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Effects of explant position and orientation, medium ph and nitrogen sources on micropropagation of blackberry

Composition of the medium and the explant origin are factors that interfere on success of micropropagation of Rubus species. For blackberry cultivar LochNess it was not investigated yet how the position and orientation of explant, pH levels and nitrogen source interfere on micropropagation. In this work, focused on the establishment of in vitro culture, variables were studied on R. fruticosus cv Loch Ness, such as the choice of the explants depending on their original position on the mother plant, pH level and nitrogen sources of the culture medium. For the first time in vitro on Rubus, the downward orientation (capogatto) of shoot tips explants was compared with the normal upward orientation. The highest weight and length values were recorded for the shoots proliferated from basal and nodal explants. For the initiation medium, the best multiplication rate were obtained in pH adjusted to 4.5. Shoot length was influenced by the nitrogen source; when associated with an increased light intensity, the complete substitution of ammonium by nitrate allowed results comparable with those obtained with the control medium containing both sources. The use of aminoacids did not improve the results. Apex orientation did not affect anatomical parameters or rooting rates of wild Rubus, but more efforts should be devoted on in vitro capogatto technique considering that advantages like reduction of plant growth regulators, cultivation on the same medium culture for more time and easily rooting can be established.

Keywords: Rubus fruticosus; R. ulmifolius; In vitro culture; Capogatto; Ammonium-nitrate balance.

Efeitos da posição e orientação do explante, fontes médias de ph e nitrogênio na micropropagação de amora-preta

A composição do meio e a origem do explante são fatores que interferem no sucesso da micropropagação de espécies de Rubus. Para a cultivar de amora-preta LochNess, ainda não foi investigado como a posição e orientação do explante, níveis de pH e fonte de nitrogênio interferem na micropropagação. Neste trabalho, focado no estabelecimento de cultivo in vitro, foram estudadas variáveis sobre R. fruticosus cv Loch Ness, como a escolha dos explantes em função de sua posição original na planta-mãe, nível de pH e fontes de nitrogênio do meio de cultura. . Pela primeira vez in vitro em Rubus, a orientação para baixo (capogatto) dos explantes das pontas dos caules foi comparada com a orientação normal para cima. Os maiores valores de peso e comprimento foram registrados para os brotos proliferados de explantes basais e nodais. Para o meio de iniciação, as melhores taxas de multiplicação foram obtidas em pH ajustado para 4,5. O comprimento da parte aérea foi influenciado pela fonte de nitrogênio; quando associada a um aumento da intensidade luminosa, a substituição completa do amônio pelo nitrato permitiu resultados comparáveis aos obtidos com o meio controle contendo as duas fontes. O uso de aminoácidos não melhorou os resultados. A orientação do ápice não afetou os parâmetros anatômicos ou as taxas de enraizamento de Rubus selvagem, mas mais esforços devem ser dedicados à técnica de capogatto in vitro considerando que vantagens como redução de reguladores de crescimento de plantas, cultivo no mesmo meio de cultura por mais tempo e enraizamento podem ser estabelecidas.

Palavras-chave: Rubus fruticosus; R. ulmifolius; Cultura in vitro; Capogatto; Balanço de nitrato de amônio.

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INTRODUCTION

In plant tissue culture, species and cultivars within the same species can differently respond to the same conditions (LIZÁRRAGA et al., 2017), therefore it is difficult to define a common *in vitro* culture protocol. In *Rubus* the ability of regeneration and multiplication of plants *in vitro* is also affected by the genotype (HUNKOVÁ et al., 2016).

The most suitable plant organ to be used as an explant source can vary according to the species and the purpose of tissue culture; the correct choice of the starting material can influence the success of all process. This is due to several, concurring reasons, such as cytological or nutritional factors, and also to the composition and levels of endogenous hormones in different plant organs (KRATATAS et al, 2018).

Rubus micropropagation results depends from the kind of propagules used as a source of explants (PELTO et al., 2001); the shoot tips were reported as the most suitable material to start tissue culture on *Rubus glaucus* (JADAN et al., 2015). The micropropagation of *Rubus* spp is a challenge, taking into account that some wild species appear highly promising for domestication and economic cropping (BUENO et al., 2018).

For *Rubus* spp., a typical form of asexual propagation is known, named simple inverted plunging, tip diving, tip layering or capogatto (HESLOP-HARRISON, 1959). The capogatto process starts with the contact of the shoot apex with the soil, followed by root differentiation and, finally, by the generation of a new plant, still connected to the mother plant. In nature, its occurrence depends on the season and it is influenced by the movement of carbohydrates and other substances within the plant (HARTMANN et al., 1990).

The tip layering or capogatto in boysenberry cuttings produced 98.4% of rooting when associated with the use of 4000 $mg.L^{-1}$ of IBA solution (TIBERTI et al., 2012); on *R. niveus* this technique turned out as the best method for propagation even without hormones, with rates of 77.9% of rooting (SILVA et al., 2012).This technique could represent an interesting, alternative method of propagation in vitro, especially for wild *Rubus* species, with the possibility of obtaining shoot rooting as *in vivo*, even without exogenous auxins added to the culture medium.

To our knowledge, in vitro capogatto protocols have not been assessed for *Rubus* species, whereas the position approaches were reported on other species. Compared with the standard vertical position, *Dimorphorchis lowii* explants gave better proliferation results if horizontally plated, in terms of shoot number (JAINOL et al., 2016).

As regards mineral nutrition, the MS medium is widely used as a substrate for several other species. Changes of its composition were reported to produce positive effects on the growth and development of some plants like raspberries, probably due to its high genetic diversity in nutritional requirements (POOTHONG et al., 2014).

The pH buffering of the culture medium usually recommended vary in the range of 5.5 – 5.8. The pH values can affect the development of the culture and its morphogenesis; low pH of the medium can control bacterial contamination itself, but also modifying the activity of antibiotics added to the medium. The acidity

of the culture medium is also related to nitrogen uptake.

In vitro nitrogen can be available as its inorganic forms (NH_4^+ cation and NO_3^- anion) but also as readily assimilable organic forms like urea, amino acids, polyamines and ureides; it strongly influences morphogenesis, cell totipotency and growth (WADA et al., 2015).

This work, focused on the *in vitro* culture establishment phase of *Rubus* spp., aimed to verify the effects of the explant depending on its original position in the mother plant and its subsequent orientation on the culture medium; pH variation and different nitrogen sources were also investigated.

MATERIALS AND METHODS

Plant material

Either *R. fruticosus* cv. Loch Ness plants, bought from a certified producer and subjected to 90 days of greenhouse acclimatization (25° C, 58% RH), or *R. ulmifolius* wild plants growing at 44º30'54.0 "N and 11º21'16.0" E were used as a source of explants. From both species, shoots were collected in late summer.

Surface decontamination and explant preparation

Shoots were detached from *R. fruticosus* cv. LochNess mother plants and cut into 3-4 cm long portions, including either apical or lateral buds, and were sterilized with NaOCl 1% plus one drop of Tween 20 for 30 minutes and were washed two times with autoclaved deionized water for 20 minutes. The shoots were also stirred for 20 minutes in a sterile solution of citric acid and ascorbic acid (50 $mg.L^{-1}$ each) to prevent explant exudation and tissue browning. The stems portions were cut into shorter segments including only one bud, that were used as initial explants.

From *R. ulmifolius* wild mother plant(s) 3 cm long apical portions, detached from procumbent shoots (Figure 1), were treated with the above described decontamination procedure and used as initial explants.

Figure 1: Natural capogatto early stages used as explant source of *Rubus ulmifolius*. Bologna, Italy, 2019.

In vitro Culture

Four experiments were performed, sharing the culture conditions described below, when not otherwise, further specified.

The basal composition of the initiation medium (IM) consisted in MS salts (modified with 1240 $mg.L^{-1}$ of NH_4NO_3 and 1270 $mg.L^{-1}$ of KNO_3) supplemented with 30 g/L sucrose, 30 $mg.L^{-1}$ reduced glutathione, 1

mg.L^{-1} BAP, 0.125 mg.L^{-1} of GA_3 and 2.6 g/L of Phytagel (pH adjusted to 5.8 with 1N KOH). After the autoclave sterilization (20 min at 121 °C, 1 bar) and before gelling, aliquots of the warm medium were pipetted into glass culture vessels, previously sterilized. The initial explants, individually placed into the culture containers closed and sealed with plastic film, were incubated in a growth chamber at 23 ± 1°C, in the dark for the first 7 days, then under a 16 h photoperiod of cold white fluorescent light (35 µmol$m^{-2}s^{-1}$) for further 30 days.

Four experiments were performed, sharing the culture conditions described above, when not otherwise, further specified.

Experiment I: explant position

This experiment aimed to analyze if initial bud explants of blackberry cv Loch Ness can exhibit different *in vitro* growth rates during the inclusion phase, depending on their original position on the donor shoot. Three treatments were evaluated (Figure 2): T1 - Apical portion: shoot apex and the distal 1.5 cm; T2 - Median portion: the 1st and 2nd buds facing downwards the apical portion; T3 - Basal portion: all the buds recovered from the 3rd one to 7th on the shoot insertion on the donor plant. After 37 days of culture in test tubes of 20 x 150 mm, containing 10 mL of IM culture medium, weight increase, multiplication rate and shoot length were evaluated.

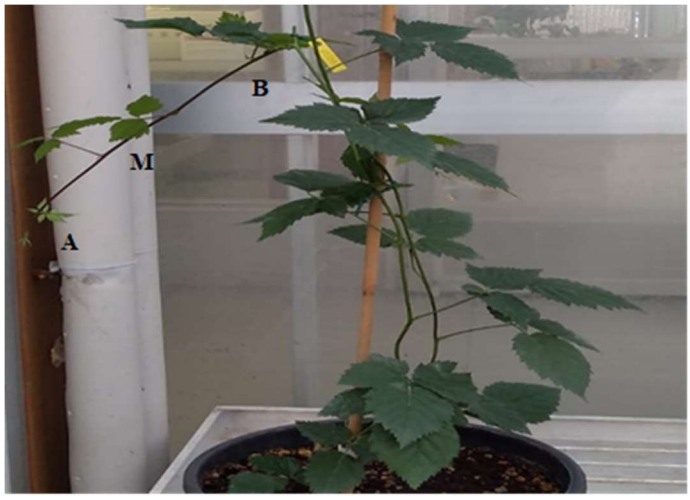

Figure 2: Positions of the explants removed from the Loch Ness plants for micropropagation. Bologna, Italy, 2019. A: Apical portion; M: Median portion; B: Basal portion.

Experiment II: explant orientation

R. ulmifolius apexes, either with the normal (tip up) or the capogatto orientation (tip down), were incubated in 60 mL jars on IM for 100 days in the growth chamber without renewing the culture medium. After this initiation phase, prolonged in order to allow *in vitro* root differentiation in the absence of exogenous auxins, the *ex vitro* acclimatization phase was directly performed, using PVC trays with 162 cm² cells, containing commercial substrate (Hochmoor®). The trays were placed in reservoirs with water, into greenhouse at temperature of 25 °C and relative humidity of 58%, according to the methodology described by Clapa et al. (2013). The trays were covered with transparent plastic to avoid the loss of water. After 30 days of acclimatization, data of plant weight and length, root length were recorded. Leaf Spad Index was

performed using a Minolta SPAD 502 portable greenness meter (Konica Minolta, Inc., Osaka, Japan) performing two measurements on the youngest expanded leaf of each shoot.

Experiment III: pH of the initiation medium

Loch Ness initial explants were cultivated in 30 x 150 mm test tubes containing 10 mL of IM culture medium, whose pH was adjusted to three values: 4.5 (T1), 5.8 (T2), 7.0 (T3). After 37 days of culture were evaluated weight increase, multiplication rate and shoot length.

Experiment IV: nitrogen source and light intensity

The experiment was performed on cv. Loch Ness explants, cultivated in 18 x 150 mm tubes containing 6 mL of culture media with different nitrogen sources. Cultures were maintained in the dark for the first week, under a 16 h photoperiod for the following 30 days. After 37 days of culture were evaluated weight increase, multiplication rate and shoot length.

Four different substrates were employed, all with the same nitrogen concentration as the control T1 (IM medium). Considering both nitrogen source and light intensity, five treatments were compared (Table 1). All treatments showed final concentrations of N and K of 610 $mg.L^{-1}$ and 1703 $mg.L^{-1}$, respectively.

Table 1: Treatments applied to Loch Ness explants in experiment IV.

Salts/Nutrients ($mg.L^{-1}$)	T1	T2	T3	T4	T5
NH_4NO_3	1240.0	0	0	0	0
KNO_3	1270.0	4403.6	4403.6	1270.0	1270.0
K_2SO_4	2700.6	0	0	2700.6	2700.6
Glutamic acid	0	0	0	4558.8	0
Glycine	0	0	0	0	2325.8
pH	5.8	5.8	5.8	5.8	5.8
Light intensity ($\mu mol\ m^{-2}s^{-1}$)	35	35	300	35	35

The experimental design of all the four experiments was completely randomized with 20 repetitions per treatment. To verify the assumptions of the statistical model, the Shapiro-Wilk normality, Bartlett homogeneity and Tukey additivity tests were performed. To verify the effects of the treatments analysis of variance (ANOVA) and, when necessary, Tukey mean tests were used. Optimal lambda of Box-Cox transformation was employed to process the longest root length in experiment II, and for all the variables of experiment III.

For explant rooting analysis over time, in experiment II, regression analysis was performed. Data analyses were performed in the R environment (R CORE TEAM, 2015), considering significance level 5%.

RESULTS

Experiment I: explant position

The best results of weight increase and shoot length were recorded for the explants obtained from the basal part of the donor shoot, while the proliferation rate was not affected by explant position (Table 2).

Table 2: Effects of original position of the explants in the culture establishment on in vitro shoot growth of *Rubus* cv Loch Ness.

Treatment	Weight increase (mg)	Shoot length (mm)	Multiplication rate (Nº)
Basal	158.57 ± 79.11 a	6.42 ± 1.65 a	1.35 ± 0.63^{ns}
Medium	97.00 ± 42.95 b	3.60 ± 1.14 b	1.20 ± 0.42
Apical	72.22 ± 40.12 b	3.48 ± 1.25 b	1.14 ± 0.36

Mean ± standard error; Different letters on columns indicate difference statistical on Tukey´s test ($p < 0.05$). ^{ns}: Does not differ statistically.

Experiment II: explant orientation

With the capogatto technique applied *in vitro* to *R. ulmifolis* explants (Table 3) no differences were observed on rooting, with 0,95 on linear model of regression analysis and anatomical parameters comparable with the normal position.

Table 3: Effects of *R. ulmifolius* explant orientation, after 100 days of culture on IM medium, followed by 30 days of acclimatization on soil.

Treatment	Plant weight (mg)	Plant length (cm)	Main root length $^{(1)}$ (cm)	SPAD value
Capogatto	2.16 ± 1.32^{ns}	8.80 ± 3.95 ^{ns}	5.68 ± 4.37 ^{ns}	28.99 ± 3.53 ^{ns}
Normal	2.39 ± 1.15	9.56 ± 2.15	6.32 ± 3.47	27.12 ± 1.96

Mean ± standard error; ^{1}: ^{ns}: not significant.

Experiment III: pH of the initiation medium

Multiplication of cv. Loch Ness was significantly affected by pH, with the highest rate obtained with pH 4.5; shoot weight and length did not differ between the treatments (Table 4).

Table 4: Effects of pH in weight, length and multiplication rate of shoots induced from nodal stems of *Rubus* cv Loch Ness after 30 days of culture on IM medium.

pH	Weight (g)	Length (mm)	Multiplication rate (Nº)
4.5	5.54 ± 4.37 ^{ns}	6.36 ± 1.58 ^{ns}	4.82 ± 2.58 a
5.8	4.55 ± 2.41	6.47 ± 1.21	3.60 ± 2.12 b
7.0	6.05 ± 4.59	6.28 ± 2.14	4.06 ± 2.68 ab

Mean ± standard error; Different letters indicate statistical difference on Tukey´s test ($p < 0.05$); ^{ns}: not significant.

Experiment IV: nitrogen source and light intensity

Nitrogen source and light intensity affected only shoot length (Table 5). Highest values were recorded for the control T1 and for the treatment T3, where ammonium had been completely replaced by nitrate with the increased light intensity.

Table 5: Axillary shoots induced from nodal stems of *Rubus* cv Loch Ness cultured on modificated MS media with four different sources of nitrogen, during initial culture.

Treatment	Weight increase (g)	Shoot length (mm)	Multiplication rate (Nº)
T1 Nitrate+Ammonium	4.99 ± 3.96 ^{ns}	6.84 ± 1.56 a	4.93 ± 2.95 ^{ns}
T2 Nitrate	4.03 ± 2.92	5.73 ± 1.68 b	4.44 ± 2.97
T3 Nitrate + Light	6.06 ± 6.07	6.59 ± 1.94 a	5.02 ± 3.77
T4 Nitrate +Glutamic Acid	4.95 ± 3.89	6.42 ± 1.71 ab	4.18 ± 2.93
T5 Nitrate+ Glycine	5.01 ± 5.57	6.52 ± 1.87 ab	4.69 ± 3.74

Mean ± standard error; Different letters indicate statistical difference on Tukey´s test ($p < 0.05$); ^{ns}: not significant.

DISCUSSION

The better performance of the basal explants on *in vitro* implantation, recorded also for other species

such as *Pfaffia glomerata* (NICOLOSO & ERIG, 2002) and *Salix vitaminalis* (REGUEIRA et al., 2018), can be explained with their higher nutrient reserves to support shoot development.

On the other hand, as observed on *Shinnersia rivularis* (KRATATAS et al., 2018), shoot tips can show a prompter reaction when transferred to *in vitro* culture, probably due to their greater number of actively dividing cells in the meristematic zones. Indeed, from previous research works on other *Rubus* genotypes, shoot tips resulted the most responsive explants. Jadan et al. (2015) showed that the use of *Rubus glaucus* apical buds provided 57% of shoot growth, against 28% obtained from axillary buds.

To explain the different behavior that we observed on *R. fruticosus*, genotype- dependent factors could be supposed, such as in *Rosa genus*, where species and cultivars showed different requirements in terms of the original explant position (PATI et al., 2006). In addition, it is likely that the shoot tip tissues could have been more severely damaged during the surface decontamination procedure than the protected meristems of the axillary buds. Indeed, the exposure to sodium hypochloride, that we preferred to the more efficient mercuric dichloride because of health concerns, was prolonged for a time (30 minutes) long enough to obtain an acceptable sterilization level.

More efforts should be devoted to on in vitro capogatto technique considering that advantages like reduction of plant growth regulators, cultivation on the same medium culture for more time than usual and easily rooting can be established. Nevertheless, these should be considered as preliminary results, since important variables such as the season of explant collection, the comparison of more culture medium with different plant growth regulators composition, size of apical portion, asepsis process and the natural dark condition of substrate were not tested in our experiment. However, it should be considered that the *in vitro* success of the capogatto technique, probably depending also on the tested *Rubus* genotype, could not necessarily reflect its *in vivo* performance, where rooting takes place while the mother tissues are still connected to the new plant.

The influence of pH medium in Blackberry micropropagation are reported on *R. chamaemorus* by Martinussen et al. (2004) that, comparing a culture medium with pH either 5.8 or 4.5, found that the last value, more similar to the soil natural conditions, could increase shoot proliferation and dry weight. Also on *Vaccinium* species Cüce et al. (2016) observed that the best pH value for multiplication rate was 4.5, increasing shoot length on *V. myrtillus* and *V. uliginosum*, and node number too on *V. arctostaphylos* and *V. uliginosum*; they reported that these plants can alter the pH during cultivation and probably the different pH value can interfere on nutrients uptake.

Considering Nitrogen sources, the success of control can be attributed to the right balance of nitrate and ammonium in the medium that can affects nitrogen uptake and pH medium. With a predominant NH_4 uptake, medium acidification occurs; on the contrary, alkalinzation takes place when the main source is NO_3 (TIAN et al., 2015). This is related to the fact that ammonium is directly assimilated by plants, causing acidification by the release of H^+ (WOODWARD et al., 2006) whereas nitrate uptake implies co-transport of H^+.

The fact that many *Rubus* species are nitrophilic and their growth and coverage positively respond to

the increase of both nitrogen supply and light intensity (WALTER et al., 2016) could be comproved by results of Treatment 3. This nitrophilic condition can be confirmed when observed that *Rubus* are associated with acidic soils, like attested by Martinussen et al. (2004) in *R. chamaemorus.* However, this overload of nitrogen supply can be toxic if the conditions are not favorable for the action of the enzymes that will originate the ammonium assimilated by the plant. One of the most important environmental factors regulating nitrate assimilation is light, which is a signal for enzyme nitrate reductase (NR) expression and activity, the first step of nitrate assimilation (LILLO et al., 2001).

Singh et al. (2017) attested that higher light intensities obtained the best results on increase of fresh and dry mass on *Acer saccharum*. The authors suggested that this increase could be related with the habitat of these plants, i.e. open and bright areas, like those where the natural growth of *Rubus* species occurs. These conditions emphasize an invasive potential of *Rubus* genus in many countries such as Argentina, Australia, New Zealand, the United States, Chile and Portugal (VARGAS-GAETE et al., 2019).

CONCLUSIONS

The choose of explant position can interfere on the results of initial phase of LochNess micropropagation, considering that basal explants are the most suitable for its in vitro establishment. The use of capogatto didn´t represent differences compared to control. LochNess showed to prefer acid substrate, what can be observed even on some in vivo natural plants. The balance of nitrate and ammonium and the absence of ammonium with increase of light demonstrated be better than other treatments to this cultivar. Gaining in-depth knowledge on mechanisms related to the metabolism of nitrogen and other nutrients would significantly contribute to understand the performance of plants in vitro.

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