CHITOSAN AND *META*-TOPOLIN SUPPLEMENTATION OF THE MULTIPLICATION MEDIA OF *VITIS VINIFERA* L.

Marcelina Krupa-Małkiewicz[1,a,*], Ireneusz Ochmian[2,b]

[1] – Department of Plant Genetics, Breeding and Biotechnology, West Pomeranian University of Technology Szczecin, Słowackiego 17 Str., 71–434 Szczecin, Poland
[a] – ORCID: 0000–0002–4333–9122
[2] – Department of Horticulture, West Pomeranian University of Technology Szczecin, Słowackiego 17 Str., 71–434 Szczecin, Poland
[b] – ORCID: 0000–0002–3606–1927
[*]corresponding author: mkrupa@zut.edu.pl

Abstract

We investigated the efficiency of chitosan (CH) and meta-*topolin (*m*T) on in vitro growth and adaptation to greenhouse conditions of Vitis vinifera cv. Johanniter and cv. Hibernal. After the initiation stage, we transferred explants to Woody Plant Media (WPM) with chitosan (molecular weight 3.33 kDa) at a concentration of 10, 20, and 40 ppm or to WPM with* m*T at a concentration of 0.5, 1.0, and 2.0 mg l–1. WPM without CH or* m*T served as a control. Among the tested combinations, WPM with 10 ppm of CH led to the maximum mean root length (12.18 cm and 12.65 cm for the Hibernal and Johanniter cultivars, respectively) and the number of new leaves (5.9 and 8.15 for the Hibernal and Johanniter cultivars, respectively). As the CH concentration in the medium increased, the length of shoots and roots decreased. We found the highest percentage of acclimatised plants on WPM (cv. Hibernal) and WPM with 40 ppm of CH (cv. Johanniter). The addition of* m*T had a negative effect on the morphological traits we measured, regardless of the concentration. Leaves of explants from WPM with* m*T were yellower and redder compared with the control. None of the explants obtained on this medium produced roots.*

Keywords: *biologically active substance, in vitro, cytokine, winegrape*

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1. Introduction

Grapes are one of the most important fruit plant species; they belong to a group of foods rich in antioxidant compounds. They are a rich source of calcium; iron; vitamins A, C, and E; amino acids; and phosphorous. Fruits and their products (wine and juice) exert many health-promoting effects, particularly a reduced risk of cardiovascular diseases and type 2 diabetes [1]. The conventional method of winegrape propagation (cutting) is time consuming: a planted winegrape needs 4–5 years to produce propagation materials by cuttings due to its long juvenile period. Winegrape juvenility is one of the principal natural problems hindering winegrape production [2]. Thus, to achieve high-quality crops it is important to determine optimal growth conditions. In addition to traditional plant propagation techniques, biotechnological methods are used. The best plant growth under *in vitro* conditions can be achieved by using an appropriately selected culture medium. In the following stages of propagation, it is often necessary to add plant growth regulators to the medium to optimise plant growth. However, adding these components can increase the costs of micropropagation [3]. Besides, the most used plant growth regulators are synthetic, which is contrary to the growing acceptance of more sustainable and eco-friendly production. Since then, many different protocols of *in vitro* winegrape propagation have been developed [2, 4, 5]. Most of them recommend the use of 6-benzyloaminopurine (BAP) as a general growth hormone. *Meta*-topolin (*m*T), or 6–(3–hydroxylbenzylamino) purine, is an aromatic cytokinin originally isolated from poplar leaves. It is a natural cytokinin that differs from BAP in that its major *O*–glucoside metabolite is translocated rapidly to different parts of explants. Hence, *m*T is widely used as a substitute for BAP to mitigate foliar senescence, hyperhydricity, and necrosis, while improving shoot proliferation, elongation, quality, rooting, and acclimatisation competence. In the available literature, *m*T has not been used for *Vitis vinifera* L. shoot multiplication.

There are only a few reports describing the application of chitosan (CH) in winegrape research. CH is a polysaccharide produced from chitin – obtained from crab shells, among other sources – which can be made soluble through alkaline or enzymatic deacetylation. This biocompatible biopolymer has become a feasible biomaterial with a variety of applications in agriculture, the food industry, cosmetology, biotechnology, and medicine [6–8]. Due to its bioactivity in plants, CH enhances plant production [9]; stimulates seed germination and shoot and root growth [10]; intensifies the photosynthetic process by enlarging chloroplasts and increasing the chlorophyll concentration [11]; and alleviates the harmful effects of abiotic stress in plants [12, 13].

We aimed to compare the effectiveness of CH and *m*T on micropropagation of winegrape explants of the *V. vinifera* cv. Hibernal and cv. Johanniter *in vitro*. We identified the optimal medium for the multiplication and rooting of *V. vinifera*.

2. Materials and Methods

2.1. Plant Material

The research material consisted of plants of two winegrape cultivars, Hibernal and Johanniter, taken from the Rajkowo Palace Vineyard. The vineyard is located in subzone 7A in north-western Poland on the Szczecin Lowlands. The shoots were taken in May 2021.

2.2. Sterilisation, Initiation and Multiplication

Shoots of *V. vinifera* cv. Hibernal and cv. Johanniter were surface sterilised with 70% (v/v) ethanol solution for 30 s and then thoroughly rinsed with sterile deionised water for 2 min. Next, shoots were soaked in a 7% (v/v) solution of sodium hypochlorite (NaOCl) for 15 min and then rinsed three times (for 2 min each) in sterile deionised water. Under a laminar

flow cabinet, winegrape shoots 17–20 mm in length were initiated on Woody Plant Medium (WPM) (Duchefa Biochemie B.V., the Netherlands) [14] without plant growth regulators. The initiated explants were transferred to fresh WPM regularly every 4 weeks for 4 months to prepare the explants for the subsequent experiments. After the end of the initiation step, the number of initiated explants and the percent of contamination and regenerated explants were estimated.

Next, the shoot explants were transferred to WPM supplemented with CH (molecular weight of 3.33 kDa) at a concentration of 10, 20, and 40 ppm, or to WPM supplemented with *m*T at a concentration of 0.5, 1.0, and 2.0 mg l^{-1}. Shoot explants transferred to WPM without *m*T and CH served as the control. Both CH and *m*T were added to WPM before autoclaving.

2.3. Culture Conditions

Each combination included 48 shoots (six shoots per flask in eight replicates). All media were supplemented with 30 g l^{-1} sucrose (Chempur, Poland) and 100 mg l^{-1} myo-inositol (Duchefa) and were solidified with 8 g l^{-1} agar (Biocorp, Poland); the pH of the media was adjusted to 5.7. The media were heated and 30 ml was poured into a 450 ml flask. It was then autoclaved at 121°C (0.1 MPa); the time depended on the volume in the vessel. All cultures were incubated in a growth room at 24 ± 2 °C with a 16 h photoperiod and a photosynthetic flux density of 40 µmol m^{-2} s^{-1} provided by Narva (Germany) emitting daylight cool white. After 6 weeks, the explants were removed and washed with distilled deionised water, and the lengths of the shoots and roots and the number of roots and leaves per plant were measured. In addition, the plant fresh and dry masses were estimated.

2.4. Chitosan

CH was obtained from the Center of Bioimmobilisation and Innovative Packaging Materials at West Pomeranian University of Technology in Szczecin, Poland. CH was prepared according to Bartkowiak [15] using the free-radical degradation process. It was purified (filtration) and characterised using high-performance liquid chromatography (SmartLine Knauer, Germany; Tessek Separon HEMA-BIO 40 column, Tessek, Czech Republic). The average degree of deacetylation of the product was 85%.

2.5. *Ex Vitro* Acclimatisation

After removing the remains of agar medium, plants with developed root systems, were transferred to plastic pots (10 cm diameter) filled with a mixture of 90% peat and 10% perlite. The pots were covered with agro-textile fabric to ensure 90% humidity and left in the growth room for 3 weeks. The plants were then transferred to the greenhouse. The average temperature was 22°C and the relative humidity was 73%. After 6 weeks of acclimatisation, the percentage survival of the plants was determined.

2.6. Determination of Colour

The pigment (colour) of leaves (from the middle part of the shoot) was measured in transmission mode using the photocolourimetric method in the CIE *L*a*b** system [16] with a CM-700d spectrophotometer (Koncia Minolta, Japan). The diameter of the measurement hole was 3 mm, the observer type 10º, and the illuminant D65 was used. The value of *a** ranges from green (–*a**) to red (+*a**). The parameter *b** describes the colour in the range from yellow (+*b**) to blue (–*b**). The value of parameter *L**, which represents monochromaticity, ranges from 0 (black) to 100 (white).

2.7. Statistical Analysis

All statistical analyses were performed using Statistica 13.0 (StatSoft, Poland). Statistical significance of the differences between means was determined by testing the homogeneity of variance and the normality of the distribution, followed by ANOVA analysis of variance with Tukey's *post hoc* test. The significance was set at $p < 0.05$.

3. Results and Discussion

According to Kinfe *et al.* [2], *in vitro* propagation of winegrapes is difficult. The response of winegrape cultivars to a specific medium is strongly dependent on the particular genotype, sterilising agent, medium, and plant growth regulators used. Sterilisation of shoot tip explants is considered to be a serious problem in all woody plants, as the explants originate from field-grown winegrapes. We used NaOCl as a sterilising agent. Diab *et al.* [17] and Kinfe *et al.* [2] found almost the same combination and concentration of the sterilant to be effective to sterilise winegrape explants. However, Melyan *et al.* [5] observed that treatment with 2% calcium hypochlorite for 7 min plus 70% ethanol for 3 min led to 75% aseptic culture establishment of the Parvana winegrape cultivar *in vitro*. We found more contaminated explants (55.17%) for the Johanniter cultivar than the Hibernal cultivar (15.11%) (Table 1). In addition, Hibernal explants initiated growth better in *in vitro* cultures and the percentage of regeneration was three times higher compared with Johanniter explants.

Table 1. The percent contamination and regeneration of *Vitis vinifera* cv. Hibernal and cv. Johanniter explants *in vitro*.

Cultivar	Number of initiated explants	Contamination [%]	Regenerated explants [%]
Hibernal	100	15.11	60
Johanniter	100	55.17	20

Note. The observations were recorded regularly for 30 days for the non-growing, infected, and healthy cultures

The mean shoot length, number of new shoots, and fresh and dry mass are given in Table 2, and all steps of winegrape micropropagation are presented in Figure 1. Of the CH concentrations applied in the experiment, 10 ppm was the most efficient in increasing the number of leaves per plant (16% and 21% more compared with the control for the Hibernal and Johanniter cultivars, respectively), root length (54% and 35% more compared with the control, for the Hibernal and Johanniter cultivars, respectively), and the shoot length of Hibernal plants (4% more compared with the control). In addition, an increase in the CH concentration in WPM had an inhibitory effect on the morphological traits of winegrape explants (Figure 2), although the differences were not statistically significant. When comparing the growth of the two cultivars *in vitro* on the control medium, the Johanniter explants were 32% taller, had 15% longer roots, and had 24% more leaves compared with the Hibernal explants. In contrast, the Hibernal explants had 37% more roots compared with the Johanniter explants (Figure 2). When we added *m*T to the medium, we noted that as the concentration increased, the shoot length, number of leaves, and fresh and dry mass decreased. In addition, plants growing on WPM with *m*T did not develop roots and showed a tendency to form calli (Figure 3).

Table 2. The effects of different Woody Plant Media (WPM) compositions on the morphological traits of *Vitis vinifera* cv. Hibernal and cv. Johanniter explants cultured *in vitro* for 6 weeks.

Medium	Shoot length [cm]	Root length [cm]	Number of roots	Number of leaves per plant	Fresh weight [g]	Dry weight [g]
Hibernal						
WPM	4.78^{bdc}	7.93^{b}	3.95^{a}	5.10^{bcd}	0.28^{ab}	0.04^{ab}
WPM + 10 ppm CH	4.93^{abcd}	12.18^{a}	2.85^{bc}	5.90^{abc}	0.31^{ab}	0.04^{ab}
WPM + 20 ppm CH	4.70^{bdc}	9.83^{b}	2.65^{bc}	5.30^{bcd}	0.28^{ab}	0.04^{ab}
WPM + 40 ppm CH	4.68^{bcd}	8.75^{b}	3.15^{ab}	5.30^{bcd}	0.31^{ab}	0.04^{ab}
WPM + 0.5 mg l^{-1} *m*T	1.48^{ef}	0.00^{c}	0.00^{d}	4.80^{bcd}	0.28^{ab}	0.03^{bc}
WPM + 1.0 mg l^{-1} *m*T	1.13^{e}	0.00^{c}	0.00^{d}	3.25^{de}	0.09^{c}	0.02^{c}
WPM + 2.0 mg l^{-1} *m*T	1.08^{f}	0.00^{c}	0.00^{d}	1.90^{e}	0.08^{c}	0.02^{c}
Johanniter						
WPM	7.00^{a}	9.38^{b}	2.50^{bc}	6.75^{ab}	0.42^{a}	0.06^{a}
WPM + 10 ppm CH	6.78^{ab}	12.65^{a}	2.05^{c}	8.15^{a}	0.33^{ab}	0.04^{ab}
WPM + 20 ppm CH	6.40^{abc}	8.53^{b}	1.80^{c}	6.95^{ab}	0.28^{ab}	0.03^{ab}
WPM + 40 ppm CH	5.68^{abc}	7.84^{b}	2.16^{bc}	6.74^{ab}	0.30^{ab}	0.03^{ab}
WPM + 0.5 mg l^{-1} *m*T	5.35^{abcd}	0.00^{c}	0.00^{d}	6.70^{ab}	0.34^{a}	0.05^{ab}
WPM + 1.0 mg l^{-1} *m*T	4.50^{cd}	0.00^{c}	0.00^{d}	5.40^{bcd}	0.09^{c}	0.03^{bc}
WPM + 2.0 mg l^{-1} *m*T	3.45^{de}	0.00^{c}	0.00^{d}	4.10^{cde}	0.19^{bc}	0.01^{bc}

Note. CH, chitosan; *m*T, *meta*-topolin. Means followed by the same letter do not differ significantly at α = 0.05 according to Tukey's multiple range test (n = 48 shoots per treatment).

There are several reports on the micropropagation of winegrape [5, 17, 18]. Most authors recommend Gamborg's B5 medium to micropropagate grape plants. In our study, we successfully used WPM. Chee and Pool [19] tested the effects of auxin and cytokinin and the photoperiod on the *in vitro* development of shoots from cultured shoot apices of Rougeon winegrapes. Similarly, Cai *et al.* [20] used B5 medium to investigate yeast extract and polysaccharide-induced elicitation responses in *V. vinifera* cell suspension cultures. Yerbolova *et al.* [18] identified the best hormonal combination of BAP and 1-naphthaleneacetic acid (NAA) in Initiation Medium (IM) for dormant bud initiation and rooting of *in vitro* plants. However, there is no information in the literature about the possibility of using CH or *m*T to micropropagate grape plants. CH as an elicitor has been used by many researchers to stimulate the growth of various plant species *in vitro*. Obsuwan *et al.* [21] used CH to promote the morphological characteristics of *Rhynchostylis giganteaprotocorms in vitro* cultures. According to Sopalun *et al.* [22], CH stimulated *in vitro* shoot formation of *Grammatophyllum speciosum*. On the other hand, Krupa-Małkiewicz and Fornal [12] confirmed the positive effect of CH as an *in vitro* growth stimulator of petunias.

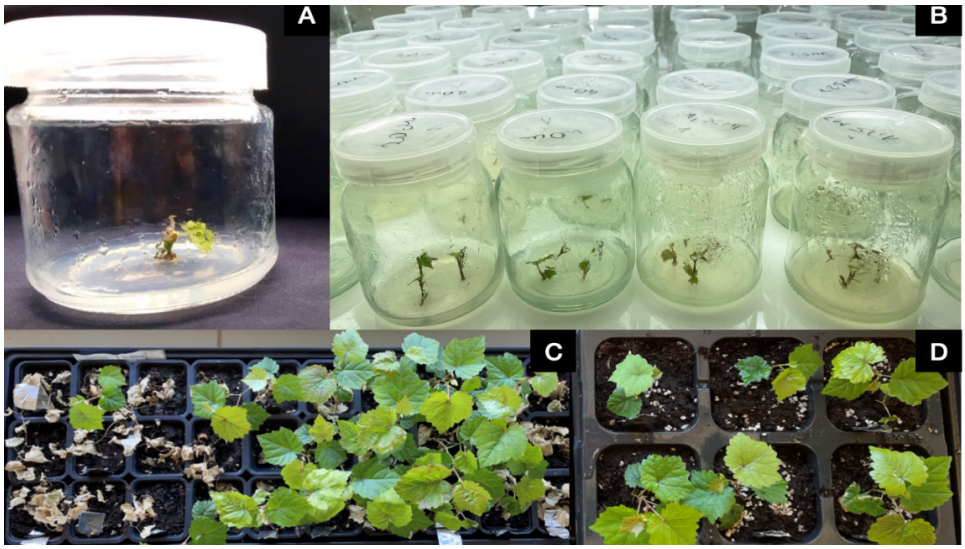

Figure 1. Stages of micropropagation of *Vitis vinifera* winegrapes. (A) Shoot initiation on Woody Plant Media (WPM) of *V. vinifera* cv. Johanniter after 1 week of culture. (B) Rooting and regeneration of explants on WPM supplemented with chitosan and *meta*-topolin at different concentrations. (C) Acclimatisation to *ex vitro* conditions in the growth room.

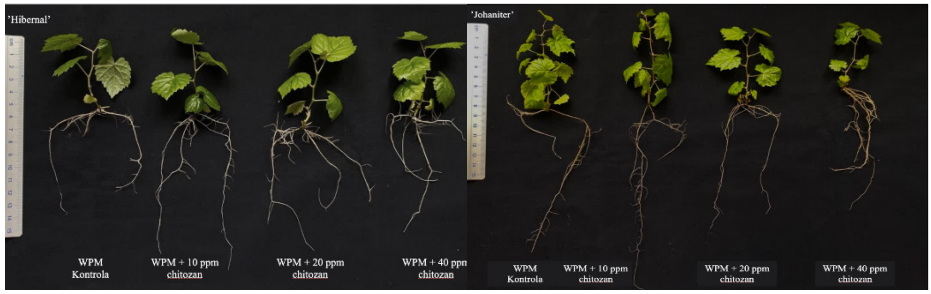

Figure 2. Shoot explants of *Vitis vinifera* cv. Hibernal and cv. Johanniter after 6 weeks on Woody Plant Media (WPM) supplemented with chitosan at a concentration of 0 (control), 10, 20, and 40 ppm.

Figure 3. Shoot explants of *Vitis vinifera* cv. Hibernal and cv. Johanniter after 6 weeks on Woody Plant Media (WPM) supplemented with *meta*-topolin (*m*T) at a concentration of 0 (control), 0.5, 1.0, and 2.0 mg l^{-1}

Table 3. The effects of chitosan (CH) and *meta*-topolin (*m*T) on the *L**, *a**, and *b** parameters of *Vitis vinifera* cv. Hibernal and cv. Johanniter cultured *in vitro*.

Medium	*L**	*a**	*b**
Hibernal			
WPM	49.98^{bc}	–8.14^{d}	21.44^{b}
WPM + 10 ppm CH	50.22^{bc}	–8.15^{d}	21.72^{b}
WPM + 20 ppm CH	52.62^{abc}	–8.62^{d}	24.76^{b}
WPM + 40 ppm CH	50.25^{bc}	–8.26^{d}	22.49^{b}
WPM + 0.5 mg l^{-1} *m*T	57.18^{a}	–0.69^{bc}	29.70^{a}
WPM + 1.0 mg l^{-1} *m*T	50.76^{bc}	–1.96^{c}	25.53^{ab}
WPM + 2.0 mg l^{-1} *m*T	52.69^{bc}	0.36^{bc}	21.70^{b}
Johanniter			
WPM	51.76^{bc}	–8.01^{d}	21.47^{b}
WPM + 10 ppm CH	51.12^{bc}	–8.56^{d}	21.75^{b}
WPM + 20 ppm CH	51.42^{bc}	–8.11^{d}	22.01^{b}
WPM + 40 ppm CH	52.07^{bc}	–7.91^{d}	21.32^{b}
WPM + 0.5 mg l^{-1} *m*T	53.17^{ab}	1.75^{ab}	24.07^{b}
WPM + 1.0 mg l^{-1} *m*T	49.89^{bc}	3.43^{a}	22.47^{b}
WPM + 2.0 mg l^{-1} *m*T	48.60^{c}	0.48^{bc}	23.27^{b}

Note. WPM, Woody Plant Media. Means followed by the same letter do not differ significantly at α = 0.05 according to Tukey's multiple range test (n = 48 shoots per treatment).

The success of micropropagation of woody and shrub plants depends on the rooting ability of the plants. Kruczek *et al.* [10] reported that 20 ppm of CH was optimal for the initiation of goji explant rhizogenesis *in vitro*. In the present study, we confirmed that 10 ppm of CH stimulated rhizogenesis of winegrape explants. Only Hibernal explants acclimatised to the *ex vitro* conditions (Figure 4). We also observed that the percent of acclimatised plants decreased as the CH concentration increased and was lower than the control (75%) by 22% (WPM + 10 ppm CH) to 99% (WPM + 40 ppm CH).

Many authors suggest that the contents of photosynthetic pigments in leaves are closely correlated to their colour [7, 23, 24]. Hence, measuring the colour provides a way to assess the condition of the plants. We assessed the colour of the studied winegrapes by determining the *L**, *a**, and *b** parameters. A change in the *L** parameter indicates darkening. There were no significant differences in leaf colour intensity (Table 3). The exceptions were Hibernal and Johanniter explants from WPM with 0.5 mg l^{-1} *m*T, which were brighter than the control (49.98 and 51.76, respectively) by 14% and 3%, respectively. Moreover, all winegrape explants grown on WPM with the addition of *m*T had redder leaves (from –1.96 for Hibernal to 3.43 for Johanniter) compared with the control (–8.14 and –8.01, respectively, for the Hibernal and Johanniter cultivars). This is shown by the value of the a^* parameter. The leaf surface colour defined by b^* indicated the location along the axis between yellow and blue; we did not find statistically significant differences. The winegrape leaves we examined in this study were less green than goji leaves *in vitro* [10] and similar in colour to strawberry leaves [11].

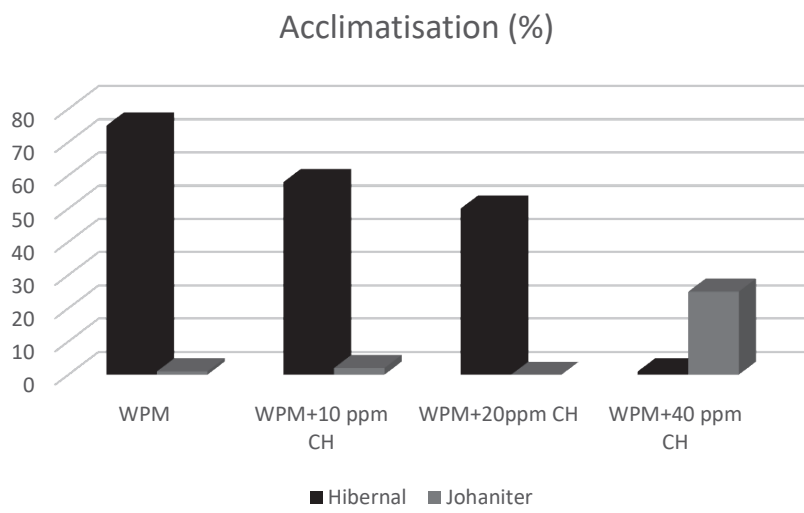

Figure 4. The percent of *Vitis vinifera* cv. Hibernal and cv. Johanniter explants that acclimatised to the *ex vitro* conditions.

4. Conclusion

The results of this study indicate that the addition of CH (with a molecular weight of 3.33 kDa) positively influences micropropagation of *V. vinifera* cv. Hibernal and cv. Johanniter. The CH treatments were more effective than the *m*T treatments in terms of stimulation of growth, independently of the concentration. CH at the concentration of 10 ppm led to taller plants, more leaves, and greater fresh and dry mass compared with the highest CH concentration. Moreover, CH at 10 ppm alongside a complex of organic substances can be used successfully to promote *V. vinifera* cv. Hibernal rooting and *ex vitro* acclimatisation.

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