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USE OF BIOFERTILIZANT BASED ON COLLAGEN HYDROLYSATE FOR CEREAL SEED TREATMENT

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Abstract

Sowing seeds of cereals (wheat, barley, rye, oats) in drought soil results in a low emergence rate of sprouts, mainly by low moist content of soil. Using collagen hydrolysate solution resulting from leather industry by-products for treatment of cereal seeds increases germination rate and energy of germination rate. To be effective, the seed treatment must ensure enough moisture at seed surface and must not dissolve into soil solution. This paper aims at presenting new collagen-based materials for cereal seed treatment, which generates an increase of the quality indicators for treated seeds. Creation of a new and advanced technology for treatment of cereal seeds, by using collagen hydrolysate, has the objectives of increasing seed quality indexes; achieving a better management of water resources during germination processes, while preserving the environment. The technologies developed for protein raw material processing and characteristics of collagen hydrolysates with bioactive properties are presented. The obtained collagen hydrolysates were analyzed in terms of average molecular weights by electrophoresis, and in terms of particle size by DLS (Dynamic Light Scattering) using a Zeta Sizer device and the amino acid composition of collagen hydrolysates, determined by high-performance liquid chromatography. The cereal seeds treated with collagen hydrolysates have been analyzed using a special high-resolution tomography device in order to observe the morphological modification of seeds in germination process and an HPLC device was used for chemical modification in seed composition during germination process. High levels of Gibberellic Acid content have been observed on the seeds treated with collagen hydrolysate in comparison with untreated seeds during germination process. High biomass of cereal seedling has been measured for seeds treated with collagen hydrolysate in comparison with untreated seeds.

Key words*: Collagen hydrolysates, Gibberellic Acid, Seed treatment.*

INTRODUCTION

Research studies have recently identified new collagen resources, especially for making bio-materials intended for the health field (Shah and Manekar, 2012; Aleman and Martinez-Alvarez, 2013; Ferraro et al., 2013; Siddiqui et al., 2013; Song et al., 2014). The protein waste from leather industry is a valuable source of organic nitrogen with potential to be released in time, useful for plant protection in condition of unfavorable conditions of soil and weather. The free amino acids content of collagen hydrolysate showed growth stimulation effect for vegetable plants (Gaidau et al., 2009; Chitu et al., 2010). Many reports were dedicated to the application of leather industry waste in agriculture (Mohammad et al., 2014; Gaidau et al., 2013) and to the improvement of collagen extraction technologies (Shanthi et al., 2013; Dettmer et al., 2013).

The application of collagen hydrolysate on seed treatment represents an innovative approach with few references in the literature.

The influence of collagen hydrolysate treatment to seeds on growth stimulation is a very important topic which can contribute to crop production increasing and agriculture sustainability.

The paper is focused on application of well characterized collagen hydrolysate in cereal seed treatment, the influence on seed growth stimulation and crop production improvement.

MATERIALS AND METHODS

Collagen hydrolysate extraction from bovine leather waste

Leather wastes originated from shaving of mineral free of chromium wet-white leathers with the following characteristics: 15.6% volatile matter, 13.6% total ash, 14% total nitrogen and 79.2% dermic substance, 11.7% metallic oxides, aqueous extract pH value of 2.6 were subjected to the chemical-enzymatic extraction of collagen hydrolysate.

The method for collagen hydrolysate extraction was similar with the method performed on wet-blue shavings (Gaidau et al., 2013; Gaidau et al., 2009): wet-white shavings were put in a vessel of 50L equipped with thermal isolation, refrigerator, automated stirring, time and pH monitoring (Figure 1) with 600% water at 80°C and with 12% $Ca(OH)_2$ (% reported at shaving weight). The extraction was made under continuous stirring at constant temperature during 2 hours and at pH of 8.5-9.0. The reaction mass was cooled down to 68-70ºC when 1% Alcalase 2.5L was added and hydrolysis was continued for 3 hours. The enzyme deactivation was done at 90ºC by stirring for 10 min. The solution was cooled over night and then the solution was filtered and packed. The schematic technology is presented in Figure 1.

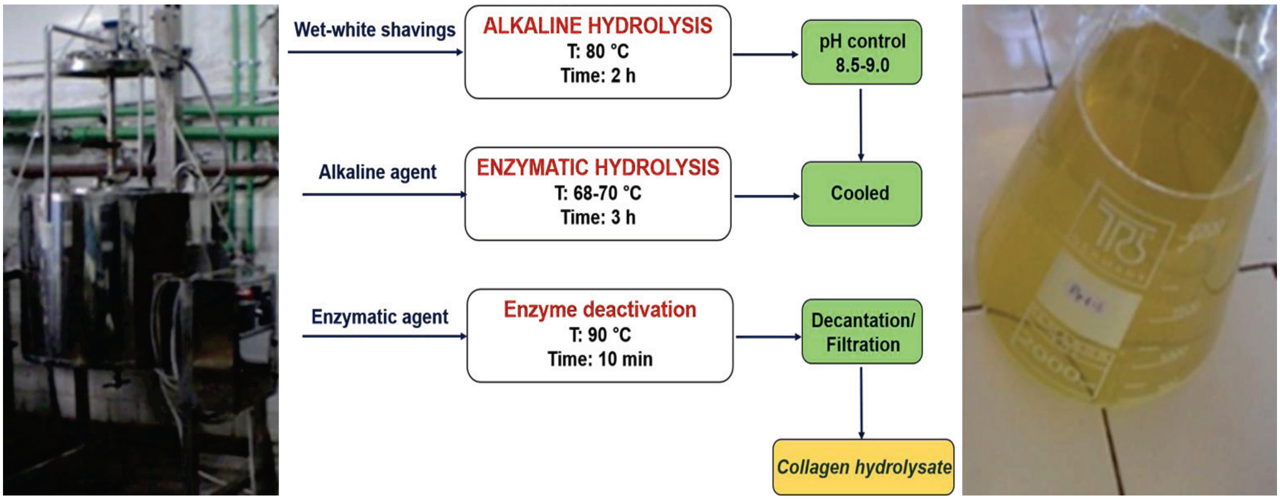

Figure 1. The flow chart for collagen hydrolysate extraction

Collagen hydrolysate characterization

The collagen hydrolysate (Figure 1) was analysed in terms of dry substance, total ash, metal oxide, calcium oxide, total nitrogen and protein substance, aminic nitrogen, sulphates, chlorides, pH, molecular weight (SDS-PAGE) and amino acid content (HPLC, Thermo Electron, Finningen Surveier with DAD detector). Particle size and distribution of collagen hydrolysate was determined with ZetaSizer device Nano ZS (Malvern, UK).

X-ray microtomography on wheat seeds

Computed tomography (CT) custom made with mini X-ray source and cheap flat panel CMOS sensor with space resolution of 10 µm/voxel was used for wheat seed morphology analyses. Subject used have been wheat seeds treated with water and with collagen hydrolysate fixed to germinate on the wet support of a filter paper. After 12 h, 24 h and 36 h the CT images were recorded.

Analysis of gibberellic acid from germinated wheat seeds

Water and methanol of HPLC grade were used for extraction of the gibberellic acid (GA3) and for preparation of HPLC mobile phase. Acetic acid of analytical purity (Merck) used for preparation 0.1M acetic acid solution (HPLC mobile phase A). Gibberellic acid of standard purity (Sigma) was used for preparation the stock standard solution (100 ppm) by dissolving in methanol / 0.1M acetic acid 50:50 (v/v) mixture. Working solutions were prepared by diluting the stock solutions with the HPLC aqueous mobile phase (A) at appropriate

concentrations. Working solutions were prepared fresh on the day of use. These solutions were filtered before injections through 0.45μm syringe filters. Fifty grams of wheat grains per sample was socked for one hour, at room temperature in a dark place, as follow: one sample with water and one sample with collagen hydrolysate, solution 7.5%. After 1 h, the seeds were incubated in a thermocabinet at 20ºC, for 24 hours. Then the sample were homogenized with 100 mL methanol 80% (v/v) for 4 hours on a rotary shaker, at 120 rpm. The extract was filtered through a Whatman filter and the methanol evaporated under vacuum. The residue was transferred into 10 mL volumetric flask with 0.1M acetic acid solution (mobile phase A) and stored at 4ºC for further analysis. The chromatographic analysis was performed on an Agilent HPLC 1100 series with quaternary pump, diode array detector and auto sampler. This equipment has a column thermostat and a degasser system.
The column used was a Zorbax Eclipse C18 (100 mm x 4.6 I.D) stainless steel analytical column with 3.5 μm particle size. Throughout this study, the mobile phases used were 0.1 M acetic acid solution (mobile phase A) and methanol (mobile phase B).
The separation was carried using a gradient elution from 0% to 100% methanol, in 15 minutes, with 1 mL/min flow rate. The chromatographic C18 column was equilibrated in mobile phase A for 30 min and was maintained at constant 25ºC. The signal of the compound was monitored at 254 nm. An injection volume of 10 μL was used for each analysis.
The standard solutions were chromatographed to determine the retention time for GA3 and to obtain a calibration curve in a linear calibration range.
Peak identification was based on retention time and spiking of the sample with gibberellic acid standard.

Quantity evaluation of biomass of wheat seedling
For experiments made in laboratory accordingly with standard of germination SR-EN- 1634/1999 about influence of treatment with collagen hydrolysate solution 7.5% on the growing rate of seedlings of wheat have been used 3 variants in 4 repetitions, each of 100 seeds of wheat. The wheat seeds have been laid on the filter paper at uniform distance among seeds. Seeds have been treated with collagen hydrolysate solution 3 l/t for variant V1, 5 l/t for variant V2 and untreated for V0.
After that the paper have been rolled and introduced into thermocabinet BINDER at 20ºC and 80% relative humidity of air for 8 days.
After 8 days samples have been extracted from thermocabinet, seedling have been removed from paper and weighing on an analytical weighing with precision ±0.01 g. For each variant 4 replicates have been measured.

RESULTS AND DISCUSSIONS

The characteristics of collagen hydrolysate for agriculture use
The results of chemical analyses showed that the collagen hydrolysate has 7.5% dry substance, 94.1% organic substances, 16.0% total nitrogen, 90.1% protein content, 1.33% aminic nitrogen, 5.8% total ash, non-detectable heavy metal oxides, 2.9% calcium oxides, 462 mg/dm^3 sulphates, 1613 mg/dm^3 chlorides and pH of 9.7.
The SDS-PAGE measurements confirmed the low molecular weight of collagen hydrolysate (sample 6) as compared to the collagen hydrolysate extracted with the same method from wet-blue shaving (sample 4). The Figure 2 presents the obtained collagen hydrolysate (sample 6) in comparison with molecular marker (MM) and other collagen hydrolysates extracted in different conditions (samples 1, 2, 3, 4 and 5) being under 17 kDa.

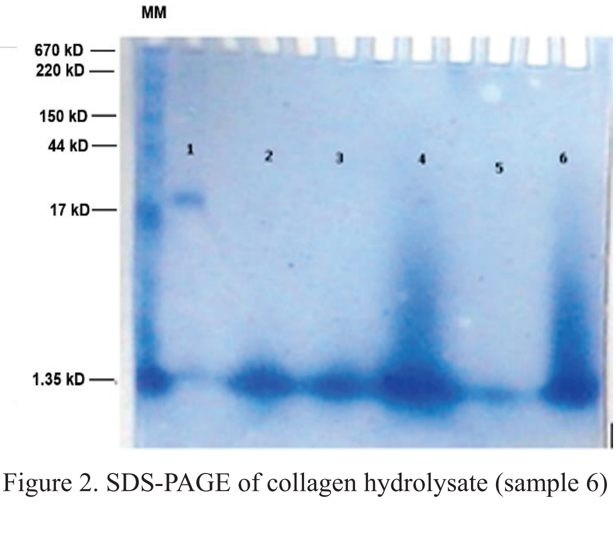

Figure 2. SDS-PAGE of collagen hydrolysate (sample 6)

The analysis of free amino acids of collagen hydrolysate showed a content of 8.339 g/100 mL collagen hydrolysate with the following composition: 0.146% glutamic acid, 1.217% glycine, 0.164% alanine, 1.150% arginine, 0.495% valine, 0.135% lysine, 5.475% histidine, 0.379% proline and 0.238% methionine. The free amino acid content provides growth stimulation and nutrition to cereal seeds and plants. The increase of seed health contributes to the quality indexes improving the cereal crop production.

The collagen hydrolysate particle size and distribution (Figure 3) were measured in comparison with other collagen hydrolysate extracted from wet-blue shavings (Figure 4) by Dynamic Light Scattering with NanoZS Zeta-Sizer (Malvern).

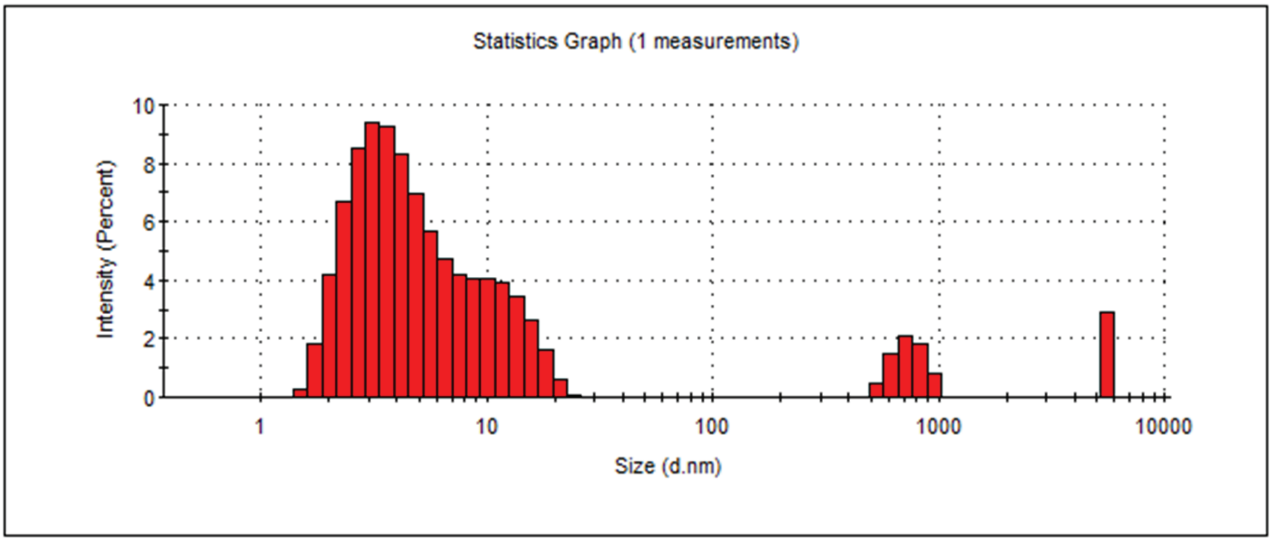

Figure 3. Collagen hydrolysate particle size and distribution (sample 6)

The particle size and distribution of collagen hydrolysates showed 3 populations of particles, the main population (85.6%-90.2%) being in the range of 4-11 nm. The other populations are of 484 nm-743 nm with a concentration of 6.7%-12.9% and the smallest population of 5.56 µm with a concentration of 1.3-2.9%. The wet-white origin collagen hydrolysate (Figure 3) is rich in small particles with 11nm medium size (90.2%) and 9.8% particles of 0.74-5.56 µm size. The collagen hydrolysate extracted with the same method (Figure 1) from wet-white and wet-blue shavings (Figure 4) have similar particle size and distributions. We suppose that this particle size and distribution ensure the best penetration and film forming properties for seed protection and plant nutrition.

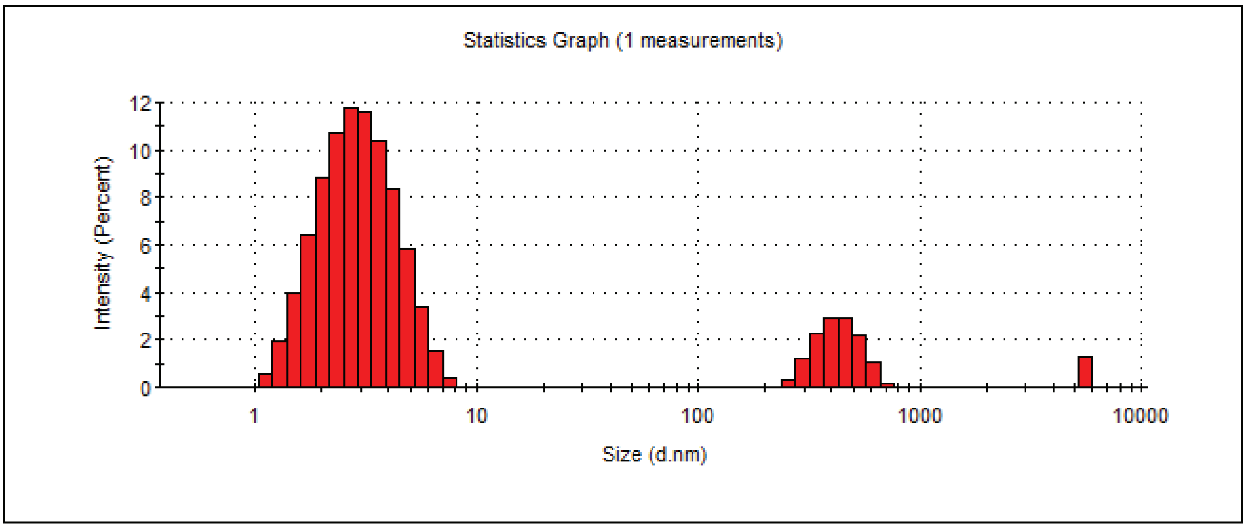

Figure 4. Collagen hydrolysate particle size and distribution (sample 4)

The results obtained indicated an acceleration of germination process of wheat seeds in first 36 h after started germination process (imbibition with water), for using treatment of collagen hydrolysate. In the germination process water penetrate the seed tissue to the *embrionum*. By imbibition with water the α-amylase enzyme are activated and protein content of wheat seed are transformed in amino acids. Amino acids are precursors of gibberellic acid which stimulate the elongation of cell and growing of coleoptile and embryonic radix (Dettmer et al., 2013). Amino acids from collagen hydrolysate penetrate the cell from wheat seed and are transformed direct in gibberellic acid.

The analysis of influence of the free amino acids of collagen hydrolysate on germination process are showed in Figure 5. The *embrionum* of wheat seed treated with collagen hydrolysate are imbibed faster than *embrionum* of untreated wheat seed (imbibed just with water). Germination starts with the uptake of water by imbibition of the dry seed, followed by embryo expansion. This usually culminates in rupture of the covering layers (testa and endosperm) and emergence of the radicle, generally considered as the completion of the germination process. Radicle protrusion at the completion of seed germination depends on embryo growth driven by water uptake (Muntean, 1995).

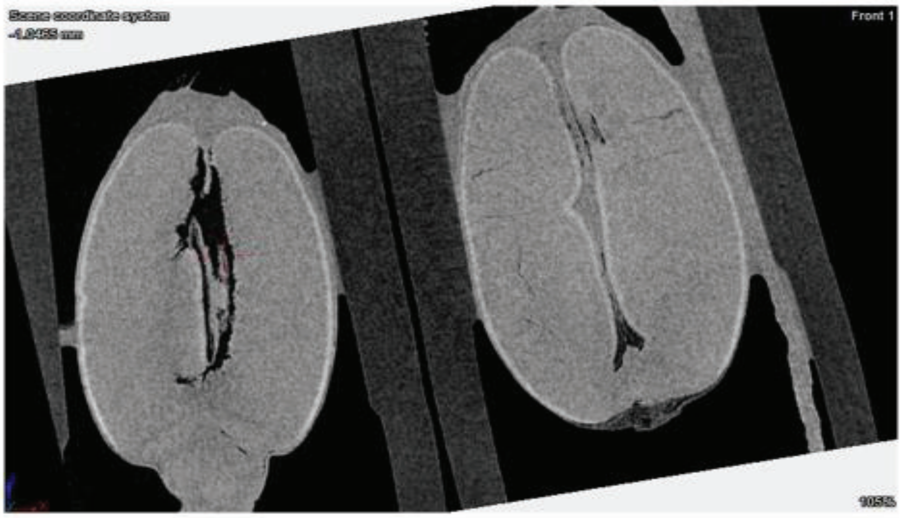

Figure 5. CT caption after 12 h of germination (left side germination of wheat seed treated with collagen hydrolysate and right side germination of wheat seed treated with pure water)

After 24 h of germination the untreated seed develops a coleoptile with length of 1.23 mm and a diameter of 0.89 mm (Figure 6).

Figure 6. CT caption after 24 h of germination of untreated wheat seed

After 24 h of germination the seed treated with collagen hydrolysate develops a coleoptile with length of 1.93 mm and a diameter of 1.45 mm, and a radicle with length of 1.15 mm and a diameter of 0.76 mm, as is presented in Figure 7.

Figure 7. CT caption after 24 h of germination of wheat seed treated with collagen hydrolysate

After 36 h of germination the untreated seed develops a coleoptile with length of 2.81 mm and a diameter of 0.69 mm, and no radicle, as is presented in Figure 8, but the seed treated with collagen hydrolysate develops a coleoptile with diameter of 1.84 mm and two radicles (Figure 9).

Figure 8. CT caption after 36 h of germination of untreated wheat seed

Figure 9. CT caption after 36 h of germination of wheat seed treated with collagen hydrolysate

The gibberellic acid analyses (Figure 10) showed higher values for the wheat seeds germinated in collagen hydrolysate (0.12 mg GA3 per 100 g germinated wheat seeds) as compared to the seeds germinated in water (0.08 mg GA3 per 100 g germinated wheat seeds), in good correlation to the computed tomography conclusions.

As seen in Figure 10, the results proved that GA3 is better released by the wheat grains after imbibition with collagen conclusions. As seen in Figure 10, the results proved that GA3 is better released by the wheat grains after imbibition with collagen hydrolysate as compare to the sample germinated in water.

The content of gibberellic acid of wheat seeds treated with collagen hydrolysate solution is higher with 50% than content of gibberellic acid of wheat seeds imbibed only with water.

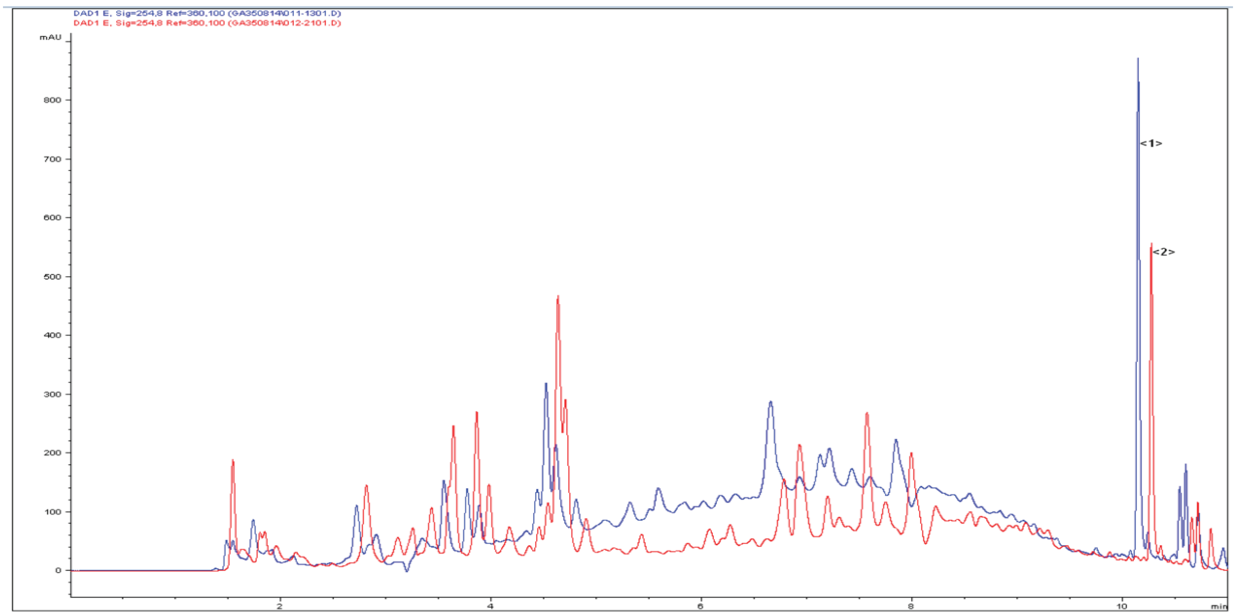

Figure 10. The chromatograms for: <1> GA3 extracted from wheat seeds germinated with collagen hydrolysate; <2> GA3 extracted from wheat seeds germinated with water

The total biomass developed by germination of 100 seeds of wheat treated with collagen hydrolysate solution 7.5% have been heavier that biomass developed by germination of 100 seeds of wheat imbibed with water (Table 1).

For growing quantity of treatment solution of collagen hydrolysate solution from 3l per ton of seed to 5l per ton the weight has risen but not significantly.

Table 1. Biomass of seedlings of wheat after 8 days of germination

Variant	R1	R2	R3	R4	Average	Difference to control	Significance
V1	17.01	15.66	16.15	14.91	15.93	4.01	***
V2	16.07	15.69	16.18	16.05	16.00	4.08	***
V0	11.82	11.72	11.6	12.54	11.92	MT	MT
					LSD 5%=	0.9326	
					LSD 1%=	1.2508	
					LS 0.1%=	1.6415	

CONCLUSIONS

Collagen hydrolysate solution resulting from leather industry by-products used for treatment of cereal seeds increases the biomass of wheat seedlings under the same conditions of seed moisture and relative humidity of air, which indicates a better performance of seeds treated with collagen hydrolysate solution under drought conditions.

A technology for extraction of collagen hydrolysate with bioactive properties has been developed.

The obtained collagen hydrolysate was analyzed in terms of average molecular weights, particle size, and the amino acid composition; the concentration of 7.5% has been selected as compatible with wheat seeds.

The high-resolution computer tomography performed on wheat seeds fixed for germination evidenced that the seeds treated with collagen hydrolysate solution have a higher growing rate than seeds imbibed just in water. Morphological modification of seeds in germination process has been observed.

A higher concentration of gibberellic acid has been extracted and quantified from the germinated seeds treated with collagen hydrolysate in comparison with untreated seeds in good correlation to the morphological modifications. High biomass of cereal seedling has been measured for seeds treated with collagen hydrolysate in comparison with untreated seeds.

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