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Seed Coating with Biowaste Materials and Biocides—Environment-Friendly Biostimulation or Threat?

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Abstract: The presented study assessed the effect of bovine (BC) and fish (FC) waste-derived collagen, poly(hexamethylenebiguanide) hydrochloride (PHMB) and waste dolomite on the emergence of seedlings, growth and development of pea (*Pisum sativum* L. 'Lasso') plants. The seed coating method was used to apply the binding agents. Some of the studied agents were showed to significantly affect the index of emergence velocity (IEV) and of emergence synchrony (IES), but not the final emergence percentage (FEP). The results showed that treatment of the pea seeds with BC, FC and PHMB had a slightly positive effect on plant growth, whereas negative effects of dolomite were observed, i.e., detrimental differences in morphological traits of stipules. Moreover, BC, FC and PHMB improved maximal efficiency of PSII (F_V/F_M) and did not negatively influence chlorophyll content. Analyses demonstrated positive effects of FC and PHMB and negative ones of BC and dolomite on elemental composition of roots and shoots of the studied plant species. We suggest that the FC and PHMB can be used as promising agents for improvement of plant growth, whereas usage of BC and dolomite should be limited.

Keywords: *Pisum sativum* L.; waste collagen; dolomite; PHMB; chlorophyll fluorescence; plant nutrition; seedling emergence

1. Introduction

The leather industry plays one of the major roles in the global economy. It also creates up to 5.4 million tons of both solid and liquid wastes each year [1]. Moreover, about 75–80% of solid wastes arise from the processing of raw hides to leather [2]. The main task of the leather industry is to obtain physically and chemically stable material. However, leather processing also generates a biodegradable polymer material (collagen) as a side product of the tanning industry [3]. The main sources of collagen in the leather industry are wastes from tanning and processing of the leather, i.e., raw trimmings, fresh and limed garments and splits. Highly valuable collagen products are typically extracted from bovine hide off-cutting wastes [4]. Another industrial source of collagen is marine fisheries, which generate a large amount of waste, including viscera and fish scales [5]. Collagen of animal origin has wide applications in food, biomedical and pharmaceutical industries [6].

Waste management is still a worldwide problem that arises from the continuous increase in the quantity and complexity of urban and industrial wastes [7]. Advances in technological and industrial processes have led to the development of new applications for waste-derived products that have diverse environmental impacts [8]. A modern approach of the circular economy connecting waste treatment and environmental protection is associated with new strategies based on the incorporation of waste-derived products into various industry branches, e.g., agriculture [9]. Industrial and agricultural wastes are commonly used as sources of plant biostimulants that support the reduction in agronomic inputs without reducing the quality of crops [10]. Depending on the substrate used for the production of biostimulants, they are composed of a wide range of bioactive compounds, including amino acids, peptides, humic acids and mineral compounds [11]. These environment-friendly substances can also counteract the effects of stresses, increasing tolerance to salinity, drought, oxidative stress and mechanical damage [12].

Two major categories of waste-derived protein-based products can be distinguished: (I) protein hydrolysates (PHs), consisting of peptides, and (II) amino acids (mostly glutamate, glutamine, proline, glycine and betaine). The major large-scale sources of PHs are epithelial and connective tissues rich in collagen and elastin, carob germ protein, alfalfa residues, wheat-condensed distiller solubles, tobacco cell wall glycoproteins and algal proteins [13]. To obtain PHs on the industrial scale, these materials are subjected to chemical, thermal and enzymatic hydrolysis [14]. It is worth noticing that animal collagen recently became an object of scientific interest due to its practical application allowing reduction in mineral fertilization and disposal of animal-processing wastes [11,15,16]. It was showed that PH-based biostimulants can improve agricultural production and satisfy global demands for environment-friendly crop management practices [17,18].

The application of dolomite is the other environment-friendly fertilization method used worldwide [19]. This mineral is composed of calcium magnesium carbonate, $CaMg(CO_3)_2$ [20]. Dolomite fertilization can positively affect physical, chemical, and biological soil properties. It is mainly used on acidic soils to reduce aluminum and iron toxicity and to increase calcium and magnesium availability [21]. Due to practical purposes, dolomite powder used for fertilization is often a by-product of dolomite production for other branches of industry [19].

Pea (*Pisum sativum* L.) belongs to Fabaceae family, which is one of the largest families of plants worldwide [22]. This nitrogen-fixing leguminous plant is characterized by less tolerance to environmental stresses as compared to cereals [23]. Moreover, pea is an important vegetable crop used worldwide, including in arid and semi-arid regions. Grain legume crops, including pea, possess high nutritional value, play significant roles in the human diet and animal feed and can make a significant contribution to addressing food and nutritional security [24].

Crop production depends on successful seed germination, which is usually the most critical stage in the plant life cycle [25]. The ability of seeds to complete germination can be improved by seed coating, which is the technique of covering seeds with chosen materials. Seed coating enhances plant establishment and growth, ensures plant protection and reduces stress [26,27]. The most commonly reported active ingredients used for the preparation of seed coatings are fungicides, pesticides, insecticides, nematicides, predator deterrents and herbicides [28]. In addition to the abovementioned agents, collagen seems to be another promising material that can be used for seed coating due to its nutritional features and pro-environmental character. It positively affects seed germination, plant growth, development, nutrient uptake, yield and overall performance under stress conditions [12,13,18]. Seed coating with collagen was showed to promote the completion of germination and survival of plants under stress conditions [29].

Although the application of protein hydrolysates has become a popular method of crop improvement [30], studies pertaining to the use of waste-derived collagen material for seed coating are still not popular. It is not known if the application of this material causes substantial positive effects on plant performance. Furthermore, animal waste-derived biostimulants have not been widely tested for detrimental side effects. It is not known if they are carriers of phytotoxic contaminants, i.e., heavy metals, especially those associated with human activity (anthropogenic metals). Besides collagens, dolomite dust and poly(hexamethylenebiguanide) hydrochloride (PHMB) biocide were not checked for their possible agricultural applications. Thus, this study was intended to answer the following questions: (1) Are animal waste-derived collagens, dolomite powder and medical biocide promising agents for seed coatings (in terms of improvement of seedling emergence, growth and development)? (2) Are these materials safe in terms of possible contamination of crops? The following main hypotheses were tested: (1) The studied coating methods positively influence pea growth and development. (2) The studied coating methods are safe in terms of heavy metal loads.

2. Materials and Methods

2.1. Plant Material

The seed material of *Pisum sativum* L. 'Lasso', was obtained from the Plant Breeding Strzelce Ltd., Co. (Strzelce, Poland; IHAR-PIB Group) and was used for all studies. The selected cultivar is an edible variant of pea originating from Belgium [31] that was registered in Poland. This cultivar has leafblades reduced to tendrils and large, foliaceous stipules. It is also characterized by very good resistance to fungal diseases.

2.2. Coating Materials

2.2.1. Waste Bovine (BC) and Fish (FC) Collagen

Bovine collagen was obtained from tanning waste (The National Research and Development Institute for Textiles and Leather, Romania). Fish collagen was obtained from the skin of *Hypophthalmichthys* sp. (*Cyprinidae*) (INVENTIA Polish Technologies, Żuławka, Poland). The chemical properties of collagen hydrolysates, including acidity, heavy metal contents and amino acid contents, were determined (Table 1). Chemical properties of the bovine and fish collagens, such as pH value, total ash content, protein content and total nitrogen content, were measured by employing quantitative tests including GC-MS method, titrimetric method (Kjeldahl method) and weight and potentiometric methods. Contents of amino acids were assayed using gas chromatography (GC) according to PB 5.4, ed. 4 (30 June 2013) accredited analytical procedure. The samples of collagen were prepared with propyl chloroformate using EZ:faast GC kit (Phenomenex Inc., Torrence, USA) according to the instructions of the manufacturer. The samples (0.1 g each) were mineralized with HNO_3 (65%, wt., Chempur, Piekary Śląskie, Poland) in Teflon vessels using Magnum II microwave mineralizer (Ertec, Wrocław, Poland). Determination of heavy metal content was carried out using the flame atomic absorption spectrometry (AAS) technique (Unicam 939, Pye-Unicam, Cambridge, UK). The content of each metal was determined using dedicated techniques: vapor generation atomic absorption spectrometry (VGAAS) for determination of As and Hg and flame atomic absoption spectrometry (FAAS) for determination of Cr, Zn, Pb, Cd and Cu. For the determination of Sn, the ICP OES analysis was used according to the procedure described in Section 2.5.4. ("Metal Content Determination").

Table 1. Chemical properties of waste bovine and fish collagen used for seed coating.

Parameter	Bovine Collagen (BC)	Fish Collagen (FC)
Acidity of 10% solution (pH)	7.27	3.46
Protein content (% DW)	77.42	89.90
Total ash content (% DW)	5.36	1.81
Total nitrogen content (% DW)	13.78	14.30
Heavy metal content (mg kg^{-1} DW)		
Cr (III)	<0.1	<0.1
Zn	<0.05	<0.05
As	<0.005	<0.05
Pb	<0.10	<0.10
Hg	<0.10	<0.10

Cd	<0.10	<0.10
Cu	<0.02	<0.02
Sn	6.60 ± 0.06	-
Amino acid content (mg kg^{-1} DW)		
Ala	820.00 ± 70.00	931.33 ± 19.50
Gly	1961.33 ± 151.28	2768.33 ± 422.89
Val	105.00 ± 13.45	148.33 ± 6.66
Leu	222.67 ± 39.00	165.67 ± 14.98
Ile	52.67 ± 6.81	74.00 ± 4.00
Thr	151.00 ± 9.64	228.33 ± 10.41
Ser	137.00 ± 19.29	275.67 ± 5.13
Pro	2248.33 ± 480.63	3606.00 ± 129.00
Asp	447.00 ± 3.46	336.67 ± 10.41
Met	50.67 ± 5.13	82.67 ± 1.53
Hyp	1166.67 ± 251.66	1773.33 ± 30.55
Glu	554.33 ± 4.04	642.00 ± 12.12
Phe	83.33 ± 3.79	140.33 ± 7.51
Lys	92.00 ± 5.29	196.33 ± 1.53

DW–dry weight.

2.2.2. Poly(hexamethylenebiguanide) Hydrochloride (PHMB) Biocide

Commercially available PHMB formulation (VANTICIL IB, 20% aqueous solution, Lonza, Arch UK Biocides Ltd., Castleford, UK) was used in this study. PHMB is a cationic biocide with exceptional antimicrobial activity, chemical stability and low toxicity [32] that is used mainly for medical purposes. Aqueous solution of PHMB is slightly opalescent, colorless to pale yellow and acidic (pH = 4.6). The desirable concentration of PHMB was obtained following dilution with deionized H_2O.

2.2.3. Dolomite (D)

The dolomite dust (Dolmic 5 SK, average grain size ca. 45 ± 0.5 μm) used in this work was obtained from commercial source (Jeleniogórskie Kopalnie Surowców Mineralnych Lipiński, Szklarska Poręba, Poland). This mineral is composed mainly of calcium magnesium carbonate ($CaMg(CO_3)_2$). The chemical properties of dolomite used in this study (Table 2) were determined in order to estimate natural traces of non-essential elements and possible anthropogenic contaminations. Properties of dolomite were determined according to the procedure described in Section 2.5.4. ("Metal Content Determination").

Table 2. Chemical composition of dolomite.

Element	Content (mg kg^{-1} DW)
Al	469.51 ± 64.73
B	16.15 ± 11.41
Ba	2.53 ± 0.24
Ca	238,280.43 ± 7383.11
Cr	2.29 ± 0.24
Cu	1.08 ± 0.07
Fe	1242.19 ± 24.96
K	465.07 ± 60.31
Mg	137,983.20 ± 4673.20
Mn	279.07 ± 8.75
Na	25.25 ± 9.77
Ni	3.28 ± 0.42

Sr	29.66 ± 0.22
Ti	10.92 ± 1.81
Zn	18.38 ± 0.53

Ag, As, Bi, Cd, Co, Ge, Hg, Li, Mo, Pb, Sb, Sc, Sn, Zr, V—not detected (below the detection threshold of used method).

2.3. Experimental Setup and Seed Coating Method

The experimental setup was designed in complete factorial manner (4 × 2) using four binding agents (deionized H_2O, bovine collagen (BC), fish collagen (FC) and poly(hexamethylenebiguanide) hydrochloride (PHMB)) and two variants of dolomite coating (+/−). Thus, the experiments included eight groups: control seeds (0), control seeds with dolomite (0 + D), bovine-collagen-treated seeds (BC), bovine-collagen-treated seeds with dolomite (BC + D), fish-collagen-treated seeds (FC), fish-collagen-treated seeds with dolomite (FC + D), poly(hexamethylenebiguanide) hydrochloride treated seeds (PHMB) and PHMB-treated seeds with dolomite (PHMB+D) (Table 3).

Table 3. The experimental setup of seed coating used in the presented study.

Abbreviation	Binding Agent	Dolomite Addition [a]	Amount of Binding Agent or Binding Agent/Dolomite Mixture Applied to 500 g Seeds (g)	Seed Weight (mg) [b]
0	-	-	-	237.92 ± 15.10
0 + D	-[c]	+	16.08	240.12 ± 12.19
BC	BC	-	15.29	241.73 ± 19.60
BC + D	BC	+	19.10	249.88 ± 13.57
FC	FC	-	26.94	251.44 ± 11.63
FC + D	FC	+	33.67	288.86 ± 20.66
PHMB	PHMB	-	27.09	244.80 ± 14.10
PHMB + D	PHMB	+	33.87	248.00 ± 13.94

[a] For 0 + D, BC + D, FC + D and PHMB + D variants, 200 g of dolomite was applied, [b] measured by weighting four lots of 100 randomly selected seeds, [c] dolomite was bound to testa by water. Abbreviations: 0—control seeds, 0 + D—control seeds with dolomite, BC—bovine-collagen-treated seeds, BC + D—BC-treated seeds with dolomite, FC—fish-collagen-treated seeds, FC + D—FC-treated seeds with dolomite, PHMB—poly(hexamethylenebiguanide) hydrochloride treated seeds, PHMB + D—PHMB-treated seeds with dolomite.

A rotary disk granulator was used to coat seeds (Faculty of Process Engineering and Environmental Protection at the Lodz University of Technology, Łódź, Poland). The granulator (500 mm of diameter) included a disk granulator, an electric motor with rotation speed regulation and the granulated deposit moistening system. Speed of 20 rpm and inclination angle of the disk granulator of 45° were found as the optimal process conditions. Seed coating (spraying) treatments consisted of 0.7% BC, 0.4% FC and 1.0% PHMB. Optimal concentration of compounds was selected based on preliminary test for efficiency of seed coating process. Additionally, dolomite was applied. In each variant, there was 5 min of coating process. The seed coating method used in the presented study is shown in Table 3.

Weight of the coated seeds (Table 3) was determined using precise balance under laboratory conditions.

2.4. Growth Conditions

The plants were grown under controlled laboratory conditions. The temperature was 23.0 ± 0.5 °C during light phase (14 h) and 20.0 ± 0.5 °C during dark phase. Relative humidity was stable at 55–65% (measured with the TFA Dostmann thermohygrometer, Wertheim-Reicholzheim, Germany). Light was supplied with red–blue–white LED lamps (Growy LED, Neonica, Łódź, Poland) and PAR at plant level was at stable at 100 μmol photons s^{-1} m^{-2} (measured with FluorPen FP100, Drasov, Czech Republic). The plants were planted in 1.5 dm^3 plastic pots (15 cm in diameter, 12 cm deep) filled with commercially available garden soil. The chemical properties of the soil (Table S1) were determined at the Regional Chemical and Agricultural Station in Łódź (Poland) according to the procedures used therein for routine soil analyses. The plants were subjected to nondestructive (chlorophyll content, fluorescence of chlorophyll *a*) and destructive (growth and metal content) analyses 28 days after sowing (Experiment 1). Seedling emergence counts were recorded daily for a period of 14 days (Experiment 2).

2.5. Experiment 1: Effects of Seed Coating on Growth and Nutrition

2.5.1. Chlorophyll Content Determination

The chlorophyll content was measured with the handheld chlorophyll content meter CCM-300 (Opti-Sciences Inc., Hudson, USA) according to the instruction of the manufacturer. The ratio of chlorophyll fluorescence (F_{735}/F_{700}) was captured according to the method proposed by Gitelson et al. [33]. Subsequently, chlorophyll content was calculated using standard equations of the device and expressed in mg m^{-2}. Chlorophyll content was measured on four locations (interveinal areas) of each fifth-floor stipule of four plants per treatment.

2.5.2. Fluorescence of Chlorophyll a Determination

The changes in polyphasic rise in fluorescence of chlorophyll *a* (OJIP) were measured with the handheld pulse amplitude modulation fluorometer, FluorPen FP100 (Photon System Instruments, Drasov, Czech Republic). The stipules were dark-adapted for 20 min prior to analysis by detachable clips. The data were recorded with the preprogrammed protocol of the device based on claims and equations (Table S2) of Strasser et al. [34]. OJIP-related parameters were measured on two locations (interveinal areas) of each fifth-floor stipule of four plants per treatment.

2.5.3. Growth Measurements

The plants were carefully washed in the laboratory, separated into shoots and roots and weighed to determine fresh weight (FW). Then, fifth-floor stipules were carefully cut off using a razor blade. Stipule area was measured with a CI-202 portable laser leaf area meter (CID Bio-Science, Camas, USA), and then the stipules were dried for at least 48 h at 60 °C to determine dry weight (DW). Subsequently, the shoots and roots were separately placed in paper envelopes, dried for 48 h at 60 °C and weighed to determine DW. Shoot DW was calculated as sum of DW of stipule and DW of shoot with excised stipule.

Specific leaf area (SLA) values were calculated as area per unit of leaf DW and expressed in cm^2 g^{-1} DW. Shoot/root ratio (S:R ratio) values were calculated on the basis of FW and DW of roots and shoots. To determine the relative growth rate (RGR), the plants were harvested at 9, 14 and 21 days after sowing. RGR values were calculated using the formula RGR = (ln mass2 × ln mass1/time) [35] and expressed in g g^{-1} DW d^{-1}.

2.5.4. Metal Content Determination

The samples (about 0.1 g) of shoots and roots of peas were placed in a Teflon vessel, and 6 cm^3 of HNO_3 (65%, wt., Chempur, Piekary Śląskie, Poland) was added. They were then mineralized using a Magnum II microwave mineralizer (Ertec, Wrocław, Poland). The mineralization process was carried out in three cycles, lasting a total of 20 min at a maximum temperature of 300 °C and a maximum pressure increasing to 45 bar, with a maximum microwave power (100%). The clear mineralizates were quantitatively transferred to 25 cm^3 flasks and supplemented with demineralized water. Inductively coupled plasma optical emission spectrometry with an ICP-OES 5110 spectrometer (Agilent, Santa Clara, USA) was employed for multielemental analysis. Commercial argon (from Hen-DuKol, Łódź, Poland) was used to generate the plasma. In the method of optical emission spectrometry with induced plasma excitation, the sample is introduced in the form of an aerosol. Measurements were made in dual view configuration.

After digestion of the samples, the ICP OES quantification procedure was performed in axial view of plasma with radio frequency power of 1400 W, sample flow 1.4 $cm^3 min^{-1}$, plasma gas flow 12 $dm^3 min^{-1}$, auxiliary gas flow 1.0 $dm^3 min^{-1}$, nebulizer gas flow 0.7 $dm^3 min^{-1}$ and integration time 3 × 10 s. The method was optimized before testing. The analytical lines and the optimal operating mode for determination of individual elements were selected. Linear fit of the calibration curve was used to determine the concentration of elements in the samples. The determinations were made using the external standard method. Calibration of the measuring method was carried out before the measurement using a series of chemical standards (reference materials) with different levels of the content of the tested component. The contents of the tested metals in the samples were read from the standard curves prepared from the multielement standard solution to ICP (metals: Ag, Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Li, Mg, Mn, Na, Ni, Pb, Se, Sr, V and Zn in 5% HNO_3, LGC Standard, Manchester, USA) and standards of individual metals (B, Bi, Ge, Mo, Sb, Sn, Ti and Zr in 2–5% HNO_3 and trace amounts of HF, Chem-Lab, Zedelgem, Belgium) by appropriately diluting the standards with 5% HNO_3 (v/v). The calibration solutions used for the determinations by ICP-OES were prepared by serial dilutions of multielement stock solutions containing 1000 mg dm^{-3} of each analyte. The concentration of the calibration solutions for ICP-OES analysis ranged from 0.003 to 200 mg dm^{-3} for the trace elements Ag, Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Li, Mg, Mn, Na, Ni, Pb, Se, Sr, V and Zn and 0.0003 to 20 mg dm^{-3} for elements B, Bi, Ge, Mo, Sb, Sn, Ti and Zr. The tests were carried out in accordance with the testing procedure.

2.6. Experiment 2: Effects of Seed Coating on Emergence of Seedlings

Emergence of seedlings was measured according to Kołodziejek et al. [36]. The coated seeds (25 per pot) were planted in the soil at four depths (0, 2, 4 and 6 cm). The pots were watered throughout the study to maintain adequate moisture. Seedlings were considered to have emerged when cotyledons were visible. Emergence counts were recorded daily for a period of 14 days. To determine quantitative emergence traits, final emergence percentage (FEP), index of emergence velocity (IEV) and index of emergence synchrony (IES) were calculated. These parameters are equal to final germination percentage, index of germination velocity (known as modified Timson's index) [37] and index of germination synchrony (Z value) [38] but pertain to seedling emergence instead of completion of germination. FEP values range from 0 to 100, and higher values denote greater emergence. IEV values range from 0 to 100, and higher values denote faster emergence. IES values range from 0 to 1, and higher values denote more synchronized emergence. FEP, IEV and IES values were calculated using preprogrammed equations of germinationmetrics package [39] run in R (×64, 3.6.2 version) [40].

2.7. Statistical Analysis

For each parameter, four independent biological replicates were used for measurements. The data were transformed using log10 transformation and were analyzed for normal distribution using Kolmogorov–Smirnov test. Equality of variances was tested with Levene's test. All data were analyzed by Student's test (differences between two variants −treated and not treated with dolomite−among single binding agent) and ANOVA followed by Tukey's post hoc test (differences between four variants−treated with different binding agents−among given treatment with dolomite). The data pertaining to emergence experiment were log10 (FEP, IEV) or arcsin (IES) transformed to meet the assumptions of normality and homogeneity of variance implicit in parametric statistical procedures. Two-way ANOVA was used to detect effects of studied factors in Experiment 1, and three-way ANOVA was used to detect effects of studied factors in Experiment 2. The analyses were performed using Statistica 13.1 (TIBCO Software, Palo Alto, USA).

3. Results

3.1. Effects of Seed Coating on Growth and Nutrition

The plants established from the BC-coated seeds showed greater accumulation of shoot FW in comparison to the control plants (0) (Figure 1). Furthermore, subtly greater FWs of FC- and PHMB-treated plants were recorded. Unexpectedly, FW and DW of both roots and shoots of all dolomite-treated plants (0 + D, BC + D, FC + D, PHMB + D) were significantly lower (reduction by ca. 50%) in comparison with the respective groups not subjected to dolomite treatment (0, BC, FC, PHMB) (Figure 1). However, reduction in weight was proportional, as shoot/root ratio of FW and DW remained unaffected (Figure S1). FW and DW of both roots and shoots were dependent on dolomite, but not on binding agent or interaction of the studied factors (Table S3). Shoot/root ratio was not affected by any of the studied factors (Table S3).

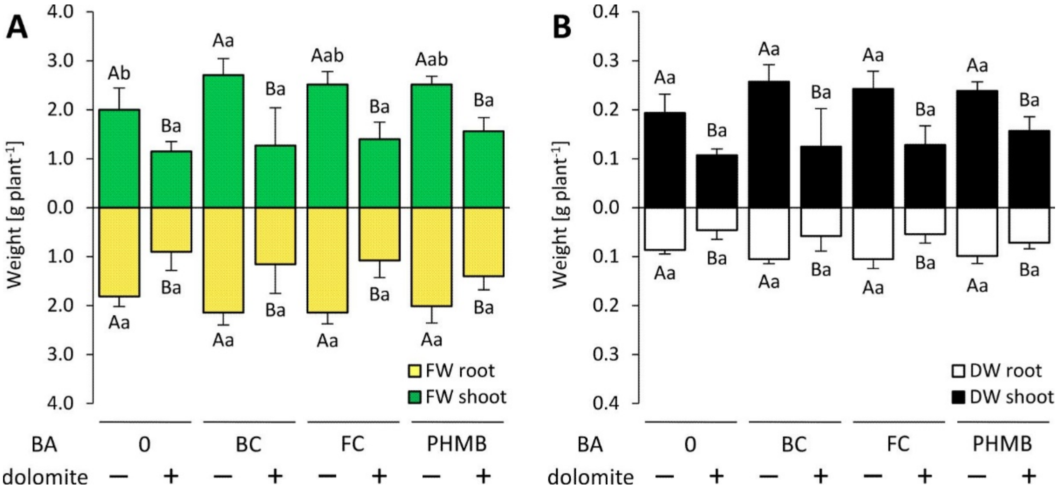
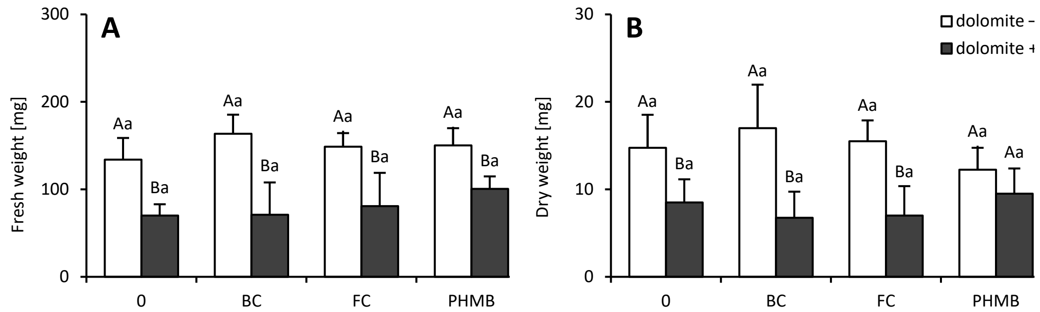


Figure 1. Fresh (FW; **A**) and dry weight (DW; **B**) of *Pisum sativum* L. plants subjected to different seed coating treatments. Values followed by different upper-case letters within the given binding agent treatment (0, BC, FC or PHMB) are significantly different ($p < 0.05$); Student's test (n = 4). Values followed by different lower-case letters within the given dolomite treatment (dolomite − or dolomite +) are significantly different ($p < 0.05$); ANOVA followed by Tukey's post hoc test (n = 4). BA—binding agent, 0—control seeds, BC—bovine-collagen-treated seeds, FC—fish-collagen-treated seeds, PHMB—poly(hexamethylenebiguanide) hydrochloride treated seeds.

Differences in plant growth were reflected in morphological traits of stipules. Fifth-floor stipules of the dolomite-coated plants (BC+D, FC+D, PHMB+D) were significantly smaller (ca. 50% reduction) than those from the plants treated only with the binding agents (BC, FC, PHMB) (Figure 2). Dolomite treatment caused strong and significant reduction in FW and DW of stipules, which coincided with a proportional reduction in their area, as was supported by measurements pertaining to SLA (Figure 2). However, the binding agent had no influence on any of the stipule-related traits (Figure 2). FW, DW and area of stipules significantly depended only on dolomite (Table S3). SLA was independent of all studied factors (Table S3).



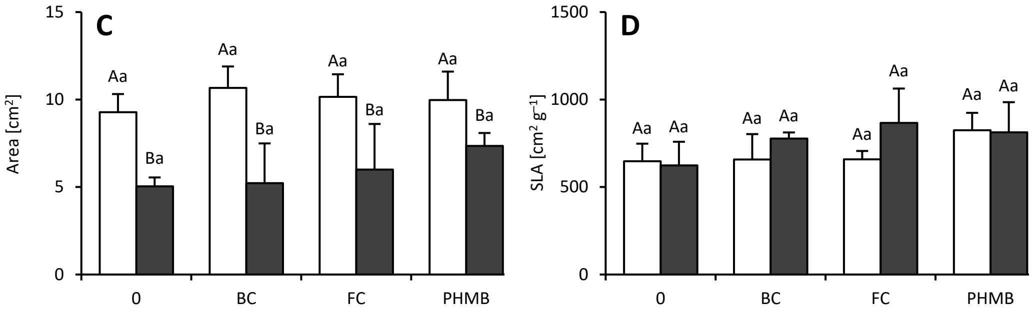


Figure 2. Stipule fresh weight (FW; **A**), dry weight (DW; **B**), area (**C**) and specific leaf area (SLA; **D**) of *Pisum sativum* L. plants subjected to different seed coating treatments. Values followed by different upper-case letters within the given binding agent treatment (0, BC, FC or PHMB) are significantly different ($p < 0.05$); Student's test (n = 4). Values followed by different lower-case letters within the given dolomite treatment (dolomite − or dolomite +) are significantly different ($p < 0.05$); ANOVA followed by Tukey's post hoc test (n = 4).

Very interestingly, dolomite coating was showed to be beneficial or detrimental for total chlorophyll content, which depended on the binding agent used for coating. The effects of BC + D and FC + D proved positive, while that of H_2O + D was negative (Figure 3). It was also linked with the functioning of photosynthetic apparatus, as BC-, FC- and PHMB-treated plants showed significantly higher values of maximum efficiency of PSII (F_V/F_M) than the control ones, and this effect was not abolished by dolomite treatment (Figure 4). Several other minor changes in chlorophyll *a* fluorescence were observed (Figure 4). Both chlorophyll content in stipules and F_V/F_M were significantly affected by binding agent and interaction of binding agent and dolomite (Table S3).

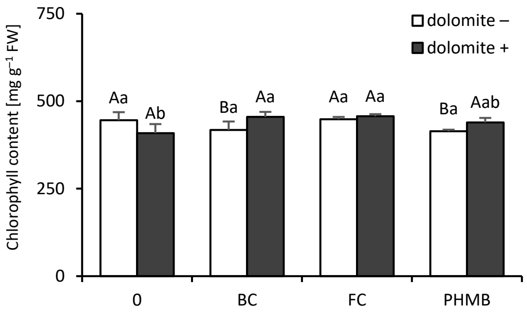


Figure 3. Chlorophyll content in stipules of *Pisum sativum* L. plants subjected to different seed coating treatments. Values followed by different upper-case letters within the given binding agent treatment (0, BC, FC or PHMB) are significantly different ($p < 0.05$); Student's test (n = 4). Values followed by different lower-case letters within the given dolomite treatment (dolomite − or dolomite +) are significantly different ($p < 0.05$); ANOVA followed by Tukey's post hoc test (n = 4).

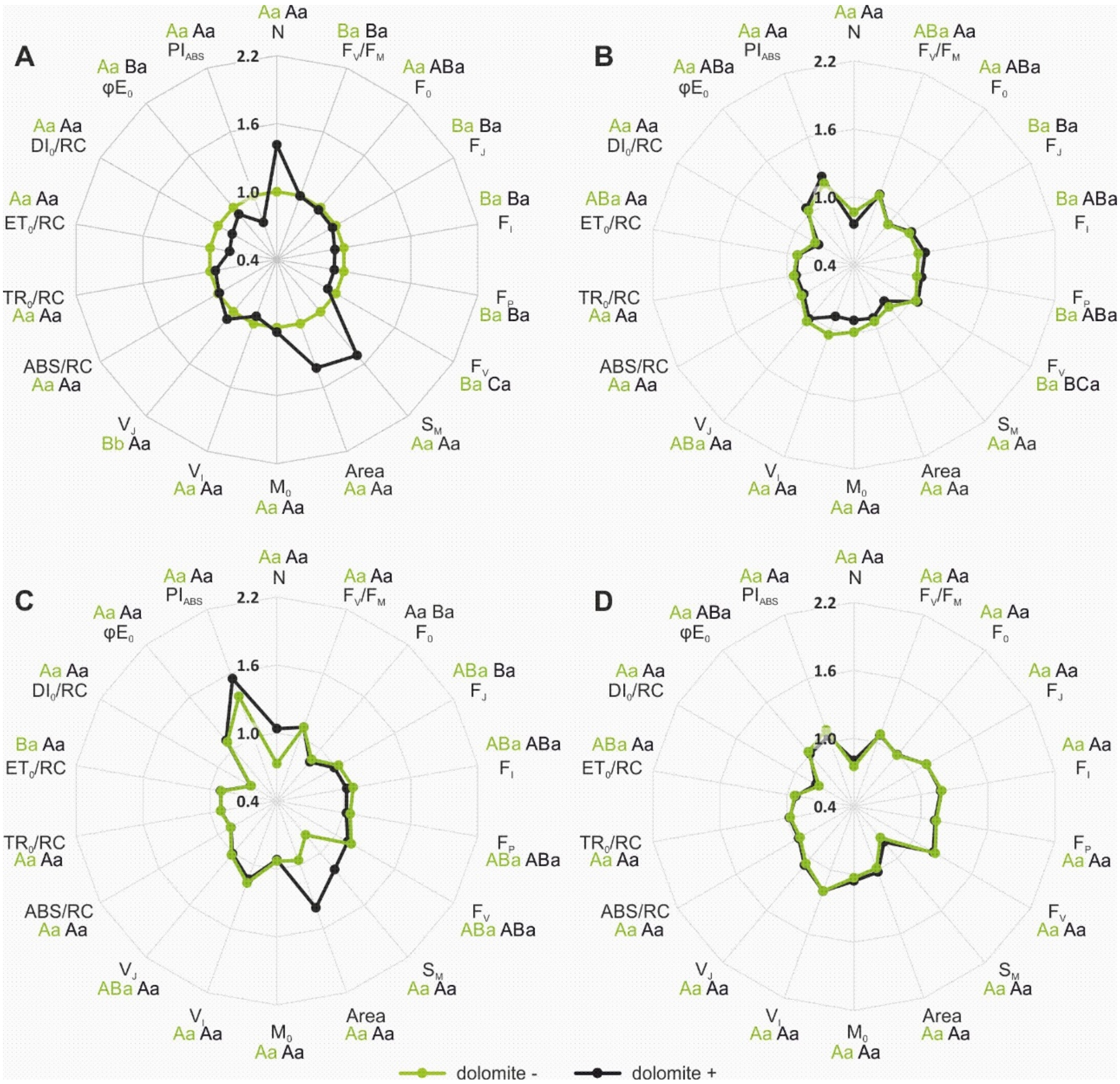


Figure 4. Relative changes of parameters from chlorophyll *a* fluorescence OJIP transient curves in *Pisum sativum* L. plants subjected to different seed coating treatments. Values followed by different upper-case letters within the given binding agent treatment (0; **A**, BC; **B**, FC; **C** or PHMB; **D**) are significantly different ($p < 0.05$; ANOVA followed by Tukey's post hoc test; n = 4); values followed by different lower-case letters within the given dolomite treatment (dolomite − or dolomite +) are significantly different ($p < 0.05$; Student's test; n = 4). Color of letter-based statistical indicators refers to each experimental variant as indicated in legend. Abbreviations: ABS/RC—photon flux absorbed by PSII antenna chlorophyll per RC at t = 0, Area—area between fluorescence curve and F_M, DI_0/RC—dissipated energy flux per RC at t = 0, ET_0/RC—electron transport flux per RC at t = 0 F_I—fluorescence intensity at I-step, F_J—fluorescence intensity at J-step, F_M (=F_P)—fluorescence intensity at *p*-step, F_0 (=F_O)—fluorescence intensity at O-step, F_V—maximal variable fluorescence, F_V/F_M—maximum quantum yield of primary PSII photochemistry, M_0—approximated initial slope of the fluorescent transient, N—number of Q_A redox turnovers until F_M is reached, PI_{ABS}—performance index of electron flux from PSII based to intersystem acceptors, S_M—standardized area above the fluorescence curve between F_0 and F_M, TR_0/RC—trapping flux leading to Q_A reduction per RC at t = 0, V_I—relative variable fluorescence at I-step, V_J—relative variable fluorescence at J-step, ϕ_{E0}—quantum yield for electron transport from Q_A to plastoquinone at t = 0.

Analyses demonstrated significant effects of the seed coating treatments on the elemental composition of the roots and shoots of pea (Figures 5, 6, S2 and S3). Regarding contents of elements in roots, only Mo and Ni remained unaffected by any treatment (Figure S2). Very interestingly, Al and Fe contents were significantly affected only by dolomite addition (BC + D, FC + D, PHMB + D), whereas contents of Cr and Sn depended only on the binding agents (BC, FC, PHMB) used for the seed coating process (Figure 5). A strongly marked fingerprint pertaining to Sn content in roots of the BC-treated plants was observed (Figure 5). Content of other elements was significantly affected by both binding agents and dolomite. Dolomite addition caused unexpected and strongly marked decrease in Mg content in roots among all the studied variants (Figure 5). The PHMB-treated plants (PHMB) were more prone to dolomite-dependent perturbations (PHMB + D) of Ca, K, Mn, Na and Sr acquisition (Figures 5 and S2). Furthermore, the plants established from dolomite-coated seeds showed greater contents of Cu (0 variant), Fe (0 and FC variant) and Ti (0 variant) (Figures 5 and S2). Content of six elements (Ca, Na, Sn, Sr, Ti and Zn) were significantly dependent on binding agent, whereas five elements were dependent on dolomite (Fe, K, Mg, Na and Sr) (Table S3). Nine of fifteen determined elements (Al, Ca, Fe, K, Mg, Mn, Na, Sr and Ti) were significantly affected by the interaction of binding agent and dolomite (Table S3).

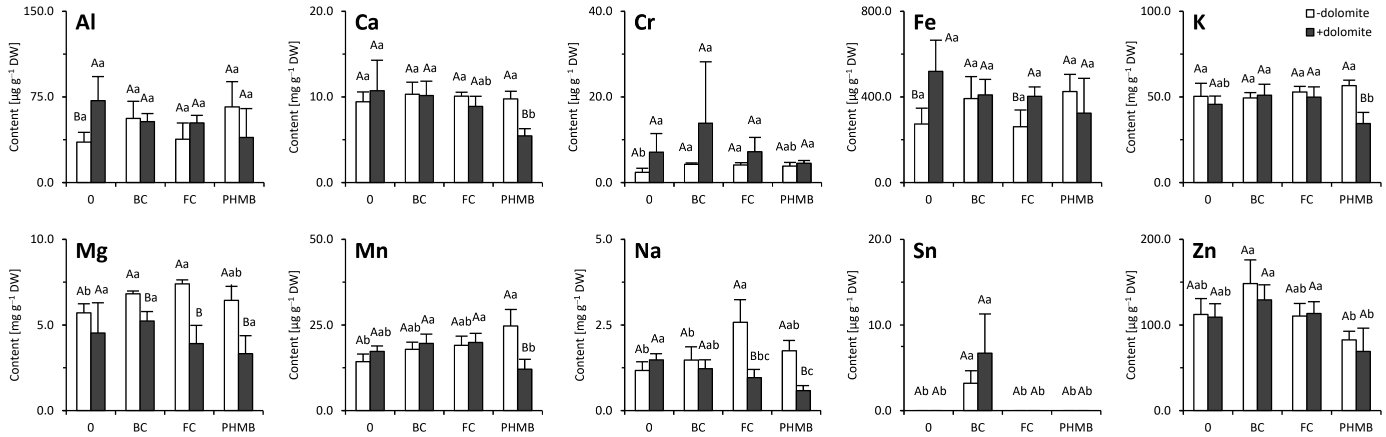
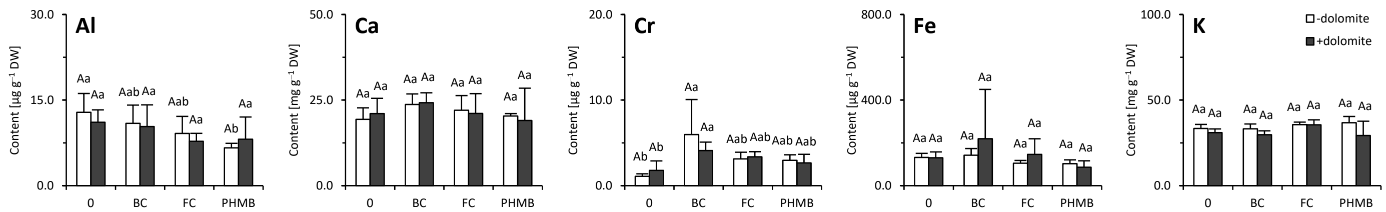


Figure 5. Elemental composition (Al, Ca, Cr, Fe, K, Mg, Mn, Na, Sn, Zn) of roots of *Pisum sativum* L. plants subjected to different seed coating treatments. Values followed by different upper-case letters within the given binding agent treatment (0, BC, FC or PHMB) are significantly different ($p < 0.05$); Student's test (n = 4). Values followed by different lower-case letters within the given dolomite treatment (dolomite − or dolomite +) are significantly different ($p < 0.05$); ANOVA followed by Tukey's post hoc test (n = 4).

Lesser changes in the content of elements were observed in the shoots. Contents of Ca, Cu, Fe, K, Mg, Mn, Ni, Sr and Ti were more or less stable and were not significantly affected by all the treatments (Figures 6 and S3). However, the binding agents caused differential effects on the accumulation of the other elements. For example, the PHMB-treated plants (PHMB) were characterized with lesser Al, Na and Zn contents than the control ones (0) (Figure 6). Similar to the results pertaining to the shoots, only the BC-treated plants (BC) accumulated Sn, which was in this case correlated with higher Cr contents (Figure 6). Significant differences in Mo contents among the dolomite-coated plants subjected to various binding agents were observed (Figure S3). Contents of eight elements were affected by binding agent (Al, Cr, Cu, Mo, Na, Sn, Ti and Zn), whereas just four elements were affected by dolomite (Cu, K, Mo and Zn) (Table S3). Interaction between studied factors did not influence the elemental composition of shoots (Table S3).



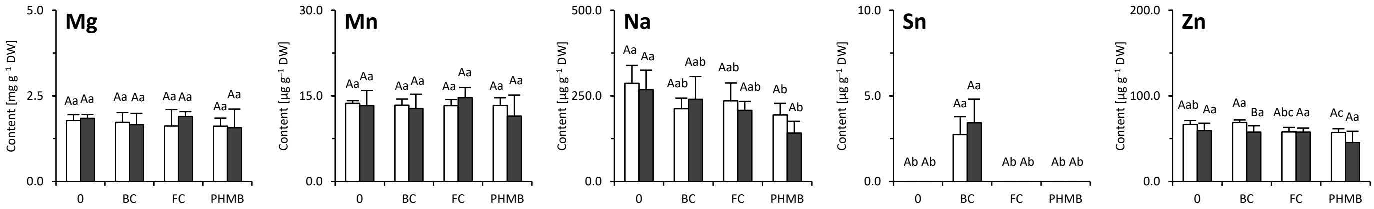


Figure 6. Elemental composition (Al, Ca, Cr, Fe, K, Mg, Mn, Na, Sn, Zn) of shoots of *Pisum sativum* L. plants subjected to different seed coating treatments. Values followed by different upper-case letters within the given binding agent treatment (0, BC, FC or PHMB) are significantly different ($p < 0.05$); Student's test (n = 4). Values followed by different lower-case letters within the given dolomite treatment (dolomite − or dolomite +) are significantly different ($p < 0.05$); ANOVA followed by Tukey's post hoc test (n = 4).

Relative growth rate measured in two intervals was reduced in FC- and PHMB-treated plants subjected to additional dolomite treatment (FC+D, PHMB+D) (9–14 days) and BC-treated ones (14–21 days) (Figure 7). RGR was significantly affected by binding agent and interaction of binding agent and dolomite (Table S3).

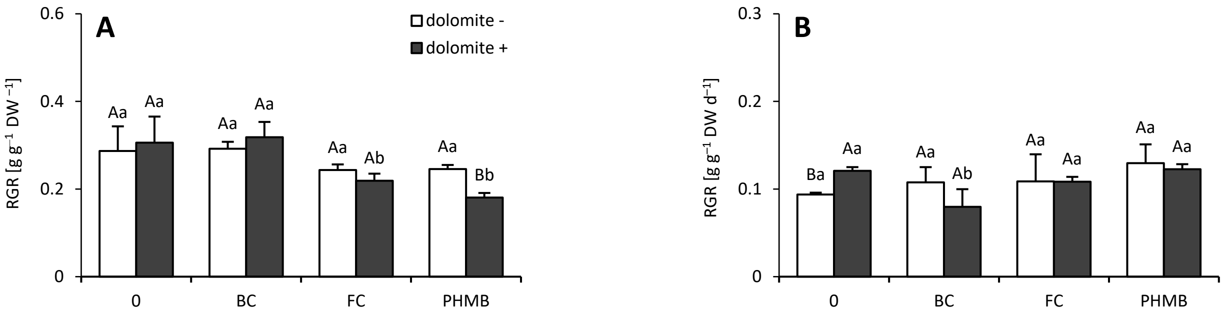


Figure 7. Relative growth ratio (RGR) of *Pisum sativum* L. plants subjected to different seed coating treatments measured at two intervals (9-14 d; **A**, 14-21 d; **B**). Values followed by different upper-case letters within the given binding agent treatment (0, BC, FC or PHMB) are significantly different ($p < 0.05$); Student's test (n = 4). Values followed by different lower-case letters within the given dolomite treatment (dolomite − or dolomite +) are significantly different ($p < 0.05$); ANOVA followed by Tukey's post hoc test (n = 4).

3.2. Effect of Seed Coating on Emergence of Seedlings

The results showed that the seed coating and sowing depth had significant effects on the seedling emergence traits (Figure 8). Differences between the FEP values were observed only for the seeds sowed on the soil surface, as a lesser percent of seedlings emerged when the seeds were treated with PHMB (Figure 8A). Based on obtained results, it can be concluded that sowing depth has a major impact on FEP values. Taking into account the IEV values, significant deceleration of emergence speed was recorded only for the BC-coated seeds at the depth of 4 cm and PHMB+D-coated ones at the depth of 6 cm (Figure 8B). The results pertaining to IES showed desynchronization of the emergence of BC- and PHMB-treated plants under the tested circumstances, synchronization of the emergence of the seedlings from FC-treated seeds from the greatest depth and dolomite-caused desynchronization of the emergence of BC- and FC-treated plants from the depth of 6 cm (Figure 8C). Sowing depth and dolomite significantly affected all measured traits pertaining to emergence (Table S4). Interactive effects were observed only for IEV and IES (Table S4). The highest values of FEP and IEV were found for the control variant and the low sowing depth.

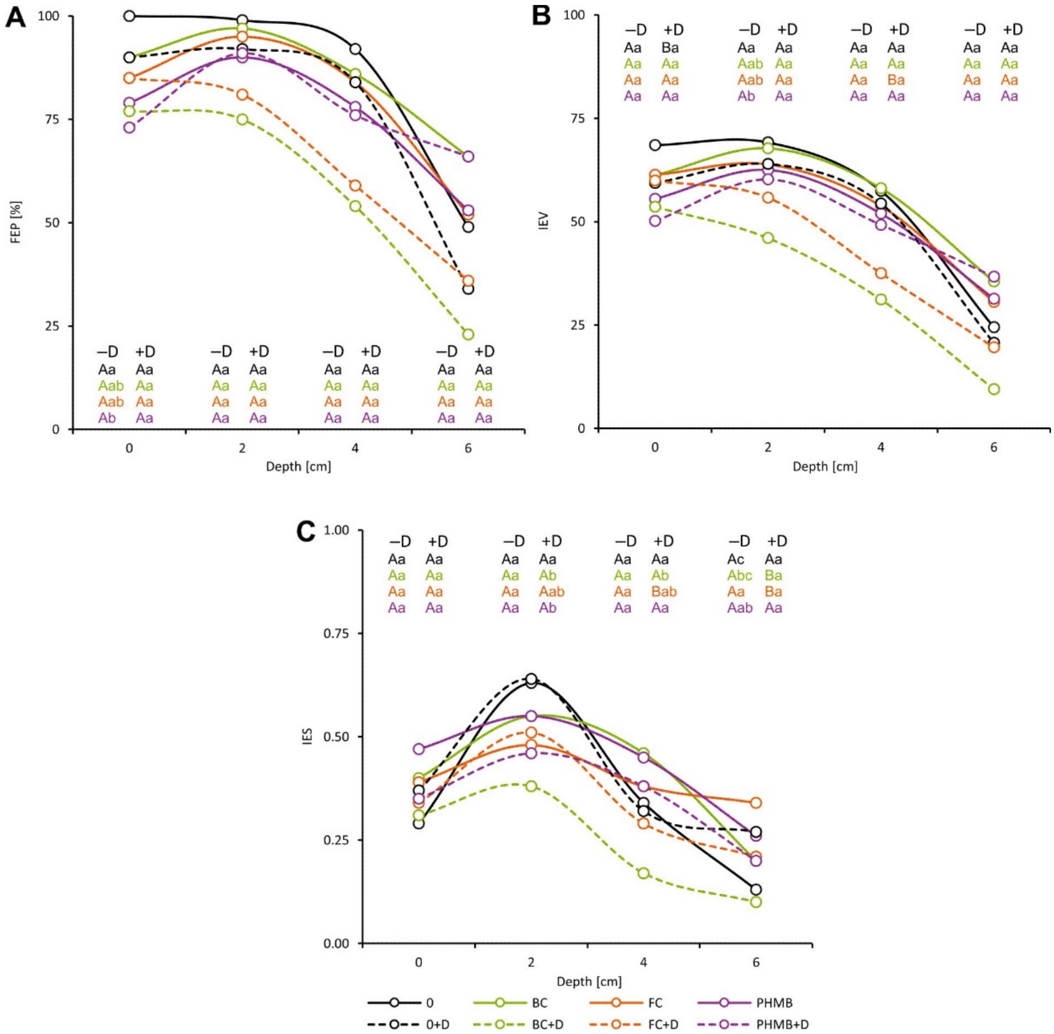


Figure 8. Final emergence percentage (FEP; **A**), index of emergence velocity (IEV, **B**) and index of emergence synchrony (IES; **C**) of *Pisum sativum* L. seedlings from seeds subjected to different coating treatments measured at four sowing depths. Values followed by different upper-case letters within the given binding agent treatment (0, BC, FC or PHMB) are significantly different ($p < 0.05$); Student's test (n = 4). Values followed by different lower-case letters within the given dolomite treatment (dolomite − or dolomite +) are significantly different ($p < 0.05$); ANOVA followed by Tukey's post hoc test (n = 4). Color of letter-based statistical indicators refers to each experimental variant as indicated in legend.

4. Discussion

To the best of our knowledge, the effects of seed coating with animal waste-derived collagen and PHMB on the growth, nutrition and emergence of plants (including pea) have not been previously elucidated. Biostimulants, including waste-derived ones, can be applied during all life stages of plants [18,41,42]. However, the only available data pertain to application methods other than seed coating, including soil drenching and foliar spray; thus, we used them as a reference for the results recorded in the presented study.

The studied collagens have different amino acid compositions, resulting from species-specific circumstances. Previous comparative evaluations confirmed interspecific differences in amino acid composition of fish-derived collagens [43], but not intraspecific, age-related ones [44]. It was also stated that two nonstandard amino acids (hydroxyproline and hydroxylysine), which are present at negligible levels in plant-derived protein hydrolysates, are common denominators of collagen-derived protein hydrolysates [30]. Furthermore, the content of total amino acids is higher in animal-derived protein hydrolysates than in plant-derived ones. Collagen-based biostimulants contain high quantities of Gly and Pro, whereas legume-based protein hydrolysates are rich in Asp and Glu, which was also observed in fish-based protein hydrolysates [30,45–47]. Moreover, a high amount of Na was detected in a protein hydrolysate from meat flour, whereas high levels of other nutrients, such as Mg, Mn, Fe and Zn, were found in the plant-derived protein hydrolysates [45]. Our results are in total agreement with these observations as hydroxyproline was the major amino acid constituent of the examined hydrolysates. It is also worth noting that fish collagen is richer in almost all detected amino acids, excluding Leu and Asp; thus, from the practical point of view, it seems to be more valuable than the bovine one.

It was reported that foliar application of animal- and plant-derived protein hydrolysates promotes the vegetative growth and yield of several fruit trees [30,48]. An analogous effect of animal-derived protein hydrolysate was observed for seedling growth and productivity of papaya plants (*Carica papaya* L.) [49]. Stimulation of growth by animal-derived biostimulant was also recorded for maize (*Zea mays* L.) [45], ornamental snapdragon plants (*Antirrhinum majus* L.) [11] and several crop species (cucumber, arugula, broccoli, tomato, pepper and maize) [50]. However, some experimental studies revealed that benefits from soil drenching and foliar spray with collagen-derived agents were minimal or nonsignificant. For example, no significant effects of the amino acid based biostimulant on the yield (measured as DW) of spinach (*Spinacia oleracea* L.) [51] or of endive plants (*Cichorium endivia* L.) were found [52]. Similarly, a study on carrot (*Daucus carota* L.), showed increased yield only in one of three tested cultivars in only one of three years of field trial [53]. However, this can be the result of mismatching of collagen-derived agents and plant nutritional demands. It was recently proposed that lack of benefits depended on the origin of protein hydrolysates (animal or vegetal), production method (chemical or enzymatic hydrolysis), target plant species, application method and environmental conditions [48]. Our study indicated only slight benefits of the tested binding agents in terms of plant growth stimulation. However, the results denoted that animal waste-derived collagens and PHMB improved maximal efficiency of PSII (F_V/F_M). Furthermore, considering trends of changes of several chlorophyll *a* fluorescence parameters in plants established from coated seeds (most notably PI_{ABS} and DI_0/RC), slight improvement of energy flux between light-harvesting pigments and reaction centers can be predicted [54]. As observed changes in chlorophyll *a* fluorescence were not additionally modified by dolomite coating, it can be hypothesized that lesser growth of dolomite-treated plants was caused by Ca-specific ion toxicity or Ca/Mg antagonism, but not perturbations in functioning of photosynthetic apparatus. Moreover, no negative effects on chlorophyll content were observed. This is in agreement with other studies showing positive effects of animal-derived substances on leaf pigmentation and/or photosynthetic efficiency [55,56]. Even if carbon skeletons from photosynthesis are not used as a resource for growth, they can be still redirected into other metabolic processes that do not consist in increment of biomass *per se*, i.e., processing of metabolites, maintaining homeostasis or fine-tuning of defense. It can be very handy under field conditions, as plants with better photosynthetic performance are more tolerant to environmental stress [57]. There is also some experimental evidence supporting coincidence of hydrolysate-triggered improvement of photochemical activity of the photosystem II and nutritional status of a plant [58]. This was confirmed by our study, as collagens and PHMB caused greater root uptake of Mg, an element with a crucial role in primary plant metabolism. Similar results were presented for white ice lettuce (*Lactuca sativa* L.) [59] fertilized with fish waste compost. Mg plays a central role in the functional properties of chlorophylls and is a co-factor and allosteric modulator for >300 enzymes [60]. This partially explains the better photosynthetic performance of the studied pea plants and probably allows optimization of transport of assimilates from source leaves to sink organs [60].

It must be pointed out, however, that in addition to the abovementioned metabolic upgrades, coating of seeds with the studied collagens entailed some weighty and adverse nutritional alternations, namely accumulation of Sn, Cr and Na. Sn is an element naturally occurring in arable soils. For example, European soils contain from <2 to 106 mg Sn kg^{-1} soil (0.1–2.6 in Poland; central Europe) [61]. Thus, BC collagen containing 6.6 mg Sn kg^{-1} soil should be treated as a serious source of phytotoxic contamination, as it caused bioaccumulation of this element in the presented study. The levels of Sn recorded in this investigation represented upper marginal concentrations of this element in plant tissues (<0.10–3.0 mg kg^{-1}) [61]. Furthermore, Sn (believed to be an element with low ability to transfer into aboveground plant organs from roots) [61] was efficiently translocated into shoots of the studied pea plants (ca. 2.5 mg kg^{-1}), which poses a potential risk to trophic chains and human nutrition. It is very probable that this results from accumulation of Sn in bovine tissues (0.02–0.08 mg kg^{-1}), as was recently reported [62]. In BC-treated plants, the contents of another anthropogenic element, Cr, were highest, which can be the result of a tanning process [63]. It is in agreement with recent studies showing that leather-derived collagen contains moderate (ca. 30–90 mg kg^{-1}) amounts of Cr due to the fact that basic Cr salt is still used as a tanning material for large-scale production of leather [64,65]. Although our results showed that bovine collagen contained only traces of Cr (III), it should not be excluded that total Cr content accounted for the high content of this element in the shoots of BC-treated plants. In summary, it should be taken into consideration that usage of bovine collagen increases agroenvironmental Sn and Cr load [64,65]. Additionally, it is widely accepted that Na^{+}-associated osmotic stress is among major abiotic hindrances to plant cultivation [66]. Therefore, ca. 100% increase in Na content in the roots of plants established from FC-coated seeds (in comparison to the control plants) suggests possible osmotic stress resulting in wide nutritional and metabolic changes in plant functioning [67]. Similar observations were reported in fish-waste-fertilized white ice lettuce (*Lactuca sativa*) [59].

It was previously shown that the application of dolomite did not affect nutrient availability, nutrient uptake or plant growth of maize (*Zea mays*) [21]. Application of dolomite alone and with another mineral fertilizer, leonardite, also did not enhance root dry matter gain [68]. However, the dolomite addition can increase plant weight, chlorophyll contents and uptake of N and K in some plant species, e.g., *Petiveria aleaceae* L. [69]. Our findings showed that usage of dolomite had even more adverse effects, as strongly reduced growth and perturbations in nutrition of pea were observed. Similar observations were presented for oil rape (*Brassica napus* L.) and spring barley (*Hordeum vulgare* L.) fertilized with industrial-waste dolomite powder [70]. However, studies also indicated that the final output of dolomite usage depends on the individual requirements of a given species [69]. Application of dolomite may cause an increase in soil alkalinity [69], which is negatively correlated with the size of dolomite dust grains [71]. As the dolomite powder we selected had particle size ca. 10 times smaller (45 μm) than the grain size that was suggested to increase soil pH (>0.4 mm) [71] and ca. 30 times smaller than the grains ineffective in this process (1.3 mm), strong local reduction in soil acidity is very probable to be a reason for the presented results. However, observed growth deceleration can be also a result of elemental imbalance due to supraoptimal load of selected elements from dolomite (most notably Al and Fe), which was strongly marked in plants treated exclusively with dolomite.

PHMB was only rarely listed as a potentially valuable compound to be applied in agriculture (mostly for postharvest preservation of fruits) [72]. The antimicrobial properties of PHMB are connected with its ability to destabilize membranes; however, it can also chelate metals due to five conjugated amines from the biguanide molecule [32]. These properties may partially explain the lesser contents of some metals (Al and Zn) and osmolytes (Na) in the shoots recorded in this study. Interestingly, moderate perturbations pertaining to element acquisition recorded in the roots were intensified by dolomite, and thus potential interaction of PHMB with Ca^{2+}, Mg^{2+} or CO_3^{2-} is very probable and needs further elucidation. It seems that PHMB is a promising compound reducing potentially detrimental phytoaccumulation of Mn, Sr, Ti and Zn under natural or artificial alkalization of the soil microhabitat.

Testing seedling emergence from different depths can give valuable information about the performance of a plant and its ability to establish using limited resources [73]. As pea was showed to have the best ability to emerge from deep soil layers among several plant species from the Fabaceae family [74], this study was intended to evaluate treatment-caused changes in the emergence speed and synchrony. In general, coating did not cause any changes in FEP, which indicates that the seeds did not suffer any substantial damage from the used chemicals or from the technological process of coating; however, this is not fully evidenced in the case of BC-treated plants, as greater FEP values resulted in greater risk of accumulation of Sn and Cr in the trophic chain [61]. Only dolomite-coated seeds showed lesser FEP, IEV and IES values, which may be the result of inhibitory [75] and/or cytotoxic effects of Ca^{2+} at the germination stage.

5. Conclusions

Treatment of the pea seeds with BC, FC and PHMB affected plant growth and development. FC and PHMB used for coating best improved plant standing. Thus, they seem to be promising substitutes for mineral fertilizers and growth regulators (hypothesis 1). However, usage of BC and dolomite powder should be limited due to their detrimental effects on plant growth and nutrition and/or due to potential risk of contamination with anthropogenic pollutants (hypothesis 2). The results indicated that growth and primary functioning of plants can be improved by seed coating with waste-derived materials. However, one can see that waste-based biologically active agents can be treated as biostimulants or anthropogenic pollutants, depending on their chemical composition. Although biostimulation (including seed-coating-based methods) has gained popularity in recent times, much effort is still needed to screen for the most valuable and environment-friendly substances. The results obtained in this study strongly suggest that potential contamination of a given substance should be coinvestigated with its biological activity. The most valuable waste-derived agents can be then treated as real biostimulants that can be efficiently applied by the seed coating method in order to improve the field performance of crops. In summary, the most promising agents should be valorized for modern agriculture, whereas those causing potential risk for environments should be used with great caution, or their usage should be abandoned.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4395/11/6/1034/s1, Table S1: Properties of soil used for plant cultivation, Table S2: Parameters derived from the OJIP transient used in this study, formulas of their calculation and definitions, Table S3: Results of two-way ANOVA (F values) examining the effects of the studied factors on measured traits of *Pisum sativum* L. plants subjected to different seed coating treatments, Table S4: Results of three-way ANOVA (F values) examining the effects of the studied factors on final emergence percentage (FEP), index of emergence velocity (IEV) and index of emergence synchrony (IES) of *Pisum sativum* L. seeds subjected to different seed coating treatments, Figure S1: Fresh weight (A) and dry weight (B) shoot/root (S:R) ratio of *Pisum sativum* L. plants subjected to different seed coating treatments, Figure S2: Elemental composition (Cu, Mo, Ni, Sr, Ti) of roots of *Pisum sativum* L. plants subjected to different seed coating treatments, Figure S3: Elemental composition (Cu, Mo, Ni, Sr, Ti) of shoots of *Pisum sativum* L. plants subjected to different seed coating treatments.

Author Contributions: M.S. proposed the study concept; M.S., M.W. and J.K. designed the study; M.S., M.W., K.S., M.L.-R. and A.O. conceived the experiments; M.W., M.S., J.K., K.S., M.L.-R. and K.Ł. analyzed the results; M.S., M.W. and J.K. prepared the first draft of the manuscript; all of the authors wrote and reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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