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Rapid clonal rooting through *in vitro* proliferation of nodal cuttings of 'Cascade' Hop

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Abstract: The demand for female hop plants (*Humulus lupulus* L.) by the brewing industry, combined with the dioecious nature of the species, presents challenges for propagation via seeds. Therefore, producers rely on vegetative propagation through rhizomes or cuttings. However, these practices may lead to certain drawbacks concerning phytosanitary quality. Consequently, micropropagation emerges as an effective alternative for producing healthy and genetically uniform hop plants. The objective of this study was to evaluate the *in vitro* culture response and *ex vitro* acclimatization of 'Cascade' hop as an alternative to conventional multi-stage micropropagation protocols, which typically include (i) *in vitro* establishment, (ii) shoot multiplication on cytokinin-rich medium with repeated subcultures, (iii) a separate rooting phase, and (iv) *ex vitro* acclimatization. Specifically, nodal cuttings from stabilized *in vitro* cultures were directly grown in five different culture media supplemented solely with auxins. The rooting media, compared with a hormone-free (HF) medium, differed in concentration and the ratio of two auxinic hormones: indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). After two months of *in vitro* culture, it was found that, despite the absence of cytokinins in the culture medium, the explants were able to sprout and produce shoots with lengths ranging from 1.22 to 2.25 cm. Rooting-wise, the medium supplemented with 1 mL L^{-1} IAA produced the highest number of roots (4.98), while the longest roots were observed in the medium supplemented with 2 mL L^{-1} IBA. The derived *in vitro* hop seedlings were transferred to agriperlite after thorough washing to remove residual culture medium. The acclimatization rate was 97.8%. Our findings suggest that for non-recalcitrant species, such as hops, the multiplication phase could potentially be eliminated, resulting in significant savings in time and cost.

Keywords: auxins; *Humulus lupulus* L.; micropropagation; nodal explant; regeneration.

1. Introduction

The United States was the first country to introduce craft beer, from where it subsequently spread worldwide (Anderson, 2023). Thanks to this novel beverage, hops, initially a simple bittering agent, have become a key factor in the production of craft beers (Legun et al., 2022; Neve, 1991). Recent findings indicate that sensory attributes are not solely determined by genotype but are also influenced by the "terroir" effect (Van Holle et al., 2021). These developments have initiated a flourishing period in the beer industry, thus encouraging brewers globally to cultivate their hops (Acosta-Rangel et al., 2021; Ruggeri et al., 2024; Jastrombek et al., 2022). Among the emerging countries in hop cultivation is Italy, as evidenced by numerous studies (Agehara et al., 2024; Carrubba et al., 2022; Marceddu et al., 2024; Gresta et al., 2023; Ruggeri et al., 2023). Hop-growing area in Italy has increased steadily in recent years, as documented by the most recent national reports and sector analyses, with hop acreage expanding from 67 ha in 2020 to 90 ha in 2024 (+34 % increase) (CREA, 2025), in clear contrast to the stagna-

tion or contraction currently reported in traditional hop-growing regions such as Germany, United States, and Czech Republic (BarthHaas, 2024). However, for the establishment of hop yards, growers face significant challenges primarily related to the procurement of plant material, especially in newly cultivated areas.

Traditionally, vegetative propagation is the most common method for multiplying hop plants, typically using stem or root cuttings taken from mature plants during the spring or autumn periods (Machado et al., 2018). However, this propagation material presents certain drawbacks, including the deterioration of plant tissues due to transportation (Rossini et al., 2021), the potential introduction of pathogens (Machado et al., 2018), and the scarcity of certified plants on the market. To overcome these limitations, *in vitro* tissue culture can serve as a viable alternative for the large-scale propagation of healthy, genetically uniform plants, regardless of the season and in limited spaces. To establish a correct micropropagation protocol, several factors must be considered, including the health status of the starting material and the optimal concentrations of sterilizing agents and/or plant growth regulators (PGRs) at various stages of the micropropagation process. To date, several *in vitro* systems have been developed for the multiplication and regeneration of hop plants, including liquid bioreactor-based protocols from organogenic nodules (Batista et al., 2000), shoot-tip and nodal micropropagation pipelines (Roy et al., 2001; Smýkalová et al., 2001; Machado et al., 2018; Clapa & Hârța, 2021), axillary-bud induction systems (Hirakawa et al., 2022), and more recent protocols optimized for specific cultivars and culture systems such as 'Cascade' and Columbus under solid and temporary-immersion conditions (Iacuzzi et al., 2023; Leto et al., 2025; Gianguzzi et al., 2025). Callus- and leaf-derived regeneration procedures, sometimes coupled with encapsulation or synthetic-seed technology, have also been proposed (Liberatore et al., 2020a, 2020b). However, several studies highlighted that the *in vitro* response is strongly genotype- and growth-regulator-dependent (De-Souza et al., 2021; Bychkova et al., 2024), so further research is required to develop robust, genotype-flexible protocols and to elucidate how culture conditions impact agronomic performance and metabolite profiles in field-grown plants.

Therefore, the present work aims to develop an *in vitro* micropropagation protocol that bypasses the multiplication phase and does not utilize cytokinin PGRs. Specifically, *in vitro* stabilized cuttings of the 'Cascade' variety were cultured *in vitro* on different culture media supplemented only with auxins. Due to the high aptitude of the 'Cascade' variety for micropropagation, the present protocol could serve as a starting point for future research.

2. Materials and Methods

2.1. Plant Material

The plant material used in the experiment originated from established *in vitro* cultures maintained at the micropropagation laboratory of the University of Palermo. The 'Cascade' cultivar, developed by the United States Department of Agriculture (USDA), exhibits high vigor, excellent yield, and distinctive aromatic characteristics with spicy citrus notes (Yakima Valley Hops, 2025). Before evaluating the different culture media, the plant material from which the microcuttings were taken was placed on a hormone-free culture medium for 45 days.

2.2. Culture Medium and Growth Conditions

To identify the optimal Plant Growth Regulator (PGR), uninodal explants were cultivated on six different culture media (Table 1). All components and reagents utilized in the trial were procured from Duchefa Biochemie Company (Haarlem, Netherlands).

The six-culture medium consisted of Murashige and Skoog (MS) macro- and micronutrients and vitamins (Murashige & Skoog, 1962), supplemented with 3.5 mL L^{-1} sucrose and 3.5 mL L^{-1} maltose as a carbon source, and 10 g L^{-1} agar as a solidifying agent. Except for the MHF culture medium, which did not contain PGRs, the other culture media differed in the type, concentration, and ratio of tested aux-

ins. The auxins chosen for testing were indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), given their successful use in various micropropagation protocols. This includes the traditional rooting medium of Roy et al. (2001) containing 5.71 µM IAA and 4.9 µM IBA, as well as newer protocols that adjusted the ratio and concentration of these auxins during rooting (Machado et al., 2018; Iacuzzi et al., 2024). All media were autoclaved at 0.11 MPa at 121 °C. Each treatment included 15 microbox trays, each containing 10 explants (a total of 150 explants per treatment). Microbox trays were incubated at 27 ±1 °C and placed under fluorescent lamps (Philips TLM 30w/84) with a photosynthetic photon flux intensity of 3 µmol m^{-2} s^{-1} (broad spectrum in the 400-700 nm range, with main emission peaks around 435, 545 and 610 nm) and a 16-h photoperiod.

Table 1. Composition of the culture media used for *in vitro* culture. All media (MHF and M1–M5) contained Murashige and Skoog (MS) basal salts and vitamins, sucrose, maltose and agar, adjusted to pH 4.5. Auxins (IAA and IBA) were supplied at the indicated concentrations in media M1–M5, whereas the hormone-free medium (MHF) contained no auxin.

Component	MHF	M1	M2	M3	M4	M5
MS salt and vitamins[1]	x	x	x	x	x	x
Succrose (mL L^{-1})	3.5	3.5	3.5	3.5	3.5	3.5
Maltose (mL L^{-1})	3.5	3.5	3.5	3.5	3.5	3.5
IAA[2] (mL L^{-1})	-	1	2	-	-	0.5
IBA[3] (mL L^{-1})	-	-	-	1	2	0.5
Agar (g L^{-1})	10	10	10	10	10	10
pH	4.5					

[1]Murashige and Skoog (Murashige and Skoog, 1926); [2]IAA: Indolo-3-acetic acid; [3]IBA: Indole-3-butyric acid. "x" = presence of Murashige and Skoog (MS) basal medium with salts and vitamins.

2.3. Data Collection

After 90 days of *in vitro* culture, the following parameters were recorded: average shoot percentage [%], average shoot number [n], average shoot length [cm], average rooting percentage [%], average root number [n], and average root length [cm]. Additionally, after 15 days of acclimatization, the rate of acclimatized plantlets was evaluated. The adopted acclimatization protocol followed the approach commonly used for hop micropropagation (*ex vitro* rooting and acclimatization on perlite or peat-based substrates under high humidity yielding survival rates of 90–100%; Machado et al., 2018; Clapa & Hârța, 2021; Mironenko et al., 2024; Bychkova et al., 2024). In detail, rooted plantlets bearing 3–4 fully expanded leaves and a well-developed root system were gently removed from the culture vessels, and the agar was carefully washed from the roots with lukewarm tap water. Plantlets were transplanted into plastic pots (Ø 12 cm) filled with sterile perlite, previously moistened with water. The pots were placed in plastic trays and covered with transparent polyethylene lids to maintain high relative humidity during the first days. Trays were kept in a growth chamber at 20 ± 1.5 °C under a 16 h photoperiod and ca. 100 µmol m^{-2} s^{-1} light. Relative humidity was gradually reduced by progressively opening the lids over 10–14 days, and shading (≈50%) was provided during the first week to limit transpiration stress. After 15 days *ex vitro*, survival rates (%) were recorded.

2.4. Statistical Analysis

Statistical analyses were performed using the MINITAB 19 package (State College, PA, USA) for Windows. The significance of the effect of the media on the measured parameters was assessed using analysis of variance (ANOVA). The significance of the difference between means was analyzed using Tukey's test ($p \leq 0.05$). Percentage data were subjected to arcsine transformation before ANOVA.

3. Results

The percentage, number, and length of shoots showed statistical differences between treatments (Table 2). Specifically, for all three parameters, the lowest values were observed in the treatment without PGR addition (MHF). The shoot percentage and number did not show statistically significant differences among the treatments with PGR-supplemented substrate. However, the M2 treatment (2 mL L^{-1} of IAA) showed the highest absolute mean values (i.e., 94% ± 0.03 and 2.04 ± 0.15, respectively; Table 2). Similarly the highest mean shoot length was recorded in the M1 treatment, even though it did not differ significantly from M3, M4, and M5 (Table 2).

Table 2. Effect of culture medium on *in vitro* shoot production of 'Cascade' hop. Percentage of explants producing shoots, mean number of shoots per explant, and mean shoot length (cm) after the culture period on the tested media (MHF and M1–M5).

Medium	Shoot Percentage [%]	Shoots [n]	Shoot length [cm]
MHF	26 ± 0.06 b	0.44 ± 0.12 b	0.79 ± 0.22 c
M1	90 ± 0.04 a	1.92 ± 0.15 a	2.25 ± 0.25 a
M2	94 ± 0.03 a	2.04 ± 0.15 a	1.22 ± 0.17 bc
M3	86 ± 0.05 a	1.80 ± 0.14 a	1.72 ± 0.24 ab
M4	90 ± 0.04 a	1.74 ± 0.18 a	2.09 ± 0.24 ab
M5	90 ± 0.04 a	1.92 ± 0.17 a	1.73 ± 0.22 ab
p	<0.001	<0.001	<0.001

Values are means ± standard error. Different letters within the same column indicate significant differences at $p \leq 0.05$.

All parameters concerning rooting showed significant statistical differences between treatments ($p < 0.001$) (Table 3). Rooting percentage, root number, and root length were significantly lower in plantlets grown on the culture medium without PGR addition (MHF) than in the other treatments. Regarding the rooting percentage, the media supplemented with auxins did not show significant differences among them (Table 3). The highest root number was observed in the M1 culture medium (Table 3). In addition, M1, M2, and M4 induced the highest root length compared to control (Table 3).

Plantlets grown on the M1 culture medium, which showed the highest shoot length, number and length of roots, and a promising rooting percentage (although not statistically different from other PGR-treated plantlets), were used to assess the next acclimatization phase.

After 15 days of acclimatization, the acclimatization rate of plantlets was evaluated, with a remarkable 97.8% survival rate.

Table 3. Effect of culture medium on *in vitro* root production of 'Cascade' hop. Rooting percentage, mean number of roots per explant, and mean root length (cm) after culture on media MHF and M1–M5.

Medium	Rooting percentage [%]	Roots [n]	Root length [cm]
MHF	16 ± 0.05 b	0.40 ± 0.13 c	0.12 ± 0.04 c
M1	72 ± 0.06 a	4.98 ± 0.52 a	0.46 ± 0.08 a
M2	64 ± 0.07 a	3.42 ± 0.51 ab	0.31 ± 0.04 b
M3	58 ± 0.07 a	2.82 ± 0.47 b	0.44 ± 0.07 a
M4	80 ± 0.05 a	3.44 ± 0.48 ab	0.54 ± 0.06 a
M5	58 ± 0.07 a	2.58 ± 0.45 b	0.30 ± 0.0a b
p	<0.001	<0.001	<0.001

Values are means ± standard error. Different letters within the same column indicate significant differences at $p \leq 0.05$.

4. Discussion

The primary outcome of this research confirms a high and remarkable survival rate, as first reported by previous studies (Clapa e Hârța, 2021). This confirms that hop is a promising species that adapts well to *in vitro* regeneration protocols. This aspect is of vital importance, especially in areas of new cultivation that often struggle to supply propagating material (Ruggeri et al., 2024). In this context, the development of a rapid and reproducible micropropagation protocol from nodal cuttings - when applied to certified, virus-tested mother plants - represents a best-practice framework to support the timely production of uniform clonal plants. Such protocols can help stabilize the supply of planting material for local nurseries and, indirectly, sustain the expansion and raw-material needs of local breweries (Van Kerckhoven et al., 2020; Iacuzzi et al., 2024).

As previously indicated by Morais et al. (2014) our study confirms that adding auxins in small doses can promote shoot elongation. Auxin acts as a signal for cell division, elongation, and differentiation, and plays a major role in apical dominance (Cao et al., 2019; Di Mambro et al., 2017). Furthermore, shoot segmentation and the consequent disruption of apical dominance in plants lead to lower auxin levels (Müller et al., 2015), thus releasing the axillary bud from inhibition during development (Alvarenga et al., 2015; Roy et al., 2001; Clapa e Hârța, 2021).

Eventually, regarding rooting rates, root number, and root lengths, the results of this study appeared to be fairly consistent with previous studies (Hirakawa e Tanno, 2022; Gianguzzi et al., 2025), confirming the promising potential of the presented protocol for hop micropropagation.

5. Conclusions

In this research, it was found that uninodal explants of the hop variety 'Cascade' can regenerate whole plants when cultured *in vitro* in cytokinin-free media. The best results in terms of shoots and roots were achieved with low IAA concentrations (1 mL L^{-1}), suggesting, along with the high acclimatization rates, that the *in vitro* regeneration method could easily replace the rhizome multiplication method. In this sense, the present work provides a basis for future investigations into micropropagation systems with reduced or even omitted use of cytokinins, which may help limit cytokinin-related disorders (e.g., hyperhydricity, excessive callusing, reduced rooting, and increased risk of somaclonal variation) while still ensuring rapid clonal multiplication from nodal cuttings in hop.

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