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Journal:	*Journal of Agricultural and Food Chemistry*
Manuscript ID	Draft
Manuscript Type:	Article
Date Submitted by the Author:	n/a
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Use of protein-based matrices as amino acids source in *in-vitro* grapevine

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Abstract

Proteins are sources of peptides and amino acids which are able to stimulate plant growth through mechanisms such as enhancement of root growth and increased availability of micronutrients. Protein-based matrices are a novel source of these raw materials since they allow their availability in small, prolonged doses, which could be of interest in *in-vitro* assays. Thus, this work aimed to evaluate the use of soy protein-based matrices in *in-vitro* cultures of cv. *Magliocco Canino,* Their influence was assessed in different media conditions in the presence or absence of zinc (an essential microelement for plant growth). The shoots were evaluated based on their growth parameters (weight increase, number of stems, number of leaves, stem and internode length). A biochemical profile of the shoots cultivated in different media was obtained by FTIR. The results highlighted the benefits of using protein-based matrices in *in-vitro* culture as shoots showed an increase in weight, number of leaves, and longer stems, also in zinc-deficient media. In conclusion, this work emphasizes the potential of protein-based matrices as stimulants for grapevine explants.

Keywords: Grapevine; *In*-vitro culture; Matrices; Proteins, Stimulants.

1. INTRODUCTION

Amino acids are organic molecules composed of amino and carboxylic groups [1]. The main route of amino acid supply to the plant is through the root, which take up both amino acids, inorganic and organic nitrogen from the soil [2,3]. Plants transform inorganic nitrogen (NO_3^- and NH_4^+) into amino acids for the consequent synthesis of proteins [4]. In this way, amino acids are present in every metabolic reaction and plant structure [5]. Highlighting the potential use of amino acid-based products for crop production, which have the complex power to protect some nutrients [6,7]. In addition, they increase the mobility of fairly mobile nutrients (i.e., calcium, zinc, iron, etc.), thereby reducing and preventing plant stress [8,9].

The synthesis of amino acids from inorganic nitrogen requires a high amount of energy [2,10] which is also critical in stressful situations (drought, disease, sudden temperature variations, etc.) when free amino acids are needed up to 100 more times [11]. This decreases the formation of proteins, therefore negatively impacting plant growth [12,13]. For this reason, the application of peptides and free amino acids may favor all plant processes in which proteins are involved. In this sense, many authors have used amino acids and peptides to promote plant growth. For example, Noroozlo et al. (2019) evaluated the stimulating effect of glycine and glutamine on lettuce growth [14]. On the other hand, Ghasemi et al. (2012) provided a solution to iron deficiency in tomato plants through the synthesis of iron-amino acid chelates [15]. It is worth mentioning that although the goal of these amino acids is the synthesis of proteins, these proteins cannot be exogenously incorporated into the plant due to their size and molecular weight [16].

The use of protein as a source of amino acids has not been fully explored due to its complex structure that requires protease digestion to obtain those amino acids

[17]. However, this digestion is ensured due to the enzymes secreted by the microbes and the plants themselves [18]. Therefore, proteins could be used as amino acid source. In addition, it has the advantage of slow digestion, so it can provide amino acids for a longer time, being efficient throughout all the crop growth without needing more nutrients [19].

Agri-food wastes can be a great alternative to providing these proteins due to their low price and high protein content [20]. Nevertheless, these residues have great amounts of water, so it is necessary to stabilize them in order to use them in plant production without detrimental effects [21]. In this sense, the formation of protein-based matrices, through the application of temperature and pressure, can be an easy and economical solution to obtain a product with a stable high protein concentration [22,23].

In-vitro culture aims to grow plants in an aseptic-controlled environment, reducing the effect of factors that affect plant growth [24]. However, the use of media has difficulty reproducing natural conditions under laboratory conditions, as well as supplying plants with what was previously obtained from the complete system [25]. The use of the protein-based matrices asset in this work can represent a source of amino acids for *in vitro* cultivated plantlets.

Zinc is a microelement, required for different plant enzymatic activity, metabolic pathways, protein synthesis, and carbohydrate metabolism, and is also involved in the synthesis of tryptophan, cell division, maintenance of membrane structure and photosynthesis [26,27]. The most characteristic symptoms of zinc deficiency in fruit trees, rosetting and little leaf, are the results of strong inhibition of internode elongation and leaf expansion [28]. In addition, zinc can also be involved in plant-

pathogen interactions. In this sense, zinc is used by plants to combat pathogens, intoxicating them and controlling their proliferation [29].

The main objective of this work was the evaluation of the use of soy protein-based matrices in *in-vitro* culture. We aimed to verify if the application of protein-based matrices could improve the performance of *in-vitro* cultivated plants. *Magliocco Canino 'Ninno'* shoots were selected. Magliocco Canino “Ninno” is a biotype present in the Calabria Region (Italy) which displays some peculiarities with respect to the standard Magliocco Canino genotype as leaves produces small berries without seeds. The productivity of this plant is very limited [25]; therefore *in-vitro* study can contribute to understanding the possible causes of its peculiarities. Thus, their influence on shoots was studied using different media with or without zinc deficiency. To this purpose, the parameters (weight increase, number of stems, number of leaves, stem, and internode length) of the shoots were monitored throughout the study. In addition, an estimation of the biochemical profile of these shoots was performed by FTIR.

2. MATERIALS & METHODS

2.1 Materials

In this study micropropagated Magliocco Canino “Ninno” shoots were used, in order to assess different substrates in a completely randomized experimental design. The culture employed as an explant source had been previously stabilized on the media tested in this experiment, further referred to as ‘Standard modQL’. Under laminar flow, with a scalpel apical shoot portions (37.7 ± 13.6 mg) were aseptically detached, including the first four visible, sub-apical internodes (2.8 ± 1.8 mm long).

A salt formulation previously tested on Magliocco Canino Ninno [28] was selected for this study (Table 1): 'mQL', i.e. a modified Quoirin and Lepoivre [30], with the NH_4NO_3 concentration further increased to 7.5 mM (Negri, unpublished). It was tested either as its standard or Zinc-deficient compositions. For all the tested media the same organic base was used at pH 5.7: sucrose (30 g L^{-1}), myoinositol (100 mg L^{-1}), thiamine (1 mg L^{-1}), nicotinic (1 mg L^{-1}), pyridoxine (1 mg L^{-1}), glycine (2 mg L^{-1}), BA (1 mg L^{-1}), IBA (0.05 mg L^{-1}) and agar (7.5 g L^{-1}). All the reagents used in the media were analytical grade.

Soy protein-based matrices were used to supply proteins to the medium. They were processed with a similar protocol to those described by Jiménez-Rosado et al. [22]. Briefly, soy and glycerol (ratio 1:1) were homogenized in a rotating mixer (Polylab QC, ThermoHaake, Germany) at 50 rpm for 10 min, and they were subsequently injected in a MiniJet Piston Injection Molding System II (ThermoHaake, Germany); parameters used: 40 °C and 90 ºC in the cylinder and mold, 600 bar of injection pressure for 20 s, and 300 bar of holding pressure for 300 s to obtain the bioplastic matrices. This system was immersed in 300 mL of ethanol for 24 h to remove the glycerol. The protein-based matrices were obtained after a freeze-drying process (LyoQuest, Telstar, Spain), with a biodegradability of 40 days, providing nitrogen, which can also be taken up by plants. The amino acid composition of the soy protein used is shown in Table 2. It should be noted that the matrices that are the object of study in this work do not contain any other component apart from the protein.

2.2 *In-vitro* culture treatments

Four different treatments were imposed: (i) standard medium (control), (ii) zinc-deficient medium (i.e., the same as the standard medium, but lacking

$ZnSO_4 \cdot 7H_2O$), (iii) standard medium with protein-based matrix and (iv) zinc-deficient medium with the protein-based matrix. Once autoclaved (20 min at 121 °C, 1 bar) into Pyrex bottles, the substrates were transferred to the laminar flow and aliquoted (10 ml) into previously sterilized, 55 ml glass tubes (30 mm diameter). After medium gelation a shoot was positioned in the center of each tube. A matrix fragment (70 mg approximately) was introduced beside the sample. Finally, each tube was non-hermetically sealed with an aluminum cap and fastened with a single layer of polyethylene film. The process was carried out under sterile conditions in a laminar flow hood.

Shoots were kept for 35 days in a culture chamber with 16 hours of light (intensity of 35 µcd) at a temperature of 26 ± 2 ºC.

2.3 Assessment of morphological and physiochemical plant traits

The different samples were hermetically preserved during their cultivation to avoid contamination of the environment. After 35 days, the plants were first dissected with a scalpel to facilitate their analysis. Later, their weight increase (final fresh weight – initial fresh weight), the number of stems and leaves, as well as the length of their stems and internodes were determined. In addition, the morphology of the leaves was observed.

Tissue zinc concentration was also determined in the different samples. For this, inductively coupled plasma-atomic emission spectroscopy (ICP-AES) was carried out. The plants were subjected to a previous digestion with 7:1 ratio of HNO_3:H_2O_2 to obtain an aqueous solution which contain the zinc present in the samples. Then, the aqueous solutions were analyzed in an ICP SpectroBlue TI (Spectro, Germany) to obtain the zinc concentration.

Finally, the leaves of the different treated shoots were also analyzed by Fourier transform infrared spectroscopy (FITR) to evaluate the biochemical profile of each shoot [31]. A MIR-ATR-XPM spectroscope (Bruker, USA) was used to obtain the absorbance profile of each sample between 4000 and 400 cm^{-1}, with a resolution of 4 cm^{-1}. To obtain the profile, an average of 100 scans was performed.

2.6 Statistical analysis

At least six replicates were performed for each analysis and system. All the data were reported as average values. An analysis of variance was performed. Tukey's post hoc test with a confidence level of 95% (p <0.05) was performed using the SPSS 18 statistical package (Excel, Microsoft, USA) to evaluate the significant differences, which were reported with different letters.

3. RESULTS & DISCUSSION

Table 3 shows the parameters obtained for the shoots submitted to the different treatments i, ii, iii, and iv. The inclusion of soy protein-based matrices increased weight and proliferation (higher number of stems and leaves). This behavior may suggest that the matrices improve plant formation due to the stimulation generated by the amino acids present in the matrix [23,32,33]. Soy protein presents high concentrations of glutamic acid (21.7%), which promotes plant development [34]; alanine (10.4%), which increases metabolic activity [35]; leucine (8.6%) and aspartic acid (8.2%), which are important in the growth, development, and flowering of plants [36]. The proteins break down into amino acids during

biodegradation [37]. These amino acids are an extra source of nitrogen and building blocks for enzymes, allowing plants to have more energy to develop [38].

Zinc deficiency produces a slight decrease in weight (43% approximately) obtained from the plants (Table 3, i (media without matrix) vs. ii (zinc deficiency media without matrix)). In addition, zinc deficiency results into a lower tissue Zn concentration and increases the percentage of deformed leaves. These results could contribute to explain the smaller fruits produced by zinc-deficient plants [39]. The inclusion of the matrix compensates in part the lower weight increases due to zinc deficiency (Table 3, ii (zinc deficiency media without matrix) vs. iv (zinc deficiency media with matrix)). This effect may be due to the presence of amino acids provided by the protein matrix that allows the stimulation of the plant for the formation of the proteins and enzymes necessary for its growth. Nevertheless, the absence of zinc prevents the generation of enzymes necessary for plant growth, such as those needed for the synthesis of tryptophan [40], making it unable to reach the levels obtained by the control plants.

Regarding tissue zinc concentration (Table 3), zinc-deficient media cause shoots to present a deficiency of this micronutrient (concentration < 11.5 ppm [41]). This deficiency may be the reason for the aforementioned defects in the shoots cultivated in this medium. The inclusion of the protein-based matrix has promoted the absorption of zinc (from the medium) by the shoots. In this way, the shoots obtained in the media with protein-based matrices (iii and iv) have a higher amount of zinc than those obtained without matrices (i and ii, respectively). In addition, it should be noted that the shoots obtained in the medium with zinc deficiency but with matrix (treatment iv) have a zinc concentration similar to the reference system (treatment i), although the matrix does not provide zinc.

Regarding the visual aspect of dissected plants (Fig. 1), zinc deficiency (treatment ii) caused higher leaf deformation (Table 3), an effect that has already been reported as a cause of micronutrient deficiency [42,43]. The inclusion of protein-based matrices decreased this effect, possibly due to the stimulant effect of amino acids and the higher energy available per plant [44]. Once again, the medium without zinc deficiency and with the incorporated matrix (treatment iii) was the one that generated the highest number of leaves, appearing the largest and least deformed (Table 3). This response suggests that the protein-based matrices incorporate amino acids into the plants, stimulating plant growth by increasing plant weight and number of leaves and less likely to be deformed, as seen in Table 3.

Finally, the biochemical profile of the leaves of differently treated plants is shown in Figure 2. The vibration band of the O-H group (3320 cm^{-1}) is observed, which is due to the residual water present in the plants. The polysaccharides (cellulose, hemicellulose and lignin) present a band between 1260 and 870 cm^{-1} due to the vibration mode of C-O-C groups. Other intense bands were found between 2970 and 2840 cm^{-1}, corresponding to the C-Hx vibrations of lipid content in the plants. In addition, the spectra show protein structures in the samples, which can be observed in the bands between 1520 and 1280 cm^{-1}, corresponding to amide groups. All these peaks have also been identified in previous works, being the typical profile of other grapevine plants [45–47]. All treatments presented the same profile, so the biochemical composition of plants was not affected by the treatment used. This may be because, although plant growth is different in each medium and treatment, all systems form the same structures and bonds.

Using protein-based matrices may be beneficial for horticultural production, particularly in addressing zinc micronutrient deficiency in plants. The use of protein-based matrices incorporates beneficial stimulants such as amino acids, improving nutrient use efficiency, therefore minimizing losses while reducing the need for fertilizer applications and consequently environmental contamination.

4. CONCLUSIONS

Protein-based matrices, especially those based on soy protein, have shown their high potential in the performance of *in-vitro* cultivated plants (treatments iii and iv). The *Magliocco Canino 'Ninno'* shoots selected for this essay presented a greater weight and number of leaves, reducing the appearance of defects and deformations when soy protein-based matrices were used (even in zinc-deficient media). All this was caused by the stimulant effect of proteins presented in these matrices. Thus, zinc deficiency symptoms were partially mitigated using protein matrices.

Controlled fertilization with protein-based matrices is a promising approach to providing nitrogen and addressing zinc micronutrient deficiencies in horticulture without the negative effects of conventional fertilization.

Nevertheless, this is a preliminary study on the use of these protein-based matrices. Future work is needed to assess the effects and mode of action of protein matrices on crops.

ACKNOWLEDGEMENTS

This work is part of a research project (PID2021-124294OB-C21) funded by MCIN/AEI/10.13039/501100011033 and by “ERDF A way of making Europe”. The authors gratefully acknowledge their financial support. In addition, the authors thank for the predoctoral grant of Mercedes Jiménez-Rosado (FPU2017/01718, MEFP) and her research stay supported by MEFP. Finally, the authors acknowledge the assistance in microanalysis services of CITIUS (University of Seville).

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417

418 # FIGURE CAPTIONS

419 **Figure 1:** Visual aspect of dissected *Magliocco Canino* plants subjected at the
420 different treatments: (i) standard medium (control), (ii) zinc-deficient medium, (iii)
421 standard medium with protein-based matrix and (iv) zinc-deficient medium with
422 the protein-based matrix in modified Quoirin and Lepoivre medium (mQL).

423 **Figure 2:** FTIR profile of the *Magliocco Canino* plants subjected at the different
424 treatments: (i) standard medium (control), (ii) zinc-deficient medium, (iii) standard
425 medium with protein-based matrix and (iv) zinc-deficient medium with the protein-
426 based matrix.

TABLES

Table 1: Composition of macro and microelements in the standard media: Quoirin and Lepoivre modified medium (mQL).

Salt	Standard mQL (µM)
$CaCl_2 \cdot 2H_2O$	-
$Ca(NO_3)_2 \cdot 4H_2O$	4801.059
KH_2PO_4	2324.914
KNO_3	17804.154
$MgSO_4 \cdot 7H_2O$	1443.367
NH_4NO_3	7495.971
$CoCl_2 \cdot 6H_2O$	0.105
$CuSO_4 \cdot 5H_2O$	0.100
FeNaEDTA	465.559
H_3BO_3	100.275
KI	0.482
$MnSO_4 \cdot H_2O$	4.497

$Na_2MoO_4 \cdot 2H_2O$	1.033
$ZnSO_4 \cdot 7H_2O$	29.907

430

431 **Table 2:** Amino acid composition of soy protein-based matrices.

Amino acids	**Concentration**	
	μmol L^{-1}	**% Amino acid in the protein**
Aspartic acid(Asp)	361.4	8.2
Threonine (Thr)	109.6	2.5
Serine (Ser)	46.2	1.0
Glutamic acid (Glu)	958.8	21.7
Glycine (Gly)	326.3	7.4
Alanine (Ala)	460.5	10.4
Cisteine (Cys)	73.7	1.7
Valine (Val)	261.3	5.9
Methionine (Met)	38.2	0.9
Isoleucine (Ile)	193.6	4.4
Leucine (Leu)	379.2	8.6
Tryptophan (Trp)	1.0	<0.1
Tirosine (Tyr)	131.3	3.0
Pheenilalamine (Phe)	186.5	4.2
Histidine (His)	59.8	1.4
Lysine (Lys)	248.1	5.6
Proline (Pro)	343.5	7.8
Arginine (Arg)	234.3	5.3

432

433 **Table 3:** Parameters (weight increase, number of stems, number of leaves, stem
434 and internode length and tissue zinc concentration) obtained for the *Magliocco*
435 *Canino* plants subjected to the different treatments ((i) standard medium (control),
436 (ii) zinc-deficient medium, (iii) standard medium with protein-based matrix and (iv)
437 zinc-deficient medium with the protein-based matrix). Different letters in the same
438 column mean significant differences (p <0.05).

Treatment	Weight increase (mg)	Number of stems	Number of leaves	Deformed leaves (%)	Stem Length (mm)	Internode Length (mm)	Tissue Zn concentration (ppm)
i	810.3 ± 83.4^{c}	6 ± 1^{a}	40 ± 7^{a}	22 ± 1^{b}	20.5 ± 1.5^{ab}	2.9 ± 0.9^{a}	25.2 ± 0.1^{b}
ii	350.5 ± 23.6^{a}	6 ± 1^{a}	39 ± 4^{a}	92 ± 1^{c}	19.0 ± 2.0^{a}	2.1 ± 0.7^{a}	8.1 ± 0.4^{a}
iii	1250.97 ± 139.0^{d}	9 ± 2^{b}	70 ± 16^{b}	14 ± 1^{a}	15.0 ± 8.9^{ab}	3.4 ± 2.1^{a}	29.3 ± 0.4^{c}
iv	498.8 ± 79.4^{b}	7 ± 1^{ab}	41 ± 10^{a}	15 ± 1^{a}	23.5 ± 1.2^{b}	2.7 ± 1.1^{a}	25.4 ± 0.2^{b}

439

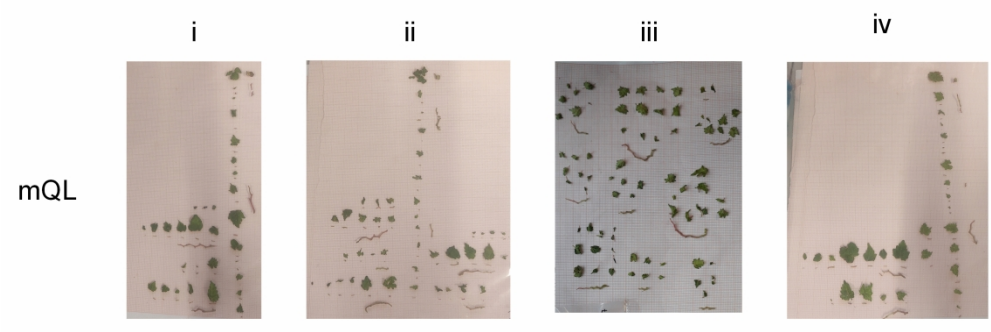

Figure 1: Visual aspect of dissected Magliocco Canino plants subjected at the different treatments: (i) standard medium (control), (ii) zinc-deficient medium, (iii) standard medium with protein-based matrix and (iv) zinc-deficient medium with the protein-based matrix in modified Quoirin and Lepoivre medium (mQL).

296x99mm (300 x 300 DPI)

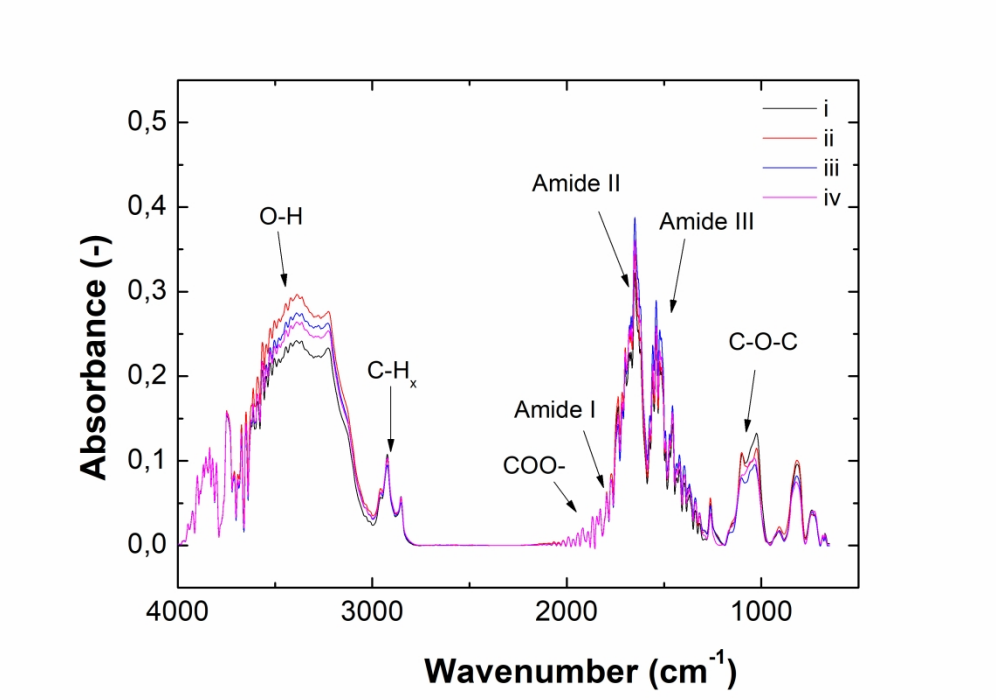

Figure 2: FTIR profile of the Magliocco Canino plants subjected at the different treatments: (i) standard medium (control), (ii) zinc-deficient medium, (iii) standard medium with protein-based matrix and (iv) zinc-deficient medium with the protein-based matrix.

296x209mm (300 x 300 DPI)