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Combined effect of rooting medium and biostimulant application on morphological traits of micropropagated hop plantlets

Establishing vigorous and healthy plants is the first requisite for the future success of a new hopyard. Although hop micropropagation has been extensively studied, little information is available on subsequent ex vitro rooting and acclimatization phase. For example, materials and practices used to obtain an excellent hop plantlet ready for the field are often not referenced. The major challenge is to produce well-rooted plants without excessively boosting vegetative growth. New substrates (alternative to peat) and treatments (alternative to synthetic plant hormones) are likely required to address this issue and to increase the sustainability of the process. With this aim, we used micropropagated plantlets of cultivar Cascade and Nugget to assess the combined effect of rooting medium and biostimulant application on root and shoot development during two hardening periods: i) acclimatization in glasshouse, and ii) acclimatization in open air. Two growing media were tested: i) a standard peat-based substrate (control treatment), and ii) an experimental substrate, consisting of a mixture of the standard substrate and woodchip compost. Extracts of seaweed *Codium fragile* (Suringar) Hariot and Indian fig opuntia (*Opuntia ficus-indica* (L.) Mill.) were used as biostimulant agent. The experiments were set up in a completely randomized design with three replicates. Survival rate, shoot and root traits as well as rhizome development were determined. At the end of the study, the foliar biostimulant significantly increased root growth (21 % in length and 46 % in weight) and rhizome length (+ 46 %), while it had a moderate effect on shoot development. Hop cultivars tested in our study highlighted a very different way to allocate plant biomass during the hardening period, with Nugget having a root-to-shoot ratio twofold higher than Cascade. Finally, even though the experimental substrate did not affect plant traits during the first acclimatization period, it had a strong negative effect during the acclimatization in open air. Our findings provide practical guidance for nursery managers seeking to produce high-quality hop plantlets using sustainable materials and treatments.

Descriptors: biostimulant, Cascade, growing media, Nugget, plant hardening

1 Introduction

Growing hops (*Humulus lupulus* L.) requires at least three years to get a full crop as well as a considerable capital investment and labor demand [1]. Starting hop plantation with uniform and disease-free plants is the first step towards a good establishment and successful management of the crop. Vegetative propagation is the traditional method for multiplying hop plants, typically involving rhizomes or herbaceous cuttings collected from mature plants in autumn or spring [2]. This technique produces genetically identical plantlets that are either directly planted or first grown in pots before field transfer after several months [3]. Sexual reproduction is instead limited to breeding purposes. In vitro propagation provides an advantageous alternative method, allowing the year-round production of healthy, uniform plants, independent of environmental conditions [4]. For hops, micropropagation has been successfully applied to large-scale plant production [5], virus elimination [6], germplasm conservation [7, 8] and genetic transformation [9]. Given the economic importance of hops, refining propagation protocols and acclimatization strategies is crucial for hop supply chain.

Recently, several studies have been conducted to develop efficient micropropagation protocols for hops [10–12]. Conversely, the post-propagation phase remains a neglected topic, especially in new growing areas, where the development of hop farming is hindered by several structural constraints and a limited number of agronomic studies [13, 14]. The major challenge is to obtain a well-rooted plant without excessively boosting vegetative growth. New substrates (alternative to peat) and treatments (alternative to synthetic plant growth regulators) are needed to overcome that issue and to increase the sustainability of the process.

Obtaining a healthy and vigorous plantlet during the hardening period is crucial for subsequent good field performance of several crops [15, 16]. Target plant measures for post-propagation phase include root and shoot biomass and their ratio, root and shoot length, and, obviously, survival rate [17]. A well-developed and

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functional root system is of utmost importance for outplanting success, because it allows plantlets to readily uptake water and nutrients, anchor themselves to the ground and tolerate or avoid drought stress [18, 19]. However, for a good morphological and physiological balance, root development should be combined with an adequate shoot growth. Root to shoot ratio (R:S) can be defined as the ratio of root to shoot weight on dry matter basis [20]. It is an important trait because a well-balanced R:S helps newly-outplanted plantlets prevent water deficits and enhances survival during drought conditions. Even though an optimal R:S is difficult to set, plantlets with higher R:S are often considered to have greater outplanting survival and quicker establishment [21].

Plant performances during acclimatization largely depend on production methods such as container and substrate properties, irrigation and fertilization management, mycorrhizal inoculation, use of plant growth regulators, mechanical conditioning, root and shoot pruning practices and management of microclimatic conditions [22]. Among them, physical (e.g., bulk density, air-filled porosity) and chemical properties (e.g., pH, cation exchange capacity, carbon to nitrogen ratio) of growing media greatly impact on the characteristics of the root system, because they can modify rhizogenesis and root growth. Bulk density and air-filled porosity determine root growing environment through their well-known inverse relationship. A low bulk density and high air-filled porosity are required in nursery production of containerized plants, including hops. This combination balances moisture and aeration of the growing media, thus improving root and shoot growth while decreasing root malformations [23]. Conversely, an increase in bulk density could reduce rhizome growth [24], which is a key morpho-physiological feature for micropropagated hop plantlets. Chemical properties of substrates, such as pH, cation exchange capacity (CEC), Carbon to Nitrogen ratio (C:N), can also have a remarkable influence on hop root growth and function. The pH of substrates affects availability of some elements, leading to symptoms of nutrient toxicity or deficiency. The optimum pH of growing medium varies with plant species, and for hops is around neutrality [25]. CEC represents the total exchangeable cations a substrate can retain per unit weight. CEC values depend upon the substrate and, in organic growing media, can vary from 39 – 60 cmol kg^{-1} of coconut coir, to 90 – 140 cmol kg^{-1} of peat, up to 160 – 180 cmol kg^{-1} of compost [26]. Growing media having lower values need an increase in the frequency of fertilizer application. Regarding C:N, it greatly influences the stability of the growing substrate. A ratio around 20 – 40 is considered ideal for potting media, while lower or higher values result in a rapid N release for plant uptake or microbial N immobilization, respectively [27].

Peat is, by far, the most used component of growing media for container production of several plant species [28]. Its everlasting success is mainly due to optimal physical characteristics (e.g., low bulk density and high air-filled porosity) and chemical properties (relatively high cation exchange capacity, a low pH and nutrient content, which can be artificially adjusted to crop-specific requirements). Moreover, peat is generally free of biological contaminants such as pests, pathogens, and weed seeds [29].

Globally, the possibility of replacing peat in growing media for nursery production is a widely-discussed topic closely linked to environmental sustainability and exploitation of natural resources [30]. In Europe, several national initiatives have been taken to reduce peat use in horticulture and replace it with alternative materials [31]. Compost, wood fibers, bark products, biochar, and coir are among the most studied growing media constituents, which might substitute peat, at least in part, in both hobby and professional sector [31–33]. However, each of these materials have shown one or more drawbacks that could significantly limit their use [34]. For example, compost and biochar exhibit high variability in their physical and chemical characteristics depending on feedstock and specific production methods [31, 35, 36]; wood-based materials often have a high C : N ratio, low water retention and possible phytotoxicity [37]; coir production is associated with i) high water consumption to remove initial salinity, ii) considerable energy use for long-distance transportation, and iii) social concerns [38].

The use of forestry by-products has gained increasing attention in recent years [39]. Forestry by-products consist of logging residues, including bark, branches, sawdust, leaves, etc. that are often left on the ground [40]. Composting forest residues is a common practice prior to use them as substrate alone or mixed with other materials, as this process alleviates many problems typically associated with wood-based growing media [41]. Although logging residues are often considered wastes, they might have a great potential as sustainable substrate or soil amendment, supporting circular economy principles [40, 42, 43].

In addition to composting, applying biostimulants to plants or rhizosphere could be an effective strategy to mitigate the negative effects associated with wood-based growing media. A plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content [44]. Numerous studies have demonstrated the beneficial effects of biostimulants when applied to field crops and horticultural species [45, 46]. Because many plant biostimulants improve root development, several substances are commonly used in nursery production to reduce transplanting shock and speed up crop establishment [47].

Seaweed extracts are a major group of plant biostimulants that, applied to soil or as foliar treatment, can significantly enhance seed germination, root development and overall plant vigour of horticultural species and field crops [48–50]. Algae-based biostimulants are rich in phytohormones and other bioactive and elicitor components, which trigger the expression of different genes, connected with defense and nutrient uptake mechanisms. Thanks to these properties, several studies showed that the application of seaweed products promoted germination rates, seedling vigor, root size and density [51, 52].

Seaweed extracts, mainly from *Ascophyllum nodosum* (L.) Le Jolis, have been rarely tested in open-field trials on mature hop plants [53–55] and, at the best of our knowledge, in only one study on the vegetative propagation of hops using herbaceous cuttings [56].

In general, while most studies on hop micropropagation have focused on plant growth regulators and their concentrations, as well as types of explants and culture media, there is a lack of information on materials and methods that could improve the performance of hop plantlets during the acclimatization [57, 58].

The aims of this study were to i) evaluate the response of two different micropropagated hop cultivars to ex vitro conditions; ii) assess the effects of biostimulant application, growing substrate and their interaction on shoot and root development during the hardening period.

2 Materials and methods

This study was conducted at the University of Tuscia (Viterbo, Italy) in spring-summer 2025.

The experiment was divided into two sub-experiments:

- sub-experiment 1 (from April 16th to May 10th) aimed to evaluate the response of hop plantlets during the acclimatization in a controlled environment (glasshouse provided with cooling and shadowing system). The average temperature during this period was 21.4 °C;
- sub-experiment 2 (from July 3rd to July 29th) aimed to evaluate the response of hop plantlets during the acclimatization in open air. The average temperature and relative humidity during this period were 24.1 °C ± 2.4 (standard deviation) and 55.8 % ± 15.8 (standard deviation).

2.1 Plant material and growth conditions during micropropagation

Plant material of cultivars Cascade and Nugget, both obtained as certified disease-free stock, was used in this study. These varieties were selected because they represent two different hop categories (bittering hop for Nugget and aroma hop for Cascade) and, at the same time, are among the most cultivated hop commercial cultivars in Italy [13, 59]. In vitro establishment has been performed following the protocol by Lupo et al. (2025) [60]. Briefly, herbaceous shoots from both cultivars were cut into uninodal microcuttings (hundred for each variety) and used as explants. Before culture, the shoots were sectioned into nodal segments (hundred for each variety), surface-sterilized in a 40 % commercial bleach solution for 10 minutes, and rinsed three times with sterile distilled water. The sterilized nodes were then placed in 20 ml tubes containing 4 ml of hormone-free half-strength MS medium [61], sucrose 1 % and 6 g L^{-1} Plantagar S-1000 (B&V). The pH of the medium was adjusted to 5.6 prior to autoclaving. Cultures were incubated at 24 ± 1 °C under a 16-hour photoperiod (40 µmol m^{-2} s^{-1}) provided by fluorescent lamps in a controlled growth chamber. For the multiplication phase, nodal segments with axillary buds were cultured on half-strength MS medium, supplemented with 2 % sucrose and 0.3 mg L^{-1} 6-benzylamminopurine (BAP). Once an adequate number of shoots was produced, apical shoots with three to four nodes (15 – 20 mm) were excised and used for in vivo rooting. The shoots were excised and placed in a Jiffy pots and covered with polyethylene film to maintain high relative humidity. After 7 days, rooted plantlets were ready for the hardening period.

2.2 Growing media and biostimulant treatment

Two growing media were tested:

- Substrate A: standard substrate (control treatment), commonly used in nursery production of hops and other horticultural species, consisting of organic sphagnum peat (60 % volume) and coconut coir (40 % volume);
- Substrate B: experimental substrate, consisting of a mix of the standard substrate (70 % volume) and woodchip compost (30 % volume). Consequently, the final composition of substrate B was 42 % peat, 28 % coir, and 30 % woodchip compost (v/v), with 18 % (v/v) peat replacement relative to the standard substrate (60 % peat).

The composition of the experimental substrate was chosen according to several studies, which indicated a moderate peat reduction [43].

The raw material used for the 30 % volume of Substrate B was virgin wood in the form of small chips from forestry operations on coniferous and broadleaf stands. The trees were felled and extracted to the landing where the chipping took place. The chipped material was mechanically separated using a rotary screen, which recovered the fine fraction (wood and soil residues, material with dimension < 4 mm). This was stored under cover in piles exceeding 80 m^3 in volume for an average storage period of two months. During this period, a fermentation/composting process was initiated with natural triggering. Before agronomic tests, the material underwent further heat treatment to inactivate any harmful organisms still present. The mixture of wood and soil material resulting from the woodchip screening process was analyzed to fully understand its main characteristics for agronomic applications. The characteristics

Table 1 Main characteristics of the virgin wood and soil mixture used to prepare the experimental substrate. The data were analyzed by Kruskal-Wallis test. The comparison was made among 20 different sieving loads, with three sampling replicates per load

Characteristic	Mean	Standard deviation	p-value
pH	7.2	0.60	> 0.05
Total Organic Carbon (db, %)	40.75	2.56	< 0.05
Organic Nitrogen (db, %)	0.40	3.98	< 0.05
C : N Ratio	82	1.24	< 0.05
CEC (meq/100 g)	6.80	0.54	> 0.05
Phosphorous (total, mg/kg)	0.90	0.05	> 0.05
Potassium (total, mg/kg)	0.54	0.05	> 0.05
Zinc (total, mg/kg)	45.64	1.38	> 0.05
Copper (total, mg/kg)	13.84	1.47	> 0.05

CEC: Cation Exchange Capacity; db: dry basis

Fig. 1 **Micropropagated hop plantlets during the acclimatization period in glasshouse**

of the fine fractions were assessed using methodologies recognized and approved by the scientific literature [62]. Reference was made to the following parameters, recognized as the factors most influencing substrate quality (table 1):

- pH;
- Organic matter (%);
- Phosphorus (mg/kg);
- Potassium (mg/kg);
- Particular metals (mg/kg).

In addition, the salmonella and Escherichia coli test were conducted with negative results.

The analyses were conducted on 60 samples taken from 20 different sifting loads, analyzing three replicates per load. Sampling was conducted randomly during the sifting process. The analyzed granulometry (particle size distribution) was as follows: 1.3 % particle size < 6 mm > 4 mm, 16.6 % < 4 mm > 2 mm, 23.9 % < 2 mm > 1 mm, 53.6 % < 1 mm > 0.06 mm, 4.6 % < 0.06 mm.

This wood and soil mixture has demonstrated characteristics that make it labeled as a simple, non-composted plant improver pursuant to the Italian Legislative Decree 75/2010. Regarding its suitability for agronomic purposes, no possible situations of phytotoxicity or other plausible environmental incompatibilities have been identified.

Extracts of seaweed *Codium fragile* (Suringar) Hariot and Indian fig opuntia (*Opuntia ficus-indica* (L.) Mill) were used as biostimulant agents. Extracts were obtained applying mechanical pressure. The weight ratio of the algae and plant pressed biomass mixtures was 5:1. Chemical analyses revealed the presence of the following constituents: 1.8 % total N, 1.0 % organic N, 0.8 % ammonium N, 0.9 % water soluble P_2O_5, 2.9 % water soluble K_2O, 2.9 % total organic C. Biostimulant treatment was applied as 1 % aqueous solution (10ml liquid extracts mixed in 1 L water). Control plants were sprayed with water only.

2.3 Sub-experiment 1: acclimatization in controlled environment

Two-hundred micropropagated plantlets were transplanted into plastic module trays to start the acclimatization period in a glass-

Table 2 **Hop plant traits as affected by foliar treatment, cultivar and growing media during the hardening period in glasshouse (means ± standard error). Within each experimental treatment, values followed by different letters are significantly different at p < 0.05**

	Root length (cm)	Root weight (mg, DM)	Shoot length (cm)	Shoot weight (mg, DM)	Leaves (n. plant^{-1})	Survival rate (%)
Foliar treatment						
Water (control)	17.7 ± 1.44 b	4.22 ± 0.51 b	3.30 ± 0.12 b	13.8 ± 1.0 b	15.1 ± 0.95 b	75.7 ± 5.17 a
Biostimulant	25.2 ± 1.44 a	7.03 ± 0.51 a	4.22 ± 0.12 a	21.4 ± 1.0 a	18.4 ± 0.95 a	88.7 ± 5.17 a
P-value	**< 0.01**	**< 0.01**	**< 0.001**	**< 0.001**	**< 0.05**	0.098
Hop cultivar						
Nugget	25.3 ± 1.44 a	6.64 ± 0.51 a	3.63 ± 0.12 a	18.3 ± 1.0 a	16.3 ± 0.95 a	87.4 ± 5.17 a
Cascade	17.6 ± 1.44 b	4.61 ± 0.51 b	3.89 ± 0.12 a	16.8 ± 1.0 a	17.2 ± 0.95 a	77.1 ± 5.17 a
P-value	**< 0.01**	**< 0.05**	0.157	0.308	0.544	0.181
Growing media						
Substrate A	20.8 ± 1.44 a	5.92 ± 0.51 a	3.92 ± 0.12 a	18.4 ± 1.0 a	17.0 ± 0.95 a	82.1 ± 5.17 a
Substrate B	22.1 ± 1.44 a	5.33 ± 0.51 a	3.60 ± 0.12 a	16.8 ± 1.0 a	16.5 ± 0.95 a	82.3 ± 5.17 a
P-value	0.563	0.432	0.087	0.260	0.714	0.978

Substrate A: standard substrate consisting in peat (60 % volume) and coconut coir (40 % volume); Substrate B: experimental substrate, consisting in a mix of the standard substrate (70 % volume) and woodchip compost (30 % volume); DM: dry matter

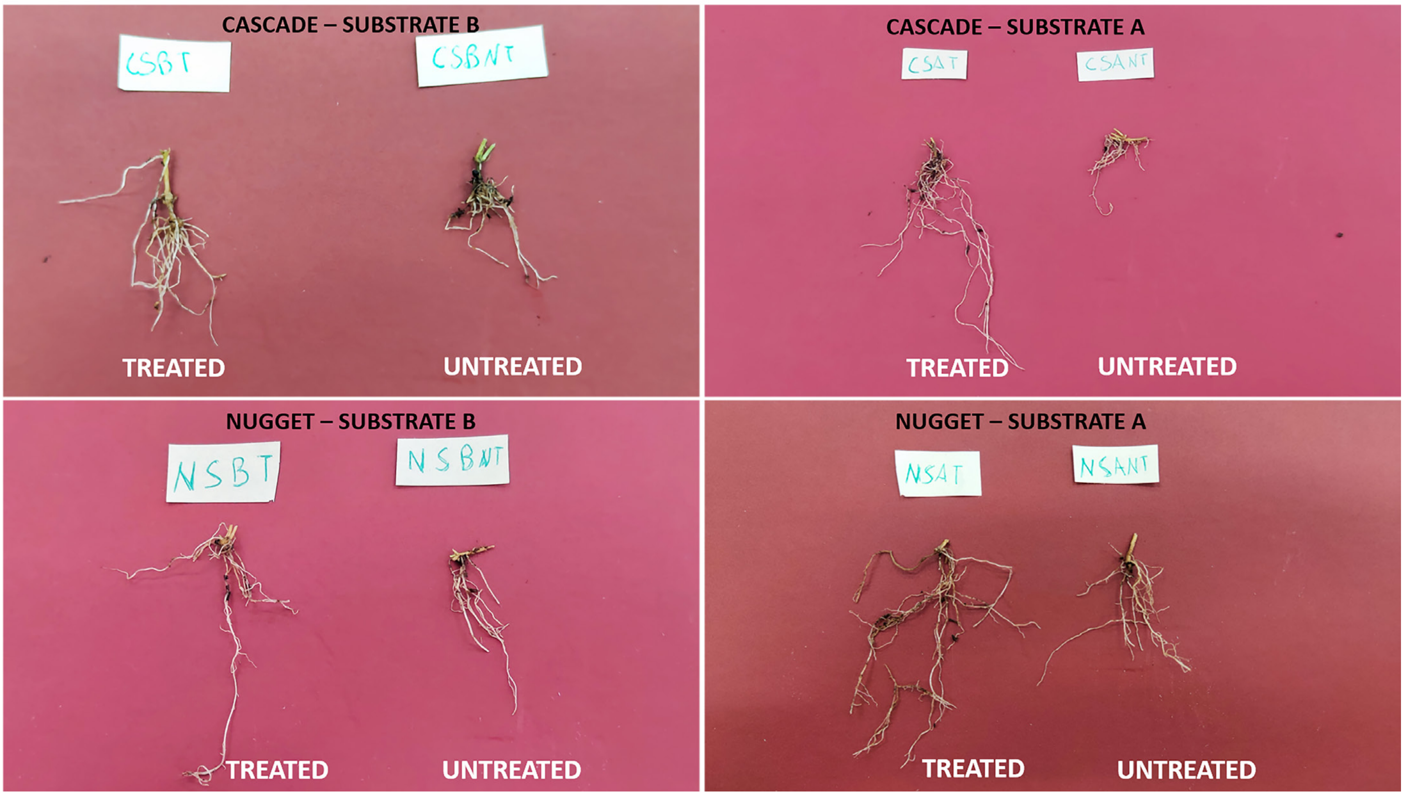

Fig. 2 **Root morphology of hop micropropagated plantlets under different experimental conditions, during the hardening period in glasshouse. Substrate A: standard substrate (control treatment), consisting in peat (60 % volume) and coconut coir (40 % volume); Substrate B: experimental substrate, made up of a mix of the standard substrate (70 % volume) and woodchip compost (30 % volume). Treated: foliar application of extracts from seaweed *Codium fragile* (Suringar) Hariot and Indian fig opuntia (*Opuntia ficus-indica* (L.) Mill). Untreated: foliar application of water**

house (fig. 1). Each tray consisted in 28 cells, and each cell had the following dimensions: 7 cm × 7 cm × 7 cm. Shadings were used to reduce irradiance during the acclimatization. Air temperature in the glasshouse varied from 18 to 29 °C.

Foliar biostimulant was applied one time only by spraying the plantlets as soon as they were moved to the glasshouse for the ex vitro phase.

Irrigation was provided as needed and, once every two weeks, it was replaced by fertigation using a 2 % solution of a liquid fertilizer containing 7 % N, 3 % P_2O_5, 6 % K_2O and enriched with micronutrients. Fertigation was applied at the dose of 15 mL per plantlet. Downy mildew was chemically controlled spraying a liquid fungicide containing 10 % Metalaxil and 40 % Folpet.

2.4 Sub-experiment 2: acclimatization in open air

After the acclimatization in the glasshouse, the plantlets were all pruned to three buds and then transplanted into greater plastic pots (dimensions: 12 cm × 12 cm × 12 cm) to start the hardening period in open air. The plants received about 6 hours of full sunlight (approximately from 7:00 AM to 1 PM) and then they were in shadow. Air temperature varied from 16 and 33 °C.

After a week from transplanting, fertigation (25 mL per plantlet) and the application of biostimulant were performed.

Experimental treatments (growing media and biostimulant application) as well as management of plantlets were the same as sub-experiment 1.

2.5 Plant growth measurements

At the end of each sub-experiment, three random plantlets per replicate were sampled (a total of 72 plantlets) and the following

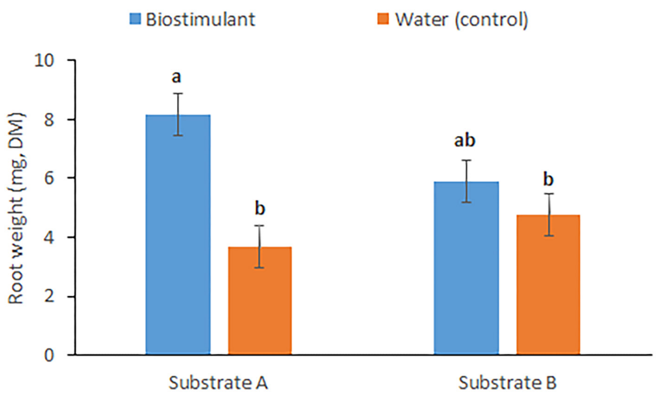

Fig. 3 **Effect of biostimulant × growing media interaction on the root dry weight. The vertical bars represent the standard error of the mean. Letters above the bars correspond to the ranking of the Tukey's test at $p < 0.05$. Substrate A: standard substrate (control treatment), consisting in peat (60 % volume) and coconut coir (40 % volume); Substrate B: experimental substrate, made up of a mix of the standard substrate (70 % volume) and woodchip compost (30 % volume)**

Table 3 **Hop plant traits as affected by foliar treatment, cultivar and growing media during the hardening period in open air (means ± standard error). Within each experimental treatment, values followed by different letters are significantly different at p < 0.05**

	Root length (cm)	Root weight (mg, DM)	Rhizome length (cm)	Shoot length (cm)	Shoot weight (mg, DM)	Leaves (n. plant^{-1})
Foliar treatment						
Water (control)	336 ± 20.9 b	65.0 ± 4.26 b	10.0 ± 0.98 b	6.74 ± 0.18 a	103.0 ± 12.2 a	15.9 ± 0.68 a
Biostimulant	408 ± 20.9 a	94.7 ± 4.26 a	14.6 ± 0.98 a	7.16 ± 0.18 a	110.0 ± 12.2 a	17.5 ± 0.68 a
P-value	**< 0.05**	**< 0.001**	**< 0.01**	0.126	0.150	0.122
Hop cultivar						
Nugget	380 ± 20.9 a	92.2 ± 4.26 a	12.9 ± 0.98 a	6.84 ± 0.18 a	85.8 ± 12.2 b	13.9 ± 0.68 b
Cascade	364 ± 20.9 a	67.5 ± 4.26 b	11.7 ± 0.98 a	7.06 ± 0.18 a	126.9 ± 12.2 a	19.5 ± 0.68 a
P-value	0.598	**< 0.01**	0.403	0.408	**< 0.05**	**< 0.001**
Growing media						
Substrate A	499 ± 20.9 a	110.0 ± 4.26 a	18.4 ± 0.98 a	7.51 ± 0.18 a	135.8 ± 12.2 a	20.2± 0.68 a
Substrate B	246 ± 20.9 b	49.7 ± 4.26 b	6.2 ± 0.98 b	6.39 ± 0.18 b	76.9 ± 12.2 b	13.2 ± 0.68 b
P-value	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.01**	**< 0.001**

Substrate A: standard substrate (control treatment), consisting in peat (60% volume) and coconut coir (40 % volume); Substrate B: experimental substrate, made up of a mix of the standard substrate (70 % volume) and woodchip compost (30 % volume); DM: dry matter.

plant traits were determined: plant survival rate, plant height, number of leaves per plant, shoot and root dry biomass. Additionally, root and rhizome length were measured by using WinRHIZO™ scanning and software (Regent Instrument Inc., Ville de Québec, QC Canada).

Survival rate was determined by counting the living plants on three different dates (April 19th, April 24th, and May 5th) and then, by dividing the total number of surviving plants by the number of plants originally planted, then multiplying by 100. Since all plants survived during sub-experiment 2, survival rate was reported only for sub-experiment 1.

Shoot length was measured by a ruler, while the number of leaves per plant were determined by counting all the leaves produced by each plant. After collecting those data, the hop plantlets were divided into two parts: roots and shoots. The roots and rhizomes were carefully washed and manually separated before analysis with WinRHIZO™ scanning and software. Root images were scanned at 600 dpi (945 pixels) resolution. Since no rhizome was still formed during sub-experiment 1, rhizome length was determined only for the sub-experiment 2. Later, to determine dry weight, roots and shoots were oven dried at 60 °C until constant weight.

2.6 Experimental design and statistical analysis

The experiment was set up in a completely randomized design with three replicates. Plants served as technical replicates, while trays as biological replicates. All the data were subjected to three-way analysis of variance (ANOVA) and means were separated using Tukey test at 95 % probability level. The analysis was performed by using the ‘Anova()’ function in the ‘car’ package of R, while the post hoc test was performed using the "emmeans" package and the "multcomp" function [63]. Statistical analysis was run separately by sub-experiment, testing the effect of the main treatments (i.e., genotype, substrate and biostimulant) and their interactions. All factors were considered as fixed.

3 Results

3.1 Sub-experiment 1: acclimatization in controlled environment

One month after imposing ex vitro conditions, biostimulant treatment significantly increased both root and shoot growth of hop plantlets (table 2), while no statistical differences from control were found for survival rate (P = 0.098). In more detail, biostimulant application enhanced: i) root length and weight by 42 % and 67 %, respectively (fig. 2); ii) plant height, dry weight and the number of leaves by 28 %, 55 %, and 21 %, respectively. Additionally, biostimulant treatment doubled the root dry weight of hop plantlets grown in the standard substrate, while no significant effect was detected on plants grown in the alternative growing medium (fig. 3; interaction P = 0.034).

Nugget showed a more developed root system than Cascade (+ 45 % in length and dry weight, P < 0.01), while no significant differences were detected in shoot system and survival rate between cultivars.

As for growing media, no significant effect was revealed for any of the analyzed plant traits.

3.2 Sub-experiment 2: acclimatization in open air

The results from this experiment are reported in table 3.

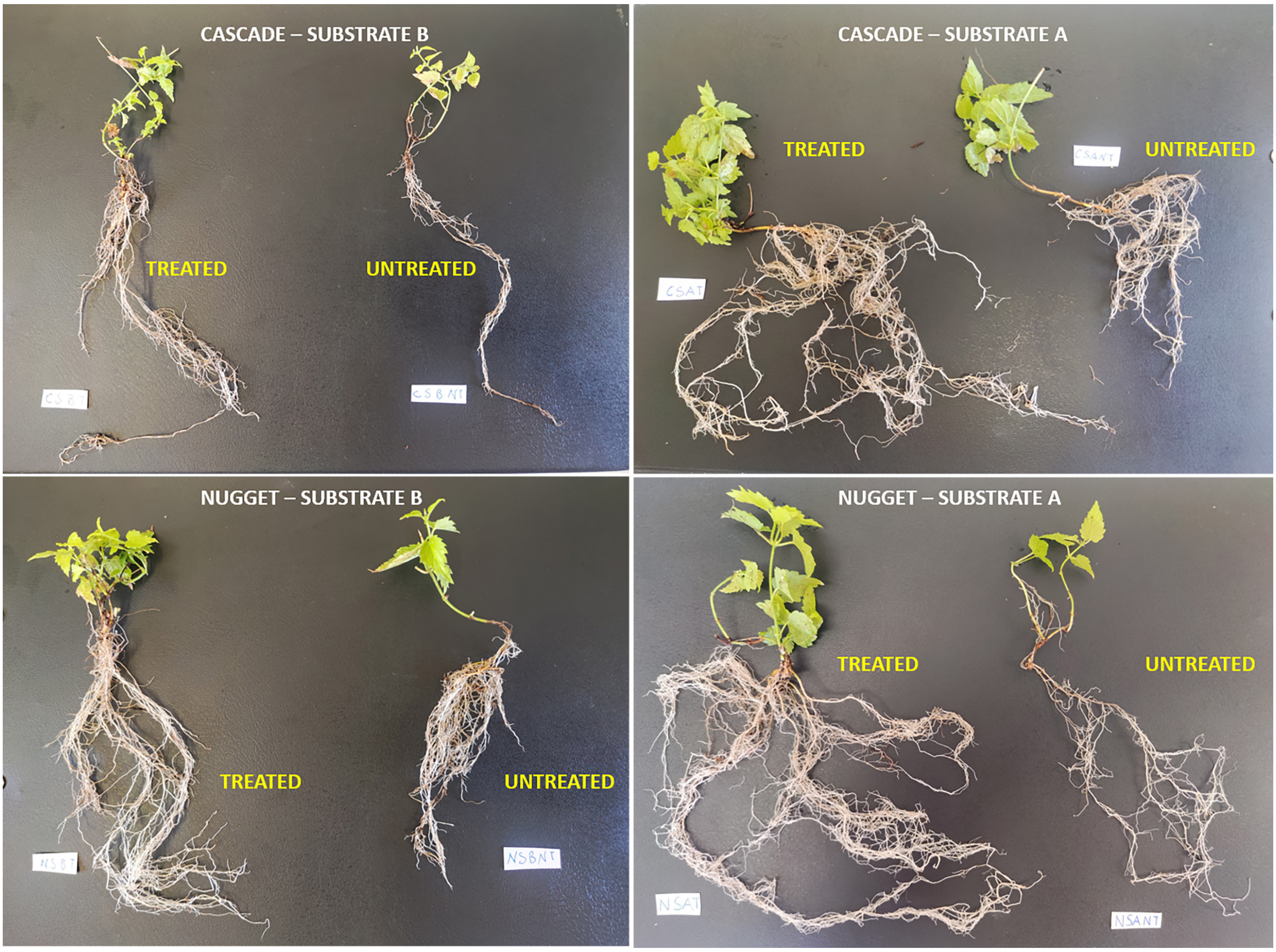

Fig. 4 **Hop micropropagated plantlets under different experimental conditions, during the hardening period in open air. Substrate A: standard substrate (control treatment), consisting in peat (60 % volume) and coconut coir (40 % volume); Substrate B: experimental substrate, made up of a mix of the standard substrate (70 % volume) and woodchip compost (30 % volume). Treated: foliar application of extracts from seaweed *Codium fragile* (Suringar) Hariot and plant *Opuntia ficus-barbarica* A. Berger. Untreated: foliar application of water**

One month after the plantlets were exposed to open air conditions, biostimulant treatment continued to significantly affect root development of hops, including rhizome length, while no statistical differences from control were found for shoot traits. In more detail, biostimulant application enhanced root length by 21 % and both root weight and rhizome length by 46 % (fig. 4). Additionally, biostimulant × cultivar interaction was found to be significant for both the number of leaves per plant ($P = 0.009$) and shoot length ($P < 0.001$). Specifically, biostimulant application significantly increased both those traits only in cultivar Nugget, while no effect was detected for cultivar Cascade (fig. 5).

While the difference in root length between cultivars was no longer significant, Nugget continued to show a root dry weight significantly greater than Cascade hops (+ 37 %, $P < 0.001$). Rhizomes were longer in Nugget than in Cascade hops, but the difference was not statistically significant (+ 10 %, $P = 0.403$). Shoot weight and the number of leaves per plant were significantly higher in cultivar Cascade than Nugget (+ 48 %, $P < 0.05$ and +40 %, $P < 0.001$, respectively).

Differently from sub-experiment 1, the growing media significantly affected all the measured plant traits, with the standard substrate that always outperformed the alternative substrate. In particular, the highest percentage increases were found for root traits (+ 100 – 120 %), particularly for rhizome length (+ 200 %).

4 Discussion

We are not aware of prior studies that jointly test the combined effect of foliar biostimulant and growing media on morphological traits of micropropagated hop cultivars.

The foliar biostimulant applied in this study had a positive and significant effect on root traits of hop plantlets in both acclimatization periods. When considering the sub-experiment 1 (acclimatization in a controlled environment), the beneficial effect of spraying a mixture of seaweed and plant extracts extended also to shoot traits. These findings are consistent with those obtained on algae-based biostimulants in general [64] as well as using the same product on durum wheat [65]. The seaweed *Codium fragile*, similarly to other macroalgae, is rich in phytohormones (e.g., cytokinin, gibberellins,

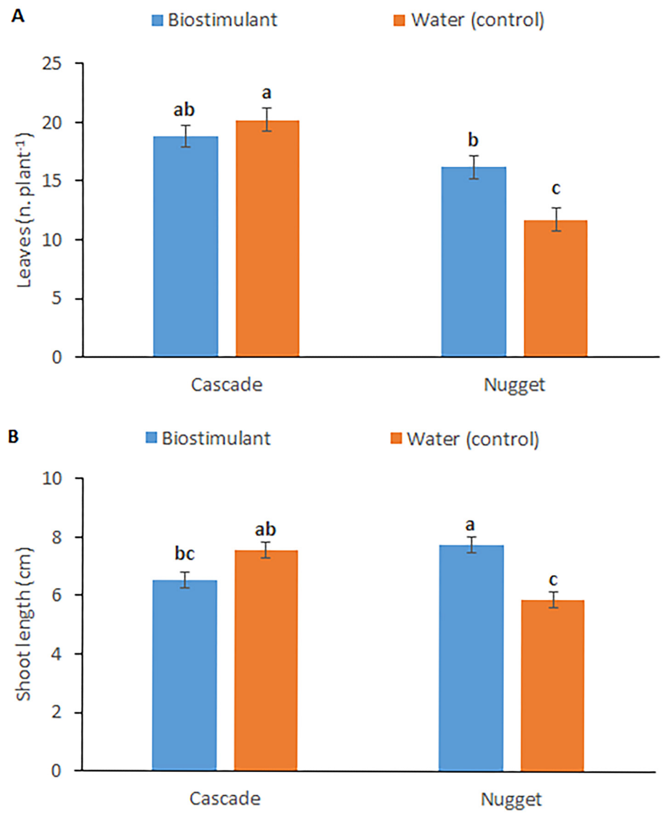

Fig. 5 Effect of biostimulant × cultivar interaction on leaves' number (A) and shoot length (B) of hop plantlet during the hardening period in open air. The vertical bars represent the standard error of the mean. Letters above the bars correspond to the ranking of the Tukey's test at p < 0.05

and auxins), essential nutrients and amino acids [66, 67]. These active ingredients were found to stimulate the development of lateral roots, formation of root buds and leaf blades as well as cell division [68, 69]. Similarly, the species belonging to the *Opuntia* genus are a source of bioactive compounds, such as minerals (e.g., magnesium, potassium, calcium and zinc), N-organic molecules and free amino acids, like phenylalanine [70, 71], which plays a fundamental role in the early growth of plants [72]. The positive effect of foliar biostimulant on shoot system decreased along with the plantlet growth, becoming not significant during the hardening in open air. This could be due to the fact that, generally, plant biostimulants are greatly effective under stress conditions, while they may be less effective when plants grow and overcome stress conditions [73, 74]. In sub-experiment A, fragile hop plantlets moved from optimal environmental conditions, like those experienced in laboratory, to those of glasshouse (e.g., sub-optimal humidity and light, variation in air temperature). Conversely, in sub-experiment B, shoot traits were collected from plants which were partly hardened to abiotic stresses. However, the effect of foliar biostimulant over the acclimatization period could be helpful in obtaining a well-rooted plant without excessively boosting the vegetative growth. This could lead to i) easier nursery management by reducing top-pruning interventions of hop plantlets; ii) greater outplanting success by increasing the ability of plantlets to overcome possible abiotic stresses.

Although survival rate of plants treated with foliar biostimulant was numerically higher than that of untreated plants, the effect was not statistically significant (table 2, P = 0.098). Brighenti et al. (2023) [56] showed similar results using a commercial algae-based biostimulant to improve vegetative propagation of two hop cultivars. Specifically, the application of biostimulant to both leaves and substrate increased survival rate by 10 to 60 percentage points, depending on hop cultivar and cutting size. However, Brighenti et al. (2023), in their study, used herbaceous cuttings of Brewer's Gold and Cascade and a substrate consisting in a mixture of 40 % carbonized rice husk and 60 % peat.

In our study, interaction between biostimulant and growing media was found to be significant for root weight. Particularly, foliar treatment doubled the root weight of hop plantlets grown in the standard substrate while it had no significant effect on those grown on the alternative substrate. A hypothesis could be the presence of phytotoxic compounds (e.g., phenolic compounds, organic acids, resins, etc.) that often characterize wood-based materials [75] and that could have severely hindered the beneficial effect of foliar biostimulant. Further analysis and tests (e.g., germination index, leaching EC/pH, phenolic assay) will be needed to confirm this hypothesis as well as a modification of the substrate through a longer composting period.

The negative effect of the alternative substrate was particularly evident during the second phase of the acclimatization period (hardening in the open air), when the plantlets were transplanted in larger pots and were actively growing. In that period, all the plant traits were negatively affected by the experimental growing medium (substrate B), especially the development of rhizome, which is the perennial storage organ that allows the plant to survive and grow in subsequent seasons.

Hop cultivars tested in our study highlighted a very different way to allocate plant biomass during the hardening period. Particularly, Nugget showed a longer and heavier root system than Cascade hops in sub-experiment A, and continued to show a greater root dry weight during sub-experiment B. Conversely, Cascade produced a higher number of leaves as compared to Nugget. In sub-experiment B (acclimatization in open air), root-to-shoot ratio of Nugget hops was twofold higher than that of cultivar Cascade (1.1 vs. 0.5), indicating a possible better morphological balance to face challenging environmental conditions (e.g., drought and/or high evaporative demand). However, the utility of using root to shoot ratio as the sole indicator of seedling quality is still questionable, as it should be supported also by data on plant hydraulics and other elements of physiology [20].

Interestingly, biostimulant application had a differential effect on hop cultivars during the hardening in open air. Indeed, foliar treatment markedly boosted the shoot system of Nugget, while it had no significant effect on Cascade. Our results are consistent with those obtained by Brighenti et al. [56], who studied the effect of a seaweed-based biostimulant on hop cutting morphology. The authors found that the number of shoots and shoot length of Cascade hops were not affected by biostimulant application, which, conversely, significantly increased shoot growth of cultivar Brewer's Gold. This significant genotype × biostimulant interaction deserves a deeper understanding and specific studies involving plant genetics and physiology.

5 Conclusions

Results from this study can help nursery managers to obtain the ideal micropropagated hop plantlet by using sustainable materials and treatments.

The foliar seaweed-plant extract improved root traits in both acclimatization periods, and it also increased several shoot traits in the glasshouse. Consequently, this biostimulant treatment may be considered in nursery production of hops, because it can originate in vitro derived plantlets less prone to transplant shock. Additionally, since the foliar biostimulant did not excessively boost shoot growth, it is unlikely to necessitate additional pruning during the ex vitro phase, although this was not measured and warrants operational testing.

Interestingly, hop cultivars differently allocated plant biomass during the hardening period, with Nugget developing a longer and heavier root biomass than Cascade, which, oppositely, produced a greater shoot biomass as compared to Nugget. Additional studies on field performance of these hop cultivars are needed to clarify which plantlet balance provides the best agronomic response.

Finally, even though the experimental substrate did not affect plant traits during the first acclimatization period (hardening in glasshouse), its marked negative effect during the acclimatization in open air suggests not to use it for micropropagated hops. However, the valuable positive features of this material (affordability, low cost, waste recycling) warrant further and deeper analyses to discover the cause of this effect and to re-formulate a new growing medium.

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