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STUDY ON *IN VITRO* PROPAGATION OF *Vaccinium myrtillus* Linn. VIA NODAL CULTURE METHOD

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ABSTRACT

Vaccinium myrtillus Linn. is an imported fruit tree with high economic value. Currently, in Vietnam there has not been any publication on *in vitro* propagation of the blueberry plant. This study is focused on *in vitro* propagation of *V. myrtillus* by nodal culture. The results showed that *in vitro* shoot growth and proliferation were the best when nodes were cultured on WPM medium supplemented with 0.5 mg/L zeatin, 30 g/L sucrose, 10 g/L agar, pH 5.8 (shoot height 4.24 cm, 1 shoot/explant, 8.60 nodes/shoot). The third nodes to sixth nodes proved to be suitable materials for *in vitro* propagation of *V. myrtillus*. The proliferation and growth of shoots on the medium without activated charcoal (plant height 3.92 cm, 1 shoot/explant, 8.60 nodes/shoot) were better than those grown on the medium supplemented with 1 g/L of activated charcoal (plant height 3.64 cm, 1 shoot/explant, 7.90 nodes/shoot). For the *in vitro* rooting stage, the WPM medium containing 1.0 mg/L IBA, 30 g/L sucrose, 10 g/L agar, pH 5.8 resulted in plant height 5.49 cm, root length 1.42 cm, root formation rate 100%. After transferring the cultivated-tissue Vaccinium myrtillus to the greenhouse, substrate with coconut fibre powder, the results were observed to be plant height 6.94 cm, root length 3.18 cm, survival rate 95%. The plantlet growth increased overtime after 4 months in the greenhouse.

Keywords: Number of nodes, number of shoots, shoot height, *Vaccinium myrtillus*, WPM medium.

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INTRODUCTION

Vaccinium myrtillus Linn. is an imported fruit tree with high economic value, because it has uses such as: providing nutrients, supplementing rich antioxidants, memory retention, female weight loss, cholesterol reduction, eyesight improvement, in the US, *V. myrtillus* is ranked one of the most nutritious fruits (Cüce et al., 2016; Pepkolaj et al., 2017; Elkiran & Avsar, 2020). Therefore, research on *in vitro* propagation of this imported, valuable tree is very necessary. There have been many publications researched on *in vitro* propagation of *V. myrtillus* (Cüce & Sökmen, 2017; Mohamed et al., 2018) and there have been also many publications researched on the chemical composition and pharmacological effects of the *V. myrtillus* fruit (Jaakola et al., 2009; Güder et al., 2015; Elkiran & Avsar, 2020). Currently, the *V. myrtillus* has been imported and cultivated in Vietnam, showing good growth and rich fruit production. However, traditional propagation by seed shows disadvantages, such as unequal growth of seedlings and low germination rate of seeds. Additionally, vegetative propagation by cuttings provides a low multiplication rate, insufficient for large scale production. Application of plant cell tissue culture for *V. myrtillus in vitro* propagation will help to overcome the limitations of traditional propagation methods and support *in vitro* plant production.

MATERIALS AND METHODS

Material

The young branches of the *V. myrtillus* plant (Fig. 1a) cultivated at the Tay Nguyen Institute for Scientific Research were selected and sterilized. The selected explants were washed with soapy water, then sterilized with 70 ^{o}C alcohol for 1 minute, and sterilized with 2% NaOCl solution for 10 minutes. The sterilisation was then cut into stem segments about 1 cm and inoculated on woody plant medium (WPM medium) (Lloyd & McCown, 1980) without supplementation of plant growth regulators for *in vitro* shoot proliferation (Fig. 1b). These *in vitro* shoots continued to grow well after transferring to the new medium without supplementation of plant growth regulators as the material source for the experiments (Fig. 1c).

Figure 1. **a.** *Vaccinium myrtillus* plant; **b.** Shoot formed from nodes after explant sterilization; **c.** Growth of shoots derived from sterilized explant after transferring new medium

Culture medium and conditions

WPM is used in all experiments. Depending on the purpose of each experiment, plant growth regulators are added, including zeatin, 6-benzyl adenine (BA), kinetin, thidiazuron (TDZ), indole-3-butyric acid (IBA). The substrates used for transferring *in vitro* plants to the greenhouse were sand, basalt soil and coconut fibre powder. For *in vitro* cultures, the photoperiod was 8 hours/day, the light intensity was 34 µmol/m^2/s^1, the temperature was 25 ± 2 ^{o}C and the air humidity was 75−85%. *Ex vitro* experiments were carried out in the greenhouse covered by a clear, white plastic roof to prevent rain, with an average air temperature of 18–21 ^{o}C, average humidity of 82%, and under sun light (Khoa Dang company provided plant growth

regulators, sucrose and agar; address: 286 D Bui Thi Xuan street, Ward 2, Da Lat city, Lam Dong province).

Methods

Effect of zeatin, BA, kinetin and TDZ on in vitro shoot growth and proliferation

The nodes of the *in vitro V. myrtillus* plant (Fig. 1c) were cultured on WPM medium supplemented with 0.0, 0.5, 1.0, 1.5, 2.0 mg/L zeatin or BA or kinetin or 0.0, 0.1, 0.5, 1.0 mg/L TDZ, 30 g/L sucrose, 10 g/L agar, pH 5.8. Each treatment had 15 nodal explants; Data were collected after 50 days of culture. Shoot height (cm), shoot number/explant, nodal number/explant and shoot morphology were observed.

Effect of position of stem node on in vitro shoot growth and proliferation

The position of nodes on the same *V. myrtillus* was numbered in order from top to bottom (the first node to the sixth node). These nodal positions were cultured on a WPM medium supplemented with 0.5 mg/L BA, 30 g/L sucrose, 10 g/L agar, pH 5.8. Each treatment had 15 nodal explants; Data were collected after 50 days of culture. Shoot height (cm), shoot number/explant, nodal number/explant and shoot morphology were observed.

Effect of medium without or with supplemented activated charcoal on in vitro shoot growth and proliferation

The nodes of the *in vitro V. myrtillus* plants (Fig. 1c) were cultured on a medium supplemented with or without 1 g/L activated charcoal. Each treatment had 15 nodal explants; Data were collected after 50 days of culture. Shoot height (cm), shoot number/explant, nodal number/explant and shoot morphology were observed.

Effect of IBA on in vitro root formation

The nodes of *in vitro V. myrtillus* plants (Fig. 1c) were cultured on WPM medium supplemented with 0.5, 1.0, 1.5, 2.0 mg/L IBA, 30 g/L sucrose, 10 g/L agar, pH 5.8. Each treatment had 15 nodal explants; Data were collected after 50 days of culture. Shoot height (cm), root length (cm), rate of root formation (%) and shoot morphology were observed.

Effect of substrate on the survival rate of the cultivated-tissue plant at the greenhouse

Cultivated-tissue *V. myrtillus* plants with a stem, leaves, roots and height about 3 cm (Figs. 2a, 2b) were cultivated on a substrate composed of sand, basalt soil, and coconut fibre powder. Each treatment had 21 nodal explants; Data were collected after 45 days of cultivation. Plant height (cm), root length (cm), survival rate (%) and plantlet morphology were observed.

Data processing

The data of the experiments were analyzed by SPSS statistical software (version 15.0) in Duncan's test and T-test (Duncan, 1955) with $P \leq 0.05$.

RESULTS AND DISCUSSION

Effect of zeatin, BA, kinetin and TDZ on in vitro shoot growth and proliferation

In vitro shoot growth and proliferation of *V. myrtillus* plant from nodes after 50 days of culture is shown in Table 1. The results showed that all nodes cultured on different media proliferated shoot; however, under the media supplemented with different plant growth regulators and at different concentrations was different shoot growth and proliferation. Medium supplemented with 0.5 mg/L zeatin was the best for shoot growth and proliferation as compared with the media supplemented with concentrations of BA, kinetin and TDZ. The highest shoot grew (4.24 cm) in the medium supplemented with 0.5 mg/L zeatin. Additionally, the shoots grown in BA at 0.5 mg/L and kinetin at 1.0 mg/L grew to heights of 4.00 cm and 3.87 cm, respectively, lower than those grown on the medium supplemented with zeatin of 0.5 mg/L. Meanwhile, shoot height on the medium supplemented with TDZ concentrations was lower than that in the medium without growth regulators. The results also showed that the nodes in the medium supplemented with zeatin,

BA and kinetin only proliferated 1 shoot/explant, while the nodes in the medium supplemented with TDZ proliferated more shoots (3.40 shoots/explant). The shoot with the highest number of nodes (8.60 nodes/shoot) grew on the medium supplemented with 0.5 mg/L zeatin. Additionally, the shoots grown in BA at 0.5 mg/L and kinetin at 1.0 mg/L grew to have 8.50 nodes/shoot and 8.10 nodes/shoot, respectively, lower than those grown on the medium supplemented with zeatin of 0.5 mg/L. Meanwhile, the number of nodes/buds in the medium supplemented with TDZ concentrations was lower than that on the medium without plant growth regulators. The results also showed that concentrations of zeatin at 0−0.5 mg/L, BA at 0–0.5 mg/L and kinetin at 0−1.0 mg/L increased shoot height and the number of nodes; however, when zeatin concentration increased to 1−2 mg/L, BA to 1−2 mg/L and kinetin to 1.5−2 mg/L, shoot height and number of nodes decreased. These results are expected; shoot height and the number of nodes were stimulated when the concentration of zeatin, BA and kinetin were low, but the opposite process inhibited growth when the concentration of zeatin, BA and kinetin were high. Meanwhile, increasing TDZ concentration (0.1−1.0 mg/L) stimulated shoot proliferation and inhibited shoot height. The results also showed that, explants in the media supplemented with plant growth regulators all produced callus, except for those grown on kinetin (Fig. 2). The shoots cultured on the medium supplemented with zeatin, BA and kinetin had brown color at the tips, while the shoots cultured on the medium supplemented with TDZ were light green (Fig. 2). Zeatin, BA, kinetin and TDZ are plant growth regulators of the cytokinin group that play an important role in plant cell tissue culture; they stimulate shoot proliferation and are commonly used in plant tissue culture in the rapid multiplication phase. Currently, in Vietnam, there are no publications on *in vitro* propagation of the blueberry plant, though there are international publications on this plant's propagation. For zeatin, publications observed that *in vitro* propagation of *V. myrtillus* and *V. uliginosum* species was successful on WPM medium supplemented with zeatin alone or in combination with IBA (Gajdošová et al., 2006; Cüce et al., 2016; Cüce el al., 2017; Mohamed et al., 2018). For BA, publications observed that *in vitro* propagation of the blueberry plant was successful on WPM medium supplemented with BA and IBA or IAA, but there were few studies (Rache et al., 2010; Melani & Adriana, 2016). For kinetin and TDZ, there are no publications using kinetin and TDZ *in in vitro* propagation of the blueberry plant. The results of this study also showed that the color at shoot tips was similar with publications of Gajdošová et al. (2006), Cüce et al. (2016), Cüce et al. (2017) and Mohamed et al. (2018).

Thus, WPM medium supplemented with 0.5 mg/L zeatin was good for *in vitro* shoot growth and proliferation of the *Vaccinium myrtillus* plant.

Figure 2. **a_1, a_2, a_3, a_4, a_5.** Shoot growth and proliferation on WPM supplemented with 0.0, 0.5, 1.0, 1.5, 2.0 mg/L zeatin; **b_1, b_2, b_3, b_4.** Shoot growth and proliferation on WPM supplemented with 0.5, 1.0, 1.5, 2.0 mg/L BA; **c_1, c_2, c_3, c_4.** Shoot growth and proliferation on WPM supplemented with 0.5, 1.0, 1.5, 2.0 mg/l kinetin; **d_1, d_2, d_3.** Shoot growth and proliferation on WPM supplemented with 0.1, 0.5, 1.0 mg/L TDZ

Table 1. Effect of zeatin, BA, kinetin and TDZ on *in vitro* shoot growth and proliferation after 50 days of culture

Growth regulator		Shoot height (cm)	Shoot number/explant	Nodal number/shoot	Shoot morphology
Substance name	Concentration (mg/L)				
Zeatin	0.0	2.72^{f*}	1.00^{c}	5.90^{fg}	Shoots grew normally and had dark green colour
	0.5	4.24^{a}	1.00^{c}	8.60^{a}	Shoots grew well, brown colour at the tips
	1.0	3.84^{c}	1.00^{c}	7.80^{cd}	Shoots grew well, brown colour at the tips
	1.5	2.78^{f}	1.00^{c}	5.90^{fg}	Shoots grew normally, shoot stems were brown
	2.0	1.67^{i}	1.00^{c}	3.60^{h}	Shoots grew weakly, shoot stems were brown, watery
BA	0.5	4.00^{b}	1.00^{c}	8.50^{ab}	Shoots were brown at the tips, grew well
	1.0	3.33^{d}	1.00^{c}	7.60^{d}	Shoots were brown at the tips, grew well
	1.5	2.19^{g}	1.00^{c}	6.30^{f}	Shoots were brown, grew normally
	2.0	1.07^{j}	1.00^{c}	3.40^{h}	Shoot stems were brown, shoots grew weakly
Kinetin	0.5	3.12^{e}	1.00^{c}	6.90^{e}	Shoots were green and grew well
	1.0	3.87^{bc}	1.00^{c}	8.10^{bc}	Shoots were green and grew well
	1.5	2.70^{f}	1.00^{c}	6.30^{f}	Shoots were green and grew normally
	2.0	1.84^{h}	1.00^{c}	5.60^{g}	Shoot stems were brown and grew weakly
TDZ	0.1	2.16^{g}	3.10^{b}	3.60^{h}	Shoots were smooth green, grew well
	0.5	1.82^{h}	3.20^{b}	3.40^{h}	Shoots were green, grew weakly
	1.0	1.58^{i}	3.40^{a}	3.10^{h}	Shoots were green, grew weakly

Note: *: Different letters (a, b,...) in the same column represent statistically significant differences with $P \leq 0.05$ in Duncan's test.

Effect of node positions on the *in vitro* shoot growth and proliferation

The nodes were inoculated on the WPM medium supplemented with 0.5 mg/L BA, 30 g/L sucrose, 10 g/L agar, pH 5.8. Shoot growth and proliferation from the first to the sixth node after 50 days of culture were shown in Table 2. The results showed that all shoots on the same plant proliferated and grew; however, proliferation was different at different nodal positions (Figs. 3a–3c). The

shoot growth and proliferation at the 3rd to 6th node were the best and there was no difference (shoot height 3.92, 3.96, 3.94 and 3.95 cm, respectively; the number of nodes 8.20, 8.50, 8.30 and 8.40 nodes/explant respectively). The first and second nodes proliferated and grew worse (shoot height 2.18 and 2.96 cm respectively; the number of node 3.90 and 6.10 nodes/explant respectively). All nodes proliferated only 1 shoot and there was no difference. The results also showed that the rate of shoot proliferation was from 90% to 100% at the nodal positions, and the proliferation rate of the nodal positions was irregular. This study was consistent with the report of Vu et al. (2015) and their observations that the nodal positions of *Anoectochilus setaceus* Blume at the 3rd to 6th nodes were suitable as propagation materials. Phan et al. (2018) studied the shoot proliferation and growth of *Anoectochilus formosanus* Hayata and observed that the 2nd to 6th node were the best as propagation materials. The 1st to 4th nodes of *Hibicus sagittifolius* Kurz was suitable as a source of material for *in vitro* propagation.

Thus, the 3rd to 6th nodes are suitable as a source of material for *in vitro* propagation of *V. myrtillus*.

Table 2. Effect of nodal positions on the *in vitro* shoot growth and proliferation

Nodal position	Shoot length (cm)	Shoot number/explant	Nodal number/shoot	Shoot morphology
1st	2.18c*	1.0a	3.90c	Shoots grew weakly and had light green
2nd	2.96b	1.0a	6.10b	Shoots grew normally and had brown colour at the tips
3rd	3.92a	1.0a	8.20a	Shoots grew well and had brown colour at the tips
4th	3.96a	1.0a	8.50a	Shoots grew well and had brown colour at the tips
5th	3.94a	1.0a	8.30a	Shoots grew well and had brown colour at the tips
6th	3.95a	1.0a	8.40a	Shoots grew well and had brown colour at the tips

Note: *: Different letters (a, b,...) in the same column represent statistically significant differences with $P \leq 0.05$ in Duncan's test.

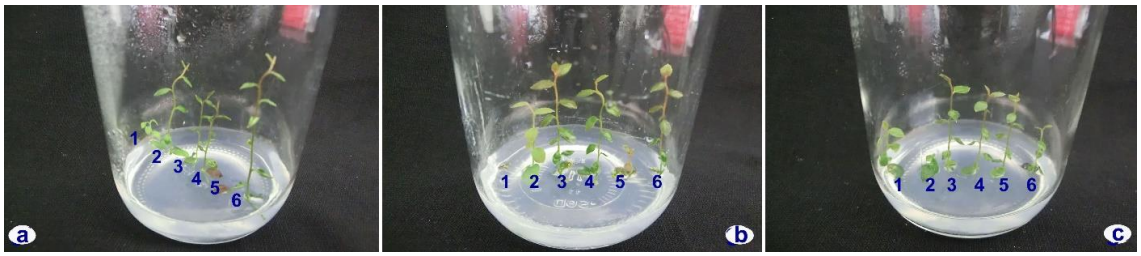

Figure 3. **a, b, c.** Shoot growth and proliferation of the first node to the sixth node on WPM medium supplemented with 0.5 mg/L BA

Effects of medium with or without the addition of activated charcoal on the *in vitro* shoot growth and proliferation

The *in vitro* nodal segments of *V. myrtillus* were cultured on the WPM medium added with these components: 0.5 mg/L BA, 30 g/L

sucrose, 10 g/L agar, and with or without activated charcoal (AC). Results after a 50-day culture showed that the shoot growth and proliferation between the medium with AC and the medium without AC were distinctly different and statistically significant (Table 3). The explant cultured on both mediums with the addition of 1 g/L AC and non-AC produced 1.0 shoot/explant. The shoot length and the number of nodes per explants on the culture medium without AC (3.92 cm, 8.6 nodes) were higher than the one with AC (3.64 cm, 7.9 nodes). Moreover, the shoots on non-AC were brown colour (Fig. 4a), strong and produced callus, which is a suitable material for *in vitro* and *ex vitro* rooting. The shoots on medium with AC medium were in dark green and did not induce callus (Fig. 4b). Activated carbon is an amorphous form of carbon, capable of absorbing gases, vapours and colloidal liquids. The addition of AC to the culture medium can promote or inhibit plant growth *in vitro*. The effects of AC include: darkening conditions in the culture medium, absorption of inhibitors in the culture medium, absorption of plant growth regulators and other organic compounds. The release of substrates is beneficial to the growth of *in vitro* plants (Pan & Staden, 1998). Te addition of AC to the culture medium can affect rooting, shoot elongation and embryogenesis (Webb et al., 1988), altering the ratio of substances present in the culture medium as well as the pH of the medium (Wann et al., 1997). Phan et al. (2017) an *in vitro* propagation study of *Hibicus sagittifolius* Kurz showed that in the medium without AC, the explants did not form roots, however, when adding 1 g/l AC into the culture medium, 100% of the explants produced roots. When *Codonopsis javanica* Blume was cultured in the medium supplemented with 1 g/L AC, 100% produced roots without regenerating shoots, while the one on the medium without AC showed 100% formed shoot clusters without forming roots (Phan & Nguyen, 2014). *Anoectochilus formosanus* Hayata grew and developed well on the medium was supplemented with 1.0 g/L or 2.0 g/L AC (Phan et al., 2018). The shoot length and rooting of *Chrysanthemum indicum* were better in the medium supplemented with 1.0 g/L AC.

Table 3. Effects of medium with or without the addition of activated charcoal on the *in vitro* shoot growth and proliferation

Medium	Shoot length (cm)	Shoot number/explant	Nodal number/explant	Shoot morphology
Activated charcoal	3.64^{b}	1.00	7.90	Shoots grew well and had brown colour at the tips
Non - activated charcoal	3.92a*	1.00	8.60	Shoots grew well and had dark green colour

Note: *Different letters in the same column represent statistically significant differences with $P \leq 0.05$ in the *T-test*.

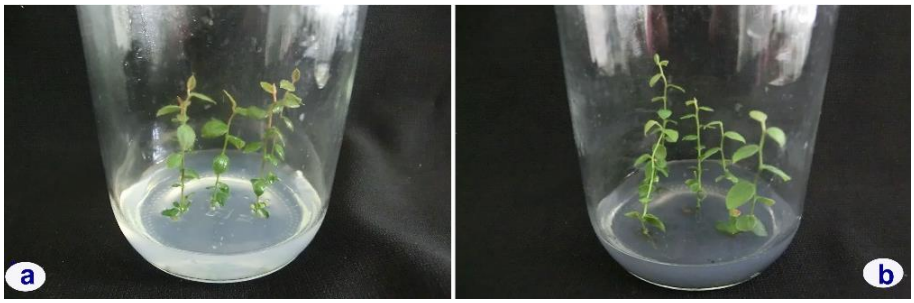

Figure 4. **a.** Shoot growth and proliferation on the medium without activated charcoal; **b.** Shoot growth and proliferation on the medium addition of activated charcoal

Thus, *V. myrtillus* cultured in WPM medium without adding activated charcoal grew better than those with 1 g/L of activated charcoal.

Effect of IBA on *in vitro* root formation

The results of the above experiments showed that explants on a medium without the addition of plant growth regulators did not form roots but just formed shoots, so in this experiment IBA was designed at random concentrations of 0.5, 1.0, 1.5 and 2.0 mg/L. The *in vitro* rooting ability of *V. myrtillus* plants after 50 days of culture is shown in Table 4. The results showed that the media had an effect on the rooting of the explant, however, media supplemented with concentrations of different IBA formed different roots. The rate of root formation in the treatment containing 0.5 mg/L IBA was the lowest, only 73.33%. The length of root decreased (1.47, 1.42, 1.21, 1.18 cm respectively) when the concentration of IBA increased (0.5, 1.0, 1.5, 2.0 mg/L). According to data root length was different between the medium supplemented with 0.5 and the medium supplemented with 1.0 mg/L IBA, but according to the analysis of data statistics, there was no difference. Similarly, according to data of the root length in the medium supplemented with 1.5 and 2.0 mg/L IBA was different, but according to the analysis of data statistics had no difference. The results also showed that the height of plants decreased (5.54, 5.49, 5.18, 4.73 cm respectively) when the concentration of IBA increased. Tree height differed between the medium supplemented with 0.5 mg/L and the medium supplemented with 1.0 mg/L IBA, but according to the analysis of data statistics, there was not different. This can be explained by how the rate of root formation and the inhibition of growth of root length and plant height increase with the concentration of IBA. The plant's morphological characteristics showed that plants cultured on the medium supplemented with 0.5 and 1.0 mg/L IBA were dark green, had white roots and grew well (Figs. $5a_1$, $5a_2$). Plants cultured on the medium contained 1.5 and 2.0 mg/L IBA grew well, were dark green and roots were brown (Figs. $5a_3$, $5a_4$). IBA is a plant growth regulators of the auxin group that stimulates the formation of roots and it is commonly used in plant tissue culture, rate of root formation depends on the concentration of IBA. Currently, there have been many publications using IBA in research of blueberry formation. Cüce & Sökmen (2015) researched *in vitro* propagation of *V. uliginosum* species and observed that root formation was the best, with a rate of 60% when shoots were cultured on the WPM medium supplemented with 0.5 mg/L IBA in combination with 1.0 mg/L activated carbon. Mohamed et al. (2018) researched *in vitro* propagation of *V. corymbosum* and observed that WPM medium supplemented with combinations of IBA (1 mg/L) and activated carbon (0.1%) obtained the highest roots, with 5.9 roots/shoot. Rache & Pacheco (2010) researched on *in vitro* propagation of *V. meridionale* species and observed that the rate of root formation reached 88−100% after 60 days of culture.

Table 4. Effect of IBA on *in vitro* root formation after 50 days of culture

IBA (mg/L)	Shoot height (cm)	Root length (cm)	Rate of root formation (%)	Plant morphology
0.5	5.54^{a*}	1.47^{a}	73.33%	Plants were dark green colour, grew strongly and roots were white
1.0	5.49^{a}	1.42^{a}	100	Plants were dark green colour, grew strongly and roots were white
1.5	5.18^{b}	1.21^{b}	100	Plants were dark green colour, grew strongly and roots were brown
2.0	4.73^{c}	1.18^{b}	100	Plants were dark green colour, grew strongly and roots were brown

Note: *Different letters (a, b,...) in the same column represent statistically significant differences with $P \leq 0.05$ in Duncan's test

Figure 5. **a_1, a_2, a_3, a_4.** *In vitro* root formation on medium supplemented with 0.5, 1.0, 1.5, 2.0 mg/L IBA

Thus, WPM medium supplemented with 1 mg/L IBA was good for *in vitro* formation of *V. myrtillus* plant.

Effect of substrate on the survival rate of the cultivated-tissue plant at the greenhouse

The ability to survive and adapt of cultivated-tissue *V. myrtillus* after 45 days of cultivation in the greenhouse is shown in Table 5. The study on the transfer of the cultivated-tissue plant to the greenhouse is an important step in plant tissue culture. The cultivated-tissue plant must adapt to the new condition when it moves from the condition of *in vitro* culture to the greenhouse. The root of plantlets has to adapt to the new substrateand. Additionally, the humidity in *in vitro* conditions is higher and more stable than that in the greenhouse. Therefore, the plantlet dies if left in the *in vitro* conditions. Thus, plantlets need to care for carefully when they move to the nursery initially. The results showed that the growth of plantlets cultivated on the substrate of coconut fibre powder was the best, with a plant height of 6.94 cm, root length of 3.18 cm and a survival rate of 95.24%. Plant height, root length, the survival rate of plantlets cultivated on substrate 1/2 coconut fibre powder mixed 1/2 sand (6.42 cm, 1.65 cm, 85.71% respectively) and those of plantlets cultivated on the substrate of 1/2 coconut fibre powder mixed 1/2 basalt soil were worse (6.49 cm, 1.31 cm, 90.48% respectively). This can be explained by how the substrate of coconut fibre powder was suitable for the growth of cultivated-tissue *V. myrtillus* in the first stage in greenhouse conditions. Morphological characteristics show that the plantlets cultivated on the substrate of coconut fibre powder were green and had good root growth (Fig. $6c_3$). Meanwhile, plantlets cultivated on the substrate of1/2 coconut fibre powder mixed with 1/2 sand and substrate of 1/2 coconut fibre powder mixed with 1/2 basalt soil were also green, but their roots grew worse (Figs. $6c_1$, $6c_2$). The plantlets grew well after 2 months, three months and four months of cultivation (Figs. 6d, 6e, 6f). Currently, there have been many publications using substrates of sand, basalt soil and coconut fibre powder cultivated cultivated-tissue plants at the greenhouse stage, besides Phan & Nguyen (2014) who have grown cultivated-tissue *Codonopsis javanica*in greenhouse conditions. Their results showed that the survival rate of plantless was 100% after 20 days of cultivation when grown on a substrate of coconut fiber powder. Phan & Nguyen (2017) studied and showed that the survival rate of cultivated-tissue *Anoectochilus formosanus* achieved 100% after 2 months of cultivation

when grown on a substrate of coconut fibre powder. Mohamed et al. (2018) showed that cultivated-tissue blueberry was cultivated successfully on soil substrate, with a survival rate of 100%. Rache & Pacheco (2010) was successful when transferring cultivated-tissue blueberries to the greenhouse.

Thus, the substrate of coconut fibre powder was good for transferring *V. myrtillus* plantlets to conditions of the greenhouse.

Table 5. Effect of substrate on the survival rate of the cultivated-tissue plant at the greenhouse after 45 days of culture

Substrate	Shoot height (cm)	Root length (cm)	Survival rate (%)	Plant morphology
1/2 coconut fiber powder + 1/2 sand	6.42^{b*}	1.65^{b}	85.71	Plants were green and grew weakly
1/2 coconut fiber powder + 1/2 basalt soil	6.49^{b}	1.31^{c}	90.48	Plants were green and grew weakly
Coconut fiber powder	6.94^{a}	3.18^{a}	95.24	Plants were green color, grew well and roots developed strongly

Note: *: Different letters (a, b,...) in the same column represent statistically significant differences with $P \leq 0.05$ in Duncan's test.

Figure 6. **a, b.** Cultivated-tissue *Vaccinium myrtillus* plantlets; **c_1.** Plantlets cultivated on the substrate of 1/2 sand combined with 1/2 coconut fiber powder; **c_2.** Plantlets cultivated on the substrate of 1/2 basalt soil combined with 1/2 coconut fibre powder; **c_3.** Plantlets cultivated on the substrate of coconut fibre powder; **d, e, f.** Growth of plantlets on the substrate of coconut fibre powder after 2 months, 3 months and 4 months of cultivation

CONCLUSION

The results of this study showed that *in vitro* shoot growth and proliferation were the best on a WPM medium supplemented with 0.5 mg/L zeatin. The third stem nodes to sixth stem nodes proved to be suitable materials for *in vitro* propagation of *V. myrtillus*. The shoots growth and proliferation on the medium without activated charcoal were

better than those on the medium supplemented with 1 g/L of activated charcoal. For *in vitro* rooting, the WPM medium containing 1.0 mg/L IBA was good for *in vitro* formation. For the *ex vitro* stage, the substrate of coconut fibre powder was the best for transferring cultivated-tissue *V. myrtillus* to the greenhouse.

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