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Rheological and Microstructural Features of Plant Culture Media Doped with Biopolymers: Influence on the Growth and Physiological Responses of *In Vitro*-Grown Shoots of *Thymus lotocephalus*

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Abstract: In vitro culture is an important biotechnological tool in plant research and an appropriate culture media is a key for a successful plant development under in vitro conditions. The use of natural compounds to improve culture media has been growing and biopolymers are interesting alternatives to synthetic compounds due to their low toxicity, biodegradability, renewability, and availability. In the present study, different culture media containing one biopolymer (chitosan, gum arabic) or a biopolymer derivative [hydroxyethyl cellulose (HEC), carboxymethyl cellulose (CMC)], at 100 or 1000 mg L^{-1}, were tested regarding their influence on the growth and physiological responses of *Thymus lotocephalus* in vitro culture. Cellulose-based biopolymers (HEC and CMC) and gum arabic were used for the first time in plant culture media. The results showed that CMC at 100 mg L^{-1} significantly improved shoot elongation while chitosan, at the highest concentration, was detrimental to *T. lotocephalus*. Concerning only the evaluated physiological parameters, all tested biopolymers and biopolymer derivatives are safe to plants as there was no evidence of stress-induced changes on *T. lotocephalus*. The rheological and microstructural features of the culture media were assessed to understand how the biopolymers and biopolymer derivatives added to the culture medium could influence shoot growth. As expected, all media presented a gel-like behaviour with minor differences in the complex viscosity at the beginning of the culture period. Most media showed increased viscosity overtime. The surface area increased with the addition of biopolymers and biopolymer derivatives to the culture media and the average pore size was considerably lower for CMC at 100 mg L^{-1}. The smaller pores of this medium might be related to a more efficient nutrients and water uptake by *T. lotocephalus* shoots, leading to a significant improvement in shoot elongation. In short, this study demonstrated that the different types of biopolymers and biopolymer derivatives added to culture medium can modify their microstructure and at the right concentrations, are harmless to *T. lotocephalus* shoots growing *in vitro*, and that CMC improves shoot length.

Keywords: carboxymethyl cellulose; chitosan; complex viscosity; gum arabic; hydroxyethyl cellulose; porosity; scanning electron microscopy; shoot elongation; surface area

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1. Introduction

Thymus lotocephalus G. Lopez & R. Morales is an aromatic plant endemic to the Algarve region (South of Portugal), traditionally used as a medicine and to condiment food. Several

studies have shown that *T. lotocephalus* produces different phytochemicals with biological activity, granting this species with antiseptic, digestive, expectorant, and antioxidant properties [1–3]. Due to its restricted distribution, it is a threatened and legally protected species [4,5] and therefore harvesting it from the wild is not a reasonable option and requires permission from the responsible authorities. As an alternative, our group has pioneered the development of an in vitro propagation protocol that contributes both to the conservation of this species and to produce plant material to be used for the extraction of bioactive compounds [3,6,7].

In vitro culture is a very useful biotechnological tool not only for fundamental research and plant conservation, but specially for industrial and agricultural purposes, being used for large scale plant multiplication, to produce secondary metabolites and for crop improvement [8]. In vitro plant cultures can serve as model systems in plant research, as most factors affecting in vitro growth and physiology resemble those limiting plant development *in vivo*. *T. lotocephalus* is a viable choice in plant research because it responds well to in vitro growth, with 100% multiplication rates [6]. However, to make the most of in vitro propagation, the growth conditions, such as culture medium composition, correct medium pH, adequate temperature, and gaseous environment, must be properly established and strictly controlled [9]. To achieve higher yield and improve plant development, plant culture medium can be supplemented with plant growth regulators and/or other additives [10,11]. Recently, the production of phenolic compounds in *T. lotocephalus* in vitro shoots was improved and the antioxidant activity enhanced by increasing sucrose concentration and incorporating different elicitors (e.g., yeast extract, salicylic acid and $AgNO_3$) into the culture medium [7].

Synthetic plant growth regulators (e.g., 6-benzylaminopurine, kinetin, indole-acetic acid and 1-naphthaleneacetic acid) and elicitors (e.g., silver nitrate) are often used to manipulate in vitro growth parameters and physiological responses, as they are more potent, chemically more stable, and economically more affordable than their natural counterparts [10]. In recent years, the general interest in the use of alternative natural products has been growing in many sectors, such as agriculture, food industry, and pharmaceutical, and biopolymer-based materials are a promising alternative to replace synthetic compounds, contributing to a sustainable development [12,13]. The intrinsic features of biopolymers, such as low toxicity, biodegradability, renewability, and availability, in many cases, rival those of many non-sustainable synthetic systems [14–17]. Though, despite their potential, the use of these materials is still at an early stage [13].

Biopolymers have been tested with multiple purposes in in vitro culture of plants, and chitosan is, by far, the most investigated. Chitosan, a linear polysaccharide derived from chitin, is a very versatile biomaterial, when comparing to other natural and synthetic polymers [18]. This biopolymer has been used to improve plant growth [19–22] and seed germination [21,23], as an elicitor of secondary metabolites [24–26], and as a disinfectant agent [27]. Conventionally, agar, a natural polysaccharide, is used to solidify in vitro plant culture media due to its gelling properties, stability, resistance to plant metabolic enzymes and high clarity [28]. Regardless of its natural origins, agar is typically the most expensive constituent of plant tissue culture media and different gums and starches have been studied throughout the years as gelling agent alternatives [28–35].

Though cellulose-based materials have been used in agriculture to improve plant growth and to increase the water retention capacity of the soils [15,17,36,37], to the best of our knowledge, there are no reports on the use of cellulose-based derivatives in plant culture media. Hence, their in vitro potential is still incognito, and research is important to expand the knowledge on how they interact with *in vitro*-grown plants. Gum arabic (a soluble fibre obtained from *Acacia senegal* and *Acacia seyal* trees) has been extensively used in the food industry and other sectors [38] and more recently, in agriculture [39], but no reports were found on its use for the in vitro culture of plants. Therefore, in the present study, two cellulose derivatives, hydroxyethyl cellulose (HEC) and carboxymethyl cellulose (CMC), and gum arabic were, for the first time, used in plant culture media to test

their effect on the growth and physiological responses of *T. lotocephalus* shoots growing *in vitro*. In addition, chitosan, a biopolymer widely used in plant in vitro culture, was also tested.

The rheological and microstructural features of semi-solid and gel-like plant culture media are expected to influence plant growth and development. However, literature is surprisingly scarce on this topic [28,30]. While rheology provides information about the viscoelastic flow behaviour and structure of materials [40], the microstructural analysis provides insights into the morphology of the system, such as roughness, pore size, surface area and other textural features [41,42]. The understanding of all these characteristics and how they affect plant development can strongly contribute to the improvement of culture media and, consequently, support the progress of in vitro plant propagation. In the present study, the rheological and microstructural features of the plant culture media were assessed by mechanical rheometry and scanning electron microscopy (SEM), respectively. In the latter case, the pore size, surface area and volume of the gelled culture media were inferred by nitrogen porosimetry, using the Brunauer–Emmett–Teller (BET) approach [43].

2. Materials and Methods

2.1. Materials

Bradford reagent, chitosan (low molecular weight, 75–85% deacetylated), dithiothreitol, H_2O_2, hydroxyethyl cellulose (HEC) (Mw of Ca. 720 KDa with a molecular substitution of 2.5 mol per mol of cellulose), nitroblue tetrazolium (NBT), polyvinylpolypyrrolidone (PVP), protein standard, 2-thiobarbituric acid (TBA), and trichloroacetic acid (TCA), were acquired from Sigma-Aldrich (Steinheim, Germany). Acetone was acquired from Fisher Scientific (Leicestershire, UK). Ethylenediaminetetraacetic acid (EDTA) was acquired from Fluka (Buchs, Switzerland). Potassium iodide (KI) was acquired from Merck (Darmstadt, Germany). Carboxymethyl cellulose sodium salt (CMC) (Mw of ca. 250 Kda with a degree of substitution ca. 0.80–0.85) and gum arabic (−100 mesh power, total ash < 4%) were purchased from VWR international (Leuven, Belgium) and Alfa Aesar (Haverhill, MA, USA), respectively. Sucrose was acquired from Panreac (Barcelona, Spain), and agar from Duchefa Biochemie B.V. (Haarlem, The Netherlands).

2.2. Plant Material, Media Preparation and In Vitro Growth Conditions

T. lotocephalus shoots growing in vitro in MS (Murashige and Skoog) [44] medium supplemented with 2% (*w/v*) sucrose and 0.6% (*w/v*) agar, [6], were used in the experiments. The cultures were maintained at 25 ± 2 °C and a 16-h photoperiod (cool white fluorescent lamps at a photon flux density of 40 μmol m^{-2} s^{-1}), with a subculture period of six weeks.

MS medium was supplemented with different biopolymers and biopolymer derivatives at two concentrations, as follows: 100 and 1000 mg L^{-1} HEC (designated henceforth as HEC100 and HEC1000, respectively); 100 and 1000 mg L^{-1} CMC (designated henceforth as CMC100 and CMC1000, respectively); 100 and 1000 mg L^{-1} chitosan (designated henceforth as Chit100 and Chit1000, respectively); and 100 and 1000 mg L^{-1} gum arabic (designated henceforth as GA100 and GA1000, respectively). MS medium without supplements was set as the control.

All media were supplemented with 2% (*w/v*) sucrose and solidified with 0.6% (*w/v*) agar, and pH was adjusted to 5.7–5.8 before autoclaving at 121 °C for 20 min. Shoots were inoculated in 500 mL-Erlenmeyer flasks containing 80 mL of the previously defined media and incubated under the conditions described above. For each medium, 12 Erlenmeyer flasks with seven shoots each were tested to assess growth and physiological parameters. In parallel, flasks with the different media, but without plant shoots, were prepared and placed next to the flasks containing shoots, for the same period and under the same light and temperature conditions, to be used in the rheological analysis.

After eight-weeks culture, the total number of shoots, the length of the longest shoot and the fresh and dry weight of the biomass produced per medium were recorded. Dry weight was determined after oven drying the shoots at 63 °C until constant weight.

2.3. Photosynthetic Pigments

Photosynthetic pigments were extracted from 25 mg of fresh plant material, in a pre-chilled mortar, with 4 mL of 100% acetone and measured in a spectrophotometer (PG Instruments, Lutterworth, UK) at 661.6, 644.8 and 470 nm. The levels of chlorophylls and carotenoids were estimated as described by Lichtenthaler [45].

2.4. Hydrogen Peroxide (H_2O_2) Content

The H_2O_2 content was measured according to Loreto and Velikova [46] with some modifications. In short, 100 mg of plant material was ground in 1 mL 0.1% (*w/v*) TCA at 4 °C and centrifuged at 12,000× *g* for 15 min. The supernatant (0.2 mL) was added to 10 mM potassium phosphate buffer (0.2 mL, pH 7.0) and 1 M KI (0.4 mL). The reaction was developed for 30 min in darkness and the H_2O_2 content was quantified at 390 nm against a set of H_2O_2 standards. The results were expressed as micromole per gram of fresh weight (FW).

2.5. Superoxide Dismutase (SOD) and Catalase (CAT) Activities, and Total Soluble Protein

The antioxidant enzymes SOD and CAT were extracted from 100 mg of plant material, in a pre-chilled mortar, with 50 mM sodium phosphate buffer (pH 7.0) supplemented with 0.1 mM EDTA, 1% (*w/v*) PVP, and 2.5 mM dithiothreitol. The mixture was centrifuged at 20,000× *g* for 10 min at 4 °C and the obtained supernatant was used in the following enzyme assays. SOD activity was determined by the reduction of NBT, according to Beauchamp and Fridovich [47]. The photo-reduction of NBT was measured at 560 nm. One unit of SOD was defined as the amount of enzyme required to inhibit NBT reduction by 50%. The CAT activity was determined by the degradation of H_2O_2, according to Aebi [48]. The H_2O_2 decomposition was monitored at 240 nm (ε = 39.4 mM^{-1} cm^{-1}). One unit of CAT was defined as the amount of enzyme required to degrade 1 µmol H_2O_2 per min. The specific activity for both enzymes was expressed as enzyme units per milligram of protein, based on the measurement of the total soluble protein as described by Bradford [49]. In short, the total soluble protein was determined at 595 nm against a linear calibration curve prepared using standard solutions of bovine serum albumin.

2.6. Determination of Lipid Peroxidation

Lipid peroxidation was measured by determining the amount of malondialdehyde (MDA) available to react with TBA [50]. Briefly, plant material (100 mg) was ground in 0.1% (*w/v*) TCA and centrifuged at 10,000× *g* for 5 min. The supernatant was added to either 20% (*w/v*) TCA or 0.5% (*w/v*) TBA in 20% (*w/v*) TCA. The mixture was heated at 95 °C for 30 min and then cooled in an ice bath, followed by centrifugation at 3000× *g* for 10 min. The absorbance of the supernatant was measured at 440, 532 and 600 nm and MDA equivalents (nmol mL^{-1}) were calculated as described by Hodges et al. [50]:

$$\text{MDA equivalents} = (A - B)/157{,}000 \times 10^6 \quad (1)$$

$$A = [(Abs_{532+TBA}) - (Abs_{600+TBA}) - (Abs_{532\text{-}TBA}) - (Abs_{600\text{-}TBA})] \quad (2)$$

$$B = [(Abs_{440+TBA}) - (Abs_{600+TBA})] \times 0.0571 \quad (3)$$

2.7. Statistical Analysis

Values are presented as means ± standard errors of ten replicates for growth parameters and five replicates for physiological parameters. A one-way analysis of variance (ANOVA) was carried out to assess differences using the SPSS statistical package for Windows (release 22.0, SPSS Inc., Chicago, CA, USA). Media supplemented with biopolymers or biopolymer derivatives were compared to the control using Dunnett's test ($p < 0.05$).

2.8. Rheological Measures

The rheological measurements were carried out on a HAAKE MARS III rheometer (Thermo Fisher Scientific, Karlsruhe, Germany) set with a plate–plate geometry (35 mm, 2 mm gap). A Peltier unit was used to ensure strict temperature control and a solvent trap was used to prevent water evaporation. The storage modulus (G'), the loss modulus (G'') and the complex viscosity (η*) were assessed by dynamic oscillatory assays performed in a frequency range of 0.05 to 50 Hz within the linear viscoelastic regime (the selected stress was 7 Pa). Measurements were performed on all culture media, with or without plant shoots, at the beginning and at the end of the culture period.

2.9. Scanning Electron Microscopy (SEM)

Samples from all media, with or without plant shoots, at the beginning and at the end of the eight-week culture period were lyophilized prior to their microscopic morphology analysis. The samples were suitably spread on a double-side carbon tape mounted onto an aluminium stub. SEM was performed using a tungsten cathode scanning electron microscope SM 6010LV/6010LA, Jeol (Tokyo, Japan). Secondary electron mode, and acceleration voltage of 1 kV and a working distance of 9 mm, was selected as the operational conditions. Uncoated samples were deposited directly on the carbon tape.

2.10. Brunauer-Emmett-Teller (BET) Method

The surface area and the average pore diameter were determined by N_2 gas adsorption isotherms using an ASAP 2000, from Micromeritics. This technique considers the BET (Brunauer, Emmett and Teller) model for the referred evaluation [43].

3. Results and Discussion

In vitro culture is a useful tool for studying and perceiving how external factors affect the growth and development of plants due to its controlled conditions and fast growth. Within these conditions, the culture medium is essential for an adequate plant development and the understanding of its rheological and microscopic features can greatly contribute to future improvements.

The present study was performed using *T. lotocephalus*, a well-established in vitro shoot culture [6,7,51]. The effect of different biopolymers and biopolymer derivatives at two concentrations, was evaluated on *T. lotocephalus* growth regarding the number of new shoots, shoot length and biomass production (Table 1). Apart from Chit1000, multiplication percentages were 100% in all tested media with no apparent morphological differences between cultures (Figure 1). Shoots were visually well developed and healthy without severe signs of hyperhydricity, a common symptom of in vitro *T. lotocephalus* shoots in some basal media commonly used [6,7].

Comparing to the control, most of the tested media presented statistically equal results in relation to the number of new shoots, 45.09. The exceptions were HEC1000 and Chit1000 that induced a significant ($p < 0.05$) reduction in the number of new shoots (27.01 and 4.73 shoots, respectively) and, consequently, in biomass production (fresh weight, 2.03 and 0.54 g, respectively, and dry weight, 0.32 and 0.10, respectively). The highest number of variations from the control occurred in the case of shoot length (4.74 cm). Shoots cultivated in the CMC100 medium presented the greater length ($p > 0.05$), 6.45 cm, and shoots from HEC1000, CMC1000, Chit1000, GA100 and GA1000 were significantly shorter ($p < 0.05$), 3.64, 3.44, 2.33, 3.79 and 3.42 cm, respectively.

Table 1. Growth parameters, total chlorophyll, and carotenoids of *Thymus lotocephalus* shoots grown in vitro (eight weeks) on media supplemented with HEC, CMC, chitosan, or gum arabic at two concentrations (100 and 1000 mg L^{-1}).

Medium	No. Shoots	Shoot Length (cm)	Fresh Weight (g)	Dry Weight (g)	Total Chlorophyll (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)
Control	45.09 ± 4.28	4.74 ± 0.26	4.64 ± 0.47	0.50 ± 0.04	1.18 ± 0.07	0.25 ± 0.01
HEC						
100	38.07 ± 3.70	4.62 ± 0.18	3.20 ± 0.32	0.46 ± 0.04	1.40 ± 0.05	0.30 ± 0.01 *
1000	27.01 ± 3.70 *	3.64 ± 0.12 *	2.03 ± 0.28 *	0.32 ± 0.03 *	1.42 ± 0.05	0.31 ± 0.01 *
CMC						
100	39.16 ± 3.20	6.45 ± 0.15 *	3.49 ± 0.34	0.47 ± 0.09	1.19 ± 0.09	0.26 ± 0.02
1000	40.09 ± 4.56	3.44 ± 0.09 *	3.32 ± 0.50	0.41 ± 0.04	1.39 ± 0.07	0.31 ± 0.01 *
Chit						
100	43.04 ± 4.25	5.21 ± 0.16	3.37 ± 0.53	0.35 ± 0.04	1.09 ± 0.09	0.24 ± 0.02
1000	4.73 ± 0.35 *	2.33 ± 0.11 *	0.54 ± 0.11 *	0.10 ± 0.01 *	0.86 ± 0.10 *	0.20 ± 0.02
GA						
100	52.89 ± 5.99	3.79 ± 0.16 *	4.63 ± 0.60	0.49 ± 0.04	1.13 ± 0.08	0.26 ± 0.02
1000	48.59 ± 5.92	3.42 ± 0.11 *	4.19 ± 0.55	0.43 ± 0.04	1.09 ± 0.03	0.25 ± 0.01

Values are expressed as the mean ± SE. For each parameter, the asterisks (*) indicate differences from the control using Dunnett's test at $p < 0.05$. HEC: hydroxyethyl cellulose; CMC: carboxymethylcellulose sodium salt; Chit: chitosan; GA: gum arabic; 100: 100 mg L^{-1}; 1000: 1000 mg L^{-1}.

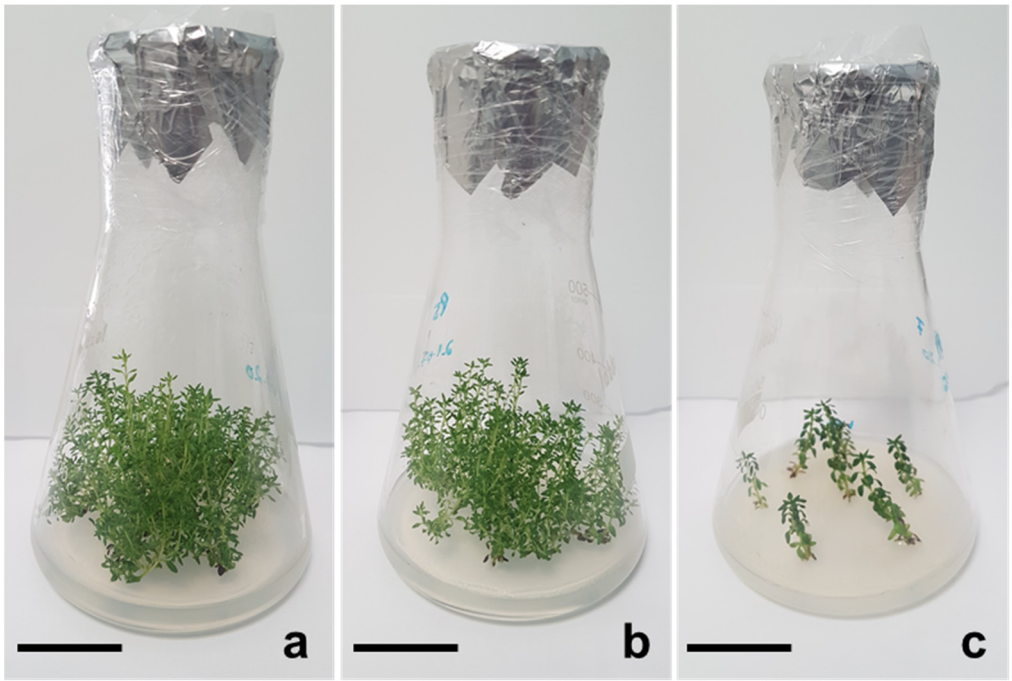

Figure 1. *Thymus lotocephalus* shoots after an eight-week culture. Control (**a**), CMC100 (**b**) and Chit1000 (**c**) media. The scale bars represent approximately 4 cm. CMC: carboxymethyl cellulose sodium salt; Chit: chitosan; 100: 100 mg L^{-1}; 1000: 1000 mg L^{-1}.

In our previous studies, *T. lotocephalus* similarly produced short shoots in vitro (between 1.9 and 3.8 cm) regardless of the constituents of the medium [6,7], and the supplementation with CMC at 100 mg L^{-1} considerably improved shoot elongation in comparison to the control, up to 6.45 cm, although this increase was not accompanied by an increase in the biomass production. Longer shoots are usually more effective in the subsequent stages of in vitro propagation, namely rooting and acclimatization [52], and by producing longer shoots in the multiplication phase, an extra phase for shoot elongation can be avoided, reducing the time and cost required to obtain a full-grown plant. The results also

demonstrate that the higher chitosan concentration used in this study was detrimental to the in vitro growth of *T. lotocephalus*, as all parameters assessed were significantly poorer than the control. For instance, the number of shoots in Chit1000 was 4.73, in comparison with the 45.09 shoots of the control. Chitosan has been reported to improve growth and as a good alternative to replace plant growth regulators in in vitro culture of several plant species, such as *Malus domestica* Borkh. 'Gala' [19], *Cattleya maxima* J. Lindley [20], *Serapias vomeracea* (Burm.f) Briq. [21], and *Ipomoea purpurea* (L.) Roth [22]. However, Largia et al. [26] also found that an increased concentration and an increased period of exposure to chitosan had a negative effect on the growth of *Bacopa monnieri* (L.). Nonetheless, the medium with lower chitosan concentration, Chit100, allowed obtaining similar plants as in the control medium.

Besides growth, physiological and biochemical parameters are also important indicators of plant development, particularly the plant stress level status [53]. Therefore, to investigate the influence of the added biopolymers to the basal medium on *T. lotocephalus* physiology and biochemistry, several stress-indicator parameters were assessed. Although plants growing in vitro have limited photosynthetic abilities, photosynthesis is a vital process that regulates plant growth and yield [54,55]. Photosynthetic pigments are essential pieces of the photosynthetic apparatus [56] and a reduction in their biosynthesis can lead to negative consequences on plant development [55]. Total chlorophyll and carotenoid contents were measured in response to the different biopolymers and biopolymer derivatives and the results are shown in Table 1. The culture media HEC100, HEC1000 and CMC1000 showed the highest values for total chlorophyll (1.4, 1.42, and 1.39 mg g^{-1} FW, respectively) and carotenoids (0.30, 0.31, and 0.31 mg g^{-1} FW, respectively), whereas Chit1000 presented the lowest for both contents (0.86 mg g^{-1} FW for total chlorophyll, and 0.20 mg g^{-1} FW for carotenoids). In general, the differences from the control were not significant, suggesting that the biopolymers had no major effect on the production of photosynthetic pigments.

Many biotic and abiotic factors can induce oxidative stress in plants through the production and accumulation of reactive oxygen species (ROS), such as H_2O_2, that are highly reactive and toxic and may interfere with multiple pathways [57–59]. Therefore, H_2O_2 levels are a good indicator of plant stress. At low concentrations, H_2O_2 works as a signal molecule in response to biotic and abiotic stresses acting as a key regulator in a broad range of physiological processes. However, at high concentrations, H_2O_2 results in oxidative stress that may cause damage to lipids, proteins, pigments, carbohydrates, and nucleic acids [57]. According to the results, there were no significant differences in the H_2O_2 content for all biopolymers and biopolymer derivatives added to the media when compared to the control, 0.35 μmol g^{-1} FW (Table 2). These stable levels may indicate that there were no stress-induced changes prompted by the biopolymers or that *T. lotocephalus* shoots had the ability to control H_2O_2 accumulation.

In fact, plants have developed defense mechanisms against oxidative stress [57]. Both enzymatic (such as SOD and CAT) and nonenzymatic (such as phenolic compounds and proline) antioxidant defense systems can scavenge ROS and thus prevent oxidative damage [57,60]. Following the enzymatic pathway, SOD is the first enzyme of the detoxification processes, which catalyzes O^{2-} to H_2O_2 and O_2, and its activity is complemented by CAT that catalyzes dismutation reactions of H_2O_2 into O_2 and H_2O [57,60]. In the present study, after eight weeks of culture, the SOD and CAT activities of *T. lotocephalus* shoots did not increase in any of the media supplemented with biopolymers or biopolymer derivatives, when compared to the control, 44.41 and 0.0186 Units mg^{-1} protein, respectively (Table 2). There was indeed a significant decrease ($p < 0.05$) in SOD activity, in HEC1000, Chit100, GA100, and GA1000 media (38.04, 19.33, 23.37 and 19.95 Units mg^{-1} protein, respectively), and in the case of CAT activity, in HEC100, CMC100, CMC1000, Chit1000, and GA100 media (0.0113, 0.0091, 0.0093, 0.0109, and 0.0089 Units mg^{-1} protein, respectively), compared to the control. The levels of SOD and CAT activities reflect the stable levels of

H_2O_2 previously observed, proving that the biopolymers did not induce stress changes in *T. lotocephalus* shoots growing *in vitro*.

Table 2. H_2O_2 content, SOD activity, CAT activity, MDA content, and total soluble protein content in *Thymus lotocephalus* shoots grown in vitro (eight weeks) on media supplemented with HEC, CMC, chitosan, or gum arabic at two concentrations (100 and 1000 mg L^{-1}).

Medium	H_2O_2 (μmol g^{-1} FW)	SOD (Units mg^{-1} Protein)	CAT (Units mg^{-1} Protein)	MDA (nmol g^{-1} FW)	Protein (mg g^{-1} FW)
Control	0.35 ± 0.04	44.41 ± 3.85	0.0186 ± 0.0017	27.53 ± 0.94	15.07 ± 1.38
HEC					
100	0.34 ± 0.09	38.04 ± 4.28	0.0113 ± 0.0005 *	18.01 ± 0.74 *	13.93 ± 1.92
1000	0.23 ± 0.02	29.10 ± 2.72 *	0.0157 ± 0.0013	26.14 ± 1.43	18.75 ± 1.87
CMC					
100	0.26 ± 0.04	36.88 ± 4.18	0.0091 ± 0.0006 *	26.47 ± 0.97	13.63 ± 2.19
1000	0.29 ± 0.08	46.28 ± 3.89	0.0093 ± 0.0015 *	14.47 ± 0.42 *	12.31 ± 0.41
Chit					
100	0.29 ± 0.03	19.33 ± 1.28 *	0.0156 ± 0.0010	15.98 ± 1.32 *	18.35 ± 1.57
1000	0.37 ± 0.03	41.83 ± 4.04	0.0109 ± 0.0009 *	24.10 ± 2.08	16.48 ± 1.97
GA					
100	0.34 ± 0.06	23.37 ± 3.91 *	0.0089 ± 0.0009 *	28.05 ± 2.63	22.50 ± 1.82 *
1000	0.21 ± 0.02	19.95 ± 4.28 *	0.0176 ± 0.0012	24.10 ± 1.16	15.23 ± 1.36

Values are expressed as the mean ± SE. For each parameter, the asterisks (*) indicate differences from the control using Dunnett's test at $p < 0.05$. CAT: catalase; CMC: carboxymethylcellulose sodium salt; Chit: chitosan; GA: gum arabic; HEC: hydroxyethyl cellulose; H_2O_2: hydrogen peroxide; MDA: malondialdehyde; SOD: superoxide dismutase; 100: 100 mg L^{-1}; 1000: 1000 mg L^{-1}.

Another important symptom of oxidative stress in plants is lipid peroxidation in cell membranes, which occurs once the capacity of the plant to scavenge ROS is surpassed and can lead to cell death. Damaged membranes release MDA, a final product of lipid peroxidation and, therefore, a viable indicator of plant stress [61,62]. The MDA content in *T. lotocephalus* shoots did not increase in the presence of biopolymers or biopolymer derivatives in the culture medium. MDA content decreased ($p < 0.05$) in HEC100, CMC1000, and Chit100 media (18.01, 14.47, and 15.98 nmol g^{-1} FW, respectively) comparing to the control medium (27.53 nmol g^{-1} FW) (Table 2). The results indicate that the oxidative damage to cellular membranes was limited and reinforce the assumption that the biopolymers did not induce stress changes to *T. lotocephalus* shoots.

Protein content can also be affected by stress factors, whether by inhibiting its synthesis or by promoting the production of other proteins involved in stress response [63,64]. This study reveals that there was a significant increase ($p > 0.05$) in the total protein content on GA100 medium (22.50 mg g^{-1} FW), while no significant differences were observed in the remaining media. In general, these obtained results are consistent with the previous contents and activities evaluated (no H_2O_2 accumulation nor an increase in MDA content or SOD and CAT activities), indicating that there was no stress induced on *T. lotocephalus* shoots as there were no major differences in the total protein content.

The preceding results proved that biopolymers and biopolymers derivatives are harmless (at appropriate concentrations) to plants and can even improve their performance, as we demonstrated with CMC that considerably stimulated shoots elongation. The fact that those biopolymers did not induce stress-changes to in vitro cultures is a major advantage to their future use. They can be synergistically explored in a safe way in the development of new biopolymer-based materials, considering their individual characteristics, to improve plant yield and/or replace synthetic materials harmful to the environment.

To understand the effect of biopolymers on culture media, the rheological and microstructural features were assessed. The viscoelastic properties of all the media prepared in this study were analyzed via linear oscillatory measurements. As shown in Figure 2, samples display an extended linear regime. The frequency sweep tests revealed that all

media are robust gel-like systems, with the storage (elastic) modulus (G′) larger than the loss (viscous) modulus (G″) and frequency-independent. All media presented identical frequency sweep patterns, as exemplified in Figure 3 for HEC100 medium. Polymer gels, like the culture media prepared here, are systems that exhibit both solid and liquid-like properties. Their interactions and physical entanglements among polymer chains induce the formation of a large inter-connected network that pervades through the whole, granting fluidity and elasticity to the systems [65].

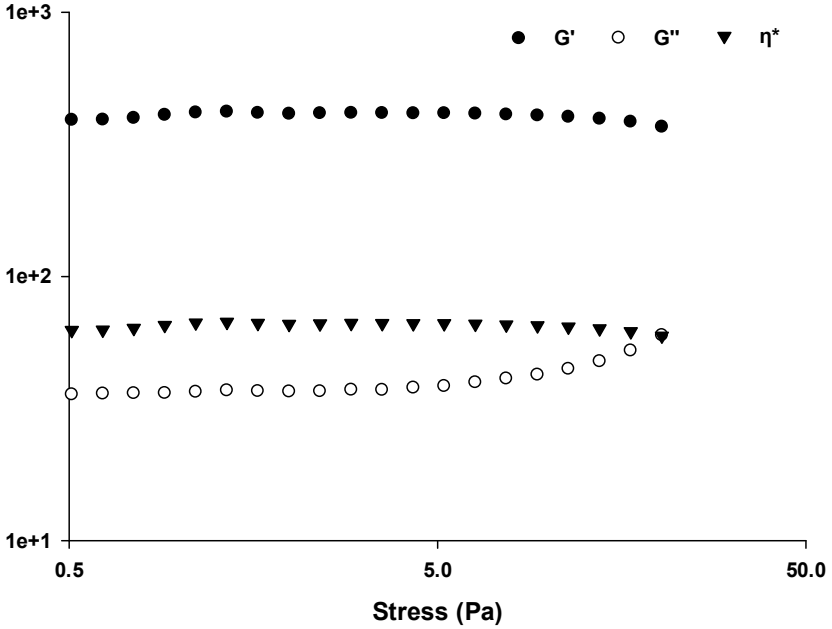

Figure 2. Stress sweep curves for G′ (Pa), G″ (Pa) and η* (Pa·s) as a function of stress at a constant frequency of 1 Hz, obtained for the medium HEC100, representative of the profiles observed in the tested samples from all media. G′: storage (elastic) modulus; G″: loss (viscous) modulus; HEC: hydroxyethyl cellulose; η*: viscosity; 100: 100 mg L^{-1}.

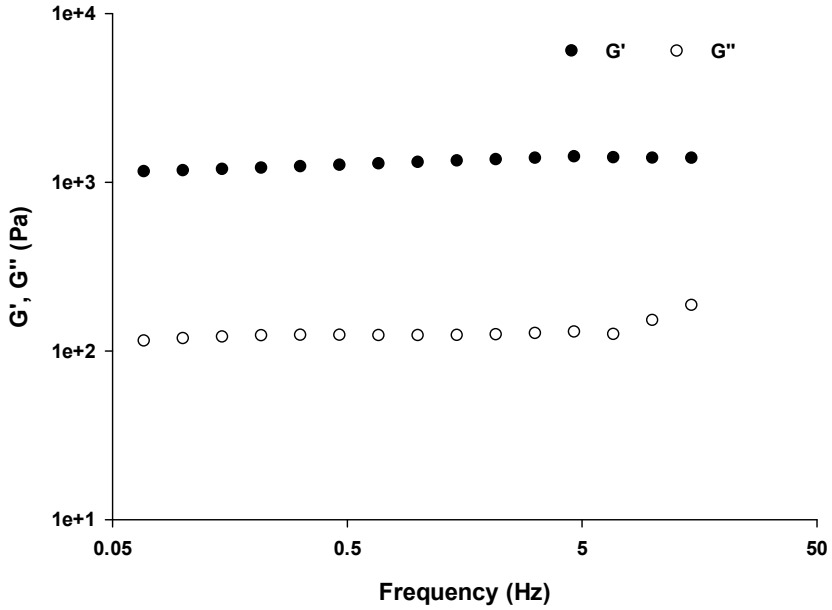

Figure 3. G′ and G″ extracted from the frequency sweep experiments at a constant frequency of 1 Hz for the medium HEC100, representative of all media. G′: storage (elastic) modulus; G″: loss (viscous) modulus; HEC: hydroxyethyl cellulose; 100: 100 mg L^{-1}.

The complex viscosity (η*) was assessed at the beginning and at the end of *T. lotocephalus* culture period, in all the media with and without growing shoots (Figure 4). Overall, the viscosity values of the culture media with and without shoots were very similar and slight differences might be attributed to artifacts related to inertia and slip effects that commonly occur in mechanical rheometry. Comparing the viscosity of the media at the beginning of the culture and after the eight-week culture period, an increase in the viscosity values is generally observed. The most pronounced differences occurred on HEC1000 (with shoots) (from 160.8 to 282.25 Pa·s), CMC100 (with shoots) (from 136.1 to 224.4 Pa·s), CMC100 (without shoots) (from 136.1 to 222.5 Pa·s) and CMC1000 (with shoots) (from 199.9 to 275.35 Pa·s) media and the exceptions were the control, GA1000 and Chit100 media, which presented similar results at the beginning and at the end of the growth period for media with and/or without shoots. The culture medium is essentially composed of water and the increase in viscosity can be related to the loss of water by evaporation during the culture period or to the intake of water by the plant during growth and along subculture period. The rate of water evaporation can vary according to the 3D-structure of the gel network that compose the medium, which in turn is sensitive to the structure and concentration of the polymers.

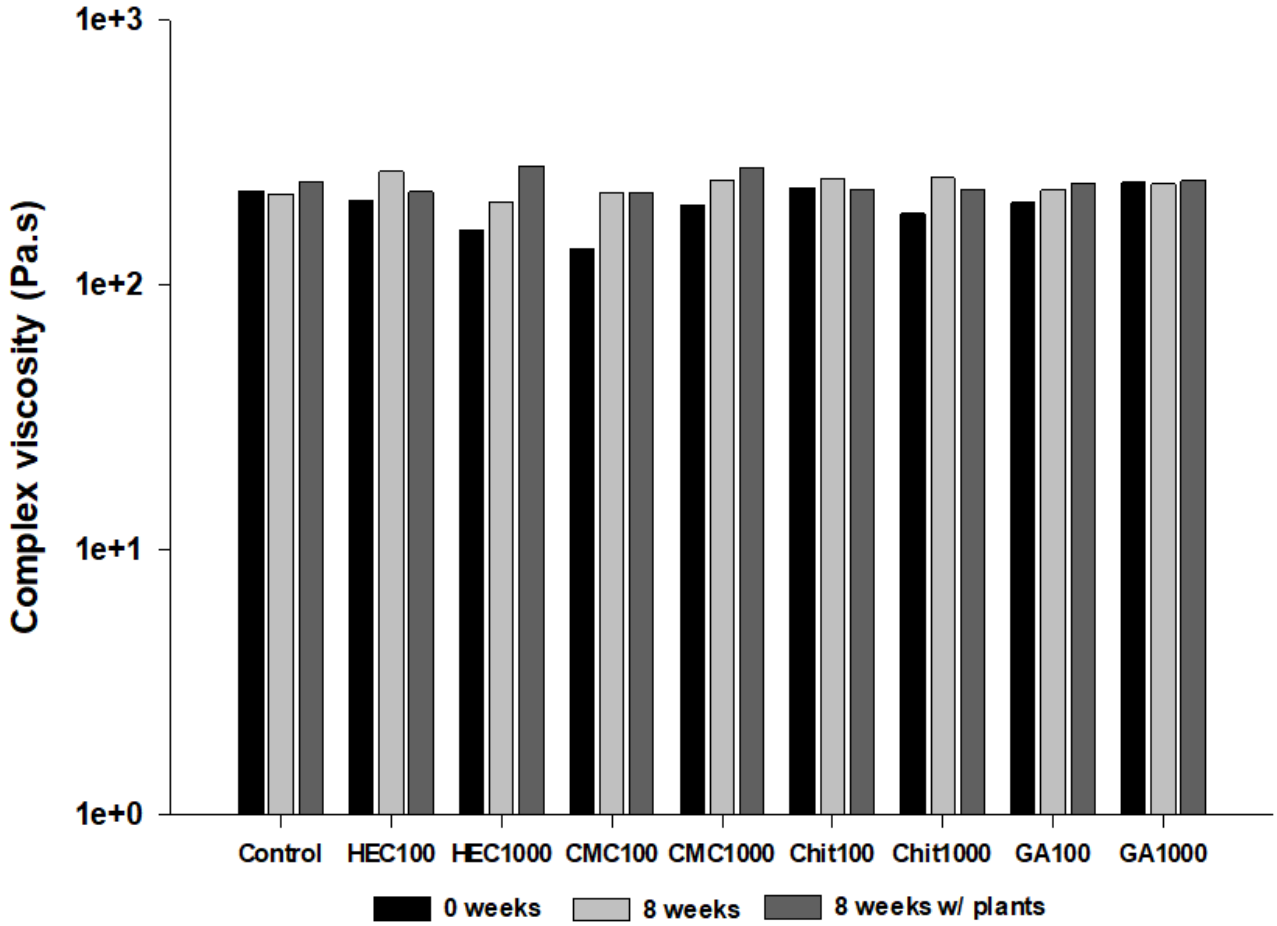

Figure 4. η* (Pa·s) extracted from the frequency sweep experiments at a constant frequency of 1 Hz for media supplemented with HEC, CMC, chitosan, or gum arabic at two concentrations, at the beginning (0 weeks) and at the end (8 weeks–without plants; 8 weeks w/plants–with plants) of the eight-week culture period. CMC: carboxymethylcellulose sodium salt; Chit: chitosan; GA: gum arabic; HEC: hydroxyethyl cellulose; η*: viscosity; 100: 100 mg L^{-1}; 1000: 1000 mg L^{-1}.

Simultaneously, in the media with shoots, due to plant's water consumption, an additional decrease of water may occur, leading to a tighter 3D gel network and higher viscosity values. This was observed on the control, HEC1000, CMC1000 and GA100 media, when comparing the final viscosity values of the media with (220.6, 206.6, 247.9, and 229.4 Pa·s, respectively) and without (245.4, 282.25, 275.4, and 243.4 Pa·s, respectively) shoots. In the cases where the viscosity was higher for media without shoots (e.g., HEC100, Chit100 and Chit1000), the biopolymers and biopolymer derivative used could have had influence on those superior values. As for the different concentrations used, HEC and chitosan presented higher viscosity values on media less concentrated, while CMC and gum arabic had higher values on more concentrated media.

To assess to the microstructure of the different media, SEM analysis was performed and the micrographs of all the media are shown in Figure 5. At the beginning of the culture period, the surface morphology of CMC100 and Chit1000 media was similar (Figure 5g,m, respectively), presenting denser and more compact surfaces compared to the control (Figure 5a). The same was observed for HEC100, HEC1000 and Chit100 (Figure 5b,c,i, respectively), while the surfaces of CMC1000, GA100 and GA1000 media were more wrinkled and creased (Figure 5h,n,o, respectively). The images captured after the eight-week culture period, demonstrated that wrinkles intensified overtime for CMC100 (Figure 5j), while the control and Chit1000 presented slight alterations (Figure 5d,p, respectively). The appearance of wrinkles overtime can be a consequence of water loss, which in turn might affect the viscosity of the culture media. Therefore, these findings are consistent with the greater increase in viscosity overtime observed for both CMC100 with and without plants in comparison to the other media (Figure 4).

The BET (Brunauer–Emmett–Teller) surface area was similar between media supplemented with CMC and chitosan, 21.42 and 20.61 $m^2\ g^{-1}$, respectively, and both are higher than the control, 11.07 $m^2\ g^{-1}$ (Table 3). However, the average pore diameter in CMC100, 1.88 nm, is considerably lower than the control, 3.52 nm, and Chit1000, 3.34 nm (Table 3). Interestingly, CMC100 was the medium that presented the longest shoots, which can be related to the medium average pore size: smaller pores may possibly contribute to a more efficient uptake of nutrients and water by the plants, resulting in an increase in translocation and consequently in longer shoots. This network of smaller pores also seems to be responsible to CMC100 higher variation in viscosity overtime (Figure 4).

Table 3. Nitrogen porosimetry for surface area and pore size measurements, using the BET approach, of the control and CMC100 and Chit1000 media. BET: Brunauer–Emmett–Teller; CMC: carboxymethylcellulose sodium salt; Chit: chitosan; 100: 100 mg L^{-1}; 1000: 1000 mg L^{-1}.

Medium	BET Surface Area ($m^2\ g^{-1}$)	Average Pore Diameter, øav (nm)
Control	11.0716	3.5176
CMC100	21.4237	1.8758
Chit1000	20.6064	3.3416

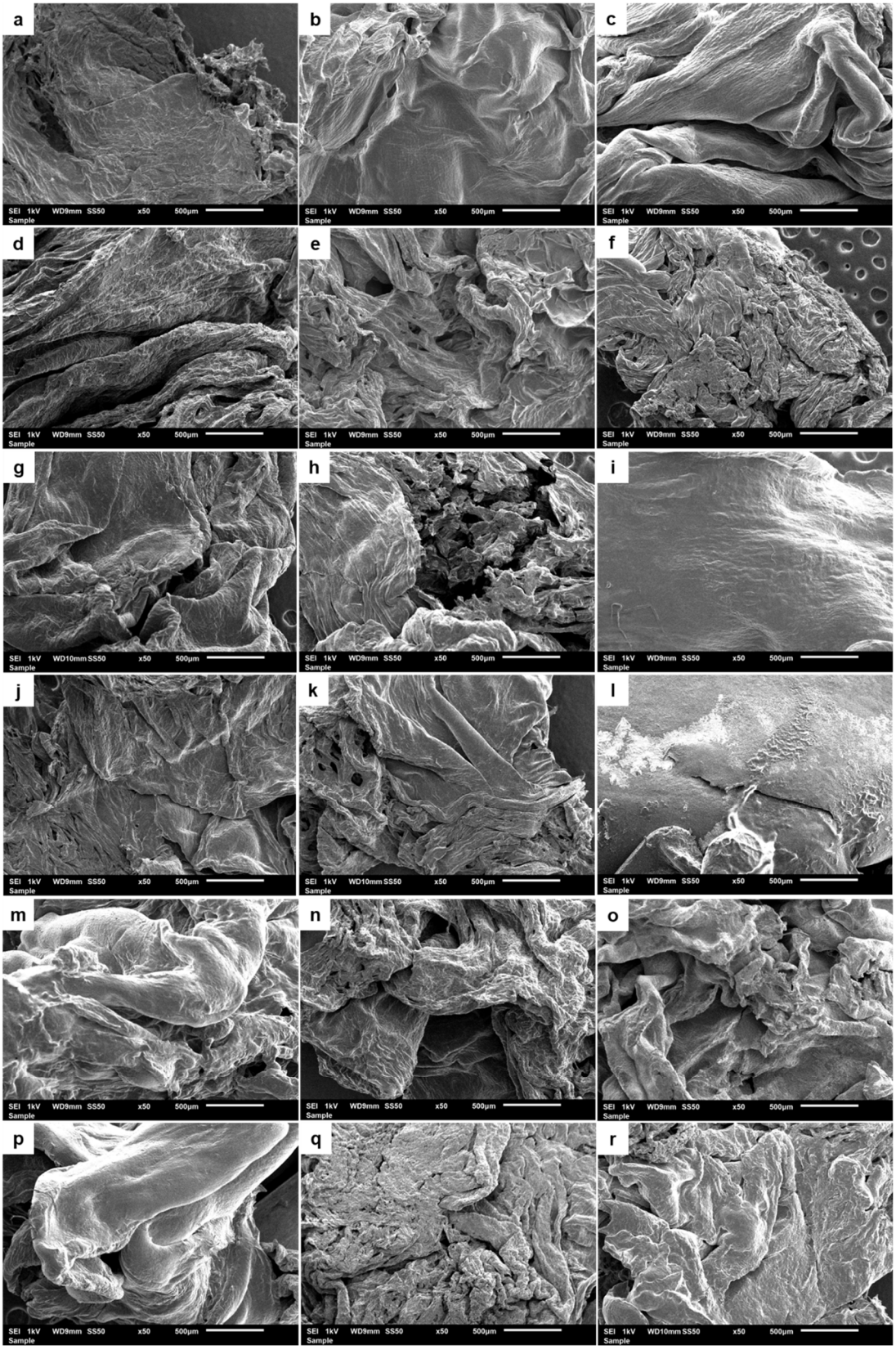

Figure 5. Scanning electron microscopy images of the control, HEC100, HEC1000, CMC100, CMC1000, Chit100, Chit1000, GA100 and GA1000 media at the beginning (**a**,**b**,**c**,**g**,**h**,**i**,**m**,**n** and **o**, respectively) and at the end (**d**,**e**,**f**,**j**,**k**,**l**,**p**,**q** and **r**, respectively) of the eight-week culture period. The scale bars represent 500 µm. Magnification: 50×. CMC: carboxymethylcellulose sodium salt; Chit: chitosan; GA: gum arabic; HEC: hydroxyethyl cellulose; 100: 100 mg L^{-1}; 1000: 1000 mg L^{-1}.

CMC is a water-soluble cellulose derivative obtained by etherification of cellulose [15]. Abdollahi et al. [66] demonstrated that there is a good compatibility between CMC and agar (which is a component of all the culture media tested in the present study) in the development of CMC-agar biocomposite films. Besides, morphological studies on super absorbent hydrogels based on CMC and polyvinylpyrrolidone crosslinked with gamma irradiation, for agricultural applications, demonstrated that these hydrogels have highly interconnected porous network structure, which facilitates the water diffusion process through the 3D matrix [37]. An improved water diffusion can result in a higher water evaporation rate, but it can also facilitate the water uptake by the plant. Consequently, these characteristics support the results obtained for CMC at 100 mg L^{-1}. Similar to our study, nanoparticles dispersions stabilized with CMC, aiming to improve seed germination and seedling growth, substantially improved root elongation of *Solanum lycopersicum* L. (tomato) seedlings, without toxic effects on plants [67].

When it comes to the application of novel products to in vitro culture of plants, there are numerous factors involved (e.g., plant species, medium components and concentrations, and growth conditions), and a generalization could be premature. Nonetheless, the evidence obtained in this work is promising for the application of biopolymers and biopolymer-based materials to the propagation of plants *in vitro*. They are harmless to plant tissues and may be able to change the culture media microstructure in a way that benefits plant growth and development. We intend to further investigate other conditions (e.g., concentrations and combinations between these biopolymers and others), to disclose synergisms that might be beneficial for the growth of different plant species.

4. Conclusions

The first aim of this study was to evaluate the influence of different biopolymers and biopolymer derivatives on the growth and physiological responses of *Thymus lotocephalus* in vitro culture. One of the main requirements when using innovative materials or methods in plant culture is the absence of harmful effects. Bearing this in mind, this study proved that all the biopolymers and biopolymer derivatives tested are physiologically safe for in vitro plant cultures and therefore can represent in the future a valuable and important resource for in vitro plant propagation. The use of biopolymers and biopolymers-based materials on in vitro plant production meet the increasing demand for more sustainable production and products and besides improving growth, these materials can also replace synthetic ones.

This study also showed that cellulose-based biopolymers, firstly tested here for in vitro plant propagation, are a promising alternative to synthetic compounds and their use in vitro should be further investigated. Though we only obtained clear beneficial results for one of the tested biopolymer derivatives (CMC) regarding the shoot length parameter, we hope this work paves the way to explore other conditions, such as different concentrations and combinations of biopolymers, in the future.

The other objective was to access the rheological and microstructural features of the prepared media. Biopolymers and biopolymer derivatives can alter the rheological and microstructural features of plant culture media and consequently influence plant development. The data suggests that a medium with smaller pores might allow for a more efficient water uptake by the plants, contributing to a higher elongation.

Due to their eco-friendly characteristics and versatility, biopolymers have an enormous potential for in vitro plant propagation that needs to be further explored. Future research on the inclusion of biopolymers in plant tissue culture medium can significantly contribute to the replacement of synthetic, non-sustainable alternatives while improving plant growth.

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and A.R.; project administration, B.M. and A.R.; funding acquisition, B.M. and A.R. All authors have read and agreed to the published version of the manuscript.

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