Research Article

Received: 5 March 2019 Revised: 15 May 2019 Accepted article published: 21 June 2019 Published online in Wiley Online Library: 24 July 2019

(wileyonlinelibrary.com) DOI 10.1002/jsfa.9881

# Bioactive properties of greenhouse-cultivated green beans (*Phaseolus vulgaris* L.) under biostimulants and water-stress effect

**Spyridon A Petropoulos,[a*] Oludemi Taofiq,[b] Ângela Fernandes,[b] Nikos Tzortzakis,[c] Ana Ciric,[d] Marina Sokovic,[d] Lillian Barros[b] and Isabel CFR Ferreira[b*]**

## Abstract

**BACKGROUND: The scarcity of irrigation water is severely affecting global crop production. In this context, biostimulants are increasingly used as alternatives means against abiotic stress conditions. In this study, phenolic compounds composition and bioactive properties of common bean (*Phaseolus vulgaris* L.) plants grown under water stress conditions and biostimulants application were investigated.**

**RESULTS: Sixteen individual phenolic compounds were detected in both pods and seeds with a notable difference in their compositional profile. A significant effect on phenolic compounds content and composition was also observed for the biostimulants tested. Regarding the antibacterial activity, pods of the second harvest and seed extracts showed significant efficacy against *Bacillus cereus*, especially in water-stressed plants, where all biostimulant treatments were more effective than positive controls. Moreover, all biostimulant treatments for seed extracts of water-stressed plants were more effective against *Staphylococcus aureus* compared with ampicillin, whereas streptomycin showed the best results. Extracts from pods of the second harvest from normally irrigated plants showed the best results against the fungi tested, except for *Penicillium verrucosum* var. *cyclopium*. Finally, no significant cytotoxic effects were detected.**

**CONCLUSION: In conclusion, the biostimulants tested increased total phenolic compounds content compared with control treatment, especially in pods of the first harvest and seeds of water-stressed plants. Moreover, bioactive properties showed a varied response in regard to irrigation and biostimulant treatment. Therefore, biostimulants can be considered as a useful means towards increasing phenolic compounds content, and they may also affect the antimicrobial properties of pods and seeds extracts.**


**Keywords:** antimicrobial properties; biostimulants; common bean; cytotoxicity; phenolic composition; water stress

## INTRODUCTION

The scarcity of irrigation water and the gradual degradation of soil and water quality are severely affecting global crop production, especially in the arid and semi-arid parts of the world.[1] Moreover, stress conditions are a key factor for horticultural crops yield, and proper management of abiotic stress factors has a pivotal role in ensuring crop sustainability and in improving quality of the final product.[2,3]

The use of biostimulants has been suggested as an effective means against the severe effects of stress factors on crops yield, and several studies also report significant effects on the chemical composition of the final product.[4] However, biostimulants do not secure an *a priori* beneficial effect on plant growth, since in several cases a negative impact has been observed, indicating that integrative studies are needed in order to define the use of such products in agriculture.[5] Moreover, the varied response of crops to different biostimulant products of similar composition complicates the situation further, since the impact of biostimulants could be dependent on the number and dose of applications, the growth stage during application, and the growing conditions, among other factors.[6] For example, the response of tomato crop to various commercially available *Ascophyllum nodosum* extracts

* *Correspondence to: SA Petropoulos, University of Thessaly, School of Agricultural Sciences, Fytokou Street, 38446, N. Ionia, Magnissia, Greece. E-mail: spetropoulos@uth.gr; or ICFR Ferreira, Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal. E-mail: iferreira@ipb.pt*

a *Department of Agriculture, University of Thessaly, Crop Production and Rural Environment, Magnissia, Greece*

b *Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Bragança, Portugal*

c *Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, Lemesos, Cyprus*

d *Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Belgrade, Serbia*

under drought stress conditions varied significantly, highlighting the importance of meticulous investigations in terms of specific crop biostimulant product combinations.[7]

The term biostimulant is defined as a growth-promoting compound, meaning that several classes of compounds could be suggested for such use, with different mechanisms of action being implemented in each case.[8] Although the use of biostimulants was first applied in organic farming with compounds of natural origin, synthetic compounds with growth promoting properties can, nowadays, also be referred to as biostimulants.[9] The main benefits of biostimulants applied directly in the soil, such as arbuscular mycorrhizal fungi, plant growth-promoting rhizobacteria and other nonpathogenic fungi, are associated with improved soil water-holding capacity and root conformation, increased root growth accompanied by more effective nutrient and water uptake, and, most importantly, with increased yield.[10] Other products, such as seaweed extracts, protein hydrolysates, and plant extracts, stimulate plant growth mostly through their bioactive ingredients, although soil improvement properties have also been reported in terms of physicochemical properties of soil matrix.[10,11]

The past decade has evidenced an increased use of biostimulants in the farming sector in general and in intensive production systems, such as horticultural cropping systems, in particular.[12,13] Apart from the evident goal for an increased yield through the use of biostimulants, their effects on product quality are equally important, and several reports have highlighted the improvement in quality of various horticultural crops, such as spinach [14], strawberry,[15] and tomato.[10] In the study of Colla *et al.*,[10] the effect of biostimulants on tomato fruit quality was focused mainly on lycopene, total soluble solids, and minerals contents, with no significant effects being observed on total phenols and ascorbic acid content. In contrast, Paradiković *et al.*[16] reported a beneficial effect of biostimulants on total phenols and vitamin C content in sweet yellow pepper (*Capsicum annuum* L.) fruit, and the application of mineral–organic fertilizers increased flavonoids content in endive (*Cichorium endivia* L.) leaves.[17]

Extracts from natural products exert various biological activities, including antimicrobial and cytotoxic effects, usually associated with the presence of bioactive phytochemicals.[18,19] Legumes are considered a good source of bioactive compounds, such as polyphenols, saponins, protein hydrolysates, and peptides, that have been associated with several beneficial health effects.[20,21] In addition, several studies have reported the presence of compounds that exhibit antifungal and antibacterial activities, with special interest in common bean seeds, including prenylated flavonoids and peptides.[18,20,22]

Considering the increased interest in sustainable means for the management of horticultural crops, the aim of the present study was to investigate the effect of various biostimulant products and water-stress conditions on the quality of greenhouse-cultivated common beans (*Phaseolus vulgaris* L.), focusing on the bioactive properties of green pods and seeds. For this purpose, four biostimulants were tested under normal irrigation and water-stress conditions, and the chemical profile of green pods and seeds was evaluated in terms of phenolic compounds content and their antimicrobial and cytotoxic properties.

## MATERIALS AND METHODS

### Plant material and growing conditions

Experiments were carried out in an unheated plastic greenhouse at the experimental farm of the University of Thessaly, Greece, during the growing period of summer–autumn 2017. Sowing took place on 11 August 2017 and common bean seeds (cv. Zargana Chrysoupolis) were sown directly in soil. A double-row sowing system was applied with a spacing of 50 cm between the rows and a plant density of 20 000 $ha^{-1}$, with each treatment consisting of six plants and replicated three times (180 plants in total). The soil at 0–30 cm depth was clay (26% sand, 32% silt, and 42% clay); pH was 8.0 (1 : 1 soil : water); organic matter content was 3.1%; calcium carbonate was 10.8%; available phosphorus (Olsen method) was 70.9 mg $kg^{-1}$; total nitrogen (Kjeldahl method) was 1.8 g $kg^{-1}$; exchangeable potassium oxide (ammonium acetate method) was 195 mg $kg^{-1}$; electrical conductivity was 0.95 dS $m^{-1}$. Two factors were applied in a split-plot factorial design, namely water stress and biostimulants. Biostimulants treatments included: (i) control (C: no biostimulants added), (ii) Nomoren (G; Anthis S.A., Greece), (iii) Twin-Antistress (TW; Microspore Hellas, Sacom Hellas, Greece), (iv) Veramin Ca (B; Microspore Hellas, Sacom Hellas, Greece), and (v) EKOprop (EK; Anthesis S.A., Greece). The detailed composition of each product was as follows. Nomoren contained 20% of arbuscular mycorrhizal fungi (*Glomus* spp.). Twin-Antistress contained natural microorganisms based on *Bacillus subtilis*, and yeast and *Ascophyllum nodosum* extracts, as well as 1% nitrogen (organic), 10% organic carbon, and 30% organic matter (<50 kDa). Veramin Ca contained an amino acid complex of vegetable origin with *Aloe vera* extract and 15.6% calcium oxide. EKOprop contained a mixture of arbuscular mycorrhizal fungi (1% *Glomus* spp.), rhizosphere symbiotic bacteria (*Bacillus* spp., *Streptomyces* spp., *Pseudomonas* spp., $1{,}6 \times 10^9$ CFU $g^{-1}$ in total), and saprophytic fungi (*Trichoderma* spp., $5 \times 10^5$ CFU $g^{-1}$).

Water-stress treatments included normally irrigated plants (W+), with irrigation taking place twice a week, and water-stressed plants (W−), where irrigation was applied once a week. In particular, irrigation treatments were scheduled with the use of tensiometers (Irrometer-Moisture Indicator; Irrometer, Riverside, CA, USA) and included: (i) normally irrigated plants (W+), where irrigation was applied approximately twice a week and when tensiometer readings were between 10 and15%; (ii) water-stressed plants (W−), where water holding was applied with irrigation being implemented approximately once a week and when tensiometer readings were between 40 and 50%. Tensiometer readings correspond to soil moisture content ranging from field capacity (0%) to dry soil (100%). Irrigation was applied through a drip irrigation system with one dripper per plant (4.0 L $h^{-1}$ of water per dripper) accounting for 350 $m^3$ $ha^{-1}$ (17.5 L of water per plant) for normally irrigated plants and 210 $m^3$ $ha^{-1}$ (10.5 L of water per plant) for water-stressed plants. Biostimulants were applied with irrigation water and according to the directions for use of each product at 10, 20, and 30 days after sowing (DAS) as follows: G treatment was applied at 5 L $ha^{-1}$ for each dose; TW was applied 5 L $ha^{-1}$ for each dose; B was applied at 500 g per 100 $L^{-1}$ water for each dose; and EK was applied at 1 kg $ha^{-1}$ for each dose. Water stress started after the second application of biostimulants (21 DAS). Harvest of pods took place at marketable maturity at 60 and 70 DAS, and seeds were collected from fully mature green pods at 103 DAS and after separating from the pericarps. Batch samples of pods and seeds were lyophilized, ground with a mortar and pestle, and stored at deep-freezing conditions (−80 °C) until further analyses.

### Extract preparation

Extraction was conducted using a magnetic stirrer plate (25 °C at 150 rpm). Briefly, 1 g of lyophilized pods and seeds materials were extracted twice during 1 h, using 30 mL of methanol–water



  

(80 : 20, v/v). The suspension was filtered through a Whatman No. 4 paper and the resulting filtrate vacuum-dried in a rotary evaporator (R-210; Büchi, Flawil, Switzerland) at 40 °C to remove methanol. The extracts obtained were further frozen and lyophilized for further analysis.

### Characterization of phenolic compounds by high-performance liquid chromatography with diode array detector–electrospray ionization mass spectrometry

The 20 mg of the extracts were redissolved in 2 mL of methanol–water (80 : 20, v/v) and filtered using nylon-membrane syringe filters (0.22 µm and 25 mm, Whatman®, Maidstone, UK) into an amber vial. Phenolic compounds were analyzed using a Dionex Ultimate 3000 (Thermo Scientific, San Jose, CA, USA) ultra-performance liquid chromatography system equipped with a diode array detector (280, 330, and 370 nm as preferred wavelengths) coupled to an electrospray ionization mass spectrometry detector. Chromatographic separation was achieved with a Spherisorb S3 ODS-2 C18 (3 µm, 4.6 mm × 150 mm; Waters, Milford, MA, USA) column maintained at 35 °C using a thermostat.[23] The binary mobile phase contained both 0.1% formic acid in water (A) and acetonitrile (B). Mass spectrometry (MS) detection was performed in negative mode, using an LTQ XL linear ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization source. Data acquisition and processing was conducted using an Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA). The individual compounds were identified by comparing their retention times, UV–visible spectra and MS fragmentation pattern with those obtained from the available commercial standards, and also with data available from already reported studies. The phenolic compounds quantification was based on calibration curves obtained from available standards and the results were expressed as micrograms per gram of extract.

### Evaluation of antimicrobial properties

*Microbial strains*

The bacterial strains used were as follows: the Gram-positive bacteria *Bacillus cereus* (food isolate), *Staphylococcus aureus* (ATCC 6538), and *Listeria monocytogenes* (NCTC 7973); and the Gram-negative bacteria *Escherichia coli* (ATCC 35210), *Enterobacter cloacae* (human isolate), and *Salmonella typhimurium* (ATCC 13311).

The fungal strains used were as follows: *Aspergillus fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus niger* (ATCC 6275), *Penicillium ochrochloron* (ATCC 9112), *Penicillium funiculosum* (ATCC 36839), *Penicillium verrucosum* var. *cyclopium* (food isolate).

*Microbial inhibition assay*

The bacterial and fungal strains used in the present work were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research 'Sinisa Stanković', University of Belgrade, Serbia. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) were determined by the microdilution method as previously described by Soković *et al.*[24] The lowest concentration without visible microbial growth is define as the MIC, the lowest concentration indicating 99.5% death of the bacteria strain is the MBC, and the lowest concentration indicating 99.5% death of the fungal strain is the MFC. Positive controls were streptomycin and ampicillin for bacterial strains and ketoconazole and bifonazole for fungal strains.

### Hepatotoxicity

The hepatotoxic activity of the extracts obtained from pods and seeds for the different treatments were evaluated by the sulforhodamine B using a primary cell culture (PLP2) prepared from a porcine liver.[25] Different concentrations of the extracts up to 400 µg mL$^{-1}$ were used and the results expressed as $GI_{50}$ values, which represent extract concentration responsible for 50% inhibition of PLP2 cell growth. Ellipticine was used as positive control.

### Statistical analysis

The experimental design was laid out in a split-plot arrangement, with each main plot consisting of water stress treatments (W+ or W–), and fully randomized subplots comprised the biostimulants treatments. Each treatment was replicated three times ($n = 3$). In order to constitute a representative and adequate sample of the treatments tested, batches of several samples of pods and seeds were taken at random from each plot, in order to obtain three different samples. These batches were then powdered to obtain homogeneous samples. For each methodology, three extractions were carried out and the analyses were performed in triplicate. Statistical analysis was conducted with the aid of Statgraphics 5.1.plus (StatPoint Technologies Inc., Warrenton, VA, USA). Data were evaluated by a two-way analysis of variance for the main effects, whereas the means of values were compared by Tukey's honestly significant difference test ($P = 0.05$).

## RESULTS AND DISCUSSION

### Comparative analysis of phenolic content in each seed and pods treatment

Bean seeds are considered a good source of bioactive compounds, such as polyphenols, protein hydrolysates, and peptides, which have been associated with several beneficial health effects.[26] In our study, phenolic compounds composition of common bean in relation to biostimulants application and water-stress conditions was investigated. The individual compounds detected in pods and seeds are listed in Table 1.

Sixteen individual phenolic compounds were detected in both pods and seeds, with two of them being classified as phenolic acids and 14 compounds as flavonoids. Peak characteristics, tentative identities, and quantification are presented in Tables 1 and 2. The main phenolic compounds found were flavonols (mainly quercetin, kaempferol glycosides), flavan-3-ols (catechin and derivatives), and flavanonol (taxifolin glycoside) derivatives, and phenolic acids (hydroxycinnamic acid derivative).

Quercetin-3-*O*-rutinoside (peak 4), kaempferol-3-*O*-rutinoside (peak 7), (+)-catechin (peak 11), and kaempferol-3-*O*-glucoside (peak 15) were positively identified according to their retention, mass, and UV–vis characteristics by comparison with commercial standards. To the best of our knowledge, catechin seems to be one of the most common phenolic compounds found in the seeds of *P. vulgaris*[27] Peak 4 (quercetin-3-*O*-rutinoside) and peak 15 (kaempferol-3-*O*-glucoside) have been previously identified in 80% (v/v) aqueous acetone extracts obtained from *P. vulgaris* L.,[28] whereas peak 7 (kaempferol-3-*O*-rutinoside) was also found in the hydromethanolic extract prepared from pods of *P. vulgaris* obtained from Almeria, Spain.[29]

Peaks 1 ([M – H]$^-$ at *m/z* 741) and 3 ([M – H]$^-$ at *m/z* 725) were identified as quercetin ($\lambda_{max}$ around 350 nm and an $MS^2$ fragment at *m/z* 301) and kaempferol ($\lambda_{max}$ around 348 nm, $MS^2$ fragment at *m/z* 285) derivatives. Both compounds (peaks 1 and 3) presented



  

**Table 1.** Retention time (Rt) wavelengths of maximum absorption in the visible region ($\lambda_{max}$), mass spectral data, and tentative identification of phenolic compounds in common bean pods (first and second harvest) and seeds

| Peak | Rt (min) | $\lambda_{max}$ (nm) | $[M-H]^-$ (*m/z*) | $MS^2$ (*m/z*) | Tentative identification |
|---|---|---|---|---|---|
| **Pods, first harvest** | | | | | |
| 1 | 14.8 | 352 | 741 | 609 (100), 301 (82) | Quercetin-*O*-pentoside-*O*-rutinoside |
| 2 | 16.1 | 352 | 595 | 301 (100) | Quercetin-3-*O*-xylosyl-glucoside |
| 3 | 17.4 | 342 | 725 | 593 (100), 285 (48) | Kaempferol-*O*-pentoside-*O*-rutinoside |
| 4 | 17.9 | 352 | 609 | 301 (100) | Quercetin-3-*O*-rutinoside |
| 5 | 18.2 | 350 | 477 | 301 (100) | Quercetin-*O*-glucuronide |
| 6 | 19.1 | 340 | 579 | 285 (100) | Kaempferol-3-*O*-xylosyl-glucoside |
| 7 | 21.2 | 340 | 593 | 285 (100) | Kaempferol-3-*O*-rutinoside |
| 8 | 22.1 | 340 | 461 | 285 (100) | Kaempferol-*O*-glucuronide |
| **Pods, second harvest** | | | | | |
| 4 | 17.9 | 352 | 609 | 301 (100) | Quercetin-3-*O*-rutinoside |
| 5 | 18.2 | 350 | 477 | 301 (100) | Quercetin-*O*-glucuronide |
| 7 | 21.2 | 340 | 593 | 285 (100) | Kaempferol-3-*O*-rutinoside |
| 8 | 22.1 | 340 | 461 | 285 (100) | Kaempferol-*O*-glucuronide |
| **Seeds** | | | | | |
| 9 | 5.9 | 325 | 367 | 193 (10), 191 (100), 173 (5), 147 (5), 129 (5) | *cis*-3-*O*-Feruloylquinic acid |
| 10 | 6.2 | 323 | 367 | 193 (10), 191 (100), 173 (5), 147 (5), 129 (5) | *trans*-3-*O*-Feruloylquinic acid |
| 11 | 7.1 | 280 | 289 | 245 (100), 205 (37), 179 (21), 125 (5) | (+)-Catechin |
| 12 | 9.2 | 286, 320 | 449 | 287 (100) | Eriodictyol-*O*-hexoside |
| 13 | 12.5 | 281 | 577 | 451 (32), 425 (100), 407 (27), 289 (11), 287 (22) | B-type (epi)catechin dimer |
| 6 | 19.2 | 343 | 579 | 285 (100) | Kaempferol-3-*O*-xylosyl-glucoside |
| 14 | 20.4 | 350 | 505 | 301 (100) | Quercetin-*O*-acetylhexoside |
| 15 | 22.6 | 343 | 447 | 285 (100) | Kaempferol-3-*O*-glucoside |
| 16 | 24.8 | 343 | 533 | 489 (100), 447 (27), 285 (5) | Kaempferol-*O*-malonylhexoside |

two $MS^2$ fragments, revealing the alternative losses of a hexosyl (*m/z* at 609 and 593; −162 u) and deoxyhexosyl-hexoside (*m/z* at 301 and 285; −308 u) residues, indicating the location of each residue on different positions of the aglycone. Moreover, the sugar moieties and location onto the aglycone could be obtained; thus, the positive identification of different rutinosides, including quercetin and kaempferol 3-*O*-rutinoside, may suggest a rutinose identity for the deoxyhexosyl-hexose. Therefore, these peaks were tentatively identified as quercetin-*O*-pentoside-*O*-rutinoside and kaempferol-*O*-pentoside-*O*-rutinoside respectively. Similar assumptions were performed to identify peaks 2 ($[M-H]^-$ at *m/z* 595) and 6 ($[M-H]^-$ at *m/z* 579), thus only one $MS^2$ fragment was identified, suggesting that the two sugars were linked together, being tentatively assigned as quercetin-*O*-pentosyl-hexoside and kaempferol-*O*-pentosyl-hexoside respectively, Pitura and Arntfield[28] presented a similar compound to peak 6, being assigned as a kaempferol-3-*O*-xylosyl-glucoside; therefore, this identity was supposed for both these compounds. Furthermore, peaks 5 ($[M-H]^-$ at *m/z* 477) and 8 ($[M-H]^-$ at *m/z* 461) presented $MS^2$ fragments with the loss of a glucuronyl (−176 u), being identified as quercetin-*O*-glucuronide and kaempferol-*O*-glucuronide respectively. Peak 5 was also identified in the hydromethanolic extract prepared from pods of *P. vulgaris* L.[29] Berger *et al.*[30] also identified two flavonol glycosides, quercetin-*O*-glucuronide and kaempferol-*O*-glucuronide, in the ultrasonicated hydromethanolic extract from green beans obtained from Germany. Peak 14 ($[M-H]^-$ at *m/z* 505), releasing a fragment at *m/z* 301 [quercetin − H]$^-$ (−162 u − 42 u, loss of acetyl and hexosyl moieties), was tentatively identified as quercetin-*O*-acetylhexoside. Peak 16 ($[M-H]^-$ at *m/z* 533) presented a pseudomolecular ion with 86 u (malonyl moiety) higher then peak 16, thus being assigned to a kaempferol-*O*-malonylhexoside. Peak 12 was assigned to eriodictyol derivatives based on their UV spectra and pseudomolecular ion $[M-H]^-$ at *m/z* 449, releasing a fragment at *m/z* 287 [eriodictyol − H]$^-$ (−162 u, loss of a hexosyl moiety), being tentatively identified as eriodictyol-*O*-hexoside.

Flavan-3-ols were also detected, corresponding to peaks 11 and 13, which showed UV spectra with $\lambda_{max}$ 279–280 nm, characteristic of these compounds; thus, peak 13 presented a pseudomolecular ion ($[M-H]^-$ at *m/z* 577) corresponding to procyanidin dimers.

Finally, peaks 9 and 10 ($[M-H]^-$ at *m/z* 367) presented MS and UV–visible characteristics of hydroxycinnamic acid derivative, being associated to feruloylquinic acids. These compounds were identified taking into account the hierarchical keys previously developed by Clifford *et al.*,[31] thus being assigned to *cis* and *trans*-3-*O*-feruloylquinic acid respectively.

A notable difference in compositional profile of pods and seeds was observed, and differences were also detected in phenolic compounds profile of pods from different harvest dates. In particular, eight phenolic compounds were detected in pods of the first harvest, whereas only four of them were also detected in the second harvest (peaks 4, 5, 7, and 8). It is worth mentioning that all the compounds detected in pods were classified as flavonoids, whereas seeds contained nine phenolic



  

**Table 2.** Quantification of phenolic compounds (μg $g^{-1}$ extract dry weight) in common bean pods (first and second harvest) and seeds

| Compound | CW+* | BW+ | EKW+ | GW+ | TWW+ | CW− | BW− | EKW− | GW− | TWW− |
|---|---|---|---|---|---|---|---|---|---|---|
| **First harvest of pods** | | | | | | | | | | |
| Quercetin-*O*-pentoside-*O*-rutinoside[A] | nd | 543.8 ± 0.5a | 447.8 ± 0.2b | 234.60 ± 0.01c | nd | nd | 447.22 ± 0.05b | 441.0 ± 0.2b | nd | 469.2 ± 0.4a |
| Quercetin-3-*O*-xylosyl-glucoside[A] | nd | 481.5 ± 0.5a | nd | nd | nd | nd | 437.0 ± 0.4v | 440.1 ± 0.2b | nd | 451.3 ± 0.5a |
| Kaempferol-*O*-pentoside-*O*-rutinoside[A] | nd | 471.4 ± 0.3a | 445.9 ± 0.1c | 234.3 ± 0.1d | 455.9 ± 0.4b | nd | 436.3 ± 0.2c | 441.5 ± 0.1bc | 643.0 ± 0.6a | 449.0 ± 0.1b |
| Quercetin-3-*O*-rutinoside[A] | 479.47 ± 0.03a | 477 ± 2a | 463.8 ± 0.2b | 247.7 ± 0.4c | 477.51 ± 0.01a | 492 ± 1b | 438.6 ± 0.4d | 463.9 ± 0.9c | 659.1 ± 0.3a | 469 ± 1c |
| Quercetin-*O*-glucuronide[A] | 608 ± 2a | 574.0 ± 0.3b | 470.4 ± 0.6d | 253.4 ± 0.3e | 522.9 ± 0.3c | 511 ± 1c | 477.9 ± 0.4d | 524 ± 1b | 653.5 ± 0.5a | 510.1 ± 0.8c |
| Kaempferol-3-*O*-xylosyl-glucoside[A] | nd | 476 ± 2a | 448.7 ± 0.1c | 238.4 ± 0.3d | 460.8 ± 0.5b | nd | 455.3 ± 0.2b | 446.8 ± 0.9c | 645.4 ± 0.1a | 455.1 ± 0.3b |
| Kaempferol-3-*O*-rutinoside[A] | 455.4 ± 0.3b | 463.4 ± 0.1a | 458.4 ± 0.2ab | 242.2 ± 0.1c | 468.5 ± 0.1a | 464.0 ± 0.2b | 436.2 ± 0.2d | 455.4 ± 0.1c | 656.8 ± 0.5a | 461.1 ± 0.4b |
| Kaempferol-*O*-glucuronide[A] | 508.1 ± 0.1a | 507.9 ± 0.5a | 458 ± 2c | 244.46 ± 0.03d | 495.2 ± 0.2b | 469.4 ± 0.6c | 471.2 ± 0.2c | 488.5 ± 0.7b | 65b.0 ± 0.8a | 489.9 ± 0.2b |
| **Total phenolic compounds** | **2051 ± 2d*** | **3995.7 ± 1.4a*** | **3193 ± 2b*** | **1695 ± 1e*** | **2881 ± 1c*** | **1936 ± 1d*** | **3599.9 ± 0.4c*** | **3701 ± 2b*** | **3908 ± 2a*** | **3755 ± 1b*** |
| **Second harvest of pods** | | | | | | | | | | |
| Quercetin-3-*O*-rutinoside[A] | 466.4 ± 0.4d | 511.1 ± 0.3a | 477.6 ± 0.8c | 498 ± 1b | 498.0 ± 0.1b | 449 ± 2d | 447.88 ± 0.03d | 468.1 ± 0.8b | 476.2 ± 0.9a | 459.3 ± 0.3c |
| Quercetin-*O*-glucuronide[A] | 520.6 ± 0.5b | 614.6 ± 0.1a | 470.9 ± 0.4d | 494 ± 1c | 523.6 ± 0.4b | 452.2 ± 0.3c | 444.10 ± 0.07d | 467.8 ± 0.7b | 509.36 ± 0.01a | 452.8 ± 0.1c |
| Kaempferol-3-*O*-rutinoside[A] | 440.69 ± 0.04d | 459.4 ± 0.2c | 457.18 ± 0.03c | 472.62 ± 0.08a | 465.7 ± 0.3b | 433.4 ± 0.1c | 435.25 ± 0.01c | 449.16 ± 0.01b | 462.51 ± 0.01a | 451.4 ± 0.1b |
| Kaempferol-*O*-glucuronide[A] | 456.96 ± 0.03c | 482.4 ± 0.4a | 459.41 ± 0.01c | 473.0 ± 0.3b | 474.1 ± 0.3b | 438.8 ± 0.7c | 441.06 ± 0.03c | 451.5 ± 0.2b | 471.9 ± 0.2a | 449.1 ± 0.1b |
| **Total phenolic compounds** | **1884.6 ± 0.1c*** | **2068 ± 1a*** | **1865.1 ± 0.5c*** | **1936.7 ± 0.3b*** | **1961.4 ± 0.3b*** | **1774 ± 3c*** | **1768.29 ± 0.03c*** | **1836.6 ± 0.3b*** | **1920 ± 1a*** | **1812.5 ± 0.2b*** |
| **Seeds** | | | | | | | | | | |
| *cis*-3-*O*-Feruloylquinic acid[B] | 194 ± 4c | 54.7 ± 0.3d | 237.8 ± 0.2b | 238 ± 6b | 265 ± 3a | 57.9 ± 0.6e | 104.1 ± 0.2d | 246 ± 11a | 177 ± 7c | 227 ± 2b |
| *trans*-3-*O*-Feruloylquinic acid[B] | 62 ± 2d | 47.9 ± 0.3e | 70.9 ± 0.2c | 121 ± 6a | 110 ± 2b | 40.8 ± 0.7e | 144 ± 2b | 108 ± 5c | 175 ± 8a | 70 ± 4d |
| (+)-Catechin[C] | 697 ± 3b | 429 ± 18e | 467 ± 5d | 640 ± 14c | 1021 ± 12a | 171 ± 3e | 1185 ± 19a | 509.6 ± 0.8d | 768 ± 20b | 716 ± 2c |
| Eriodictyol-*O*-hexoside[D] | 221 ± 6a | 191 ± 10c | 213.2 ± 0.8b | 169 ± 1d | 215 ± 1b | 161 ± 4d | 188 ± 6b | 181.5 ± 0.8c | 226 ± 1a | 178 ± 1c |
| B-type (epi)catechin dimer[C] | 1425 ± 25a | 1085 ± 18b | 933 ± 28c | 841 ± 44d | 716 ± 1e | 858 ± 4d | 1471 ± 34b | 683 ± 6e | 1931 ± 23a | 1309 ± 14c |
| Kaempferol-3-*O*-xylosyl-glucoside[A] | nd | 463.9 ± 0.4b | nd | 476.5 ± 0.5a | 466.2 ± 0.1b | nd | nd | nd | nd | nd |
| Quercetin-*O*-acetylhexoside[A] | 464.1 ± 0.2a | 452.6 ± 0.2b | 442.9 ± 0.2c | 467.5 ± 0.3a | 466.9 ± 0.4a | 461.6 ± 0.1b | 467.5 ± 0.2a | 455.6 ± 0.3c | 456.4 ± 0.1c | 470.8 ± 0.1a |
| Kaempferol-3-*O*-glucoside[A] | nd | 469.6 ± 0.1a | nd | 469.4 ± 0.3a | nd | nd | nd | nd | nd | nd |
| Kaempferol-*O*-malonylhexoside[A] | nd | 468.9 ± 0.1b | nd | 480.4 ± 0.1a | nd | nd | nd | nd | nd | nd |
| **Total phenolic acids** | **256 ± 3d*** | **103 ± 1e*** | **308.7 ± 0.4c*** | **358.7 ± 0.2b** | **375 ± 2a*** | **99 ± 1d*** | **248 ± 2c*** | **354 ± 7a*** | **352 ± 2a** | **297 ± 2b*** |
| **Total flavonoids** | **2807 ± 22c*** | **3560 ± 46a*** | **2057 ± 34d*** | **3544 ± 59a*** | **2885 ± 10b*** | **1651 ± 6e*** | **3312 ± 47b*** | **1829 ± 6d*** | **3381 ± 44a*** | **2674 ± 15c*** |
| **Total phenolic compounds** | **3063 ± 24d*** | **3663 ± 45b*** | **2366 ± 34e*** | **3902 ± 58a*** | **3260 ± 12c*** | **1750 ± 4e*** | **3560 ± 49b*** | **2183.1 ± 0.4d*** | **3733 ± 46a*** | **2972 ± 16c*** |

nd: not detected. calibration curve used: A, quercetin-3-*O*-glucoside ($y = 34\,843x - 160\,173$; $R^2 = 0.999$; LOD = 0.21 μg $mL^{-1}$; LOQ = 0.71 μg $mL^{-1}$); B, ferulic acid ($y = 633\,126x - 185\,462$, $R^2 = 0.999$; LOD = 0.20 μg $mL^{-1}$; LOQ = 1.01 μg $mL^{-1}$); C, catechin ($y = 84\,950x - 23\,200$, $R^2 = 0.999$; 0.40 μg $mL^{-1}$; LOQ = 1.33 μg $mL^{-1}$); D, taxifolin ($y = 203\,766x - 208\,383$, $R^2 = 0.999$; LOD = 0.60 μg $mL^{-1}$; LOQ = 2.02 μg $mL^{-1}$). Means in the same row and the same water treatment followed by different Latin letters are significantly different according to Tukey's honestly significant difference (HSD) test ($P = 0.05$); The asterisk symbol (*) indicates significant differences between means of the same row and the same biostimulants treatment according to Tukey's HSD test ($P = 0.05$).
*W+: normally irrigated plants; W−: water-stressed plants; C: control treatment (no biostimulants added); B: Veramin Ca; EK: EKOprop; G: Nomoren; TW: Twin-Antistress.

compounds in total, including two phenolic acids (peaks 9 and 10) and seven flavonoids (peaks 6 and 11–16), none of which were detected in pods, with the exception of the compound of peak 6.

A significant effect on phenolic compounds content and composition was also observed for the biostimulants tested (Table 2). In the first harvest of pods, the highest content of total phenolic compounds was detected in the B and G treatments for normally irrigated and water-stressed plants respectively. Moreover, phenolic compounds content was higher in normally irrigated plants in the C and B treatments, whereas for the rest of the biostimulant treatments the water stress resulted in an increase of phenolic compounds content. Regarding the second harvest of pods, phenolic compounds content was higher in normally irrigated plants than water-stressed ones. Moreover, total phenolic compounds content was higher in the first harvest than in the second harvest, regardless of the biostimulants and water treatments, with differences ranging between 162 $\mu g\, g^{-1}$ extract for the C treatment of water-stressed plants and 1988 $\mu g\, g^{-1}$ extract for the G treatment of water-stressed plants, with the only exception being the application of the G treatment in normally irrigated plants, where total phenolic compounds content was lower by 241.7 $\mu g\, g^{-1}$ extract. Regarding seeds, the highest content of total phenolic compounds and of most of the individual compounds was observed in the G treatment, regardless of the irrigation regime. Flavonoids content was the highest in the B and G treatments for normally irrigated plants, as well as in the G treatment for water-stressed plants, whereas phenolic acids were most abundant in the TW (normally irrigated plants) and EK and G treatments (water-stressed plants). Concerning individual compounds, the most abundant compounds were B-type (epi)catechin dimer (values ranged between 683 $\mu g\, g^{-1}$ extract for EKW– treatment and 1931 $\mu g\, g^{-1}$ extract for GW– treatment) and (+)-catechin (values ranged between 171 $\mu g\, g^{-1}$ extract for CW– treatment and 1021 $\mu g\, g^{-1}$ extract for TWW– treatment) in both normally irrigated and water-stressed plants, although differences were observed among the biostimulant treatments tested. Moreover, biostimulant application resulted in an increase in the proportion of both compounds in relation to total phenolic compounds content under water stress conditions, especially for the G treatment, where the relative percentage of these compounds was almost doubled (increase from 37.9% to 72.3%) in water-stressed plants. According to Kumar *et al.*,[32] the bioactive compounds content of soybean seeds, and especially phenolic acids content, may decline with increasing maturity. Moreover, the use of *Ecklonia maxima* seaweed extract as biostimulant has been reported to reduce total phenols and ascorbic acid content in leaves of *Brassica rapa* L. subsp. *sylvestris* L. Janch. var. *esculenta* Hort. plants.[33] The same trend was observed in our study, where a decrease in total phenolic compounds content was observed between pods of different harvests for normally irrigated or water-stressed plants with no biostimulant use. In contrast, Kałużewicz *et al.*[34] observed an increase of total phenols, phenolic acids, quercetin, and kaempferol content in broccoli heads after the application of an amino-acid-based biostimulant and/or a combination of amino-acid-based biostimulant with *Ascophyllum nodosum* filtrates. However, this increasing trend was not consistent throughout the years, and the authors suggested a combined effect of growing conditions and biostimulants application, whereas the beneficial effect of seaweed filtrates on total phenols content was attributed to osmotic stress induced by the high content of *A. nodosum* filtrates in mannitol.[34] Similar results have been reported for total phenols and flavonoids contents of soybean seeds after the application of two synthetic biostimulants (Atonik and Tytanit), where, despite the different response being observed during a 3 year study, the application of biostimulants in a high single doses gave consistently the best results.[9] Polyphenols and flavonoids content of seeds from three soybean cultivars was also affected by natural biostimulants application such as Fylloton (a complex of amino acids and seaweed extracts) in a dose and cultivar-dependent manner.[35] The beneficial effect of biostimulants on phenolic compounds composition and content of snap bean pods was also reported in the case of garlic clove extracts and associated with its high content in phytohormones such as jasmonic and salicylic acids.[36] In our study, the prolonged effect of water stress (pods of the second harvest and seeds) resulted in a decrease of phenolic compounds content, whereas the G treatment showed the highest increase compared with the control, regardless of the irrigation treatment.

### Bioactive properties of seeds and pods extracts

Common bean seeds are appreciated as a valuable food source with noteworthy antimicrobial properties.[20,37,38] In our study, pods and seed extracts of common bean were tested against three Gram-negative bacteria (*E. coli*, *S. typhimurium*, and *E. cloacae*), and three Gram-positive bacteria (*S. aureus*, *B. cereus*, and *L. monocytogenes*) (Table 3). Pods (second harvest) and seeds extracts showed significant efficacy against *B. cereus* in most cases, especially for water-stressed plants, where all biostimulant treatments were more effective than positive control compounds (streptomycin and ampicillin). Moreover, all biostimulant treatments for seed extracts of water-stressed plants were more effective against *S. aureus* compared with ampicillin, whereas streptomycin showed the best results. The inhibitory and bactericidal effects of pods and seed extracts against *L. monocytogenes* and *E. cloacae* were less profound, and only selected treatments were more effective than streptomycin (e.g. EK treatment from pods of the first harvest of water-stressed plants and TW treatment from seeds of water-stressed plants), whereas in most cases the extracts tested showed better results against ampicillin. The same treatments were also more effective than streptomycin against *E. coli*, whereas pods and seed extracts from water-stressed plants were more effective than ampicillin. None of the extracts tested showed better efficacy than streptomycin against *S. typhimurium*, whereas almost all treatments had better efficacy than ampicillin. When comparing biostimulants with control treatment (no use of biostimulants) a varied response was observed depending on the bacteria tested and the irrigation regime. Isolated peptides from Chinese lima beans (*Phaseolus lunatus* L.) were found effective against various bacteria (e.g. *Bacillus megaterium*, *B. subtilis*, *Proteus vulgaris*, and *Mycobacterium phlei*), whereas defensins obtained from *Vigna unguiculata* seeds showed defensive properties against *E. coli*.[39] According to Godlewska *et al.*,[40] aqueous extracts obtained after boiling of Baltic seaweeds (*Polysiphonia*, *Ulva*, and *Cladophora*) showed significant *in vitro* inhibitory effects against *E. coli* but did not affect *S. aureus* cultures. Moreover, Gan *et al.*[41] suggested that pigmented seed coats of various legumes may be used as natural food preservatives and the high antibacterial efficacy was positively correlated with flavonoids and proanthocyanidins content. In our study, this trend was not confirmed, indicating that other compounds apart from polyphenols (e.g. bioactive peptides) are responsible for antibacterial activity of seed extracts.[20,42]



  

**Table 3.** Antibacterial activity of common bean pods (first and second harvest) and seeds extracts (MIC and MBC mg $mL^{-1}$)

| | | *B. cereus* | *S. aureus* | *L. monocytogenes* | *E. coli* | *E. cloacae* | *S. typhimurium* |
|---|---|---|---|---|---|---|---|
| **First harvest of pods** | | | | | | | |
| CW+* | MIC | 0.15 | 0.60 | 0.30 | 0.15 | 0.30 | 0.30 |
| | MBC | 0.20 | 0.80 | 0.40 | 0.20 | 0.40 | 0.40 |
| BW+ | MIC | 0.075 | 0.30 | 0.20 | 0.15 | 0.20 | 0.20 |
| | MBC | 0.10 | 0.40 | 0.40 | 0.30 | 0.40 | 0.40 |
| EKW+ | MIC | 0.05 | 0.30 | 0.30 | 0.30 | 0.30 | 0.60 |
| | MBC | 0.10 | 0.40 | 0.40 | 0.40 | 0.40 | 1.20 |
| GW+ | MIC | 0.05 | 0.20 | 0.20 | 0.15 | 0.20 | 0.20 |
| | MBC | 0.10 | 0.40 | 0.40 | 0.20 | 0.40 | 0.40 |
| TWW+ | MIC | 0.15 | 0.60 | 0.40 | 0.20 | 0.40 | 0.60 |
| | MBC | 0.20 | 0.80 | 0.80 | 0.40 | 0.80 | 0.80 |
| CW− | MIC | 0.20 | 0.60 | 0.30 | 0.20 | 0.30 | 0.30 |
| | MBC | 0.40 | 0.80 | 0.40 | 0.40 | 0.40 | 0.40 |
| BW− | MIC | 0.05 | 0.30 | 0.30 | 0.30 | 0.20 | 0.30 |
| | MBC | 0.10 | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 |
| EKW− | MIC | 0.10 | 0.30 | 0.15 | 0.15 | 0.10 | 0.20 |
| | MBC | 0.20 | 0.40 | 0.20 | 0.20 | 0.20 | 0.40 |
| GW− | MIC | 0.15 | 0.40 | 0.30 | 0.30 | 0.40 | 0.60 |
| | MBC | 0.20 | 0.80 | 0.40 | 0.40 | 0.80 | 0.80 |
| TWW− | MIC | 0.10 | 0.30 | 0.20 | 0.15 | 0.20 | 0.20 |
| | MBC | 0.20 | 0.40 | 0.40 | 0.20 | 0.40 | 0.40 |
| **Second harvest of pods** | | | | | | | |
| CW+ | MIC | 0.15 | 0.60 | 0.30 | 0.40 | 0.30 | 0.30 |
| | MBC | 0.20 | 0.80 | 0.40 | 0.80 | 0.40 | 0.40 |
| BW+ | MIC | 0.10 | 0.40 | 0.20 | 0.15 | 0.30 | 0.30 |
| | MBC | 0.20 | 0.80 | 0.40 | 0.20 | 0.40 | 0.40 |
| EKW+ | MIC | 0.05 | 0.30 | 0.20 | 0.30 | 0.20 | 0.20 |
| | MBC | 0.10 | 0.40 | 0.40 | 0.80 | 0.40 | 0.40 |
| GW+ | MIC | 0.10 | 0.30 | 0.20 | 0.40 | 0.20 | 0.30 |
| | MBC | 0.20 | 0.40 | 0.40 | 0.80 | 0.40 | 0.40 |
| TWW+ | MIC | 0.075 | 0.20 | 0.30 | 0.40 | 0.40 | 0.60 |
| | MBC | 0.10 | 0.80 | 0.40 | 0.80 | 0.80 | 0.80 |
| CW− | MIC | 0.15 | 0.60 | 0.40 | 0.20 | 0.40 | 0.40 |
| | MBC | 0.20 | 0.80 | 0.80 | 0.40 | 0.80 | 0.80 |
| BW− | MIC | 0.10 | 0.30 | 0.40 | 0.30 | 0.30 | 0.20 |
| | MBC | 0.20 | 0.40 | 0.80 | 0.40 | 0.40 | 0.40 |
| EKW− | MIC | 0.15 | 0.60 | 0.30 | 0.20 | 0.30 | 0.40 |
| | MBC | 0.20 | 0.80 | 0.40 | 0.40 | 0.40 | 0.80 |
| GW− | MIC | 0.075 | 0.60 | 0.40 | 0.20 | 0.40 | 0.40 |
| | MBC | 0.10 | 0.80 | 0.80 | 0.40 | 0.80 | 0.80 |
| TWW− | MIC | 0.10 | 0.40 | 0.40 | 0.20 | 0.30 | 0.30 |
| | MBC | 0.20 | 0.80 | 0.80 | 0.40 | 0.40 | 0.40 |
| **Seeds** | | | | | | | |
| CW+ | MIC | 0.05 | 0.40 | 0.30 | 0.20 | 0.30 | 0.30 |
| | MBC | 0.10 | 0.80 | 0.40 | 0.30 | 0.40 | 0.40 |
| BW+ | MIC | 0.025 | 0.30 | 0.20 | 0.10 | 0.20 | 0.20 |
| | MBC | 0.05 | 0.40 | 0.40 | 0.20 | 0.40 | 0.40 |
| EKW+ | MIC | 0.20 | 0.60 | 0.40 | 0.40 | 0.30 | 0.30 |
| | MBC | 0.40 | 0.80 | 0.60 | 0.80 | 0.40 | 0.40 |
| GW+ | MIC | 0.025 | 0.20 | 0.20 | 0.15 | 0.20 | 0.20 |
| | MBC | 0.05 | 0.40 | 0.40 | 0.20 | 0.40 | 0.40 |
| TWW+ | MIC | 0.05 | 0.20 | 0.30 | 0.20 | 0.30 | 0.30 |
| | MBC | 0.10 | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 |
| CW− | MIC | 0.10 | 0.40 | 0.30 | 0.15 | 0.20 | 0.30 |
| | MBC | 0.20 | 0.80 | 0.40 | 0.20 | 0.40 | 0.40 |
| BW− | MIC | 0.025 | 0.20 | 0.30 | 0.20 | 0.30 | 0.40 |
| | MBC | 0.05 | 0.40 | 0.40 | 0.40 | 0.40 | 0.80 |



  

**Table 3.** Continued

| | | *B. cereus* | *S. aureus* | *L. monocytogenes* | *E. coli* | *E. cloacae* | *S. typhimurium* |
|---|---|---|---|---|---|---|---|
| EKW− | MIC | 0.025 | 0.20 | 0.15 | 0.15 | 0.20 | 0.20 |
| | MBC | 0.05 | 0.40 | 0.40 | 0.20 | 0.40 | 0.40 |
| GW− | MIC | 0.05 | 0.20 | 0.30 | 0.20 | 0.30 | 0.30 |
| | MBC | 0.10 | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 |
| TWW− | MIC | 0.025 | 0.20 | 0.20 | 0.10 | 0.15 | 0.20 |
| | MBC | 0.05 | 0.40 | 0.30 | 0.20 | 0.20 | 0.40 |
| Streptomycin | MIC | 0.10 | 0.04 | 0.20 | 0.20 | 0.20 | 0.20 |
| | MBC | 0.20 | 0.10 | 0.30 | 0.30 | 0.30 | 0.30 |
| Ampicillin | MIC | 0.25 | 0.25 | 0.40 | 0.40 | 0.25 | 0.75 |
| | MBC | 0.40 | 0.45 | 0.50 | 0.50 | 0.50 | 1.20 |

*B. cereus*: *Bacillus cereus* (food isolate); *S. aureus*: *Staphylococcus aureus* (ATCC 6538); *Listeria monocytogenes*: *L. monocytogenes* (NCTC 7973); *E. coli*: *Escherichia coli* (ATCC 35210); *E. cloacae*: *Enterobacter cloacae* (human isolate); *S. typhimurium*: *Salmonella typhimurium* (ATCC 13311).
MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.
*W+: normally irrigated plants; W−: water-stressed plants; C: control treatment (no biostimulants added); B: Veramin Ca; EK: EKOprop; G: Nomoren; TW: Twin-Antistress.

Antifungal effects of common bean pods and seeds extracts against three *Aspergillus* and three *Penicillium* species are presented in Table 4. Extracts from pods of the first harvest from normally irrigated plants showed the best results against the fungi tested, except for *P. verrucosum* var. *cyclopium*, where MIC and MFC values were similar to those of water-stressed plants. The application of biostimulants in pods of the first harvest had a varied effect on antifungal properties depending on the irrigation treatment and the fungi tested, with pod extracts having better or similar effects to positive controls (ketoconazole and bifonazole). Considering that in our study the total phenols content was higher in pods of the first harvest obtained from normally irrigated plants, regardless of biostimulant treatment, this could partly explain this trend. Moreover, the comparison of biostimulants with control treatment (no use of biostimulants) showed a varied response that was dependent on the fungi tested and the irrigation regime. However, other compounds may also exhibit antifungal activities, as has already been reported in the literature. In particular, Carvalho *et al.*[43] suggested that antimicrobial peptides isolated from cowpea (*V. unguiculata*) seeds were effective against various pathogenic fungi. In addition, a chitinase isolated from Canadian cranberry beans showed inhibitory effects against plant pathogenic fungi,[44] and antifungal properties were also exerted by defensins isolated from *P. vulgaris* seeds.[38] The use of biostimulants may also confer antifungal properties to crops; for example, the use of algae extracts may have a stimulating effect on plant growth and confer protective properties against plant pathogens in strawberry plants.[45] However, to the best of our knowledge, this is the first report regarding the effect of biostimulants on antifungal properties of common bean pods and seeds extracts.

### Cytotoxic effects of seeds and pods extracts

Regarding the cytotoxic effects of pods and seeds extracts against porcine liver primary culture (PLP2 cell lines), the results of the present study showed no significant cytotoxic effect, with $GI_{50}$ values higher than 400 μg $mL^{-1}$, regardless of the irrigation regime and the biostimulants treatment. The presence of antinutritional factors, such as tripsin inhibitors, lectins, and tannins, in legumes has been associated with toxicity effects. However, de Mejia *et al.*[46] reported low cytotoxicity for protein extracts of common beans seeds, a finding that is in agreement with our results. Moreover, Wong and Ng suggested that lunatisin, an antimicrobial peptide obtained from lima beans (*P. lunatus* L.), showed no cytotoxicity against mammalian splenocytes.[47]

## CONCLUSION

In conclusion, the biostimulants tested increased total phenolic compounds content compared with control treatment, especially in pods of the first harvest and seeds of water-stressed plants, whereas significant differences were also observed between biostimulant treatments. Moreover, total phenolic compounds content was higher in the first harvest than in the second harvest, regardless of the biostimulants and water treatments, with the only exception being the application of the G treatment in normally irrigated plants. It seems that the prolonged effect of water stress (pods of the second harvest and seeds) resulted in a decrease of phenolic compounds content, whereas the G treatment showed the highest increase compared with the control, regardless of the irrigation treatment. Bioactive properties showed a varied response in regard to irrigation and biostimulant treatment, which did not seem to be associated with phenolic compounds content, indicating that antimicrobial properties could be related to other bioactive compounds not assessed in the present study. In particular, pods of the second harvest and seed extracts were effective against *B. cereus* when plants were subjected to water-stress and biostimulant treatments; seed extracts from water-stressed plants treated with biostimulants were effective against *S. aureus*; and pods of the second harvest from normally irrigated plants showed the best results against the fungi tested, except for *P. verrucosum* var. *cyclopium*. Moreover, no cytotoxic effects against the PLP2 cell lines tested were observed, regardless of the irrigation regime and the biostimulants treatments. Therefore, biostimulants can be considered as a useful means towards increasing bioactive compounds content and phenolic compounds of pods and seeds of common beans grown under drought conditions, thus increasing the quality and the added value of the final product. At the same time, they may affect the antimicrobial properties of pods and seeds extracts, which may find uses in the pharmaceutical and food industries as natural antimicrobial and food preservation agents in substitution of artificial ones, and increase the added value of the crop final products.



  

**Table 4.** Antifungal activity of common bean pods (first and second harvest) and seeds extracts (MIC and MFC mg mL$^{-1}$)

| | | *A. fumigatus* | *A. ochraceus* | *A. niger* | *P. funiculosum* | *P. ochrochloron* | *P. v. cyclopium* |
|---|---|---|---|---|---|---|---|
| **First harvest of pods** | | | | | | | |
| CW+* | MIC | 0.10 | 0.075 | 0.20 | 0.075 | 0.15 | 0.10 |
| | MFC | 0.20 | 0.20 | 0.40 | 0.10 | 0.20 | 0.20 |
| BW+ | MIC | 0.20 | 0.05 | 0.10 | 0.10 | 0.10 | 0.15 |
| | MFC | 0.40 | 0.10 | 0.20 | 0.20 | 0.20 | 0.20 |
| EKW+ | MIC | 0.60 | 0.10 | 0.30 | 0.40 | 0.10 | 0.05 |
| | MFC | 0.80 | 0.20 | 0.80 | 0.80 | 0.20 | 0.10 |
| GW+ | MIC | 0.10 | 0.05 | 0.15 | 0.10 | 0.075 | 0.10 |
| | MFC | 0.20 | 0.10 | 0.20 | 0.20 | 0.15 | 0.15 |
| TWW+ | MIC | 0.40 | 0.30 | 0.80 | 0.20 | 0.40 | 0.40 |
| | MFC | 0.80 | 0.40 | 1.20 | 0.40 | 0.80 | 0.80 |
| CW− | MIC | 0.20 | 0.10 | 0.40 | 0.10 | 0.20 | 0.10 |
| | MFC | 0.40 | 0.20 | 0.80 | 0.20 | 0.40 | 0.20 |
| BW− | MIC | 0.60 | 0.20 | 0.40 | 0.60 | 0.30 | 0.10 |
| | MFC | 0.80 | 0.40 | 0.80 | 1.20 | 0.40 | 0.20 |
| EKW− | MIC | 0.20 | 0.10 | 0.40 | 0.20 | 0.20 | 0.30 |
| | MFC | 0.40 | 0.20 | 0.80 | 0.40 | 0.40 | 0.40 |
| GW− | MIC | 0.80 | 0.40 | 0.80 | 0.40 | 0.60 | 0.40 |
| | MFC | 1.20 | 0.80 | 1.20 | 0.80 | 1.20 | 0.80 |
| TWW− | MIC | 0.30 | 0.10 | 0.40 | 0.20 | 0.30 | 0.30 |
| | MFC | 0.60 | 0.20 | 0.80 | 0.40 | 0.40 | 0.60 |
| **Second harvest of pods** | | | | | | | |
| CW+ | MIC | 0.40 | 0.40 | – | 0.40 | 0.60 | 0.40 |
| | MFC | 0.80 | 0.80 | – | 0.80 | 1.20 | 0.80 |
| BW+ | MIC | 0.20 | 0.30 | 0.60 | 0.30 | 0.40 | 0.40 |
| | MFC | 0.40 | 0.40 | 1.20 | 0.60 | 0.80 | 0.80 |
| EKW+ | MIC | 0.30 | 0.40 | – | 0.60 | 0.60 | 0.40 |
| | MFC | 0.60 | 0.60 | – | 1.20 | 1.20 | 0.80 |
| GW+ | MIC | 0.60 | 0.40 | – | 0.60 | 0.60 | 0.40 |
| | MFC | 1.20 | 0.80 | – | 1.20 | 1.20 | 0.80 |
| TWW+ | MIC | 0.60 | 0.40 | 0.80 | 0.40 | 0.80 | 0.40 |
| | MFC | 1.20 | 0.80 | 1.20 | 0.80 | 1.20 | 0.80 |
| CW− | MIC | 0.40 | 0.40 | – | 0.80 | 0.80 | 0.80 |
| | MFC | 0.60 | 0.60 | – | 1.20 | 1.20 | 1.20 |
| BW− | MIC | 0.20 | 0.60 | – | 0.80 | 0.80 | 0.40 |
| | MFC | 0.60 | 0.80 | – | 1.20 | 1.20 | 0.80 |
| EKW− | MIC | 0.30 | 0.40 | – | 0.40 | 0.60 | 0.40 |
| | MFC | 0.40 | 0.80 | – | 0.80 | 1.20 | 0.80 |
| GW− | MIC | 0.80 | 0.80 | – | 0.40 | 0.80 | 0.40 |
| | MFC | 1.20 | 1.20 | – | 0.80 | 1.20 | 0.80 |
| TWW− | MIC | 0.30 | 0.20 | 0.60 | 0.40 | 0.60 | 0.40 |
| | MFC | 0.40 | 0.40 | 1.20 | 0.80 | 1.20 | 0.80 |
| **Seeds** | | | | | | | |
| CW+ | MIC | 0.20 | 0.15 | 0.20 | 0.40 | 0.40 | 0.40 |
| | MFC | 0.40 | 0.20 | 0.40 | 0.60 | 0.60 | 0.80 |
| BW+ | MIC | 0.10 | 0.075 | 0.20 | 0.30 | 0.30 | 0.10 |
| | MFC | 0.20 | 0.10 | 0.40 | 0.40 | 0.40 | 0.20 |
| EKW+ | MIC | 0.10 | 0.10 | 0.30 | 0.40 | 0.40 | 0.40 |
| | MFC | 0.20 | 0.20 | 0.40 | 0.60 | 0.80 | 0.80 |
| GW+ | MIC | 0.40 | 0.20 | 0.40 | 0.60 | 0.60 | 0.60 |
| | MFC | 0.60 | 0.40 | 0.80 | 0.80 | 0.80 | 0.80 |
| TWW+ | MIC | 0.40 | 0.30 | 0.40 | 0.40 | 0.40 | 0.40 |
| | MFC | 0.80 | 0.40 | 0.60 | 0.60 | 0.80 | 0.60 |
| CW− | MIC | 0.40 | 0.20 | 0.30 | 0.15 | 0.20 | 0.30 |
| | MFC | 0.60 | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 |
| BW− | MIC | 0.40 | 0.20 | 0.20 | 0.40 | 0.40 | 0.40 |
| | MFC | 0.60 | 0.40 | 0.40 | 0.80 | 0.60 | 0.80 |
| EKW− | MIC | 0.40 | 0.20 | 0.60 | 0.40 | 0.40 | 0.40 |

**Table 4.** Continued

| | | *A. fumigatus* | *A. ochraceus* | *A. niger* | *P. funiculosum* | *P. ochrochloron* | *P. v. cyclopium* |
|---|---|---|---|---|---|---|---|
| | MFC | 0.80 | 0.40 | 0.80 | 0.80 | 0.80 | 0.80 |
| GW− | MIC | 0.20 | 0.20 | 0.60 | 0.30 | 0.40 | 0.30 |
| | MFC | 0.40 | 0.30 | 0.80 | 0.60 | 0.80 | 0.60 |
| TWW− | MIC | 0.40 | 0.20 | 0.40 | 0.30 | 0.20 | 0.20 |
| | MFC | 0.80 | 0.40 | 0.80 | 0.40 | 0.40 | 0.40 |
| Ketoconazole | MIC | 0.25 | 0.20 | 0.20 | 0.20 | 2.50 | 0.20 |
| | MFC | 0.50 | 0.50 | 0.50 | 0.50 | 3.50 | 0.30 |
| Bifonazole | MIC | 0.15 | 0.10 | 0.15 | 0.20 | 0.20 | 0.10 |
| | MFC | 0.20 | 0.20 | 0.20 | 0.25 | 0.25 | 0.20 |

*A. fumigatus*: *Aspergillus fumigatus* (ATCC 1022); *A. ochraceus*: *Aspergillus ochraceus* (ATCC 12066); *A. niger*: *Aspergillus niger* (ATCC 6275); *P. ochrochloron*: *Penicillium ochrochloron* (ATCC 9112); *P. funiculosum*: *Penicillium funiculosum* (ATCC 36839); *P. v. cyclopium*: *Penicillium verrucosum* var. *cyclopium* (food isolate); —: no activity.
*W+: normally irrigated plants; W−: water-stressed plants; C: control treatment (no biostimulants added); B: Veramin Ca; EK: EKOprop; G: Nomoren; TW: Twin-Antistress.

## ACKNOWLEDGEMENTS

We are grateful to the Foundation for Science and Technology (FCT, Portugal) and FEDER under Programme PT2020 for financial support to CIMO (UID/AGR/00690/2013), A. Fernandes and L. Barros contract. We are also grateful to the FEDER–Interreg España–Portugal Programme for financial support, through the project 0377_Iberphenol_6_E, and to the Ministry of Education, Science and Technological Development, grant no. 173032.

## CONFLICT OF INTEREST

None.

## REFERENCES

1 Postel SL, Entering an era of water scarcity: the challenges ahead. *Ecol Appl* **10**:941–948 (2000).
2 Rouphael Y, Kyriacou MC, Petropoulos SA, De Pascale S and Colla G, Improving vegetable quality in controlled environments. *Sci Hortic (Amsterdam)* **234**:275–289 (2018).
3 Rouphael Y, Petropoulos SA, Cardarelli M and Colla G, Salinity as eustressor for enhancing quality of vegetables. *Sci Hortic (Amsterdam)* **234**:361–369 (2018).
4 Battacharyya D, Babgohari MZ, Rathor P and Prithiviraj B, Biostimulants in horticulture. *Sci Hortic (Amsterdam)* **196**:39–48 (2015).
5 Bitterlich M, Rouphael Y, Graefe J and Franken P, Arbuscular mycorrhizas: a promising component of plant production systems provided favorable conditions for their growth. *Front Plant Sci* **9**:1–6 (2018).
6 Ertani A, Pizzeghello D, Francioso O, Sambo P, Sanchez-Cortes S and Nardi S, *Capsicum chinensis* L. growth and nutraceutical properties are enhanced by biostimulants in a long-term period: chemical and metabolomic approaches. *Front Plant Sci* **5**:1–12 (2014).
7 Goñi O, Quille P and Connell SO, *Ascophyllum nodosum* extract biostimulants and their role in enhancing tolerance to drought stress in tomato plants. *Plant Physiol Biochem* **126**:63–73 (2018).
8 du Jardin P, Plant biostimulants: definition, concept, main categories and regulation. *Sci Hortic (Amsterdam)* **196**:3–14 (2015).
9 Szparaga A, Kocira S, Kocira A, Czerwińska E, Świeca M, Lorencowicz E *et al.*, Modification of growth, yield, and the nutraceutical and antioxidative potential of soybean through the use of synthetic biostimulants. *Front Plant Sci* **9**:1–20 (2018).
10 Colla G, Cardarelli M, Bonini P and Rouphael Y, Foliar applications of protein hydrolysate, plant and seaweed extracts increase yield but differentially modulate fruit quality of greenhouse tomato. *HortScience* **52**:1214–1220 (2017).
11 Chrysargyris A, Xylia P, Anastasiou M, Pantelides I and Tzortzakis N, Effects of *Ascophyllum nodosum* seaweed extracts on lettuce growth, physiology and fresh-cut salad storage under potassium deficiency. *J Sci Food Agric* **98**:5861–5872 (2018).
12 Bulgari R, Cocetta G, Trivellini A, Vernieri P and Ferrante A, Biostimulants and crop responses: a review. *Biol Agric Hortic* **31**:1–17 (2015).
13 Van Oosten MJ, Pepe O, De Pascale S, Silletti S and Maggio A, The role of biostimulants and bioeffectors as alleviators of abiotic stress in crop plants. *Chem Biol Technol Agric* **4**:1–12 (2017).
14 Kumari R, Kaur I and Bhatnagar AK, Effect of aqueous extract of *Sargassum johnstonii* Setchell & Gardner on growth, yield and quality of *Lycopersicon esculentum* Mill. *J Appl Phycol* **23**:623–633 (2011).
15 Weber N, Schmitzer V, Jakopic J and Stampar F, First fruit in season: seaweed extract and silicon advance organic strawberry (*Fragaria×ananassa* Duch.) fruit formation and yield. *Sci Hortic (Amsterdam)* **242**:103–109 (2018).
16 Paradiković N, Vinković T, Vrček IV, Žuntar I, Bojić M and Medić-Šarić M, Effect of natural biostimulants on yield and nutritional quality: an example of sweet yellow pepper (*Capsicum annuum* L.) plants. *J Sci Food Agric* **91**:2146–2152 (2011).
17 Gajc-Wolska J, Spiżewski T and Grabowska A, The effect of seaweed extracts on the yield and quality parameters of broccoli (*Brassica oleracea* var. *cymosa* L.) in open field production. *Acta Hortic* **1009**:83–89 (2013).
18 Ververidis F, Trantas E, Douglas C, Vollmer G, Kretzschmar G and Panopoulos N, Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part I: chemical diversity, impacts on plant biology and human health. *Biotechnol J* **2**:1214–1234 (2007).
19 Ahuja I, Kissen R and Bones AM, Phytoalexins in defense against pathogens. *Trends Plant Sci* **17**:73–90 (2012).
20 Araya-Cloutier C, den Besten HMW, Aisyah S, Gruppen H and Vincken J-P, The position of prenylation of isoflavonoids and stilbenoids from legumes (Fabaceae) modulates the antimicrobial activity against Gram positive pathogens. *Food Chem* **226**:193–201 (2017).
21 Luna-Vital DA, Mojica L, de Mejía EG, Mendoza S and Loarca-Piña G, Biological potential of protein hydrolysates and peptides from common bean (*Phaseolus vulgaris* L.): a review. *Food Res Int* **76**:39–50 (2015).
22 Wong JH, Ip DCW, Ng TB, Chan YS, Fang F and Pan WL, A defensin-like peptide from *Phaseolus vulgaris* cv. 'King Pole Bean'. *Food Chem* **135**:408–414 (2012).
23 Bessada SMF, Barreira JCM, Barros L, Ferreira ICFR and Oliveira MBPP, Phenolic profile and antioxidant activity of *Coleostephus myconis* (L.) Rchb.f.: an underexploited and highly disseminated species. *Ind Crops Prod* **89**:45–51 (2016).
24 Soković M, Glamočlija J, Marin PD, Brkić D and van Griensven LJLD, Antibacterial effects of the essential oils of commonly consumed medicinal herbs using an *in vitro* model. *Molecules* **15**:7532–7546 (2010).
25 Abreu RMV, Ferreira ICFR, Calhelha RC, Lima RT, Vasconcelos MH, Adega F *et al.*, Anti-hepatocellular carcinoma activity using human HepG2 cells and hepatotoxicity of 6-substituted methyl 3-aminothieno[3,2-*b*]pyridine-2- carboxylate derivatives: *in vitro*

  

evaluation, cell cycle analysis and QSAR studies. *Eur J Med Chem* **46**:5800–5806 (2011).
26 do Evangelho JA, Vanier LN, Pinto VZ, De Berrios JJ, Dias ARG and Zavareze EDR, Black bean (*Phaseolus vulgaris* L.) protein hydrolysates: physicochemical and functional properties. *Food Chem* **214**:460–467 (2017).
27 Mendoza-Sánchez M, Pérez-Ramírez IF, Wall-Medrano A, Martinez-Gonzalez AI, Gallegos-Corona MA and Reynoso-Camacho R, Chemically induced common bean (*Phaseolus vulgaris* L.) sprouts ameliorate dyslipidemia by lipid intestinal absorption inhibition. *J Funct Foods* **52**:54–62 (2019).
28 Pitura K and Arntfield SD, Characteristics of flavonol glycosides in bean (*Phaseolus vulgaris* L.) seed coats. *Food Chem* **272**:26–32 (2019).
29 Abu-Reidah IM, Arráez-Román D, Lozano-Sánchez J, Segura-Carretero A and Fernández-Gutiérrez A, Phytochemical characterisation of green beans (*Phaseolus vulgaris* L.) by using high-performance liquid chromatography coupled with time-of-flight mass spectrometry. *Phytochem Anal* **24**:105–116 (2013).
30 Berger M, Küchler T, Maaßen A, Busch-Stockfisch M and Steinhart H, Correlations of ingredients with sensory attributes in green beans and peas under different storage conditions. *Food Chem* **103**:875–884 (2007).
31 Clifford MN, Johnston KL, Knight S and Kuhnert N, Hierarchical scheme for LC–MS identification of chlorogenic acids. *J Agric Food Chem* **51**:2900–2911 (2003).
32 Kumar V, Rani A, Dixit AK, Bhatnagar D and Chauhan GS, Relative changes in tocopherols, isoflavones, total phenolic content, and antioxidative activity in soybean seeds at different reproductive stages. *J Agric Food Chem* **57**:2705–2710 (2009).
33 Di Stasio E, Rouphael Y, Colla G, Raimondi G, Giordano M, Pannico A *et al.*, The influence of *Ecklonia maxima* seaweed extract on growth, photosynthetic activity and mineral composition of *Brassica rapa* L. subsp. *sylvestris* under nutrient stress conditions. *Eur J Hortic Sci* **82**:286–293 (2017).
34 Kałużewicz A, Gąsecka M and Spiżewski T, Influence of biostimulants on phenolic content in broccoli heads directly after harvest and after storage. *Folia Hortic* **29**:221–230 (2017).
35 Kocira S, Szparaga A, Kocira A, Czerwińska E, Wójotowicz A, Bronowicka-Mielniczuk U *et al.*, Modeling biometric traits, yield and nutritional and antioxidant properties of seeds of three soybean cultivars through the application of biostimulant containing seaweed and amino acids. *Front Plant Sci* **9**:1–18 (2018).
36 Elzaawely AA, Ahmed ME, Maswada HF, Asem A and Xuan TD, Growth traits, physiological parameters and hormonal status of snap bean (*Phaseolus vulgaris* L.) sprayed with garlic cloves extract. *Arch Agron Soil Sci* **64**:1068–1082 (2018).
37 Los FGB, Zielinski AAF, Wojeicchowski JP, Nogueira A and Demiate IM, Beans (*Phaseolus vulgaris* L.): whole seeds with complex chemical composition. *Curr Opin Food Sci* **19**:63–71 (2018).
38 Mello ÉDO, Santos IS, Carvalho ADO, Souza LSD and Souza-Filho GAD, Functional expression and activity of the recombinant antifungal defensin $PvD_1$r from *Phaseolus vulgaris* L. (common bean) seeds. *BMC Biochem* **15**:7 (2014).
39 dos Santos IS, Carvalho AO, de Souza-Filho GA, do Nascimento VV, Machado OLT and Gomes VM, Purification of a defensin isolated from *Vigna unguiculata* seeds, its functional expression in *Escherichia coli*, and assessment of its insect $\alpha$-amylase inhibitory activity. *Protein Expr Purif* **71**:8–15 (2010).
40 Godlewska K, Michalak I, Tuhy A and Chojnacka K, Plant growth biostimulants based on different methods of seaweed extraction with water. *Biomed Res Int* **2016**:1–11 (2016).
41 Gan R-Y, Deng Z-Q, Yan A-X, Prasad N, Lui W, Chan C-L *et al.*, Pigmented edible bean coats as natural sources of polyphenols with antioxidant and antibacterial effects. *LWT – Food Sci Technol* **73**:168–177 (2016).
42 Ariza-Ortega T d J, Zenón-Briones EY, Castrejón-Flores JL, Yáñez-Fernández J, Gómez-Gómez Y d l M and Oliver-Salvador M d C, Angiotensin-I-converting enzyme inhibitory, antimicrobial, and antioxidant effect of bioactive peptides obtained from different varieties of common beans (*Phaseolus vulgaris* L.) with *in vivo* antihypertensive activity in spontaneously hypertensive r. *Eur Food Res Technol* **239**:785–794 (2014).
43 Carvalho AO, Machado OLT, Da Cunha M, Santos IS and Gomes VM, Antimicrobial peptides and immunolocalization of a LTP in *Vigna unguiculata* seeds. *Plant Physiol Biochem* **39**:137–146 (2001).
44 Wang S, Shao B, Fu H and Rao P, Isolation of a thermostable legume chitinase and study on the antifungal activity. *Appl Microbiol Biotechnol* **85**:313–321 (2009).
45 Righini H, Roberti R and Baraldi E, Use of algae in strawberry management. *J Appl Phycol* **30**:3551–3564 (2018).
46 de Mejia EG, Valadez-Vega MDC, Reynoso-Camacho R and Loarca-Pina G, Tannins, trypsin inhibitors and lectin cytotoxicity in tepary (*Phaseolus acutifolius*) and common (*Phaseolus vulgaris*) beans. *Plant Foods Hum Nutr* **60**:137–145 (2005).
47 Wong JH and Ng TB, Lunatusin, a trypsin-stable antimicrobial peptide from lima beans (*Phaseolus lunatus* L.). *Peptides* **26**:2086–2092 (2005).



  