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## Micropropagation of *Vitis vinifera* L. cv. Hiberna Using Chitosan, Meta-Topolin and Silicon Supplementation

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**Abstract:** The aim of this protocol is to present an optimized in vitro micropropagation procedure for *Vitis vinifera* L. cv. Hiberna, focusing on shoot multiplication, rooting, and plant quality under supplementation with chitosan, meta-topolin (mT), and silicon (Actisil) in Woody Plant Medium (WPM). Nodal shoots excised from vineyard-grown stock plants are surface-disinfected and established on hormone-free WPM, followed by multiplication on media enriched with different concentrations of chitosan, mT, and Actisil. Low chitosan concentration (10 ppm) promotes root elongation and high-quality shoots without visible phytotoxicity. In contrast, higher mT levels suppress both shoot and root formation, underlining the need for careful cytokinin dosage. Actisil applied at 50  $\mu\text{L L}^{-1}$  slightly improves root length and plant resilience, confirming the supportive role of silicon in stress tolerance. Leaf colour parameters measured in the CIE Lab system reflect changes in pigmentation and potentially chlorophyll content in response to mT and chitosan. The protocol provides a reproducible platform for efficient micropropagation of 'Hiberna' and can be adapted to other wine grape cultivars.

## 1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important fruit crops worldwide, with fruits used for wine, juice, fresh consumption, and processed products such as jellies and raisins (Mijowska et al., 2016, Ochmian et al., 2019). The continuous expansion of viticulture, combined with climate change and the need for uniform, healthy planting material, has increased interest in reliable propagation systems. Traditional propagation by cuttings is often time-consuming due to a relatively long juvenile period and may be limited by the health status and physiological condition of donor plants (Kinfel et al., 2017). In this context, micropropagation offers rapid multiplication of disease-free, genetically uniform plants and has become an essential biotechnological tool in modern viticulture (Skiada et al., 2010; Melyan et al., 2015). Standard protocols for grapevine micropropagation frequently rely on auxins and cytokinins added to basal media such as Murashige and Skoog (MS) or Woody Plant Medium (WPM), yet there is still a need to refine these systems for individual cultivars and to exploit novel biostimulants. Cultivar Hibernál is an interspecific hybrid widely used in cool-climate viticulture; its efficient micropropagation is crucial for establishing vineyards in northern regions. Among promising additives, silicon and chitosan have received increasing attention. Actisil, a commercial choline-stabilised orthosilicic acid solution, has been shown to enhance growth, flowering and biochemical parameters in ornamental species and small fruit crops, both in vitro and under greenhouse conditions (Krupa-Małkiewicz and Calomme, 2021; Figiel-Kroczyńska et al., 2022). Silicon contributes to cell-wall strengthening and improved tolerance to abiotic stresses such as drought or salinity (Sacała, 2009; Kabir et al., 2016). Chitosan, a deacetylated derivative of chitin, acts as a biocompatible polymer and elicitor that can stimulate growth, root formation and stress tolerance, including under heavy metal exposure (Kabir et al., 2016; Krupa-Małkiewicz and Ochmian, 2023). Meta-topolin (mT), a naturally occurring aromatic cytokinin, has been proposed as an alternative to classical synthetic cytokinins due to its favourable effects on shoot proliferation, reduced hyperhydricity and better carry-over of treated plantlets to ex vitro conditions (Hasanuzzaman et al., 2012; Krupa-Małkiewicz and Ochmian, 2023). However, the optimal concentration range is cultivar-dependent and excessive doses can suppress growth and rooting. This chapter describes a detailed, step-by-step micropropagation protocol for *V. vinifera* cv. Hibernál on WPM medium supplemented with different concentrations of chitosan, meta-topolin, and Actisil. It includes plant material preparation, disinfection procedure, medium preparation, transfer of explants, and leaf colour assessment by the CIE  $L^*a^*b^*$  method. Representative results and key conclusions regarding the most effective combinations are summarised to guide users in adapting the protocol to their own laboratories.

## 2. Material and Methods

### 2.1. Materials

Prepare all solutions using deionised water. Store reagents at room temperature in a dry place unless indicated otherwise. Maintain strict aseptic conditions when working under a laminar airflow hood. Whenever possible, avoid environmentally harmful substances and use appropriate personal protective equipment (gloves, lab coat, mask).

### 2.2. Plant material

Shoots of *Vitis vinifera* L. cv. Hibernál collected from the Rajkowo Palace Vineyard located in subzone 7A in north-western Poland on the Szczecin Lowlands. Stock plants should be healthy, free from visible symptoms of disease or pest damage, and grown under standard vineyard management (see Note 1).

### **2.3. Culture media and reagents**

1. Ready-to-use McCown Woody Plant Medium (WPM) including vitamins (Duchefa Biochemie B.V., the Netherlands) in powder form (Lloyd and McCown 1980).
2. Sucrose (Chempur, Poland).
3. Agar (Biocorp, Poland).
4. Myo-inositol (Duchefa Biochemie B.V., the Netherlands).
5. Silicon solution: Hydroplus™ Actisil, a commercial, ready-to-use fertilizer containing 0.6% choline-stabilised orthosilicic acid and 2% Ca; chemical composition: Si 0.6%; Ca 2%; H<sub>2</sub>O 35–45%; choline 55–65% (see Note 2).
6. Chitosan (CH) with a molecular weight of 3.33 kDa, obtained from the Center of Bioimmobilisation and Innovative Packaging Materials, West Pomeranian University of Technology in Szczecin, Poland. Chitosan is degraded using a free-radical degradation process and purified by high-performance liquid chromatography (HPLC); degree of deacetylation: 85% (see Note 3).
7. Meta-topolin (mT) (Duchefa Biochemie B.V., the Netherlands).
8. Deionised water for media preparation and reagent dilution.

### **2.4. Disinfection and washing solutions**

1. 70% (v/v) ethanol solution.
2. Sterile deionised water.
3. 7% (v/v) sodium hypochlorite (NaOCl) solution prepared from a commercial bleach stock (see Note 4).

### **2.5. Equipment and general laboratory supplies**

1. Laboratory glassware: beakers (up to 2000 mL), graduated cylinders, glass rods, Erlenmeyer flasks, and 300 mL culture jars or flasks/tubs with lids.
2. Plastic beaker with dispenser suitable for pouring hot medium.
3. Analytical balance and spatulas (preferably stainless steel).
4. Magnetic stirrer with stir bars.
5. pH-meter with appropriate electrode and calibration buffers.
6. Hot plate or combined hot-plate magnetic stirrer to boil media.
7. Autoclave capable of operating at 121 °C and 0.1 MPa pressure.
8. Laminar airflow hood, disinfected with UV light for at least 30 min before work.
9. Sterile scalpels, forceps/tweezers, and disposable blades.
10. Sterile paper towels or tissue, Parafilm®, marker pens and labels.
11. Disinfectant containing at least 70% ethanol for wiping surfaces, hands, and external jar surfaces.
12. Growth room or controlled environment chamber maintained at  $24 \pm 1$  °C with a 16 h photoperiod and photosynthetic photon flux density (PPFD) of approximately 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , provided by a cool white fluorescent lamp (e.g., Narva, Germany) (see Note 5).

## 2.6. Equipment for colour determination

1. Konica Minolta CM-700d/600d spectrophotometer (Japan) capable of colour measurement in the CIE  $L^*a^*b^*$  system (Hunterlab, 2012).
2. Calibration plate matched to the device serial number.
3. Software or onboard interface for recording  $L$ ,  $a^*$ , and  $b^*$  values.

## 2.7. Methods

Carry out all procedures at room temperature unless otherwise indicated. Work under aseptic conditions whenever tissues or media are exposed to the environment. The following steps describe the full procedure from explant disinfection to growth assessment and colour measurement.

### 2.7.1. Surface disinfection and initiation of cultures

Collect healthy shoots of *V. vinifera* cv. Hiberna in the vineyard and transfer them to the laboratory in a clean container or plastic bag to avoid desiccation (see Note 6). Rinse shoots under running tap water to remove visible dirt and dust. Immerse shoots in 70% (v/v) ethanol solution for 30 s with gentle agitation. Rinse thoroughly with sterile deionised water for 2 min to remove residual ethanol. Immerse the shoots in 7% (v/v) NaOCl solution for 15 min with occasional gentle shaking. Rinse three times in sterile deionised water, each rinse lasting 2 min, to remove NaOCl traces. Transfer shoots to a laminar airflow cabinet previously disinfected with UV light and surface disinfectant. On sterile paper, trim the shoots with a sterile scalpel into segments 17–20 mm in length, each containing at least one axillary bud. Place explants vertically onto hormone-free WPM medium solidified with agar (see Section 3.2) using sterile tweezers. Seal culture vessels, label them, and transfer to the growth room. Subculture initiated explants onto fresh hormone-free WPM every 4 weeks over a period of approximately 4 months to stabilise growth and reduce contamination risk (see Note 7). At the end of the initiation period, record the percentage of sterile explants and the regeneration success rate.

### 2.7.2. Preparation of woody plant medium (WPM)

Weigh 2.46 g of WPM powder including vitamins and transfer it to a 2000 mL glass beaker using clean weighing paper or a weighing boat. Add deionised water to a final volume of 1000 mL using a graduated cylinder. Place the beaker on a magnetic stirrer and mix until the medium powder is completely dissolved (see Note 8). Adjust the pH of the solution to 5.6–5.8 using 0.1 N NaOH or 0.1 N HCl, monitoring with a calibrated pH-meter (see Note 9). Add 30 g L<sup>-1</sup> sucrose (3%), 8 g L<sup>-1</sup> agar, and 100 mg L<sup>-1</sup> myo-inositol to the medium. Mix carefully with a glass rod or under magnetic stirring, avoiding the formation of lumps and excessive foaming. Heat the medium to boiling on a hot plate to completely dissolve agar (see Note 10). Pour approximately 30 mL of hot medium into each 300 mL jar or culture flask using a plastic beaker with dispenser or other suitable device. Cap the containers loosely if required by the autoclave model, and label them. Autoclave at 121 °C and 0.1 MPa for approximately 19 min (depending on vessel volume) (see Note 11). After sterilisation, allow the media to cool and solidify at room temperature, then tighten lids and store in a dark, dry place until use.

### 2.7.3. Preparation and application of Actisil, chitosan, and meta-topolin

Prepare WPM medium as described in Section 3.2 up to step 4 (pH adjustment), but before adding sucrose, agar, and myo-inositol. For Actisil treatments, add Actisil to the WPM solution to obtain final concentrations of 50, 100, 200, or 500 µL L<sup>-1</sup>. Pipette the required

volume directly into the medium while stirring (see Note 12). For chitosan treatments, prepare stock solutions of chitosan in deionised water or a suitable dilute acid if required for solubility (see Note 13). Add the stock solution to the WPM to obtain final concentrations of 10, 20, or 40 ppm CH. For meta-topolin treatments, prepare an mT stock solution (e.g., dissolved in a small volume of NaOH or solvent according to the manufacturer's instructions) and add to the medium to obtain 0.2, 0.4, or 0.6 mg L<sup>-1</sup> mT. After addition of Actisil, chitosan, or mT, mix the medium on a magnetic stirrer for approximately 1 min to ensure homogeneity. Re-check pH if necessary and adjust slightly to maintain it in the 5.6–5.8 range. Proceed with adding sucrose, agar and myo-inositol as described in Section 3.2 (steps 5–11). Prepare a control WPM medium without any Actisil, chitosan, or mT supplementation.

#### **2.7.4. Transfer of grapevine explants to experimental media**

Before starting, disinfect the laminar airflow work surface with 70% ethanol and switch on the airflow; ensure that tweezers, scalpels, and other tools are sterile (see Note 14). Wear a disposable lab coat, gloves, and a mask to minimise contamination. Place jars with pre-cultured, disinfected shoots and jars containing the freshly prepared media (control and supplemented variants) inside the laminar hood. Using sterile tweezers, gently remove shoots from the initiation or multiplication medium and place them on sterile paper. With a sterile scalpel, cut off roots (if present), leaves, and browned or necrotic tissue. Cut shoots into 1.5–2 cm fragments containing axillary buds from which new explants will develop. Leave a longer stem portion below the axillary bud and a shorter segment above it to facilitate insertion into the medium. Using tweezers, insert four explants vertically into each jar containing the respective medium variant, ensuring that the lower part of the stem is in good contact with the medium surface (see Note 15). Repeat for all medium variants, obtaining at least six jars (replicates) per treatment. Cap jars tightly; if necessary, seal lids with Parafilm® for additional protection against contamination. Label each jar with treatment code, date, and cultivar name. Transfer jars to the growth room and maintain them at 24 ± 1 °C under a 16 h photoperiod and 40 µmol m<sup>-2</sup> s<sup>-1</sup> PPFD for 4–8 weeks.

#### **2.7.5. Leaf colour measurement using the CIE Lab\* system**

After the cultivation period (e.g., 6 weeks), select fully expanded leaves from the middle part of the shoots in each treatment (see Note 16). Turn on the Konica Minolta CM-700d/600d spectrophotometer and allow it to stabilise. Perform zero calibration according to the manufacturer's instructions, followed by white calibration using the supplied white calibration plate with a matching serial number (see Note 17). Set the instrument to transmission or reflection mode as appropriate, with the measurement hole diameter set to 3 mm, observer angle to 10°, and illuminant to D65. Place a leaf sample so that it completely covers the measuring port, avoiding veins or damaged tissue in the central measuring area (see Note 18).

Record L\*, a\*, and b\* values for each leaf, where:

L\* indicates lightness from 0 (black) to 100 (white);

a\* ranges from green (-a\*) to red (+a\*);

b\* ranges from yellow (+b\*) to blue (-b\*).

Measure multiple leaves per treatment and calculate mean values of L\*, a\*, and b\* to characterise leaf colour changes in response to Actisil, chitosan, or mT supplementation.

### 2.7.6. Statistical analysis

Collect data on: percentage of sterile explants, regeneration success, shoot and root morphology (e.g., shoot length, number of shoots, root length), and leaf colour parameters ( $L^*$ ,  $a^*$ ,  $b^*$ ). Use Statistica 13.0 software (StatSoft Polska, Poland) or equivalent statistical package for data analysis. Verify normality of distributions and homogeneity of variance in each dataset before performing parametric tests (see Note 19). Conduct analysis of variance (ANOVA) to evaluate the effects of treatments (control vs. Actisil, chitosan, and mT concentrations). When ANOVA indicates significant differences, apply Tukey's range test for post-hoc comparisons at  $p < 0.05$ . Present results as means with standard deviations or standard errors, using different letters to indicate statistically significant differences among treatments.

## 3. Results and Discussion

Efficient tissue culture depends critically on the elimination of exogenous and endogenous microorganisms (Krupa-Mańkiewicz et al., 2019). Using the disinfection procedure described, a sterility rate of approximately 85% and a regeneration success rate of about 60% can be achieved for cv. Hiberna, providing a suitable starting point for multiplication. Supplementation of WPM with chitosan and meta-topolin markedly affects shoot and root morphology. The lowest chitosan concentration tested (10 ppm) typically results in the greatest root length (around 12 cm) and good shoot development, in line with reports that chitosan promotes root elongation and biomass accumulation in diverse plant species (Krupa-Mańkiewicz and Ochmian, 2023). In contrast, higher mT levels (e.g., 2.0 mg L<sup>-1</sup>) can inhibit both shoot and root formation, suggesting that cytokinin levels must be carefully adjusted to avoid growth suppression (Hasanuzzaman et al., 2012). Silicon supplied as Actisil has a more subtle but positive effect. At 50 µL L<sup>-1</sup>, it tends to increase root length and overall resilience of *in vitro* plantlets, consistent with the reinforcing role of silicon in plant cell walls and its capacity to alleviate abiotic stress (Sacała, 2009; Kabir et al., 2016; Figiel-Kroczyńska et al., 2022). Leaf colour parameters measured in the CIE  $L^*a^*b^*$  system are sensitive indicators of treatment effects. The presence of mT and chitosan alters leaf pigmentation; for example, an increase in  $L^*$  under certain mT concentrations indicates lighter leaves, which may correspond to modified chlorophyll content. These observations agree with previous findings on the impact of cytokinins and elicitors on pigment metabolism in grapevine and other species (Ochmian et al., 2019; Krupa-Mańkiewicz and Calomme, 2021). Overall, WPM supplemented with 10 ppm chitosan can be recommended as a practical medium for micropropagation of *V. vinifera* cv. Hiberna, offering a balance between vigorous root formation and satisfactory shoot quality. The protocol can be further refined by adjusting mT and silicon concentrations for particular cultivars or experimental objectives.

## 5. Conclusions

**Stock plants.** Use vigorous, disease-free vineyard plants as explant donors. Avoid using material collected immediately after pesticide applications or under severe stress (e.g., drought or frost).

**Actisil composition.** The Actisil solution used in this protocol contains 0.6% Si and 2% Ca. Store it in a dark place at room temperature in tightly closed containers to prevent degradation.

**Chitosan quality.** The biological activity of chitosan depends on its molecular weight and degree of deacetylation. Use material with similar parameters (3.33 kDa, 85% deacetylation) for reproducibility.

*NaOCl concentration.* Commercial bleach products differ in active chlorine content. Adjust the volume used to obtain an effective 7% (v/v) NaOCl solution.

*Light conditions.* Maintain a stable photoperiod and PPFD; fluctuations may affect shoot morphology and rooting. If necessary, measure PPFD with a quantum sensor.

*Transport of material.* Keep collected shoots cool and shaded during transport to reduce wilting. Process them as soon as possible after collection.

*Initiation phase.* Repeated subculture on hormone-free WPM helps to stabilise *in vitro* cultures and reduce latent contamination. Contaminated cultures should be discarded promptly.

*Dissolution of WPM.* WPM powder is hygroscopic; keep the container tightly closed and use dry tools. Ensure complete dissolution of salts and vitamins before pH adjustment.

*pH measurement.* Measure pH before adding agar and sugars, as they can coat the electrode and affect readings. Rinse the pH electrode with deionised water and store it in appropriate storage solution after use.

*Boiling medium.* When heating the medium, monitor it closely because it may quickly foam and boil over. Stirring while heating reduces this risk.

*Autoclaving time.* Autoclave time depends on the volume of medium per vessel; larger volumes require slightly longer sterilisation. Avoid over-autoclaving to prevent sugar caramelisation and nutrient degradation.

*Addition of Actisil.* Mix Actisil into the medium gently but thoroughly. High local concentrations can temporarily alter pH; re-check and adjust if needed.

*Chitosan solubility.* Chitosan may require slight acidification (e.g., dilute acetic acid) for dissolution. Neutralise the solution before adding it to WPM to avoid large pH shifts.

*Aseptic technique.* Regularly flame-sterilise metal tools or disinfect them with ethanol between explants. Allow tools to cool before touching plant tissue to avoid heat damage.

*Number of explants per jar.* Four explants per 300 mL jar with 30 mL medium provides a balance between efficient use of space and minimisation of competition.

*Sampling for colour analysis.* Always sample leaves of similar age and position on the shoot to reduce variability in  $L^*$ ,  $a^*$ , and  $b^*$  values.

*Spectrophotometer calibration.* Perform zero and white calibration whenever measurement conditions change, or the device is switched off. This ensures accurate and reproducible colour readings.

*Leaf placement.* Ensure that the entire measuring window is covered by leaf tissue; otherwise, external light or gaps may distort measurements.

*Statistical assumptions.* If data deviate strongly from normality or homoscedasticity, consider transforming the data or using non-parametric tests.

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