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Faculty of Tropical AgriSciences

Department of Crop Sciences and Agroforestry

# Assessment of genetic fidelity of micropropagated plants and in vitro polyploidization in Monarda didyma L.

## - A medicinal plant

Master Thesis

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**Declaration**

I confirm, that I worked out this Master thesis "Assessment of genetic fidelity of micropropagated plants and in vitro polyploidization of *Monarda didyma* L. - A medicinal plant" alone and that I have used only literature that is cited and mentioned in references. I agree this work to be placed in the library of CULS Prague and was accessible to study purposes.

Prague April 2014

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Hrdličková Michaela

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**Abstract**

Crimson beebalm [*Monarda didyma* L.] is a medicinal plant belonging to family *Lamiaceae*, native to North America. Crimson beebalm has a high content of thymohydroquinone, dithymoquinone and thymoquinone. The main objective of this thesis was the development of an appropriate protocol for propagation of Crimson beebalm (*Monarda didyma* L.) *in vitro* by using nodal segments and to obtain tetraploid plants (2n=64 chromozomes) from diploid plants (2n=32) by *in vitro* induced mitotic polyploidization. For micropropagation the nodal segments were cultured on basal MS (Murashige and Skoog, 1962) medium supplemented with different concentrations of 6-benzylaminopurine (BAP), kinetin (KIN), indolyl-acetic acid (IAA) and naphthalene acetic acid (NAA) and with cytokinins/auxins combination of BAP with IAA and KIN with NAA for shoot and root induction. For the polyploidization nodal segments of Monarda were exposed to 40, 60 and 80 μM oryzalin for 24 and 48 h. Genetic fidelity in regenerated plants was assessed using RAPD (Randomly Amplified Polymorphic DNA) markers. The highest multiplication rate was obtained from MS medium containing 0.5 mg/l of KIN (1.90±0.31 shoots per plant) and 1.5 mg/l of KIN (5.60±2.16 new nodes on longer shoots). The best root induction was achieved on medium supplemented with 1.0 mg/l IAA (6.70 ± 4.84 roots per plant). Cultivation time was 60 days. The percentage of survival of plantlets in *ex vitro* conditions was 30 %. Tetraploid plants were obtained in concentration 40 and 60 μM of oryzalin with treatment duration 24 h. Triploid plant was obtained in concentration 60 μM of oryzalin with treatment duration 48 h. In total the polyploidization efficiency was 1.92%. Analysis of RAPD primers assessed the genetic stability in micropropagated and polyploid plants.

Key words: Diploid · Flow cytometry · Genetic fidelity · *In vitro* · *Lamiaceae* · Micropropagation · *Monarda didyma* L. · Oryzalin · Polyploidization · Root induction · Shoot induction · Tetraploid

**Abstrakt**

Zavinutka podvojná [*Monarda didyma* L.] je lečivá rostlina patřící do čeledi Hluchavkovitých, která pochází ze Severní Ameriky. Zavinutka podvojná má vysoký obsah thymolhydrochinonu, dithymochinonu a obsahuje značné množství thymochinonu. Hlavním cílem této práce bylo stanovení vhodného protokolu pro množení zavinutky podvojné (*Monarda didyma* L.) v *in vitro* podmínkách za použití nodálních segmentů a získání tetraploidních rostlin (2n=64 chromozomů) z diploidních (2n=32) za použití indukované mitotické polyploidie *in vitro*. Pro mikropropagaci byly použity nodální segmenty, které byly kultivovány na MS (Murashige a Skoog, 1962) mediu doplněném různými koncentracemi 6-benzylaminopurinu (BAP), kinetinu (KIN), indolyl-3-octové kyseliny (IAA) a α – naftyloctové kyseliny (NAA) a kombinací cytokininů a auxinů, BAP s IAA a KIN s NAA, pro indukci výhonů a kořenů. Pro polyploidizaci byly nodalní segmenty vystaveny působení roztoku oryzalinu a to v koncentracích 40, 60 a 80 μM po dobu 24 a 48hodin. Genetická stabilita u regenerovaných rostlin byla stanovena použitím RAPD (Randomly Amplified Polymorphic DNA) markerů. Nejlepších výsledků pro tvorbu výhonů bylo dosaženo u MS média obsahující 0,5 mg/l KIN (1,90±0,31 výhonů na rostlinu) a 1,5 mg/l KIN (5,60±2,16 nodálních segmentů na delším výhonu). Nejvyšší tvorba kořenů byla dosažena při kultivaci na MS médiu s 1,0 mg/l IAA (6,70 ± 4,84 kořenů na rostlinu). Doba kultivace byla 60 dní. Podíl přežitých sazenic v *ex vitro* podmínkách byl 30%. Tetraploidní rostliny byly získány v koncentracích oryzalinu 40 a 60μM po dobu 24 hodin. Triploidní rostlina byla získána v koncentraci 60μM oryzalinu po dobu působení 48hodin. Celková úspěšnost polyploidizace byla 1,92%. Analýza RAPD primerů stanovila genetickou stabilitu u *in vitro* namnožených i u polyploidních rostlin.

Klíčová slova: Diploid · Flow cytometrie · Genetická stabilita · *In vitro* · Indukce výhodnu · *Lamiaceae* · Kořenová indukce · Mikropropagace · *Monarda didyma* L. · Oryzalin · Poplyploidizace · Tetraploid

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**List of the contractions used in the thesis**

AFLP - amplified fragment lenght polymorphism

ANOVA - Analysis of variance

BAP - 6-benzylaminopurine

CULS - Czech University of Life Sciences Prague

DAPI - 4'.6-diamidino-2-phenylindole

DMSO - dimethylsulfoxide

DNA - deoxyribonucleic acid

EDTA - ethylendiaminetetraacetic acid

FCM - Flow-cytometer

GA3 - giberellic acid

IAA - indole-3-acetic acid

ISSR - inter-single sequence repeat

KIN - kinetin

MS - Murashige and Skoog (1962) medium

NAA - α -Naphthaleneacetic acid

PCR - polymerase chain reaction

PGR - plant growth regulator

RAPD - random amplified polymorphic deoxyribonucleic acid

Tween 20 - polyoxyethylenesorbitan monolaurate

# 1. Introduction

Crimson beebalm (*Monarda didyma* L.) is a perennial herb belonging to the family *Lamiaceae*. It is native to eastern region of the USA (McClintock and Epling, 1942). In the middle of 18th century, Crimson beebalm was introduced as an ornamental plant in to Europe (Small, 2006).

In this plant are used aboveground parts containing active substances, thymochinon, thymohydrochinon and dithymochinon with wide treatment effects (Táborský et al., 2012). In addition to medicinal use, Crimson beebalm is used as a tea called Oswego tea and chilled beverages, also it is added to foods as a supplement as a substitute of sage. Essential oil, called bergamot is added as flavoring ingredient for perfumes (Small, 2006). It is reproduced *in vivo* mainly vegetatively by underground rhizomes, from which grow new stems, after is well reproduce by stem cuttings (Small, 2006).

Another method of multiplication can be used *in vitro* method micropropagation, which is one of the modern methods for plant propagation, in which a very small part of the parent plant derives a large number of individuals with same genetic information (Pavlová, 1992). In the available literature there are not scientific researches about micropropagation of this genus. Therefore we will investigate the most suitable *in vitro* propagation protocol for the production of *Monarda didyma* for production of this species. Determination of the optimal protocol for micropropagation of Crimson beebalm could help in the propagation of this species and even new cultivated varieties.

Literature about breeding of this species is limited. So we are interested in using *in vitro* technologies, like an *in vitro* introduction, micropropagation and conservation of *Monarda didyma*, genomic stability or variability of *in vitro* maintained plants have not been assessed yet. Genetic stability of micropropagated plants is desirable to obtain true-to-type plants. To the breeding programs belongs also polyploidization, which can serve the plants with higher yield or plants where are increased content of substances, like in this case for medicinal purposes.

However potential somaclonal variability obtained *in vitro* cultivation can serve as an important tool during breeding and selection of obtained variable somaclones some valuable traits such as resistance to pests and diseases.

Tissue cultures of medicinal plants are used also for producing active compounds for pharmaceutical industries (Sidhu, 2010).

This thesis is therefore focused on propagation of *Monarda didyma* plants by nodal segments and assessment of genetic stability in regenerates using molecular RAPD markers. Other goal is *in vitro* polyploidizatin of this medicinal plant.

# 2. Literature Review

## 2.1. Taxonomy and relative species

Linnaeus (1753) described the genus Monarda with five species from Canada, Virginia and Pennsylvania. Then the study of the genus Monarda was started around 1923 by Elizabeth McClintock and Carl Epling (McClintoc and Epling 1942). Genus was named after the Spanish botanist Nicholas de Monardes (Small, 2006). McClintock and Epling (1942) divided the genus into two subgenera *Eumonarda* a *Chelyctis*. They are different in the number of choromozoms. Subgenus *Eumonarda* has a basic chromosome number (n) 18 and subgenus *Chelyctis* has 11 chromosomes (Scora, 1967). Bushnell (1936) reported that chromosome number for *Monarda didyma* and *M. clinopodia* is (n) 16, but McClintock (in Scora, 1967) reported (n) 18 chromosomes in the subgenus Monarda. Scora (1967) reported (n) 18 for *Monarda didyma* and *M. clinopodia*, and (n) 16 for M. x media. Eumonarda may comprise two, sometimes more odd-whorls. These include *M.didyma, M. media, M. clinipodia* and varieties of the genus *M. fistulosa* (Scora, 1966).

The whole genus is widespread throughout North America, from the Rocky Mountains to the Atlantic coast, from Canada to Mexico (Prather et al., 2002), from mountainous areas in height 2000 m to the coastal habitats along the Atlantic coast in the south United States. We will find her at the edge of the jungle in Veracruz and Chihuahua Desert. In Texas, these plants survive on saline soils (Scora, 1967).

Monarda consists about 16 species and numerous subspecies distributed throughout much of North America (Whitten, 1981). Most species are perennial, but there are also annual species (Prather et al., 2002). In addition, there are other species and their hybrids which are grown for its ornamental purposes (Small, 2006).

In the mountains of eastern Tennessee and western North Carolina grow only three species: *Monarda didyma* L., *M.clinopodia* and *M.fistulosa*. *Monarda* x *media* Willdenow also occurs in these mountains, but it is a natural hybrid resulting from the hybridization and backcrossing the previous three species. These three parental species are different in color and the size of the crown (Whitten, 1981). For example, *M. clinopodia* has small white flowers and *M. didyma* has a large Damselfly flowers. This could be related to the ploidy level (McClintock and Epling, 1942).

*Monarda fistulosa* comes from California and Mexico. Flowers are purple and have a spicy flavor similar to thyme (Rausch and Lotz, 2005). It contains essential oil, which has

antibacterial activity, inhibiting the growth of mycoplasma and fungi candida. It also stimulates the antibiotic sensitivity of many Gram-negative bacteria. For its effects is designed as an ingredient burdock oil, which is used to treat ringworm called Seborrhea, whose main symptom is a dysfunction of the sebaceous glands in various areas of the skin. Most are affected by this disease people aged 17-24 years.

*Monarda punctata* grows in North America from the eastern and central United States to northern Mexico (Small, 2006). Flowers are spotted and yellow. Aroma of its leaves is comparable with the scent of oregano (Rausch and Lotz, 2005).
In the twenties was cultivated for the extraction of thymol and also used for medical purposes. Leaf extract was added to soft drinks. From this plant is pressed medicinal oil (Ottová, 2013).

*Monarda austromontana* is a slow growing annual from occurring in Mexico. Its taste is similar to the taste of oregano. This Monarda was used after many years by indigenous peoples of northern Mexico and south-east USA spicing dishes and tea calming the stomach (Ottová, 2013).

*Monarda citriodora* contains fragrant essential oils which can be substitute for true bergamot oil, which is typical for taste of Early Grey tea (Ottová, 2013).
Hybrids of *Monarda dydima* and *Monarda fistulosa* are resistant to rusts (Ottová, 2013).
The cultivar "Marshalls Delight" is a triploid and therefore is sterile. It is therefore possible to reproduce only vegetatively (Small, 2006).

### 2.1.1. *Monarda didyma* L.

Crimson Beebalm, otherwise known as Oswego-tea or Bergamot (Gleason and Cronquist, 1991), is a perennial herb, belonging to the mint family (*Laminaceae*). McClintock and Epling (1942) put it in the subgenus Eumonarda. It comes from the eastern region of the U.S.A. (Small, 2006). It is widespread in the area from New York to the northern Georgia. It grows in all altitudes, but most often in elevation from 1,000 to 2,000 meters above sea level, along the river coast, in seepage areas and in ditches along roads (McClintock and Epling, 1942).

Typical for the plant growth is lush and often becomes invasive plants for weed infestation of garden (Small, 2006). Usually begins to flower in early June, in full flower is about the third week in July and the end of flowering is approximately the third week in August (Egler, 1973).

It exists also a number of garden varieties, flowering variety of many colors that are created by crossing and breeding, but not so much used for therapeutic purposes or for tea preparation. These are the varieties for example 'Marshall's Delight' blooming bright pink (Rausch and Lotz, 2005). However it is triploid, thus is sterile. It is therefore possible to reproduce it only vegetatively (Small, 2006). Another variety is the 'Beauty of Cobham', which blooms luminously purplish pink. Flowers 'Croftway Pink' are the color of salmon, 'Schneewittchen' blooms white and deep red flower varieties 'Adam' and 'Gardeview Scarlet' ( Rausch and Lotz 2005).

Taborský et al. (2012) state that two distinct populations of *Monarda didyma* to two chemotypes, chemotype 1 and chemotype 2, differing content of the main components, which are carvacrol and thymol.

### 2.1.2. Origin and geographical distribution

Monarda comes from the eastern region of the U.S. (Fig. 1.). You can find it from the state Michigan to New York and from North Georgia to southern Tennessee. In 1754 was introduced as an ornamental plant in Europe. In half of 18th century, pioneer colonists brought the seeds of the Crimson Beebalm to England and from there were spread across Europe (Fig. 2.). Tentatively was grown on Crimea for oil, which is used as a flavoring ingredient in perfume (Small, 2006). In the eastern parts of North America are two forms of Crimson Beebalm, with narrow and broad leaves. Both forms can be found in New York and Pennsylvania, rarely to be found in the New England states in the Northeast U.S. Maryland, New Jersey, Michigan and Ohio. However, it seems that only narrow leaved forms of Crimson Beebalm occur in Virginia, North Carolina and Tennessee (McClintock and Epling 1942).

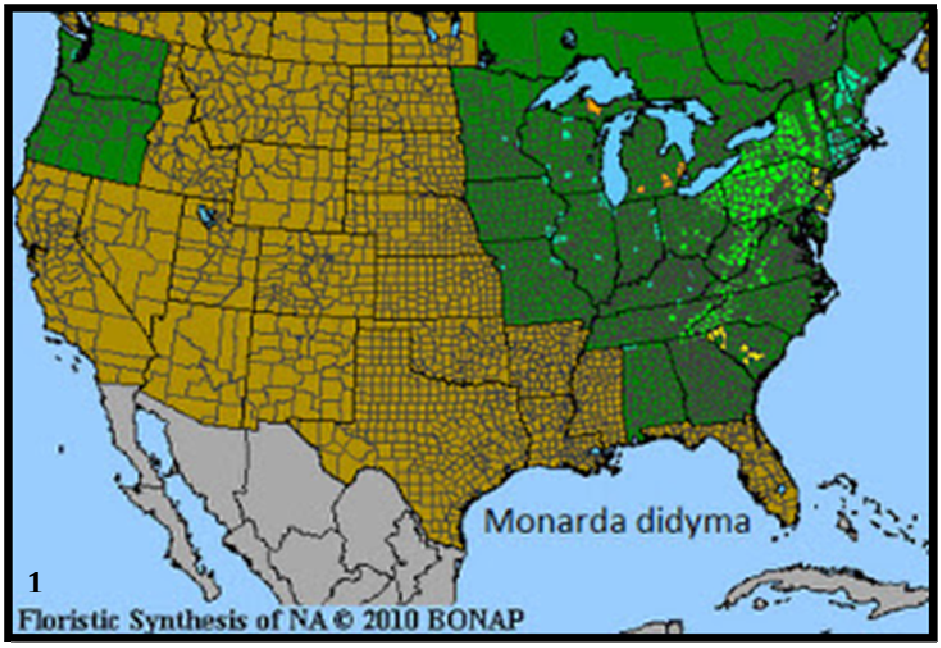



**Figure 1.** Distribution of Monarda didyma in USA.

**Source**: Restoring The Landscape With Native Plants (2011)

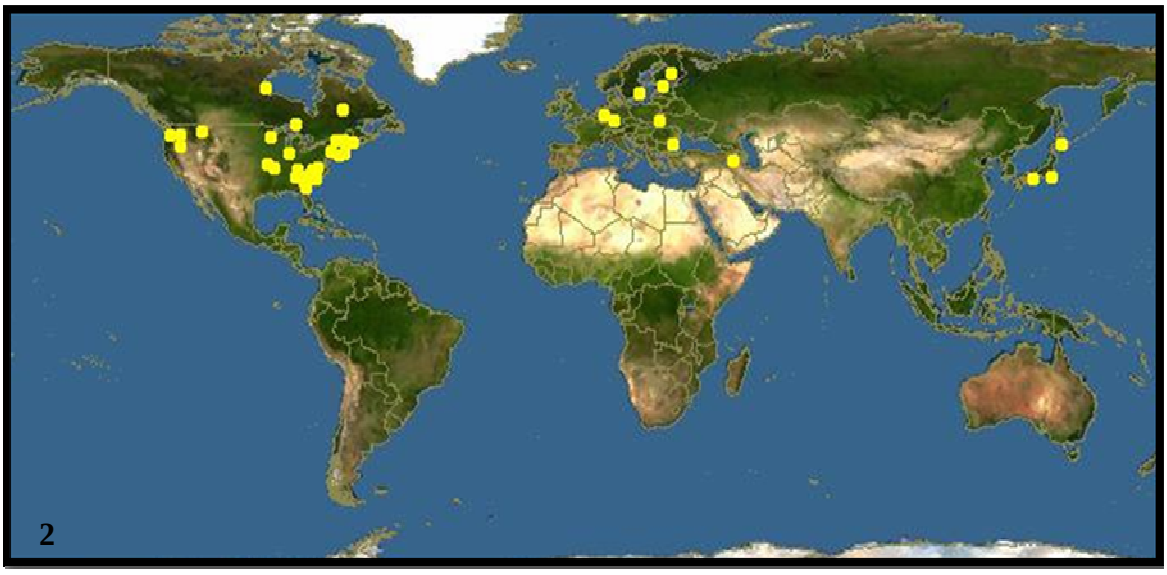



**Figure 2.** Distribution of Monarda spp. in the world

**Source:** Discove life (2014)

### 2.1.3. Botanical and morphological description

Crimson Beebalm is often found in thickets and along riverbanks. It forms clumps, sometimes up to as much 1 m in diameter (Fig.3.). In the wild in North America (for example, in the southern provinces Ontario and Quebec) it is possible to find plants that have escaped from cultivation (Small, 2006).

**Roots:** The root system of plants is flat, richly branched and breadth fast growing, soon forming dense stands (Rausch and Lotz, 2005). Form a trailing perennial rhizomes, from which spring grow new stems (McClintock and Epling, 1942).

**Stem:** The stem is about 70-150 cm high, usually branched, but may also be not branched, often hairy at the nodes (Fig.4) (Gleason and Cronquist, 1991).

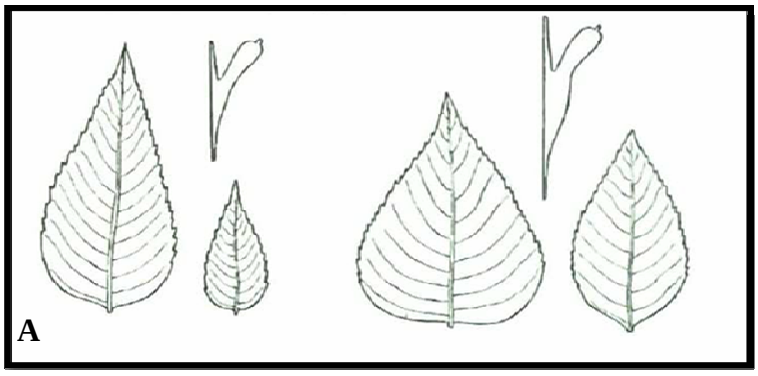
**Leaves:** Monarda leaves are thin, ovate or rhombic-ovate, almost lanceolate, 7-15 cm long and 2.5-6 cm wide, pointed, at the edge serrated, the base nearly rounded (Gleason and Cronquist, 1991) with long petioles about 1-4 cm (McClintock and Epling, 1942). The bracts are lanceolate, usually red dotted (Gleason and Cronquist, 1991). The upper leaves and bracts have often red or bronze color (Small, 2006). Differences were observed variation in the size and shape of leaves (Fig. 5). There are plants with broad leaves 3.5 to 8.5 cm long and 6-13.5 cm and plants with narrow leaves, wide range 3-5.5 cm long and 8-14.5 cm (McClintock and Epling, 1942).

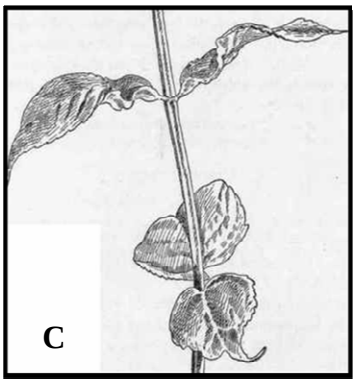
**Figure 3**. Whole plant

**Source:** Landy (2007)

**Figure 4.** Stem

**Source**: Baskauf (2004)





**A.** On the left side are narrow leaves and on the right side are broad leaf forms of *Monarda didyma*; **Source**: McClintock and Epling (1942)

**B.** Leaf of *Monarda* spp.; **Source:** Fenwick (2011)

**C**. Position of the leaves; **Source**: Theodora (1893)

**Figure 5.** A-C: Leaves

**Flowers:** Flowers are scentless and pollinated by birds. The inflorescence consist cymes, usually only one major with average 1.5-3 cm wide. Crimson Beebalm can also consist of two cymes. The outer bracts are sessile or nearly sessile (McClintock and Epling, 1942), narrowly pointed, bent outwards (Whitten, 1981), green or colored in the same color of the crown. They are hairy, with the same kind of hair as having leaves (McClintock and Epling, 1942). Calyx is a large 10-14 mm, glabrous or slightly pubescent, almost in the esophagus glabrous, teeth 1-2 mm long, finely pubescent, not glandular, above the base triangular (Gleason and Cronquist, 1991). The tubular corolla suddenly expands upward

(Whitten, 1981), has a scarlet to crimson color, rarely deep red purple. It is 3-4 cm long and extends in a straight upper lip and the tape, twisted lower lip (Whitten, 1981). The upper lip is nearly straight, long about half of the corolla tube (Gleason and Cronquist, 1991), is not densely hairy on the top and at the mouth of the cup is bald (Fig.6) (Egler, 1973).

The rods are placed 3-6 mm deep (McClintock and Epling, 1942). Threads are connected to the crown near the mouth, with two anthers, which are connected and stand under upper lip as the pistil. Adult flowers bloom from the center to the periphery. In the sea level 1,200 m.n.m. this plant blooms between 30th June and 30 July (Whitten, 1981). It was observed correlation length and width of the crown of leaves. Broad leaves form of Monarda has a double crown 35 mm long in diameter and narrow leaves form has it 39 mm long in diamete. Small-flowered Monarda forms are exceptional. Size of the crown is less than 35 mm and not sudden flare up crown (McClintock and Epling, 1942). Cultivars have a diverse coloration (Small, 2006). The most effective pollinator of this species is the Ruby – Throated Hummingbird (*Archilochus colubris*), lured by the relatively big amount of sweet nectar in the flowers, which contains mainly sucrose, small amounts of fructose and glucose (Whitten, 1981).


**A.** Flowers of *Monarda didyma*.

**Source:** Ottová (2013)


**B.** Calyx of *Monarda* spp.

**Source:** Fenwick (2011)


**C.** Crown of Crimson Beebalm

**Source:** Fenwick (2011)

**Figure 6**. **A-C:** Flowers

## 2.2. The use and importance

Small (2006) reported that in the past people used the *Monarda didyma* as a medicinal herbs for colds and sore throat. Essential oils from the leaves have been used to bronchial infections, colds, flu, flatulence, kidney problems, nausea, inflammation, pneumonia and during the attack internal parasites. Fresh tea prepared from the leaves is considered as a tranquilizer. Indians from the Cherokee tribe used *Monarda didyma* and *Monarda fistulosa* to treat "female problems", colds, flatulence, headache, colic, fever, heart disease, weak stomach, indigestion, bleeding from the nose, hysteria and insomnia. Currently, this plant is used mainly for tea called Eswego -tea (Prather et al., 2002).

Further, Small (2006) states that it is a good mix leaves of crimson beebalm with other herbs or possibly in preparation chilled bevarage with lemon slices. Young shoots and fresh leaves have a pungent citrus aroma and bitter taste. Very well it is suited as a supplement drinks wine, soft drinks, juices, jellies and cheeses.

The leaves are best suited to pork and veal meat. They are also used as a substitute for sage in the stuffing and meat dishes. Leaves for immediate processing can be collected at any time. The leaves and flowers are used to dry, which should be harvested in mid-summer. Essential oils, which are obtained from Crimson Beebalm, called bergamot oil, are added as a flavoring ingredient in perfumes. Plants for obtaining oil are harvested in full bloom. The essential oil is obtained by steam distillation in which steam was passed through fresh chopped pulp and oil, together with the steam condenses (Small, 2006).

## 2.3. Chemical composition

Crimson Beebalm contains substantial quantities of essential oil. The essential oil is obtained by steam distillation. The yield from the fresh leaves is around 0.3-1.0 % of dried leaves about 2.7 to 3.1 % (Small, 2006).

It also contains a considerable amount of thymoquinone (TQ), where were detected anticancer properties, anti-epileptic, antimicrobial, antioxidant, hepatoprotective and immunomodulatory effects. Together with TQ are found in essential oil compounds thymohydroquinone (THQ) and dithymoquinone (DTQ). For THQ were detected antibacterial, antifungal, anti-inflammatory and antioxidant properties. DTQ shows cytotoxicity to human tumor lines. These compounds were found in several genera of the *Lamiaceae* family such as Agastache, Coridothymus, Monarda, Origanum, Satureja, Thymbra and Thymus. Chemotype 2 of *Monarda didyma* exhibits more quantity TQ

and THQ than chemotype 1, more often in flowers than in the leaves and stems (Taborský et al., 2012). Taborský et.al. (2012) reported the results, whose showed that detectable amounts of these compounds have been found in three species of both Monarda (*M. didyma, M. media, and M.menthifolia*) and Thymus (*T. pulegioides, T. serpyllum, and T. vulgaris*) genera, two Satureja (*S. hortensis and S. montana*) species. Nigela sativa seeds, which are generally considered as the main natural source of both of these compounds, contain THQ 530 $mg.kg^{-1}$ and TQ 1881 $mg.kg^{-1}$. Therefore *M. didyma* and *M. media* can be recommended as new prospective natural sources of THQ and TQ for pharmaceutical industries.

## 2.4. Ecology

### 2.4.1. Cultivation

*Monarda didyma* grows in clusters. Underground shoots extend from the centers of clusters in all directions and soon new stems grow. It is characterized by lush growth. After 3-4 years is able to expand by more than one meter and often becomes invasive plant. It is good if crimson beebalm grows in rich, moist, light, granular (Page, 1980) and slightly acid soils, with a pH of about 5.5-6.5 (Hayward, 1983) with a high content of organic matter. Roots do not grow deep. In hot days, it should not be dry and in winter, when it is cultured in heavy soils tend to death (Page, 1980). Each cluster should be spaced at least 25 cm between plants for enough air flow. The mulch is recommended to use needles rather than grass (Small, 2006). In hot summer suits to them slight shadow (Goode, 1984) and in the dry season should be enough moisture (Kublick, 1990). Best of bloom is in the sun with moist soil, but will grow well in dry or semi-shade. Plants should be protected from the fierce midday sun (Rausch and Lotz, 2005).

For its unpretentiousness, tall stature and long flowering period is well suited to common perennial beds or before the trees. Monarda likes a sunny position, but is satisfied even worse by exposure to or even partial shadow. She is doing the best quality and nutritious in drained soil. Winters are not afraid, is accustomed to the cold. If you start with Monarda growing from seed sowing is done in May and June, planting into the soil in July-August (Ottová, 2013).

### 2.4.2. Diseases

Monarda is susceptible to powdery mildew diseases, especially when grown in areas without good air circulation and rusts. If it is attacked by rust it is better after flowering cut the plant to 8 cm and the attacked part burn to prevent the spread of infection. There can be used a fungicides. It is important to remember that the parts of plants treated by fungicides are not edible (Small, 2006). Leslie's (1992) writes that the genus Monarda is prone the mold. It also states that *M. fistulosa* a *M. didyma* are remarkably resistant.

Plants in the genus *Monarda* produce complex essential oils that contain antifungal compounds. Gwinn et al. (2010) had research were identified selections of *Monarda* that can reduce *Rhizoctonia* damping-off of tomato. They reported that *Monarda clinopodia*, *Monarda didyma* and *Monarda fistulosa* have positive influence to reduction of *Rhizoctonia* damping-off of tomato.

### 2.4.3. Propagation

## 2.4.3.1. Generative propagation

Reproduction by seeds is not so common, but it is possible, mainly for breeding. Generative reproduction of the daughter cells, prevents sexual process. This means the fusion of two gametes to form a zygote. This process prevents pollination (Novák and Skalický, 2008). Generative propagation method is essential for breeding, because in seminal generation affects the combination of parental characteristics and in underpinning the desired clones can be further vegetative reproduction, at which the elimination heterozygosity and conservation of the F1 generation (Horáčková a Domkářová, 2003).

## 2.4.3.2. Vegetative propagation

Plants form, especially in the autumn, creeping underground rhizomes growing to the width from which grow the stems on spring (Small, 2006). In this way, the plant herself easiest and fastest multiplies. Therefore, if we want multiply Monarda is the best multiply it by dividing clumps in the spring by nursery plants. Separation should be repeated after several years for restoration of its vitality (Garland, 1979). The divisions should be planted slightly deeper than growing the parent plant (Lima, 1986). Also, these plants can be propagated by stem cuttings. Cuttings of young stems, removing in spring or in summer, can be successfully used to propagation (Lesli's, 1992). By using hormonal

stimulants cuttings take root in 10-14 days. For reproduction can be used also multiple shoots growth peaks, which can take root in light soils. During the first year, the plants are well established and remain productive for 5-7 years. It is recommended in the first year of growth the flowers top cut away before blossom, for supporting vegetative growth (Small, 2006).

## 2.5. *In vitro* micropropagation

Micropropagation is one of the options vegetative plant propagation *in vitro*. From the very small portion of the parent plants will produce a large number of small subsidiary cells (Altman and Loberant, 2010).

*In vitro* refers to a method of culturing plant material. This means the cultivation the cultures in glass under sterile conditions on a nutrient media. Cultivation of plant material *in vitro* is primarily used for micropropagation, but also to sanitation plant material, studying physiological processes, producing secondary metabolites and genetic manipulation (Pavlová, 1992). *In vitro* cultures of plants represent sterile cultivation of isolated parts of plants aseptically grown or surface-sterilized. Then are stored in sterile environment and cultured (Sakongo, 1997).

In the early 60's (1954) was conducted initial successful regeneration of plants from one cell (Pavlová, 1992). For many species, micropropagation is cost-effective way to achieve high quality and large amounts of plant material compared to conventional vegetative propagation (Altman and Loberant, 2010).

On the based of assumption of growth and development explant cultures, totipotency of plant cells, which is the ability of plant somatic cells and tissues to develop into whole new plant (Sidhu, 2010). These cells are provided with complete genetic information and structural and metabolic apparatus, therefore, they have the ability to grow, divide and differentiate. The growth and development of explants in vitro are affected by the genotype of the plant material, but also by the composition of the nutrient medium and the physical conditions of cultivation. The process of cultivation in vitro can be optimized and there we can eliminate pathogens (Pavlová, 1992).

In the technological process, there are preferred nodal cultures, shoot tips and axial buds. They are organized structures of the stem and are cultivated for the formation of new shoots. During using of nodal culture, nod is explanted with close upper and lower portions of stem, axial bud or a part or the whole petiole with the leaf blade. When is used the culture of shoot tips, there is implanted shoot tip of shoot. These cultures are most

demanding on the composition of media, but may be more influenced by the content of growth substances in medium (Pavlová, 1992).

The advantages of *in vitro* micropropagation are higher rate of multiplication, environment can be controlled, plants are available during whole year, production of clones with desired characteristics. It is used also for conservation of threatened plant species and preservation of genetic material (Sidhu, 2010). The objective of this technique is to obtain a large number of genetically identical plants, identical to the starting material. Cloning conditions *in vitro* culture (micropropagation) can already calculate the common methods of clonal (vegetative) reproduction of plants (Novák, 1990).

### 2.5.1. *In vitro* micropropagation in *Lamiaceae*

The plants from *Lamiaceae* family are commonly used as aromatic herbs for flavouring foods, beverages, confectionary and medicinal products. Essential oils produced by *Thymus* spp. are important for example in cosmetic products, but for the medicinal purposes too, due to their antiseptic and antimicrobial properties. Beneficial uses of tissue culture for the purpose of extraction of secondary metabolites include evading of collection of endangered wild species, production of secondary metabolites irrespective of seasonal and climatic conditions, and rapid production of secondary metabolites due to rapid growth of cultures *in vitro* (Arikat et al., 2004).

To meet increasing business demands, big range of aromatic plants is desirable for the global pharmaceutical and aromatherapeutical industries (Lange, 2004). The rapidness of tissue culture techniques can be advantageous for the continuous provision of a plantlet stock for field cultivation (Reddy et al., 2001) and may further compliment breeding programmes. We will therefore investigate the most suitable *in vitro* propagation protocol for the production of *Monarda didyma* for production of this species. To our knowledge, there are no scientific reports on the micropropagation of this species.

*In vitro* initiation of plants in *Lamiaceae* is a very useful tool for production for *Salvia officinalis* (Gostin, 2008), *Salvia fruticosa* (Arikat et al., 2004), *Lavandula pedunculata* (Zuzarte et al., 2010), *Lavandula latifolia* (Sánchez-Gras et al., 1996), *Ocimum basilicum* (Dode et al., 2003).

### 2.5.2. Plant growth regulators (PGRs)

Growth hormones regulate physiological and morphological processes in plants and are also known as phytohormones. PGRs are synthesized by plants, thus many plant species can grow without external medium supplements. Phytohormones can be added into

cultivation medium to improve plant growth and to better metabolite synthesis. Nevertheless, inadequate amount of growth hormones can cause morphological and physioloical abnormalities (Sidhu, 2010). In table 1 are shown types of hormones and their functions in plant.

**Table 1.** PGRs and their functions in plants

| Types of hormones | Functions | PGRs | References |
|---|---|---|---|
| **Cytokinins** | cell division, shoot formation, development and proliferation | BAP<br>KIN<br>ZEA | Rout et al.,2000<br>Bhojwani et al., 1966<br>Rayns et al., 1993 |
| **Auxins** | cell division, bud formation and root differentiation | IAA<br>NAA<br>IBA | Rout et al.,2000<br>Bhojwani et al., 1966<br>Rayns et al., 1993 |
| **Gibberellins** | extension, growth and flowering | $GA_3$ | Bhojwani et al., 1966 |

## 2.6. Plant breeding

Plant breeding is playing important role for the improvement of the plant's characteristics. The aim of plant breeding is to get better quality, diversity and production of important crops in agriculture or horticulture and also to develop plants which are in better conformity to human needs.

It can be done by many different techniques from selection of plants with tempting characteristics for propagation to more complex molecular techniques. Breeding of new varieties is the process of crossbreeding and subsequent selection. This process can be accomplished through remote hybridization, polyploidy (increasing number of chromosomes), heterosis (increasing value of parameters F1 hybrids), and mutation induced by ionizing radiation or chemical compounds (Ondřej and Drobník, 2002).

### 2.6.1. Polyploidy

Changes in the number of chromosomes in plants play an important role in their evolution. It is reported that about 30 to 35% of the angiosperm plant species and about 70% of grass species are polyploid. The natural (spontaneous) polyploidy, which occurs freely acting natural influences in the plant kingdom is quite popular. Polyploid species are important agricultural crops in human nutrition like a potatoes, wheat, oilseed rape or in animal nutrition like oats, alfalfa, etc.. These polyploid species originated naturally as a result of evolution. (Briggs, 1967; Chloupek, 1992; Novak, 1990)

Polyploidy is defined as the property of three or more complete sets of chromosomes. Most of eukaryotic species are diploid. They have two sets of chromosomes, one set inherited from each parent (Ramsey and Schemske, 1998).

Polyploidy is an important option of chromosome evolution in many eukaryote species. Polyploids differ from their diploid originators in morphological, ecological, physiological and cytological characteristics. As a result, they have broader ecological tolerances and have a different geographical distribution (Dewitte et al., 2009).

According to their origin, polyploids are classified as autopolyploids and allopolyploids (Stebbins, 1950, cit. Dhooghe et al. 2011). An autopolyploid originates from the doubling of the chromosome number of a one diploid species which concludes in two or more pairs of homologous chromosomes. An allopolyploid is obtained after hybridization between different species and consequent polyploidization (Dhooghe et al., 2011). Grant (1981) discussed multivalent frequency in an autotetraploid line of Zea mays (x = 10). However, recent molecular investigations have demonstrated that diploid corn is itself a segmental allopolyploid (Gaut et al., 1997). At present, no autopolyploid genome is under the genetic control enjoyed by allopolyploid crops, such as cotton (Gossypium), tobacco (Nicotiana), wheat (Triticum), Brassica, Arabidopsis suecica, or corn (Zea mays), a segmental allopolyploid (Gaut et al., 1997).

Polyploidy as a breeding method is mostly used by many cross-pollinated species. It is particularly suitable for those species which have a smaller number of chromosomes and are used for vegetative plant parts (tuber, aboveground mass, etc.) and which consist a large number of seeds (Rod et al., 1982; Udall et al., 2006).

Polyploidy makes increased production capacity vegetative parts of plants (larger cells, larger plant organs), but reduces seed production (for disturbances in the formation of gametes and zygotes). Polyploid species generally forms less pollen and tend to be less fertile (Chloupek, 1992; Udall et al., 2006).

The consequences of polyploidy can be genetic. There is more talents in the locus, the result is more of the same chromosome pair of alleles in gametes and in zygotes and their sets can be homozygous and heterozygous. Genetic variability increases with changed genetic background. Faults develop in segregation and in dislocation of chromosomes in meiosis, which leads to the formation of defects and sterile gametes. Crossing tetraploids with diploids produce triploids, sterile or reduced fertility (Briggs et al., 1967; Rod et al., 1982)

Other results are morphological changes. For polyploids is typical an increase in volume of the cell nucleus, the cytoplasm and the whole cell. The vegetative parts of plants increase, but it is not proportional to the degree of polyploidy. Polyploid plants are characterized by changes in the number of leaf stomata, the number of chloroplasts, enlarged clamping cells, larger flowers, pollen and fruits. They have also bigger and stronger trichomes (Chloupek, 1992). Pires et al. (2004) have generated 20 allopolyploid lines of Brassica napus that are genetically identical. Yet, after just a few generations, these lines show significant differences in flowering time. Morphological changes are used for preliminary identification of polyploids in breeding.

Futhermore, there are changes in environmental requirements. Polyploids have higher demands on the temperature during germination, higher moisture requirements, lower transpiration rate and slower assimilation and respiration (Chloupek, 1992).

Increasing the chromosome numbers can sometimes increase concentration of certain secondary metabolites. This is especially useful to improve the production of natural plant products (Dhawan et al., 1996). For example in *Salvia miltiorrhiza* was reported higher contents of tanshinones in tetraploid plants than that in their diploid counterparts (Gao et al., 1996).

Polyploids usually have a longer growing season, often greater endurance and greater cold resistence, but often more sensitive to leaf diseases (Chloupek, 1992; Rod et al., 1982).

In polyploid species exist polyploid limit, which is the optimal number of chromosomes for a positive manifestation of the trait or feature (Briggs et al., 1967).

Ploidy is represented by the notation x, where a nucleus with two complete sets of chromosomes is referred to as a diploid (2x), three complete sets of chromosomes is a triploid (3x), four complete sets a tetraploid (4x), five complete sets a pentaploid (5x). The

highest level of ploidy nowadays known is in angiosperms an 80-ploid (80x*) Sedum suaveolens* Kimnach from family *Crassulaceae* (Otto et al., 2000).

In plants, polyploidy represents a major mechanism of adaptation and speciation. It is estimated that a high percentage of angiosperms are polyploids and also many cultivated agricultural crops, flowers and fruits are polyploids. Agronomic crops such as wheat (*Triticum aestivum L*.) (Udall et al., 2006), potato (*Solanum tuberosum*) (McGregor et al., 2000), cotton (*Gossypium* spp.) (Udall, 2006), alfalfa (*Medicago sativa*) (Udall et al., 2006) and sugar cane (*Saccharum* spp.) (Cordeiro et al., 2000) may also be polyploids. Polyploids often have improved horticultural or agronomic traits such as larger fruit, thicker leaves and robust stems (Kehr, 1996). Polyploid plants are also found in the ornamental horticulture industry and they may have thicker flower petals that last longer than their diploid counter-parts (Kehr, 1996). Common polyploid bedding plants include dahlia (*Dahlia* spp. Cav.), pansies (Viola spp. L.) and chrysanthemum (*Chrysanthemum* spp. L.). Polyploid plants frequently occur plants used in the fruit industry. Polyploid fruit crops include sour cherries (*Prunus cerasus*), strawberries (*Fragaria* spp.), kiwi (*Actinidia deliciosa*), blueberries (*Vaccinium* spp.) (Ahokas, 1999; Tavaud et al., 2004; Udall et al., 2006).

There are two mechanisms used for production of polyploid. Mitotic polyploidization, which is based on the doubling of somatic tissues and meiotic polyploidization which generates 2n gametes (Ramskey and Schemske, 1998).

### 2.6.1.1. Mitotic polyploidization

Mitotic polyploidization is based on the doubling of somatic tissues and is created by using of mitosis spindle inhibitors as colchicines, oryzalin or trifluralin (Wan et al., 1991).

The first applications of mitotic polyploidization were induced on plants established in soil in the 1930s (Blakeslee and Avery, 1937), when the colchicine was the most commonly used mitotic inhibitor. Mitotic polyploidization in vivo was successful induced for sugar and fodder beet, ryegrass and red clover (Dewey, 1980, cit. Dhooghe et al, 2011). Blakeslee (1939) reported the successful polyploids induction using colchicine in 41 different species, 24 genera and 14 families of flowering plants included *Viola tricolor*, *Petunia axillaris* and *Datura* sp.

The first in vitro chromosome doubling was in experiment with tobacco (Murashige and Nakano, 1966). *In vitro* induction of polyploidy became more popular with the development of plant tissue culture.

### 2.6.1.2. Meiotic polyploidization

Meiotic polyploidization generates 2n gametes (Ramskey and Schemske, 1998). Nowadays, the interest of using 2n gametes in plant breeding is increasing, sexual polyploids have been demonstrated to be very useful for crop improvement (Ramanna and Jacobsen, 2003). Meiotic chromosome doubling results in larger genetic variability and heterozygosity than does mitotic chromosome doubling (Carputo et al., 2003). Doubling of chromosomes can go to results in mixoploids with different ploidy levels in different plant cells or organs (Cohen and Yao, 1996).

### 2.6.1.3. Antimitotic agents

The change of the plant's cell cycle can be the method of synthetic production of doubled chromosomes *in vitro* plants (Dhooghe et al., 2011), which can be done by using antimitotic agents. The plant cell cycle is divided in 4 phases to a G1-phase, an S-phase, a G2-phase and an M-phase (mitosis) (Francis, 2007). Individual phases of the cell cycle can be affected by different chemical compounds. The most used mitotic inhibitors are those that operate in metaphase. During metaphase, a mitotic spindle of microtubules appears from the microtubule organizing centre. This spindle is essential for correct polar migration of chromosomes during the anaphase (Dhooghe et al., 2011).

Antimitotic agents like colchicine, colcemid, acenaphthene, dinitroanilines (oryzalin, trifluralin, benfluralin, dinitramin), phosphoroamidates, benzamides, benzoic acid disturb the metaphase by connecting with the alfa and beta dimmers, thus reducing the attachment of new dimmers on the assembly side of the microtubule, without reducing degradation of the microtubule at the disassembly end (Devine el a., 1993; Vaughn, 2000). The result is that disassembly is faster than assembly and microtubules are depolymerized. The most commonly used mitotic inhibitor is colchicine. Colchicine is an alkaloid that effectively stops mitosis at the anaphase stage. It is extracted from the seeds and bulbs of Colchicum autumnale, a wild meadow saffron and Colchicum luteum (Eigsti and justin, 1955).

Nevertheless, colchicine causes effects such as sterility, abnormal growth, chromosome losses and gene mutation in many plants species (Luckett, 1989, cit. Dhooghe

et al., 2011). Additionally, colchicine is very toxic to humans due to its high affinity to microtubules of animal cells (Morejohn et al., 1984). Many authors have reported the successful use of colchicine to convert tetraploids from diploids in many plants: Zantedeschia (Cohen and Yao, 1996), *Punica granatum* (Shao et al., 2003), *Gerbera jamesoni*i (Gantait et al., 2011), *Ocimum basilicum* (Omidbaigi et al., 2010) etc.

Other usually used antimitotic agents are herbicides. Herbicides have more affinity for plant tubulin dimers than colchicine. Therefor they can be used in lower concentrations then colchicine (Morejohn et al., 1987). Herbicides are not as toxic as colchicine. A dinitroaniline herbicide (3.5-dinitro-N4, N4-dipropylsulphate), Oryzalin, has a much greater affinity for plant than animal microtubules. Oryzalin has the important advantage, because is less hazardous to human health (Morejohn et al., 1987). For these reasons mitosis-inhibiting herbicides were considered like alternative for colchicine (Bartels and Hilton, 1973, cit. Dhooghe et al., 2011). Polyploids of many plants were achieved through oryzalin treatment: Rosa (Kermani et al., 2003), Spathiphyllum (Eeckhaut et al., 2004), Lilium (Takamura et al., 2002), *Solanum L*. (Chauvin et al., 2003), *Pyrus L*. (Bouvier,2002).

Bouvier et al. (2002) reported that 200 μM – 300 μM concentrations of oryzalin were required to induce polyploidy in *Pyrus L*. In *Solanum L*. the most effective treatment for producing tetraploids was a 24 hour treatment with 28.8 μM oryzalin solution (Chauvin et al., 2003). The optimal oryzalin concentration and treatment duration for polyploid induction varies among species and must be determined by the experiences.

### 2.6.1.4. Methods of polyploidy detection

Determination of polyploidy can be done by several methods. We can use flow cytometry, chromosome counting and evaluation of morphological or anatomical parameters.

**Flow cytometry:** In plant genomes studies, flow cytometry is a relatively quick, convenience and dependable tool for various applications ranging from basic research to breeding uses. Flow cytometry can be used for the following purposes: nuclear genome size determination, which can be achieved by measuring the nuclei of sample in comparison to a reference standard, after for the cell cycle analysis to study cell populations in G1, S and G2 phases and for ploidy level determination after interploidy crosses, haploidization and polyploidization treatment (Galbraith et al. 1997; Eeckhaut et al. 2005).

Flow cytometry is the most used method for detecting of polyploidy. The most concrete method is combination of flow cytometry and chromosome counting. Morphological or anatomical parameters can be used as primary selection criteria of polyploids (Dhooghe et al., 2010).

In case of flow cytometry, there is determined relative DNA density in cell nuclei and level of ploidy in tested samples (Greplová et al., 2003). Analyses of nuclear DNA are based on relative intensity fluorescence of cell nuclei stained by DNA fluorochrome (Fig.9). The final histogram shows the dominant peak belonging to nuclei in G1 phases of cell cycle.

Flow cytometry is a rapid method for ploidy testing and permits the examination of large amount samples in relatively very short time (Doležel et al., 2007). Flow cytometry does not involve roots or acclimatized plants so it is possible to analyze the samples of polyploidized plants in very early stage of development which is very important to save time (Väinölä, 2000).

**Chromosome counting:** This is a concrete method for determination of the real chromosome number (Doležel et al., 2007). For this method, the best types of tissue parts are root tips about 2 cm long, which are the most suitable source of mitotic cells for counting of chromosome. Other suitable sources are young buds, flower buds, leaves and callus. Chromozomes in root tips were observed in *Ullucus tuberosus* after polyploidization of diploid plants to octaploids (Fig.7) (Viehmannová et al., 2011)

**Evaluation of morphological or anatomical parameters:** In indirect polyploidy detecting methods are the morphological and anatomical evaluations (Viehmannová, 2009). They are simple, but often inexact. The commonly used characteristics are related to the stomata and determination of stomata size (Cohen and Yao, 1996). In general, diploids have smaller stomata than their tetraploid derivatives. Larger stomata of induced polyploids were observed in: *Zizyphus jujuba* (Gu et al., 2005), *Pyrus pyrifolia* (Kadota and Niimi, 2002) and *Nepenthes gracilis* (Fig.8.) (Fong, 2008) and many others. Stomatal density can be also changed by induced polyploidization. Yang et al. (2006) reported lower density of stomata in polyploids of *Vitis vinifera*. However, confirmation of polyploidy by flow cytometry or by direct counting is necessary, when morphological parameters are used as primary selection criteria (Zhang et al., 2010).

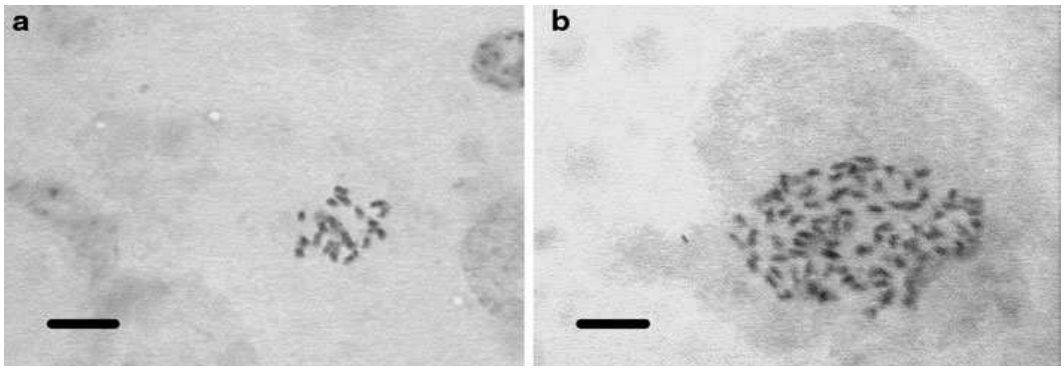



**Figure 7**. Chromozomes of root tips in *Ullucus tuberosus*.a- diploid (2n = 2x = 24); b- octoploid (2n = 8x = 96)

**Source:** Viehmannová et al. (2011)

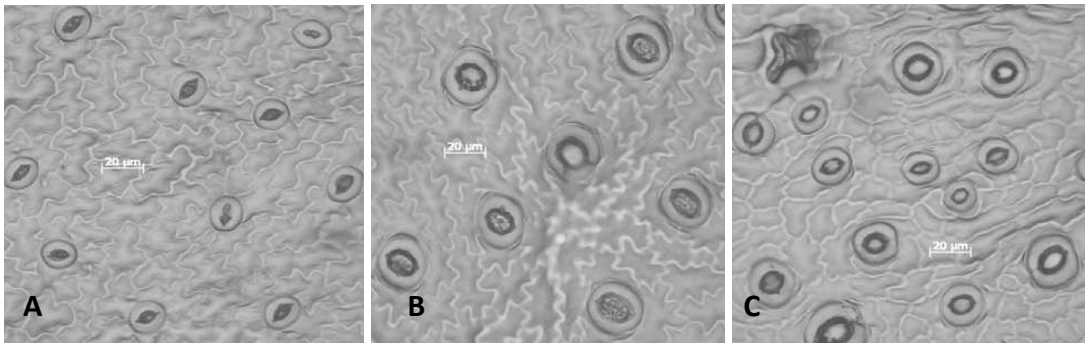



**Figure 8**. Stomata cells of *Nepenthes gracilis* from abaxial leaf imprints of (A) diploids, (B) tetraploids and (C) mixoploids (bar= 50 μm).

**Source:** Fong (2008)

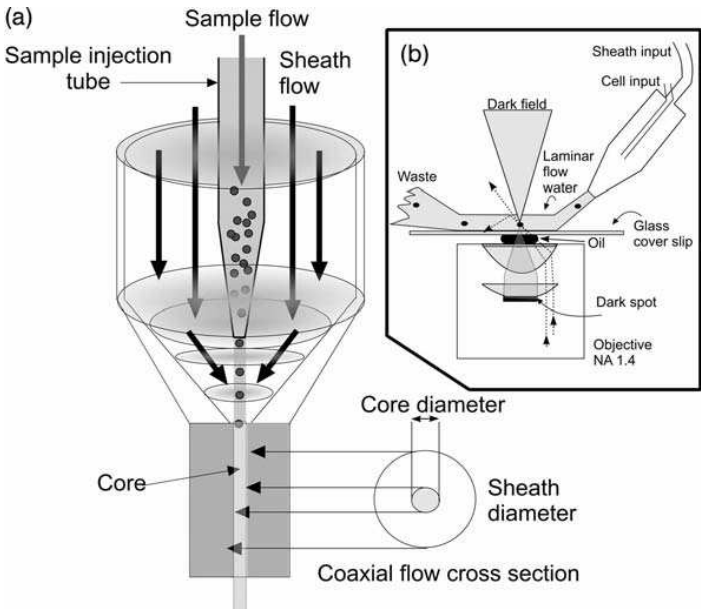



**Figure 9.FCM(a)**A functional view of a flow chamber in a flow cytometer. The fluid sheath flowing through a large area is forced under pressure into a much smaller orifice. Placed at the center of the flow cell, an injection tube injects the sample (i.e. cells and other particles) into the centre of the flowing stream, whereby forcing the cells to undergo hydrodynamic focusing. A coaxial cross-section of the sheath and core is also shown. **(b)** An alternative flow cell based on an axial flow system typically used in microscope based flow cytometers. In these instruments the laminar stream flows across a coverglass to a waste collector on the opposite side. Of note is the dark field objective. The central obscuration in the real focal plane of the objective produces a dark cone in the illumination field and since it extends above the object plane contains only the fluorescence and scattered light signal in the absence of the illumination signal. NA Ľ numerical aperture;**Source:** Doležal et al. (2007)

## 2.7. Evaluation of genetic stability in *in vitro* regenerants using molecular markers

Plant genetic techniques belong to the molecular biotechnology covering the range of interest basic chromosome analysis. This field includes chromosome analysis, mutagenesis techniques, recombinant DNA techniques, DNA sequencing, molecular breeding, DNA purification and separation techniques, polymerase chain reaction (PCR) and its variation, genetic variability analysis, polymorphismus analysis and analyses of gene functions. These techniques which are used for plant identification are designed to detect the presence of specific DNA sequences (Mondini et al., 2009).

The two aspects of genetic diversity which are important in the study of plant species, particularly those that are rare, are the level of diversity and how the diversity is partitioned within and among populations. The level of genetic variation within populations and species is of interest because it can affect the ability to adapt to changing environmental conditions (Kimbal et al., 2001).

The use of molecular markers to evaluate neutral genetic variation becomes an important tool to study population genetics (Rodrigues et al., 2013). Molecular marker technologies are based on polymorphism in protein (Mondini et al., 2009).Gel electrophoresis is used for separating fragments of nucleic acid in the gel medium, of different sizes and conformation. The size of fragments in a given experimental bands are assessed comparison bands in the DNA ladder that have migrated at a similar rate (Žiarovská et al., 2012).

Polyploidy is one of the dynamic processes in plant evolution that lead to gene flow and complex genome reorganization (Soltis and Soltis, 1995). Genome evolution of polyploids is based on multiple genetic interactions, such as recombination, gene conversion, concerted evolution, intergenomic chromosomal exchanges, cytonuclear stabilization or gene silencing, for example (Wendel, 2000). These major forces behind genome reorganization generate novel gene expression combinations that lead to acquisition of new functions (Seehausen, 2004).

The Polymerase chain reaction method was developed and firstly used in 1983 by Kary Mullis, which was then awarded for the Nobel Prize in chemistry. Currently it is very common technique used in biological and in medical research as well (Kumar and Gurusubramanian, 2011). PCR is a method used mainly for copying specific sequences

of nucleotides in DNA or in RNA sequences. It can be copied up to kilobases in length. PCR depends on the ability of a thermo stable DNA polymerase to extend primers. For carrying out of PCR it is needed template DNA, nucleoside tri-phosphates, primers and Taq DNA polymerase. The template is the DNA sequence, which we want to copy. Primers select the region of the template by annealing with their complementary sequences. The Taq DNA polymerase is a temperature tolerant enzyme, which catalyze the synthesis of DNA (Žiarovská et al., 2012). According to Kumar and Gurusubramanian (2011)the primers, which are short DNA fragments and DNA polymerase are the key components to enable selective and repeated amplification.

PCR method is based on thermal cycling, repeating of cycles of heating and cooling of the reaction for DNA melting and enzymatic DNA replication.

The DNA sequencing is a process of determination of the identity and position of each nucleotide in a molecule of DNA. This is provided for final information on a given molecule, this information is needed for identification, characterization and for taxonomy. Genetic technologies provide specific information of important agronomic traits and their use in plant breeding (Žiarovská et al., 2012).

Advances in molecular biology technologies have provided the basis for exposing unlimited numbers of DNA markers. Over the last ten years, PCR became a widespread technique for several unusual genetic chemical analysis based on selective amplification of DNA. The popularity of PCR is primarily done by its apparent simplicity and high probability of success, but needs DNA sequence information. The findings of PCR with random primers can be used to increase a set of randomly distributed loci in any genome, which is facilitated the development of genetic markers for a variety of purposes (Kumar and Gurusubramanian, 2011).

The DNA based markers are based on non PCR (Polymerase chain reaction) includes RFLP (restriction fragment length polymorphism) and on PCR includes RAPD (Random amplified polymorphic deoxyribonucleic acid), AFLP (Amplified fragment length polymorphism), ISSR (Inter simple sequence repeats), SSR (Simple sequence repeat) (Kumar et al., 2009).

Randomly Amplified Polymorphic DNA (RAPD), is a type of PCR reaction. The segments of DNA that are amplified are random. The RAPD is a commonly used molecular marker in genetic diversity studies. The knowledge about DNA sequence for the targeted gene is not required, because the primers will bind somewhere in the sequence, but it is not known exactly where. This is the reason why is that method

popular for comparing the DNA of biological systems that have not had the attention of the scientific community. The main reason for the success is the achievement of a large number of genetic markers that require small amounts of DNA without the requirement for cloning, sequencing or any other form of the molecular characterisation of the genome of the species in question (Kumar and Gurusubramanian, 2011).

RAPD detects nucletide sequence polymorphisms in DNA by using a single primer of random nucleotid sequence. RAPDs are DNA segments amplified by the PCR by using short primer of random sequence. RAPD has few advantages as high genomic abundance, can be used for samples with less amount of DNA, is ideal for automation and is relatively faster. It has also some disadvantages as dominant markers, it cannot be used across the species, no reproducible (Žiarovská et al., 2012).

Inter Simple Sequence Repeats polymorphisms are found between microsatellite repeats. In this technique, primer will target multiple loci due to known abundance of repeat sequences in the genom. The advantages of ISSRs are for example: variation can be found at several loci simultaneously, is microsatellite sequence-specific and is reliable DNA profiling, mainly for closely related species. The disadvantages are possible non homology of similar size fragments, reproducibility and also dominant markers (Žiarovská et al., 2012).

Among various molecular tools, ISSRs have gained increasing interest, because they have greater reliability and reproducibility of banding patterns when compared to RAPD primers (Culley and Wolfe, 2001), and at the same time, the cost of the analyses is relatively lower than that of some other markers such as AFLPs and microsatellites (Rodrigues et al., 2013).

Nowadays, an IPBS (Inter-Primer Binding Site) method had appeared in genetics journals. According to Radová (2012) in recent genetics research, there was described a retrotransposon-based molecular marker technique, which is used to identify the plant varieties. IPBS is a universal and efficient method from identification of polymorphism (Kalendar et al, 2010). The same author describes that the IPBS technique has proved to be a powerful DNA fingerprinting technology.

In table 2 are showen exmaples of used molecular marker techniques used in *Lamiaceae* family**.**

**Table 2:** The molecular marker techniques used in *Lamiaceae* family**.**

| Specie | Used plant part | Molecular Marker Technique |
|---|---|---|
| ***Ocimum* L. spp** | fresh leaves | **RAPD** (Vieira et al., 2003) |
| ***Mesona chinensis*** | fresh leaves | **RAPD** (Zhang et al., 2012) |
| ***Eremostachys superba*** | dried leaves | **RAPD** (Verma et al., 2006) |
| ***Phlomis lycia*** | dried leaves | **RAPD** (Yuzbasioglu et al., 2008) |
| ***Salvia fruticosa*** | fresh leaves | **RAPD** (Skoula et al., 1999) |
| ***Thymus caespititius*** | frozen leaves | **ISSR** (Trindade et al., 2009)<br>**RAPD** (Trindade et al., 2009) |
| ***Cunila galioides*** | dried leaves | **RAPD** (Fracaro et al., 2005) |
| ***Cleome gynandra*** | fresh leaves | **RAPD** (Rathore et al., 2014)<br>**SCoT** (Rathore et al., 2014) |
| ***Mentha cervina*** | silica gel-dried leaves | **ISSS** (Rodrigues et al., 2013) |
| ***Thymus* L spp.** | fresh leaves | **AFLP** (Sostaric et al., 2012) |
| ***Monarda fistulosa*** | freah leaves | **ISSR** (Kimbal et al., 2001) |

# 3. Aim of the thesis

The main objective of this work was development of an appropriate protocol for micropropagation of *Monarda didyma* and detection of genetic stability of *in vitro* regenerants by molecular markers. Another objective was to obtain polyploid (tetraploid) plants of *Monarda didyma* by mitotic polyploidization *in vitro*.

Final result serves to confirm or refute following hypotheses:

$H^1$: Plant growth regulators (cytokins and auxins) in cultivation medium for micropropagation of *Monarda didyma* have influence to aboveground and underground parts of plants.
$H^2$: Plant growth regulators in cultivation medium have not influence to genetic stability of micropropagated plants of *Monarda didyma*.
$H^3$: Oryzalin is effective antimitotic agent for polyploidization of *Lamiaceae*.

# 4. Materials and Methods

Micropropagation and polyploidization were carried out in the Laboratory of plant tissue culture of the Department of Crop Science and Agroforestry in the Faculty of tropical agriculture of the Czech University of Life Sciences in 2013 – 2014.

Molecular markers were carried out in Laboratory of molecular biology at Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Department of Genetics and Plant Breeding in 2014.

## 4.1. Plant material

The plant material for this thesis was obtained from colection of Faculty of AgriScience, which was established in *in vitro* conditions. *Monarda didyma* (n=16) chemotype 1 was chosen for its big amount of TQ in essential oil (Táborský et al., 2012) as it is mentioned before in capter 2.3. Chemical composition. Nodal segments of *Monarda didyma* were cultivated on MS (Murashige and Skoog, 1962) medium in cultivation box under 16/8 h photoperiod with illumination 2500 lx and temperature 25/20°C.

Before starting the experiment, it was necessary to multiply the plant material by using micropropagation using nodal segments.

## 4.2. Methodology of micropropagation

### 4.2.1. Preparation of aseptic condition

Before starting the experiment were prepared all the necessary tools for sterilization and wrapping the petri dishes, scalpel and tweezers in aluminum foil and then placed into all the hot air sterilizer for 3 hours at 160 ° C. The medium was prepared in test tubes, size 15 x 2.5 cm were needed autoclaved for 20 min at 120 ° C. All work was carried out in a sterile flowbox, which was sterilized in the following manner. Flowbox was cleaned with 70% ethanol and the UV lamp was turned on for at least 1h, before the experiment was over 20 min turned on the fan without UV lamp. Before starting the work had to be properly washed everything put into the box 70% ethanol on the surface. To maintain hygiene regulations lab were devoid of dirt and hands. Sterilization of instruments and follow-up firing was recommended for each use during operations.

### 4.2.2. Preparation of cultivation medium

For the multiplication of plants in vitro was used MS medium without growth regulators. Composition of MS (Murashige and Skoog, 1962) medium is shown in Appendix Table 12. For multiplication was used basic MS medium without addition of PGRs and for the actual experiment micropropagation has been used MS medium with PGRs.

To prepare the MS medium was needed glass cylinder, beaker, pH meter, microwave ovens, and scales. The chemicals were needed solutions for media preparation A-V, then 1M KOH and ascorbic acid, which are used to adjust the pH to 5.7. Furthermore, the need distilled water, sucrose, myo-inositol and agar. The required amount of solutions, sucrose, myo-inositol and agar is also provided in the Annex in Appendix Table 12.

All media components were weighed on scales or measured out in a graduated cylinder and dissolved in water. Then the pH value was adjusted to 5.7. Subsequently, to the mixture was added agar, supplemented with water to the final volume. Complete medium was heated up to complete dissolution of the agar in medium. Still warm culture medium was filled into the test tubes. They were closed with a plastic lid, and then the tubes with the medium were autoclaved for 20 min at 120 ° C.

### 4.2.3. Micropropagation

After the successful propagation of healthy material was started micropropagation, where were used only nodal segments (Fig.10.) around 1 cm long. Nodal segments were cultivated on MS medium with additions cytocinins (BAP, KIN) to promote the growth of shoots and auxins (IAA, NAA) to induce roots, both in six variants, and as a control variant was used basal MS medium without PGRs. Variants of cultivation media are placed in Table 3. In each variant were cultivated 20 plants. Nodal segments were placed into test tubes and were cultured 60 days. The cultures were incubated at 25/20 ± 0,3 °C under a 16/8 h light regime in cultivation box (POL-EKO ILW350/350 STD), with 2500 lx light intensity provided by cool white fluorescent lamps (Philips LT5 14 W/840). Plants were measured every week and after 60 days were evaluated variants of each other.

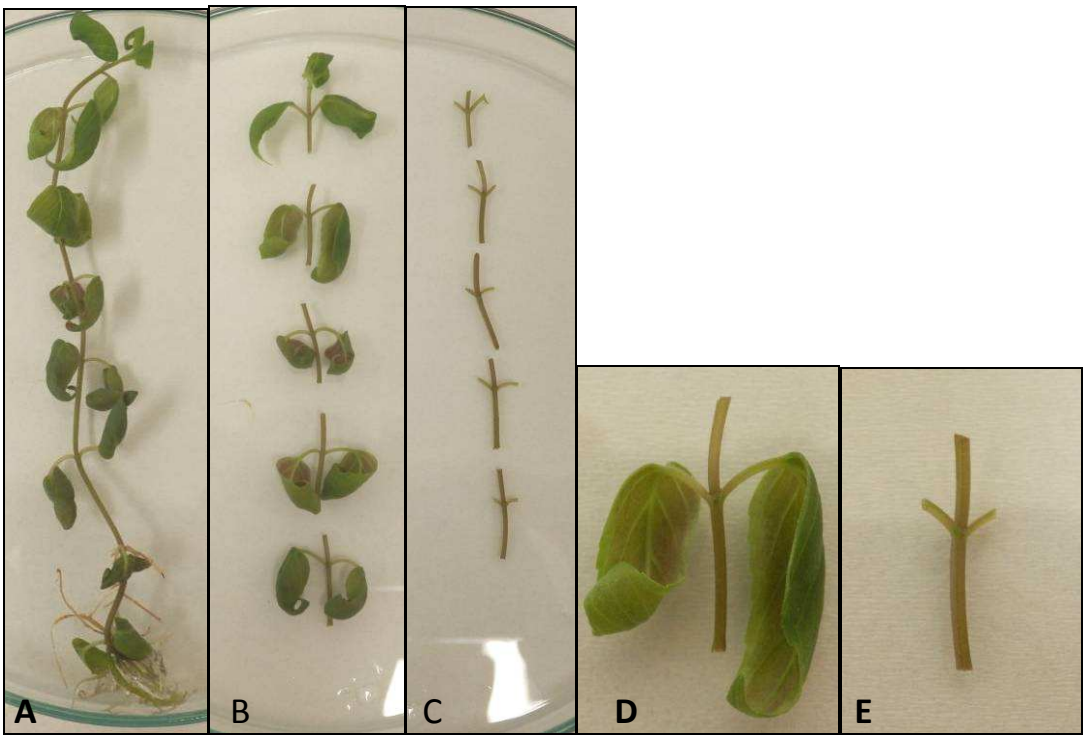



**Figure 10.** The propagation of plant by nodal segments; A- whole plant, B- plant devided into the nodes with leaves, C- nodal segments without leaves, D,E- detail of nodes (Ø 1cm)
**Source:** Author (2014)

**Table 3**. List of variants of the media

| Treat-ment | Type of medium | PGRs | | | | Concentration of PGRs mg/l | Sucrose g/l | Agar g/l | pH |
|---|---|---|---|---|---|---|---|---|---|
| Co | MS | ---- | ---- | ---- | ---- | ---------- | 30 | 8 | 5.7 |
| A1 | MS | BAP | | | | 0.5 | 30 | 8 | 5.7 |
| A2 | MS | BAP | | | | 1.0 | 30 | 8 | 5.7 |
| A3 | MS | BAP | | | | 1.5 | 30 | 8 | 5.7 |
| A4 | MS | | KIN | | | 0.5 | 30 | 8 | 5.7 |
| A5 | MS | | KIN | | | 1.0 | 30 | 8 | 5.7 |
| A6 | MS | | KIN | | | 1.5 | 30 | 8 | 5.7 |
| A7 | MS | | | IAA | | 0.5 | 30 | 8 | 5.7 |
| A8 | MS | | | IAA | | 1.0 | 30 | 8 | 5.7 |
| A9 | MS | | | IAA | | 1.5 | 30 | 8 | 5.7 |
| A10 | MS | | | | NAA | 0.5 | 30 | 8 | 5.7 |
| A11 | MS | | | | NAA | 1.0 | 30 | 8 | 5.7 |
| A12 | MS | | | | NAA | 1.5 | 30 | 8 | 5.7 |

Also we tried cultivation of nodal segment on MS medium supplemented with combination of PGRs. Variants of cultivation media are placed in Table 4. As a control variant was used basal MS medium without PGRs. In each variant were cultivated 10 plants. Culture conditions were the same as in the variants before.

Plants were measured after first month and after second month. These variants were evaluated of each other.

**Table 4.** List of variants of cultivation media with combination of PGRs

| Treatment | Type of medium | PGRs | Concentration of PGRs mg/l | Sucrose g/l | Agar g/l | pH |
|---|---|---|---|---|---|---|
| 0 | MS | ---- | ---- | 30 | 8 | 5.7 |
| 1 | MS | IAA<br>BAP | 0.5<br>1.0 | 30 | 8 | 5.7 |
| 2 | MS | IAA<br>BAP | 0.5<br>1.5 | 30 | 8 | 5.7 |
| 3 | MS | NAA<br>KIN | 1.0<br>1.0 | 30 | 8 | 5.7 |
| 4 | MS | NAA<br>KIN | 1.0<br>1.5 | 30 | 8 | 5.7 |

### 4.2.4. Transfer to *ex vitro* condition

After evaluation of *in vitro* micropropagation, the *in vitro* rooted plants (6-10 cm high) were removed from the culture medium, roots were washed for remove all agar and transferred to plastic pots (5x5 cm). The plastic pots contain sand:soil:peat moss: vermiculite (1:1:1:1; v/v) mixture. This substrate was sterilized. The plants were maintained in a greenhouse covered with polythene bags under high humidity, for 1 week and slowly weaned to lower humidity. The percentage of ex vitro survival was evaluated after 5 weeks.

## 4.3. Methodology of polyploidozation

As an antimitotic agent was used oryzalin. Stock solution of oryzalin (10mM) was prepared as follows: weighed 0.0346 g of oryzalin was dissolved in 10 ml of dimethylsulfoxide (DMSO) in a sterile flask. DMSO has a sterile function therefore no sterilization is necessary.

Nodal segments of *Monarda didyma* were tested in subsequent oryzalin concentrations: 40, 60 and 80 μM/l during 2 exposure times 24 and 48 hours. Nodal explants on MS medium in the flasks were completely immersed in oryzalin solution during whole exposure time. After the exposure time, nodal segments were removed, three times washed in sterilized distilled water and cultivated on MS medium without growth regulators in the test tubes (Fig. 11).

The percentage of survival of treated explants was recorded after 30 days.

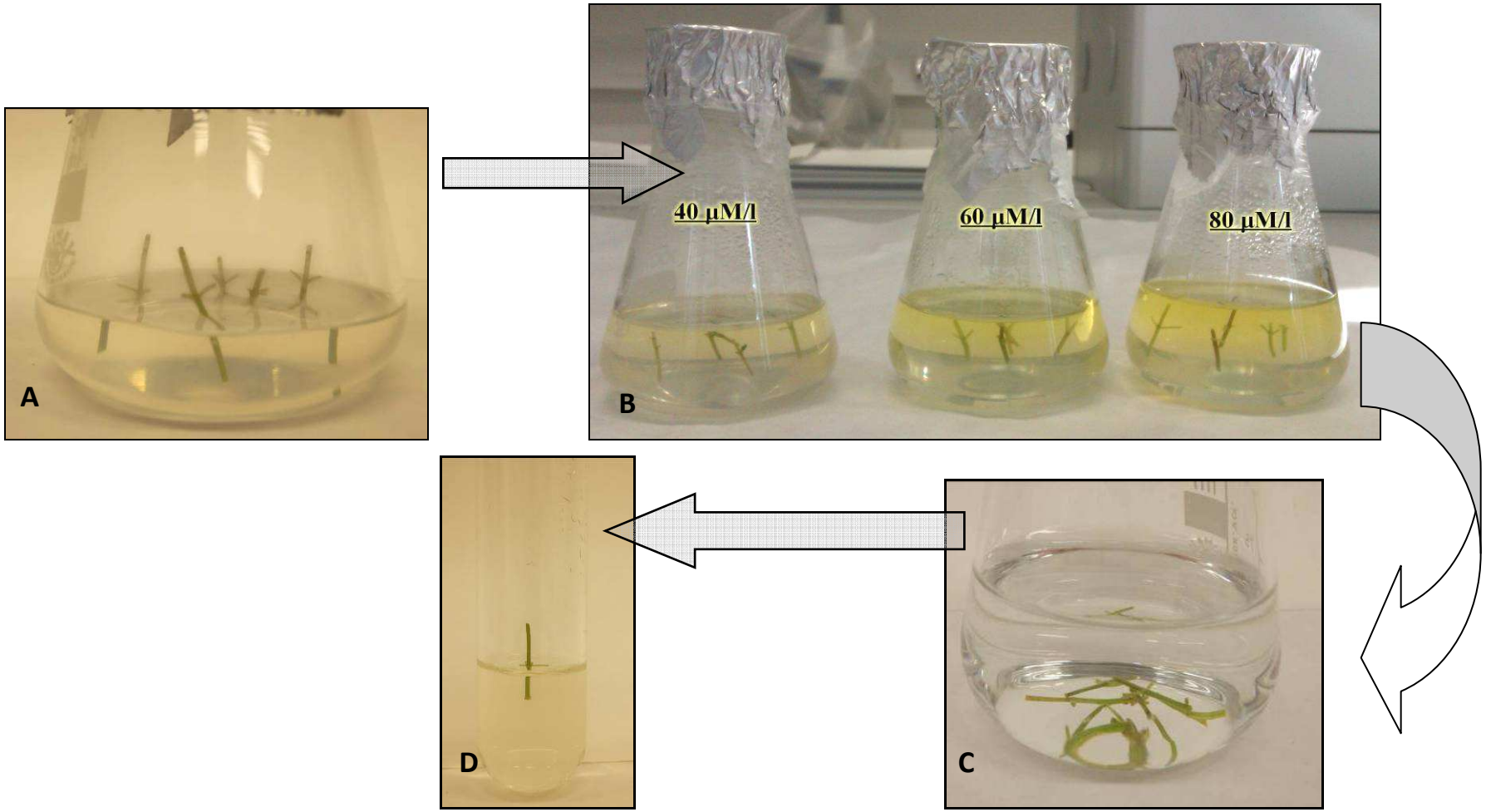



**Figure 11**. Treatment of oryzalin to nodal segments; A- nodal segments in medium, B- nodal segments immersed in oryzalin treatment, C- washing of nodal segments in distilled water, D- cultures in MS medium in test tubes

**Source:** Author (2014)

### 4.3.1. Methodology of detection of ploidy level

#### Flow-cytometry

DNA-ploidy levels were established using flow cytometry. Small pieces of leaf tissue were chopped in a Petri dish containing 500 µl of Otto I buffer (0.1 M citric acid, 0.5% Tween 20). The crude suspension of isolated nuclei was filtered through a 50-µm nylon mesh. 1 ml of Otto II buffer (0.4 M Na2HPO4.12H2O), supplemented with AT-selective fluorescent dye DAPI (4´.6-diamidino-2-phenylindole) in final concentration of 2 µg/ml, was added. Relative fluorescence intensity of at least 3,000 nuclei was recorded using Partec PAS flow cytometer (Partec GmbH, Münster, Germany) equipped with a high-pressure mercury arc lamp. Data were analyzed using the FlowMax software (Partec, GmbH, Münster, Germany). The gain of the instrument was adjusted so that the peak representing control plant G0/G1 nuclei appeared on channel 100.
Analyses were carried out in the Centre of Plant Structural and Functional Genomics in Institute of Experimental Botany AS ČR in Olomouc.

#### Direct counting of chromosomes

For ploidy level detection was used also direct counting of chromosoms in root tips.
Rooted plants were removed from the cultivation media and roots were washed under the running water. The roots were soaked for 60 min in a solution of paradichlorbenzen, then were rinsed in water. Subsequently roots were soaked for 30 minutes in glacial acetic acid (98%). Then the roots were taken on a watch glass and acetoorseine dye was added and heated over a flame. Root tips were removed and placed on a microscope slide and acetoorcein was added and covered with a cover slip. There was put pressure on the sample. Subsequently, the slide was observed under light microscope Nikon ECLIPSE 50i (Nikon, Tokyo, Japan), type magnification 1000 x. As an evaluation software was used NIC Elements Advanced Research 3.10 from Laboratory Imaging (Prague, Czech Republic).

#### Stomata studies

Only one diploid (control plant) was used for stomata study. Leaves from each plant were sampled. A small area on the underside of the leaves surfaces was smeared with nail polish.

After the nail polish solution dried, the nail polish impression was removed using a strip of transparent adhesive cellulose tape. The underside epidermis imprints was form on the tape. The tape was placed on a microscope slide and observed under light microscope Nikon ECLIPSE 50i (Nikon, Tokyo, Japan), type magnification 400x. As an evaluation software was used NIC Elements Advanced Research 3.10 from Laboratory Imaging (Prague, Czech Republic).

## 4.4. Methodology of establishment genetic stability

### 4.4.1. Isolation of DNA

Genomic DNA was isolated from the fresh leaves of *in vitro* cultivated plants of *M. didyma,* where were taken the samples about 100 mg weight. For DNA extraction was used optimized protocol of Rogers et al. (1994).

The samples were homogenized under liquid nitrogen with help of pestle and mortar. Into the homogenized material was added the 2 x CTAB solution (2 % CTAB; 100 mmol.dm$^{-3}$ Tris, pH 8; 20 mmol.dm$^{-3}$ EDTA, pH 8; 1.4 mol.dm$^{-3}$ NaCl; 1 % PVP) and incubated at 65 °C for 90 min. Into the sample was added chloroform / isoamyl alcohol (24: 1) in a ratio 1: 1 for precipitation and centrifugated for 10 minutes at 10,000 rpm. The supernatant was transferred into the new 1.5 ml reaction tube and there was added 1/5 amount of 5 % CTAB (5 % CTAB, 0.35 mol.dm$^{-3}$ NaCl). Into the sample was added again chloroform / isoamyl alcohol (24: 1) in a ratio 1: 1 for second precipitation and centrifugated for 10 minutes at 10,000 rpm. The supernatant was transferred into the new 1.5 ml reaction tube and there was added 2/3 amount of isopropanol. These tubes were kept in freezer at -20 °C for 12 hours. After freezing the tubes were centrifugated for 10 minutes at 10,000 rpm. We removed the supernatant and dried the precipitate, followed by addition of TE solution (10 mmol.dm$^{-3}$ Tris, pH 8; 1 mmol.dm$^{-3}$ EDTA, pH 8; 1 mol.dm$^{-3}$ NaCl) and it was incubated at 65 °C for 20 min. For the other precipitation were added 2 amounts of 96% of ethanol and incubated in freezer at -20 °C for 12 hours, followed by centrifugation for 10 minutes at 10,000 rpm. The supernatant was removed and pellet was dried. Into the tubes with dried pellet was added the 80 % cold ethanol, centrifugated for 5 minutes at 10,000 rpm. Drying in a desiccator for 30 min. Into the tubes was added 1x TE solution (10 mmol.dm$^{-3}$ Tris, pH 8; 1 mmol.dm$^{-3}$ EDTA, pH 8) for rehydratation.

### 4.4.2. Optimization of condition of polymerase chain reaction for RAPD analysis

In reaction mixture, there were optimized concentration of single components to determine suitable conditions of polymerase chain reaction for RAPD analysis.

**Amount of DNA:** To determine optimal DNA amount were used following amounts of isolated DNA 50 and 100 ng in 25 μl of reaction mixture.

**Concentration of primers:** To determine optimal primers concentration were used concentrations of the primers OPB-4 and OPB-14: 0.8; 1.2; 2 a 4 $\mu mol.dm^{-3}$.

**Concentration of *$MgCl_2$***: To determine optimal concentration of *$MgCl_2$* were used following concentration: 1.5; 2; 2.5 a 3 $mmol.dm^{-3}$ .

**Concentration of deoxyribonucleotide**: Tested concentrations were 0.1; 0.2; 0.3 $mmol.dm^{-3}$.

**Amount of Taq polymerase:** To determine optimal amount of Taq polymerase were added following amounts 0.1; 0.2; 0.3 a 0.4 μl to the reaction mixture with concentration of Taq polymerase 5 U. $\mu l^{-1}$.

**Temperaturefor primer establishment:** To determine optimal temperature for primers establishment were tested tree temperatures: 35, 36 a 37 °C.

All optimization reactions were repeated three times to prove result repeatability of RAPD analysis. In polymerase chain reaction, there were also tested the negative control-samples without DNA to determine the effect of components concentration on primer multimers synthesis in reaction mixture.

### 4.4.3. RAPD anylysis

Conditions for RAPD analysis were determined on the basis of the optimization of polymerase chain reaction as mentioned here above. All reactions were realized in 25 μl reaction mixture containing: 1x Dream Taq PCR MasterMix (Thermo Scientific); 3 $mmol.dm^{-3}$ of *$MgCl_2$;* 0.1$mmol.dm^{-3}$ of deoxyribonucleotide; 4 $\mu mol.dm^{-3}$ of primer and 50ng od DNA.

A set of two RAPD primers were used for screening, OPB-4 (GGACTGGAGT) and OPB-14 (TCCGCTCTGG) (Operon Technologies Inc., Alameda,CA, USA).

Time and temperature profile was as follows: 2 minutes at 94 °C for initial denaturation, 45 cycles of 1 minute at at 94°C for denaturation, 1 minute at 36°C for primer establishment, 2 minutes at 72 °C for polymerization and last step 7 minutes at 72 °C for polymerization in MyCycler™ (Biorad) device.

Electrophoretic separation of amplified products was performed on 2% agarose gel in 1x TBE buffer. Gels were run for about 3 – 3.5 hours at 55 V and visualized with a UV transilluminator. Gel pictures were recorded using the CSL-MICRODOC System (CLEAVER, Great Britain). Fragment size was determined by comparsion with the DNA standard 250 bp ladder. RAPD fragments were established as a presence or absence of bands in the gel profile.

## 4.5. Statistical analysis

All statistical analyses were performed using the SPSS 20.0 statistical software package (SPSS Inc, Chicago, IL, USA). Kolmogorov-Smirnov test and Levene's test were used to check data normality and homogeneity of variance, respectively. One-way ANOVA test was used to compare groups. Tukey post-hoc test was applied in case of homogeneity of variance, otherwise Games-Howell post-hoc test. Some variables were not normally distributed, even after square root or log transformations. Therefore, non-parametric test (Kruskal-Wallis) was used to check for differences among groups. For all tests, differences were considered significant at $P<0.05$. Descriptive statistics were also calculated.

# 5. Results and discussion

## 5.1. Micropropagation

### Shoot proliferation

The effect of BAP and KIN on the morphogenetic responses of the explants after 30 and 60 days of culture is summarized in the table 5 and 6.

In first month of cultivation, the shoot regeneration in the MS medium with addition of KIN was from 85 to 100% and in the variants with BAP from 65 to 95%, but in the second month of cultivation the percentage of regeneration decreased in variants of culture media with KIN, there was 75-100% and in variants with BAP was only 35-90%.

The reduction of the percentage of regeneration was due to death or hyperhydricity of explants. Higher percentage of hyperhydricity was reported in explants cultivated in higher concentration of BAP 1.0 and 1.5 mg/l.

The reduce of the percentage of regeneration was reported also in control variant (from 85 to 65%), which was done by methylation (oxidation) of the explants, which turned brown and died.

The main criterion of micropropagation by nodal segments is the new shoots and nodes induction per explant (coefficient of micropropagation). Nodal segments of *Monarda didyma* have two axial buds, from which regenerate two shoots as a rule one is longer than the second shoot (Fig.12A). But sometimes only one shoot regenerates (Fig.12B).

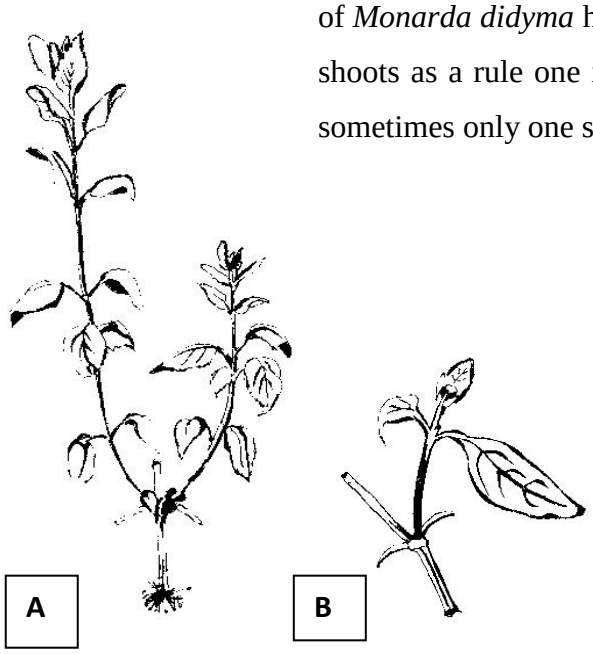



**Figure 12**. Shoots and nodes induction per explant. A. two shoots regenerated, B. one shoot regenerated

**Source**: Author (2014)

After the first month of culture in terms of the number of shoots the best option appeared with the addition of 0.5 mg/l KIN in MS medium, where were induced in average 1.90±0.31 shoots per explantat, but it is not significant difference with the other treatments (1.25±0.91 – 1.75±0.55 shoots per explantat) including the control (1.65±0.75) at level P = 0.05. But after the second month of culture, the average number of shoots increased in most variants (0.70±0.98 – 1.90±0.31) and between some variant is a significant difference.After the first and second month of culture, the low number of shoots was produced in the higher concentration (1.0 and 1.5 mg/l) of both citokynins (KIN and BAP).

Higher number of new nodes on longer shoot, after first month of culture, was induced in the treatment containing 0.5 mg/l KIN (3.15±1.14) and after second month of culture, in the treatment containing 1.5 mg/l KIN (5.60±2.16) and in both cases existed a significant difference compared to most of treatments. In second month of cultivation the number of new node and length of longer shoot were increased in some variants with KIN by 80 and 160%, respectivly.

In explants influenced by BAP the largest number of new nodes in longer shoots was produced in the lowest concentration (0.5 $mg.l^{-1}$) namely 3.75±2.38.

In general, plants regenerated in media supplemented with BAP, where were shorter plants compared to the plants regenerated in media supplemented with KIN.

Plants influenced by KIN had in average longer internodes, thin shoot and longer leaves. Plants influenced by BAP were stronger, had smaller leaves and in the base of shoot was formed callus.

Within each variant and between variants, there was a great morphological variability visually (Fig.15), so in all variant was made the test of genetic stability of regenerated plants by using RAPD makers (see chapter 5.4. Evaluation of genetic stability).

According to available information, there are not any studies with micropropagation of *Monarda didyma*, therefore the results of this study are compared with the results obtained in other species the families *Laminaceae*. The maximum regeneration (93.88%) and maximum number of shoots (6.09 ± 0.05) , with average length 3.83 ± 0.11 cm, was achieved with medium containing 1.0 mg/l BA after 4 weeks of culture in *Ocimum kilimandscharicum* Guerke (Saha et al., 2010). These authors confirmed that at higher concentration of BA reduces the percentage of the number of shoots. Mehta et al. (2012) in *Mentha piperita* L. reporte better results in percentage of regeneration (40-80%), number of shoots (2.28 to 3.42) and shoot length (3.31

to 6.56 cm) in medium supplemented with BAP than KIN (30-75% regeneration of shoots, number 1.28 to 2.42 and from 4.92 to 6.47 cm). For nodal segments with *Salvia fruticosa* Mill Arik et al. (2004 ) get better results in terms of shooting percentage, number of shoots and shoot height in medium with BA than with KIN. Also Coelho et al. (2012) reported that the best results proliferation were obtained in *Thymus lotocephalus* G. López & R. Morale swith BA than KIN, but in explants was observed high percentage of hyperhidric shoots. The same results were achieved by Hembrom et al. (2006) in *Pogostemon heyneanus* Benth. Explants on control medium in first month of culture had rapid regeneration capacity, but the explants on MS medium with cytokinins (mainly containing KIN) showed intensive growth in the end of second month of culture (Fig. 13,14). The most intensive growth of explants was evident in all cases in the seven and eight weeks of culture except the explants cultured in higher concentrations of BAP (1 and 1.5 mg/l). KIN in higher and in lower concentration spositively affects growth and development *Monarda didyma*, but high concentration of BAP has a negative effect on shoot growth in *Monarda didyma*.

**Table 5.** Influence of cytokynins to the shoot proliferation after 30 days of cultivation

**Source:** Author (2014)

| variant | PGRs concentration (mg/l) | Shoot regeneration (%) | Shoots per explant (No) * | Longer shoot (cm) * | Shorter shoot (cm) * | Nodes on longer shoot (No) * | Nodes on shorter shoot (No) * |
|---|---|---|---|---|---|---|---|
| Control | - | 85 | 1,65±0,75 [a] | 3,10±2,61 [b] | 1,06±1,34 [a] | 1,95±1,54 [ab] | 1,05±1,15 [a] |
| KIN | 0,5 | 100 | 1,90±0,31 [a] | 3,30±1,71 [b] | 1,19±1,15 [a] | 3,15±1,14 [b] | 1,40±1,35 [a] |
| | 1,0 | 85 | 1,60±0,75 [a] | 2,98±2,15 [b] | 1,40±1,61 [a] | 2,45±1,61 [bc] | 1,60±1,43 [a] |
| | 1,5 | 100 | 1,75±0,55 [a] | 3,53±2,17 [b] | 1,23±1,39 [a] | 3,10±1,12 [b] | 1,45±1,47 [a] |
| BAP | 0,5 | 95 | 1,60±0,68 [a] | 1,93±1,35 [b] | 0,89±0,80 [a] | 1,90±1,68 [ab] | 0,95±0,89 [a] |
| | 1,0 | 70 | 1,25±0,91 [a] | 1,78±3,00 [bc] | 0,63±0,79 [a] | 1,20±1,47 [ac] | 0,75±0,97 [a] |
| | 1,5 | 65 | 1,30±0,98 [a] | 0,53±0,65 [ac] | 0,36±0,38 [a] | 0,70±1,03 [a] | 0,55±1,05 [a] |

* Numbers followed by the same letter are not significantly different (Kruskal-Wallis test, $p < 0.05$)

**Table 6.** Influence of cytokynins to the shoot proliferation after 60 days of cultivation

**Source:** Author (2014)

| variant | PGRs concentration mg/l | Shoot regeneration (%) | Shoots per explant (No) * | Longer shoot (cm) * | Shorter shoot (cm) * | Nodes on longer shoot (No) * | Nodes on shorter shoot (No) * |
|---|---|---|---|---|---|---|---|
| Control | - | 65 | 1,30±0,98 $^{ab}$ | 5,58±5,21 $^{ab}$ | 1,98±2,95 $^{ab}$ | 3,15±2,83 $^{ab}$ | 1,60±1,69 $^{a}$ |
| KIN | 0,5 | 100 | 1,90±0,31 $^{b}$ | 8,59±4,83 $^{b}$ | 3,12±3,90 $^{b}$ | 5,40±2,09 $^{bd}$ | 2,45±2,33 $^{a}$ |
| | 1,0 | 75 | 1,50±0,89 $^{ab}$ | 8,02±5,73 $^{b}$ | 3,43±3,63 $^{b}$ | 4,35±2,96 $^{bc}$ | 2,45±2,26 $^{a}$ |
| | 1,5 | 100 | 1,80±0,41 $^{bc}$ | 8,12±4,47 $^{b}$ | 3,11±3,68 $^{b}$ | 5,60±2,16 $^{bd}$ | 2,80±2,88 $^{a}$ |
| BAP | 0,5 | 90 | 1,80±0,62 $^{b}$ | 5,34±4,15 $^{bc}$ | 2,24±2,45 $^{b}$ | 3,75±2,38 $^{ad}$ | 2,00±1,30 $^{a}$ |
| | 1,0 | 50 | 0,95±0,99 $^{ac}$ | 2,79±4,31 $^{ac}$ | 0,87±1,40 $^{bc}$ | 2,20±2,80 $^{ac}$ | 1,05±1,43 $^{a}$ |
| | 1,5 | 35 | 0,70±0,98 $^{a}$ | 0,83±2,16 $^{a}$ | 0,32±0,50 $^{ac}$ | 1,00±1,95 $^{a}$ | 0,55±0,89 $^{a}$ |

* Numbers followed by the same letter are not significantly different (Kruskal-Wallis test, $p < 0.05$)

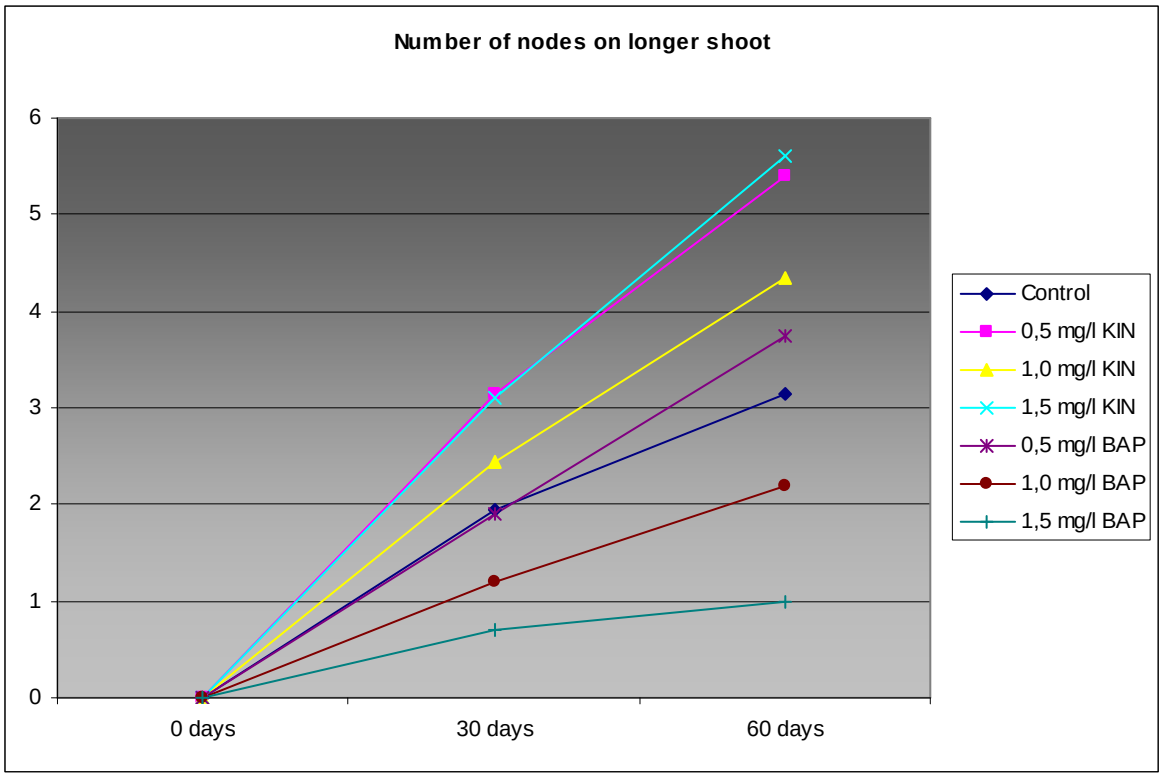



**Figure 13.** Graph of number of nodes produced on longer shoot

**Source:** Author (2014)

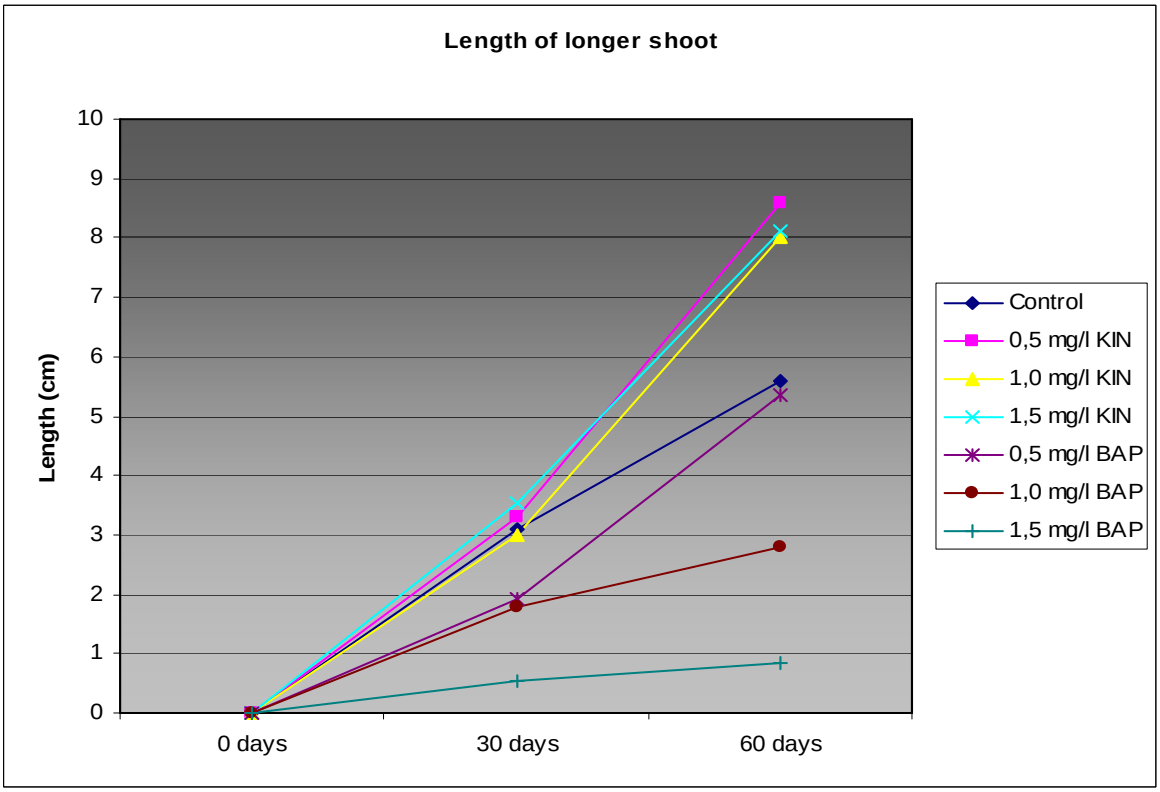
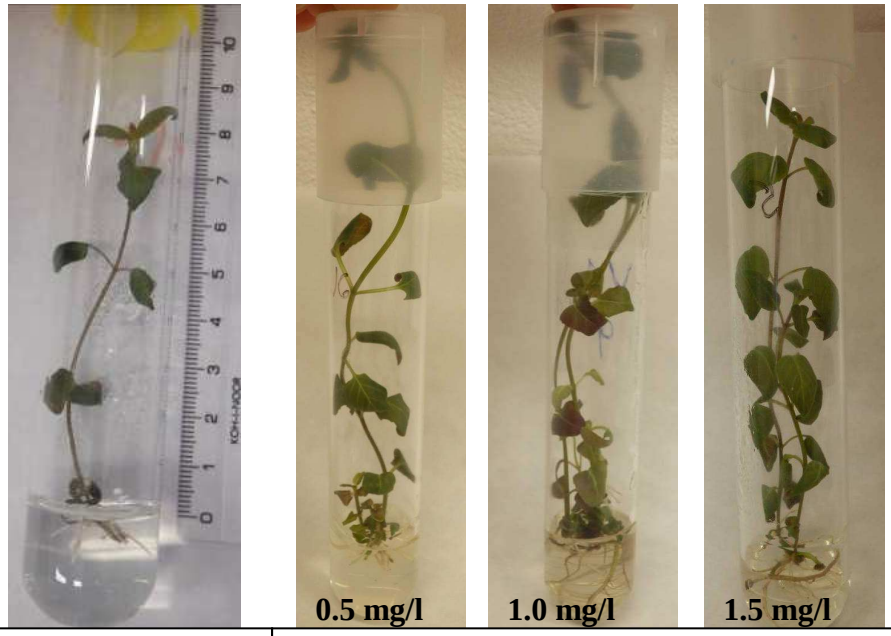


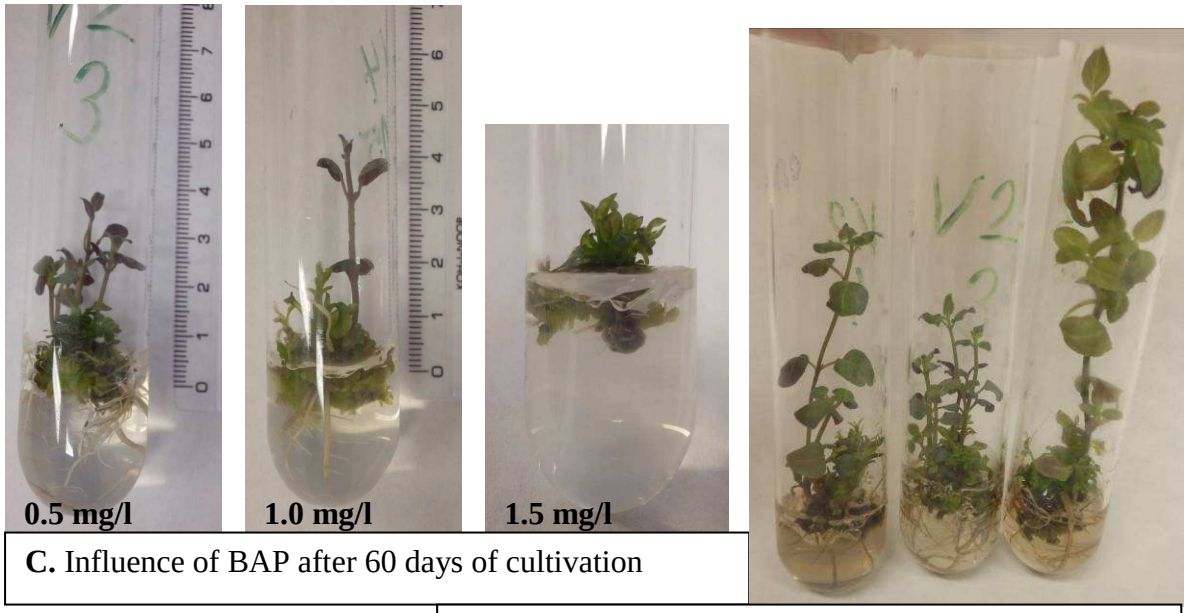

**Figure 14.** Graph of the length of longer shoot

**Source:** Author (2014)




**A.** Control plant

**B.** Influence of KIN after 60 days of cultivation




**C.** Influence of BAP after 60 days of cultivation

D. Differences in culture medium with 0.5 mg/l BAP

**Figure 15.** Influence of cytokinins on the growth and development of shoots after 60 days of cultivation, A-D.

**Source:** Author (2014)

**Roots induction**

The explants cultured on basal MS medium spontaneously induced roots, but in the treatment additioned with auxins was showed higher frequency and number of rooting (Tab. 7, Fig. 16,17).

In the first month, the percentage of roots induction was low (20-50%), beside variant of MS medium supplemented with 1.0 mg/l IAA, where the frequency of rooting was 70%. In the second month of cultivation, the percentage of roots induction increased by 15% in total frequency of rooting 65% in control variant. In variants of media with IAA roots induction increased more then 55%, where in variant supplemented with 1.5 mg. $l^{-1}$ IAA the frequency of rooting was up to 100%. In the treatment with addition of NAA, the frequency of rooting increased up to 50%, where in the variant with 0.5 mg/l NAA the frequency of rooting was 80%.

The explants in the treatment with lower concentration 1.0 mg/l of IAA induced a larger number of roots per explant (5.10±5.02) and here exist a significant difference compared to most variants, but after second month of cultivation the number of roots per explant increased in all variants. In control variant increased the number of roots much as 74% (from 1.50 to 5.75 roots per plant), the number of roots increased by 78% in variants with IAA (from 1.00 to 4.65 roots per plant) and by 91% in variants with NAA (from 0.55 to 6.00 roots per plant), but a significant differences do not exist between all treatmets containing PRGs for root induction. However, the most of roots (6.70±4.84 roots per plant) were induced in MS medium with 1.0 mg/l IAA.

Also the average of longest root was achieved after the second month of cultivation. The longest roots (0.84±0.66 cm) were induced in variant with 0.5 mg/l IAA, significant differences exist compared to other variants beside variants with IAA.

The number and length of the roots per explant increased several times after the fifth and sixth week of culture (Fig. 16, 17). The results show that for root proliferation in *Monarda didyma* is appropriate to add auxins to cultivation medium, mainly in lower doses and it is neccessary longer time of cultivation (60 days) then shorter time period (30 days).

In comparison between medium with IAA and NAA, explants preferably produced roots in a medium with IAA (Fig.18).

Prakash and Staden (2007) reported in *Hoslundia opposita* Vahl (*Laminaceae*) 68% of rooting on MS medium supplemented with 3.6 µM of IAA after 30 days of culture. In the same variant were obtained 5.3 roots per shoot with verage length of root 3.6 cm.

In *Salvia fruticosa* Mill. Arikat et al (2004) observed 70% of rooting in 2.9 µM IAA a 30% in 2.7 µM NAA. Same authors obtained 2.6 number of roots and 1.43 cm root length in concentration 2.9 µM IAA and 1 root with root length 0.25 cm in concentration 2.7 µM NAA. In higher concentrations of IAA and NAA is inhibited induction of roots (Arikat et al., 2004; Prakash and Staden, 2007). These results confirm the results gained in this study.

In other works and other species of the family *Lamiaceae* for induction of roots was used IBA instead IAA and NAA. Saha et all., 2010 in *Ocimum kilimandscharicum* Guerke reported 25% rooting in nodal explants in concentration 1.5 mg/l IBA with number of roots 0.8 and root length 0.40 cm. But in ½ MS (MS half strength) medium in the same concentration of IBA obtained higher percentage of root induction (81%), a larger number of roots (3.18) and longer roots (1.24 cm). Mehta et all. (2012), have also achieved better results in concentration 2.0 mg/l IBA, 90% induction of roots, 3.6 number of roots and root length was 1.68 cm. In the same concentration Bouhouche and Ksiksi (2007) in *Teucrium stocksianum* Boiss, other medicinal plant from family *Lamiaceae*, achieved 65% rooting with average number of roots 5.2 per explnat. But in the same concentration in v ½ MS mediu root proliferation was 100% and number of roots per explant were 19.2.

It seems that the family *Lamiaceae* species respond better at lower concentrations of salt (1/2 MS media). Figure 19 shows a diagram of for micropropagation *Monarda didyma* - phenotypic response of cultures in *in vitro* conditions.

**Table 7.** Influence of auxins to the frequency of rooting, number of roots per explant and length of roots.

**Source:** Author (2014)

| variant | PGRs concentration (mg/l) | Frequency of rooting (%) | Roots per explant (No) * | Length of roots (average cm) * | Frequency of rooting (%) * | Roots per explant (No) * | Length of roots (average cm) * |
|---|---|---|---|---|---|---|---|
| | | First month | | | Second month | | |
| Control | - | 50 | 1,50±2,12 $^{ab}$ | 0,24±0,30 $^{be}$ | 65 | 5,75±4,89 $^{a}$ | 0,73±0,69 $^{acde}$ |
| IAA | 0,5 | 35 | 1,00±1,41 $^{bce}$ | 0,30±0,44 $^{abd}$ | 80 | 4,65±3,20 $^{a}$ | 0,84±0,66 $^{be}$ |
| | 1,0 | 70 | 5,10±5,02 $^{b}$ | 0,37±0,33 $^{b}$ | 85 | 6,70±4,84 $^{a}$ | 0,71±0,45 $^{bd}$ |
| | 1,5 | 45 | 1,30±1,59 $^{bcde}$ | 0,14±0,17 $^{abc}$ | 100 | 4,95±2,11 $^{a}$ | 0,49±0,24 $^{bc}$ |
| NAA | 0,5 | 30 | 0,55±1,23 $^{ac}$ | 0,11±0,20 $^{acde}$ | 80 | 6,00±4,48 $^{a}$ | 0,49±0,31 $^{ab}$ |
| | 1,0 | 35 | 0,8±1,36 $^{ae}$ | 0,11±0,16 $^{ab}$ | 60 | 2,60±3,32 $^{a}$ | 0,27±0,27 $^{ac}$ |
| | 1,5 | 20 | 0,50±1,15 $^{ad}$ | 0,07±0,15 $^{ae}$ | 65 | 3,05±2,39 $^{a}$ | 0,25±0,23 $^{a}$ |

* Numbers followed by the same letter are not significantly different (Games-Howell test, $p < 0.05$)

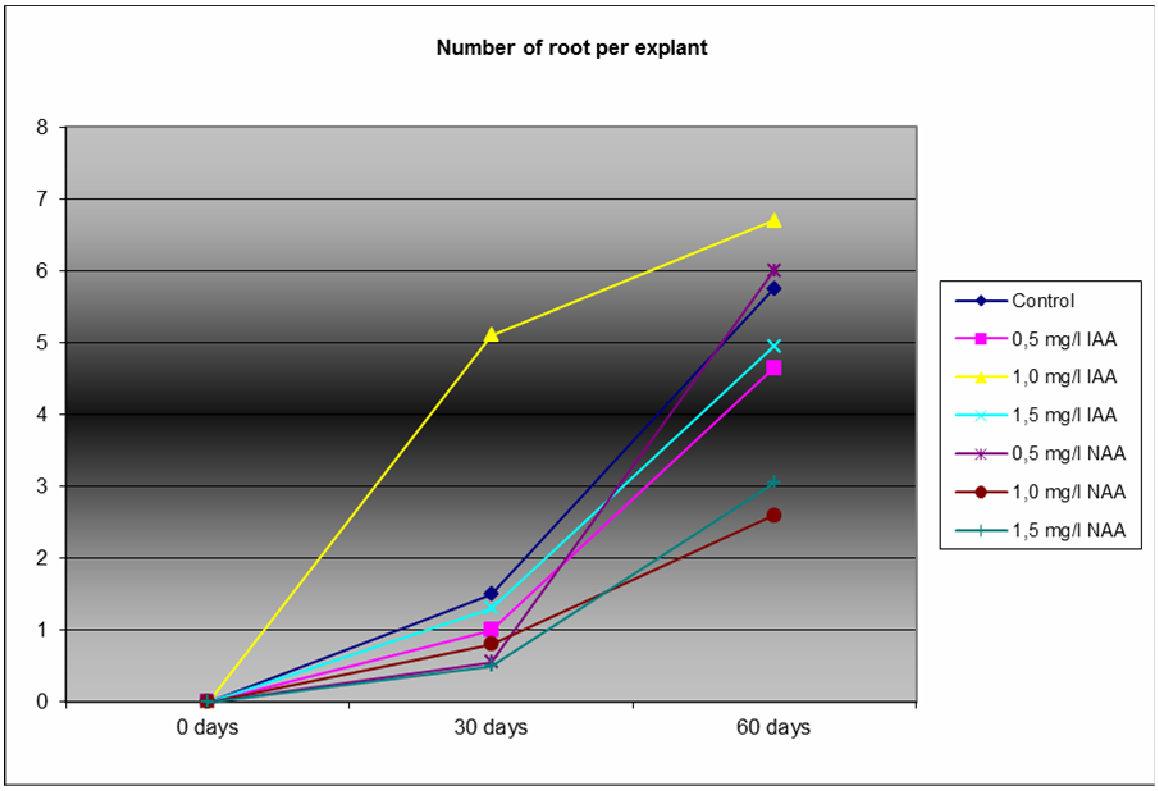



**Figure 16.** Graph of number of root per explat

**Source:** Author (2014)

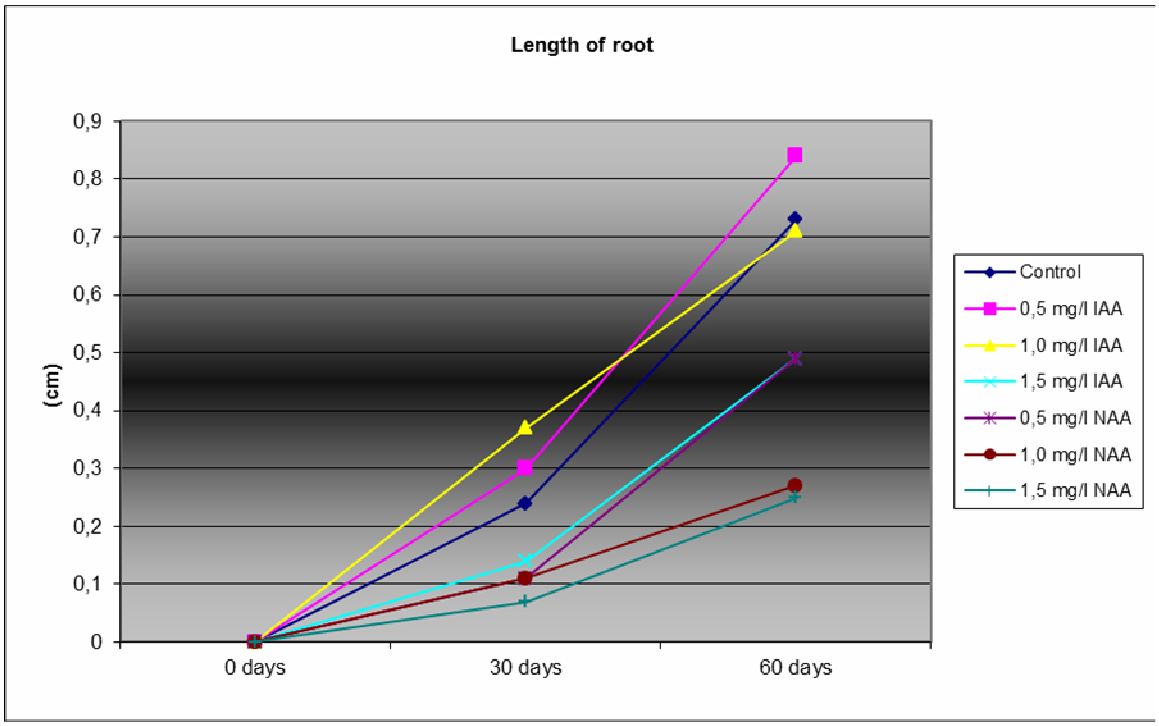
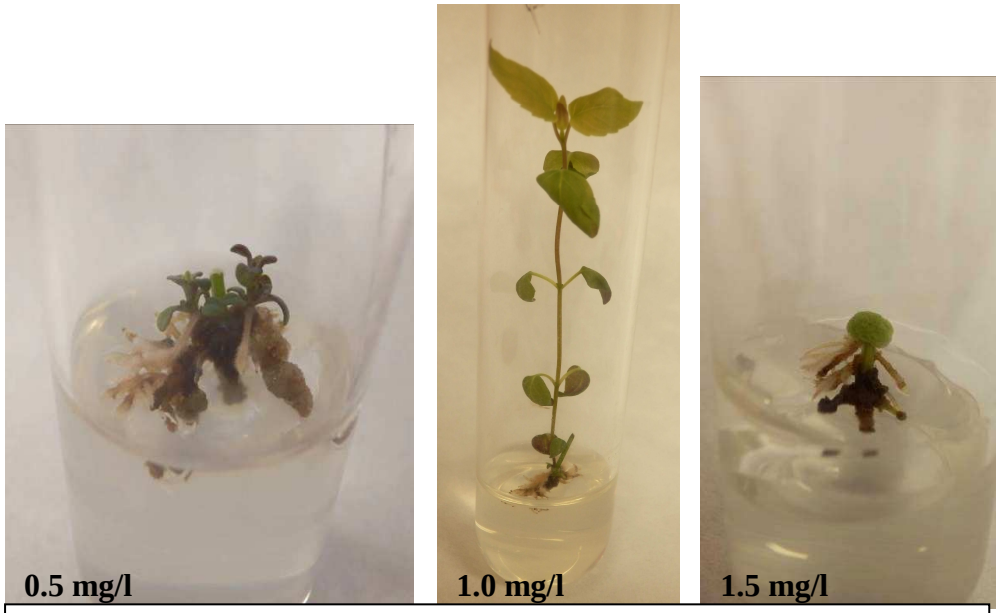
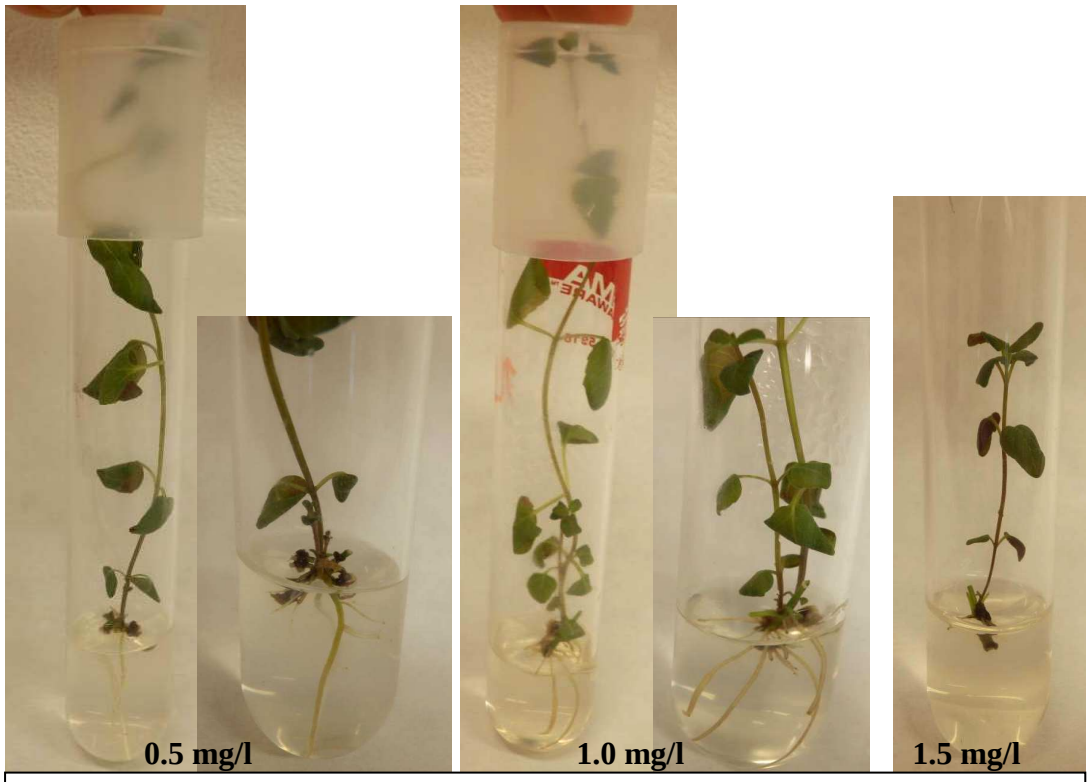



**Figure 17.** Graph of length of root

**Source:** Author (2014)




A. Influence of NAA after 60 days of cultivation




B. Influence of IAA after 60 days of cultivation

**Figure 18**. Influence of auxins on the growth and development of shoots after 60 days of cultivation. A-B.

**Source:** Author

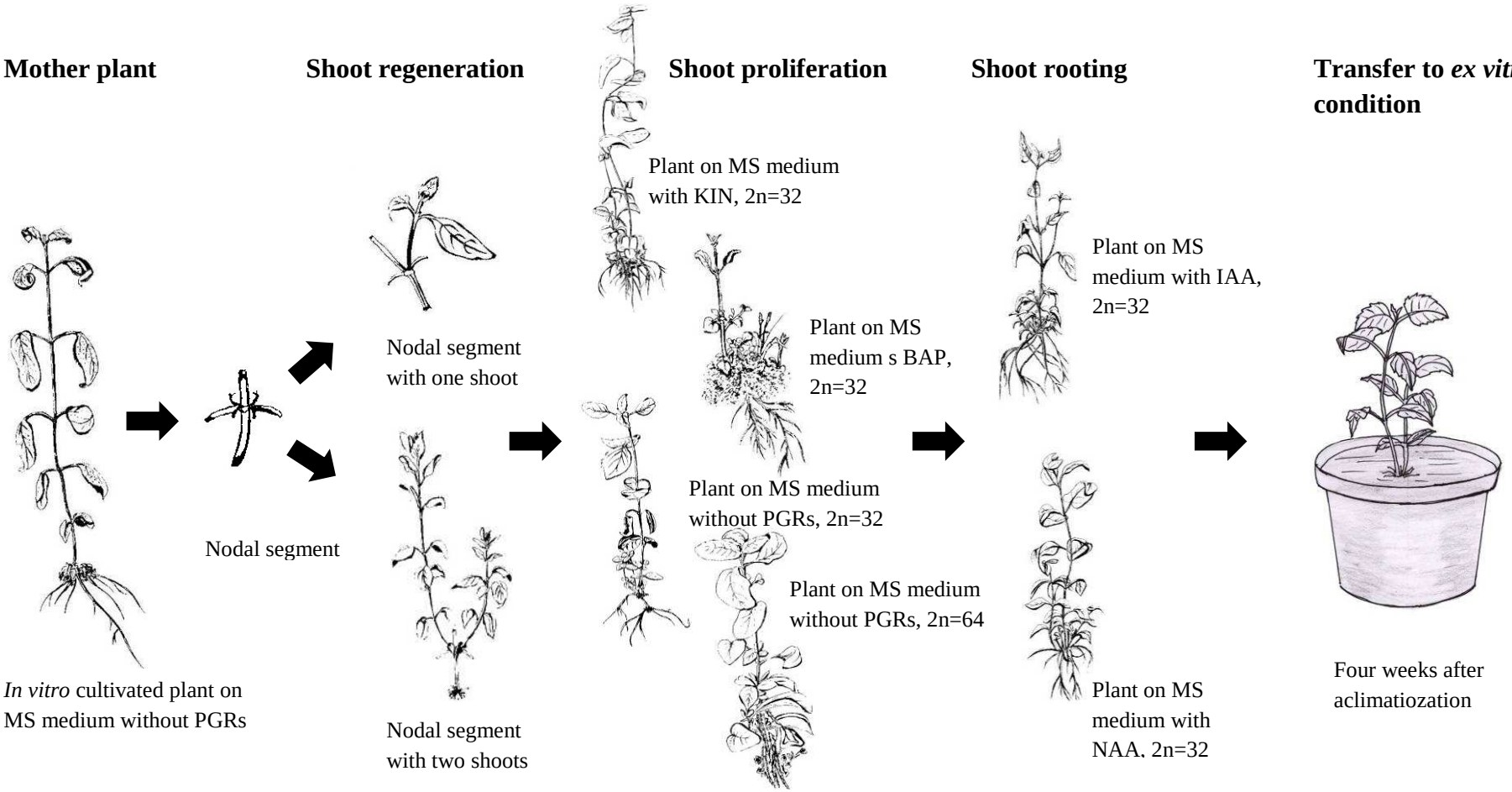



**Figure 19**. Diagram of for micropropagation *Monarda didyma* - phenotypic response of cultures in *in vitro* conditions, diploid (2n = 32) and polyploid plants (2n = 64) after 60 days of culture.

**Source:** Author (2014)

**Interaction of Cytokinins and Auxins**

In the terms of all observed parameters (number shoot, longert shoot, shoter shoot, number of nodes per explants on longer and shoter shoot, number roots and length of roots) after the first month of cultivation, there was a statistically significant differences among some treatments (Tab. 8). But after second month of cultivation there were not statistically significant differences among all treatments (Tab.9). Though the statistically significant differences does not exist between the variants, explants cultivated on MS medium without PGRs (controle variant) achieved better results in the most of observed (Fig.20,21). In comparison of interaction efficiency of IAA and BAP or NAA and KIN, the better results were obtained in combination of 0.5 mg/l IAA with 1.5 mg/l BAP. In this concentration the longest shoots were obtained (4.2±4.03 cm), the largest number of nodes on longer shoot (3.80±3.36) and on the shorter shoot (1.40±1.35) and the higer number of roots per explant (3.80±5.61). Within each vaianty between individual explants were observed physiological and morphological variability during cultivation. In some variants or explants was observed formation of callus. After the second month, the percentage of regeneration and roots induction ranged from 50 to 80% compared to variants with the addition of PGRs alone, where the percentage recovery ranged from 35-100%.

Interraction of cytokinins and auxins (IAA and BAP, NAA and KIN) in MS medium seems to be no appropriate for micropropagation of *Monarda didyma*, because the better results were obtained from cultures sultivated on MS medium supplemented by cytikinins and auxins alone.

In *Thymus lotocephalus* (other species in family *Lamiaceae*) Coelho et al., 2012 obtained better results ( number of new shoots per explantat) in concentration 0.5 mg/l BA with 0.2 mg/l IAA, but in 79% explants observed the hyperhydricity. To the same results came Bouhouch and Ksiksi (2007) in *Teucrium stocksianum* Boiss. (*Lamiaceae*) in concentration 0.5 mg/l IAA with 3.0 mg/l KIN in monitoring of effect of different combinations of plant growth regulators on shoot production. On the medium containing 2.22 µM BA and 0.49 µM IBA, Hembrom et al. (2006) reported a larger number of shoots per node and 80% of explants inducing shoots in *Pogostemon heyneanus* Benth. (*Lamiaceae*) compared to the variants with addition of cytokinins alone (BA and KIN) or in combination of cytokinins (BA with Kin). The cytokinins in combination with auxins commonly induce callogenesis in explants (Niwa et al., 2002; Manyra et al., 2009).

According to the results in *Monarda didyma* it is better cultivate the nodal explants on medium with individual content of BAP, KIN, IAA and NAA, where were getting better results.

**Table 8**. Interaction between cytokinins and auxins after 30 days of cultivation
**Source:** Author (2014)

| Variant -PGRs concentration (mg/l) | Shoot regeneration (%) | Shoots per explant (No) * | Longer shoot (cm) ** | Shorter shoot (cm) * | Nodes on longer shoot (No) * | Nodes on shorter shoot (No) * | Frequency of rooting (%) | Roots per explant (No) * | Length of roots (average cm) * |
|---|---|---|---|---|---|---|---|---|---|
| Control | 90 | 1,90±0,32 $^{b}$ | 3,21±2,36 $^{bc}$ | 0,91±1,12 $^{b}$ | 2,20±1,55 $^{bc}$ | 1,10±1,10 $^{b}$ | 50 | 2,30±2,54 $^{a}$ | 0,28±0,26 $^{a}$ |
| 0,5IAA+1,0BAP | 90 | 1,30±0,67 $^{bc}$ | 1,36±2,34 $^{ac}$ | 0,19±0,25 $^{bc}$ | 0,90±1,29 $^{ab}$ | 0,10±0,32 $^{ab}$ | 20 | 0,40±0,97 $^{a}$ | 0,17±0,38 $^{a}$ |
| 0,5IAA+1,5BAP | 80 | 1,50±0,85 $^{abc}$ | 1,90±1,73 $^{ac}$ | 0,69±0,83 $^{abc}$ | 1,80±1,75 $^{ab}$ | 0,80±0,92 $^{ab}$ | 40 | 1,10±1,91 $^{a}$ | 0,26±0,35 $^{a}$ |
| 1,0NAA+1,0KIN | 90 | 1,50±0,85 $^{ab}$ | 0,44±0,45 $^{a}$ | 0,19±0,14 $^{bc}$ | 0,30±0,67 $^{a}$ | 0,00±0,00 $^{a}$ | 30 | 0,80±1,40 $^{a}$ | 0,26±0,46 $^{a}$ |
| 1,0NAA+1,5KIN | 70 | 0,80±0,79 $^{ac}$ | 0,52±0,60 $^{a}$ | 0,07±0,16 $^{ac}$ | 0,40±0,70 $^{ac}$ | 0,00±0,00 $^{a}$ | 40 | 1,70±2,75 $^{a}$ | 0,37±0,49 $^{a}$ |

* Numbers followed by the same letter are not significantly different (Kruskal-Wallis test, $p < 0.05$); ** Numbers followed by the same letter are not significantly different (Games-Howell test, $p < 0.05$)

**Table 9**. Interaction between cytokinins and auxins after 60 days of cultivation
**Source:** Author (2014)

| Variant -PGRs concentration (mg/l) | Shoot regeneration (%) | Shoots per explant (No) *** | Longer shoot (cm) *** | Shorter shoot (cm) ** | Nodes on longer shoot (No) ** | Nodes on shorter shoot (No) * | Frequency of rooting (%) | Roots per explant (No) *** | Lenght of roots (average cm) * |
|---|---|---|---|---|---|---|---|---|---|
| Control | 70 | 1,40±0,97 $^{a}$ | 5,36±4,86 $^{a}$ | 1,61±2,20 $^{a}$ | 3,20±2,94 $^{a}$ | 1,50±1,51 $^{a}$ | 65 | 6,80±5,27 $^{a}$ | 0,61±0,51 $^{a}$ |
| 0,5IAA+1,0BAP | 80 | 1,40±0,84 $^{a}$ | 1,78±3,05 $^{a}$ | 0,48±0,61 $^{a}$ | 1,40±1,90 $^{a}$ | 0,40±0,70 $^{a}$ | 30 | 1,40±2,37 $^{a}$ | 0,28±0,46 $^{a}$ |
| 0,5IAA+1,5BAP | 60 | 1,20±1,03 $^{a}$ | 4,2±4,03 $^{a}$ | 1,13±1,18 $^{a}$ | 3,80±3,36 $^{a}$ | 1,40±1,35 $^{a}$ | 50 | 3,80±5,61 $^{a}$ | 0,83±0,95 $^{a}$ |
| 1,0NAA+1,0KIN | 70 | 1,20±1,03 $^{a}$ | 1,12±1,44 $^{a}$ | 0,53±0,77 $^{a}$ | 1,20±1,23 $^{a}$ | 0,70±1,25 $^{a}$ | 40 | 3,70±5,10 $^{a}$ | 0,46±0,70 $^{a}$ |
| 1,0NAA+1,5KIN | 50 | 0,80±0,92 $^{a}$ | 1,83±4,16 $^{a}$ | 0,12±0,26 $^{a}$ | 1,10±1,73 $^{a}$ | 0,20±0,42 $^{a}$ | 30 | 3,40±6,19 $^{a}$ | 0,30±0,59 $^{a}$ |

* Numbers followed by the same letter are not significantly different (Kruskal-Wallis test, $p < 0.05$); ** Numbers followed by the same letter are not significantly different (Games-Howell test, $p < 0.05$); *** Numbers followed by the same letter are not significantly different (Tukey test, $p < 0.05$)


**Figure 20.** Graph of interaction of cytokinins and auxins to the length of longer shoot

**Source:** Author (2014)

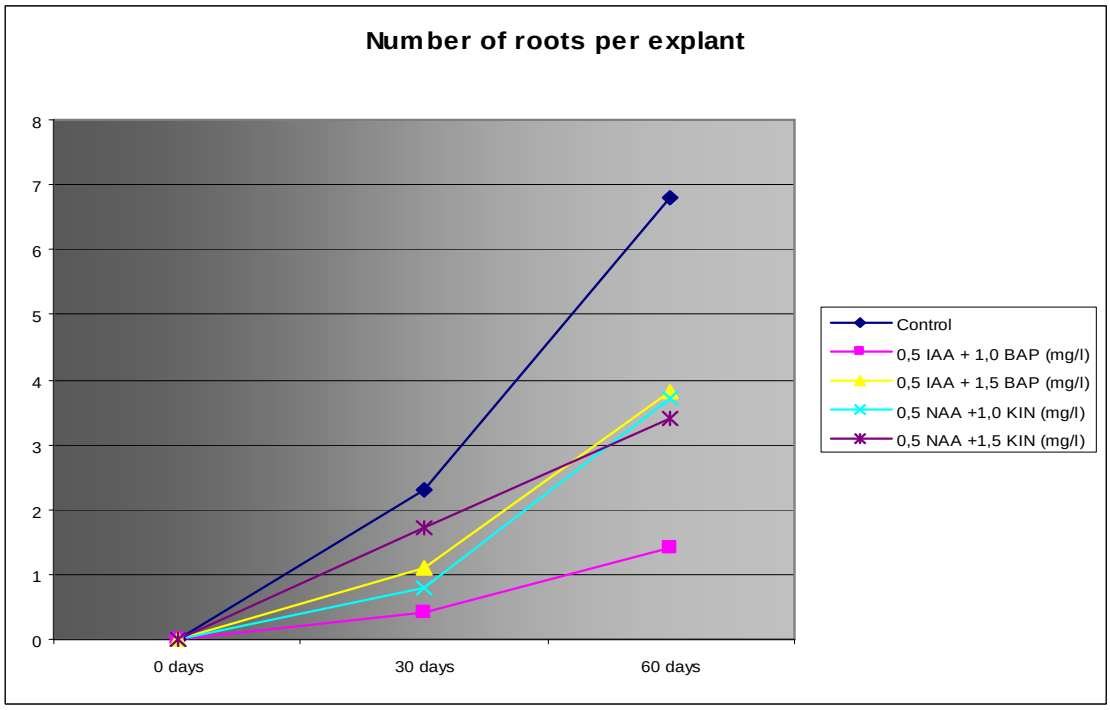



**Figure 21.** Graph of interaction of cytokinins and auxins to the number of roots per explnat

**Source:** Author (2014)

### 5.2. Transfer to ex vitro condition

*In vitro* rooted plants (76% average from all variants) were acclimatized in *ex vitro* conditions with the success rate30%. The plants did not show morphological differences. From the all tested variant the best acclimatized plants were from variant of culture medium supplemented with 1.0 and 1.5 $mg.l^{-1}$ KIN a 1.5 $mg.l^{-1}$ IAA (Fig.22.). The successful transfer depends on the vitality, size of plants, humid condition and temperature. Stronger plants had a better option of survival. Better results were obtained in other species of *Lamiaceae* family: *Thymus lotocephalus* (93.33%) (Coelho et al., 2012), *Salvia stenophylla* (75%) (Masarurwa et al., 2010), *Salvia africana-lutea* (88%) (Mankunga et al., 2008). The results demonstrate that the Crimson Beebalm is not a plant resistant and very adaptable to changes of growing conditions. There have to big caution during transferring. It will be necessary to prepare protocol for better transfer *in vitro* plants to *ex vitro* conditions.

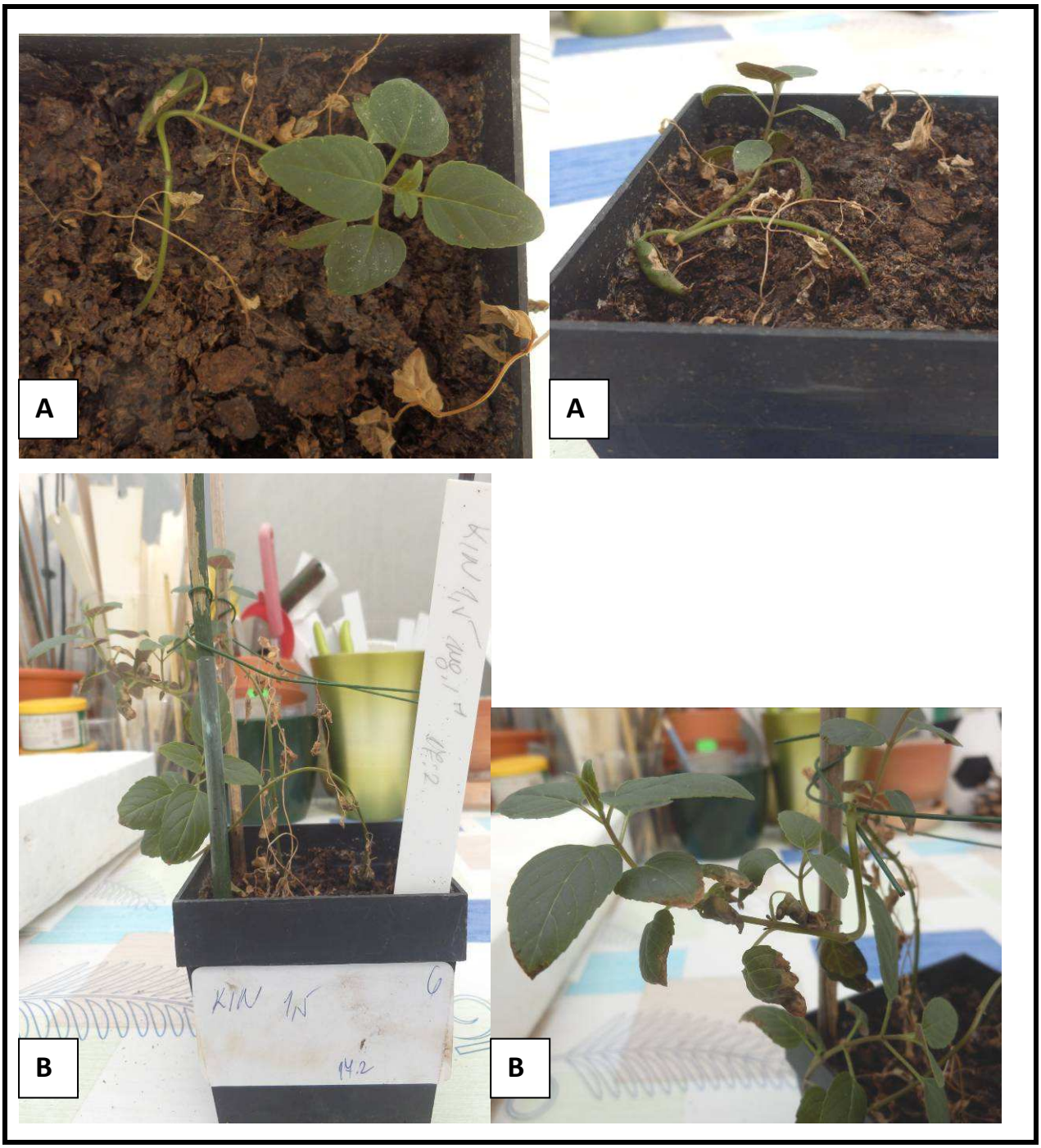



**Figure 22.** The best transferred plants; A - plants from treatment with IAA 1.5 mg/l; B - plants from treatment with KIN 1.5 mg/l

**Source:** Author (2014)

### 5.3. Induction of polyploidy and detection of ploidy level

Nodal segments exposed to 40, 60 and 80 μM of oryzalin for 24 and 48 h were cultivated for 3 weeks on MS medium and the cultures were incubated at 25/20 ± 0,3 °C under a 16/8 h light regime in cultivation box with (POL-EKO ILW350/350 STD), with 2500 lux light intensity provided by cool white fluorescent lamps (Philips LT5 14 W/840). Then the viable explants were multiplicated to the next generation by using nodal segments. The percentage of viable plants was until 24.3 % (Tab. 10).

The detection of ploidy level by using flow cytometry was carried out 2 months after the oryzalin treatment. 39 influenced and 3 control samples were analysed on flow cytometer. Flow cytometric analysis showed the relative DNA content in histogram with a peak corresponding to G0/G1 nuclei of the control plant (diploid) (Fig. 23A.). From 39 influenced plants we obtained 1 tetraploid from treatment of 40μM of oryzalin for 24h, 3 tetrapoids from tratment of 60μM of oryzalin for 24h (Fig. 23B.) and 1 triploid from treatment of 60μM of oryzalin for 48h (Fig. 23C). Polyploidization efficiency (%) according to the variants ranged from 1.4 to 4.3%.

Morphological changes were observed in 2 samples compared to control plants. Both samples treated in 60 μM of oryzalin for 24 and 48 hours had significantly shorter internodes, more leaves per explant and different shape of leaves (Fig. 24,25.) compared to the control plant (Fig. 26).

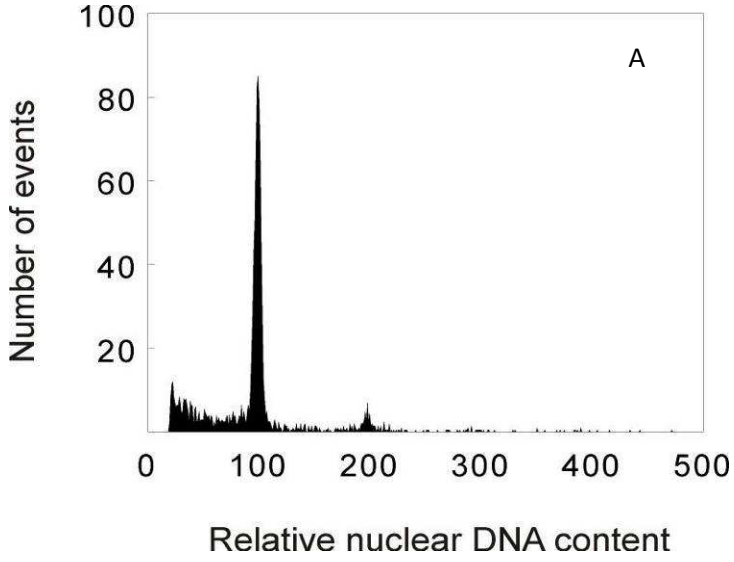
Changes were obtained also in the size of stomata in the bottom part of leaves of tetraploid plant (60/24) compared to diploid plant (control plant) (Fig.27). On the average, diploids and tetraploids have a mean (stomata) length of 31.42 ± 1.53 μm and 45.39 ± 2.74 μm, respectively.Average size of stomata of control plant was 349.3$\mu m^2$ and 425.09 $\mu m^2$ of tetraploid plant.

From direct counting of chromosomes we did not obtain any relevant figure for processing. We achieved only reprezentative figure of chromosomes in the cell of control plant (diploid) (Fig.28).

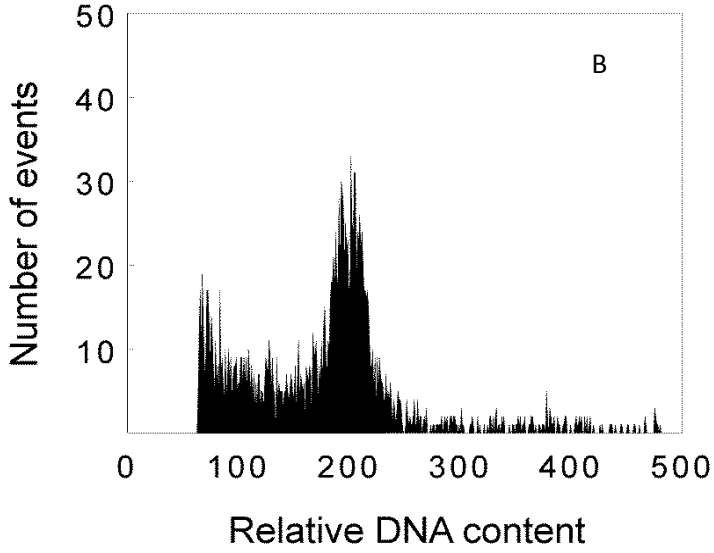
The purpose of polyploidization was to increase content of substance and the control samples of *in vitro* cultivated plants have a 0.742 mg of thymoquinone per gram of material, but in polyploids has not yet been done. It will be the subject of further study. According preliminary results from *in vitro* cultivated plants, content of substances is less in comparison with plants cultivated *in vivo*. It will be compared *in vitro* plants (controle and polyploid). Then the same will be done after aclimatization of polyploids in *in vivo.*

**Table 10.** Effect of *in vitro* oryzalin treatment on the survival rate and number of polyploids in *Monarda didyma*

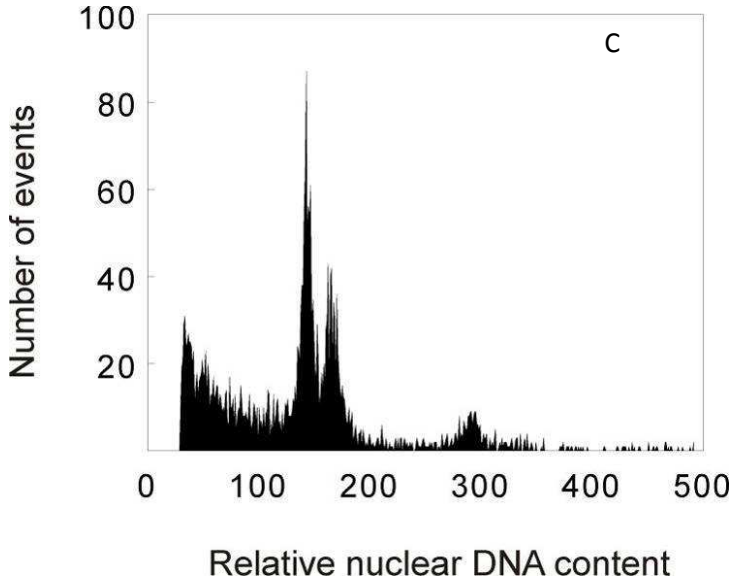
| Treatment | Concentration (µM) | Treatment duration (h) | No. of explants examined | Survival rate (%) | No. of polyploids | Polyploidization efficiency (%) |
|---|---|---|---|---|---|---|
| Oryzalin | 40 | 24 | 70 | 11.4 | 1 | 1.4 |
| | | 48 | 30 | 0 | 0 | 0 |
| | 60 | 24 | 70 | 24.3 | 3 | 4.3 |
| | | 48 | 30 | 7.1 | 1 | 3.3 |
| | 80 | 24 | 30 | 12.9 | 0 | 0 |
| | | 48 | 30 | 0 | 0 | 0 |
| Total | | | 260 | | 5 | 1.92 |




**A.** Histogram of relative DNA content with a peak corresponding to G0/G1 nuclei of the control plant on channel 100




**B.** Histogram of relative DNA content with a peak corresponding to G0/G1 nuclei of the plant sample 60 μM for 24 hours, likely polyploid (tetraploid) plant on channel 200




**C.** Histogram of relative DNA content with a peak corresponding to G0/G1 nuclei of the plant sample 60 μM for 48 hours, likely polyploid (triploid) plant on channel 150

**Figure 23.** Histograms of ploidy level
**Source:** Author (2014)

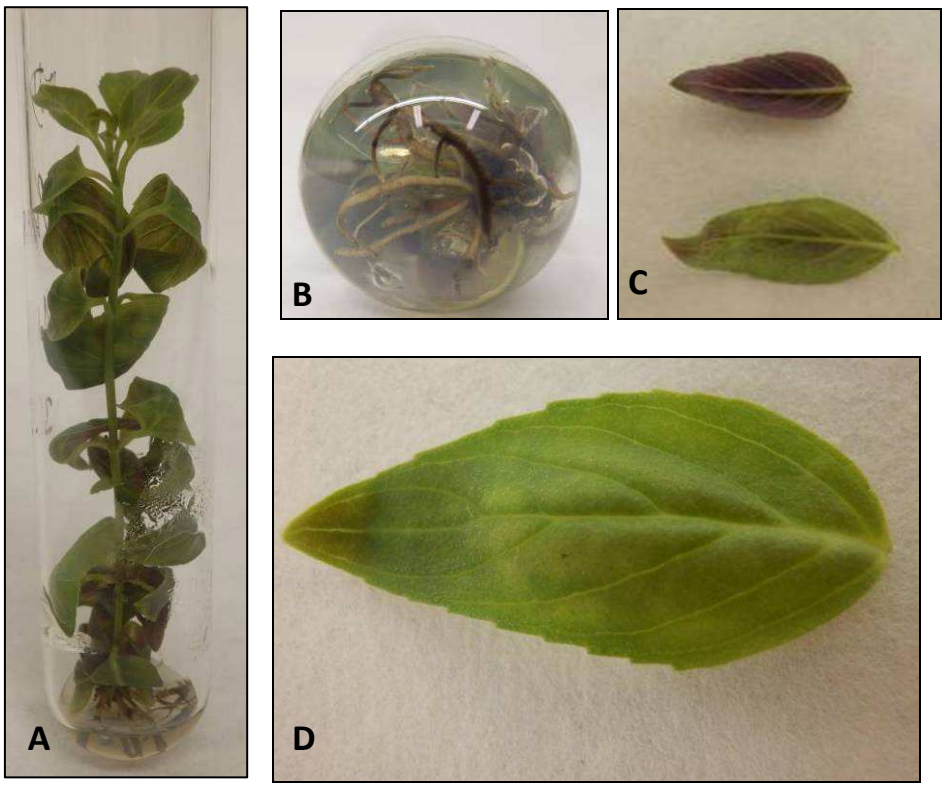



**Figure 24.** Polyploid plant - 60 µM/48 hours; A- whole plant (8.5cm), B- roots. C- leaves from the top and bottom side, D - leaf (1.6 cm)
**Source:** Author (2014)

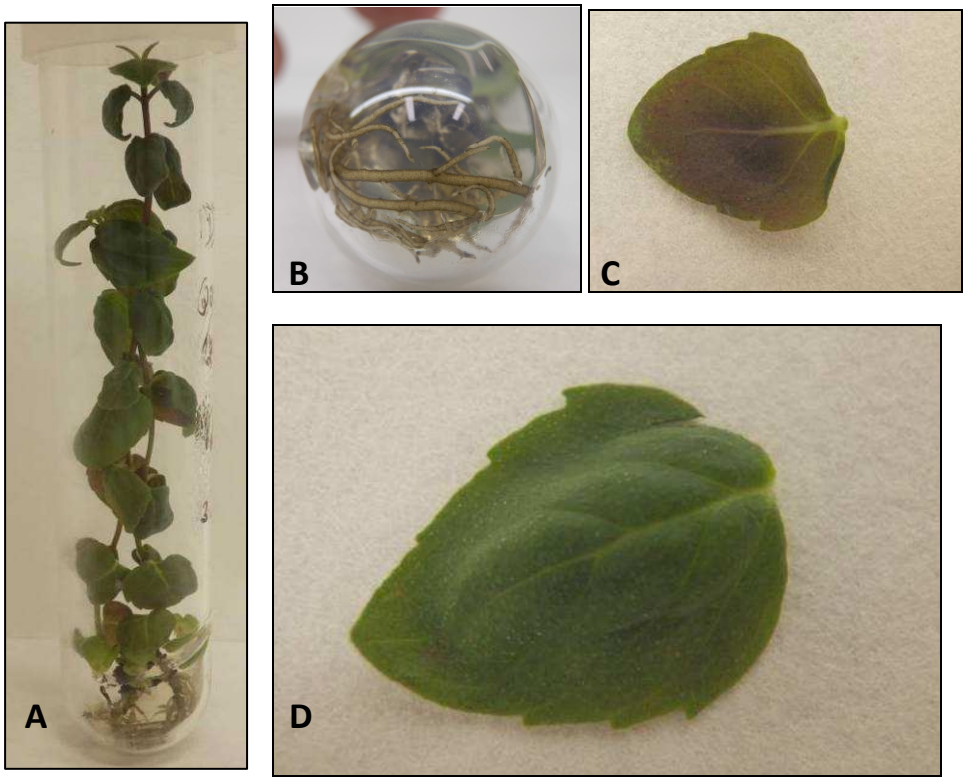



**Figure 25.** Polyploid plant - 60 µM/24 hours; A- whole plant (10.2cm), B- roots. C- leaves from the bottom side, D - leaf from the top side (0.9 cm)
**Source:** Author (2014)

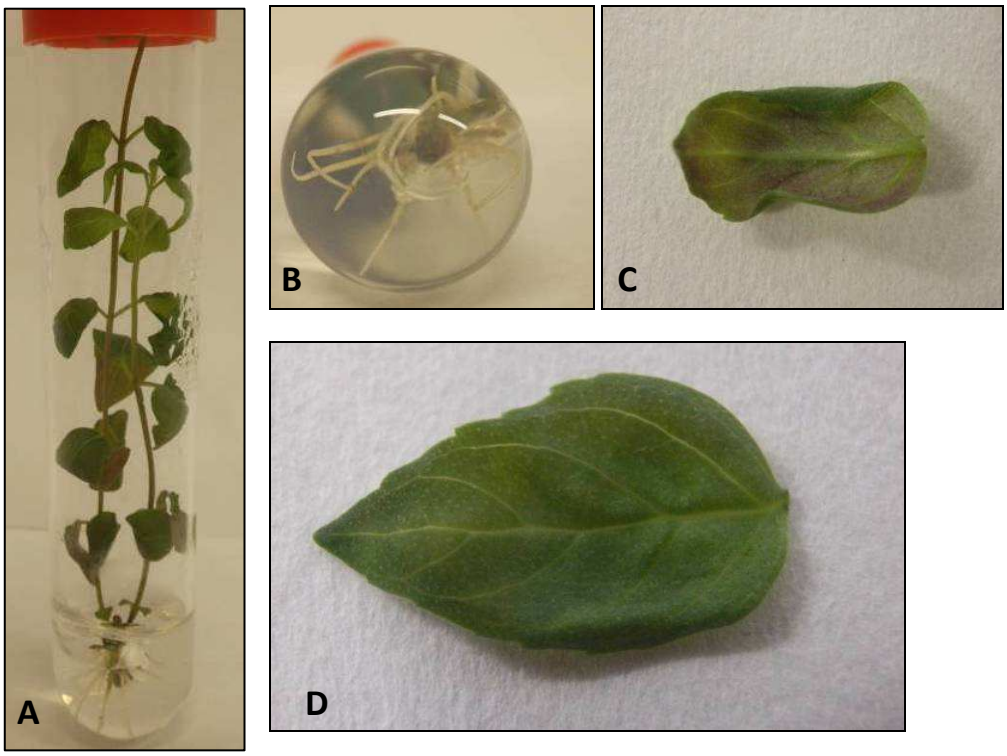



**Figure 26.** Control plant; A- whole plant (11.3cm), B- roots, C- leaves from the bottom side, D - leaf from the top side (1.2 cm)
**Source:** Author (2014)

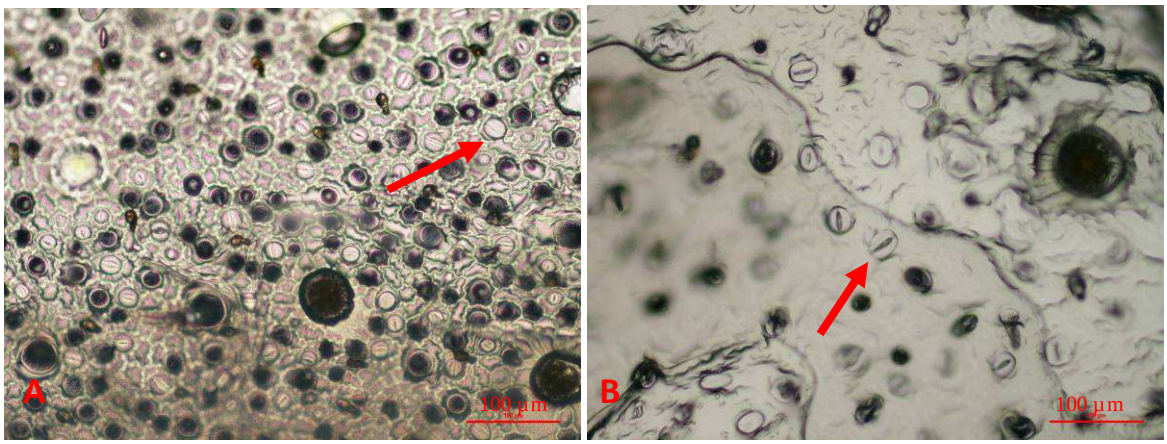



**Figure 27**. Comparision of stomata size in diploid plant (A) and tetraploid plant 60/24 (B)

**Source**: Autor (2014)

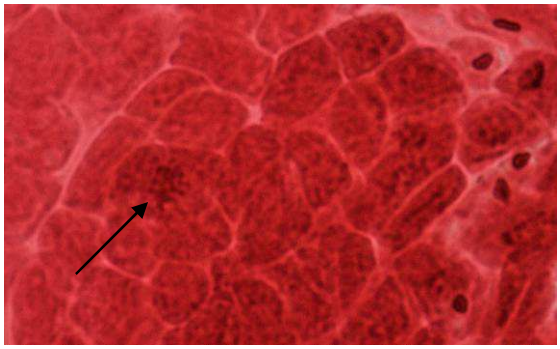


**Figure 28**. Chromosomes of diploid plant

**Source:**Author (2014)

There is no available information about induction of polyploidy in *Monarda* spp. or in *Lamiaceae* family by oryzalin treatment. From the same family *Lamiaceae* was induced polyploids in Basil (*Ocimum basilicum* L.). Omidbaigi et al. (2010) used for polyploids induction colchicine as an antimitotic agent. Treatment of 0.5 % colchicine showed the highest efficiency for tetraploid induction by treatment of growing point of seedlings, at the emergence of cotyledone leaves stage. At induced tetraploids in basil were showed larger stomatas and pollen grains, increase in chloroplast number in guard cells and decrease in stomata density, compared to diploid control plants.

The polyploidization efficiency of *Monarda didyma* in total was very low 1.92%. Viehmannová et al. (2009) reached in *Smallanthus sonchifolius* Robinson efficiency 3.33% by using oryzalin treatment. In case of *Ullucus tuberosus* Caldas, there was polyploidy efficiency only 2.5% (Viehmannová et al., (2012). Fong (2008) reported the percentage of tetraploid induction low: 1.39% in colchicine and 0.70% oryzalin treatments, respectively.

From other family *Apiaceae* in medicinal plant *Centella asiatica* L. was induced polyploids by using in vitro-grown shoot-tips. The ploidy levels of regenerated plants in *ex vitro* conditions were determined by flow cytometry and by determining chromosome counts. Treating shoot-tips with colchicine concentrations ranging from 0.05-0.20 % for 12 and 24 h promoted induction of tetraploids. Tetraploid plants demonstrated significantly longer stomata and a higher stomatal index compared to those of the diploid control plants (Kaensaksiri et al., 2011).

Oryzalin as a antimitotic agent was used in study of Allum et al. (2004), where were also exposed to nodal segments of *Rosa rugosa* Thunb.. Most successful was treatment 5 μM for 12 hours. Other Rosa species produced most tetraploids after same oryzalin concentration as in *Rosa rugosa* Thunb., indeed exposure time was longer (24 h) (Kermani et al., 2003). Sakhanokho et al. (2009) reported successful use of oryzalin treatment in *Hedychium muluense* Smith (*Zingiberaceae*). The most efficient treatment was that callus was exposed to 60 μM oryzalin for 72 h. Viehmannová et al. (2009) reported successful oryzalin treatments, which were applied on nodal segments of *Smallanthus sonchifolius* Robinson from family *Asteraceae*. The treatment of 25 μM oryzalin for 48 h had a highest efficiency for tetraploid induction. Polyploidy inducing protocol according was also used for *Ullucus tuberosus* Caldas, *Basellaceae* family, by using nodal segments. The most efficient treatment was 20 μM oryzalin for 24 h and to 25 μM oryzalin for 48 h (Viehmannová et al., 2012).

Fong (2008) reported that the stomata size of tetraploids of *Nepenthes gracilis* (Korth.) were significantly larger than stomata of diploids, while the stomata frequency was lower in tetraploids than diploids. On the average, diploids and tetraploids have a mean length of 31.42 ± 1.53 μm and 45.39 ± 2.74 μm, respectively. In the case of *Coffea canephora* (Pierre ex A.Froehner) was noticed a significant reduction in stomatal frequency from diploid to tetraploid level which was due to larger epidermal cell size and less stomatal differentiation at the tetraploid level (Mishra, 1997). The stomata length and width in *Gerbera jamesonii* (Bolus cv. Sciella) were significantly higher in tetraploids (34.55 and 28.59 μm) over that of the diploids (27.41 and 26.45 μm) (Saikat et al., 2011).

### 5.4. Evaluation of genetic stability

The amplified DNA products were screened in twelve plants obtained from each variant of micropropagtion (A1-A12), two polyploid (2n=4x) plants (P1-2) and compared with DNA template of control plant (Co). A set of 2 RAPD primers were screened. In total, out of these two primers were produced a total of 12 clear and distinct bands. Results of the analysis (Tab. 11) revealed that there were not differences between treatments. The amplified products of primers OPB-4 and OPB-14 were monomorphic across all regenerated plants and were similar to the control. During cultivation the micropropagated plants was no mutation. Primer OPT-4 produced 6 scorable bands in range 400-1000 bp and primer OPB-14 produced also 6 scorable bands in range 250-750 bp. Figure 29 and 30 show monomorphic patterns obtained with RAPD primers.

**Table 11**: RAPD primers used for detecting the genetic stability in regenerants of *M.didyma* and the bands generated (bp= base pairs)

| Primer code | Primer sequence 5´- 3´ | No. of scorable bands per primer (total/polymorphic) | No. of total bands | Frequency of monomorphic band | Approximate size range (bp) |
|---|---|---|---|---|---|
| OPB-4 | GG AC TG GA GT | 6/0 | 90 | 100 % | 400-1000 |
| OPB-14 | TC CG CT CT GG | 6/0 | 90 | 100 % | 250-750 |
| Total | - | 12 | 180 | - | - |

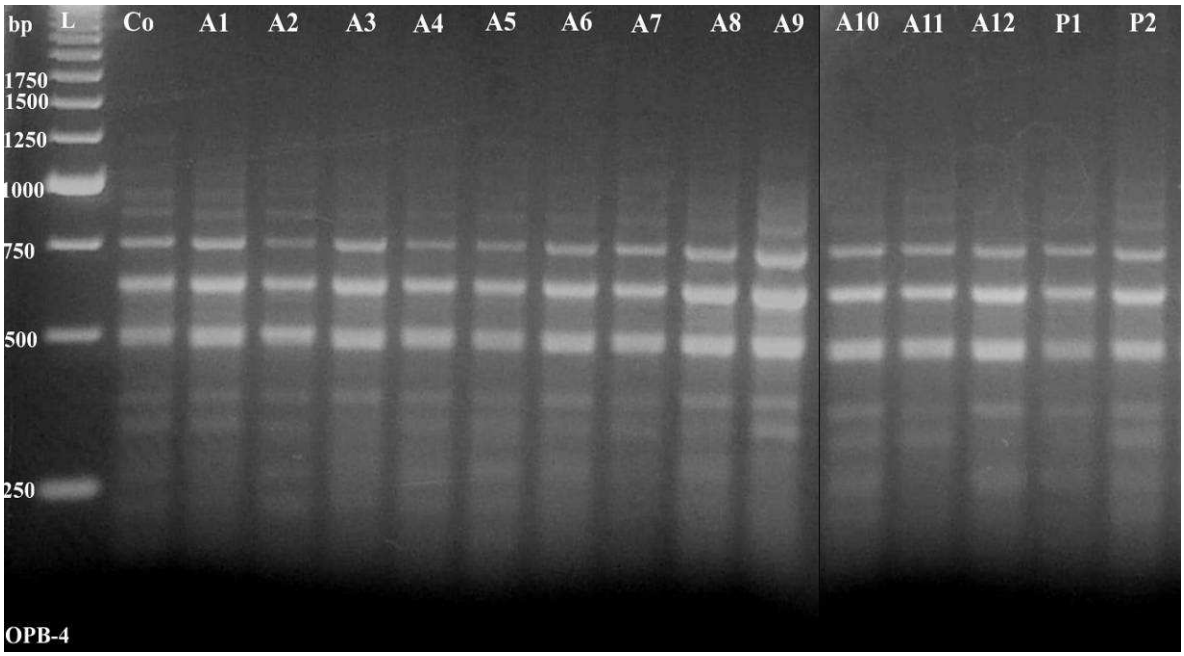



**Figure 29.** Monomorphic profile using primer OPB-4. bp: base pairs; L: 250bp DNA ladder; Co: control; A1-P2: regenerants.

**Source:** Author (2014)

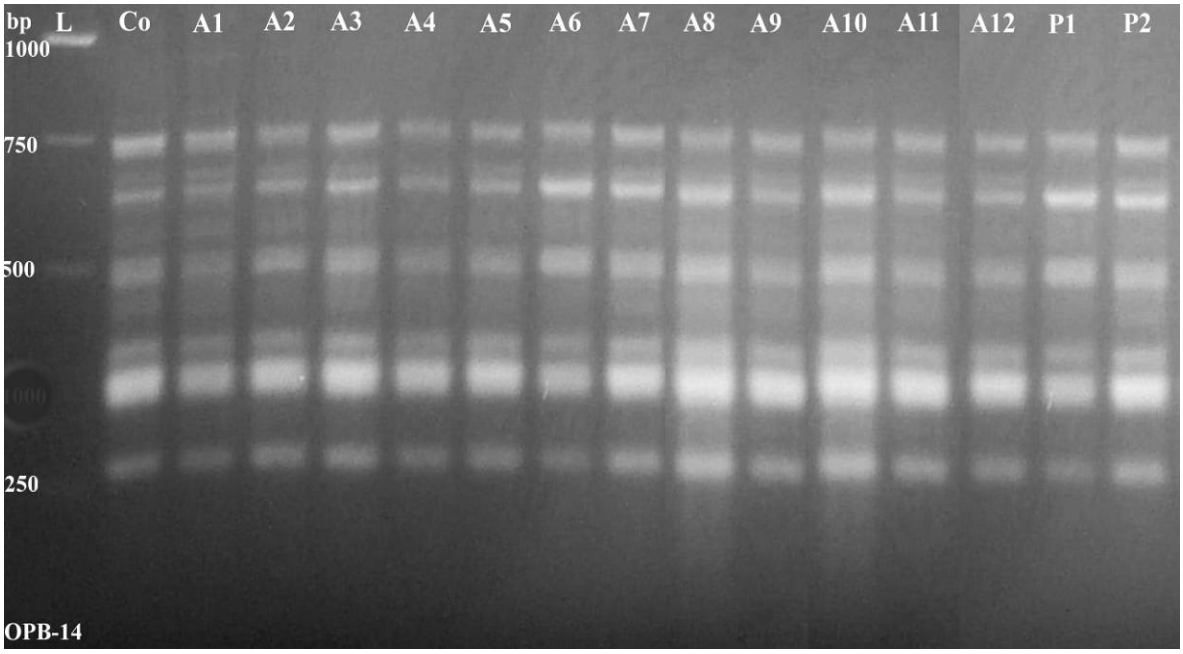



**Figure 30.** Monomorphic profile using primer OPB-14. bp: base pairs; L: 250bp DNA ladder; Co: control; A1-P2: regenerants.

**Source:** Author (2014)

In literature, genetic stability or variability of plants propagated via direct organogenesis is assessed by molecular marker techniques.

Martins et al. (2004) established RAPD and ISSR analysis for evaluation of somatoclonal variation. For analysis were used plantlets obtaind from in vitro cultures cultivated on MS medium with addition of PRGs - IBA, IAA. A total of 64 RAPD and 10 ISSR tested primers showed monomorphic amplification profiles. These results suggest that the culture conditions used for branching proliferartion are appropriate for clonal propagation of almond plantlets.

RAPD markers were used to determined the genetic stability of micropropagated shoots of *Pinus thunbergii* Parl. During this analysis exhibited no deviation in RAPD banding patterns among the tested shoots. These shoots were cultivated on LP medium with BAP. These results show that the regenetrants from our plant micropropagation system are genetically stable (Goto et al., 1998).

Rout et al. (1998) used RAPD markers for detection of variation in *in vivo* and *in vitro* cultivated plants. All RAPD profiles from micropropagated plants were monomorphic. No variation was detected within the micropropagated plants. In vitro plants were cultivated on MS medium supplemented with BAP and IAA.

RAPD markers were also used for detection of clonal fidelity in microrhizomes of Turmeric, which were cultivated on MS medium with BA and NAA. RAPD profiles revealed that all the bands produced by microrhizome induced plants were monomorphic and similar to the mother plant for all the primers tested (Archana et al., 2013).

In genus Monarda was applicated molecular markers only in *Monarda fistulosa*, where were tested genetic variation between *M. fistulosa* var. fitulosa and var. brevis. Kimbal et al. (2001) tested ISSR markers for establishment of diversity from the leaves, which were kept fresh at 4°C. This study utilizing ISSR markers indicated that these two varieties are distinct.

Genetic fidelity of plants propagated via direct organogenesis have been proved using ISSR and RAPD markers in following plant species: *Gerbera jamesonii* Bolus (Bhatia et al., 2011), Simmondsia chinensis (Link) Schneider (Kumar et al., 2011), Olea europaea L. (Leva and Petruccelli, 2012), Guadua angustifolia Kunth (Nadha et al., 2011), Aloe vera L. (Rathore et al., 2011) and in many others.

In case of kiwifruit (*Actinidia deliciosa*) were compared RAPD and SSR molecular markers to detection of genetic variability. There were analyzed *in vivo* and *in vitro* kept plants. Both DNA based techniques were able to amplify all of the genotypes, but only SSR

markers could detect genetic variation induced in micropropagated plants. Palombi et al. (2002) conclude that when the tissue culture technique is used, the analysis of somatoclonal variability could require more than one DNA - based technique.

Somaclonal variability obtained with multiplication of plants through direct organogenesis can be influenced by rate of multiplication, number of subcultures, plant genotype, concentrations of PGRs and the rate of initial explants (with a large number of multiplication cycles the final rate of variants in the population will be very high (Bairu et al., 2006; Skirvin et al., 1994).

# 6. Conclusion

*Monarda didyma* L. is a very promising plant in terms of *in vitro* propagation, it shows high ability to respond to PGR supplements on shoot proliferation and root induction. Formation of shoots and new nodes at low concentration of cytokinins was found however treatments of higher concentrations can have a negative influence. All auxin treatments were very effective in root induction especially their higher concentrations (1.0 mg/l IAA).

RAPD molecular analysis demonstrated that the regenerated plants from nodal segments cultivated on MS medium with different supplements of PGRs have genetic stability, which is adequate for the multiplication of identical plants in *in vitro* cultures.

The obtained results can be used for the rapid multiplication newly gained varieties in breeding programs. By using somatic polyploidization *in vitro* were obtained triploid (2n=48 chromosome) and tetraploid (2n=64) plants. These obtained polyploids will be the subject of research in terms of content of thymohydroquinone, dithymoquinone and thymoquinone.

Results from *in vitro* micropropagation, the genetic stability of *in vitro* propagated plants and *in vitro* polyploidy are primary in this species.

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# 8. Appendix

**Table 12.** The composition of the MS medium

**Source:** Murashige and Skoog (1962)

| Medium Murashige – Skoog | | | |
|---|---|---|---|
| Stock solutions for 1 liter of distilled water | | Batch size to 1 liter of stock solution | On the 1 liter to measure (pH 5,7) |
| A | $NH_4NO_3$ | 16,5 g | 100 ml |
| | $KNO_3$ | 19 g | |
| | $CaCl_2$ | 3,3 g | |
| | $MgSO_4 \times 7H_2O$ | 3,7 g | |
| | $KH_2PO_4$ | 1,7 g | |
| B | $H_3BO_3$ | 0,62 g | 10 ml |
| | $MnSO_4 \times 4 H_2O$ | 2,23 g | |
| | $ZnSO_4 \times 4 H_2O$ | 0,86 g | |
| C | KI | 0,083 g | 10 ml |
| | $Na_2MoO_4 \times 4H_2O$ | 0,025 g | |
| D | $CuSO_4 \times 5 H_2O$ | 0,0025 g | 10 ml |
| | CoCl x 6 2O | 0,0025 g | |
| E | Na2EDTA | 3,72 g | 10 ml |
| | $FeSO_4 \times 7 H_2O$ | 2,78 g | |
| V | nicotin acid | 0,05 g | 10 ml |
| | pyridoxin | 0,05 g | |
| | thiamin | 0,01 g | |
| | glycin | 0,2 g | |

Direct batch size to the medium:

Myoinositol 0,1 g

Sucrose 30 g

Agar 8 g