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Effect of Plant Growth Regulators on Different Explants and Explant Size of Yellow Everlasting (*Helichrysum pallasii* Sprengel Ledeb.) Under *in Vitro* Conditions

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Abstract
In this study, different explants (leaf, stem and root) and explant size (0.5, 1, 1.5 and 2 cm) of yellow everlasting, *Helichrysum pallasii* (Sprengel) Ledeb., cultured under in vitro conditions including kinetin, 2,4-D, TDZ and zeatin concentrations. Concentrations of 0.5 mg l^{-1} kinetin + 0.5 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) and 1mg l^{-1} kinetin (KIN) + 1 mg l^{-1} 2,4-D were used for callus induction. Concentrations of 1 mg l^{-1} thidiazuron (TDZ) and 1 mg l^{-1} thidiazuron (TDZ) + 0.1 mg l^{-1} zeatin (ZEA) were used for inducing shoot regeneration. MS medium without any plant grow regulators was preferred as control group. Rooting medium was preferred 1 mg l^{-1} NAA. In callus regeneration, highest regeneration rates were found as respectively 86.6%, 53.3% and 35.5% from root, stem and leaf explants with 1 cm explant length while the lowest rate (4.4%) found in control group from leaf explant with 2 cm explant length. It was obtained from the medium containing the highest shoot regeneration and from root (24.6%), stem (20.6%) and leaf (16%) explants with an explant length of 1 cm. Similarly, rooting rate from leaf, root and stem explants were found as respectively 43.8%, 32.3% and 21.9% with the 1 cm explant length obtained from 1 mg l^{-1} TDZ + 0.1 mg l^{-1} ZEA medium. In conclusion, 1 cm of explant length produced the highest regeneration rate in all source of explants. Also, 1 mg l^{-1} TDZ combined with 0.1 mg l^{-1} ZEA were more effective than 1 mg l^{-1} TDZ treatment alone in shoot regeneration.

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1. Introduction

Humans have relied on medicinal plants for ages to produce medications, flavors, agrochemicals, biopesticides, scents, colors, and food additives Nonetheless, the relevance of medical plants is increasing in tandem with the international medicinal plant trade (Phillipson, 1990; Balandrin and Klocke, 1988; Rao and Ravishankar, 2002). This increase in importance may bring problems such as desultory harvesting like excessive wild collection which could lead to endanger natural habitat of medicinal plant species. In order to prevent desultory harvesting, cultivation methods need to be enhanced. Enhanced cultivation methods likely cames into prominence because world population is rising swiftly. Tissue culture methods in a large scale are alternative cultivation methods as biochemical source (Sajc et al., 2000). Moreover, plant tissue culture methods are effective alternative ways to produce seconder metabolites while the traditional method is predicated on whole plant extraction. Seconder metabolite extraction from medicinal plants with tissue culture ensures some advantages such as biochemical production in an environmental controlled medium and free of diseases. Besides, establishment of standardized and steady production systems is available with plant tissue culture methods in seconder metabolite production (Fowler, 1985). There is an increased demand for natural products derived from medicinal plants and possible plant cell cultures are usually preferred for medicinal and aromatic plants that are in use for drug production for a long time (Kieran et al., 1997).

In plant products produced on a commercial basis, secondary metabolites are common. Alkaloids, glycosides, and essential oils are the three primary groups into which secondary metabolites can be divided. The essential oils are comprised of up of terpenoid combinations that are mostly used as solvents, flavoring agents, and scent. Glycosides include phenolics, saponins, flavonoids, cyanogenic glycosides, and tannins, which are utilized as medicines, dyes, and food colors. The alkaloids are a diverse group of components that includes approximately 4000 known structures. Most of the discovered alkaloids are naturally plant-originated substances that are physiologically effective in humans hence pharmaceutical industry's interest gradually increase on alkaloid substances (Shuler, 1981).

The genus *Helichrysum* Miller, which contains 500 species and is a member of the Asteraceae family, which is widely distributed throughout the world, is represented in the Turkish flora by 27 taxa, 15 of which are endemic. *Helichrysum pallasii* (Sprengel) Ledeb. grows on steppes and rocky slopes at altitudes ranging from 1700 to 3600 meters. It is widely recognized as a significant source of secondary metabolites, primarily for the components of its essential oils. (Davis, 1975; Davis et al., 1988; Yurteri et al., 2021). In Türkiye and other parts of the world, the plants are commonly used in traditional medicine, preferably as an herbal tea, for a variety of medicinal effects (Sala et al., 2003; Tepe et al., 2005). Several studies were conducted to optimize tissue and cell cultures in vitro conditions on *Helichyrsum* ssp. Perrini et al. (2009) reported that BAP (1 mg l^{-1}) alone or combined with IBA (0.2 mg l^{-1}) demonstrated much better shoot regeneration with a lower (20 g l^{-1}) sucrose content in *Helichrysum italicum* (Roth) G. Don ssp *microphyllum* (Willd.) Nyman. Root formation was achieved the best in the medium supplemented with IBA 0.2 mg l^{-1}. Also, Morone-Fortunato et al. (2010) obtained similar results with previous study conducted by Perrini et al. (2009) using BM enriched media with 6-benzylaminopurine+indole-3-butyric acid (BAP 1 mg l^{-1} + IBA 0.2 mg l^{-1}), with sucrose (20 g l^{-1}) in *Helichrysum italicum* (Roth) G. Don ssp. *italicum*. Dimitrova and Nacheva (2018) found that 5µM Kinetin in DKW medium was the best in enhancing multiplication rate of *Helichrysum italicum* (Roth) G. Don. The shoots were rooted in IBA medium a significant percentage of rooting on the control treatment and the nutrient medium was obtained combined with IBA. Sophie Clasquin and Max Henry (2002) reported that 2,4-D was vital for initiating primary callus regeneration in *Helichrysum arenarium* L.

Based on the above considerations, *in vitro* tissue and cell culture of *H. pallasii* has never been studied before. Utilizing plant materials which can ensure botanical and chemical stability over the increasing process seems to be of crucial importance. The aim of the present study was to determine the effects of kinetin (KIN) and zeatin (ZEA) on shoot regeneration and 2,4-kinetin with Dichlorophenoxyacetic acid (2,4-D) on callus formation in *H. pallasi* and to determine the essential oil content of obtained calluses.

2. Materials and Methods

2.1. Plant material and sterilization

Specimens of *Helichrysum pallasii* (Sprengel) Ledeb. (Boissier, 1875) were collected from its natural habitat during field studies in Altıntaş, Köse, Gümüşhane, Black Sea Region, Türkiye (40°15'26.4708" N-39°37'14.6064" E) (Figure 1.)

Figure 1. Specimens of *H. pallasii* (Sprengel) Ledeb. from its natural habitat in a rocky place

Undamaged healthy seeds were used and manually picked prior to surface sterilization. The seeds were treated with 70% ethanol for 5 minutes followed by Tween-40 treatment for 10 minutes. After these stabilization process, sterilization was completed using 20% NaOCl for 10 minutes followed by rinsing them 3 times with sterile distilled water for 5 minutes.

2.2. Culture medium and culture conditions

The pH of the medium was adjusted to 5.7 before adding agar and autoclave sterilization at 121°C for 20 minutes. The seeds were planted to MS medium (Murashige and Skoog, 1962), supplemented with 30 g L^{-1} sucrose and 7.5 g L^{-1} agar, including 5 seeds of each petri dish. Afterwards, different explants were taken from obtained plants. Stem, leaf, and root tip explants were cutted off respectively 0.5 cm (E1), 1 cm (E2), 1.5 cm (E3) and 2 cm (E4) length from regenerated plantlets from seeds in MS medium. Callus regeneration was induced with plant growth regulators (PGRs) of 0.5 mg l^{-1} kinetin + 0.5 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) (C2) and 1 mg l^{-1} kinetin + 1 mg l^{-1} 2,4-D (C3) and MS medium without any plant growth regulators as control group (C1). In order to induce shoot regeneration, 1 mg l^{-1} TDZ (M2) and 1 mg l^{-1} TDZ + 0.1 mg l^{-1} zeatin (M3) were used. MS medium without any plant grow regulators was preferred as control group (M1). Finally, the shoots were transferred into rooting media including 1 mg l^{-1} NAA. All cultures were incubated at two different incubation conditions to induce regeneration. First, explants were incubated at 26°C, 16 hours light and 8 hours dark conditions, 300 lux light source in growth chamber. In the second incubation condition, explants were exposed to dark condition at 26°C.

2.3. Statistical analysis

The study was conducted in 3 replications with 5 explants in each group in a completely randomized factorial experiment design.

Significance was evaluated by variance analysis (ANOVA), and the variations between the means were compared with JMP data analysis tool using Tukey's multiple post-hoc comparisons check.

3. Results and Discussion

3.1. *In vitro* plant propagation

Based on the obtained results, significant developmental differences were observed between different explants parts. Mostly, all factors in root explants were found to have significant (p<0.01) at Tukey's post hoc test. Also, all explant lengths that were cutted off from root explants demonstrated the best results on both callus and shoot development (Table 1 and 2).

3.1.1. Callus regeneration

In line with the findings; all explant parts in particular demonstrated different responses to the application of plant growth regulators. Root explants showed the highest regeneration rate compared to leaf and stem explant parts (Table 1).

In leaf explants, effects of explant length on callus regeneration were not found significant. The highest regeneration rate (35.5%) was obtained in M3 medium using 1 cm explant length. This trend was followed by C2 medium using 1 cm explant length treatment as 26,6% regeneration rate. The lowest regeneration rate (4.4%) was obtained from C1 medium using 2 cm explant length (Table 1.). Similar results were obtained by Pretto and Santarem (2000) in *Hypericum perforatum.* They stated that kinetin with 2,4-D concentrations showed a significant callus growth from leaf explants.

In stem explants, similar callus regeneration rate was observed as well as leaf explants. Maximum callus regeneration rates were found in C3 medium as 53.3% and 38.8% (respectively 1 and 0.5 cm explant length). Minimum callus regeneration rate (6.6%) was obtained in C1 medium with 0.5 cm explant length (Table 1).

Table 1. Effects of different plant growth regulators using different explant parts on callus regeneration rate (%) in *H. pallasii* (Sprengel) Ledeb.

Explant part	Medium	Explant length			
		E1	**E2**	**E3**	**E4**
	C1	13.3 de	13.3 de	14.4 c-e	4.4 e
Leaf	**C2**	12.2 de	26.6 ab	10.0 de	14.4 c-e
	C3	18.8 b-d	35.5 a	24.4 bc	15.5 cd
p<0.0001**	p<0.0137*	p<0.1383			
	C1	6.6 g	12.2 e-g	7.7 g	10.0 fg
Stem	**C2**	20.0 d-f	33.3 bc	13.3 e-g	16.6 d-g
	C3	38.8 b	53.3 a	27.7 b-d	22.2 c-e
p<0.0001 **	p<0.0038 **	p<0.0612			
	C1	34.4 c-e	58.8 bc	44.4 b-e	28.8 de
Root	**C2**	32.2 c-e	52.2 b-d	41.1 b-e	22.2 e
	C3	47.7 b-e	86.6 a	62.2 ab	47.7 b-e
p<0.0001 **	p<0.0004 **	p<0.0003 **			

C1: control (MS), C2: 0.5 mg l^{-1} kinetin + 0.5 mg l^{-1} 2,4-D, C3: 1 mg l^{-1} kinetin + 1 mg l^{-1} 2,4-D.
E1: 0.5 cm explant length, E2: 1 cm explant length , E3: 1.5 cm explant length, E4: 2 cm explant length.
*** p < 0.01 Tukey's at 0.01 level of significance * p < 0.05 Tukey's at 0.01 level of significance.*

The best results were obtained in root explants. All factors (medium, explant part and length) were found to have significant according to Tukey's at 0.01 level of significance (p<0.01). C3 medium with 1 cm (E2) explant length demonstrated the highest callus regeneration rate (86.6%). Although the highest results obtained from E2 explant size, there was no difference in level of significance with E3 explant size. Explant lengths of 1 (E2) and 1.5 cm (E3) were better to regenerate. Interestingly, lowest callus regeneration rate was observed in C2 medium with 2 cm (E4)

explant rate while all C1 medium results were higher than that (Table 1).

Increasing KIN and 2,4-D concentrations enhanced callus regeneration better compared with lower concentrations. Our data in this direction confirms studies of Clasquin and Henry (2002) and Giovannini et al. (2003). Researchers reported that 2,4-D treatment alone or modified improves callus growth rate in *Helichrysum* ssp.

3.1.2. Shoot regeneration

The highest shoot regeneration rates were obtained in M3 media (Figure 2) with 1 cm explant length in root, stem and leaf explants respectively 24.6%, 20.6% and 16%. Lowest shoot regeneration rates were observed in M1 medium in leaf, stem and root explants respectively 1.3%, 1.6% and 3.3% (Table 2).

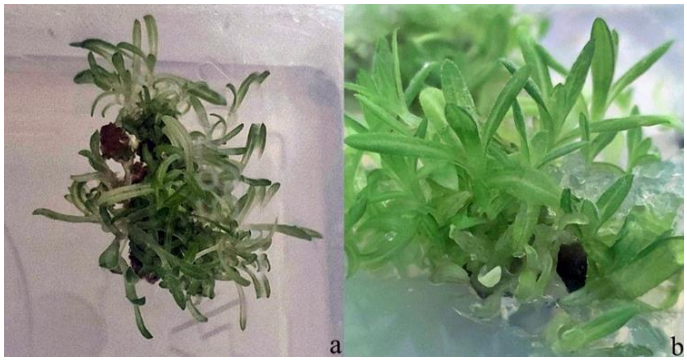

Figure 2. Shoot regeneration from root explant in M3 medium (a) and stem explant in M2 medium (b)

Explant length treatment was found significant only in root explants ($p < 0.05$) and the highest regeneration rates were obtained from root explants compared to other explant parts. M2 and M3 medium with 1 cm explant length demonstrated highest regeneration rate (26.6%) while lowest regeneration rate was 3.3% in M1 medium with 2 cm explant length. Mostly, 1 mg l^{-1} TDZ + 0.1 mg l^{-1} ZEA treatment was more efficient when compared to 1 mg l^{-1} TDZ treatment alone.

Table 2. Effects of different plant growth regulators using different explant parts on shoot regeneration rate (%) in *H. pallasii* (Sprengel) Ledeb.

Explant part	Medium	Explant length			
		E1	**E2**	**E3**	**E4**
	M1	6.3 cd	4.0 c-e	6.6 bc	1.3 e
Leaf	**M2**	1.6 e	11.0 b	2.0 de	1.6 e
	M3	4.3 c-e	16.0 a	8.0 bc	4.3 c-e
$p<0.0001$ **	$p<0.2567$	$p<0.1171$			
	M1	2.0 d	1.6 d	2.0 d	2.0 d
Stem	**M2**	3.3 cd	10.3 b	6.6 c	2.6 d
	M3	11.3 b	20.6 a	12.0 b	5.0 cd
$p<0.0001$ **	$p<0.0137$ **	$p<0.1383$			
	M1	6.0 cd	9.0 cd	5.0 cd	3.3 d
Root	**M2**	12.6 bc	24.6 a	12.6 bc	6.0 cd
	M3	7.0 cd	24.6 a	18.3 ab	12.6 bc
$p<0.0001$ **	$p<0.0324$ *	$p<0.0426$ *			

M1: control (MS), M2: 1 mg l^{-1} TDZ, M3: 1 mg l^{-1} TDZ + 0,1 mg l^{-1} ZEA.
E1: 0.5 cm explant length, E2: 1 cm explant length, E3: 1.5 cm explant length, E4: 2 cm explant length.
***p < 0.01 Tukey's at 0.01 level of significance *p < 0.05 Tukey's at 0.01 level of significance.*

Zeatin treatment combined with cytokinin's produced more shoot regeneration. Our results are in agreement with Dimitrova and Nacheva (2018).

3.1.3. Rooting from explants

Analysis of variance (ANOVA) showed that effects of medium and explant length on rooting percentages were highly significant (Table 3).

The highest rooting percentage was obtained from leaf explants with 1 cm explant length in M3 medium, while, the lowest rooting percentage obtained from root explants with 2 cm explant length in M1 medium. Among the explant lengths, 1 cm (E2) explant lengths were more efficient in formation new roots (Table 3). Moreover, M3 medium treatments demonstrated highest rooting percentages as well as its regeneration effect on shoots (Table 2, Table 3). This effect was thought that efficient shoot regeneration could lead a successful root formation when combined with coherent PGRs concentrations.

Table 3. Effects of 1 mg l^{-1} NAA treatment on rooting rates (%) on different explant parts of *H. pallasii* (Sprengel) Ledeb.

Explant part	Medium	Explant length			
		E1	**E2**	**E3**	**E4**
	M1	4.6 fg	6.2 fg	0.8 g	1.1 g
Leaf	**M2**	7 fg	18.8 d	23.1 cd	10.3 ef
	M3	28.6 bc	43.8 a	34.1 b	17.9 de
$p<0.1312$	$p<0.0020$**	$p<0.0001$*			
	M1	1.5 f	4.5 ef	1.8 f	2.1 f
Stem	**M2**	3.4 f	7.4 de	9 cd	7.8 c-e
	M3	17.6 b	21.9 a	17.1 b	11.2 c
$p<0.3292$	$p<0.0009$*	$p<0.0001$*			
	M1	7.9 c	6.1 cd	2.8 cd	0.7 d
Root	**M2**	3 cd	3.4 cd	5.3 cd	6.4 cd
	M3	27.3 ab	32.3 a	21.1 b	7.4 c
$p<0.3761$	$p<0.0115$*	$p<0.0001$*			

M1: control (MS), M2: 1 mg l^{-1} TDZ, M3: 1 mg l^{-1} TDZ + 0,1 mg l^{-1} ZEA.
E1: 0.5 cm explant length, E2: 1 cm explant length, E3: 1.5 cm explant length, E4: 2 cm explant length.
***p < 0.01 Tukey's at 0.01 level of significance *p < 0.05 Tukey's at 0.01 level of significance.*

1 mg l^{-1} NAA treatment found effective in rooting. Giovannini et al. (2003) stated that zeatin treatment alone (0.91 and 4.56 µM) or combined with IAA (10 µM) were ineffective rooting of *Helichrysum italicum*.

4. Conclusion

Based on obtained results, higher kinetin and 2,4-D treatment (C3 medium) was found to be more effective in incuding calli. Also, zeatin combination with thidiazuron enhanced shoot regeneration than thidiazuron treatment alone. Moreover, source of explant and explant size were found to be an important factor in plant regeneration as a result of this study. Tissue culture studies are few in *Helichrysum* species, more research is needed to uncover regeneration possibilities and in vitro protocols of this plant.

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