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Establishment of indigenous garlic varieties *in vitro* under influence of growth regulator and light

Uvođenje autohtonih ekotipova češnjaka *in vitro* pod utjecajem regulatora rasta i svjetlosti

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ABSTRACT

Garlic is valuable crop that is used not only for human consumption, but also in pharmaceutical, food and cosmetics industries. Indigenous cultivars have specific beneficial properties that could be permanently lost due to cultivation of new cultivars. Aim of this study was to established two indigenous garlic cultivars *in vitro* under influence of growth regulator and light. Garlic varieties Slavonian winter (Croatia) and Vincek (Slovenia) were introduced on medium supplemented with two different concentrations of BAP (1 and 1,5 mg/L) and grown under two types of light (FLUO and LED). Results showed there were no significant influence of light type but concentration of growth regulator significantly influenced *in vitro* development of garlic microshoots of both investigated cultivars. The best treatment for Vincek garlic explants was nutrient medium supplemented with 1.5 mg/L BAP and LED lights, while for Slavonian winter garlic the best treatment showed to be FLUO light and 1,5 mg/L BAP.

Keywords: 6-benzyl aminopurine (BAP), *Allium sativum*, LED (Light Emitting Diode), Slavonian winter, Vincek

SAŽETAK

Češnjak je dragocjena kultura koja se koristi osim za ljudsku prehranu, i u farmaceutskoj, prehrambenoj i kozmetičkoj industriji. Autohtone sorte imaju specifična korisna svojstva koja bi mogla biti trajno izgubljena uzgojem novih kultivara. Cilj ovog istraživanja bio je uspostaviti *in vitro* protokol za uzgoj dvije autohtone sorte češnjaka pod utjecajem regulatora rasta i svjetla. U *in vitro* kulturu uvedene su autohtone sorte češnjaka Slavonski ozimi (Hrvatska) i Vincek (Slovenija) na dvije različite varijante hranjive podloge (1 i 1,5 mg/L BAP-a) te su uzgajane pod dvije varijante osvjetljenja (FLUO i LED). Rezultati su pokazali da nije bilo značajnog utjecaja vrste svjetla, ali je koncentracija regulatora rasta značajno utjecala na *in vitro* razvoj mikroizdanaka češnjaka obje ispitivane sorte. Eksplantati češnjaka Vincek postigli su bolje rezultate na tretmanu hranjive podloge s dodatkom 1,5 mg/L BAP-a i LED svjetla, dok se za Slavonski ozimi češnjak pokazao najbolji tretman FLUO svjetlo i 1,5 mg/L BAP-a.

Ključne riječi: 6-benzil aminopurin (BAP), *Allium sativum*, LED svjetla, Slavonski ozimi, Vincek

INTRODUCTION

Garlic (lat. *Allium sativum* L.) is an annual or perennial vegetable crop which belongs to the family Alliaceae and the genus *Allium*. It is assumed that is native to Central Asia and that it originates from the area of western China. Garlic contains over 150 biologically active substances, including minerals, vitamins and amino acids. It is rich in manganese, selenium, phosphorus, vitamin C and vitamin B6 and contains essential oils with sulfur, which gives them a specific aroma. Also, garlic has many medicinal properties and antioxidant, antimicrobial and antiviral effects (Parađiković et al., 2012; Mikaili et al., 2013). As garlic production spread globally and due to the diversity of climatic and edaphic conditions of each region there was the inevitable development of numerous indigenous cultivars specific to a certain narrow area. With technological progress and improvements in plant breeding, new high yielding cultivars took place over indigenous species. Matotan (2007) reports that in Croatia a numerous Croatian vegetable gene fond has been permanently lost while many are endangered. Slavonian winter garlic is indigenous cultivar that poses high antioxidant effect as well as protective effects on human cells (Parađiković et al, 2012; Vinković et al., 2020). To preserve gene pool of such indigenous species it is necessary to collect, multiplicate and properly conserve their germplasm. Garlic is a plant species that is grown vegetatively through cloves. The main disadvantage of vegetative propagation by planting cloves directly in the field is the occurrence of primary and secondary virus infection, which results in yield and quality reduction as well as a shortening of bulb storage period (Mehta et al., 2013). Also, Robledo-Paz and Tovar-Soto (2012) state that the garlic production through cloves has numerous disadvantages such as low multiplication rate (5 to 10 per year), expensive and short-term storage that requires large space, transmission of pathogens from one generation and cultivation area to another, ultimately reducing quality and yields up to 70%. As an alternative to classical vegetative or clonal propagation with cloves, tissue and cell culture techniques have emerged. Such technique besides allowing mass multiplication regardless the season, enables improved production of plants with beneficial secondary metabolites and its isolation (Mantovska et al., 2019) which is especially noteworthy for garlic. Light play important role in plant development, not only for photosynthesis that shows a strong dependence on the quality and quantity of light but also influencing processes on molecular, biochemical and morphological basis. *In vitro* propagation is most convenient method to study its impact, especially as todays researches aspire more and more on implementation of photoautotrophic propagation with LED lights (Batista et al., 2018). Higher photosynthetic photon efficacy (PPE) and ranges between 80 and 100% of the PAR efficiency of LED lamps justify the use of LED lights in preference to previously used HPS or Fls (Cavallaro et al., 2022). Ramírez-Mosqueda et al. (2017) studied the effect of LED diodes with different wavelength of light on Stevia rebaudiana morphogenesis *in vitro*. Compared with Fluo lighting, they reported higher proliferation rate and increased content of photosynthetic pigments on plants cultured under LED lighting. LED lighting increased photosynthesis rate, pigments content and growth characteristics of tomato plantlets (Naznin et al., 2019) and also its yield, fruit weight and stem diameter (Vinković et al., 2016). Besides light, successful *in vitro* establishment depends on specificity of the genotype (Yancheva et al., 2018) as well as on explant type and sterilization method (Stanisavljević et al., 2017). Therefore, the aim of this study was to establish *in vitro* culture of two indigenous garlic cultivars and to determine the influence of different spectrum of light as well as hormone concentration on the initial growth of garlic explants *in vitro*.

MATERIAL AND METHODS

Research was conducted in the year 2020. in the Laboratory for Vegetables, Floriculture, Medicinal, Spicy and Aromatic Herbs at Faculty of Agrobiotechnical sciences Osijek.

Plant material

Two indigenous garlic ecotypes were included in the research - Slavonian winter (Croatia) and Vincek (Slovenia). Both ecotypes were collected during 2019 from local growers in Slavonia and Slovenia and in fall cultivated on faculty demonstrational-educational fields due to plant material multiplication. For *in vitro* establishment garlic bulbs of both ecotypes harvested in July 2020 were used.

Trial design and in vitro micropropagation

The cleaned garlic cloves were rinsed under running tap water for 30 minutes and then immersed in a solution of 70% ethanol for 1 minute followed by surface sterilization with 2% NaClO with the addition of a few drops of detergent for 20 minutes. After rinsing with autoclaved water, the plant material was ready for explants isolation. Isolation was performed in a laminar air flow cabinet under sterile conditions. The isolated explants were young sprouts from garlic cloves. To ensure the minimum contamination, isolated explants were sterilized for 10 minutes in 2% NaClO solution with addition of few drops of detergent followed by washing with autoclaved water several times. Garlic sprotus were cultured in test tubes on basal Linsmaier and Skoog (1965) nutrient medium without addition of a plant hormones (Table 1). After 2 weeks uncontaminated garlic sprouts were subcultured to fresh medium. Sprouts were cut in 2-3 pieces and cultured on Linsmaier and Skoog (1965) nutrient medium supplemented with 1 mg/L 6-benzyl aminopurine (BAP) or 1.5 mg/L BAP, while both variants of medium were supplemented with 0.1 mg/L indole-3-butyric acid (IBA). Experiment consisted of 60 explants in three replicates per treatment of each garlic cultivar arranged into 4 treatments: A - growth regulators (1 and 1.5 mg/L BAP) and B - light (FLUO white and LED red and blue (1:1)). After 4 weeks, on each explant were measured following parameters: microshoots length and fresh weight, multiplication and survival rate.

Data analysis

Statistical analysis was performed in SAS 9.4 program and differences between treatments were evaluated according to Fisher test.

RESULTS AND DISCUSSION

Table 1 shows influence of different light type, FLUO white light and LED (red and blue) light on garlic development. Statistical analysis showed there was no significant influence of light type on all investigated parameters at Vincek cultivar, while at Slavonian winter cultivar, only multiplication rate was influenced by light. Apart from technical advantages of LED lighting systems such as ability to control spectral composition and long operating lifetime, this type of system may have positive effect on plant growth and development *in vitro* (Gupta and Jatothu, 2013). Takagi and Qu (1995) reported positive effect of far red light on *in vitro* garlic bulb formation, also red light shortness the time of garlic callus induction and callus proliferation (Ma et al., 2011). Shoot proliferation of highbush blueberry was under positive influence of different LED light spectra compering to FLUO light (Hung et al., 2016), while Bantis et al. (2018) reported morphological, physiological and phytochemical response of pomegranate seedlings to LED light. In *in vitro* propagation LED lights show to be more efficient than FLUO lights, but material and culture vessel type can disturb amount of photosynthetically active photons reaching the plant surface (Batista et al., 2018).

Furthermore, Cioć et al. (2019) reported statistically difference in morphological properties of gerbera plantlets *in vitro* only when intensity of LED lights was higher than intensity of FLUO. In this study, garlic explants were cultured under same intensity of FLUO and LED lights and in glass tubes with cotton caps, placed in racks that could easily affect the amount of light. Therefore, there were no statistically differences among light types, but also microshoot weight was higher at both cultivar on LED lightning.

According to data analysis, concentration of growth regulator significantly influenced *in vitro* development of garlic microshoots of both investigated cultivars. As plant growth regulators, especially cytokinins, auxins and its interaction, play important role in proper *in vitro* plant development (Gaspar et al., 1996) such results were expected but response of different garlic cultivars can often vary greatly. In this study, at both cultivars

microshoot length and survival rate were greater at higher concentration of BAP growth regulator. Chauhan et al. (2015) also reported higher garlic shoot length at 1.5 mg/L BAP concentration, while Ayed et al. (2018) at same concentration reported lower values. Microshoot weight and multiplication rate of each garlic cultivar showed opposite results in response to concentration of growth regulator. At lower BAP concentration microshoot weight of Vincek cultivar was significantly higher, while of Slavonian winter cultivar was significantly lower (Table 1). Different response of garlic cultivars to various treatments of growth regulators was reported among many authors (Scotton et al., 2013; Haider et al., 2015; Metwally et al., 2020) which confirms the need for *in vitro* process optimization of each individual cultivar.

Table 1. Influence of different light on indigenous garlic *in vitro* development

Light type	Microshoot length	Microshoot weight	Multiplication rate	Survival rate
		Vincek		
FLUO	84,38[a]	0,70[a]	3,52[a]	33[a]
LED	84,00[a]	0,86[a]	3,41[a]	41,50[a]
		Slavonian winter		
FLUO	137,03[a]	0,75[a]	1,83[b]	80,50[a]
LED	145,67[a]	0,87[a]	2,06[a]	71,50[a]

[a,b,c] The values in the same column of the table marked with different letters differ significantly at level ($P < 0,05$)

Indigenous garlic varieties were established in *in vitro* culture supplementing the media with different concentrations of BAP growth regulator and cultured under two type of lights. Although, both garlic cultivars were successfully established on all treatments, diverse response of each cultivar was recorded. Interaction of light and growth regulator significantly influenced all investigated parameters at both investigated cultivars (Figure 1). According to Cavallaro et al. (2022) light and growth regulators can alter the effect of each other on plant growth. Generally, at optimal cytokinin concentration shoot proliferation is better under white light, while at lower concentration more axillary shoots develop under red light. In our investigation, highest number of axillary shoots at Vincek variety was recorded at lower BAP concentration and FLUO white light, while at the same treatment the lowest values were recorded for Slavonian winter. Vincek garlic explants cultured at nutrient medium supplemented with higher cytokine concentration and placed under LED lights (red and blue) resulted with highest microshoot length and weight and survival rate. Similar results were obtained by Bhaya and Al-RazzaqSalim (2019) who reported positive interaction of plant growth regulators and light type on banana micropropagation. Number of multiplicated shoots and shoot fresh and dry weight were significantly superior on treatments with higher concentrations of cytokinins BAP and TDZ and cultured under LED lights.

Table 2. Influence of different concentration of growth regulator BAP on indigenous garlic *in vitro* development

Growth regulator concentration	Microshoot length	Microshoot weight	Multiplication rate	Survival rate
		Vincek		
BAP 1	81,66[b]	0,74[a]	3,89[a]	25[b]
BAP 1,5	87,08[a]	0,65[b]	3,17[b]	41[a]
		Slavonian winter		
BAP 1	126,95[b]	0,67[b]	1,87[a]	69[b]
BAP 1,5	155,75[a]	0,94[a]	2,02[a]	83[a]

[a,b,c] The values in the same column of the table marked with different letters differ significantly at level ($P < 0,05$)

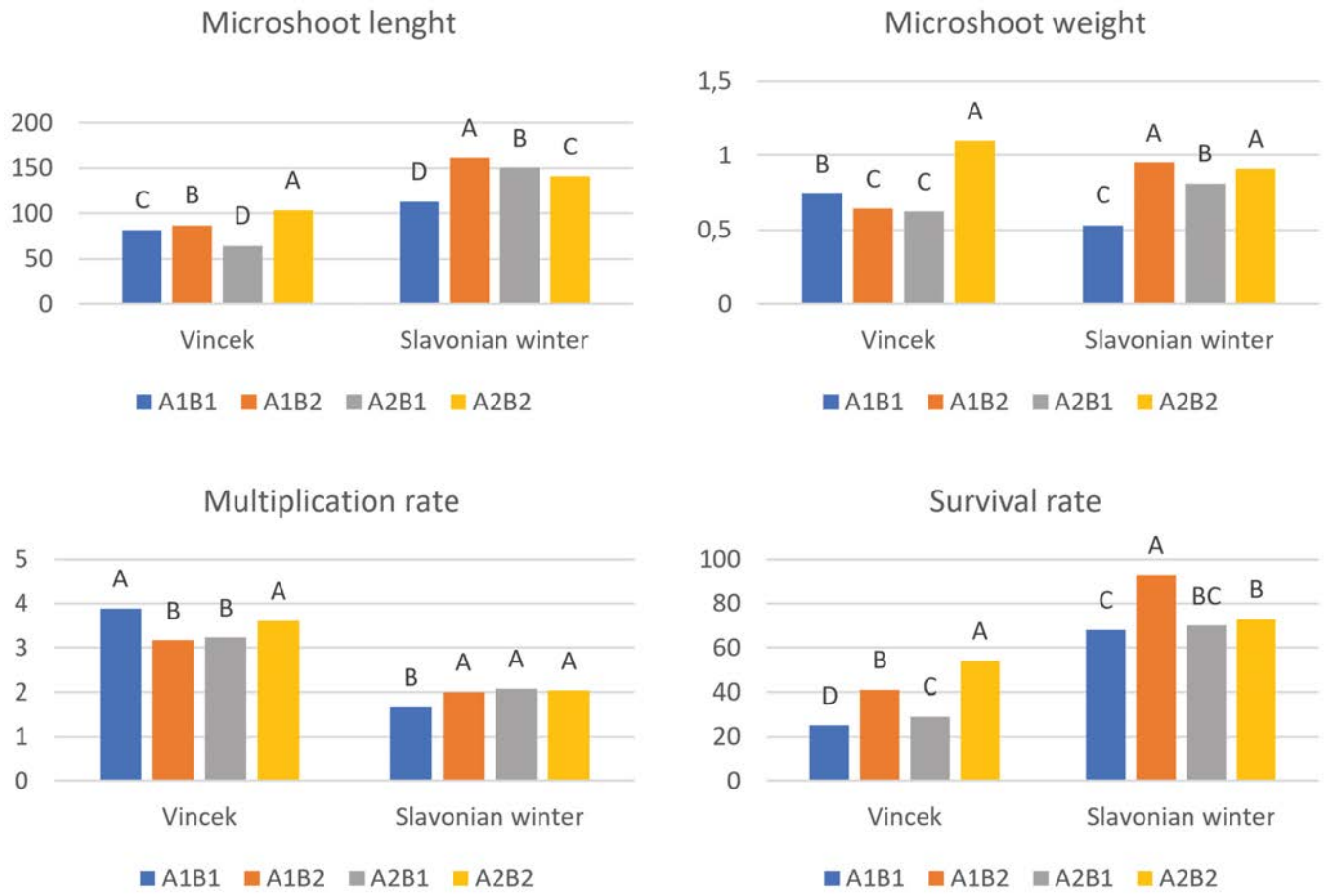

Figure 1. Influence of growth regulator and light treatments on indigenous garlic *in vitro* development. Values represent means ± standard deviations. Different letters denote significant differences ($P < 0.05$) among treatments. A1B1 – 1 mg/L BAP, FLUO; A1B2 - 1 mg/L BAP, LED; A2B1 – 1.5 mg/L BAP, FLUO; A2B2 – 1.5 mg/L BAP, LED

Panizza et al. (1994) reports that effect of cytokinins applications was predominant over the light quality on lavandin axillary proliferation, while in cytokine free medium, shoot number was enhanced under blue, white and red light at low photon fluence rates. For Slavonian winter garlic the best treatment showed to be LED light and 1 mg/L BAP whereas, the highest values of microshoot length and weight and survival rate were recorded. Different response of two investigated garlic can also be associated with its genotype as each of these garlic varieties have been grown for more than a half century in same narrow geographical area in Croatia and Slovenia each being adopted to specific microclimate.

Regarding garlic cultivar, both investigated cultivars are indigenous winter varieties which have been planted for decades by local people in the area of Slavonia, Croatia (Slavonian winter) and Podravska region in Slovenia (Vincek). Main morphological differences among cultivars are in bulb size and number of cloves. Vincek bulb is lager with 8 – 10 cloves per bulb, while Slavonian winter bulb is more compact with smaller 12 - 16 cloves. Differences between investigated cultivars are recorded on all parameters except microshoot weight. Although microshoot length of Slavonian winter garlic was significantly higher, there was no difference in microshoot weight between cultivar (Figure 2).

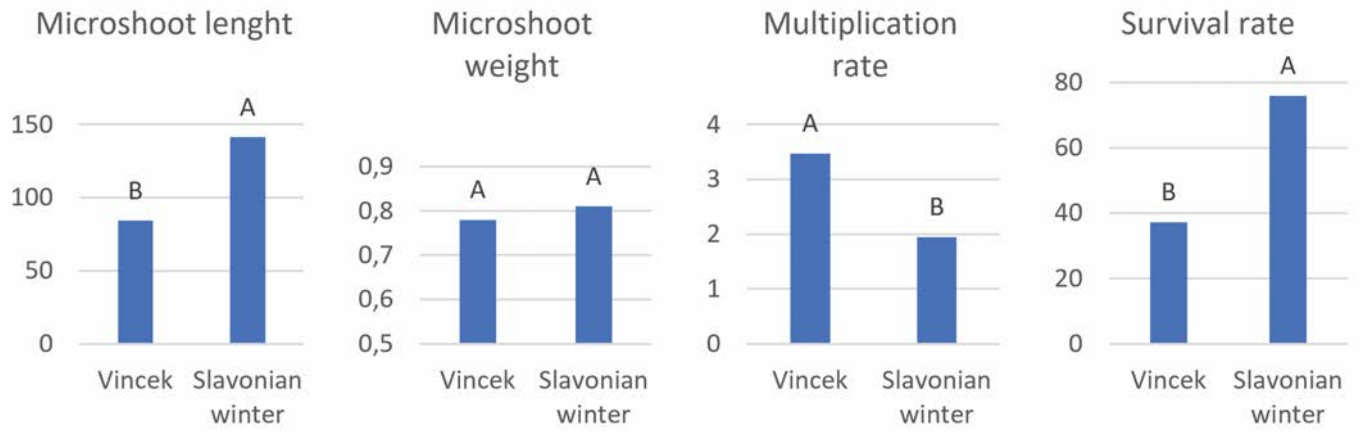

Figure 2. Influence of growth regulator and light treatments on garlic cultivar. Values represent means ± standard deviations. Different letters denote significant differences ($P < 0.05$) among treatments.

CONCLUSION

Biotechnological approach is a good method to obtain indigenous cultivars of various plants including garlic. Indigenous garlic species are valuable source of genetic traits associated with good adaptation to environmental conditions of specific region. This study shows it is possible to establish Slavonian winter and Vincek cultivar *in vitro* and thus preserve valuable resource of indigenous species. In general, the microshoot length and weight and survival rate of both investigated cultivars were highest under LED light. However, those results at Slavonian winter garlic were recorded on treatment supplemented with 1 mg/L BAP, while at Vincek garlic with 1.5 mg/L BAP. The difference in the results obtained between the examined garlic cultivars, related to the influence of light and hormones on *in vitro* growth, can be attributed to their genetic basis, while the percentage of survival rate in culture can be due to different health conditions of explants. Based on this research, as well on the researches of many authors, it can be concluded that certain cultivars require specific protocol for obtaining the best plants *in vitro*.

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