RESEARCH ARTICLE

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Micropropagation of *Helichrysum italicum* (ROTH) G. Don – a medicinal plant with ornamental value

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ABSTRACT

Helichrysum italicum (Roth) G. Don is a Mediterranean vegetal species from the *Asteraceae* family. The ornamental value of the flowers and the properties of its essential oils contribute to its popularity. The aim of the present study was to compare the effect of different cytokinins on the multiplication of *Helichrysum italicum* and to develop a reliable protocol for *in vitro* micropropagation of this medicinal and ornamental plant. Nutrient media based on both MS (Murashige and Skoog, 1962) or DKW (Driver and Kuniyuki, 1984) formulations were used. They were enriched with different cytokinins - BAP, Kinetin, or Zeatin (5µM). The best multiplication rate was achieved on DKW medium, supplemented with 5µM Kinetin. A high percentage of rooting was achieved on the control treatment and the nutrient media, supplemented with IBA.

Key words: tissue culture, ornamental and medicinal plants, multiplication rate

Introduction

Medicinal plants are the oldest source of pharmacologically active compounds and have played a dominant role in the introduction of new therapeutic agents. The international trade of medicinal plants is growing phenomenally, often detrimental to their natural habitats. Indiscriminate harvesting leads to extinction of natural populations which are still the only source of raw material. Cultivation is one way to prevent the biodiversity and natural habitats of medicinal plants. In the search for alternatives to the production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Rao and Ravishankar, 2002). *In vitro* propagation of medicinal plants with enriched bioactive principles and cell culture methodologies for selective metabolite production is found to be highly useful for commercial production of medicinally important compounds. Advances in plant cell cultures could provide new means for cost-effective, commercial production of rare or exotic plants, their cells, and the chemicals that they produce. These new technologies will serve to extend and enhance the continued usefulness of higher plants as renewable sources of chemicals, especially medicinal compounds (Vanisree et al., 2004). The genotypes were found to influence the *in vitro* performance and the chemical composition of the essential oils (Morone-Fortunato et al., 2010), so it is very important to develop reliable methods for propagation of true to true type plants.

The genus *Helichrysum* Miller, belonging to the family of *Asteraceae* is an important source of secondary metabolites and most of the species have been studied for their content of essential oils (Lawrence, 1998; Angioni et al., 2003; Appendino et al., 2007). *Helichrysum italicum* (Roth) G. Don is widespread in the Mediterranean area, where it grows as a small perennial shrub restricted to dry cliffs and open sandy soil habitats. The ornamental value conferred by its distinctive yellow scented flowers. The flower heads contain essential oils and other useful secondary metabolites. Its extracts are used in popular medicine in the Mediterranean region. *Helichrysum italicum* is known for its antiinflammatory, antiallergic, and antimicrobialactivity (Mastelicet. al., 2005). The oil is widely used for cosmetic and medical purposes and extracted from all green parts of the plant.

The scarce availability of cultivated materials, lack of agronomic practices and extreme variability of spontaneous ecotypes with very different content of oil and secondary metabolites material require the development of modern methods for propagation and cultivation. Therefore, clonal micropropagation offers opportunities for rapid reproduction of certain valuable genotypes. Although plant propagation via

shoot regeneration has been achieved in a vast array of plant species, studies on *H. italicum* tissue culture are rather limited (Giovannini et al., 2003). Giovannini et al. (2008) reported about the regeneration of *Helichrysum stoechas* plants through transformed hairy roots.

The aim of the present study was to compare the effect of different cytokinins on the multiplication of *Helichrysum italicum* and to develop a reliable protocol for *in vitro* micropropagation of this medicinal and ornamental plant.

Materials and Methods

The research was conducted at the Research Laboratory of Plant Biotechnology in Fruit growing Institute of Plovdiv.

Explant source was one-year-old plant. After surface sterilization, nodal segments with one axillary bud were placed in test tubes onto nutrient media based on both MS (Murashige and Skoog, 1962) or DKW (Driver and Kuniyuki, 1984) formulations. They were enriched with different cytokinins (5 µM): 6-benzylaminopurine (BAP) or Kinetin (KIN) or Zeatin, 0.005 µM IBA, 30.0 g L^{-1} sucrose and 6.5 g L^{-1}Phytoagar (Duchefa). The pH of the media was adjusted to 5.6 before autoclaving. All aseptic shoots were transferred on the corresponding nutrient media. Uniformly developed shoots (10-15 mm with two leaves) were used for the multiplication experiment. The same media without growth regulators served as a control (labeled as MS0 and DKW0). The study was carried out in round polypropylene microboxes with antibacterial filter (600 mL, $SacO_2$, Belgium, white filter, gas exchange rate – 10 GE/day) and in each microbox on 100 ml of culture medium, 10 shoot tips were set. The explants were subcultured every 3 weeks on the same nutrient media. After four passages growth, parameters were recorded.

For the rooting microcuttings (20 mm)were obtained from the multiplication stage and were transferred to the MS basal medium with half strength of macroelements, supplemented with IBA or NAA (0.1 mg/l or 0.2 mg/l). The same nutrient medium without growth regulators served as a control. The plantlets were cultivated in the same vessels mentioned above. In each vessel, onto 100 ml nutrient medium ten shoots were cultivated. The rooting percentage and growth parameters were recorded after 3weeks.

In vitro cultures were kept in a growth chamber at 22±2°C under 16-h photoperiod (fluorescent tubes OSRAM 40 W, 40 µmol m^{-2} s^{-1} PPFD).

Statistical analyses were carried out by one-way ANOVA using the Tukey test to validate the different significance at $P \leq 0.05$.

Results and Discussion

During the stage of multiplication, it is important to obtain the maximum number of usable new shoots. One of the most important indicators during this period is a multiplication coefficient, which represents the number of newly shoots with length more than 5 mm from one set of multiplication. In explants, maintained without growth regulator slow multiplication rate was observed (Table 1.). The use of cytokinines resulted in higher multiplication rate. Shoots grown on the all cytokinin enriched media showed significant differences in length and number of shoot /leaves compared to the hormone free control (Table 1). The multiplication and growth of *H. italicum* axillary buds was found to vary in a narrow range (2,5 - 3,4) at equimolar concentration (5 µM) of the three cytokinins studied.

The highest number of newly developed shoots was achieved on DKW nutrient medium, supplemented with Kinetin. Good multiplication rate (3.2) was recorded on both MS and DKW media, supplemented with BAP. We also observed the good growth response with the highest fresh and dry weight of the plantlets in cultivation on MS medium with BAP. These results are in accordance with Perrini et al. (2009b) who reported that the multiplication and growth of *H. italicum* ssp. microphyllum axillary buds vary with the concentration of the growth regulator and the highest level of BAP (1 mg L^{-1})alone or with IBA (0.2 mgL^{-1}) produced the better number of shoots (respectively 6.3 and 8.8). Giovannini et al (2008) obtained shoot induction from micropropagated leaf tissue on a medium supplemented with IAA in case of *H. italicum* and on a medium with thidiazuron in case of *H. stoechas*.

A significant difference on shoots length (14.4-18.35 mm average length), number of leaves and number of shoots among the culture medium variants enriched with growth regulators were not observed. On the other hand in culture medium without growth regulators, explants reached 21-25mm length without proliferation.

RESEARCH ARTICLE

Table 1. *Growth parameters of Helichrysum italicumafter 3 weeks in multiplicationstage.*

Variant	Number of shoots	Length of shoots (mm)	Number of leaves	FW of plant (mg)	DW of plant (mg)
MS0	1,50 ± 0,59 b	25,21 ± 3,16 a	13,90 ±1,96a	64,56 cd	9,96 cd
DKW0	1,60±0,51 b	21,40 ± 3,16 a	14.83±1.07 a	63,27 cd	9,91 cd
MS+K	2,90 ± 0,37 a	18,35 ± 3,36 ab	9,21 ± 1,49 b	93,08 bcd	12,75 bc
DKW+K	3,40 ± 0,42 a	17,50 ± 2,11 ab	8,42 ± 0,91 b	102,77 bcd	11,41 bc
MS+Z	3,30 ± 0,51 a	14,54 ± 0,91 b	7,82 ± 0,42 b	104,35 bcd	11,37 bc
DKW+Z	2,50 ± 0,34 a	14,96 ± 2,15 ab	8,61 ± 0,87 b	55,45 d	7,46 d
MS+BAP	3,20 ± 0,35 a	14,47 ± 0,74 b	8,35 ± 0,50 b	226,26 a	19,13 a
DKW+BAP	3,20 ± 0,51 a	14,91 ± 1,33 b	9,20 ± 0,86 b	126,58 b	13,93 b

Significant differences in fresh mass of plantlets (about four times) were reported among the different variants of treatment. The highest fresh mass (226 mg per plantlets) were recorded on the nutrient medium MS with BAP and the lowest (55.45 mg per plantlets) in plantlets grown onto DKW medium with zeatin. On the one hand, the high fresh mass could show better growth, but on the other hand, it could be an indicator of some physiological problems such as vitrification, for example. In fact, in the variant with the maximum fresh plant mass (MS with BAP), single hyperhydric leaves were found at the base of the shoot clump (Figure 1).

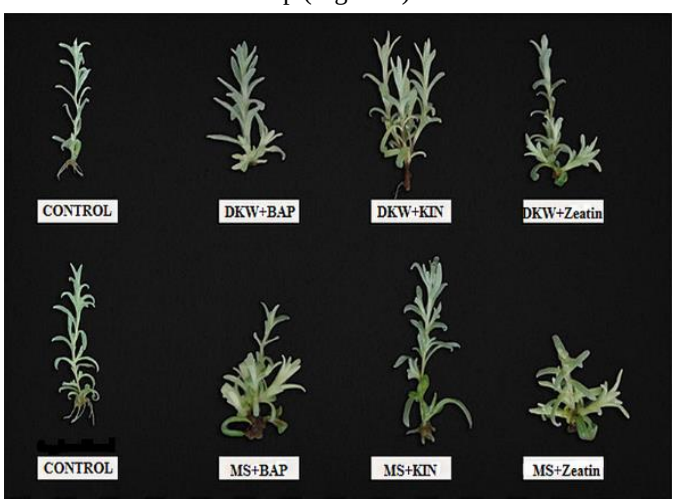

Figure1. *Micropropagation of Helichrysum italicum in multiplication stage.*

Morone-Fortunato et al. (2010) reported about significant differences in the percentage of proliferation, number of shoots/explantant growth among the investigated *H. italicum* ssp.*italicum* genotypes.

In their study of the micropropagation of *Helichrysum arenarium* onto the modified MS medium enriched with 6-benzyladenine (1.0 mg L^{-1}) Pawełczak and Bryksa-Godzisz (2008) observed the highest number of shoots (15.85 – 17.59 shoots/explantant). Also, a very high propagation coefficient was recorded in the *in vitro* propagation of *Helichrysum arenariumon* MS media with a supplement of 4 mg L^{-1} KIN,0.3mg L^{-1} BAP and 0.1mg·dm-3NAA, respectively 16.10 and 23.35 of shoots per explantant (Sawilska, Figas 2006). These large differences in the multiplication coefficient are probably due to the different genotype, but may also be related to the cultivation conditions. It is known that lower air humidity reduces the multiplication factor. It is possible that such an effect was also observed in our culture microboxes with improved gas exchange rate with the environment, but good growth habitus and healthy plants confirmed the positive effect of this type of vessels.

Very good rootinduction (between 60 and 100%) was observed in all tested media (Figure 3), however, The maximum rooting of shoots (almost 100 %) was achieved on hormone-free half-strength MS medium (Figure 2). The number of roots per explant was always higher in media enriched with auxins IBA or NAA compared to the treatment without growth regulators, with the exception of the variant with 0,2 mgL^{-1} NAA (Table 2).

Table 2. *Growth parameters of Helichrysum italicumafter 3 weeks in rooting stage.*

Variant	Control	0,1 mg/l IBA	0,2 mg/l IBA	0,1 mg/l NAA	0,2 mg/l NAA
Number of roots per plant	4,62 ± 1,04 ab	7,34 ± 0,11 a	7,90 ± 0,48 a	6,01 ± 0,34 ab	3,05 ± 0,53 b
Number of leaves per plant	22,73 ± 1,25 a	22,56 ± 2,03 a	23,83 ± 2,23a	20,73 ± 2,49 a	21,33 ± 0,84 a
Number of shoots	3,27 ± 0,89 a	2,60 ± 0,61 a	3,66 ± 1,21 a	2,93 ± 1,18 a	2,86 ± 0,14 a
Length of the shoot (mm)	12,34 ± 3,40 a	17,01 ± 1,74 a	15,39 ± 4,32 a	19,00 ± 3,93 a	16,38 ± 1,36 a
Length of root (mm)	8,01 ± 0,63 a	6,34 ± 0,58 b	5,92 ± 0,16 b	3,69 ± 0,35 c	3,12 ± 0,59 c
FW of 1 plant on the ground (mg)	70,0 ± 3,00 b	84,0 ± 4,80 b	108 ± 11,0 ab	126 ± 20 a	99 ± 75 ab
FW of roots (mg)	4,0 ± 2,00 a	3,00 ± 0,80 a	3,0 ± 0,2 a	3,0 ± 0,2 a	3,0 ± 2,0 a
DW of 1 plant on the ground (mg)	9,0 ± 0,00 c	1,09 ± 0,50 abc	13,0 ± 1,0 ab	14,0 ± 2,0 a	10 ± 0,8 bc
DW of roots (mg)	0,2 ± 0,10 a	0,40 ± 0,10 a	0,4 ± 0,0 a	0,3 ± 0,0 a	0,2 ± 0,0 a

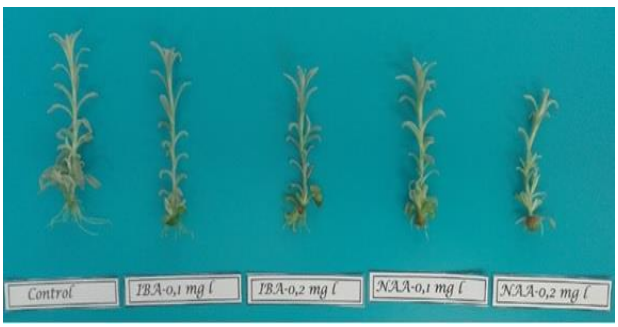

Figure 2. *Rooting stage of shoots Helichrysum italicum.*

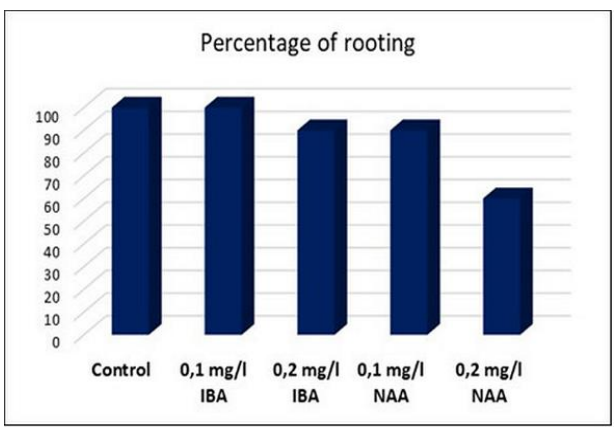

Figure 3. *Effect of Helichrysum italicum on rooting percent.*

Our results are similar to the results obtained by Perrini et al. (2009b). They reported that the highest rooting percent (86%) of *Helichrysum italicum* was achieved on medium, supplemented with IBA, and the lowest – on medium with NAA (36%). Good rooting ability were also reported for *H. italicum* (Giovannini et al., 2003; Perrini et al. (2009a) and other *Helichrysum* species - *Helichrysum arenarium* - Magdalena Tomaszewska-Sowa, Anna Figas (2014), Anna Figas et al. (2016). Morone-Fortunato et al. (2010) studied twenty genotypes and found significant genotypes effect on the rooting percentage and number of new roots. The rooting percentage varied between 50and 100%, but one genotype showed a lower rate of rooting (16.67%), further decreasing with the addition of IBA (8.33%). Indeed, the culture medium enriched with IBA, increased rooting percentages in only three genotypes. The authors suggested that the different response of the genotypes probably referred to a different endogenous content of endogenous hormones.

These results indicate that nutrient media and culture conditions must be carefully refined to achieve optimal growth of the relevant clones with the desired properties such as oil composition and other secondary metabolites.

Conclusions

Research on the *in vitro* performance of *Helichrysum italicum* is important for evaluating the potential of the large-scale tissue culture propagation of the planting material of relevant clones with the desired properties such as oil composition and other secondary metabolites.

The growing demand for *Helichrysum* planting material with important agronomic qualities justifies the need to develop modern propagation methods. *In vitro* reproduction of *Helichrysum* has many advantages over seed propagation, mainly rapid and unlimited production, the ability to achieve year-round true-to-true type planting material.

Helichrysum italicum plants have been successfully micropropagated on both MS and DKW media with different cytokinins BAP, TDZ, Zeatin. The best multiplication rate with good plantlets quality was achieved on DKW medium, supplemented with 5µM Kinetin.

High rooting percentage was achieved on all tested nutrient media, but the nutrient media supplemented with IBA (0.1 - 0.2 mgL^{-1}) resulted in the higher shoot and root length and the higher number of roots.

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