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Micropropagation and Establishment of *Humulus lupulus* L. Plantlets Under Field Conditions at Southern Brazil

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Abstract

Tissue culture technique can be an important approach for the rapid propagation of *Humulus lupulus* L. (hop plant). The present study evaluated different culture media for hop plants (cv. Columbus) in vitro shoots multiplication, as well as ex vitro rooting and plantlets establishment under field conditions at Southern Brazil. The culture medium containing the plant growth regulator thidiazuron (TDZ) resulted in greater in vitro shoots multiplications (five shoots per explant). For microcuttings ex vitro rooting, indole-3-butyric acid (IBA) application at a concentration of 3000 mg L^{-1} resulted in higher rooting and survival percentages during acclimatization. After 90 days of acclimatization, the plantlets were transplanted under field conditions and had their development and productivity evaluated 122 days after transplanting. Micropropagated plants achieved 100% survival under field conditions. Nitrogen supply is highly necessary for hop plants initial development at Southern Brazil. Micropropagation is a viable technique for quality plantlet production of *H. lupulus* cv. Columbus.

Keywords: hops, in vitro multiplication, microcutting, acclimatization

1. Introduction

Humulus lupulus L. (Cannabaceae) is an herbaceous, dioecious plant species, popularly known as hop plant. The species female inflorescences, named cones or hops, contain lupulin glands, structures producing alpha and beta acids and essential oils. These substances confer remarkable importance to the hops in the brewing industry, since they provide bitterness and aroma to beer (Neve, 1991; Taniguchi et al., 2014).

Currently, the largest hops producing countries are Germany and the United States. Brazil, in turn, is characterized for being a significant importer. In 2010, the country imported 5% from all hops exported by Germany (Biendl et al., 2014). This outstanding economic potential has brought interest in developing hop plants cultivation with adapted genetic materials for different Brazilian regions. Therefore, germplasm introduction and evaluation is one of the first steps to make this specialty crop economically viable to meet the domestic market demands.

Plant propagation is one of the main limiting factors for introducing a new crop, since the production of quality plantlets is a fundamental feature for success in the subsequent cultivation stages. Commercially, *H. lupulus* is vegetatively propagated, either by rhizomes segments or herbaceous stem cuttings. Nevertheless, tissue culture techniques can also be useful tools for hop plants multiplication. Some protocols for micropropagation of the species have already been established and proved to be viable (Batista et al., 1996, 2000, 2008).

The ultimate success in plant micropropagation lies in establishing an efficient protocol from *in vitro* regeneration to acclimatization stage, which is usually different for each species or even for each cultivar. Different culture media have been proposed for *H. lupulus in vitro* organogenesis. (Roy et al., 2001; Skof et al., 2007; Peredo et al., 2009; Gatica-Arias & Weber, 2013). Culture media should provide macro and micronutrients, as well as vitamins, carbohydrates and plant growth regulators, necessary for plant regeneration.

Because micropropagation is a high-cost technique, exclusion of the *in vitro* rooting step can be used, whenever feasible. In *Leptadenia reticulata*, the depletion of this stage reduced the time and the cost for quality plantlets

production (Shekhawat et al., 2006). E*x vitro* rooting of microcuttings is also a viable alternative for species that present poor rooting rates in regular macropropagation (Machado et al., 2011). However, microcutting techniques must be optimized and adapted to each species or cultivar to increase rooting performance in valuable plant species (Osman et al., 2013). It is also important, when introducing this technique into a new crop or a new region, to evaluate the plantlets performance under field conditions.

Although hop plants tissue culture has proven to be feasibly used for research, breeding and large-scale plantlet production, micropropagation-produced plantlets performance under field conditions has not been reported.

Aiming the establishment of *H. lupulus* (cv. Columbus) micropropagated plantlets under field conditions, the present study intended to: (i) evaluate different culture media for shoots *in vitro* multiplication; (ii) promote microcuttings *ex vitro* rooting; and (iii) evaluate micropropagated plants establishment and initial growth with and without nitrogen application under field conditions at southern Brazil. To the best of our knowledge, the present study is one of the first scientific approaches for the propagation and establishment of commercial hops cultivation in Brazil.

2. Method

2.1 Plant Material

The explants sources for *in vitro* culture were 1-year old *H. lupulus* (cv. Columbus) plants cultivated under greenhouse conditions. The explants consisted of nodal segments approximately 1.0 cm in length.

2.2 Culture Media and Shoots Regeneration

After washed in running tap water, nodal segments were surface-disinfected by submersion in 70% (v/v) ethanol for 25 seconds, followed by treatment with 2% (v/v) sodium hypochlorite for 20 minutes, and then rinsed three times with autoclave-sterilized deionized water. Following asepsis, the explants were placed in glass flasks (30 × 80 mm) containing 20 mL of autoclave-sterilized (120 °C at 1.5 atm for 20 minutes) MS culture medium (Murashige & Skoog, 1962).

Shoots about 2.0 cm in length were isolated from the *in vitro* established nodal segments and placed in glass flasks (240 mL volume) containing 20 mL of different culture media. Four culture media previously used in hop plants micropropagation were evaluated: M1-MS medium (Murashige & Skoog, 1962) plus 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol and 7 g L^{-1} agar (Vetec®) (Gatica-Arias & Weber, 2013); M2-MS medium plus vitamins from Gamborg's B5 medium (Gamborg et al., 1968), 20 g L^{-1} glucose, 100 mg L^{-1} myo-inositol, 1 mg L^{-1} benzylaminopurine (BAP), and 7 g L^{-1} agar (Vetec®) (Skof et al., 2007); M3-MS medium plus 20 g L^{-1} glucose, 100 mg L^{-1} myo-inositol, 4.4 mg L^{-1} BAP, 0.1 mg L^{-1} calcium pantothenate, 3 g L^{-1} agar (Vetec®) and 1 g L^{-1} GelRite (Šuštar-Vozlič et al., 1999); and M4-MS medium plus 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol, 0.0547 mg L^{-1} indoleacetic acid (IAA), 0.2189 mg L^{-1} thidiazuron (TDZ) and 7 g L^{-1} agar (Vetec®) (Roy et al., 2001).

All culture were kept in a growth chamber with 25±2 °C temperature, 16-h photoperiod and a photon flux density of 45 μmol m^{-2} s^{-1}.

Two subcultures were performed every 30 days. For the culture media experiment, the following variables were measured: number of new shoots per explant, average number of leaves on new shoots, average new shoots length (height), percentage of rooted shoots and percentage of callus formation on explants. The experimental design was completely randomized, with five replications and 14 explants per plot.

2.3 Ex vitro Rooting and Acclimatization

Hop plants *in vitro* grown shoots were used to make microcuttings approximately 4 cm long, with two pairs of leaves. Microcuttings had their bases immersed for 10 seconds in hydroalcoholic solutions (50%) of indole-3-butyric acid (IBA) in the following concentrations: 0 (control); 500; 1000; 2000; 3000 and 4000 mg L^{-1}. After treatments, microcuttings were immediately planted into 120cm³ plastic tubes containing Tropstrato® commercial substrate (Vida Verde-Tecnologia em Substratos™, Brazil) and kept in intermittent misting chamber during 15 days. After this period, plants were transferred to a greenhouse with daily manual irrigation.

Greenhouse inside temperature and relative humidity monitoring was performed using a thermo-hygrometer. Mean maximum temperature was 27.38 °C and mean minimum was 14.12 °C while the mean maximum humidity was 93.9% and the mean minimum was 66.9%.

After 42 days from planting, rooting and survival percentages and plantlets aboveground length were measured. The experiment was conducted in a completely randomized design, with four replications and 25 microcuttings per plot.

2.4 Plantlets Establishment Under Field Conditions

At 90 days after acclimatization stage, micropropagated plantlets were transplanted to the field. Plants were spaced 1m apart within the rows and the rows were spaced 3 m (1,333 plants ha^{-1}). Hop plants were conducted in a training system with 6 m height trellis wires. Two baling twines were stretched from the overhead trellis wires to the ground for each plant. The twines were attached to a small stake at the ground level. Four vigorous vines were selected from each plant (2 vines per twine). The experimental area was located at the Agraria Foundation for Agricultural Research, an agro-industrial cooperative located in the district of Entre Rios, in Guarapuava city, State of Parana, Brazil.

Soil samples were collected from the experimental area and the results were: pH = 4.7; Al^{3+} = 0.23 $cmol_c$ dm^{-3}; $H^{+}+Al^{3+}$ = 7.2 $cmol_c$ dm^{-3}; C^{2+} = 2.9 $cmol_c$ dm^{-3}; Mg^{2+} = 1.3 $cmol_c$ dm^{-3}; K^{+} = 0.22 $cmol_c$ dm^{-3}; P = 7.6 mg dm^{-3}; C = 26.88 g dm^{-3}; base saturation = 38.36%. Soil correction was performed according to CQFS (2004) recommendations.

The effects of nitrogen on micropropagated hop plants establishment and initial growth under field conditions was assessed by employing two treatments: T1: application of 30 kg N ha^{-1}; and T2: control treatment without nitrogen. For the treatment with nitrogen, the nutrient source was urea (45% N) and it was applied by sowing at the time of transplanting plantlets into the field. The experiment was performed under a randomized complete block design, with 5 replication and 10 plants per plot.

After 122 days from transplanting into the field, the following variables were measured: dry leaf yield, dry stem yield, dry cone yield, plant length (height) and leaf area. Leaves and cones were separated manually from the stems and subsequently dried at 65 °C during 48 hours in a dryer with forced air circulation for assessment of cones, leaf and stem dry weight. Plant height was determined by the distance from the ground level to the highest point of the plant on the training twines.

Fifty 0.785 cm^2 leaf discs were weighted from each experimental plot and the leaf area was then calculated as proportion of total leaves weight. Following, Physiological indexes were calculated based on these data, as described by Radford (1967). Leaf area index (LAI) was determined by the ratio between leaf area and soil area occupied by the plants (as a function of plant spacing). Leaf area ratio (LAR) was defined as the quotient between the leaf area and plant total dry weight. Specific leaf area (SLA) represents the relation between leaf area and leaf dry weight and, leaf weight ratio (LWR) was calculated by the proportion of leaves dry weight on total plant dry weight.

2.5 Statistical Analysis

Data from all experiments were initially submitted to analysis of variances homogeneity by the Bartlett's test. For the experiments with culture media and nitrogen application, the means were compared by the Tukey's Test at 5% probability. For the *ex vitro* rooting experiment, polynomial regression analysis was performed. Percentage values (rooting, survival and callus formation) were *arcsin* $\sqrt{x/100}$ transformed before the ANOVA was performed. All analyses were carried out using the statistical software Assistat (Silva & Azevedo, 2016).

3. Results

3.1 Culture Media and Plant Regeneration

Differences in culture media exerted significant influence over the analyzed variables. M4 medium promoted the highest number of shoots per explant (approximately 5 shoots per explant), whereas M1 and M3 media showed the lowest number (approximately 1 shoot per explant). The number of leaves on new shoots was also higher in the M4 medium, reaching about 11 leaves per shoot. TDZ-supplemented media was previously reported as promoting higher numbers of leaves and nodes on explants in a micropropagation system developed for *H. lupulus* (Roy et al., 2001). In other species, such as *Rauvolfia serpentine*, TDZ was also effective in *in vitro* multiplication (Alatar, 2015).

Regarding shoots length, M1 medium provided the highest values (2.7cm on average), differing statistically from the other evaluated media. Plants grown on M2 medium showed the lowest shoot length (1.8 cm on average) although not statistically different from M3 medium (Table 1). For the G × N15 hybrid (almond tree × peach tree), it was similarly reported that the highest shoots length *in vitro* were achieved on culture media without plant growth regulators supplementation, which may be explained mainly by the cytokinin effects on boosting shoots emission, inducing plants to produce a higher number of nutrients sinks with lower individual growth and elongation (Arab et al., 2014). Additionally, cytokinins are known to reduce apical dominance and induce adventitious shoots formation from meristematic explants (Resmi & Nair, 2011).

Explants rooting was observed only on M1 and M3 culture media (Table 1). Adventitious shoots and roots formation on *in vitro* cultivated explants is strongly related to the cytokinin/auxin balance (Su et al., 2011), and, accordingly, it was observed lower shoot multiplication rates on the media that induced rooting.

The highest percentage ($P < 0.05$) of callus formation on new shoots occurred on M4 medium (90.2%). M1 medium induced no callus formation (Table 1). These results are likely to be related to the lack of plant growth regulators on M1 medium and the presence of TDZ in M4 medium. Among the several external factors influencing callus formation in hops tissue culture, plant growth regulators play a fundamental role. It has already been reported that hop plants explants show no callus formation with the absence of these compounds in culture media (Fortes & Pais, 2000).

Table 1. Number of new shoots per explant, number of leaves on new shoots, new shoots length (height), shoots rooting percentage and callus formation percentage on *Humulus lupulus* (cv. Columbus) nodal segments *in vitro* cultivated using different culture media

Culture media	Shoots number	Leaves number	Heigth (cm)	Rooting (%)	Callus (%)
M1	1.4 c	5.8 c	2.7 a	32.1 b	0.0 d
M2	2.0 b	8.1 b	1.8 c	0.0 c	57.7 b
M3	1.1 c	4.6 d	2.0 bc	39.0 a	43.9 c
M4	5.2 a	10.8 a	2.1 b	0.0 c	90.2 a
CV (%)	8.57	2.04	4.45	14.78	7.37

Note. Means followed by same letter in the column are not statistically different according to Tukey´s test ($P < 0.05$). CV = coefficient of variation.

3.2 Ex vitro Rooting and Acclimatization

Microcuttings rooting percentages as a function of IBA concentrations were adjusted in a quadratic behavior. The maximum efficiency concentration, reaching about 100% rooting, was estimated at 2844.8 mg L^{-1} IBA, according to the regression equation (Figure 1A). In the control treatment, rooting percentage was about 73%. As observed in *Prunus serrulata* stem cuttings (Fragoso et al., 2017), when endogenous auxin content is insufficient, the application of synthetic auxins like IBA is important to promote a favorable hormonal balance for adventitious rooting promotion. The ideal auxin concentration to be applied exogenously depends on the species being studied. For *Passiflora foetida* microcuttings, for example, 97% rooting was obtained at a lower concentration (300 mg L^{-1}) than the ideal one estimated for *H. lupulus* in the present study (Shekhawat et al., 2015).

The relatively high rooting percentage on microcuttings under the control treatment can be explained by the fact that the adventitious rooting in stem cuttings is also strongly influenced by the natural endogenous auxin produced by the leaves and buds, which moves naturally to the cuttings base promoting cells division and differentiation into a new root system (Zuffellato-Ribas & Rodrigues, 2001; Olatunji et al., 2017).

Plantlets length and survival, similarly to plantletss rooting, were adjusted in quadratic curves according to the IBA concentrations. Maximum survival after 42 days acclimatization was estimated at a concentration of about 2925 mg L^{-1} IBA (Figure 1B), whereas maximum length (22.1 cm) is predictable to be achieved at a 2863 mg L^{-1} IBA concentration (Figure 1C), according to second degree regression equations. Therefore, concentrations about 3000 mg L^{-1} IBA can be recommended for *ex vitro* rooting of *H. lupulus* cv. Columbus microcuttings.

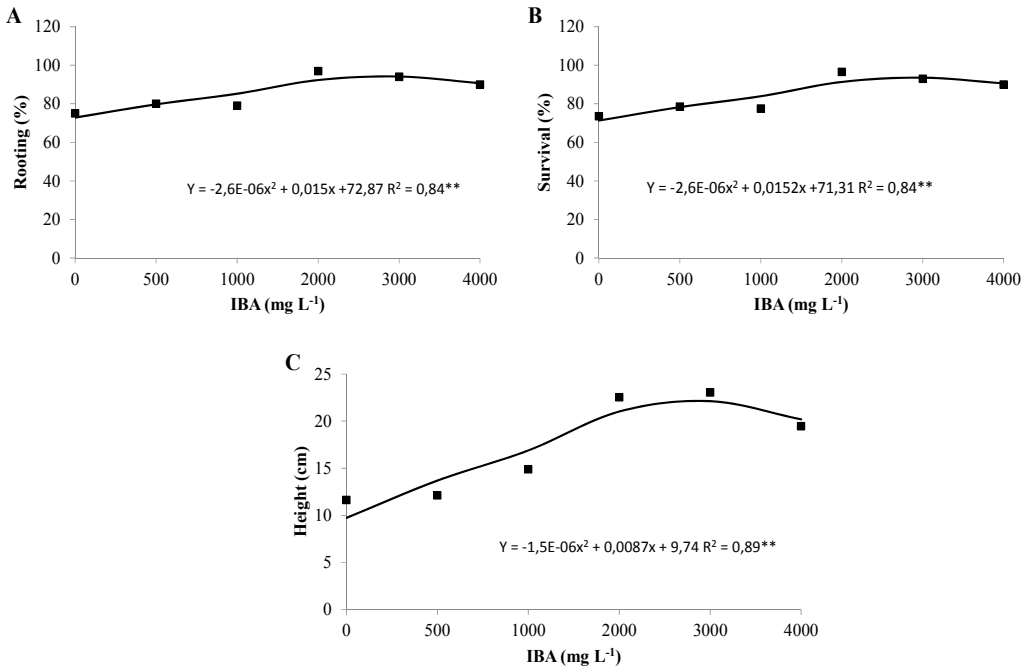

Figure 1. IBA effects on rooting (A) and survival (B) percentages and length (height) (C) of *Humulus lupulus* cv. Columbus microcuttings after 42 days acclimatizatization

3.3 Establishment Under Field Conditions

Although all transplanted plantlets survived, in general, hop plants showed relatively reduced growth, which was expected, as hop production usually only get to its maximum value after three years from planting, when the plants reach their full growth (National Hop Association of England, 2001).

Considering the treatments, plants presented better performance in all variables when nitrogen was applied. When treated with 30 kg ha^{-1} N, plants showed an average dry leaf yield of 138.4 kg ha^{-1} compared to 21.9 kg ha^{-1} when no nitrogen was applied. Similar behavior was observed for dry stem yield and dry cone yield, which were remarkably higher in plants that received nitrogen. Dry cone yield reached 61.1 kg ha^{-1} in the nitrogen-treated plants (Table 2). Previous studies have shown that hop cones yield is highly influenced by nitrogen supply to plants (Bavec et al., 2003). Biomass accumulation is dependent on plant's ability to maximize photosynthesis, which, among other factors, is related to nutritional aspects (Mandal et al., 2013).

As the biomass yields, physiological indices LAI, SLA, LWR and LAR were significantly superior in plants treated with nitrogen at transplanting, evidencing the highest growth of plants (Table 2). Taken together, these indexes represent the quality of plants photosynthetic apparatus and help to explain in terms of light and CO_2 harvesting capacity, the better productive performance of nitrogen-supplemented plants.

Table 2. Dry leaf yield, dry stem yield, dry cone yield and physiological indexes from Humulus lupulus cv. Columbus micropropagated plants according to nitrogen presence or absence under fiel conditions. Guarapuava, Paraná State, Brazil

Nitrogen (30 kg ha^{-1})	DLY (kg ha^{-1})	DSY (kg ha^{-1})	DCY (kg ha^{-1})	Height (cm)	LAI	SLA ($cm^2 g^{-1}$)	LAR ($cm^2 g^{-1}$)	LWR
Absence	21.9 b	12.3 b	4.0 b	86.8 b	0.027 b	125.8 b	79.8 b	0.640 b
Presence	138.4 a	53.8 a	61.1 a	228.3 a	0.219 a	158.8 a	114.5 a	0.721 a
CV (%)	11.99	15.73	59.63	12.05	8.53	8.14	5.77	4.87

Note. Means followed by same letter in the column are not statistically different according to Tukey's test ($P < 0.05$). DLY = dry leaf yield. DSY = dry stem yield. DCY = dry cone yield. LAI = leaf area index. SLA = specific leaf area. LAR = leaf area ratio. LWR = leaf weight ratio. CV = coefficient of variation.

4. Conclusions

M4 culture medium (containing TDZ) provides higher adventitious shoots multiplication rates on *in vitro* cultivated *H. lupulus* (cv. Columbus) nodal segments. *Ex vitro* rooting and acclimatization of hop plants microcuttings can be successfully performed using IBA concentrations about 3000 mg L^{-1}. Nitrogen application (30 kg ha^{-1}) at the time of transplanting plantlets promotes greater development and dry cones yield under Southern Brazil conditions. Micropropagation is a viable technique for quality plantlets production of *H. lupulus* cv. Columbus.

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