VALORIZATION OF COLLAGEN AND KERATIN BY-PRODUCTS FROM LEATHER INDUSTRY TO INCREASE THE QUALITY OF PRODUCTION FROM A CHERRY ORCHARD

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Received: 22.05.2024 Accepted: 13.09.2024 https://doi.org/10.24264/lfj.24.3.2

VALORIZATION OF COLLAGEN AND KERATIN BY-PRODUCTS FROM LEATHER INDUSTRY TO INCREASE THE QUALITY OF PRODUCTION FROM A CHERRY ORCHARD

ABSTRACT. This paper presents the results of valorization of the protein by-products recovered from the leather processing industry in the horticultural field. Collagen and keratin extracts were the starting point for protein combinations containing nanometric particles that indicate the presence of amino acids and oligopeptides, recognized for the effects of biostimulation, nutrition and systemic protection of plants. The protein extracts associated with plant extracts for the development of a biofungicidal product, with the double action of antifungal protection of plants and stimulation of agricultural production, were tested in a cherry orchard. The test results of two of the variants of the biofungicidal product show better fruit quality indicators and increased production, both compared to a standard treatment and compared to a variant to which no specific treatment was applied.

KEY WORDS: byproducts, collagen, keratin, orchard production

VALORIZAREA SUBPRODUSELOR DE COLAGEN ȘI CHERATINĂ DIN INDUSTRIA DE PIELĂRIE PENTRU CREȘTEREA CALITĂȚII PRODUCȚIEI DINTR-O LIVADĂ DE CIREȘI

REZUMAT. Această lucrare prezintă rezultate ale valorificării în domeniul horticol a sub-produselor proteice recuperate din industria de prelucrare a pielii. S-au realizat extracte de colagen și cheratină din care s-au obținut combinații proteice cu conținut de particule nanometrice care indică prezența aminoacizilor și a oligopeptidelor, recunoscute pentru efectele de biostimulare, nutriție și protecție sistemică a plantelor. Extractele proteice asociate cu extracte vegetale pentru dezvoltarea unui produs biofungicid, cu dublă acțiune de protecție antifungică a plantelor și de stimulare a producției agricole, au fost testate într-o livadă de cireși. Rezultatele testelor a două dintre variantele de produs biofungicid prezintă indicatori de calitate a fructelor mai buni și producții sporite, atât comparativ cu un tratament standard, cât și comparativ cu o variantă la care nu s-a aplicat un tratament specific.

CUVINTE CHEIE: produse secundare, colagen, cheratină, producția livezii

LA VALORISATION DES SOUS-PRODUITS DE COLLAGÈNE ET DE KÉRATINE DE L'INDUSTRIE DU CUIR POUR AUGMENTER LA QUALITÉ DE LA PRODUCTION D'UN VERGER DE CERISIER

RÉSUMÉ. Ce travail présente les résultats de la valorisation dans le domaine horticole des sous-produits protéiques récupérés de l'industrie de transformation du cuir. Des extraits de collagène et de kératine ont été réalisés à partir desquels des combinaisons de protéines ont été obtenues avec une teneur en particules nanométriques indiquant la présence d'acides aminés et d'oligopeptides, reconnus pour les effets de biostimulation, de nutrition et de protection systémique des plantes. Les extraits protéiques associés à des extraits de plantes pour l'élaboration d'un produit biofongicide, à double action de protection antifongique des plantes et de stimulation de la production agricole, ont été testés dans la cerisaie. Les résultats des tests de deux des variantes du produit biofongicide montrent de meilleurs indicateurs de qualité des fruits et une production accrue, tant par rapport à un traitement standard que par rapport à une variante à laquelle aucun traitement spécifique n'a été appliqué.

MOTS CLÉS : produits secondaires, collagène, kératine, production fruitière

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INTRODUCTION

The BIO-PLANT-Protect project as presented in a previous work [1] is a European cooperation between Romania and Poland to capitalize animal and vegetable waste in circular agriculture and to develop new bio-fungicidal compositions with bivalent activity: protection against pathogens and bio-stimulation of germination and growth of plants.

The increasingly intensive promotion of organic agriculture keeps up-to-date research for the recovery of proteins (primarily nitrogen carriers) from various secondary sources and their validation for use in plant culture [2].

The natural leather processing industry, through the specificity of its processes, is an important source of protein by-products, collagen and keratin, which can be exploited for agricultural use [3], due to the high nitrogen content and the wide spectrum of amino acids with potential of protection against abiotic stress and stimulation of plant growth [4].

Many research studies are still in progress, both for improving the processes of obtaining hydrolysates of collagen and keratin [5, 6], and those related to the use of these hydrolysates to increase the yields of agricultural crops [7, 8]. The extraction of collagen as gelatin is also currently being studied [9].

The previous results of the BIO-PLANT-Protect project demonstrated the antifungal effect of the developed products and the ability to bio-stimulate the germination of horticultural plant seeds [10, 11].

In the present research protein combinations based on gelatin, collagen hydrolysates and keratin hydrolysate dedicated to association with plant extracts with fungicidal effects, were tested in the orchard on fruit of the cherry species, to evaluate the nutritional potential of the protein components.

This paper presents the protein extracts made from leather byproducts such as gelatin, collagen hydrolyzate and keratin hydrolysate, and the effects of treatments in the cherry orchard with the protein combinations recovered from the leather industry.

The cherry orchard tests were carried out with two types of protein combinations recovered from the leather industry, a treatment with a protein combination consisting of gelatin and collagen hydrolyzate extracted from untanned leather and a treatment with a protein combination consisting from gelatin, collagen hydrolyzate extracted from tanned leather and keratin hydrolyzate extracted from sheep wool.

The cherry orchard tests have shown that treatment with the experimental version with keratin content has determined the highest values of fruit weight, firmness, pH and of the total soluble substance content, compared to an untreated control and with Standard treatment. Significantly higher productions, compared to an untreated control and with Standard treatment, were obtained with both experimental treatments.

EXPERIMENTAL

Materials and Methods

Materials

Bovine leather and sheep wool by-products have been used for collagen and keratin extracts as gelatin and collagen and keratin hydrolysates: residual semi-processed bovine leather for collagen extraction was collected from the leather processing pilot station of INCDTP – Division: Leather and Footwear Research Institute, chopped, and preserved by freezing; wool for keratin extraction was purchased from sheep farmers and degreased at the INCDTP – Division: Leather and Footwear Research Institute.

Analytical grade chemical reagents: hydrated calcium oxide (CaO CaOH, MW = 81.371 g/mol) was purchased from Cristal R Chim SRL (Bucharest, Romania); ammonia potassium hydroxide and oxalic acid were purchased from Chimreactiv SRL (Bucharest, Romania); propionic acid was purchased from Sigma-Aldrich (Bucharest, Romania).

Enzymes: for collagen hydrolysis, Alcalase 2.4 L (protease from *Bacillus*

licheniformis with 2.4 U/g activity); for keratin hydrolysis, Protamex® (an endo-protease from *Bacillus* spp. with 1.5 U/g activity) was purchased from Novozymes (Atasehir, Turkey).

Methods

Gelatin and collagen hydrolysates from residual untanned leather by-products were prepared by thermal and enzymatic hydrolysis. Also, collagen hydrolysate was prepared by alkaline and enzymatic hydrolysis of residual bovine-tanned leather. Keratin hydrolysate was prepared by alkaline and enzymatic hydrolysis from degreased residual sheep wool. For pH adjustment propionic acid was used in collagen extraction and oxalic acid in keratin extraction. The protein extraction processes are shown in Figure 1.

The protein extracts and their combinations were analyzed physico-chemically to evaluate the most significant characteristics, for applications in the agricultural field: molecular weight, nanometric particle size distribution, amino acid content profile.

Methods for Characterizing Protein Extracts and Their Combinations

The average molecular mass of gelatin was determined by SDS Page electrophoresis in Mini-PROTEAN ® Tetra Cell 4 gel handcasting system (Tank, hand casting stand and accessories), with processing on the Gel Documentation Imaging Bio-Print XT4 which includes a scientific CCD camera with a Super Resolution of 5.5 megapixels; HPLC for the amino acid composition of gelatin, collagen, and keratin hydrolysates by using an Amino Acid Analyzer LC 3000 (Sykam GmbH, Eresig, Germany), equipped with a polymeric cation exchanger column, post-column ninhydrin derivatization at 125°C, and photometric measurement at 570 nm, with monitoring the results by Chromatography-Software ChromStar 6.0 (SCPA GmbH, Bremen, Germany); Dynamic Light Scattering (DLS) using a ZetaSizer Nano ZS (Malvern, UK) for the analysis of nanometric particle size and their distribution.

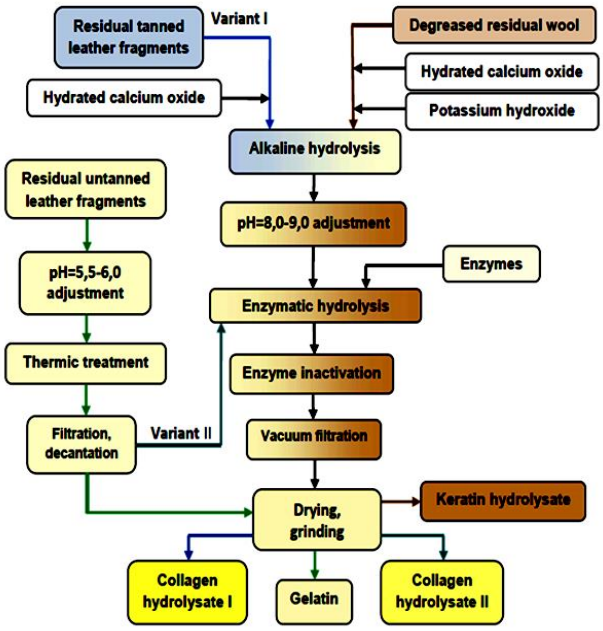

Figure 1. Protein extraction processes

The combined protein extracts, as part of a biopesticide prototype, were tested in the orchard on fruit of the cherry species, the Skeena variety, at Research and Development Institute for Fruit Growing, Mărăcineni, Pitești, Romania, to follow the effect of the

application of biopesticides with ambivalent activity, antifungal and fertilization, on the production and quality of the fruits.

The experiment was carried out with 3 repetitions in a completely randomized block, with 5 trees/repetition, to which the following treatments were applied: (1) untreated control; (2) treatment with the biopesticide prototype containing gelatin and collagen hydrolysate extracted from untanned leather; (3) treatment with the biopesticide prototype containing gelatin extracted from untanned leather, collagen hydrolysate extracted from tanned leather and keratin hydrolysate; (4) treatment with a standard product (Serenade® ASO).

Finally, the following indicators were analyzed: mass, pulp firmness, pH, total soluble matter content and fruit production. The observations were of a numerical type, with the aim of obtaining data and characteristic parameters for the objective evaluation of the fruiting processes.

The statistical method used to evaluate the differences between the averages of the determined indicators was the analysis of variance, and the differences between the variants were highlighted using the Duncan test for a confidence level α=0.05. The data were processed using the SPSS 14.0.0 program. The effect of the tested products was represented graphically.

RESULTS AND DISCUSSIONS

The average molecular mass of gelatin was determined by SDS Page electrophoresis with processing of the migration gel on the viewing camera. Figure 2 shows the migration gel, with the gelatin sample on line 12, the buffer on line 14 and the marker on line 15, detected automatically, as well as the details recorded by the viewing camera for the gelatin sample on line 12.

After processing the data recorded by the viewing camera, an average molecular weight of gelatin of 127 kDa was obtained, in accordance with the amino nitrogen content (< 0.2% of total nitrogen).

The amino acid profile and content in protein extracts determined by HPLC is shown in Figure 3.

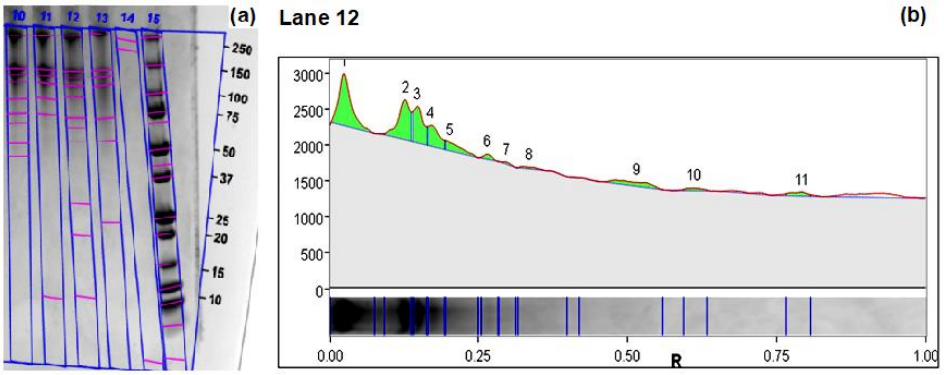

Figure 2. SDS Page electrophoresis of gelatin: (a) the gel after decolorization, (b) the molecular mass profile of the peptides, shown by the viewing camera

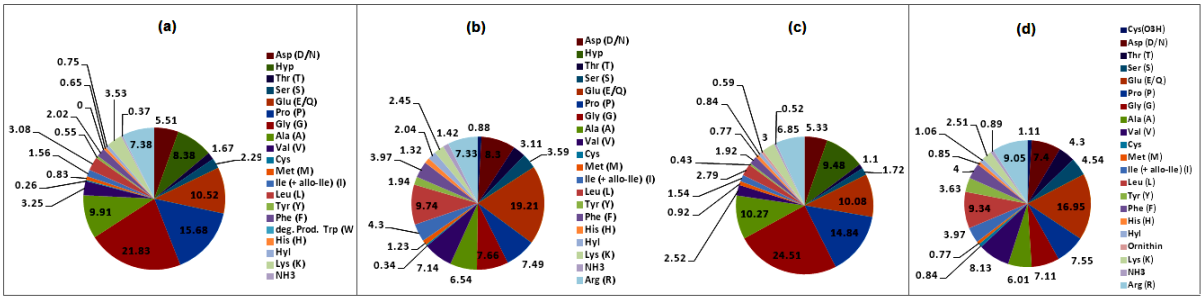

Figure 3. Amino acid profile of protein extracts: (a) gelatin extracted from untanned leather, collagen hydrolysate extracted from tanned leather, (c) collagen hydrolysate extracted from untanned leather, (d) keratin hydrolysate

A very wide profile of amino acids, including essential ones, capable of penetrating cell membranes is observed.

Figure 3 shows as a characteristic the presence of hydroxyproline in the collagen extracts, while the keratin extract is characterized by the presence of cysteine, which through the supply of sulfur (phytonutrient) can improve the yield and quality of crops.

The presence of traces of cysteine in gelatin and collagen hydrolyzate obtained from the same skin resource, is the result of the fact that the skin from which they were extracted, being a by-product, had processing deficiencies in the repair stage and therefore traces of hair remained which were degraded in the hydrolytic processes.

Moreover, it is observed that collagen extracts, obtained from different processes or sources, have significantly different compositions for certain amino acids because the fractured peptide chains were different, depending on the processing stage of the hides. The differences between the gelatin and the collagen hydrolyzate resulting from the residues left after gelatin extraction were recorded, in the content of threonine, serine, proline, valine, tyrosine. Also, the differences between the collagen hydrolyzate extracted from tanned leather and the collagen hydrolyzate resulting from the residues left after gelatin extraction were recorded, in the content of threonine, serine, tyrosine.

The two combinations of protein extracts, made for the biopesticide prototype, were analyzed by DLS confirming the content of nanometric particles, Figure 4.

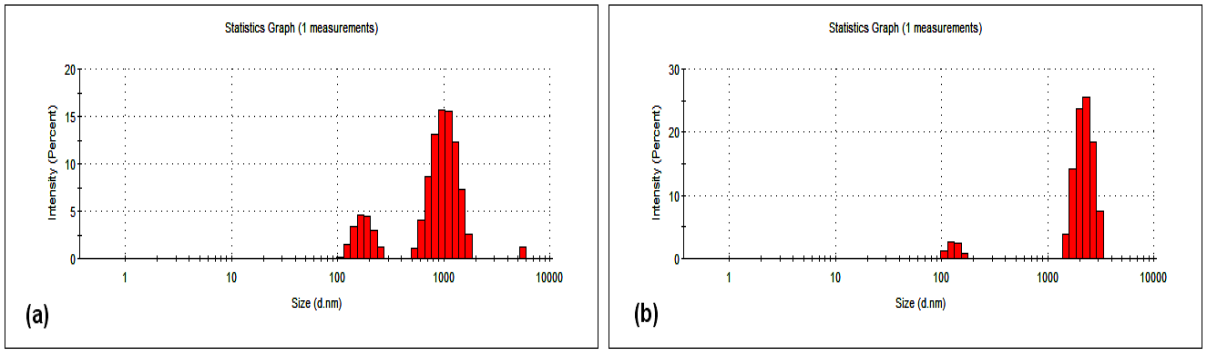

Figure 4. Particle size distribution in protein combinations: (a) gelatin and collagen hydrolysate; (b) gelatin, collagen hydrolysate and keratin hydrolysate

Figure 4 presents the histograms of the average results of three measurements performed for each of the two protein combinations tested.

The reflected light intensity measurements indicate the existence of both small and medium particle populations (100-1000 nm), as well as particle populations larger than 1000 nm. In the protein combination (a) we find a population of 14% particles of 100-200 nm, a population of 85% particles of 500-1500 nm and 1% particles of 5560 nm, which differentiates it from protein combination (b) in which we find a population of 7% particles of 100-200 nm and a population of 93% particles of 1500-3000 nm.

The content of nanometric particles is associated with the presence of amino acids and oligopeptides recognized for the effects of biostimulation, nutrition and systemic protection of plants.

The favorable effect of treatments in the orchard with protein components recovered from the leather industry was confirmed by the results of the tests initiated in the cherry orchard. Figure 5 presents the histograms of the measurements of the main quality indicators of the fruits harvested from the experimental lots.

The analysis of the histograms in Figure 5 indicated that the values respected the condition of normality of distribution for the indicators of fruit weight, pulp firmness, total soluble substance content and fruit production. In the case of pH, a slight positive asymmetry was observed, due to the

presence of very high values, present in small numbers, as well as a vaulting generated by the accumulation of most of the values in the central area.

According to the Multiple Comparative Test Duncan, the largest fruit weight, 9.2 g, was recorded for the variant (2), treatment with the biopesticide prototype containing gelatin and collagen hydrolysate extracted from untanned leather, followed by (4), treatment with a standard product (Serenade® ASO) and variant (3), treatment with the biopesticide prototype containing gelatin extracted from untanned leather, collagen hydrolysate extracted from tanned leather and keratin hydrolysate, to which a fruit weight of 9.0 g was recorded. The lowest fruit weight of 8.4 g was recorded for (1) untreated control.

The firmest fruits were those harvested from variants (3) and (2), respectively 72.9 units and 72.1 units (tissue test Shore HPE II Fff Bareiss). A significantly smaller firmness than those in variant (2) and variant (3), similar to the untreated control, was found in the resulting fruits after the standard treatment.

The least acidic fruits, with a pH of 3.8, were harvested from treatment variants (3) and (2), while the fruits harvested after the standard treatment (4) and those of the untreated control (1) presented more acidic fruits.

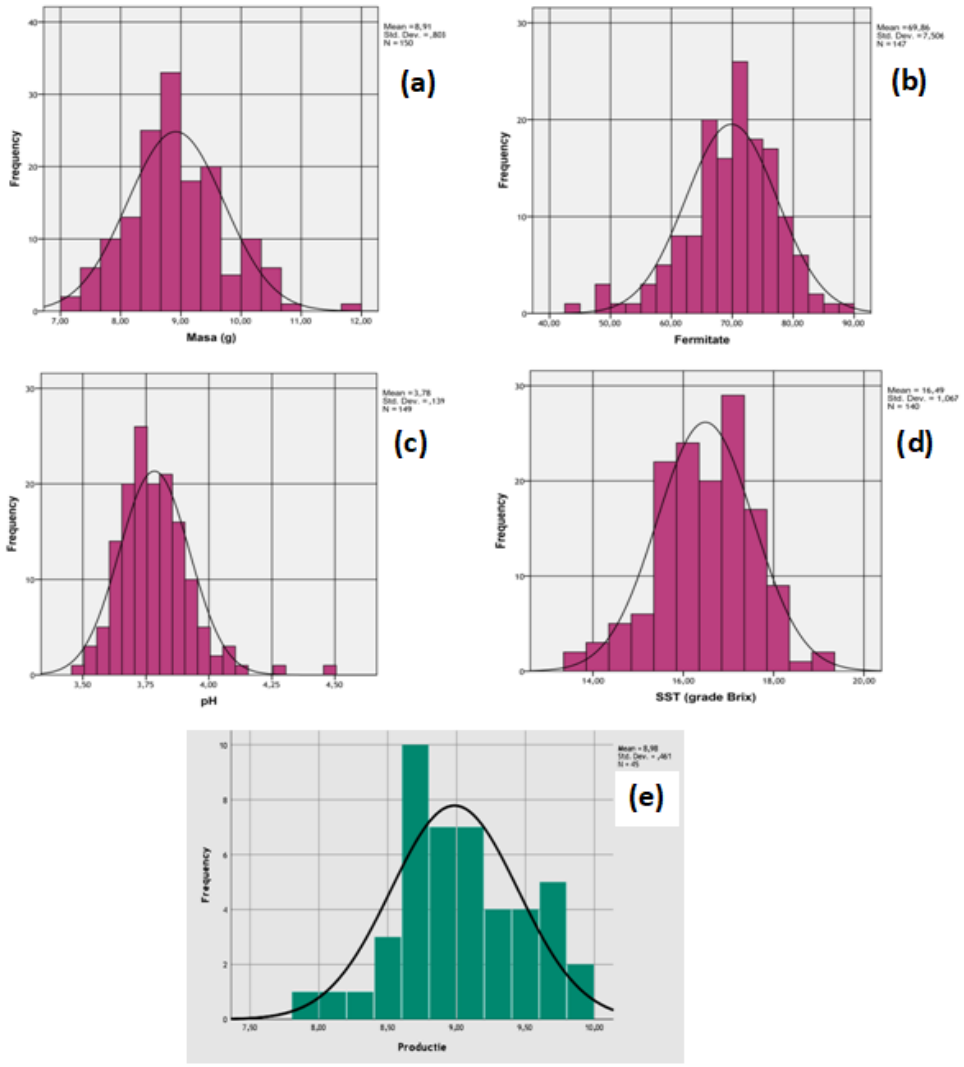

Figure 5. Histograms of recorded values for: (a) fruit mass; (b) pulp firmness; (c) the pH of the juice; (d) the total soluble matter content of the fruit; (e) fruit production

Both variants of experimental treatments (2) and (3) have significantly and similarly increased the total soluble substance content.

In Figure 6, the fruit production is presented for the tested treatment variants: 1 – the untreated control, 2 – treatment with the biopesticide prototype containing a protein combination consisting of gelatin and collagen hydrolysate extracted from untanned leather, 3 – treatment with the biopesticide prototype containing a protein combination consisting of gelatin extracted from untanned leather, collagen hydrolysate extracted from tanned leather and keratin hydrolysate, 4 – treatment with a standard product (Serenade® ASO).

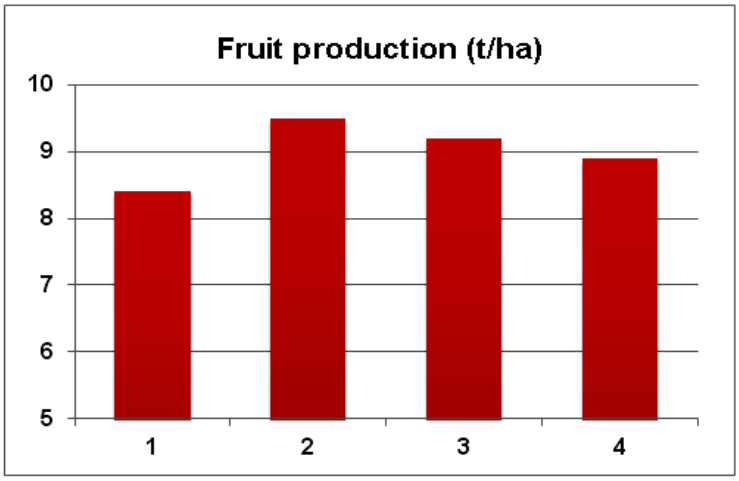

Figure 6. The influence of treatments on fruit production: 1) untreated control; 2) gelatin and collagen hydrolysate extracted from untanned leather; 3) gelatin, collagen hydrolysate extracted from tanned leather and keratin hydrolysate; 4) standard product (Serenade® ASO)

Cherry orchard tests have shown that treatment with experimental version (2) has determined the highest values of fruit weight (at 9.2 g), firmness (at 72.1 units), pH (3.8) and of the total soluble substance content (16.7˚Brix). However, the effects produced by the treatment with the experimental version (3) on the weight of the fruit were similar. Productions close to the maximum 9.5 t/ha, obtained with treatment (2) were also obtained in (3) fertilization version, 9.2 t/ha, both being significantly higher, compared to the untreated witness and with Standard treatment.

In order to establish the reproducibility of the results obtained in last season in the cherry orchard, a new set of tests is underway in the present season, and the results will be presented in subsequent publications.

CONCLUSIONS

The composites based on protein hydrolysates and gelatins have amino acids and polypeptides suitable for the immediate and delayed release of organic nitrogen necessary for plant stimulation and nutrition.

The treatments containing protein combinations was shown to increase fruit quality parameters as well fruit production level compared to both the untreated control and the standard treatment.

The use of collagen and keratin from the by-products of the leather industry for agricultural applications is a viable alternative to synthetic products.

Acknowledgements

This work was supported by the grants of the Romanian National Authority for Scientific Research and Innovation CCCDI - UEFISCDI, project BIO-PLANT-Protect, PN-III-

P3-3.5-EUK-2019-0250 (contract no. 262/2021) and the Institutional Performance project 4PFE/2020.

REFERENCES

1. Niculescu, M.-D., Dumitriu, B.-G., Cristea, S., Miu, L., Lasoń-Rydel, M., Sobczyk, J., Alexe, C.-A., Eureka Project Bio-Plant-Protect as Model Knowledge Transfer Between Academic, Research and Industry, *Leather and Footwear Journal*, **2023**, 23, 4, 279-286, https://doi.org/10.24264/lfj.23.4.6.
2. Zhao, L., Mu, S., Wang, W., Gu, H., Toxicity Evaluation of Collagen Hydrolysates from Chrome Shavings and Their Potential Use in the Preparation of Amino Acid Fertilizer for Crop Growth, *J Leather Sci Eng*, **2022**, 4, 1, 1-23, https://doi.org/10.1186/s42825-021-00072-1.
3. Ștefan, D.S., Bosomoiu, M., Constantinescu, R.R., Ignat, M., Composite Polymers from Leather Waste to Produce Smart Fertilizers, *Polymers*, **2021**, 13, 24, 4351, https://doi.org/10.3390/polym13244351.
4. Abdelkader, M., Voronina, L., Baratova, L., Shelepova, O., Zargar, M., Puchkov, M., Loktionova, E., Amantayev, B., Kipshakbaeva, A., Arinov, B., Biostimulants-Based Amino Acids Augment Physio-Biochemical Responses and Promote Salinity Tolerance of Lettuce Plants (*Lactuca sativa* L.), *Horticulturae*, **2023**, 9, 7, 807, https://doi.org/10.3390/horticulturae9070807.
5. Szopa, D., Skrzypczak, D., Izydorczyk, G., Chojnacka, K., Moustakas, K., Witek-Krowiak, A., Waste Valorization towards Industrial Products through Chemo- and Enzymatic- Hydrolysis, *Bioengineered*, **2023**, 14, 1, 2184480, https://doi.org/10.1080/21655979.2023.2184480.
6. Mengistu, A., Angassa, K., Tessema, I., Optimization of Keratin Hydrolysate Extraction from Tannery Sheep Hair Waste, *Int J Chem Eng*, **2023**, ID 9293505, 1-18, https://doi.org/10.1155/2023/9293505.
7. Ambrosini, S., Sega, D., Santi, C., Zamboni, A., Varanini, Z., Pandolfini, T., Evaluation of the Potential Use of a Collagen-Based Protein Hydrolysate as a Plant Multi-Stress Protectant, *Front Plant Sci*, **2021**, 12, 600623, https://doi.org/10.3389/fpls.2021.600623.
8. Chen, H., Gao, S., Li, Y., Xu, H.J., Li, W., Wang, J., Zhang, Y., Valorization of Livestock Keratin Waste: Application in Agricultural Fields, *Int J Environ Res Public Health*, **2022**, 19, 11, 6681, https://doi.org/10.3390/ijerph19116681.
9. Ramli, R.A., Mohamad Razali, U.H., Izzreen Mohd Noor, N.Q., Optimization of Extraction Conditions of Gelatin from Buffalo (*Bubalus bubalis*) Skins Using Response Surface Methodology, *Heliyon,* **2023**, 9, 3, e14367, https://doi.org/10.1016/j.heliyon.2023.e14367.
10. Cristea, S., Ivan, E., Jurcoane, Ș., Dumitriu, B., Perișoara, A., Niculescu, M.-D., Olariu, L., *In vitro* Antifungal Activity of Some Biopesticide Prototypes on the Fungus *Monilinia* spp., Proceedings of the 7th International Congress on Innovative Aspects for Leather Industry – IAFLI **2023**, 11.
11. Cristea, S., Niculescu, M.-D., Perișoara, A., Ivan, E., Stanca, M., Alexe, C.-A., Tihauan, B.-M., Olariu, L., Germination Study of Some Protein-Based Gels Obtained from By-Products from the Leather Industry on Tomato and Pepper Seeds, *Gels*, **2024**, 10, 1, 75, https://doi.org/10.3390/gels10010075.