80]. The results of these studies have shown that exogenous application of these bioproducts can result in improved stress tolerance of crop plants [40, 44]. Previous studies by our team, on the use of aqueous extracts of allelopathic plants including *Artemisia absinthium* L., *Levisticum officinale* Koch. and *Arctium lappa* L. in a 3-year soybean crop proved that their application increased plant yield. Additionally, the botanical extracts analyzed were shown to modify the biochemical indices of soybean. In the search for new plant species that show biostimulatory potential, the seeds *Linum usitatissimum* L. have been proposed. This was due to the results of our research, which proved that water extracts, obtained from common flax, were effective in improving germination and emergence of various crops, as well as reducing microbial contamination [37, 18, 19, 74]. In addition, our results showed the effectiveness of flaxseed macerates in soybean cultivation, but the issue was the efficiency of ecological cold extraction of biologically active compounds [39].

Extrusion is most commonly used to extract biologically active compounds from flaxseeds, but this method has the disadvantage of high loss of vitamins, phospholipids, phytosterols, and antioxidants [35]. Solvent extraction is a solution to this problem, but the use of acetone, methanol, and petroleum ether, among others, may decrease the biological activity of the extracts and contribute to environmental pollution. Therefore, an ecological approach seems to be the use of water or supercritical fluid (e.g., CO_2) as a solvent as well as the application of microwave and ultrasonic-assisted extraction [81].

Despite the concentration of many biologically active compounds and the use of the flaxseed plant for cosmetic, food, medical, and pharmaceutical purposes, there is no information in the available literature on its potential agronomic use. On the other hand, many arguments indicate that flax could be an ideal candidate for the creation of biostimulant preparations, given appropriate extraction methods. This is particularly relevant for soybean crops. Therefore, the use of these products, especially those of natural origin, seems to be necessary for sustainable soybean production [72].

The objective of the present study was to evaluate the biostimulant potential of an aqueous flaxseed extract, designed and produced to increase the soybean yield. It was hypothesized that the aqueous flaxseed extract could be considered a new potential biostimulating tool for plant growth.

Material and methods

Biostimulants production and characterization

Material: Seeds of *Linum usitatissimum* L. were purchased from biofarming (Runo Poland, PL-EKO 07 EU Organic Farming). The extraction was conducted according to the protocol described in our previous research [76]. For the infusion preparation 5 g of ground material was added to 250 mL of distilled water (temperature 95°C) and maintained in a water bath for 30 min (the constant temperature in a sample solution 95°C). The mixture was left for 48 h at the temperature of 4 °C. Then, the resulted suspension was centrifuged at 4250×*g* for 5 min and filtered (Whatman qualitative filter paper, Grade 1) (Fig. 1).

Mineral composition of the infusion

The extracts (1 mL) were mineralized with 65% nitric acid using the microwave digestion system under high pressure and increasing temperature algorithm up to 220 °C. During the digestion procedure Ethos One—microwave digestion system (Milestone, Italy) was used. After the mineralization procedure all samples were filled up to 50 mL with extra pure deionization water (< 0.07 µS cm^{-1}). The measurements were performed by Inductively coupled plasma optical emission spectrometers (ICP-OES, Thermo iCAP Dual 6500, USA). Each measurement

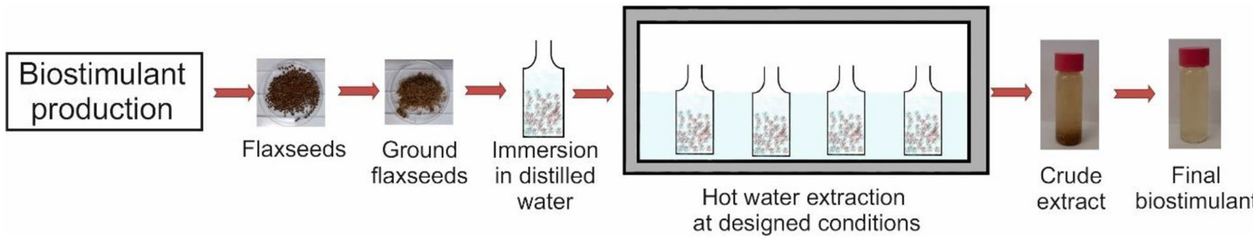


Fig. 1 Schematic diagram of the biostimulant production

was made in two spectral: radial and axial, across and along with horizontally burning argon plasma and concentrations were calculated using calibration curves. The certified reference material was used when obtaining recoveries for individual elements, as in Fig. 2 [82].

Qualitative–quantitative analysis of sugars in the extract

The sugar content in the obtained product was evaluated according to the EN 12630, 1999 standard [26] and method described by Pereira da Costa and Conte-Junior [46] (HPLC system). The chromatographic equipment SYKAM (Eresing, Germany) consisting of sample injector S5250, pump system S1125, column oven S4120 and RI detector S3590 was used. Separation was carried out using Sugar-D column (4.6 mm I.D. × 250 mm; COSMOSIL). The separation was achieved with a mobile phase of 75% ACN in water in isocratic mode. The flow rate was 0.5 mL min^{-1} at column temperature set at 30 °C. The volume of injected sample was 20 µL and 20 min was needed to complete the analysis.

Protein amino acids composition in infusions from seeds of *L. usitatissimum* L.

The evaluation of amino acids has been carried out with the use of the amino acids analyser AAA 400 (Ingos, Prague, Czech Republic) with UV–Vis detector. The separation of amino acids has been achieved using 0.37 × 450 mm ion exchange column thermostated at 60 °C. The detection of amino acids has been conducted at following wavelength 570 nm but the proline at 440 nm. The time analysis was of 90 min.

Determination of fatty acids in infusions from seeds of *L. usitatissimum* L.

The esterified sample was used to determine the fatty acid composition. The sample was prepared according to Zhang et al. [84] using Varian 450-GC gas chromatograph with a flame ionization detector (FID) equipped with SelectTM Biodiesel, capillary column 30 mm (length) × 0.32 mm (ID) × 0.25 µm film thickness. Helium was used as the carrier gas at the flow rate of 1.5 mL min^{-1}.

Application of the infusions from seeds of *L. usitatissimum* L. in field trials—plant material and growth conditions

Soybean plants and seeds (cv. Abelina) originated from field experiment (2017–2019) conducted in Perespa (50° 66′ N; 23° 63′ E, Poland). The experiment was designed and performed in a random block system in four replications, on experimental plots with the size of 15 m^2. Plants were grown on soil classified as Gleyic Phaeozems (pH in 1 M KCl 7.3–7.4). The average level of available nutrients in 100 g of soil was as follows: 12.6–14.2 mg P_2O, 15.2–17.1 mg K_2O, 6.3–6.8 mg Mg and 8.1–9.1 mg

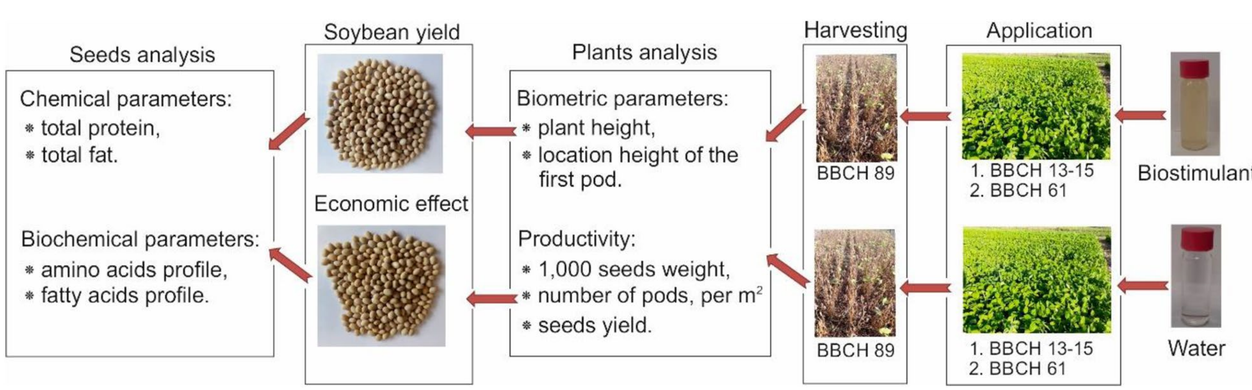


Fig. 2 Schematic diagram of the biostimulant prototyping

$N{-}NO_3 + N{-}NH_4$. *Triticum aestivum* L. was used as the previous crop. Seeds were sown on the 2 May 2017, 2018, and 2019 with 4.0 cm gaps in rows with 30 cm spacing. No herbicides were used, and weeds were removed mechanically and manually. The fertilization and irrigation were not carried out. In each growing season, plants were treated with the extracts from *Linum usitatissimum* L. Product was applied in the form of double plant spraying (250 L ha^{-1}) at the BBCH 13–15 and BBCH 61 (BBCH—Biologische Bundesanstalt, Bundessortenamt and CHemical industry—scale of the phenological development stages) developmental stages of soybean. Combinations with plants sprayed with water used for extract preparation served as the control (Fig. 2). Spraying was performed with the Pilmet 412 LUX (Unia, Grudziądz, Poland) sprayer equipped with nozzles air-induction flat fan nozzles 6MSC (working pressure 0.30 MPa). After the pods have matured (BBCH 89), plants were harvested. Dates and dosage of application were chosen based on results of our earlier experiments addressing the use of natural and synthetic biostimulants in soybean cultivation [77].

Analysis of soybean plants

Biometric traits of soybean plants were determined: the plants height and the location height of the first pod. The productivity of the crop was also evaluated by testing the weight of 1,000 seeds, the number of pods per m^2, and yield.

Protein content and amino acids composition in soybean seeds

The total protein content in seeds from control crops and crops with tested biostimulant was measured by the Kjeldahl method [7], Official Method 992.23, 979.09). Hydrolysis of protein into amino acids has been carried out according to Davies and Thomas [20]. The sample (70 g) was placed in hydrolyzer tube (Ingos) with 20 mL of 6 M HCl. After that the tube was clamped and the sample was kept at 110 °C for 20 h. After cooling to room temperature (RT), the hydrolysate was filtered through a G-5 glass filter, the solvents were evaporated and the residues were redissolved in citric buffer at pH 2.2. Before the HPLC analysis, samples were filtered (0.22 µm filter). The evaluation of amino acids has been performed using the amino acids analyser AAA 400 (Ingos, Prague, Czech Republic) with photometric detector (0.37×450 mm ion exchange column, 60 °C). The amino acids have been detected at 570 nm but the proline at 440 nm. The time analysis was of 90 min (Fig. 2).

Fat content and determination of fatty acids in soybean seeds

The total fat content in the seeds of the control and experimental groups was analyzed by the acid hydrolysis method ([7], Official Method 922.86). For the fatty acids evaluation the GC column was set Select™ Biodiesel, 30 m (length) × 0.32 mm (ID) × 0.25 µm film thickness. Helium was used as the carrier gas [84] (Fig. 2).

Evaluation of economic effect of the application of *L. usitatissimum* L. extract in soybean field cultivation—partial budget analysis

Partial budget analysis (PBA) was assessed to evaluate the net economic benefits. The economic effect of *Linum usitatissimum* L. extracts application was computed based on the value of yield increase resulting from the use of products and costs of their application in soybean cultivation [76]. Income growth (EUR ha^{-1}) resulting from the use of biostimulants was calculated as a difference between value of yield increase and costs of the use. The value of yield increase (EUR ha^{-1}) was evaluated as a product of average price of soybean seeds in a given study year and a difference between the seed yield from the combination with extracts application and seed yield from the control combination. Costs of the products treatment (EUR ha^{-1}) were computed as a sum of four parameters: cost of extracts purchase, cost of water used for the treatment, cost of performing the treatment, and cost of human labor for extract preparation. The average purchase price for seeds was determined from the market offers (311.12 EUR t^{-1}). The cost of *Linum usitatissimum* L. seeds (1.12 EUR kg^{-1}) was taken from herbal store (Runo Poland). The cost of water was the average price of 1 m^3 (1.50 EUR m^{-3}) in the Lubelskie Province. It was estimated that it would take an hour of human labor to prepare the extract for 1 hectare of the crop. Cost of human labor for bioproduct preparation (10 EUR ha^{-1}). The cost of the procedure was the average price of the plant spraying service (17.56 EUR ha^{-1}).

Statistical analysis

All tests of extracts and soybean plants and seeds were performed in 3 replicates. The normality of the data distribution was assessed using the Shapiro–Wilk test. The results obtained from the studies were subjected to statistical analysis using one-way ANOVA. Determination of the significance of differences between mean values was made, based on Tukey's confidence intervals (significance level $p \le 0.05$). Statistica 13.3 software (TIBCO Software Inc., USA) was used for statistical analysis.

Results

Chemical composition of infusion

The study showed that flaxseed infusions were rich in micro- and macro-nutrients (Figs. 3 and 4). As a result of hot water extraction, obtained preparations were characterized by high concentrations of K (90.19 mg mL^{-1}), Ca (26.83 mg mL^{-1}), Mg (12.43 mg mL^{-1}), P (7.37 mg mL^{-1}), and S (25.99 mg mL^{-1}).

The evaluation of micronutrients content showed that in the studied botanical preparation, there were significant concentrations of Zn (0.154 mg mL^{-1}) and Cu (0.133 mg mL^{-1}). The presence of Fe (0.021 mg mL^{-1}) and Mn (0.035 mg mL^{-1}), was also demonstrated. However, no presence of toxic elements was found in the extracts. The study also revealed the presence of beneficial elements for plants in the form of Na (32.85 mg mL^{-1}) and Al (0.002 mg mL^{-1}).

The sucrose content was the highest at 0.764 mg mL^{-1}. In contrast, the glucose content was more than 47% lower at 0.401 mg mL^{-1}. It was also found that the flaxseed infusions did not contain fructose (Fig. 5).

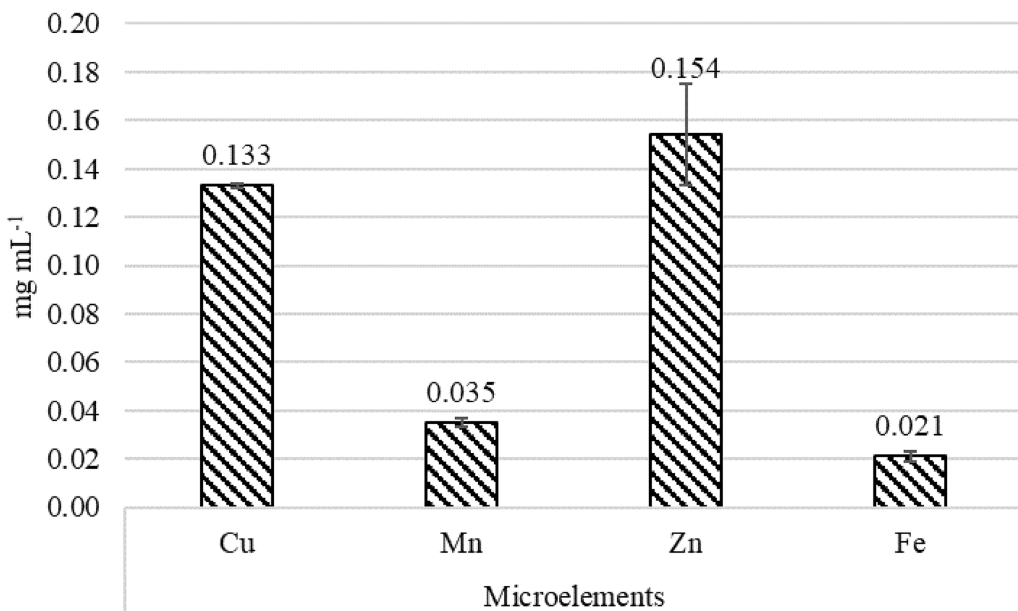


Fig. 3 Microelements in the flaxseed infusion; mean and standard deviation

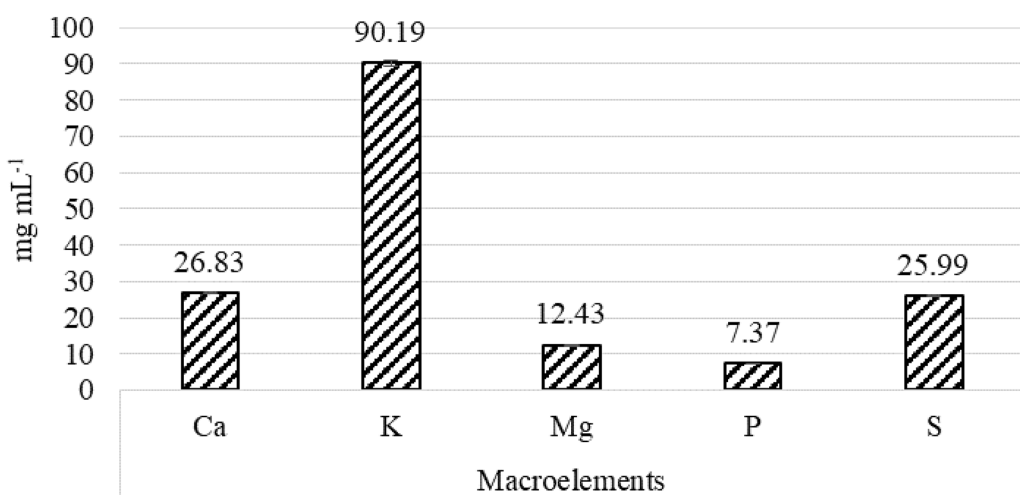


Fig. 4 Content of macroelements in the flaxseed extract; mean and standard deviation

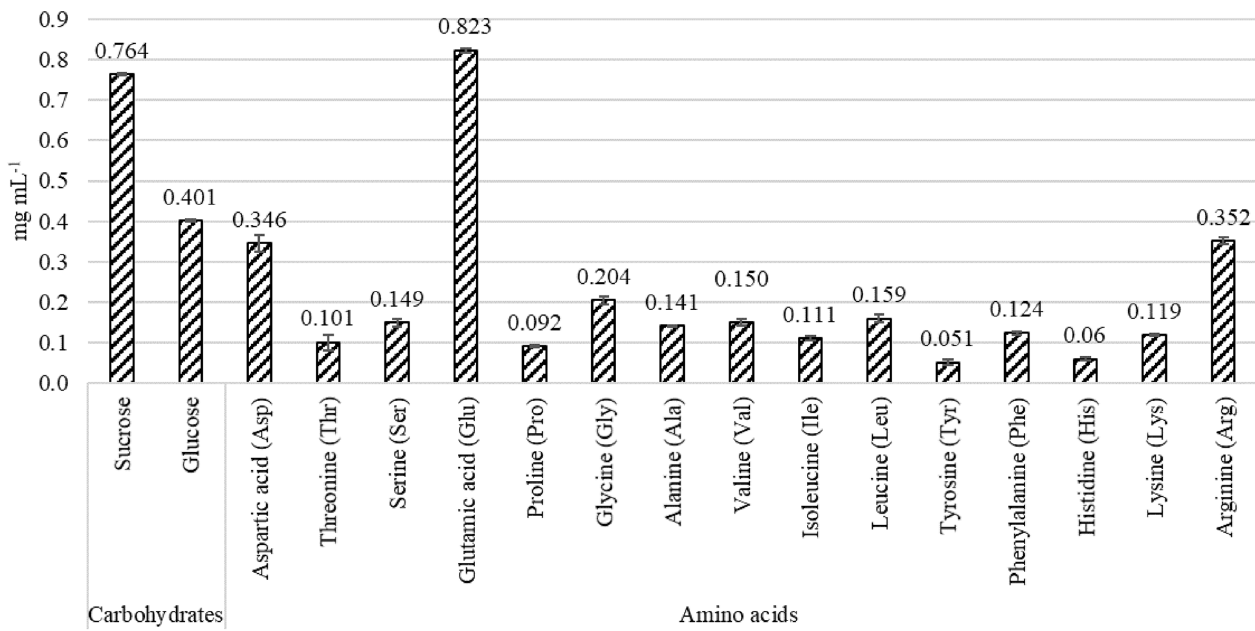


Fig. 5 Carbohydrates and amino acids in the water extract from *L. usitatissimum* L.; mean and standard deviation

The results also showed that the botanical flaxseed extract had a diverse amino acids profile (Fig. 5). Dominant amounts were found for three amino acids: glutamic acid (0.823 mg mL^{-1}), arginine (0.352 mg mL^{-1}), and aspartic acid (0.346 mg mL^{-1}). On the other hand, the concentrations of tyrosine (0.051 mg mL^{-1}), proline (0.092 mg mL^{-1}), and histidine (0.060 mg mL^{-1}) were the lowest among the entire pool of amino acids present in the extract (Fig. 5).

Results of testing fatty acids in fat extracted from flaxseed are presented in Table 1.

In the bioproducts, 0.08% fat was extracted. The analysis showed that among the fatty acids identified, oleic acid and elaidic acid (0.0217 g 100 g^{-1}), and hexadecanoic acid (0.0180 g 100 g^{-1}) had the highest proportion.

Table 1 Fatty acids in the water extract from *L. usitatissimum* L. (mean ± standard deviation)

Compound	Fatty acids (g 100 g^{-1})
Octanoic acid (C8:0)	0.0006 ± 0.0000
Decanoic acid (C10:0)	0.0007 ± 0.0000
Dodecanoic acid (C12:0)	0.0011 ± 0.0001
Tetradecanoic acid (C14:0)	0.0032 ± 0.0002
Myristoleic acid (C14:1n9)	0.0001 ± 0.0000
Pentadecanoic acid (C15:0)	0.0003 ± 0.0000
Hexadecanoic acid (C16:0)	0.0180 ± 0.0021
Palmitoleic acid (C16:1n7)	0.0003 ± 0.0000
Heptadecanoic acid (C17:0)	0.0003 ± 0.0000
Octadecanoic acid (C18:0)	0.0086 ± 0.0002
Oleic acid (C18:1n9c) + elaidic acid (C18:1n9t)	0.0217 ± 0.0014
Linoleic acid (C18:2n6c) + linoelaidic acid (C18:2n6t)	0.0031 ± 0.0002
α-Linolenic acid (C18:3n3)	0.0026 ± 0.0001
Eicosanoic acid (C20:0)	0.0003 ± 0.0000
Docosanoic acid (C22:0)	0.0004 ± 0.0000
Tricosanoic acid (C23:0)	0.0001 ± 0.0000
Tetracosanoic acid (C24:0)	0.0002 ± 0.0000

Extract application affected morphological characteristics and yields of soybean

Results of a field study, that analyzed the effect of extracts, showed that foliar application determined morphological traits in soybean (Fig. 6). It was found that soybean plants grew higher as a result of infusion application (124.1 cm, 126.3 cm, 118.3 cm, respectively, in 2017, 2018, 2019). In the first and second year of the field experiment, it was noted that the height of plants from the crop in which the biostimulant was tested was approximately 12% higher compared to control plants. Lower differences occurred in 2019, when changes reached about 5%.

Analysis of another biometric trait proved that in case of the height of the first pod (parameter important for combine harvesting of soybeans), the application of linseed infusions increased this parameter (increase of over 40, 26 and 23%, respectively, in 2017, 2018, 2019, relative to control plants).

Additionally, soybean plants responded to the application of extract by changing the number of pods per

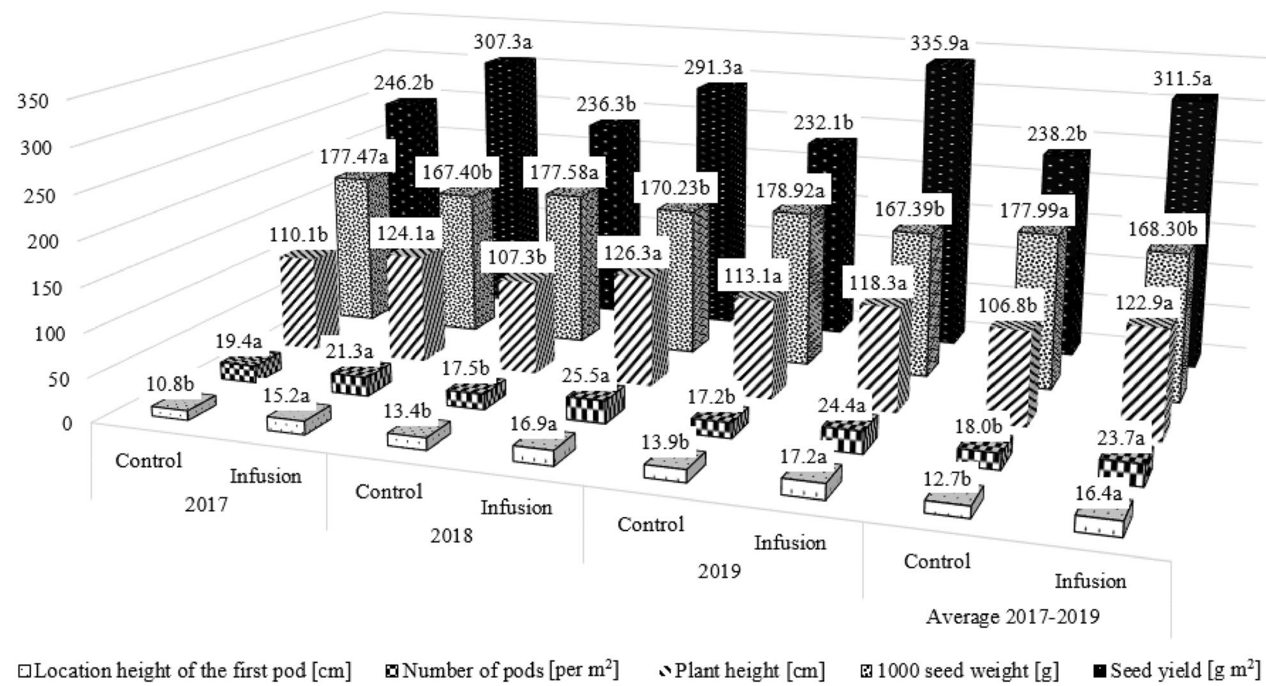


Fig. 6 The effect of the water extracts from *Linum usitatissimum* L. treatment on biometric traits and yield of soybean; Values not sharing a common letter indicate significant difference at $p \leq 0.05$

m^2. It was found that this structural element of soybean yield was improved by more than 30% by the application of *Linum usitatissimum* L. extracts.

The observation of higher yields after the application of the tested bioproducts in relation to the control crop was a confirmation of the biostimulatory effect. It is worth noting that trends in terms of yield improvement were recorded in all years of field studies. In the first and second year of the field experiment, it was shown the increase in the yield of soybeans treated with the bioproduct (by more than 24 and 23% compared to the control crop). On the other hand, the greatest productivity was observed after application of the extract in the third year of the field experiment (an increase of more than 44% with respect to control crops). Analysis of the average yield from the three-year experiment proved that the effect of the biostimulant application was an average improvement in soybean yield of 30%. However, the field studies showed that the botanical extract affected the reduction of 1000 seed weight. The weight of 1000 seed of soybean was reduced by 5.6, 4.1 and 6.4% compared to the control samples in the respective years of the field experiment. This yield index was significantly higher in control seeds.

Application of the novel biostimulants changed the content of protein and amino acid profiles

The assay demonstrated that using linseed extract in soybean cultivation affected contents of total protein in the seeds. Application resulted in slight reduction in protein contents against the control (approx. 2%) (Fig. 7).

A qualitative analysis of soybean crops demonstrated that the seeds contained wildly different levels of amino acids (Table 2).

When applied to soybean crops, infusions led to higher contents of individual amino acids against the control. The infusion-sprayed crops exhibited an increase in glutamic acid content of over 9%. Aspartic acid was the second most abundant compound in the seeds (its levels were 8% higher after infusion treatments compared to the control seeds). A similar correlation was observed for leucine (13%) and proline (24%). The least abundant amino acids were histidine and tyrosine, however, their content was higher in the soybean seeds collected from the extract-treated groups compared to the water-treated control group.

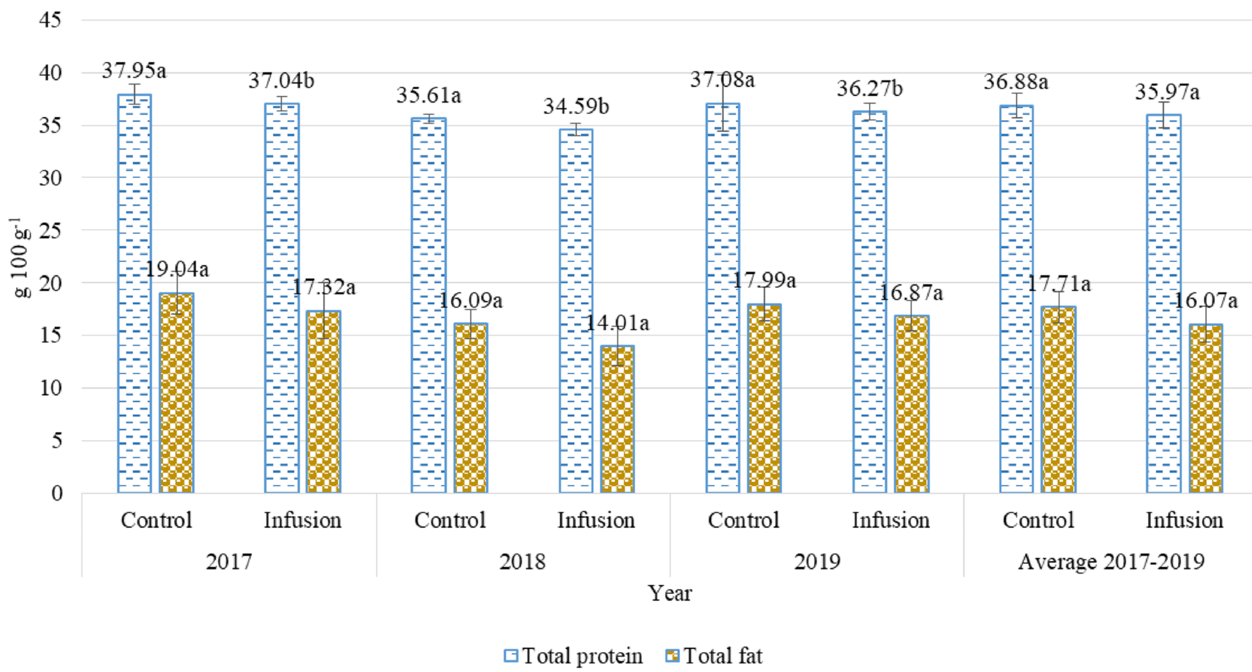


Fig. 7 The effect of the *L. usitatissimum* L. extracts treatment on total protein and total fat in soybean seeds (mean ± standard deviation). Values not sharing a common letter indicate significant difference at $p \le 0.05$

Table 2 The effect of the *L. usitatissimum* L. extracts treatment on amino acids concentration in soybean seeds (mean ± SD)

Amino acids (mg/g)	2017		2018		2019		Average 2017–2019	
	Control	Infusion	Control	Infusion	Control	Infusion	Control	Infusion
Aspartic acid (Asp)	31.9 ± 1.9a	34.5 ± 2.0a	28.2 ± 1.8a	30.6 ± 2.0a	30.5 ± 1.9a	33.3 ± 2.0a	30.2 ± 2.3b	32.8 ± 2.5a
Threonine (Thr)	12.2 ± 0.7a	13.1 ± 0.7a	10.6 ± 0.6a	11.6 ± 0.8a	11.4 ± 0.7a	12.2 ± 0.8a	11.4 ± 0.9a	12.3 ± 0.9a
Serine (Ser)	15.2 ± 0.8a	16.4 ± 1.0a	13.3 ± 0.8a	14.5 ± 1.0a	14.1 ± 1.0a	15.6 ± 1.0a	14.2 ± 1.1b	15.5 ± 1.2a
Glutamic acid (Glu)	52.4 ± 2.7a	56.7 ± 3.3a	45.6 ± 2.7a	50.3 ± 3.1a	49.0 ± 2.8a	53.8 ± 3.1a	49.0 ± 3.8b	53.6 ± 3.9a
Proline (Pro)	16.6 ± 0.9b	20.7 ± 1.1a	14.4 ± 0.9b	18.4 ± 1.1a	15.8 ± 0.9b	19.1 ± 1.0a	15.6 ± 1.2b	19.4 ± 1.4a
Glycine (Gly)	12.0 ± 0.7a	13.5 ± 0.8a	10.6 ± 0.6a	11.9 ± 0.7a	11.6 ± 0.7a	12.7 ± 0.7a	11.4 ± 0.8b	12.7 ± 0.9a
Alanine (Ala)	12.2 ± 0.7a	13.6 ± 0.7a	10.8 ± 0.7a	12.2 ± 0.8a	11.5 ± 0.6a	12.9 ± 0.8a	11.5 ± 0.8b	12.9 ± 0.9a
Valine (Val)	13.8 ± 0.8a	15.5 ± 0.8a	12.1 ± 0.7a	13.8 ± 0.9a	13.1 ± 0.8a	14.5 ± 0.9a	13.0 ± 1.0b	14.6 ± 1.0a
Isoleucine (Ile)	12.1 ± 0.7a	13.6 ± 0.9a	10.7 ± 0.6a	12.3 ± 0.8a	11.4 ± 0.7a	12.8 ± 0.7a	11.4 ± 0.8b	12.9 ± 0.9a
Leucine (Leu)	20.6 ± 1.2b	23.7 ± 1.3a	17.9 ± 1.1b	21.1 ± 1.4a	20.0 ± 1.3a	21.8 ± 1.3a	19.5 ± 1.6b	22.2 ± 1.6a
Tyrosine (Tyr)	9.1 ± 0.6a	9.9 ± 0.6a	8.0 ± 0.5a	8.9 ± 0.5a	8.7 ± 0.5a	9.4 ± 0.5a	8.6 ± 0.7b	9.4 ± 0.6a
Phenylalanine (Phe)	13.9 ± 0.8a	15.4 ± 0.9a	12.2 ± 0.8a	13.8 ± 0.8a	12.9 ± 0.8a	14.6 ± 0.8a	13.0 ± 1.0b	14.6 ± 1.0a
Histidine (His)	7.9 ± 0.5a	8.67 ± 0.5a	6.9 ± 0.4a	7.67 ± 0.4a	7.4 ± 0.5a	8.17 ± 0.5a	7.4 ± 0.6b	8.17 ± 0.6a
Lysine (Lys)	18.8 ± 1.1b	21.5 ± 1.3a	16.7 ± 1.1a	19.1 ± 1.3a	17.9 ± 1.1a	20.3 ± 1.1a	17.8 ± 1.3b	20.3 ± 1.5a
Arginine (Arg)	19.7 ± 1.2a	21.5 ± 1.3a	17.2 ± 1.1a	19.2 ± 1.2a	19.2 ± 1.1a	20.8 ± 1.3a	18.7 ± 1.5b	20.5 ± 1.5a

Values not sharing a common letter indicate significant difference at *$p \le 0.05$*

The biostimulants affected fat content and fatty acid levels in soybean seeds

The results regarding fatty acid profiles in soybeans show a multivariate soybean response to the application of linseed extracts (Table 3).

Some of the investigated fatty acids increased in level, while others decreased compared to the control seeds. Hexanoic acid (33.16 mg g^{-1}, 25.09 mg g^{-1}, 25.69 mg g^{-1}), octanoic acid, and cis-5,8,11,14,17-eicosapentaenoic acid were detected exclusively in

Table 3 The effect of the *L. usitatissimum* L. extracts treatment on fatty acids concentration in soybean seeds (mean ± SD)

Fatty acids (mg/g)	2017		2018		2019		Average 2017–2019	
	Control	Infusion	Control	Infusion	Control	Infusion	Control	Infusion
Hexanoic acid (C6:0)	< LLD	0.19 ± 0.02	< LLD	0.14 ± 0.02	< LLD	0.15 ± 0.02	< LLD	0.16 ± 0.03
Octanoic acid (C8:0)	< LLD	0.37 ± 0.06	< LLD	0.28 ± 0.05	< LLD	0.28 ± 0.05	< LLD	0.31 ± 0.07
Tetradecanoic acid (C14:0)	0.20 ± 0.02a	0.10 ± 0.02b	0.16 ± 0.03a	0.08 ± 0.01b	0.15 ± 0.02a	0.09 ± 0.01b	0.17 ± 0.03a	0.09 ± 0.01b
Hexadecanoic acid (C16:0)	27.07 ± 3.52a	33.16 ± 3.65a	20.21 ± 1.37b	25.09 ± 2.61a	20.19 ± 2.73a	25.69 ± 3.92a	22.49 ± 4.15b	27.98 ± 4.90a
Palmitoleic acid (C16:1n7)	0.12 ± 0.01a	0.14 ± 0.02a	0.10 ± 0.02a	0.12 ± 0.03a	0.11 ± 0.01a	0.13 ± 0.01a	0.11 ± 0.01b	0.13 ± 0.02a
Heptadecanoic acid (C17:0)	0.17 ± 0.03b	0.30 ± 0.03a	0.13 ± 0.03b	0.24 ± 0.03a	0.15 ± 0.02b	0.24 ± 0.04a	0.15 ± 0.03b	0.26 ± 0.04a
Octadecanoic acid (C18:0)	7.70 ± 1.20b	10.45 ± 0.77a	5.94 ± 0.99b	8.62 ± 0.99a	6.58 ± 1.00a	8.35 ± 1.34a	6.74 ± 1.20b	9.14 ± 1.35a
Oleic acid (C18:1n9c) + elaidic acid (C18:1n9t)	45.33 ± 4.64a	51.29 ± 9.34a	40.67 ± 6.44a	44.72 ± 3.13a	42.91 ± 7.60a	49.31 ± 6.15a	42.97 ± 5.85a	48.44 ± 6.50a
Linoleic acid (C18:2n6c) + linoelaidic acid (C18:2n6t)	107.85 ± 10.99a	86.59 ± 5.81b	91.31 ± 10.11a	69.30 ± 10.52a	100.06 ± 12.95a	75.20 ± 9.94a	99.74 ± 12.21a	77.03 ± 10.90b
α-Linolenic acid (C18:3n3)	14.11 ± 2.33a	7.82 ± 1.35b	11.83 ± 1.17a	6.30 ± 0.83b	11.80 ± 1.24a	6.19 ± 0.52b	12.58 ± 1.84a	6.77 ± 1.15b
Eicosanoic acid (C20:0)	0.78 ± 0.12b	1.03 ± 0.08a	0.59 ± 0.10b	0.82 ± 0.09a	0.64 ± 0.13a	0.79 ± 0.09a	0.67 ± 0.13b	0.88 ± 0.13a
Cetoleic acid (C20:1n9)	0.46 ± 0.03	< LLD	0.35 ± 0.05	< LLD	0.33 ± 0.02	< LLD	0.38 ± 0.07	< LLD
Eicosadienoic acid (C20:2n6)	0.07 ± 0.01a	0.08 ± 0.01a	0.06 ± 0.01a	0.06 ± 0.01a	0.05 ± 0.01a	0.07 ± 0.01a	0.06 ± 0.01a	0.07 ± 0.01a
Heneicosanoic acid (C21:0)	0.04 ± 0.01a	0.06 ± 0.01a	0.04 ± 0.01a	0.04 ± 0.01a	0.04 ± 0.01a	0.05 ± 0.01a	0.04 ± 0.01a	0.05 ± 0.01a
Eicosapentaenoic acid (C20:5n3	< LLD	0.45 ± 0.04	< LLD	0.35 ± 0.07	< LLD	0.31 ± 0.02	< LLD	0.37 ± 0.07
Docosanoic acid (C22:0)	0.81 ± 0.11b	1.39 ± 0.11a	0.69 ± 0.13b	1.09 ± 0.16a	0.72 ± 0.11b	1.12 ± 0.12a	0.74 ± 0.12b	1.20 ± 0.18a
Erucic acid (C22:1n9)	0.05 ± 0.01b	1.79 ± 0.13a	0.04 ± 0.01b	1.39 ± 0.21a	0.03 ± 0.01b	1.26 ± 0.17a	0.04 ± 0.01b	1.48 ± 0.28a
Tricosanoic acid (C23:0)	0.04 ± 0.01b	0.10 ± 0.01a	0.04 ± 0.01b	0.08 ± 0.01a	0.04 ± 0.01b	0.09 ± 0.02a	0.04 ± 0.01b	0.09 ± 0.02a
Tetracosanoic acid (C24:0)	0.24 ± 0.03	< LLD	0.19 ± 0.03	< LLD	0.20 ± 0.01	< LLD	0.21 ± 0.03	< LLD

< LLD – below limit of detection; Values not sharing a common letter indicate significant difference at $p \leq 0.05$

seeds from the infusion-treated crops. The control soybean samples were the only ones found to contain tetracosanoic acid (0.24 mg g^{-1}, 0.19 mg g^{-1} and 0.20 mg g^{-1}). Using linseed extracts in soybean cultivation also led to a pronounced decrease in linoleic acid, and linolelaidic acid contents. The amount of α-linolenic acid in soybeans was reduced upon the application of linseed infusions against the control group. The results obtained for cis-13,16-docosadienoic acid were particularly interesting. None was detected in the control and infusion-treated seeds. The results also showed that the seed fat content was modulated by the use of linseed-derived biostimulant (Fig. 7).

The economic efficiency of using this biostimulant product

The application of flaxseed extract increased soybean yield. The results of the 3-year experiment showed that in each year a higher income was obtained after the application of the extract compared to the control in which the preparation was not applied. This was due to the fact that the costs of production and use of the biostimulant were lower than the income resulting from the increase in soybean yield.

The highest profit was recorded in the last year of the study and amounted to 300.20 EUR ha^{-1}. The average income during the study period was 172.15 EUR ha^{-1} (Fig. 8).

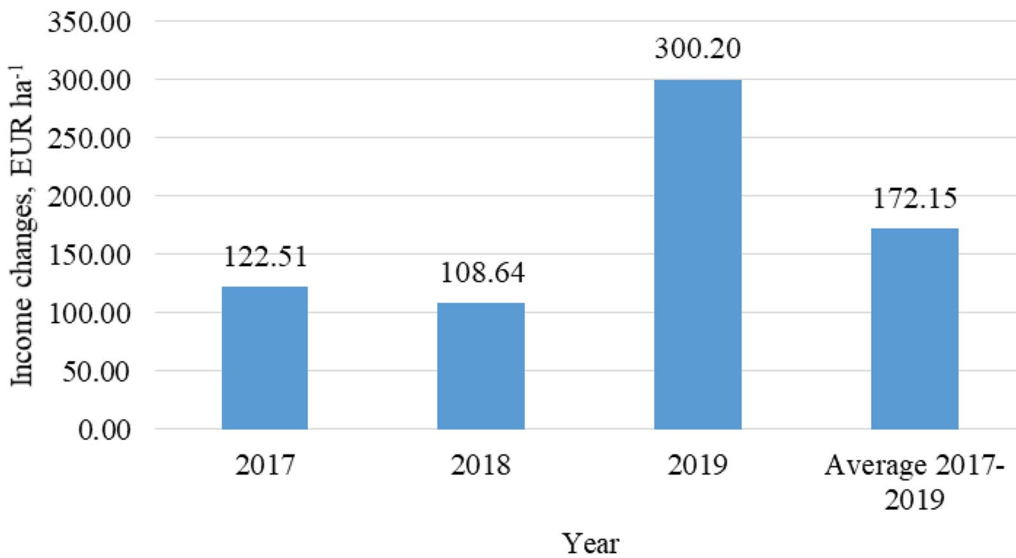


Fig. 8 The added returns incurred by the biostimulant applications

Discussion

Currently, there is a growing interest in studying the potential of plant extracts in agriculture [6, 8]. Many research results indicate that plant extracts can act as plant resistance inducers [16] and biostimulants [17, 28, 83]. However, learning more about the potential of plant extracts requires large-scale research to prove that they can be used as effective biostimulants [15].

The tested infusions were rich in macro- and micro-elements, as well as sugars, amino acids, and fatty acids. This composition suggested that they would induce a positive effect in the soybean plants on the agronomical, physiological, biochemical and qualitative responses.

The high content of amino acids in flaxseed infusions indicates a possible biostimulatory effect, since this group of compounds is most commonly used in biostimulation of crop plants [70, 78]. Amino acids are considered to be a precursor of polyamines [79] as well as chlorophyll synthesis in developing leaves [29].

Carbohydrates, considered key molecules for plants, have also been identified in the chemical composition of infusions [48]. They are believed to have a number of functions in plants, including structural, signaling, transport, and storage, among others. The biostimulatory effects of carbohydrates may be due to their high polymerization potential, effects on osmotic balance, and stabilization of membranes and proteins [34, 48]. Flax extracts contained high concentrations of soluble carbohydrates (glucose and fructose), which may indicate a biostimulatory effect, due to the fact that they play an extremely important role in plant metabolism and growth [66].

Evaluation of the chemical composition of flaxseed water extract also revealed an abundance of mineral compounds. Micronutrients and macronutrients are used in many plant physiological processes [55]. The uptake of these compounds by plants depends on their availability after biostimulant treatments [10, 62]. However, according to the definition of biostimulants, the effects of these products cannot be attributed to the concentrations of micro- and macro-elements, as they are not fertilizers. However, their presence can have a kind of synergistic effect together with other components of plant extracts, intensifying primary or secondary metabolism in crop plants [10, 42]. In addition, the presence of beneficial elements for plants, found in the extracts, indicates their possible effect on photosynthetic traits of plants under both optimal and stressful conditions [49].

The botanical extracts were also assessed for the fatty acids content, which play important roles in plant defense, including pathways associated with phytohormones and a number of mediator signaling pathways. Several studies even indicate that plants can efficiently use linoleic acid for jasmonic acid biosynthesis. This process is associated with improved plant growth and defense mechanisms [58, 59, 61]. The fatty acids present in the composition of botanical infusions may exhibit biostimulatory effects due to the fact that they act as activators of many plant defense mechanisms through activation of the enzymatic apparatus [27, 68].

Application of water extracts of common flaxseed was a determinant of yield and structural elements of soybean yield. In our study, there was an increase in the number of pods per m^2, which resulted in a higher yield with a slightly reduced level of 1000 seed weight. The application of infusion helped the soybean plants to form and fill their pods (positive effect on yield) [21]. This agronomic practice resulted in an increase not only in plant height, but also in the height of setting of the first pod. These morphological parameters are important not only for the mechanical harvesting of soybeans (large distance from the soil surface), but also affect the yield potential of the plants [60, 71]. The stimulating effect of the infusions may have been due to improved plant growth development and increased biomass accumulation [73].

The results of our study are consistent with those presented in the literature indicating that plant extracts can modify the multielemental profile of root vegetables. Extracts from plants such as the valerian, common nettle, tall goldenrod, and milk gowan have been shown to promote accumulation of elements in the edible parts of vegetables [2]. Rouphael and Colla [63] state that the nutritional and mineral status of crops treated with biostimulants (including plant extracts) suggests that this mechanism can originate from an increased "nutrient acquisition response".

Our study also showed that the fatty acid content, as well as total fat, fluctuated between soybean crops treated with the flaxseed extract. Unfortunately, the lack of previous studies by other authors left little opportunity to compare results. The accumulation of unsaturated fatty acids is inherently linked to the membrane fluidity and plant adaptation to environmental stress. Thus, the elevated concentrations of these acids detected in the soybean may have been caused by the linseed extract, which increased soybean tolerance to abiotic stress factors. In a study by Puglisi et al. [51], the application of biostimulants based on humic-like substances was shown to increase fatty acids levels in microalgae. However, the researchers were unable to identify any potential modes of action underlying this response. Liang et al. [41] state that amino acids present in biostimulants may also modify levels of different fatty acids in crop yields, since certain amino acids synthesized in plants contain a branched aliphatic chain as part of their structure. According to literature reports, 18-carbon acids are the predominant form of fatty acids in most plants [3, 33]. Our study showed that application of botanical biostimulants increased 18-carbon acid levels in soybean seeds. This is of particular importance in the context of these acids are precursors of a large pool of biologically active compounds.

Our study of biostimulant use in soybean cultivation concluded with an economic analysis of the income generated from the production—a deciding factor for farmers choosing a product. The study demonstrated that of linseed extract was effective in improving yields, which translated directly to increased returns in actual field conditions. This is corroborated by our previous study on how the treatment with *Artemisia absinthium* derived biostimulants impacted income from soy cultivation. The analysis of the economic returns, yields, and costs of treatment in a 3-year field trial showed that the foliar administration of *Artemisia absinthium* infusions and macerates led to the highest increases in soybean yields, which in turn ensured stable profits for the producers [76]. These gains are, admittedly, not spectacular when compared with the economic performance of commercial biostimulants applied to bean crops. Treatment of bean crops with Kelpak SL increased the average income by more than EUR 600 per hectare, whereas the same parameter amounted to over EUR 300 for Terra Sorb [38]. While it is true that this analysis concerned a different crop, it still must be stated that further work will be needed to improve the performance of the novel biostimulants. The solution may lie in changing the method of administration or extraction. Nevertheless, the established economic benefits are sufficient to qualify the extract examined herein as biostimulating products.

The results proved that the biological activity of flaxseed infusions is sufficient for it to be classified as ecological biostimulants. The prototyped extract complied with the requirements set out in the definition of these bioproducts (Regulation (EU) 2019/1009).

Summary and conclusions

Based on the study, it was concluded that solid–liquid extraction could be considered as a green alternative for prototyping a novel and ecological biostimulant. This study demonstrated also the potential of flaxseed extract, extracted by aqueous hot extraction, as a biostimulant in soybean cultivation due to its ability to improve plant growth, yield, and nutritional status. This therefore indicates that biostimulants can be obtained from resources readily available in nature such as medicinal, allelopathic or herbal plants. Thus, our study will make an important contribution to the search for new sustainable solutions for agriculture.

Based on the chemical characterization of common flaxseed infusions, there was a significant presence of biologically active compounds including amino acids and fatty acids, mineral compounds that could support the beneficial effects found in the cultivated soybean. The soybean crops responded positively to foliar application of the extract, showing enhancement on agronomic, morphologic, and biochemical levels. The growth and yields of soybeans improved after the tested infusion was administered. The results confirmed that the tested biostimulant is not only an environmentally friendly product, but also capable of increasing the growth and productivity of soybeans, thus intensification farmers' profitability. Application of the flaxseed extracts induced primary and secondary metabolism, while also promoting the accumulation of fatty acids and amino acids in the seeds. Our analysis of soybean parameters showed, in many cases, variable but coordinated biochemical responses, indicating that the botanical extracts could be considered effective biostimulants. However, in order to consistently harness these benefits of plant biostimulants, the plants must be sprayed during specific phases of phenological development so as not to disturb the allelopathic interactions between the plants. Nevertheless, hypotheses based on the current state of knowledge have been presented in an attempt to explain the observed changes at multiple levels, including the agronomic, physiological, and biochemical ones. However, in order to fully understand the biostimulatory potential of flaxseed extracts, further research is necessary, also in the aspect of possible mitigation of negative effects of biotic and abiotic stresses on crop plants.

Supplementary Information

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Additional file 1. Figure S1. Rainfall in the months of field research realization in 2017–2019 and multi-year average.

Additional file 2. Figure S2. Temperature in the months of field research realization in 2017–2019 and multi-year average.

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Conceptualization, AS and SK; methodology, AS, SK, IK, and GZ; formal analysis, AS and SK; investigation, SK, IK, and GZ; writing—original draft, AS and SK; writing—review and editing, SK and AS; visualization, SK; resources, SK, IK and GZ.

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Declarations

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