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Micropropagation of *Monarda fistulosa* L. plants by axillary bud proliferation

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The procedure of micropropagation for efficient and stable conservation of *Monarda fistulosa* L., a medicinally important plant belonging to the Lamiaceae family, was developed. Our research consisted of the introduction *in vitro* culture of the precious genotype from a biochemical point of view, identified as a result of multiple analyzes carried out by the researchers of the Scientific Medicine Research Center of the 'Nicolae Testemitanu' State University of Medicine and Pharmacy. This genotype is characterized by an increased content of secondary metabolites (thymol and carvacrol). Axillary bud proliferation was initiated from nodal explants grown on ½ Murashige-Skoog (MS) nutrient medium supplemented with various cytokinins, such as 6-benzylaminopurine (BA), 6-(α, α-dimethylallylamino)-purine (2iP) and cytokinin-like growth regulators – thidiazuron (TDZ) with a concentration of 0.5 mg.L^{-1}. After 6 weeks of culture on a nutrient medium, containing 0.5 mg.L^{-1} BA was determined the maximum number of axillary shoots per explant (2.75 ±0.86) and the highest number of internodes per shoot (11.66 ±1.30). At the same time, the rhizogenesis was induced on that medium that allows *in vitro* micropropagation by a single stage. The advantages of the elaborated procedure are reducing reagents and energy expenses for *in vitro* cultivation by two times and increasing the coefficient of multiplication from 13 to 32.

Keywords: *Monarda fistulosa*, medicinal plant, cytokinin, micropropagation, axillary bud

Introduction

Monarda fistulosa L. (wild bergamot) from the Lamiaceae family is a newly introduced perennial aromatic, medicinal and decorative plant found in the wild flora of North America (Bodrug, 1993). Essential oils obtained from the aerial part of the respective species show bactericidal, fungicidal, anthelmintic, immunomodulatory and antiseborrheic activity (Ivankovic et al., 2006; Inouye et al., 2009; Zhilyakova et al., 2009; Grzeszczuk, 2020; Yezerska et al., 2021). The study of the effects of the ethanolic extracts and the essential oil also demonstrated ovicidal, insecticidal, and antifeedant properties (Elisovetcaia et al., 2018). For the extraction of essential oils, this species is cultivated in Canada, the USA, some European countries, the Caucasus, Crimea as well as in the Republic of Moldova (Bodrug, 1993). The research carried out on *M. fistulosa* plants cultivated in the Republic of Moldova

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demonstrated the presence of an increased content of thymol and carvacrol (Casian et al., 2013; Casian et al., 2017). For these reasons, the plants of this species deserve to be recognized as medicinal plants equally with the official species such as *Thymus vulgaris* L. and *Origanum vulgare* L. subsp. *hirtum* (Casian et al., 2017; Oparin et al., 2000). The presence of significant amounts of thymoquinone may determine a greater spectrum of its therapeutic activity (Casian et al., 2017). The ratio of the components and the yield of the essential oil from different organs of *M. fistulosa* plants may vary depending on genotype and geographic origin (Mazza et al., 1992). Research carried out within the Scientific Center for Drug Research (SCDR) of 'Nicolae Testemitanu' State University of Medicine and Pharmacy (Chisinau, Moldova) demonstrated that the maximum essential oil content was determined in leaves and inflorescences, and the lowest in stems (Casian et al., 2017). As a result of multiple analyses, the researchers of the Scientific Center identified a genotype characterized by an increased content of active principles. At the same time, in the active phase of development, the appearance of powdery mildew was also observed on the surface of the leaves of cultivated plants. The disease affects the leaves, causing them to drop, and respectively affects vigor, resistance to stress over time, and optimal performance. Being a perennial plant, the spores overwinter in buds and on debris, which is released in the spring to continue the disease. Similar problems have been observed by other researchers (Davidson, 2007; Xu et al., 2022). Taking into account that there are practically no plants free of viruses and pests, our research consisted of the introduction *in vitro* culture of the precious genotype from the biochemical point of view.

It is known that the multiplication of *M. fistulosa* plants by seeds remains the most frequently used technique. But, they may not produce identical clones, which is a disadvantage when the intention is to generate plants similar in their clonal fidelity.

On the other hand, plant multiplication through *in vitro* cultures maintains the plant genotype, to produce disease-free plants, increases biomass turnover rate, reduces growth duration, and uses limited space with controlled environmental conditions. Also, plant tissue culture techniques are an effective tool used to plant improvement and secondary metabolite production (George et al., 2008; Grigoriadou et al., 2019).

The number of papers describing the cloning of *M. fistulosa* plants is not so large and the data are fragmentary. Our research aimed to develop an *in vitro* micropropagation procedure for efficient and stable conservation of *M. fistulosa*. This allows us to continuously supply clone plants that will be used as standard material in the field of medicinal plant research.

Material and methodology

Plant material and sterilization methods

Young shoots of 5–6 cm, with several lateral buds, taken from a single plant of *Monarda fistulosa*, grown in the SCDR served as plant material for the research. After leaf removal, stem segments with accompanying lateral buds were washed first in running tap water, then in sterile water with a few drops of Tween-40 and bleach solution for 15 min. Finally, the plant material was rinsed three times with sterile distilled water.

Culture media and conditions

Aseptically prepared nodal segments of approximately 0.5–0.8 cm were vertically implanted in ½ Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.2% activated charcoal (used to reduce tissue oxidation due to phenolic compounds during growth explants), 3% (w/v) sucrose and 0.6% (w/v) agar. The culture media were adjusted to pH 5.8 before autoclaving at 121 °C for 22 min. Cultures were incubated at 25 ±2 °C under a 16/8-hour photoperiod (light/dark cycle) provided by cool white fluorescent lamps (approximately 20–40 $\mu mol.m^{-2}.s^{-1}$).

Shoot induction, multiplication and rooting

After the establishment of the *in vitro* culture of *M. fistulosa* on the ½ MS medium modified supplemented with 0.2% activated charcoal, there was used ½ MS medium absent of cytokinins (control) and supplemented with different cytokinins at concentrations of 0.5 $mg.L^{-1}$ such as 6-benzylaminopurine (BA), 6-(α, α-dimethylallylamino)-purine (2iP) and cytokinin-like growth regulators – thidiazuron (TDZ). The effects of different cytokinins in the modified ½ MS culture medium on the initiation of axillary bud development, multiple shoot induction, shoot elongation and rhizogenesis initiation were evaluated. The number of shoots and shoot length were recorded, after 6 weeks of culture. Multiplication coefficient, calculated as the number of new segments (for subculturing) obtained per explant.

Statistical analysis

Data analysis for the determination of standard deviation and significant differences was performed by Statgraphics Plus 5.0 software.

Results and discussion

The micropropagation methods have been widely applied for the rapid propagation of many other medicinal plant species (Debnath et al., 2006; Grigoriadou et al., 2019; Tsoktouridis et al., 2019). One of the most widely used micropropagation strategies is the proliferation and growth of axillary buds. Lateral shoots developed from axillary buds can be separated at the subculture stage and each can be cultivated as a separate explant, thereby increasing the proliferation rate. This method is also considered the most reliable way to produce plants genetically identical to the raw material. Shoot tips and axillary buds represent organized meristems, which are less prone to genetic changes, being more resistant than disorganized ones (Ngezahayo and Liu, 2014; Krishna et al., 2016).

The establishment of *in vitro* culture from *M. fistulosa* was a rather difficult procedure due to contaminations and the sensitivity of the plant tissue to high concentrations of NaOCl. The disinfected explants were placed on ½ MS medium lacking plant growth regulators. To ensure sterility the explants were cultivated for 2 weeks. The stabilization medium was excellent and produced shoots, which were later used in our experiments.

The next step was to test the effects of different plant growth regulators on the initiation and development of axillary buds from explants. One of the most important plant growth regulators are cytokinins that induce the development of axillary buds in a wide spectrum of plants (George et al., 2008; Shimizu-Sato et al., 2009; Aremu et al., 2014). The normally dormant axillary buds are induced to elongate by cytokinins (George et al., 2008; Aremu et al., 2014). Such treatment effectively removes the dominance of apical meristems so that they produce axillary shoots, often in large numbers. These shoots are used as miniature cuttings for further plant multiplication (George et al., 2008). The general pattern of response depends on the used cytokinins such as BA, 2iP, and TDZ shown in Figure 1.

After 3–6 days of inoculation of the explants on the cultivation media containing cytokinins, we observed the initiation of direct formation of single or multiple shoots depending on the absence or presence of the tested cytokinins.

The highest frequency of shoot induction was determined by the presence of BA (2.75 ±0.86 auxiliary shoots per explant) (Table 1).

The highest number of internodes per shoot (11.66 ±1.30) was also obtained on ½ MS medium supplemented with BA, while the maximum shoot length (18.42 ±2.65 cm) was determined on the medium supplemented with 2iP (Table 1). Therefore, BA in the medium significantly improved the frequency of axillary bud induction as well as shoot number compared to ½ MS medium without cytokinin (control). The higher frequency of bud induction and the number of shoots obtained from nodal segments exposed to BA was the result of the high number of bud induction and shoots induced from axillary buds on newly formed shoots. Our results confirm studies, where ½ MS medium

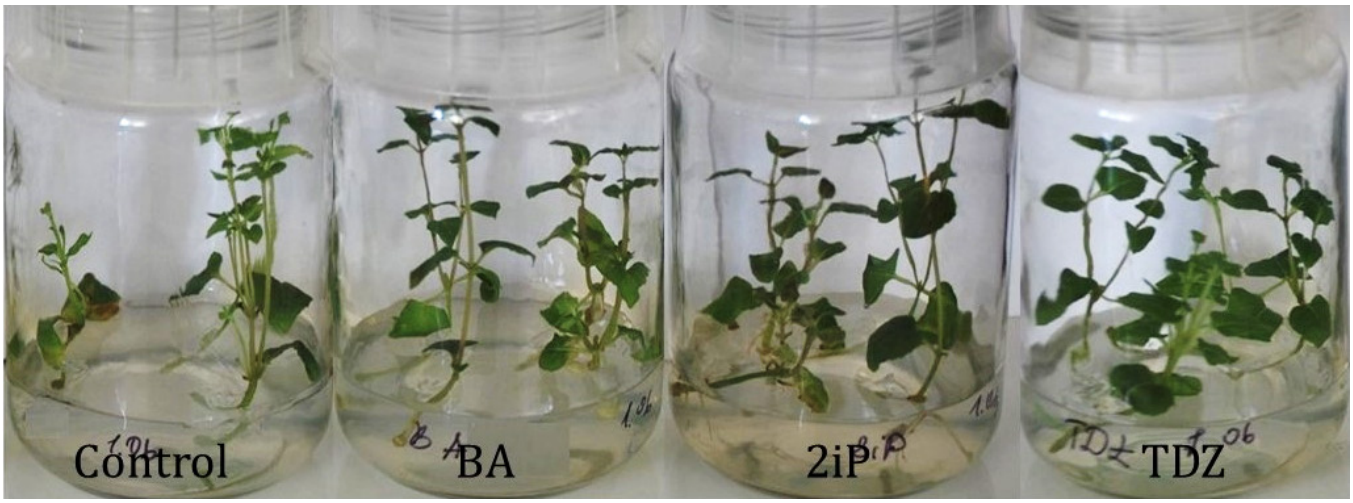

Figure 1 The plants of *Monarda fistulosa* L. grown in MS medium with different cytokinins
Control – absent of cytokinins; BA, 2iP and TDZ with a concentration of 0.5 mg.L^{-1}

Table 1 Effect of different cytokinins in MS medium on morphologic parameters of *Monarda fistulosa* L. plants

Variants	Control	BA	2iP	TDZ
Number of axillary shoots	1.8 ±0.42	2.75 ±0.86	2.18 ±0.60	1.5 ±0.52
Number of internodes	7.3 ±1.34	11.66 ±1.30	9.9 ±1.04	6.7 ±1.16
Length of shoots, cm	15.25 ±1.95	15.38 ±2.81	18.42 ±2.65	12.15 ±2.78
Multiplication coefficient	13	32	21	10
Number of roots	6.1 ±1.52	5.3 ±1.15	7.9 ±2.02	–
Length of roots, cm	4.4 ±2.01	3.2 ±1.13	2.7 ±0.95	–

BA – 6-benzylaminopurine; 2iP – 6-(α, α-dimethylallylamino)-purine; TDZ – thidiazuron

supplemented with BA, was the growth medium that stimulates multiple shoot formation in several plants, including species of the Lamiaceae family (Raja and Arockiasamy, 2008; Papafotiou and Martini, 2016; Islam et al., 2017).

The research carried out by Hrdlickov et al. (2014), demonstrated that the highest multiplication rate of the species *M. didyma* was obtained on MS supplemented with 0.5 mg.L^{-1} Kn (1.90 ±0.31 shoots per explant) and 1.5 mg.L^{-1} Kn (5.6 ±2.16 nodes per explant), practically 2 times less compared to our achievements. On the other hand, in the medium supplemented with TDZ, a retardation of the development of both the number of axillary shoots and their length was observed, which also influenced the number of internodes (Table 1).

In parallel, in three variants, including the control variant, the development of axillary buds and the elongation of shoots, as well as the initiation of rhizogenesis were established (Figure 2, Table 1).

Rooting of shoots is a very important part of any *in vitro* micropropagation scheme. A few plant species form adventitious roots on shoots at the stage of adventitious or axillary shoot elongation, but it is usually necessary to adopt a separate rooting procedure using special media or methods to induce root formation (George et al., 2008). Unlike the variant supplemented with TDZ, which provides for the initiation of direct shoot formation on a nutrient medium and the induction of rhizogenesis on another medium (two stages of *in vitro* seedling), the control variant, and those supplemented with BA and 2iP provide for *in vitro* cultivation only on a single nutrient medium (in a single stage) (Figure 2). Table 1 shows the effect of cytokinins on root formation. Except for TDZ, root induction occurred

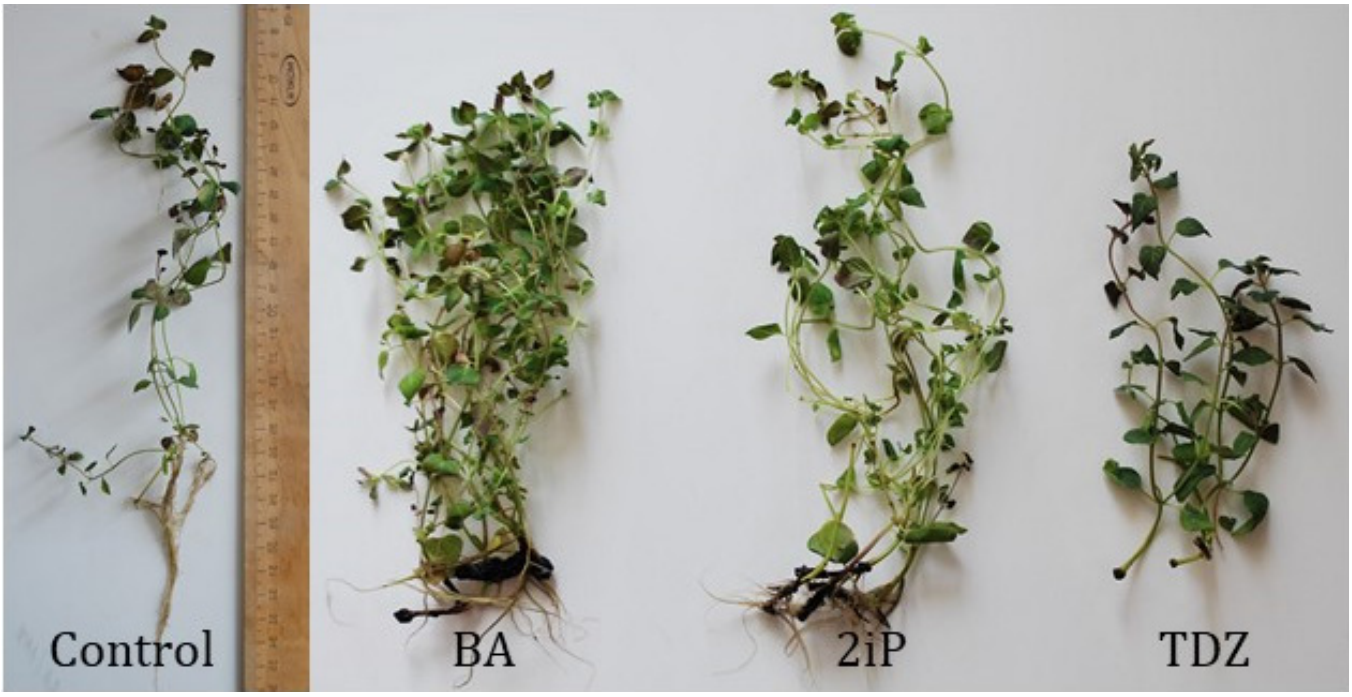

Figure 2 The plants of *Monarda fistulosa* L. at the final subcultivation stage, after 6 weeks of culture

in the rest of the variants. The addition of TDZ to the culture medium decreased not only shoot length but also root formation. The highest number of roots was observed in the presence of 2iP (7.9 ±2.02 roots per explant), followed by control (6.1 ±1.52 roots per explant). Instead, in the control variant, plants with the longest roots were obtained (4.4 ±2.01 cm). In this way, the elaborated micropropagation process allows for to reduction of two times the costs of reagents and energy resources for *in vitro* cultivation, increasing the multiplication coefficient from 13 to 32. Our further research will consist in presenting the results of the effects of the tested cytokinins on biomass accumulation and comparing the active principles accumulated in plants grown under artificial conditions with those grown under natural conditions.

Conclusions

Our research demonstrated that nodal explants of *Monarda fistulosa* possess a high potential for *in vitro* micropropagation through axillary bud proliferation. The procedure presented in this study is an efficient, rapid, and simple technique for axillary bud initiation and shoot proliferation on a relatively simple medium. The micropropagation of *Monarda fistulosa* plants using ½ MS nutrient medium containing 0.5 mg.L^{-1} BA, offers the possibility to reduce the procedure expenses and to increase the number of plants multiplied 3 times during a subcultivation.

Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

This article doesn't contain any studies that would require an ethical statement.

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