DESENVOLVIMENTO DE UM SISTEMA DE MULTIPLICAÇÃO *IN VITRO* PARA *RUBUS LOGANOBACCUS* L.

DEVELOPMENT OF AN *IN VITRO* MULTIPLICATION SYSTEM FOR *RUBUS LOGANOBACCUS* L.

ایجاد سیستم ازدیاد درون شیشه ای برای *Rubus loganobaccus* L.

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RESUMO

A variedade *Rubus loganobaccus* L. é um híbrido produzido a partir de um cruzamento entre *Rubus idaeus* e *Rubus ursinus*, conhecido por seus frutos exóticos e distintos. No entanto, ainda não foi introduzido um método comercial de multiplicação econômico, cuja micropropagação como uma técnica confiável poderia ser um procedimento econômico eficiente para gerar materiais vegetais uniformes. Nesse caso, um experimento realizado com gomos de plantas explanta os tratamentos de desinfecção mais adequados, encontrados entre 1 e 5 min em cloreto de mercúrio a 0,1%, no qual gomos saudáveis cresceram sem contaminação por fungos / bactérias. A maior porcentagem de enraizamento (96,66%) e a menor porcentagem de enraizamento (60%) foram observadas nas concentrações de fitohormônios em MS + BA (1 mg·L⁻¹) IBA + (0,1 mg·L⁻¹) e MS + 2ip (1 mg·L⁻¹) + IBA (0,1 mg·L⁻¹), respectivamente. O comprimento médio máximo e mínimo da raiz foram de 3 cm e 1 cm, respectivamente. No estágio de aclimação da raiz da planta, a porcentagem máxima de vasos sobrevividos e frescos 96,43%, o número médio máximo de ramificações 8,73, o número médio mínimo de ramificações 3,13 nos tratamentos MS + BA (1 mg·L⁻¹) + IBA (0,1 mg·L⁻¹) e meio MS livre de hormônios, respectivamente, o comprimento máximo de ramo 5,22 cm e o comprimento mínimo de ramo 1,71 cm em MS + 2ip (2 mg·L⁻¹) + IBA (0,1 mg·L⁻¹) e AN + BA (1 mg·L⁻¹) + IBA (0,1 mg·L⁻¹), respectivamente, foram observados. No geral, esse protocolo aborda de forma abrangente todo o processo de micropropagação e pode ser ainda mais utilizado na propagação comercial de *Rubus loganobaccus* e produzir materiais vegetais com vantagens superiores aos métodos convencionais.

Palavras-chave: Hormônios, desinfecção, ácido indol -3-butírico (AIB), meios de crescimento.

ABSTRACT

The *Rubus loganobaccus* L. variety is a hybrid produced from a cross between *Rubus idaeus* and *Rubus ursinus*, which is well-known for its exatic distinguished fruits. However, a commercial cost-effective multiplication method has not been introduced yet, which micropropagation as a reliable technique could be an efficient economic procedure to generate uniform plant materials. In this case, an experiment carried out using plant buds explants the most appropriate disinfection treatments found out to be 1 to 5 min in 0.1% mercuric chloride in which healthy buds grew with no fungal/bacterial contamination. The highest rooting percentage (96.66%) and the lowest rooting percentage (60%) were observed in concentrations of phytohormones in MS + BA (1 mg·L⁻¹) IBA + (0.1 mg·L⁻¹) and MS + 2ip (1 mg·L⁻¹) + IBA (0.1 mg·L⁻¹), respectively. The maximum and the minimum average root length were 3 cm and 1 cm, respectively. In the acclimatization stage of the plant root, the maximum percentage of survived and fresh pots 96.43%, the maximum average number of branching 8.73, the minimum average number of branching 3.13 in treatments MS + BA (1 mg·L⁻¹) + IBA (0.1 mg·L⁻¹) and hormone-free MS medium, respectively, the maximum branch length 5.22 cm, and the minimum branch length 1.71 cm in MS + 2ip (2 mg·L⁻¹) + IBA (0.1 mg·L⁻¹) and AN + BA (1 mg·L⁻¹) + IBA (0.1 mg·L⁻¹), respectively, were observed. Overall, this protocol comprehensively addresses the whole process of micropropagation and can be further be

used in commercial propagation of *Rubus loganobaccus* and produce plant materials with superior advantages over conventional methods.

Keywords: Hormones, Disinfection, indole -3- butyric acid (IBA), growth media.

چکیده

واریته *Rubus loganobaccus* L. هیبرید حاصل شده از تلاقی بین *Rubus idaeus* و *Rubus ursinus* می‌باشد که از جهت تولید میوه‌های بسیار جالب توجه شناخته شده است. اگرچه تا کنون روشی مناسب تجاری و از لحاظ اقتصادی به صرفه ارائه نگردیده که از این جهت ریز ازدیادی به عنوان روشی مطمئن می‌تواند به منظور تولید مواد گیاهی به شکلی کارآمد و اقتصادی مورد استفاده قرار گیرد. در همین زمینه آزمایشی صورت گرفت که با استفاده از جوانه‌های گیاه مناسب‌ترین تیمار گندزدایی قرار گیری نمونه‌ها به مدت 1 الی 5 دقیقه در 1% کلرید جیوه مشاهده گردید که در آن جوانه‌های سالم می‌توانند عاری از آلودگی‌های قارچی/باکتریایی رشد نمایند. بالاترین درصد ریشه‌زایی (96.66%) و کمترین درصد ریشه‌زایی (60%) به ترتیب در غلظت‌های $MS + BA (1 \text{ mg} \cdot \text{L}^{-1}) + IBA (0.1 \text{ mg} \cdot \text{L}^{-1})$ و $MS + 2ip (1 \text{ mg} \cdot \text{L}^{-1}) + IBA (0.1 \text{ mg} \cdot \text{L}^{-1})$ مشاهده گردید. بالاترین و کمترین میانگی طول ریشه نیز به ترتیب 3 و 1 سانتی‌متر مشاهده گردید. در مرحله عادتدهی ریشه‌های به شرایط بیرون نیز بالاترین درصد گیاهچه نجات یافته حداکثر 96/43% حداکثر تعداد میانگین تعداد شاخه جانبی 8/73، حداقل پمیانگین تعداد شاخه جانبی 3/13 به ترتیب در تیمارهای $MS + BA (1 \text{ mg} \cdot \text{L}^{-1}) + IBA (0.1 \text{ mg} \cdot \text{L}^{-1})$ و محیط کشت MS عاری از هورمون به دست آمد، حداکثر طول شاخه جانبی 5/22 سانتی‌متر و حداقل طول شاخه جانبی 1/71 سانتی‌متر به ترتیب در تیمارهای $2ip (2 \text{ mg} \cdot \text{L}^{-1}) + IBA (0.1 \text{ mg} \cdot \text{L}^{-1})$ و $MS + BA (1 \text{ mg} \cdot \text{L}^{-1}) + IBA (0.1 \text{ mg} \cdot \text{L}^{-1})$ مشاهده گردید. بطورکلی، پروتوکل به دست آمده به صورتی جامع کل فرآیند ریزازدیادی این گیاه را مد نظر قرار داده و می‌تواند بیشتر برای ازدیاد تجاری *Rubus loganobaccus* و تولید مواد گیاهی یا مزایای برتر در مقابل روش‌های سنتی مورد استفاده قرارگیرد.

کلمات کلیدی: هورمون، گندزدای، ایندول-3-بوتریک اسید (IBA)، محیط رشد

1. INTRODUCTION:

The *Rubus loganobaccus* variety is a hybrid produced by *Rubus idaeus* and *Rubus ursinus*. The generic name is loganberry and belongs to the family of Rosaceae. Its fruit is red, called the raspberry bear, and it is used as a nutraceutical containing vitamins, fibers, and antioxidants and antimicrobial compounds. The fruits of this plant in European regions are known as healthy snacks (Arencibia, 2013).

This genus is one of the most diverse species in plants, with approximately 740 species (Jennings, 1988). Berries exist not only as fresh fruits but are also generally consumed as frozen fruits, or they are converted into juice, wine, jams, soups, soft foods, and tisanes. In addition, they are used in ice cream and cream of cakes. They are also served beside main courses to make food healthier. The fruit of this plant is rich in vitamin C. One cup of this fruit fulfills 88% of our daily need of vitamin C. Because of its vitamin C, it has antioxidant properties and has the ability to neutralize free

radicals (Lee, 2016). Consumption of *Rubus* sp. has grown substantially over the last twenty years. In 1990, the area under cultivation in North America was 4385 hectares, and nearly 90 percent of the product was consumed in the processing industry. (Finn, 2012). Near the valley in Oregon, America food remnants of raspberry have been found that date back to 8000 BCE. The oral tradition of The American Indians (Indigenous peoples of the Americas) has long enjoyed the use of red and black raspberries, and similar fruits in order to take advantage of their medicinal and nutritional properties. (Hummer, 2007). The Rosaceae genus in Iran has 8 herbaceous and shrub often acanaceous species commonly known as raspberries, and often, it is growing in the forest and non-forest shaded areas. Species of Raspberry in Iran are *R. raddeanus*, *R. ochthodes*, *R. hyrcanus*, and *R. persicus*, which in addition to Iran also grow in Russia, Talesh, and the Caucasus (Mozaffarian, 2007). In *Rubus loganobaccus* species, the plant climbs up to 5 m high (through supporting

vegetation). Primocane stems are rounded, or rarely angled, glabrous or with sparse fluff, or non-glandular pilose hairs, prickles -36 mm long, not confined to stem angles. Flowering takes place at the angle of the younger stems. (Evans *et al.*, 2007).

Primocane leaves in plants pinnately compound with 3, 5 or 7 leaflets. The leaflets are 5.5 to 8 cm long and 3.5 to 6.5 cm wide. The underpart of leaves is fluffed and generally broadly-ovate to elliptic. The petioles are 3 to 8.5 cm in length. (Evans, 2007) Floricane leaves at the base of the petals contain 3 to 5 leaflets. The length of the leaflets is often 4 to 10.5 cm and 2.5 to 10.5 cm wide. The petiole is 3 to 6.5 cm in length. The inflorescence is subcorymbose with 6 to 12 flowers. The fruits are ovoid to oblong and initially, are green, and when they ripen, they turn dark red to dull black color. Flowering mainly happens late in the spring and summer. (Evans, 2007) Tissue culture is the best way to reduce propagation costs some of the plants (due to mass production) and increases the production of secondary metabolites in medicinal plants. In-plant propagation by tissue culture techniques, which is known as micropropagation, cultivation of apical or axillary buds is used in a suitable and nutrient culture medium. Laboratory plants that have been propagation with this method have been healthy in most species and are more similar and nearer to the actual species.

Micropropagation methods can produce many plants in a short time and can be profitable for off-season production with better market opportunities. Tissue culture provides a quick method for the propagation of fructiferous products and can produce a large number of virus-free, genetically similar plants in a relatively short time and little space without being affected by seasonal variation. The use of *in vitro* micropropagation is more rational and more economical. Methods of biotechnology in the cultivation and production of new raspberry cultivars that are better adapted to local soil and climatic conditions are valuable. The first experiments were carried out for the *in vitro* cultivation of raspberries in 1970. There is also information on how to use this method in Bulgaria. The application of *in vitro* propagation has been documented in a large number of *rubus* cultivars. However, micropropagation requires a lot of experimental research to optimize the conditions in all its stages (Stelkunova, 1970).

2. MATERIALS AND METHODS:

To carry out micropropagation, the plant was obtained from the "CFD" Co. located in Tehran. Several branches that had a leaf (There was an axillary bud in the axil of a leaf) were separated by scissors from the pots. Brushing on branches was done to remove soil and dirt on the branches and leaves. Then the branches were cut with scissors so that each part contains an axillary bud. The parts were placed on a filter and placed on a plastic beaker under running water for 5 hours (Figure 1).

The parts containing the buds were removed with the aid of a forceps and surgical blade. They were then placed in 2 ml vials after being infected. Approximately 2 to 3 weeks later, the buds evolved on isolated parts as a rosette of leaflets. The growth room temperature in which the vials were stored was 24 °C for the day and 18 °C for the night. 16 hours of light and 8 hours of darkness, the intensity of light was 4 to 5 thousand luxuries. (Figure 2). The buds were divided into two groups and were placed into two autoclaved glass containers. There were 2 treatments at this stage:

- 1) 1 to 5 minutes in 0.1% mercuric chloride
 - 2) 2 to 7 minutes in 0.1% mercuric chloride.
- In the rooting stage, for each rooting treatment, there were 6 containers (except for treatment 5) in which 5 explants were planted in each glass. The 6 treatments prepared for rooting are shown in Table 1.

In this article, the treatments using the acronym LB are contracted form of the tested loganberry. After the rooting stage, in the respective six treatments and after spending the required time (2 to 3 weeks) for sufficient growth of the plant and its roots (in the growth room), the plants were transferred to the pots filled with soil and kept under the greenhouse condition. In this stage, the plants were removed from the intended treatments (jelly) with the aid of forceps, and after washing the roots with water and removing the jellies, sticking to them (to prevent contamination in the pots), they were transferred to the pots. The plants were planted in a pot and placed in a shaded house where the temperature of the day was 27 °C and the night time temperature was 18 °C and relative humidity was 40 to 50%. To avoid dehydration, plants were covered with transparent plastic covers, which were gradually removed. After 3 weeks of acclimatization, plants continued to grow without

cover and in a normal mode. 12 treatments were prepared in accordance with Table 2 in order to obtain the best suitable breeding environment (Proliferation = the number of branches per plant) and the best environment for producing the highest possible branches for this plant. In each container, five explants were planted that, after adequate growth, the plants of each treatment were removed from the sterile containers by forceps and measured with the ruler. The longest branch of each plant was measured. Statistical analysis was performed using SPSS software using Duncan's test.

3. RESULTS AND DISCUSSION:

1.3 Results in primary sterility:

Of the 27 buds in treatment 1, all were healthy and free from fungal and bacterial contamination, and all germinated. Of the 28 buds in treatment 2, there was one fungal contamination, and 22 germinated. (Table 3; Figure 1).

2.3 Rooting results:

The highest percentage of rooting was observed in LB2, and LB6 treatments, and the lowest percentage of rooting was observed in LB4 treatment. The highest mean root length in LB1 and LB2 was by 2-3 cm. The lowest mean root length in LB4 and LB6 was by 1 cm. LB2 and LB3 treatments were produced medium-thick branches and were produced in terms of the thickness of thin branches in LB1, LB4, LB5, and LB6 treatments. In terms of root density, only roots of LB4 treatment had moderate density. In the remnant treatments (LB1, LB2, LB3, LB5, and LB6) in LB2, LB3, and LB5 treatments, secondary roots were produced, and in LB1, LB4 and LB6 treatments, the secondary roots were not produced (Table 4). As shown in Graphic 2, the highest percentage of rooting (96.66 %) is in treatments 2 and 6. The lowest percentage of rooting (60) is in treatment 4 (Table 4; Figure 2).

A container containing rooting treatment (2) b)
 Container containing rooting treatment (1) c)
 Container containing rooting treatment (3) d)
 Container containing rooting treatment (4) e)
 Container containing rooting treatment (5) f)
 Container containing rooting treatment (6).

3.3 The result of soil root acclimatization

In the stage of acclimatization of the roots of the plants, the highest percentage of survived and fresh pots in treatment 6 was seen (96.43). The lowest percentages of survived and fresh were seen in treatment 2 (45/45). The

highest percentage of droopy pots in treatment 2 was seen (54/55). The lowest percentage of droopy pots was seen in treatment 6 (3.57%). The quality of plants grown in the pots was good, and the most extended plant stem length (in pots) was for treatment 6. The lowest plant stem length (in pots) belonged to treatment 2 (Table 5; Figures 3, 4, 10).

4.3 Branching

In all treatments, plants in the containers, in addition to the micropropagation coefficient (branching) and the measurement of the length of the branches, were examined in terms of being survived and fresh, being green, branching thickness, and rooting potential (Figure 5 and 6). In the branching stage, 12 treatments were used, with the highest average branching (8.73), which belongs to LB2 treatment.

The lowest branching average (3.13) is for LB10 treatment. In LB1, LB3, LB4, LB5, LB6, LB7, LB8, LB9, and LB12 treatment roots were not produced, and in LB2 treatment, 87.33% of the plants did not produce root, and 12.97% of the plants produced root. In all treatments, different thicknesses of the branches were seen from very thin, thin, medium to thick. In LB1, LB2, LB3, LB7, LB8, and LB9, LB10, LB11, and LB12 treatments, 100% of plants' leaves were green. In LB4, LB5, and LB6 treatments, all leaves were not green, and a small percentage was yellow. In all treatments, 100% of the plants were fresh, and drooping was not seen (Tables 6 and 7; Figure 11).

At first, *in vitro* propagation was conducted by Broome and Zimmerman (1978) on Blackberry Bramble and Harper (1978) on Black raspberry. Since then, several reports have been published in the *in vitro* propagation of *Rubus* species. Zimmerman *et al.*, (1986) concluded in their study that the best medium for cultivating tissue of different cultivars of raspberry was MS medium (Klokonos, 1986). In a 1985 study, Welander reported that a very poor proliferation rate was obtained from only shoot tips, but better results were achieved with nodal segments. (Welander, 1985).

In a study by Stoevska *et al.* on *Rubus Idaeus* in 1995, apical and axillary buds on active-growing, one-year shoots of Alena Shopska and Samodiva (two cultivars of *Rubus Idaeus*) plants have been used. This study was conducted to determine the possibility of micropropagation. The initial development of the explants and propagation was done in 2 media.

The medium of V2 (Anderson) with 1,

2.5, and 5 mg·L⁻¹ concentrations of BAP and MS medium with concentrations of 0.1, 0.5, and 2 of IBA was used. Medium V1 was found to be more appropriate. The best propagation coefficient for the Alena Shopska variety was 6.86 ± 0.13, and for the samodiva variety, it was 5.53 ± 0.24. They also concluded that the length of the branches decreased as BAP concentration increased. The percentage of rooted shoots extremely was affected by the composition of the nutrient medium and the concentration of IBA. The use of higher Auxin doses of IBA 2 mg·L⁻¹ resulted in the formation of undesirable calluses in the root of the micro-cutting, and the developed roots remained short and backward (Stoevskaya et al., 1995).

In a study conducted by Valentina Isac and et al. in 2012 on the micropropagation of several cultivars of *Rubus Idaeus*, it includes: (Ruvi, Citria, Gradina, Heritage, Cayuga, The Latham, Malling Promise, Malling Exploit, Willamette, Gustar, Vely, Autumn Bliss and Opal). Transfer explants were grown on the original medium to a nutrient medium with 3.0 mg·L⁻¹ BA and 0.1 mg·L⁻¹ IBA to stimulate differentiation of axillary buds. The raspberry micropropagation takes place over 25-30 days with the MR of 15 to 41.9 (plantlets / explant), depending on the variety. It is relevant that in *in-vitro* routine micropropagation work, more than 2/3 of raspberry varieties have had MR higher than 6 reaching a maximum of 41.9 shoots per initial explant in 'Ruvi' (Valentina Isac, 2012).

In a study, conducted by Jafari Najaf-Abadi and her colleague in 2009 in Iran, to provide the best conditions for *in vitro* propagation of the thornless trailing blackberry, axillary buds were used as explants. Explants were cultured on MS medium containing 2 mg·L⁻¹ BA. In a two-weeks period afterward, to proliferate shoots, 12 various treatment with culture medias MS and AN and hormonal concentrations 3 of BA (0, 2 and 3 mg·L⁻¹), single or with GA3 (0, 0.2, 0.5 and 1 mg·L⁻¹) were used. The most significant number of branches with an average of 3.33 and the maximum branch length with an average of 5.87 cm was produced in medium containing 2 mg·L⁻¹ BA and 0.5 mg·L⁻¹ GA3 and in the experiment involving the application of various levels of IBA (0, 0.5, 1 and 2 mg·L⁻¹) added to MS medium used to evaluated rooting formation in plantlets. A concentration of 2 mg·L⁻¹ IBA creates a more significant number of roots and maximum root length. In this medium, four roots with a root length average of 7.83 cm were produced. Of the hardened *in vitro*

produced plants approx. after 30 days, 85% of rooted micro-cutting tolerated the open-field condition and completely adopted. In order to increase the growth of axillary buds and reduce the apical dominance in shoot cultures, one or more cytokinins are commonly incorporated into the medium at the propagation stage (Jafari Najaf-Abadi and et al., 2009). Bobrowski et al. (1996) reported that the best medium to propagate branch in blackberry was obtained in MS medium with BA (1.0 and 2.0 mg·L⁻¹). But GA3 didn't improve the propagation rate.

In research on *Rubus* species reported that if BA concentration decreased by 2 mg·L⁻¹, the propagation of axillary buds would not increase, and when the concentration of BA increases to 4 mg·L⁻¹, the propagation of axillary buds decreases. They found that GA3 was essential for propagation in three of the four species used and had significant effects on the complex hormone system. It was known that GA3 could synergistically act with auxins (Wei, 1992).

In 2005, Villa and others selected blackberry axillary buds as explants, and after sterilization, they transferred MS culture medium to 2 mg·L⁻¹ BA. The greatest number of branches with an average of 3.33 and the maximum branch length with an average of 5.87 cm was produced in medium containing 2 mg·L⁻¹ BA and 0.5 mg·L⁻¹ GA3 (Villa and et al., 2005). Gonzales et al. (2000) conducted a study on three species of blueberries (*Vaccinium* cultivar), blackberry (Smoothstem cultivar), and raspberry (Gradina cultivar). Blueberry germination was achieved in WPM with MS vitamins for 15 days and then 30 days in the same culture medium with Zeitin mM 18. The best result was obtained in the same culture medium as mM 25 Zip. (Gonzales et al., 2000).

Jin-Hu Wu et al. (2009) investigated the factors affecting the efficiency of micropropagation of 32 selections of *Rubus*. They studied the pre-treatment and early culture phases. Using a Chilling temperature at 4°C in a 6-week period as a pre-treatment led to the enhancement of establishing stage under *in vitro* condition; it was witnessed that 65% of cultures post chilling had normal healthy growth. Hormonal factors such as BA found to be the most appropriate among the three media considered to improve branch development, 3 to 7 branches, or developed plantlets in average recorded per nodal explant. Modification of the level of BA from 0.5 mg·L⁻¹ to 3 mg·L⁻¹ in a sub-culture to another positively improved the

difficulties involved in *Rubus* micropropagation with long-term exposure to the high levels of BA. The decrease in chlorosis accompanied by enhancement of the quality of plant materials through lowering the concentration of MS macro- and micro-elements to one-third, with combination with 0.49 μM IBA and 0.05% activated charcoal, ultimately subjected to the low light intensity ($17 \mu\text{mol m}^{-2} \text{s}^{-1}$). Since response to *in vitro* condition varied considerably, therefore various micropropagation protocols were applied depending on the genotype (Jin-Hu *et al.*, 2009).

In a study conducted by Djurdjina Ruzic and his colleague in 2006, the blackberry cultivar Čačanska bestrna was successfully micropropagated. Buds from the branches cut during dormancy (end of January) were used as the initial explants. MS media with BA and IBA or NAA and GA3 were used for the propagation stage. The most significant values of the propagation index were obtained on medium within BA 1.0, IBA 0.1, and GA3 0.1 $\text{mg}\cdot\text{L}^{-1}$. The branches had small nodular callus and a high number of bud rudiments in the basal section of branches (Ruzic, 2006).

In a study, Alexandru (2016) presented a protocol for the regeneration of *Rubus loganobaccus* by *in vitro* micropropagation. The Apical buds were used as the explants in the MS culture medium. Branch regeneration was done on MS 100% media gelled with plant agar with different concentration of BAP (0.3; 0.4; 0.5; 0.6 $\text{mg}\cdot\text{L}^{-1}$). Results were obtained on 0.3 and 0.4 $\text{mg}\cdot\text{L}^{-1}$ concentration of BAP, at higher concentrations (0.5, 0.6 $\text{mg}\cdot\text{L}^{-1}$) the buds inhibited. The highest branch regeneration results were obtained on MS medium containing BAP (0.4 $\text{mg}\cdot\text{L}^{-1}$), generating a number of 8 branches per test tube. When BAP concentration increased above 0.5 $\text{mg}\cdot\text{L}^{-1}$, the propagation rate of branches decreased (Mîrza Alexandru, 2016). Georgieva *et al.* (2016) in a protocol for the propagation of berries by Bioreactor, reported that propagation (in MS medium supplement 0.05 BAP $\text{mg}\cdot\text{L}^{-1}$, 1 IBA and 0.03 GA3), $\text{mg}\cdot\text{L}^{-1}$, 130 $\text{mg}\cdot\text{L}^{-1}$, sucrose) and rooting (MS without hormones supplement 30 gr l sucrose) in a solid medium strawberry plants propagation coefficient for 4 weeks was 1.9 (Georgieva, 2016).

In 2006, Zawadzka and Orlikowska conducted a study on the factors affecting the micropropagation of the five cultivars of Raspberry Red (Beskid, Canby, Malling Seedling, Norna, Veten) using MS culture

medium. There was no significant difference in the number of leaves obtained from branches cultured on media containing TDZ or BAP. The regeneration medium contained 0.1 $\text{mg}\cdot\text{L}^{-1}$ TDZ. At higher concentrations, TDZ caused leaf blackening or induced hyperhydration, usually followed with necrosis of the adventitious branches. From the three auxins tested, IBA (IBA, IAA, and NAA) effectively induced micropropagation in all of the cultivars, and NAA gave poor results with all of the cultivars tested (Zawadzka, 2006).

In a study conducted by Jafari Najaf Abadi and her colleagues in 2011 in Iran to study the micropropagation of thornless raspberries by using axillary bud explants, axillary buds were chosen as explants. After sterilization, they transferred the MS culture medium to 2 $\text{mg}\cdot\text{L}^{-1}$ BA. After 2 weeks, the explants were transferred to a proliferation culture medium containing 12 growth regulators of BA (0, 2 and 3 $\text{mg}\cdot\text{L}^{-1}$) and GA3 (0, 0.2, 0.5, and 1 $\text{mg}\cdot\text{L}^{-1}$). The greatest number of branches with an average of 3.33 and the maximum branch length with an average of 5.87 cm was produced in medium containing 2 $\text{mg}\cdot\text{L}^{-1}$ BA and 0.5 $\text{mg}\cdot\text{L}^{-1}$ GA3. In rooting treatment, basic MS culture medium with 4 IBA concentrations was used. The concentration of 2 $\text{mg}\cdot\text{L}^{-1}$ IBA produced the highest (4 roots) and the longest root (7.83 mm long) (Jafari Najafabadi, 2009).

Mozaffarzadeh *et al.* (2014) in Iran, the effect of culture medium on the micropropagation of the *Rubus idaeus* raspberry plant using axillary buds in two different culture medium was studied. The axillary buds were cultured on MS medium, and after one-month elongated branches were cut and transferred to the different culture mediums (MS and ½ MS supplement BA hormone 1 $\text{mg}\cdot\text{L}^{-1}$ and GA3 0.5 $\text{mg}\cdot\text{L}^{-1}$) in four replications for proliferation experiments. After one month, the proliferation traits such as a number of nodes and stem length were measured in different treatments, and the average of the traits was compared using variance analysis. The best medium for micropropagation of this plant was MS medium (Mozaffarzadeh, 2009). Jin-Hu *et al.*, in a 2009 study, concluded that thornless genotypes were easier to start than the bramble genotypes because there was little contamination. Apical buds were more straightforward to cultivate than axillary buds because the cultures that started from the apical buds produced several branches at a faster rate than those produced by axillary buds. However, axillary buds are still reliable

explants source, especially if the initiation cultures stage is developed for multi-branch evolution. (Jin-Hu *et al.*, 2009).

In order to establish the optimal conditions for micropropagation in two raspberry cultivars Raspberry (Opal, Cayuga), the results showed that generally, stimulation of multiple shoots or bud formation is achieved by culturing explants on medium supplemented with relatively high levels of cytokinins. TDZ has been shown to promote branch regeneration in woody plant species. The TDZ induced organogenesis via a reduced dominance of the apical meristem, resulting in the formation of adventitious and/or axillary buds directly on the cultured branch tips. Cytokinins act early during branching initiation to control meristem activity and GAs act at later stages, regulating cell division and expansion to control shoot elongation.

Various factors can influence the response of explants culture, including the maternal genotypes, its growing conditions, and the time of the choice of the explants, the culture medium, and the culture conditions. In general, the replication of selected explants is not only influenced by different species of plants but also in a particular species, and different genotypes have different replication. Generally, it is better that young tissue of living cells should be used as explants because young tissue-forming cells have small vacuole, and the ratio of the nucleus to the whole cell is high (Omid, 2015). Applying appropriate methods for selecting and sterilizing healthy explants can significantly affect the success of the plant tissue culture project. Two of the most important physical factors affecting cultivars are heat and light. In general, more cultures are kept at a temperature of 20 to 28 °C. It should be noted that in many species, there is a direct relationship between the proper temperature of the tissue culture and the proper temperature of the maternal plant in normal conditions. (Omid, 2015).

Light is used as an indispensable factor in the differentiation and morphogenesis cultivars. The most common light used in tissue culture is light from a fluorescent lamp that has some red light in its range. One of the reasons for using a Fluorescent lamp in tissue culture is low heat production. Photoperiod can be effective in controlling sleep and germination of cells, and its effect is similar to the effect of cytokinins. Although tissue culture of genotypes and different plant species has different responses to light, a photoperiod of 16 hours is recommended in most cultures for regeneration (stemming). In

some experiments, the adverse effects of light on the root have been reported. Seedlings obtained from tissue culture have an incomplete cuticle and often have an inappropriate root system. When these seedlings are transported to the soil, they quickly undergo water shortage, growth retardation, and even death. To resolve this problem, it is essential to create proper roots and to acclimatize to the soil conditions (Omid, 2015).

5. CONCLUSIONS:

Factors affecting the micropropagation process in *Rubus loganobaccus* vary remarkably, which in this study, the phytohormonal factor found to be a significant factor in branching. Additionally, rooting as another critical step in the production of micropropagated plants showed a significant dependency on hormonal treatments. In general, in this multi-purpose study, a considerably effective disinfection method for *Rubus loganobaccus* bud explants was developed in addition to a multiplication system with high branching and the rooting rate, which can be employed in commercial propagation programs. All the abovementioned outcomes could be advantageous when it comes to production of propagation material of *Rubus loganobaccus* and enable the producer to lessen their costs while increasing their rate as well as financial benefits.

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Table 1. Specification of rooting treatments

Treatment No.	Treatment name	Specifications of each treatment
1	LB1	MS – H
2	LB2	MS+ 1/2 NH ₄ NO ₃ +1/2K(NO ₃)
3	LB3	MS+1/2 NH ₄ NO ₃ +1/2K(NO ₃) + IBA(0.1) mg
4	LB4	MS+1/2 NH ₄ NO ₃ +1/2K(NO ₃) + IBA(0.1) mg + sucrose
5	LB5	MS+1/2 NH ₄ NO ₃ +1/2K(NO ₃) + IBA(0.5) mg
6	LB6	MS+1/2 NH ₄ NO ₃ +1/2K(NO ₃) + IBA(1) mg

Introduction of treatments Table 1:

Treatment No. 1 (LB1): MS-H: (Murashige and Skoog medium) - Hormone

Treatment No. 2 (LB2): MS + 1/2 NH₄NO₃ + 1/2(NO₃)₂

Treatment No. 3 (LB3): MS + 1/2 NH₄NO₃ + 1/2(NO₃)₂ and indole -3- butyric acid (IBA) 0.1mg

Treatment No. 4 (LB4): MS + 1/2 NH₄NO₃ + 1/2(NO₃)₂ and indole -3- butyric acid (IBA) 0.1mg + sucrose

Treatment No. 5 (LB5): MS + 1/2 NH₄NO₃ + 1/2(NO₃)₂ and indole -3- butyric acid (IBA) 0.5mg

Treatment No. 6 (LB6): MS + 1/2 NH₄NO₃ + 1/2(NO₃)₂ and indole -3- butyric acid (IBA) 1mg

Table 2. Information on twelve branching treatments

Treatment No.	Treatment name	Specifications of each treatment
1	LB1	MS + BA(0.5) mg 2ip(0.5) mg IBA(0.1) mg
2	LB2	MS + BA(1) mg IBA(0.1) mg
3	LB3	MS + BA(2) mg IBA(0.1) mg
4	LB4	MS + 2ip(1) mg IBA(0.1) mg
5	LB5	MS + 2ip(2) mg IBA(0.1) mg
6	LB6	MS + BA(1) mg 2ip(1) mg IBA(0.1) mg
7	LB7	MS + BA(0.5) mg 2ip(0.5) mg IBA(0.5) mg
8	LB8	MS + BA(0.5) mg 2ip(0.5) mg IBA(0.1) mg GA(0.5) mg
9	LB9	IBA(0.1) mg GA(0.5) mg BA(1) mg 2ip(1) mg + MS
10	LB10	MS – H
11	LB11	IBA(0.1) mg BA(1) mg + (AN)
12	LB12	IBA(0.1) mg BA(0.5) mg 2ip(0.5) mg + (AN)

Introduction of treatments Table 2:

Treatment No. 1 (LB1): Murashige and Skoog medium (MS) + Benzyl adenine (BA) 0.5 mg, 2ip (isopentenylidenine) 0.5mg, indole -3- butyric acid (IBA) 0.1 mg

Treatment No. 2 (LB2): Murashige and Skoog medium (MS) + Benzyl adenine (BA) 1 mg, indole -3- butyric acid (IBA) 0.1 mg

Treatment No. 3 (LB3): Murashige and Skoog medium (MS) + Benzyl adenine (BA) 2 mg, indole -3- butyric acid (IBA) 0.1 mg

Treatment No. 4 (LB4): Murashige and Skoog medium (MS) + 2ip (isopentenylidenine) 1mg, indole -3- butyric acid (IBA) 0.1 mg

Treatment No. 5 (LB5): Murashige and Skoog medium (MS) + 2ip (isopentenylidenine) 2mg, indole -3- butyric acid (IBA) 0.1 mg

Treatment No. 6 (LB6): Murashige and Skoog medium (MS) + Benzyl adenine (BA) 1 mg, 2ip (isopentenylidenine) 1 mg, indole -3- butyric acid (IBA) 0.1 mg

Treatment No. 7 (LB7): Murashige and Skoog medium (MS) + Benzyl adenine (BA) 0.5 mg, 2ip (isopentenylidenine) 0.5mg, indole -3- butyric acid (IBA) 0.5 mg

Treatment No. 8 (LB8): Murashige and Skoog medium (MS) +Benzyl adenine (BA) 0.5 mg, 2ip (isopentenylidenine) 0.5mg, indole -3- butyric acid (IBA) 0.1 mg, Gibberellic acid (GA) 0.5 mg

Treatment No. 9 (LB9): Murashige and Skoog medium (MS) + indole -3- butyric acid (IBA) 0.1 mg Gibberellic acid (GA) 0.5 mg, Benzyl adenine (BA) 1 mg, 2ip(isopentenylidenine)1 mg

Treatment No. 10 (LB10): Murashige and Skoog medium (MS) – Hormone

Treatment No. 11 (LB11): Anderson (AN) + indole -3- butyric acid (IBA) 0.1 mg Benzyl adenine (BA) 1 mg

Treatment No. 12 (LB12): Anderson (AN) + indole -3- butyric acid (IBA) 0.1 mg Benzyl adenine (BA) 0.5 mg 2ip (isopentenylidenine) 0.5 mg

Table 3. Sterile treatment results

Treatment name	number	Duration of stay in treatment	Contamination (bacterial, fungal)	Final Health (Germinated Number)
1	27	5 minutes	--	27
2	28	7 minutes	1	22 (5 were not germinated)

Table 4. *The results of the effects of rooting treatments*

Treatment name	Average of rooting	Percentage of rooting	The average length of roots	Branch thickness	roots density	secondary roots
LB1	23	76.66	2-3 _{cm}	Thin	High	Not have
LB2	29	96.66	3 _{cm}	medium-thick	High	have
LB3	28	93.33	1-2 _{cm}	medium-thick	High	have
LB4	18	60	1 _{cm}	Thin	Medium	Not have
LB5	32	91.42	1-2 _{cm}	Thin	High	have
LB6	29	96.66	1 _{cm}	Thin	High	Not have

Table 5. *Root acclimatization results*

Rooting treatment No.	Total number of pots containing this treatment	Number of survived and fresh pots	Percentage of survived and fresh pots	Number of droopy pots	Percentage of droopy pots	Quality	Plant stem length
1	25	15	60	10	40	Good	1-3 _{cm}
2	22	10	45.45	12	54/55	Good	1-2 _{cm}
3	27	25	92,60	2	7/40	Good	3-5 _{cm}
4	19	17	89.47	2	10/53	Good	1-3 _{cm}
5	32	26	81/25	6	18/75	Good	3-5 _{cm}
6	28	27	96/43	1	3/57	Good	2-6 _{cm}

Table 6. Branching treatments results

Treatment name	Average branching	Rooting / lack of root	Branch thickness	Green / yellow plant	Fresh/ droopy plant
LB1	7.76	No Root 100	Thin 23.30	Green 100	Fresh 100
			Medium 51.93		
LB2	8.73	No Root 87.03 Rooting 12.97	Very thin 45.80	Green 100	Fresh 100
			Thin 41.22		
			Medium 12.97		
LB3	6.28	No Root 100	Thin 47.77	Green 100	Fresh 100
			Medium 52.22		
LB4	7.03	No Root 100	Thin 40.75	Green 93.83	Fresh 100
			Medium 37.91		
			Thin to medium 21.32	Yellow 6.11	
LB5	6.10	No Root 100	Thin 75.26	Green 96.31 Yellow 3.68	Fresh 100
			Medium 11.57		
			Thick 13.15		
LB6	6.25	No Root 100	Thin 51.2	Green 75.2 Yellow 24.8	Fresh 100
			Thick 48.8		
LB7	6.40	No Root 100	Medium 100	Green 100	Fresh 100
LB8	3.88	No Root 100	16.49 Thin	Green 100	Fresh 100
			83.5 Medium		
LB9	3.16	No Root 100	36.84 Thin	Green 100	Fresh 100
			63.15 Medium		
LB10	3.13	Long and multiple roots 100	84.04 Medium	Green 100	Fresh 100
			15.95 Thick		
LB11	4.56	No Root 100	36.84 Medium	Green 100	Fresh 100
			63.15 Thick		
LB12	5.28	No Root 100	16.66 Thin	Green 100	Fresh 100
			83.33 Medium		

Table 7 - Branch length results

Raw	Treatment name	The average branch length of each treatment
1	LB1	3.11 cm
2	LB2	3 cm
3	LB3	1.86 cm
4	LB4	2.46 cm
5	LB5	1.71 cm
6	LB6	2 cm
7	LB7	2.33 cm
8	LB8	2.2 cm
9	LB9	1.8 cm
10	LB10	4.48 cm
11	LB11	5.22 cm
12	LB12	1.8 cm

Figure 1. Deployment graphic in sterile treatments **Figure 2. Comparison of treatment rooting percentage**

Figure 3. Comparison of rooting treatments acclimatization in soil

Figure 4. Comparison of rooting and acclimatization percentage of the root in the soil

Figure 5 : Comparison of the percentage of branching of the treatments

Figure 6: Comparing of branch length of branching treatments

Figure 7. Plant specimen in a pot within the greenhouse

Figure 8. Initial parts (containing buds) planted in vials before and after the production of leaflets.

Figure 9. Rooting images in relevant treatment

Figure 10. Rooting treatments at the stage of acclimatization to the soil.

Figure 11. Containers were containing branching treatment (1 to 12) after branching.